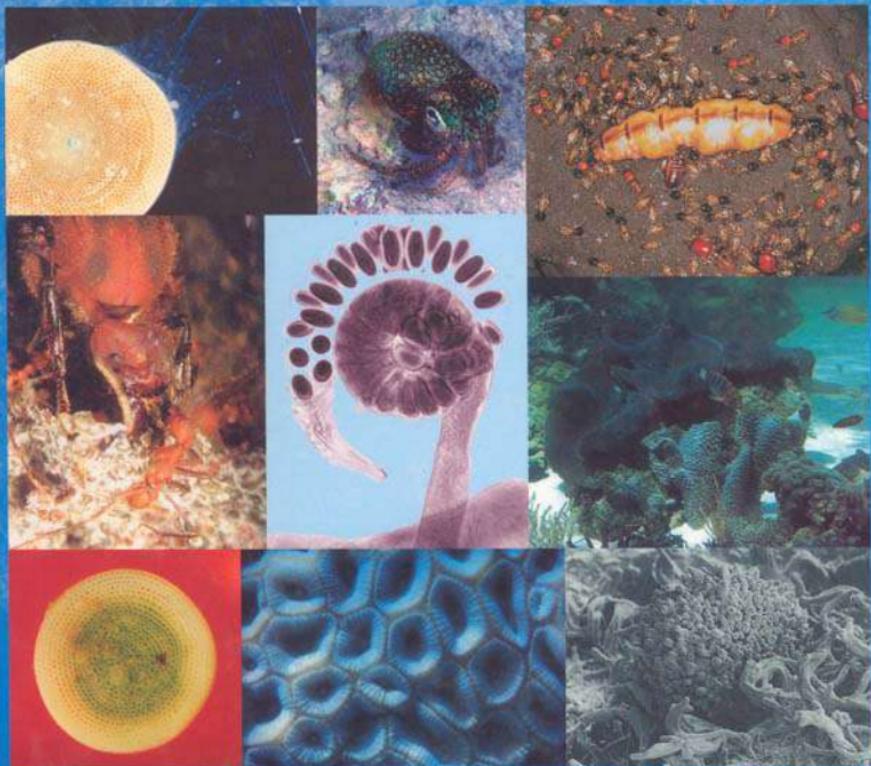


**CELLULAR ORIGIN AND LIFE IN
EXTREME HABITATS**

**Symbiosis:
Mechanisms and Model Systems**

Edited by
Joseph Seckbach



Kluwer Academic Publishers

SYMBIOSIS

Cellular Origin and Life in Extreme Habitats

Volume 4

Symbiosis:

Mechanisms and Model Systems

Edited by

Joseph Seckbach

Hebrew University of Jerusalem, Israel

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This book is dedicated to:

Professor Raphael Ikan (The Hebrew University of Jerusalem) my dear personal friend, colleague, symbiotic partner in publications (Chemistry Lexicon, 1991, 1999), with all my best wishes for health and happiness.

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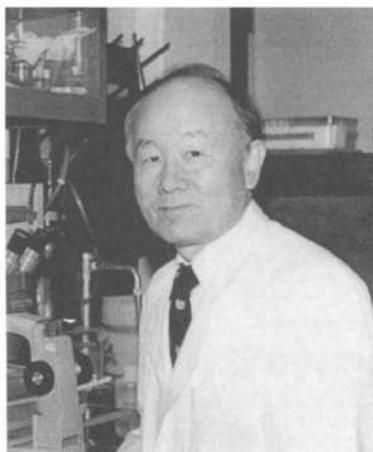
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Dr. **Kwang W. Jeon** is a Professor in the Department of Biochemistry at the University of Tennessee (Knoxville). He received his Ph.D. from the University of London, England (1964). His major research interests are to elucidate host-symbiont relationships at cellular and molecular levels and to formulate a comprehensive theory for the integration of newly acquired symbionts into host cells leading to the acquisition of new cell components and changes in cellular phenotypic characters. Dr. Jeon's experimental model is an amoeba-bacteria symbiosis that was recently established under laboratory conditions. The symbiotic bacteria were originally parasitic and harmful to the host but changed to required cell components within a few years and both the host and symbionts have changed to adapt themselves to stabilize the symbiotic relationship. His current aims are to elucidate the mechanisms for the development of host's dependence on their symbionts for survival. Symbiotic bacteria have been found to suppress the expression of one of the amoeba's housekeeping genes but to supplement the gene product for the host's use, thus forcing the host to become dependent on them. His studies also include the roles of symbiont-synthesized proteins in the symbiotic interactions, the genetic basis for the parasite-to-symbiont transition, and possible roles of plasmid DNAs of the symbionts in the establishment and maintenance of endosymbiosis.

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Foreword

By compiling this Symbiosis volume with contributions by more than 50 active scholars, Prof. Seckbach has made a great contribution to the field of symbiosis research. He has made it much easier for specialists and students of “symbiology” to survey the current status of research on symbiosis in various model systems, ranging from bacterial association to that of higher animals. It has been reported that there are more than 2,000 biologists worldwide, who are directly or indirectly involved in the study of symbiosis and the research output is voluminous. Unfortunately, however, the topic of “symbiosis” has not been a catchword in biology and there are only a couple of periodicals devoted to covering symbiosis research. Therefore, a volume such as this one is a welcome addition to the literature on symbiosis.

As is generally recognized, “symbiosis” is widespread in the biosphere and has contributed significantly to making the nature of life on Earth what it is now, both by being largely responsible for generating the eukaryotic cellular organization and by being the major source of genetic novelty for cell variation. While there are many cases of permanent partner associations, new symbiotic relationships are constantly formed and broken, some of them being cyclic. In keeping with advances in cell and molecular biology, the study of complex interactions among symbiotic partners has progressed to dealing with such associations in much more detail at the cellular and molecular levels. Thus, for example, symbiont-induced or enhanced gene expression is known to occur widely and in some cases symbiont integration is found to cause differentiation of cells and tissues leading to new morphogenesis. However, there are still many questions not fully answered, such as the mechanism for the initial survival of symbionts in or on hosts, especially when symbionts manage to survive in a “hostile” environment such as inside professional phagocytes whose given roles are to destroy exogenous intruders. In such cases, symbionts produce a large amount of stress proteins that apparently play important roles in the survival of symbionts, but the detailed mechanism is not known.

Also, the reason for mutual dependence by symbiotic partners in obligatory symbiosis is not clearly known. In some symbiotic relationships, such dependence is formed within a relatively short period of time. However, it is not clear if the development of dependence is genetic, physiological, or combination of both. New findings such as gene silencing by double-stranded RNA might help us in solving such puzzles.

With his earlier multi-authored books such as “*Evolutionary Pathways and Enigmatic Algae*” and of the book series “*Cellular Origin and Life in Extreme Habitats*” in the first volume “*Enigmatic Microorganisms and Life in Extreme Environments*” and in the second book “*Journey to Diverse Microbial Worlds*” Prof. Seckbach already contributed much to the study of symbiosis. We all know that compiling a multi-authored volume is not an easy task. However, with his zeal and skill, Prof. Seckbach succeeded in bringing together a large number of authors to cooperate for the current volume. It is regrettable that the limited space for each chapter has made a sacrifice for depth unavoidable. However, I am confident that this volume will not only provide up-to-date summaries on symbiosis research but also it will spur further advances in the study of the fascinating subject of symbiosis.

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Biodata of **Joseph Seckbach**, editor of this *Symbiosis* volume, and the chief editor of the book-series of **COLE** (Cellular Origin and Life in Extreme Habitats).

Dr. Joseph Seckbach, a retired academician from the Hebrew University, earned his Ph.D. from the University of Chicago (1965) and spent his postdoctoral years in the Division of Biology at Caltech (Pasadena, CA). Then he headed a team at the University of California, Los Angeles (UCLA) searching for extraterrestrial life. He has been with the Hebrew University of Jerusalem since 1970 and performed algal research and taught Biological courses. He spent sabbatical periods in Tübingen (Germany) as a DAAD Fellowship recipient, at UCLA and Harvard University. At Louisiana State University (LSU, Baton Rouge), he served (1997/1998) as the first selected occupant of the John P. Laborde endowed Chair for the Louisiana Sea Grant and Technology Transfer, and as a visiting Professor in the Department of Life Sciences.

Dr. Joseph Seckbach edited (and contributed several chapters to) the following books

- The “Cyanidium book” entitled *Evolutionary Pathways and Enigmatic Algae: Cyanidium caldarium (Rhodophyta) and Related Cells*. Published by Kluwer Academic Publishers, The Netherlands (1994) <http://www.wkap.nl/bookcc.htm/0-7923-2635-0>.
- COLE Book # 1. *Enigmatic Microorganisms and Life in Extreme Environments*, published by Kluwer (1999) <http://www.wkap.nl/bookcc.htm/0-7923-5492-3>.
- COLE Book # 2. *Journey to Diverse Microbial Worlds: Adaptation to Exotic Environments*, published by Kluwer (2000) <http://www.wkap.nl/bookcc.htm/0-7923-6020-6>.
- COLE Book # 3. *The New Science of Astrobiology* (J. Chela-Flores, author) pub. by Kluwer (2001).
- COLE Book # 4. *Symbiosis*, published by Kluwer (2001).
- *From Symbiosis to Eukaryotism: Endocytobiology VII* (E. Wagner, and J. Seckbach, eds.) Proceeding of the Endocytobiology VII meeting in Freiburg, Germany. Published by the University of Freiburg and Geneva (1999) <http://www.biologie.Uni-freiburg.de/ise/proceedings.htm>.
- *Algae and Extreme Environments*, Proceeding of Trebon (Czech Republic) conference, (edited by J. Elster, J. Seckbach, W.F. Vincent and O. Lhotsky) published by Nova Hedwigia, Berlin – Stuttgart (2001).
- He is the co-author (with author R. Ikan) of the Hebrew-language publication *Chemistry Lexicon* (1991, 1999)

Among his publications are books, scientific articles concerning plant ferritin (phytoferritin), cellular ultrastructure, evolution, acidophilic algae, and life in extreme environments. He has also edited and translated books of popular science. Dr. Seckbach's recent interest is in the field of enigmatic microorganisms and life in extreme environments

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“Two are better than one: because they have a good reward for their labor.....and a threefold cord is not quickly broken.”

Ecclesiastes 4 verses 9, 12

PREFACE: LIVING TOGETHER ONE INSIDE THE OTHER

This book is the fourth volume in the series Cellular Origin and Life in Extreme Habitats (“COLE”) edited by J. Seckbach and published by Kluwer Academic Publishers (Dordrecht, The Netherlands). Here we make a new evaluation of the ubiquitous symbiotic forms of life. In eight sections, nearly fifty contributors present current reviews covering the wide sphere of knowledge on symbiotic systems. Our experts examine various aspects of symbiosis beginning with ecological associations and origin of cells to several lines of the organelles’ sources and cellular features, and up to mutual associations between various organisms. Most of the manuscripts were reviewed and revised later by their authors to suit the scientific level of this book

In this volume we review much of the symbiotic field, which encompasses different organisms living together, usually one inside another in fully harmonious and mutualistic relationships, and with recognized selective advantages.. The nature of symbiosis has been a matter of academic discussion for more than a century (since the publication by Anton DeBary’s “*Die Erscheinung der Symbiose*, Verlag K.J. Trübner, Strassburg, 1879”). Besides contributing to basic studies, knowledge of life system on the planet, this area also has other practical applications, such as the biotechnological aim to develop symbiotic N₂-fixers of non-leguminous species, which would save on the use of nitrogen fertilizers and could improve soil and crops.

Symbiologists have generally accepted the idea that symbiotic interactions between various cells of different species led to the first eukaryotic cell. Following that theory, it is assumed that a larger cell (the “host”) engulfed free-living (prokaryotic or eukaryotic) microorganisms (the “endosymbionts”). Over a period of time and after one or more gene exchanges (translocation from symbiont to the host and vice versa), these endosymbiotic microorganisms became intracellular organelles (e.g., chloroplasts and mitochondria). One may call the mathematical equivalent of this theory as “One plus One (plus One) equals One.” Thus, the complex eukaryotic cell contains different prokaryotic (sometimes also eukaryotic symbionts) constituents and therefore contains heterogeneous sources of genomes (of the host and endosymbionts). In this volume as well as in chapters of the COLE series and elsewhere in the literature, a few authors question or oppose the endosymbiotic cellular evolutionary concept. In their rebuttal, the proponents of direct filiation (Eukaryogenetic autogenous compartmentalization) point out a mechanism resembling Darwin’s natural selection that took place during transition from the prokaryotic phase to the nucleated cell.

Also in this volume one can find new symbiosiotic associations among representatives of the three domains of Life (Archaea, Bacteria, and within Eukarya). The chapters review relationships between Archaea, Bacteria, and even the human body, which harbors billions of microorganisms. In many cases, the established host organisms with their permanent endosymbiotic “tenants” are not able to live without these obligative dwellers that supply metabolites and energy to their host “landlords.” A good example is the ruminant-rumen microorganisms (bacteria, protists, and fungi) that live in symbiotic association within specialized chambers of various mammals.

From the wonderful world of symbiosis come new discoveries day after day. For example, some insects are now known to harbor endosymbiotic protists in their guts. These protists themselves contain symbiotic prokaryotes and fungi (see chapters dealing with insects and other animals). The fungi being heterotrophs by nature play a vital role in symbiotic associations (above and below the ground). They associate intimately with plants (such as in mycorrhizae or in roots of the legume family) and algae (as lichens), and one may speak about “fungi that fly in the air” by taking a “free ride” on their insect hosts. Indeed, a symbiotic association could be compared to a Russian “babushka” doll with one figure inside another (in fact, such complex combinations have been observed among termite symbionts). Von Dohlen has even reported recently (*Nature* **416**, 433-436, 2001) that modern bacteria (γ -proteobacteria) live symbiotically inside other bacteria (β -proteobacteria), whereas the “host” bacterium is in turn an endosymbiont of a plant sap-feeding mealybug. This type of triple cellular association allows gene products to be exchanged more freely between species.

The purpose of this volume is to introduce the teacher, researcher, scholar, and student as well as the “open-minded” and science-oriented reader to the global importance of symbiosis and to new aspects of symbiosiotic relationships among living organisms. Evidence of the growth of this field is the existence of the International Symbiosis Society (ISS) and International Society of Endocytobiology (ISE).

It is our hope that the windows opened in this volume exposing life in various symbiotic contexts will lead to further discoveries, increasing our understanding of the diversity of microbial life occurring in intimate association with other (higher) organisms.

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Acknowledgements

I would like to thank our contributors and mainly to the “early birds” who submitted their chapters in time, I admire their understanding and patience. I express my gratefulness to the board members for displaying interest in this project and for always being available to review the manuscripts for this volume. I have to stress my gratitude to Professors Aharon Oren (Hebrew University), Kwang Jeon (University of Tennessee, TN), Paul Nardon (INSA, Lyon, France), and Jim F. White (Rutgers University, US) for their extraordinary interest and involvement in this book.

Thanks are due to the following Professors for supporting this volume in various ways, including reviewing chapters, suggesting the book cover’s photos and its title. Paola Bonfante (University Torino, Italy), Nick J. Brewin (JIC, Norwick, UK), Kurt R. Buck (Monterey Bay Aquarium, CA), Russell L. Chapman (LSU, LA), Cameron Currie, (University Washington, Tacoma WA) for his picture contribution for the cover, David Day (University of Western Australia), Zvy Dubinsky (Bar Ilan University, Israel) for his photo for the book cover. Betsey Dyer (Wheaton College, Norton, MA, USA), David Garbary (St. Francis Xavier University, Nova Scotia, Canada) for his original suggestion of our book title, Manuela Giovanetti (University of Pisa, IT), John J. Lee (City College of CUNY, NY) for his photo for the book cover, Robert Lichtwardt (University of Kansas, Lawrence, KS) for his extraordinarily interest and contribution of the photo for the book cover, Michele K. Nishiguchi (NMS University, Las Cruces, NM) for her photo for the book cover, Dennis Searcy (University of Massachusetts, Amherst, MA), Hainfried Schenk (Tübingen University, Germany), David Secord (University of Washington, Tacoma WA) for his suggestion of various titles for our volume. Hugh Wilcox (Ashland, OR), Johannes Hackstein (Catholic University of Nijmegen, NL) for his illustration for the cover, and to Douglas Zook (Boston University) for his constant involvement during the process of this project.

I give my appreciation to the faithful members of Kluwer Academic Publishers for their full cooperation during all the steps of this volume. Last but not least, gratefulness and gratitude are due to my wife Fern for her encouragement and patience as she watched this volume become a reality.

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Legends for the photos to the Book Cover of SYMBIOSIS:

MECHANISMS AND MODEL SYSTEMS

J. SECKBACH (EDITOR)

Centerpiece:

The fungus is *Palavascia sphaeromae* (Trichomycetes, Eccrinales), a symbiont of the intertidal marine isopod, *Sphaeroma quadridentatum*. This specimen came from the Atlantic shores of North Carolina, USA, but the fungus has been found in Sphaeromidae from the Mediterranean and Pacific Ocean as well. The fungus grows attached to the hindgut cuticle of isopods, but does not sporulate until it projects from the anus. This is an unusual feature in Eccrinales. The attached photo shows a coiled tip of a thallus projecting from the hindgut and producing sporangiospores. Since the isopod hosts aggregate in moist microhabitats during periods of low tide in order to avoid desiccation, it is believed the production of sporangiospores outside of the gut permits the spores to be ingested directly by other isopods during this time of intimate aggregation. **Professor Robert Lichtwardt** (The University of Kansas, Lawrence, KS, USA) contributed this photo.

Figure 1 (12:00 O'clock):

The common bobtail squid, *Euprymna tasmanica* (Cephalopoda: Sepiolidae) from Australia. The sepiolid squid contains a bacteriogenic light organ in the mantle cavity, which emits light used in anti-predatory behavior. **Dr. Michele K. Nishiguchi** (New Mexico State University, Las Cruces, NM, USA) contributed this photo.

Figure 2 (1:00 o'clock):

The royal cell of a fungus-growing termite, *Macrotermes* sp., from Uganda, opened to show the large queen with extended, swollen abdomen, the smaller king and four other castes in attendance: major and minor soldiers, with major and minor workers. Fungal tissues ingested by the termites are rich in nitrogen and may provide enzymes, which enhance polysaccharide digestion. The symbiosis is obligate for both partners. M. Leponce took this photo. This photo was published in *Termites: Evolution, Sociality, Symbioses, Ecology*. Edited by T. Abe, D.E. Bignell and M. Higashi (2000). Kluwer Academic Publishers, Dordrecht, The Netherlands. **Professor D. E. Bignell** (Queen Mary, University of London, UK) contributed this photo.

Figure 3 (3:00 o'clock):

Great Barrier Reef, Australia. All of the depicted organisms harbor symbiotic algae. Such include the giant clam *Tridacna*, and the various corals. These symbionts provide the energy for the entire coral ecosystem. **Professor Z. Dubinsky** (Bar Ilan University, Ramat Gan, Israel) contributed this photo.

Figure 4 (at 5:00 o'clock):

Highly magnified scanning electron micrograph of the surface of the fungus-comb of the African fungus-growing termite *Macrotermes subhyalinus*, showing mycelium growing on composted dry grass (periphery) and a cluster of fungal conidia forming a single nodule (center). The N-rich conidia are eaten by younger worker termites and mixed with new forage; older workers consume the mycelium as a food. Micrograph, R. Leuthold. This photo was published in *Termites: Evolution, Sociality, Symbioses, Ecology*. Edited by T. Abe, DE Bignell and M. Higashi (2000). Kluwer Academic Publishers, Dordrecht, The Netherlands. **Professor D. E. Bignell** (Queen Mary, University of London, UK) contributed this photo.

Figure 5 (at 6:00 o'clock):

The scleractinian coral *Favia* from Eilat, Red Sea, Israel. The host animal harbors endocellular microalgae, the zooxanthellae at densities $\sim 10^6 \text{ cm}^{-2}$. This photo shows the coral during daylight where the tentacles are withdrawn exposing the algae to the light. At night the tentacles are extended to capture nutrient-rich zooplankton prey. In the mutualistic coral-algae symbiosis the algae provide the energy whereas the coral supplies essential nutrients derived from prey digestion. **Professor Z. Dubinsky** (Bar Ilan University, Ramat Gan, Israel) contributed this photo.

Figure 6 (7 O'clock)

The foraminiferan (Protozoa) *Parasorites* serves as a host for endosymbiotic green algae. Contributed by **Professor J.J. Lee** (City College of CUNY, NY. USA).

Figure 7 (9 O'clock)

The fungal garden and the ants are mutualistic dependent. This photo shows a leaf-cutting ant worker and queen on the fungus garden. Compare this figure to the termite queen in figure 2. Contribution of **Dr. C. Currie** (The University of Kansas, Lawrence, KS, USA).

Figure 8(11:00 o'clock)

Amphisorus hemprichii, a coin-like sorted foraminiferan which appears olive brown because it has endosymbiotic dinoflagellates. A network of spider web-like pseudopodium is visible emerging from the right side of the shell. Contributed by **Professor J.J. Lee** (City College of CUNY, NY. USA).

I. General Aspects

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Biodata of **Douglas Zook** author of “*Prioritizing symbiosis to sustain biodiversity: Are symbionts Keystone species?*”

Dr. Douglas Zook is an Associate Professor of Science Education and Biology at Boston University. He received his Ph.D. in Biology from Clark University with follow-up Fulbright-supported research at the University of Tübingen, Germany. Dr. Zook teaches intensive graduate courses in Symbiosis, Global Ecology, and Teaching Methods. Previous research focused on *Cyanophora paradoxa* as a model symbiotic system for studying plastid evolution, bacterial influences in lichens. Besides directing the international Microcosmos Biology Education effort and the masters program in Science Education at Boston University, Dr. Zook is currently the president of the international symbiosis society.

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PRIORITIZING SYMBIOSIS TO SUSTAIN BIODIVERSITY: *Are Symbionts Keystone Species?*

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1. Introduction

The numbers of species in the eukarya domain, particularly plants and animals, continues on the wane beyond expected extinction rates commonly ascertained in geological history (Reid and Miller, 1989). Rapidly increasing human populations coupled with a demand for resource-intensive lifestyles has resulted in a fragmentation of non-anthropogenic landscapes, direct removal of habitats that support a diversity of life, and threats to fundamental support mechanisms, such as an enhanced global temperature increase, an ozone and sulfur dioxide-dominated troposphere, and toxic metal leakage into hydrological systems. This potpourri of documented effects has forced the rapid emergence of a biological-sociopolitical discipline, conservation biology.

Originally defined as a crisis-discipline in that researchers must often quickly compile data to help stave off the suddenly recognized demise of an organism (Soulé, 1985), more recent emphasis points to a need to be more preventive and prioritize conservation efforts. Limited funds, the spreading scope of biodiversity loss, and competing concerns within the human cultures and economies of various countries all dictate that new or altered approaches which can result in species preservation must be considered.

Within this framework, the term of “keystone” species first introduced in concept, albeit not in name, in a classic paper by Robert Paine (1966), is gathering still more attention. Definitions of keystone species appear to be ephemeral, but combining Power et al. (1996) and Bush (2000) is straightforward: Those species with a disproportionately large effect on its ecosystem relative to its biomass and abundance. Thus, dominant grasses, essential in many food webs are important due to their role in energy flow, or oak trees, which provide a sheltered community for scores of species but have a substantial biomass, would not be considered keystones. Often keystone species have such a profound effect that its removal would render numerous other species threatened or eventually extinct. Conversely, identifying and preserving these recognized keystones may mean the conserving of significant biodiversity. Organisms dependent on the keystones would not have to be protected directly, rather the focus would be on the keystone organism. Management policies would be more enlightened with greater potential for success. As Primack (1993, p. 47) notes, “Selective logging in

rainforests would preserve fig vines and fig trees even though other trees were being removed.”

2. Keystone Examples

The ecological literature has well-documented examples of keystone species, almost exclusively in mega-fauna and -flora. However, many species that are commonly accepted as keystone species actually show contradictory data. These include the predatory *Pisaster* sea stars of the northwest USA coast whose loss would invoke a cascade effect of other species loss in a web, but other predators such as *Nucella spp.* whelks may actually effectively substitute (Navarette and Menge, 1996); *Enhydra lutris* (sea otter) is said to be a keystone predator because it culls various invertebrates such as sea urchins, which in turn would over graze the macro-algae (Estes and Duggans, 1995), yet reintroduced otter populations in the Semichi Islands of Alaska have not shown significant changes in urchin and kelp populations (Konar, 2000); and, *Castor canadensis* (North American beavers) dramatically alter water flow and thus nutrients that support a wide diversity of life, but Donkor and Fryxell (1999) investigated the effects of beaver browsing in boreal forests and saw little indication of beaver enhancement of dominant plant species. African elephants, flying foxes, and fig trees are among many others considered “keystone species.”

Clearly, specific criteria for identifying keystones remains elusive. Moreover, in tropical rainforests, there is likely a variety of plants that are critical as trophic resources at different seasons. For example, one recent study (Peres, 2000) proposes two legumes in Amazonia which produce gums upon which a variety of arboreal vertebrates depend. Often, as shown by Fauth (1999) who observed newt and salamander roles in vernal ponds, a species that is a keystone in one habitat may not be in a similar geographical habitat. Jordan et al. (1999) developed an index for characterizing keystone species based on their role in food web networks, while Hurlbert (1997) reinforces the conclusions of Mills et al. (1993) by arguing that keystone concepts are ill-defined and can contribute little to ecological analysis. Power et al. (1996) appropriately emphasize the notion that keystone species can be very “context dependent.”

3. Symbionts as Keystones

Ambiguities in the defining, characterizing and measuring of keystones could be perhaps lessened if ecologists and conservation biologists concentrated more on microbial systems, particularly in the context of keystone system processors (Bond, 1993), i.e., species that are the main promoters of nutrient cycling or energy transfers. In this context, I propose another line of thought that is already intuitively established, further testable, and yet substantially ignored in the keystone debate: Microbial symbionts from many symbiotic systems are less ambiguous than mega-organism counterparts and should be considered archetypal keystone species. Symbiosis is here defined as the acquisition of one organism by another and through subsequent intimacy build new structures and new metabolism (Douglas, 1994; Zook, 1998). Focusing on

how many of the earth's biomes have evolved and persist, we can discover the "cone effect," of some symbionts -- that is, certain infections of one organism into other quite different organisms lead to massive expressions such as forests and reefs, and thus to an expanding array of life. Many of these symbiont species are inconspicuous in hidden or limited biomass, yet their biodiversity effects are profound and far-reaching, qualifying them as priority keystone species.

3.1 DINOFLAGELLATE SymbIONTS OF CORALS

Consider, for example, the dinoflagellate *Symbiodinium spp.* as a keystone species: Thousands of species from across the kingdoms are the direct result of the prodigious exoskeleton of the coral-dinoflagellate symbiosis, which forms a massive and growing substrate and nutrient collecting zone, i.e. reef. As much as 900 grams of Carbon per meter squared per year are estimated to be fixed by coral-dinoflagellate reefs and its inhabitants (Bush, 2000). Reefs can also be considered as sinks for over 100 million tons of Carbon each year (Kinsey and Hopley, 1991) as well as substantial calcium.

Without the acquisition of its compatible symbiotic dinoflagellates, coral polyps excrete calcium carbonate in a greatly reduced fashion, seldom exceeding the size of a fist. But, in reef-building (hermatypic) corals, the symbionts sequestered and encysted within specialized host membranes (symbiosomes) are known to transfer up to 95% of their photosynthate to the host animal (Muscatine, 1990). The symbionts rapidly acquire and assimilate more carbon dioxide, shifting the carbonate equilibrium toward consistent limestone precipitation (Barnes and Chalker, 1990; Goreau, 1959). It is a profound irony that one of the most biogenic and influential earth systems in the biosphere, the coral reef, is likely the result of active alkaline leakage which is in turn influenced by increased rates of photosynthesis (Gattuso et al., 1999; Kleypas, 1997) from limited dinoflagellate variants that cannot be seen with the naked eye. The selective advantage for the coral in producing such an exoskeleton is likely that the animal has new, preferred substrates for additional polyp development.

Symbiodinium-based reef building corals provide the primary shelter for many organisms (Crossland et al., 1991) This dependency of the coral reef community on the *Symbiodinium* and a variety of related infecting dinoflagellates (Trench and Schoenberg, 1980; Trench, 1997) has become more evident in recent years with continued coral algal bleaching events. By comparing the health and diversity of a coral reef prior to the onset of symbiont bleaching with the reef at various periods after bleaching, clear, observable data emerges.

Increased photic zone water temperature likely due to higher emissions of greenhouse gases such as carbon dioxide and water vapor cause the symbionts to leave the coral tissue, i.e. to bleach out. The thermal thresholds for the symbiotic dinoflagellates range from 28 to approximately 30 degrees C. (Hoegh-Guldberg, 1999). The actual de-pigmentation is due to the production of oxygen free radicals that result from a combination of greater light intensity in higher than normal water temperatures. The algae essentially become less sensitive to photoinhibition (Jones et al., 1998; Hoegh-Guldberg, 1999). Consequently, we can record a striking demise of coral health and distribution and predict resultant losses in the abundance of diversity of a variety of other micro and macro-algae, and a host of fishes and invertebrates that depend on

these photosynthesizers. This is clear and unambiguous evidence of the keystone status of this microbial symbiont.

3.2 MYCORRHIZAL SYMBIANTS OF PLANTS

The powerful symbiotic influence on diversity in the oceanic realm is of course paralleled in terrestrial environments by the subterranean distribution of mycorrhizal hyphae. Mycorrhizae are prime candidates for keystone status as indicated by Perry et al., (1990), albeit their total biomass in any given hectare of soil can be surprisingly high. There is growing evidence, especially from fossil analysis of the Rhynie Chert strata in Scotland (Taylor et al., 1995), that plants as such have never been truly isolated as a taxonomic entity, but made the transition from marine Charophycean algae to early land plants through the infection and later maintenance of fungi (Pyrozinski & Malloch, 1975; Selosse and Tacon, 1998). This dynamic co-evolution, possibly expressed in a radiation of branches from over 400 million years ago, is now the sine qua non of current forests, wetlands, grasslands, and savannas of the world. In the blossoming research into mycorrhizal associations with plants, there are growing indications that a major selection factor for the distribution, abundance, and diversity of plants may be these subterranean fungi (Read, 1999) which serve as essential conduits into plant roots for phosphorus, trace metals, and ammonium ions (Smith and Read, 1997).

For example, recent reports show complex successional events with mycorrhizae over the life of a plant community and even a single plant, as well as the transfer of photosynthate not only from the plant leaves to the symbiotic fungi but in some cases through the fungal hyphae into its mycelial fan in the soil and directly into another nearby plant, sometimes of a differing species (Perry et al., 1989; Simard, 1995). Such interactions strongly suggest that plant guilds may compete for the most advantageous mycorrhizae or that mycorrhizae by its ubiquitous soil outreach select optimal symbionts and, in effect, may determine plant distribution and diversity (Read, 1999). It follows, then, that certain mycorrhizal fungi species that commonly associate with dominant trees, shrubs or grasses should be considered keystone species. Preserving these fungi and their soil habitats may be as critical, for example, as forestry management analysis above ground level.

3.3 TREBOUXIA SYMBIANTS OF LICHENS

Lichens are estimated to cover as much as eight per cent of the actual terrestrial land surfaces of the planet (Smith and Douglas, 1987). The result of intimacy among specific fungal and algal and sometimes prokaryotic cells, lichen thalli have a remarkable impact on the numbers and distribution of organisms (Galloway, 1992), particularly in rainforests and tundra. For example, lichens infected with cyanobacteria such as *Nostoc*, *Calothrix*, and *Gloecapsa* are prolific in the tropical rainforest canopy (Wolseley, 1991), as well as temperate rainforests (Galloway, 1995; Malcolm and Galloway, 1997). Often sequestered in specialized regions known as "cephalodia," the *Nostoc* filaments convert gaseous nitrogen to ammonium ions within specialized, thick-walled, non-photosynthetic spheres along their trichome, known as "heterocysts." The leaking of nitrogenous compounds from these lichens as well as direct assimilation after degradation and death may be a significant contribution to nitrogen cycling among

tropical canopy species (Forman, 1975; Huss-Danell, 1977) and temperate rainforests (Green et al., 1980), and thus may have a significant synergistic effect on rainforest organism viability and diversity.

Other biomes such as the northern tundra show an even more direct effect, as megafauna such as *Rangifer tarandus* and more specifically its vast rumen microbiota feed directly on various fruticose lichens, especially of the *Cladina* genera. While hundreds of different fungi can be in a lichenized state, there is only a relatively small number of microbial algae and cyanobacterial species that associate in the lichens. Therefore, the most widespread lichenized eukaryotic photobiont, *Trebouxia spp.*, as well as *Nostoc* cyanobionts could be considered major keystone species. Without their infection and association with a wide range of fungi, numerous other organisms would not be able to emerge, and the regions' high biodiversity would predictably drop prominently.

3.4 GUT MICROFLORA SYMBIONTS OF RUMINANTS AND TERMITES

Recognizing the strong trophic linkage of reindeer and lichen serves further to underscore the importance of symbiosis in the diverse assemblage of communities which we erroneously see as individuals, mammals. Nearly all mammals depend on extracellular symbionts, substantially anaerobic or microaerophilic, within specialized digestive chambers.

It is likely that the rumen fermentation vats of all hoofed mammals may have evolved specifically as a co-evolving accommodation to a required mind-boggling diversity and biomass of heterotrophic protists, eubacteria, and Archaea (Hume and Warner, 1980), most of which are involved with cellulose metabolism (Forsberg et al., 1997). Similarly, the large intestine of primates is substantially a hive of diverse life, wherein prokaryotes serve as vitamin producers, pH regulators, enzyme producers, compactors and even to a lesser degree nutrient recyclers (Wilson, 1997). Many microbial symbionts of these megafauna can be considered powerful keystone species, for their metabolism is a foundation that underpins an expanding inverted pyramid of diverse animals and protists. Indeed, a traditional view of identifying predatory mammals as potential keystones may be misconceived, for it is their internal symbiotic microflora that better fit the keystone definition.

A frequently unrecognized counterpart to rumen and intestinal symbioses is the “gut symbioses” of termites and other insects. Dozens of protist species within the termite gut frequently make up a third of the insect’s body weight, and bacteria number more than 10^9 per milliliter of gut fluid (Breznak, 1982). Thus, the termite hindgut can be considered one of the most diverse and dense communities per unit area on earth. Its impact reaches far beyond merely keeping an insect alive, for the whole consortium of multi-kingdomed creatures within the gut “paunch” are primary cellulose metabolizers (Kane, 1997). They ultimately change the face of ecosystems by rapidly converting remnant plant biomass to viable topsoil and subsequent re-colonization by diverse plants and invertebrates.

3.5 MYCETOCYTES OF APHIDS

Such far-reaching insect symbioses are not relegated to only specialized “paunch” containing forms. Insects such as aphids feature specialized mycetocyte cells which harbor an array of specific bacteria and even yeast involved with vitamin synthesis and amino acid production with subsequent transfer to the host insect (Moran and Telang, 1998). Such symbionts are vertically transferred, i.e. they are passed on to the next aphid generation through complex interactions within the developing insect eggs (Douglas, 1989). Indeed, these bacterial symbionts have not likely seen the light of day in over 200 million years -- since the first stages of aphid evolution. While aphids are commonly categorized as agricultural pests, they are nevertheless a crucial food source to numerous organisms in a myriad of webs distributed globally. Thus, in both cases, the termite and the aphids, protist and bacterial symbionts may be important keystone species. Conservation measures designed to maintain the conditions for the continued proliferation of paunch- or mycetome-microflora may mean substantial biodiversity enhancement.

3.6 OTHER SYMBIONTS

While some symbiotic partnerships, such as the water fern *Azolla* which houses nitrogen-fixing *Anabaena*, are prominent due primarily to their vast economic importance, greatly enhancing *Oryza sativa* production (Shi and Hall, 1988) rather than a significant non-anthropogenic-based functions, other nitrogen fixers such as *Rhizobium* with legumes and *Frankia* with actinorhizal plants have powerful ecological impacts and directly underpin growth and diversity. As described by Bond (1993) studies by Vitousek and Walker (1989) showing the major biodiversity increase after the establishment of a nitrogen-fixing shrub in lava regions of Hawaii, indicate a potential keystone role.

More recent research shows that many common grasses, particularly of the *Festuca* genera, are not only root-infected by partner vesicular-arbuscular mycorrhizae but may depend on other specialized fungi that intracellularly infect the grass leaf and even the grass seed, conveying protection against herbivory (Clay, 1990, White, 1994). While the obligate or facultative status of these fungi in relation to the grasses is still unclear, research may in the future show that entire trophic webs in grassland and Savannah biomes are based on these symbiotic tussocks. Nitrogen-fixing bacteria and cyanobacteria, as well as certain endophytic fungi are limited in biomass and distribution and, therefore, may be considered keystones when one examines the dependency that a vast array of organisms have on these infections.

4. Summary

Identifying keystone species appears to be pivotal in the strategies designed to prioritize important ecological zones and preserve high levels of biodiversity. Yet, definitions and the means of determining keystones are shifting and ambiguous. Microbial symbionts such as the dinoflagellate *Symbiodinium* spp. in hermatypic corals and

selected fungal species in mycorrhizal plant associations are more clear and critical keystone species. The research agenda in the conservation biology and ecology disciplines can no longer afford to be limited to traditional mega-flora and -fauna perspectives. Indeed, symbiotic systems appear to be a cornerstone of biodiversity.

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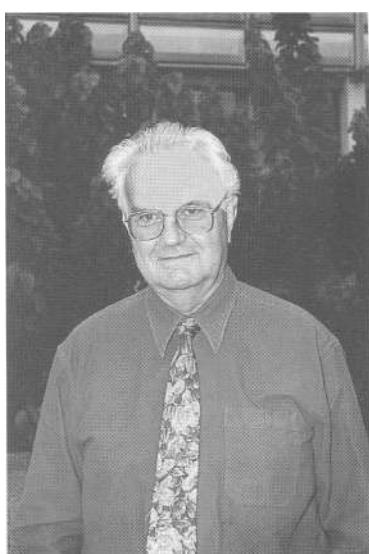
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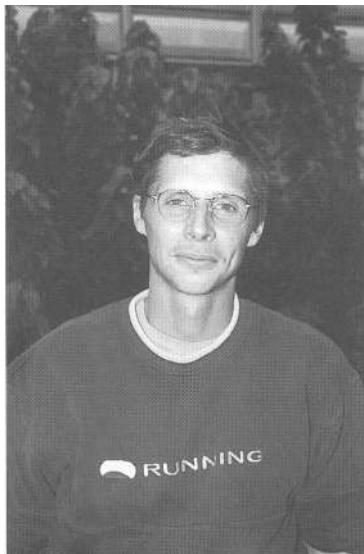
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MORPHOLOGICAL ASPECTS OF SYMBIOSIS

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1. Introduction and terminology

Most animals and numerous plants live in close association with microorganisms, and the concept of symbiosis has long been discussed (De Bary, 1879 - Smith, 1992 - Saffo, 1992 - Margulis, 1993 - Schenk, 1993 - Nardon and Nardon, 1998). In this chapter, we retain the simple definition of De Bary: "Symbiosis is the permanent association between two or more specifically distinct organisms, at least during a part of the life cycle." The typical parasitic and pathogenic interactions will not be included, because it is often difficult to distinguish between weakly aggressive and neutral associations. An example is the *α-proteobacterium* *Wolbachia*, often associated with insects and other invertebrates, and considered either as a parasite or as a neutral partner, according to Werren (1997). In general, one partner is bigger than the other(s) and is termed "the host", while the smaller partners are called the "symbiotes", or "symbionts". From an epistemological and historical point of view, "symbiote" is the correct term and not "symbiont" (Hertig *et al.*, 1937 – Steinhaus , 1949).

Symbiologists are sometimes not consistent, and the nomenclature changes according to authors. Here we propose to use the following terms to define different aspects of symbiosis:

Ecto or exosymbiosis: The partners remain external to each other, even if they are very intricately associated, as in lichens. In *ectosymbiosis* the smaller partner is an *ectosymbiote*.

Endosymbiosis: The smaller associates, *endosymbiotes*, are inside the host but remain extracellular. Most of the time *endosymbiotes* are in the digestive tract, or inside special organs.

Endocytobiosis: Intracellular symbiosis as defined by Schwemmler (1980). The *endocytobiotes* can be temporarily extracellular if there is a migration phenomenon, for instance to invade ovaries.

Symbosome: Endocytobiotes are generally inside a host's vacuoles and the structure is termed a symbosome (Ahn *et al.*, 1990). Symbosomes must be considered as cell components. They contain one or more endocytobiotes (see the corresponding chapter in this book).

In endocytobiosis, most of the time, endocytobiotes are only harbored in special cells: *bacteriocytes* (inhabited with bacteria), *mycetocytes* (inhabited with yeast or fungi), *virocytes* (inhabited with viruses), *algocytes* (inhabited with algae or a plasts).

These cells are often aggregated and form organs called bacteriomes or mycetomes. Special organs for viruses are not yet known. Protozoa are often intracellular at some stages of their life cycles, notably *Sporozoa* and *Microspora* (Bannister, 1979). But they do not form a special organ and are generally pathogenic. Nevertheless their host cells could be called “*protistocytes*”.

In this chapter, we first show that symbiosis is not a biological curiosity but a common phenomenon in all biotopes on the earth. In the last years we observed a renewal of interest in this field. Second, we demonstrate the fact that symbiosis is a powerful factor for biodiversity, at the morphological level, inducing or favouring the formation of different structures playing a role in adaptation. As Moran and Telang (1998) stated “Endosymbiosis appears to facilitate diversification within ecological niches that would otherwise be inadequate”. Third, comparing different models, we try to put forward, beyond the morphological diversity, the presence of common features, laws, or mechanisms implicated in host-symbiote relationships. In this short review it is impossible to present all the models, and the reader is referred to recent publications for further details: Jeon (1983) - Werner (1992) - Margulis (1993) - Nardon and Grenier (1993) - Sato *et al.* (1993) - Douglas (1994) - Nardon and Nardon (1998) - Seckbach (1999) - Moran (2000).

2. The partners in ectosymbiosis

A large number of ectosymbiotic associations of various types have been described, involving protozoa, algae, fungi, plants, and animals.

2.1. ECTOSYMBIOSES INVOLVING FUNGI

2.1.1. Lichens

Lichenologists (Franck and De Bary) developed the concept of symbiosis in the 19th century. Lichen is an association between a fungus (= mycobiont) according to Werner (1992) and an alga (= photobiont) or a cyanobacterium. The two partners show different degrees of intimacy. In the less complicated lichens, like *Collema*, the alga and fungus are simply disposed side by side. In the most intricate lichens, the fungus may develop special devices, the haustoria, to penetrate the gonidia. In all cases the association constitutes the thallus (see Honegger, 1991).

The fungus generates spores that germinate and find an alga to form new lichen. *In vitro* assays show that the fungus alone may form a thallus, but, to be perfect, the presence of alga is necessary. What is intriguing is the fact that symbiosis creates a new structure, that is perfectly stable from one generation to the next, genetically and epigenetically controlled (the role of alga), probably thanks to signal exchanges between both partners.

We put forward two facts: First, the thallus, for a given association, has always the same morphology and cytology (Des Abbayes, 1963 – Werner, 1992). We can distinguish four types of lichens: crustose, leprose, foliose and fruticose. The crustose type (*Collema pulposum*) is flat, tightly fixed to the substrate, and with a homogeneous structure. The leprose type is a primitive crustose type with granular structure (*Lepraria*). The foliose type (*Parmelia*) presents four distinct layers: the upper cortex,

the photobiont layer, the mycobiont layer, and the lower cortex with the rhizines, the organs of fixation to the substrate. The fruticose type is represented by *Usnea longissima* showing a radial symmetry. Pendant on trees, it is the largest lichen with a length of several metres.

The second is the fact that in lichens both partners are modified (Galun, 1990). For instance, filamentous cyanobacteria in *Scytonema* are changed to a unicellular form without heterocytes (specialized cells fixing atmospheric nitrogen). There are also modifications in the cell wall (in *Trebouxia*). The plasticity of cyanobacteria has been well studied (even if the mechanism is yet unknown), and they are involved in numerous symbioses (see Bergman *et al.*, 1999).

2.1.2. Ectomycorrhizae

Ectomycorrhizae represent symbiotic associations between fungi and root plants, the fungus mycelium coating young roots. Hyphae can also penetrate into the roots, but they are always extracellular (with few exceptions), forming the Hartig net (Werner, 1992). The structure of ectomycorrhiza is essentially determined by the fungal species rather than by the host plant. The root cap and the meristem remain uninfected. In some plants, like *Pinus radiata*, the ectomycorrhizae do not induce morphological changes in the root. But in other trees, like *Betula*, modifications appear and establish a particularly close contact between partners. The number of root cells remain unchanged, but they swell, giving rise to a club-shaped form to the swollen root tips.

2.1.3. Other type of fungi/plants associations

A great number of plants have pathogenic and symbiotic relations with fungi (see Werner, 1992 – Anderson *et al.*, 1984 – Pirozynski and Hawksworth, 1988). An interesting model is that of grasses associated with Clavicipitaceous fungal endophytes. The hyphae penetrate grass tissues, and, depending on the species, become either a parasite, as *Claviceps*, which sterilizes the grass ovaries, or a symbiote, as *Balancia*, which produces alkaloids protecting the grass against herbivorous animals. In *Epichloe* infection, a stroma is formed with plant and fungal tissues. The mesophyll cells may be hypertrophied and then the stroma appears as an enlargement of the stalk.

2.1.4. Fungi/Insects ectosymbiosis

Fungi are not only frequently associated with plants but also with animals, especially in insects: Thus, some ants, termites, Coleoptera, and wood-wasps cultivate fungi and feed their larvae.

Despite the non-permanent contact between an insect and the fungus, some special devices can differentiate in the host. In the wood-boring Coleoptera (Scolytidae and Platypodidae) about 36 genera cultivate symbiotic fungi in galleries excavated by the mother (Francke-Grosmann, 1967 – Batra, 1967). The fungus is inseminated in the galleries by adult insects. What is curious is the fact that, in Scolytidae, the spores are transported inside depressions of the cuticle associated with setae (Levieux *et al.*, 1991), located on the elytra, on the mandibles or on the sides of pronotum. The Platypodidae have special fungus-carrying organs, the mycangia (Nakashima, 1975), which are cavities associated with glandular cells. They are present either in female or male, or in both sexes (Beaver, 1989; Haanstad and Norris, 1985).

Lymexilidae (Coleoptera) and wood-wasps (Hymenoptera) also possess mycangia. These mycangia are absent in non-symbiotic species, and they are considered as possible products of symbiosis. It is fantastic to imagine how these mycangia could have been formed, probably during coevolution of the partners.

Another case of ectosymbiosis with fungi is the association of leaf-cutting ants (*Acromyrmex*, *Atta*) with a cultured fungus: *Attamyces bromotificus* (Cherret *et al.*, 1989). This type of symbiosis is obligate for the two associates. The fungus is the food for larvae and adults. It has never been discovered outside attine nests. The queen transports the fungus in a special pocket when she establishes a new colony.

Other examples of ectosymbiosis are the fungus-growing termites (Noirot, 1980 – Wood and Thomas, 1989). In each nest of Macrotermiteinae there is a comb on which grows the specific fungus *Termitomyces*. We also find a *Xylaria*, but only in small quantities. The comb is mainly built from fresh fecal material. What is curious is the characteristic form of the comb, unique to each species of termite.

2.2. ECTOSYMBIOSIS IN ANTS/PLANTS

In the tropical zones, several species of ants live in tight association with trees like *Acacia* or epiphytes (Jolivet, 1986). Some special devices can differentiate in host plants, as domacia [Figure 1]. Domacia are specific structures inhabited by ants when they are not at work. It is not known if these domacia were preexisting to ants or not, but they are a “symbiotic organ”, useful both to the tree (a water reserve) and to the insect (a shelter).

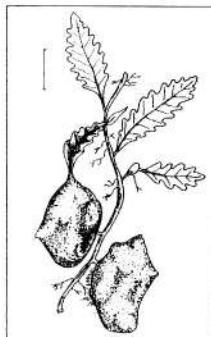


Figure 1 Domacia on the fern-ant epiphyte *Soleno-pteris* sp. The ants stay inside the modified rhizoma. Bar = 2 cm. (from Rauh 1955, with permission of *Annals Soc. Entomol. Fr.*)

2.3. ECTOSYMBIOSES INVOLVING PROTOZOAN OR ALGAE

In some biotopes such as benthos or tropical planktonic communities, the silica walls of diatoms are colonized by various ectosymbionts or epibionts: cyanobacteria, dinoflagellates, ciliates and even diatoms (as *Nitzschia*). Furthermore some ciliate species also possess ectosymbionts, like diatoms. We may also have dinoflagellates fixed upon foraminiferan. All combinations seem possible (Taylor, 1982). The specificity is not so great, and we may suppose weak interactions between host and ectosymbionts.

In one particular biotope, the intestine of termites, and particularly in the paunch of the hindgut, some endosymbiotic protozoan, like *Joenia annectens*, are covered with bacterial ectosymbionts, (Radek *et al.*, 1992 – 1996 ; see also Ohkuma in this book). *Mixotricha paradoxa* is a flagellate in the hindgut of *Mastodermes darwinensis*. It possesses a large number of bacterial ectosymbionts which are small spirochetes (about 500,000) and less numerous large spirochetes. At the base of spirochetes, also attached to the host cell, are short rod-shaped bacteria (Cleveland and Grimstone, 1964 - *in Margulis*, 1993). It must be emphasized that the attachment of these ectosymbionts is not random but they form regular rows and their undulations are coordinated, so that they play a crucial role in the protozoan motility. In a devescovinid flagellate from a Florida termite, two bacterial ectosymbionts are also regularly attached on the surface (Tamm; 1980). Freeze-fracture and thin-section electron microscopy show that surface specializations in both partners occur at the junctional complexes. Rod bacteria lie in pockets of the eukaryotic membrane which are coated by dense material and contain numerous intramembrane particles. Fusiform bacteria are attached along ridges of the flagellate surface. The surface of the rod bacteria in contact with the medium bears a thick glycocalyx and flagella. We have an architecture. Who is the architect? The control of junction assembly by the cortical bacteria is probable, but how does it work? In *Joenia*, removal of the ectosymbionts with antibiotics, shows that the specialized contact sites are present in the absence of bacteria. But nothing remains of the former attachment sites in axenic *Devescovina* cells. In this case, these sites are perhaps induced by bacterial contact. But there does not seem to exist a general mechanism for bacterial attachment in these symbiotic associations (Radek *et al.*, 1996).

2.4. ECTOSYMBIOSE IN INVERTEBRATES

The pompeii worm *Alvinella pompejana* is an Annelid living in deep-sea hydrothermal vents (Gaill *et al.*, 1987 – Cary *et al.*, 1997). The worms are in a tube, and do not seem to have intracellular bacteria, but the teguments of the worm are covered with microorganisms of various types belonging to the **ε -proteobacteria** group. Four types are visible on the dorsal part: (a) rod-shaped bacteria linked to the cuticle of the worm by thin filaments; (b) small spiral-curved bacteria, (c) bacteria with appendages, and (d) filamentous bacteria, which are most abundant. In *A. pompejana* the role of the bacterial community has not yet been elucidated and no specific morphological changes have been described.

3. The partners in endosymbiosis

The intestinal symbioses are probably the most widespread endosymbioses in animals. We may also have associations in special organs and diverse others forms of symbiosis with prokaryotes or eukaryotes. The endosymbionts remain extracellular.

3.1. SYMBIOSSES IN THE INTESTINE

We shall limit our discussion to termites and to mammals that are phylogenetically very distinct.

3.1.1. Mammals

From the point of view of symbiosis, we can distinguish two groups of mammals: monogastrics and ruminants.

Monogastric mammals. They are nonruminants, such as humans and rodents. According to Savage (1977), a normal human organism is composed of over 10^{14} cells, of which only about 10 % being animal cells. What we call a human, at least from a zoological point of view, is really a consortium of more than 300 different species. The associated microorganisms are principally bacteria living in the gastrointestinal tract (Ducluzeau and Raibaud, 1979), or yeasts. We cannot describe this complex microecosystem in detail here, but we emphasize that it is not a uniform biotope. The physical, chemical and cytological characteristics change all along the digestive tract, and the microbiota also changes: in mouse, short bacilli (1-2 μm) are attached on the stomachal epithelium, whereas in the duodenum there are filamentous (more than 8 μm long) bacteria. Different niches exist and 40 % of the wet weight of fresh feces of the mouse is from bacteria.

Endosymbiontes are in the lumen or in the alimentary bolus, or are attached to the gut epithelium. Some long bacteria are unique to the small bowel of mammals and have never been found elsewhere. Comparison of holoxenic normal intestine with axenic ones (deprived of endosymbiontes) shows that microorganisms can modify the appearance of the digestive tract. In the ileum of axenic rats, enterocytes have microvilli longer than those in axenic rats. Furthermore the intestinal wall of an axenic rat is thinner and weaker than the normal wall, and the number of mucous cells is greater in the xenic epithelium. Another difference concerns the Peyer organs, which are atrophied in the absence of symbiontes. These organ play an important role in immunodefence of an organism (secretion of immunoglobulines).

Ruminants mammals. They are polygastric, and the anterior intestinal tract is greatly modified to facilitate symbiosis [figure 2]. The principal compartment is the rumen. It reaches 250 l in the cow and represents 70-75 % of all the intestinal volume. The rumen is probably the most complex of all the known biotopes with a temperature of 40°C, pH 6.7 and a redox potential of -350 mV under anaerobiosis conditions. The bacterial endosymbionte population represents more than 200 species, among which about 30 are specific to the rumen. Most are strict anaerobic bacteria and are attached to the mucous epithelium and the vegetal particles. Their number is estimated to be 10^{10} cells/ml of rumen content. Other endosymbiontes are protozoa, essentially ciliates (10^4 to 10^6 cells/ml) which can ingest solid particles. Finally fungi are less numerous (10^3 to 10^7 spores/ml) (Hespell *et al.*, 1997).

3.1.2. Termites

All the termite species are not associated with fungi. Most of them live in symbiosis with bacteria and/or protozoa, located in their digestive tract. More recently, in lower termites, yeasts have also been found (Prillinger *et al.*, 1996). The hindgut is well developed and forms a paunch containing both bacteria and protozoa in the lower termites, which are wood-eating insects. But only bacteria are found in the higher termites (*Termitidae*), which are humivorous (Noirot, 1992). The density of symbiotic flagellates ranges from 10^5 to 10^7 cells per ml of gut fluid (To *et al.*, 1980) but it may reach $10^{10}/\text{ml}$ (Breznak, 1982-1984). These bacteria are either free in the gut lumen, or attached to the alimentary bolus or the gut wall, or associated with flagellates (see

Mixotricha). In this book, Ohkuma describes several methanogen species; they show a distinct spatial distribution in the gut. In the termite *Procubitermes aburiensis*, bacteria of the hindgut are attached to specific cuticular spines elaborated from the gut wall. Each spine is linked to a specialized root cell which exhibits a bundle of parallel microtubules extending from the apex to the base of the cell (Bignell *et al.*, 1980). It can be assimilated to a symbiotic organ, but we do not know how it has been formed.

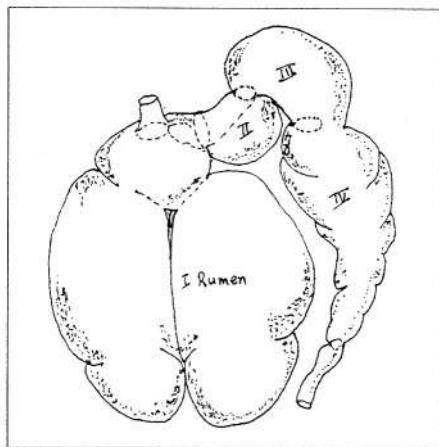


Figure 2. Anterior intestine of a ruminant. II: reticulum, III: bomasum, IV: abomasum (the primitive stomach).

3.2. LUMINOUS ORGANS

The luminous organs are present in various animals: marine fishes, squids, crustaceans, nematodes and pyrosomes. The luminescence is due to the presence of bacteria (at least 10^7 cells/ml) which remain extracellular. We have chosen the squid model, *Euprymna scolopes*, colonized with highly luminous and specific bacteria: *Vibrio fisheri* (McFall-Ngai and Ruby, 1991, 1998). As described by the authors, "This symbiosis offers unique opportunities to understand the processes underlying the colonization of animal epithelia by benign bacteria".

The complex light-emitting organ is located in the center of the mantle cavity. In the adult *Euprymna* the organ is composed of two lobes with three distinct epithelium-lined crypts that house the *Vibrio*. As an accessory tissue there is a thick reflector which directs luminescence ventrally (Ruby, 1996).

The *Vibrio* is not heritable and the infection from the environment must be repeated for each generation. A mechanism of recognition is probably present to ensure the specificity of the association. At hatching, the organ bears two lateral pairs of appendages that facilitate the capture of *Vibrio* (Doino and McFall-Ngai, 1995). The crypts are connected by a pore to the surface of the organ. Bacteria enter the pores and reach the crypts where they are first attached to microvilli. They multiply and finally fill the space entirely (McFall-Ngai, 1999). Each day, at dawn, the host expels 90-95 % of its bacteria in the seawater. Being established, symbiosis induces modifications in both

the host and the endosymbiont (McFall-Ngai, 1999). The latter undergoes several changes in its morphology and physiology. Between 12 and 24 h after the initiation of symbiosis, the *Vibrio* loses the polar flagella characteristic of free *Vibrio* in the surrounding seawater. Furthermore, the endosymbiont decreases about 8 fold in volume and is more fluorescent.

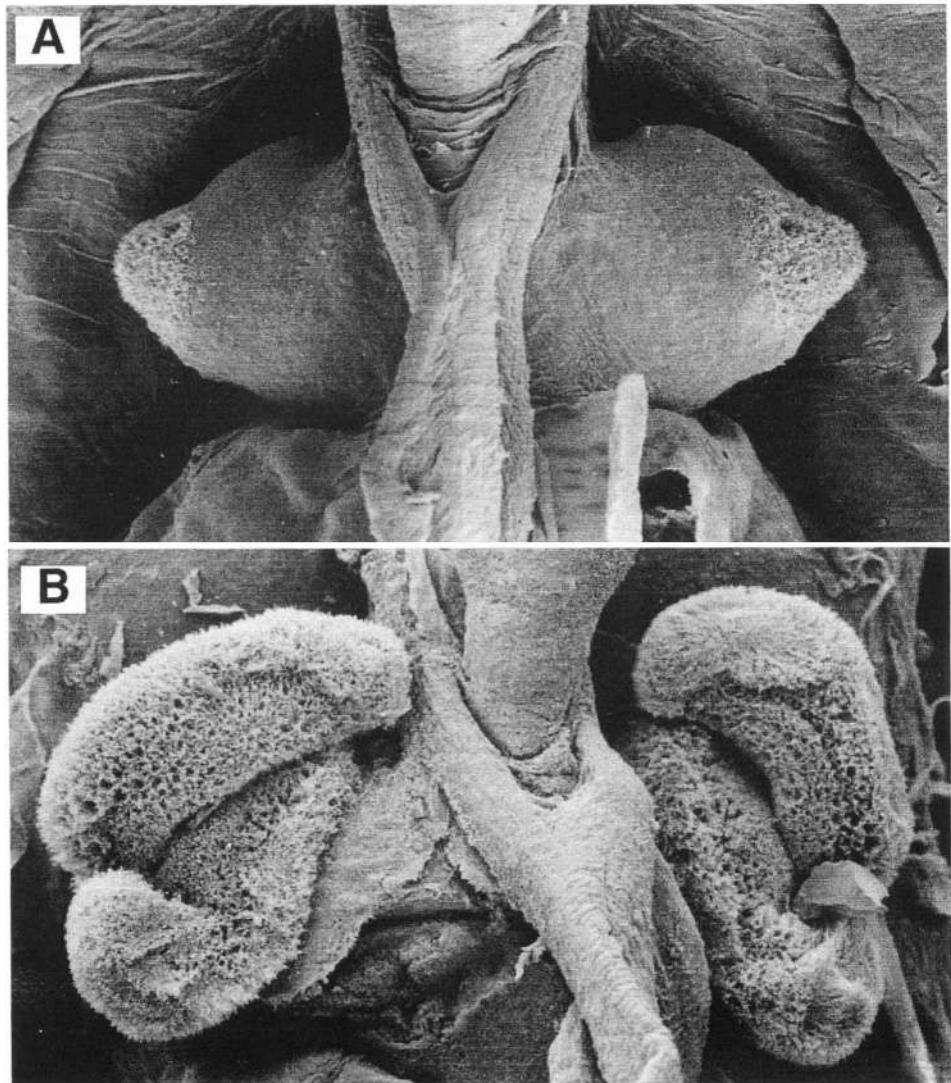


Figure 3. light organ of the squid *Euprymna scolopes*. A: ciliated and microvillous field on each lateral face of the light organ in 4-days old symbiotic animals. B: the same at hatching in absence of *Vibrio*.

The squid also shows important changes in the light organ, triggered by the bacteria. The structures elaborated before infection regress over a period of 4 days if the colonization is successful. This is particularly spectacular for the lateral appendages [figures 3A and 3B]. In uninfected squids these structures are retained. We have here an example which strongly suggests that symbionts interfere with the normal program of development of a juvenile host. Only 12 hours of contact is necessary to induce cell death and tissue regressions of the external ciliated epithelium (McFall-Ngai, 1998). The epithelial cells in the crypts do not undergo cell death but exhibit a fourfold increase in volume over the first few days after colonization. The microvillar density also increases fourfold.

3.3. OTHER TYPES OF ENDOSYMBIOSIS

Endosymbiosis in plants does not seem to be a general rule, but in animals no invertebrate taxon appears to be entirely symbiont-free. For instance, symbioses between eukaryotes and bacteria are known in protozoans, sponges, cnidarians, nematodes, turbellarians, vestimentifera, molluscs, and arthropods. Here, we shall consider only the insect galls of plants, the echinoderm and sponge endosymbioses.

3.3.1. Insect galls

Some mite species induce the formation of plant galls but most of the time they are pathogenic, whereas insect galls are generally tolerated by the plants, if they are not too numerous. Among the gall-forming insects are cynipid Hymenoptera and cecidomyiid Diptera. The larvae are inside the gall, whose morphology is specific to the insect (Folliot, 1977) [figure 4]. Some aphids are also gall-forming insects. They live inside the gall whose morphology is specific: coral-shaped for *Tuberaphis taiwana* (Aoki and Kurosu, 1998), or as a spiral pouch for aphids living on the petiole of poplar leaves. The mechanism of gall induction is not yet fully elucidated; it is a fascinating problem of morphogenesis. In *Quercus* alone, more than 800 galls are known. The plant shows hyperplasia, hypertrophy and dedifferentiation of cells (Cambar, 1953 – Forrest, 1987 – Pirozynski, 1991).

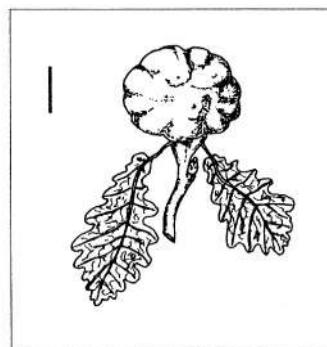


Figure 4. A gall induced on oak tree by cynipid larvae (*Biorhiza pallida*). Bar = 1 cm (from Folliot, 1977, with permission of *Annals Soc. Entomol. Fr.*).

3.3.2. Echinoderms

A study of Echinoderms in New Zealand and in British Isles, showed that among samples of 38 and 88 species, 17 and 63 species, respectively, harbored Gram-negative subcuticular bacteria (SCB). Their number is very important: about 10^9 cells/g ash-free dry weight of host tissue (Kelly *et al.*, 1995 – Kelly and McKenzie, 1995). The SCB can be classified into two major morphological types. One type lacks capsules and is usually spiral. It is most abundant. The other kind is rod-shaped, with capsules or vacuoles. The two types can be found together in the same host of some species.

3.3.3. Sponges

Some sponges are composed of 21% of animal cells, 38% of extracellular bacteria and 41% of intercellular substances (Bertrand and Vacelet, 1971). The study of symbiotic extracellular bacteria in four species of the sponge genus *Plakina* shows that the number and composition of bacterial types allow one to discriminate between species, and the pattern of distribution of bacteria may help to characterize the genus *Plakina*. The abundant bacteria are often grouped in zona devoid of collagen. Eight morphological types are described (Muricy *et al.*, 1999). Microorganisms living with sponges are diverse: heterotrophic bacteria, cyanobacteria, zoothorella, diatoms and even fungi. Specialized organs are not formed, despite the fact that these associations are probably very ancient. Furthermore, sponges can also possess endocytobiontes located in bacteriocytes (Vacelet, 1970). This “symbiocosm” is therefore very complex

4. The partners in endocytobiosis

In the preceding sections concerning ectosymbiosis and endosymbiosis, we tried to present evidence for both the biodiversity of symbiosis and the convergent phenomena. Endocytobiosis is more widespread and more diverse, only a few models will be described. Here again we will describe common processes at the cytological level, or the possible other great differences. Among the interesting traits to analyze, we have the nature of the partners, the location of endocytobiontes at cellular and organismic levels, their variability, their destruction, the structure of hosting organs (bacteriocytes, mycetocytes), and more generally all morphological consequences of symbiosis.

4.1 PROTOZOAN AS HOSTS

4.1.1. Bacteria

Most Protozoa live in symbiosis with bacteria of algae. *Amoeba proteus* is a wonderful model where the establishment of an endocytobiosis has been studied (Jeon, 1995). The amoeba was infected by pathogenic γ -proteobacteria in the first step, but, along the evolution of the association, the same pathogenic bacterium became not only harmless, but obligate for the amoeba, and this occurred during only one year of observation (Jeon, 1987). Therefore endocytobiosis is not necessarily a very old association, despite the fact that the bacteria appear to be very well integrated to the physiology of the host cell. About 42,000 bacteria are enclosed within host-generated vesicles, the symbiosomes (see corresponding chapter) (Ahn and Jeon, 1979). In this model, the physiological influence of endocytobiontes is crucial (Jeon, 1983), but the morphological

changes are very discrete (Jeon and Lorch, 1967), perhaps because the symbiosis is very recent. The infected amoebae become smaller in size and yellowish in color.

The ciliate hypotrich *Euplotes* may have numerous endocytobiontes, harmful, neutral or beneficial. *Omikron* is a Gram-negative, obligate bacterium. The curved bacilli are spread into the cytoplasm (Heckmann, 1983 – Görtz and Brigge, 1998). Another ciliate. *Cyclidium porcatum* exhibits a unique tripartite structure consisting of hydrogenosomes mixed with methanogene archaeabacteria and eubacteria (Esteban *et al.*, 1993). This structure is stable, indicating that the ciliate is an anaerobic consortium of three different functional partners.

In the above species and in many others, symbiotic bacteria are located in the cytoplasm, generally in symbiosomes. But, in other examples, curiously, the endocytobionte invades the nucleus. Many are parasites, but *Holospora obtusa*, which belongs to the α sub-group of proteobacteria, is rather neutral for its host, the ciliate *Paramecium caudatum*. In the host macronucleus the symbiont is not surrounded by a membrane. It does not infect the micronucleus, and has a peculiar life-cycle (Görtz, 1983 - Dohra and Fujishima, 1993 – Kawai and Fujishima, 2000). The infectious form (13 μm long) penetrates into the ciliate cytoplasme by ingestion in a digestive vacuole. Then, the bacterium leaves the vacuole and is transported within the macronucleus for *H. obtusa*, or the micronucleus for *H. elegans* (Maier *et al.*, 1990), where they divide to be transformed in short bacilli (1.2 μm long). The latter no longer divide and elongate to differentiate into the infectious long form. Therefore we note the great morphological plasticity of these endocytobiontes.

The colonization of nuclei is also observed in other protozoan species. Görtz (1983-1986) proposes the term “endonucleobiosis” to designate this fact. The invaders are not all bacteria. In Ciliate nuclei, parasitic algae may exist, or other protozoa like flagellates in *Stentor* where they induce a strong hypertrophy of the nucleus. More generally, endonuclear symbiontes cause morphological changes in infected nuclei: shape, size and location within the cell, but the effects depend on the host. Both endonuclear and cytoplasmic symbiontes may coexist, for instance in *Paramecium bursaria*.

4.1.2. Plastidic protozoa and algae symbioses

Plastidic protozoa is the name proposed for protozoa harboring functional plastids (=chloroplasts) in their cytoplasm (Laval-Peuto, 1992). This phenomenon is widespread and the plastids come from various classes of algae. In foraminifera they mainly arise from diatoms or chlorophytes. In the cytoplasm, symbiotic plastids are located at the cell periphery and their number varies. In the large oligotrich ciliates there are several hundred plastids per cell (more than 300 in *Tontonia appendiculariformis*). Three membranes generally enclose the plastids. The outer one, or periplastidial, is thought to be a product of the ciliate-host, which protects the plastids from digestion.

The association between the ciliate *Mesodinium rubrum* and chloroplasts of Cryptomonad is one of the most integrated symbiosis. Each ciliate harbors 10 to 100 chloroplasts responsible for the maroon color. It also exhibits some modifications, interpreted as adaptations to the presence of the chloroplasts: the oral cone is reduced, the “mouth” is vestigial and the oral tentacles are lacking, as compared to the closely related non-symbiotic species. Furthermore, the ciliate swims with the oral end posterior and has a phototactic behavior (Taylor, 1982).

We have to distinguish between the presence of plastids, always limited in time, and true symbiosis with algae. Algae are able to reproduce and to be maintained in the strain.

Paramecium bursaria normally live symbiotically with several hundred intracellular *Chlorella* (Karakashian, 1975). They divide and are hereditary. The algae are within individual vacuoles. They divide at a rate always compatible with the *Paramecium* growth.

Some algal endocytobiontes show little or no apparent morphological adaptations to their associations. But others exhibit modifications from their close relatives, such as reduction of wall structure and loss of flagella (in Foraminifera).

4.2. MARINE AND FRESHWATER INVERTEBRATES AS HOSTS

4.2.1. Prokaryotes as endocytobiontes

Sponges. In the sponges a great number of bacterial endosymbiontes are present, outside the cells (see above). But there are also bacteriocytes with endocytobiontes as in *Verongia* (Vacelet, 1970). These bacteriocytes are large cells (10 to 32 μm in diameter) compared to other cells of the sponge (5 to 13 μm). The number of bacteriocytes is variable. They are not localized anywhere, but preferably near the surface of the animal and esculum. In another sponge, *Petrosia ficiformis*, cytobiontes are in large membrane-bounded vacuoles. Several types of microorganisms are seen (Bigliardi *et al.*, 1993). In a symbiosis with cyanobacteria, Sara *et al.* (1998) show that the sponge presents changes in its morphology due to the cyanobacterium: larger size, modified shape and surface skeleton and decreasing number of pores. Perhaps these changes result from a better adaptation to photosynthesis. Intracellular endocytobiontes are present in some species (Vacelet, 1970).

Echinoderms. In echinoderms, we may have the same situation as in sponges: the presence of bacteriocytes in supplement of the numerous extracellular endosymbiontes. An example is *Ophiocoma ballensi* (Ophiolepid) which possesses bacteriocytes within the connective tissue of the tube feet (Kelly *et al.*, 1995).

Bivalve molluscs. Bivalve molluscs living in deep-sea hydrothermal vents, possess endocytobiotic chemolithotrophic bacteria. The two principal models are Vesicomyid clams *Calyptogena* and the Mytilid *Bathymodiolus* (Nelson and Fisher, 1995). Such symbiosis is obligate. The bacteria are inside vacuoles which can be interconnected (Fiala-Médioni *et al.*, 1990). They are highly integrated and colonize the gill cells. A study of *Calyptogena magnifica* shows that most of the gill tissue is composed of bacteriocytes. The endocytobiontes never invade all the cells but are located under the ciliated zone. The external surface of each bacteriocyte is fringed by well-developed microvilli. The Gram-negative bacteria are rod-shaped and often grouped in clusters limited by a double membrane (Fiala-Médioni and Métivier, 1986). A closer observation allows the distinction of two types of associations.

First, in the mytilid *Bathymodiolus thermophilus*, bacteria only colonize the apical part of cells, which are not modified in their basal area. Second, in Vesicomyids *Calyptogena magnifica*, *C. phaseoliformis* and *C. laubieri*, host cells are completely transformed into bacteriocytes. In both types lipid inclusions and numerous lysosomes are also present (Fiala-Médioni and Le Pennec, 1987).

The most remarkable characteristic of all mollusc species living on the sites of deep hydrothermal vents of the East Pacific Ridge is the well-developed gill which is the main organ of nutrition, via the endocytobiontes (Fiala-Médioni, 1988). This is particularly true for the vesicomyid *Calyptogena magnifica*, which has a reduced intestinal tract and reduced labial palps, and the absence of gut transit of particles. Furthermore the bacteriocytes and gill cells possess abundant microvilli in contact with the surrounding environment. Concerning the mytilid *Bathymodiolus*, the intestine is functional, with a transit of particles. The labial palps are well developed, but microvilli are reserved to particular cells. In this species the role of bacteriocytes seems to complete and not to replace the role of the gut.

Solemya borealis is a bivalve living in anoxic sediments and contains a high concentration of chemoautotrophic bacteria in the gill. They are Gram-negative and rod-shaped endocytobiontes. Bacteriocytes are confined to the region proximal to the ciliated edge of the gill, and are flanked by intercalary cells (symbiont free). The gill is extremely hypertrophied and lacks an intestine (Conway *et al.*, 1992). Despite the notable difference in the habitat, the symbiosis appears morphologically very close to that in *Calyptogena*.

Two mytilid species (Cavanaugh, 1993) harbor another type of endocytobiontes. Gram-negative coccoid to rod-shaped and metanotroph. These bacteria contain stacked intracytoplasmic membrane arrays. They are engulfed in vacuoles surrounded by a bacterial membrane. The bacteriocytes are interspersed with symbiont-free intercalary cells.

Concerning the biodiversity of symbiosis, the comparison between lucinid bivalves *Linga pensylvanica* and *Codakia orbicularis* is interesting since they both have the same sulfur-oxidizing bacteria, and they both live in the same tropical sea-grass beds. However, they exhibit different structures: absence of granule-cells in *Linga* while they are abundant in *Codakia*; numerous lysosomes in bacteriocytes of *Linga*, which do not exist in *Codakia*; complexity of the intercalary cells in *Linga* and different locations within bacteriocytes. The latter groups only occupy in *Codakia* the most superficial one third of the lateral zone, while in *Linga* bacteriocytes are located all along the lateral zone, intermingled with intercalary cells (Gros *et al.*, 1996).

The gastropod *Ifremeria nautilaei* lives in hydrothermal vent and possesses symbiotic chemoautotrophic bacteria in bacteriocytes (30 to 50 μm long) which comprise the majority of cells in the long ctenidial filaments. Each endocytobionte is in a vacuole, all vacuoles seem to be interconnected. As in bivalves, the host's stomach is reduced in size. From these different examples, it seems that the reduced intestine is an adaptation to the environment and especially to the presence of endocytobiontes (Windoffer and Giere, 1997).

Vestimentiferan worm Riftia pachyptila. This vestimentiferan is probably the most extensively studied animals living in deep-sea vents and seeps. It is a long worm (2 m or more), and its anterior end, the obturacular region, has a highly vascularized organ, the branchial plume, which is equivalent to a gill. The remainder of the worm is protected by a thick walled tube. What is unusual about this worm is that it has no mouth, no gut and no anus. However, it possesses an organ, the trophosome, which accounts for about 16 % of the animal's wet weight and consists of bacteriocytes, associated cells and blood vessels (Cavanaugh *et al.*, 1981 – Nelson and Fisher, 1995). The trophosome is also characterized by important sulphur quantities (Truchet *et al.*, 1998). The density of

bacteria is $10^{10} - 10^{11}$ cells per gram. The properties of bacteriocytes change during the time of association with the bacteria that are finally lysed, liberating nutritive metabolites for the worm (Bosh and Grassé, 1984 a and b). Symbiosis has completely modified the structures, and therefore the modalities of nutrition, probably by a coadaptation process. This is one of the best examples of morphological innovation induced by symbiosis.

4.2.2. Algae or plants as endocytobiontes

Molluscs. Several mollusc species harbor plastids or algae as endocytobiontes. The marine mollusc *Elysia viridis* lives in symbiosis with chloroplasts of the alga *Codium fragile*. These algae are located in digestive cells, in vacuoles bounded by a host membrane which persists throughout (Hawes, 1979).

Coelenterates. The best-studied species of Coelenterate is the freshwater *Hydra viridis* (Muscatine *et al.*, 1975 a and b – Muscatine and McNeil, 1989 – Rahat, 1990, 1992). In nature they harbor endocellular algae (*Chlorella*) inside digestive cells, the cause of their green coloration. The brown hydra has no algae. When symbiotic, each digestive cell harbors about 12-20 *Chlorellae* in individual vacuoles. They are preferentially located at the base of host cells. The green algae are not directly captured by *Hydra*, but they have to be ingested by small crustaceans which are preys for *Hydra*. So, the algae are primarily filtered by these crustaceans which introduce the algae in the coelenteron where they are phagocytized by digestive cells of *Hydra*. Some algae are digested rapidly, or after some weeks, while others survive and reproduce. The reasons for such differences are not yet well understood. It seems that the regulation of algae is made at the endodermal level. Rahat (1990) introduced the notion of “preadaptation”. Controlled by the host, *Chlorella* becomes heritable and a stable symbiosis is thus established. In general, in algal-invertebrate symbiosis, the wall of algae may be more or less modified. But in the *Hydra/Chlorella* symbiosis, no significant change has been observed.

4.3. PLANTS AS HOSTS

Despite their autotrophy, many plants are associated with bacteria or fungi, thus improving their nutrition.

4.3.1. Endomycorrhizae

In contrast to ectomycorrhizae, in endomycorrhizae hyphae of the symbiotic fungus penetrate into the root cells where they differentiate as vesicles and arbuscules mycorrhizae (VAM). This allows an increased surface of contact between the two partners (Meyer, 1967). When the VAM penetrate cells, the host plasmalemma is never ruptured and surrounds all branches of the arbuscule. There is no direct contact with the cytoplasm. The penetration of the fungus is marked by a considerable increase (23 fold) in the amount of cytoplasm and a regression of the vacuole typical of plant cells. The nucleus swells up and the chromatin is less condensed. Regression of the golgi apparatus is also observed. When the arbuscule degenerates, the cytoplasm decreases (Dexheimer and Gerard, 1990 – Berta *et al.*, 1990). It seems evident that these structures (vesicles and arbuscules) facilitate the exchange of metabolites. Finally, the VAM stimulate the production of adventitious roots (Scannerini and Bonfante-Fasolo, 1990).

4.3.2. *Rhizobium* symbioses

Rhizobium is a soil bacterium, capable of triggering a root modification in legumes, that leads to the formation of a nodule. This is the best-known model of symbiosis, where a lot of signals are exchanged between the partners, and the controlling genes have been identified. Not only *Rhizobium*, but also related bacteria of the soil, like *Bradyrhizobium* and *Azorhizobium* (*Rhizobia*), may elicit the formation of specialized organs in which symbiotic bacteria are able to convert atmospheric nitrogen into ammonia as a nitrogen source. Nodule formation is a multistep process (Van Rhijn and Vanderleyden, 1995 – Schauer et al., 1999 – Oke and Long, 1999):

- *Rhizobia* move toward roots by positive chemotaxis to plant root exudates (amino acids, flavonoides...)
- Bacteria attach to the root surface, but only on young growing root hairs. The specificity is not strict, since *R. leguminosarum* may infect *Pisum*, *Vicia*, *Lathyrus* and *Lens* species. But *R. loti* is limited to *Lotus*. The adherence of *Rhizobium* is mediated by lectins on the host and special polysaccharide molecules present on the bacterial cell surface.
- The attachment of *Rhizobia* on young root hairs triggers characteristic deforming and curling via Nod Factors (lipochitin oligosaccharides), secreted by *Rhizobia*, under the control of nodulation genes.
- The young curled root hairs entrap bacteria in a pocket where a local lesion is formed by hydrolysis of the cell wall.
- *Rhizobium* enters roots by invagination of the plasma membrane.
- The bacterial infection induces a reaction of the plant, that forms a growing tube with cell wall material: the infection thread which is filled with growing bacteria surrounded by a mucopolysaccharide matrix.
- The infection thread carries bacteria from roots to the primordial nodules, that are formed in the root cortex where cell division is induced. The location of the nodule primordium depends on the plant species. It functions as a meristem.
- Cells differentiate and the primordium gives rise to the mature nodule, which consists of two types of tissues, the peripheral and central tissues. Four zones can be distinguished: meristem, invasion zone, infected zone and degenerative zone. The bacteria are released in the invasion zone, in cells which elongate and increase in size while bacteria proliferate.

Not only the plant but also the endocytobiontes are modified by symbiosis. When they are delivered in the nodule cells, inside vacuoles, they there are transformed into bacteroids, the form capable of fixing atmospheric nitrogen. From rod-shaped they become y-shaped bacteria. The volume increases 4 - 7 times, and they no longer divide. Numerous inclusions appear in bacteroids. Their location is perfectly controlled by the plant, and they are never found either in meristematic cells, or in the vascular zone. In this example the induction phenomena are spectacular, as in the luminous organ of the squid.

4.3.3. Other symbioses

Sesbania rostrata is a tropical legume plant which possesses nodules both on roots and on stems. The endocytobionte is a *Rhizobium* and the nitrogen fixation is very active.

Frankia is an actinomycete bacterium which can live in symbiosis with non-legume plants, where they induce the formation of nodules. They penetrate into curved root

hairs, and form infectious filaments, that penetrate some cells of the cortex where they become ramified and entangled. In *Alnus*, vesicles are differentiated. This first primordial nodule is transformed in mature nodule with the formation of new lateral root hairs.

The *Gunnera* are perennial rhizomatous plants with extremely long petioles (up to 2 m). They live in symbiosis with the cyanobacterium *Nostoc punctiforme* which is predominantly intracellular in all *Gunnera* species. They are located in the rhizomatous tissues (Meyer, 1967 – Osborne *et al.*, 1991). It seems that unusual red glands formed at the base of the first pair of cotyledons are the major site of entry for endocytobiontes. Later, such red glands are formed at the base of each petiole.

TABLE 1. Examples of different types of endocytobiosis in insects.

Symbionts	Host species	Location	References
Viruses			
S	<i>Drosophila simulans</i>	Epidermal cells	Louis, 1990
Polydnavirus	Parasitoid wasps	Oviduc calyx	Stoltz and Vinson, 1977
C	<i>Drosophila melanogaster</i>	Various tissues	DeBuron and Beckage, 1998
Bacteria			
Spiroplasma	<i>Antonina cravii</i>	Various tissues	Fukatsu and Nikoh, 2000
Flavobacterium	Cockroaches	Fat body	Bandi <i>et al.</i> , 1994
α -proteobacteria (<i>Wolbachia</i>)	<i>Callosobruchus chinensis</i>	Various tissues	Kondo <i>et al.</i> , 1999
"	<i>Nasonia</i>	Various tissues	Breeuwer and Werren, 1990
"	<i>Glossina sp.</i>	Various tissues	Chang <i>et al.</i> , 2000
γ - and β -proteobacteria	mealybugs	Bacteriome	Louis, 1967; Fukatsu and Nikoh, 2000
γ -proteobacteria	<i>Sitophilus</i>	Bacteriome around anterior gut	Nardon, 1971
"	<i>Camponotus</i>	Bacteriocytes in gut epithelium	Schröder <i>et al.</i> , 1996
"	<i>Cimex lectularius</i> (P)	2 bacteriomes, fat body	(a), Hypsa and Aksoy, 1997
"	Psyllid (P)	1 central bacteriome	Thao <i>et al.</i> , 2000
"	<i>Glossina</i> (P)	Bacteriome around ant. gut	Aksoy <i>et al.</i> , 2000
"	<i>Glossina</i> (S)	Midgut cells	Aksoy <i>et al.</i> , 2000
"	<i>Acyrtosiphon pisum</i> (P) (S)	Dispersed bacteriocytes in body cavity	Baumann <i>et al.</i> , 1997
Unknown bacteria			
"	<i>Euscelis plebejus</i>	Paired bacteriome in fat body	(b)
"	<i>Oryzaephilus surinamensis</i>	4 bacteriomes around midgut	(b)
"	Bostrichidae	Gut bacteriomes	(b)
"	<i>Lycus linearis</i>	Fat body	(b)
"	<i>Lagria hirta</i>	Dorsal bacteriomes	(b)
"	Chrysomelidae	Midgut ceca	Nardon and Grenier, 1989; (a)
"	<i>Coccotrypes dactyliperda</i>	4 Malpighian tubules	Buchner, 1965
"	<i>Lixus</i>	Gut evaginations	(a)
"	<i>Balaninus</i>	Posterior bacteriocytes	(a)
"	<i>Apion</i>	2 Malpighian tubules	(a)
"	<i>Aspidapion</i>	Midgut	(a)
Yeast			
Pyrenomycetes	planthoppers	Fat body	Noda <i>et al.</i> , 1995
Candida	Cerambicidae	Gut mycetome	(a)
Torulopsis	Anobiidae	Gut mycetome	Bismanis, 1976
"	<i>Astegopterix styraci</i>	Fat body	Fukatsu <i>et al.</i> , 1994
"	<i>Pimpla turionellae</i>	Various tissues	Middendorf & Ruthmann, 1984

(a): Buchner, 1965; (b): in Schwemmler and Gasner, 1989.

4.4. INSECTS AS HOSTS

Among animals, insects are the most-studied from the symbiosis point of view. At the present time, only some families seem devoid of endocytobiontes (for instance the Carabidae) (Buchner, 1965 - Nardon and Grenier, 1989 – Nardon and Nardon, 1998). The main types of partners of insects in symbiosis are viruses, yeasts and bacteria [briefly summarized in table 1].

4.4.1. Viruses as endocytobiontes

It is not usual to consider viruses as symbionts and there are some examples in insects where viruses can be non-pathogenic (Reisser, 1992) or useful, and heritable. Homoptera are frequently vectors of plant viruses, but they are not affected themselves. Some viruses can replicate in their vectors and can be vertically transmitted through the oocytes (Louis, 1990).

The S virus of *Drosophila simulans* (DSV) is a causative agent of some abnormalities such as the absence of bristles in the adult (Louis *et al.*, 1988).

Some parasitoid wasps (Hymenoptera Braconidae and Ichneumonidae) like *Cotesia congregata* carry virus particles (polydnavirus) that are injected into the host (a caterpillar of *Manduca sexta* for instance) with the eggs. Viral DNA sequences become integrated in the genome of the wasp. As a consequence, the host is immunosuppressed and the parasite is not killed (Beckage, 1998). The virus particles are formed in the ovarian calyx, between the ovary and the oviduct. The nuclei of calyx cells house virions. When the cells lyse, these virions are released into the calyx lumen, where the eggs are contaminated. Even if they are not very spectacular, the viruses of insects induce ultrastructural modifications in host cells, both in the nucleus (disappearance of nucleoli, rupture of nuclear envelope), and in hypertrophied infected cells with swollen endoplasmic reticulum (formation of annulate lamellae, enlargement and vacuolization of mitochondria) (De Buron and Beckage, 1998). These effects vary according to the pathogenicity of viruses.

4.4.2. Yeasts as endocytobiontes

They are principally known in Homoptera and in Coleoptera (for more details see Houk and Griffiths, 1980 - Nardon and Grenier, 1989). In planthoppers, mycetocytes are part of the fat body and harbor yeasts of the *Candida* genus (Eya *et al.*, 1989). In scales yeast-like endocytobiontes are described (Tremblay, 1997). In *Ceroplastes rusci* their number per insect is estimated to be 60,000 – 70,000. These yeast-like microorganisms can freely float in the hemolymph of the host and occasionally in fat cells. But in *Sphaerolecanium prunastri* and *Eulecanium tiliae*, endocytobiontes are localized in large and polyploid mycetocytes, which are probably transformed fat cells.

In Coleoptera yeasts are present in numerous species of Cerambycidae (*Candida*) and in Anobiidae (*Torulopsis*) (Bismanis, 1976). There is a close resemblance between these two families. Mycetomes are associated with the intestine which forms evaginations at the beginning of the larval midgut. The number of lobes depends on species. In these intestinal protrusions not all the cells are infected [figure 5A].

Non-infected epithelial cells have a brush border while mycetocytes are giant cells without microvilli. Their cytoplasm is filled with symbiotic yeasts enclosed in vacuoles, and some of them are expelled into the gut lumen, as in the cerambycids *Rhagium inquisitor* (Ekblon, 1931) and *Criocephalus rusticus* (Riba and Chararas, 1976). Therefore yeasts exhibit two phases, intracellular and extracellular, an intermediate situation between endocytobiosis and endosymbiosis. Inside mycetocytes yeasts can be located either at the apex (Ekblon, 1931) as in *Rhagium*, or at their base as in *Leptura rubra* (Heitz, 1927). At the end of larval growth mycetomes disintegrate and some yeasts penetrate the new imaginal epithelium and induce the formation of smaller adult mycetomes. In the female symbionts multiply in the midgut and invade the transmission devices of the ovipositor [figure 5B]. This is a strange adaptation to assume the transmission of yeasts to the progeny. When an egg is laid, the apparatus functions as a syringe and a drop containing yeasts is propelled on the egg. When the young larva emerges from the egg, it eats yeasts with the egg envelope and becomes infected. It is assumed that a long time was needed for such coadaptation occurred between these insects and yeasts.

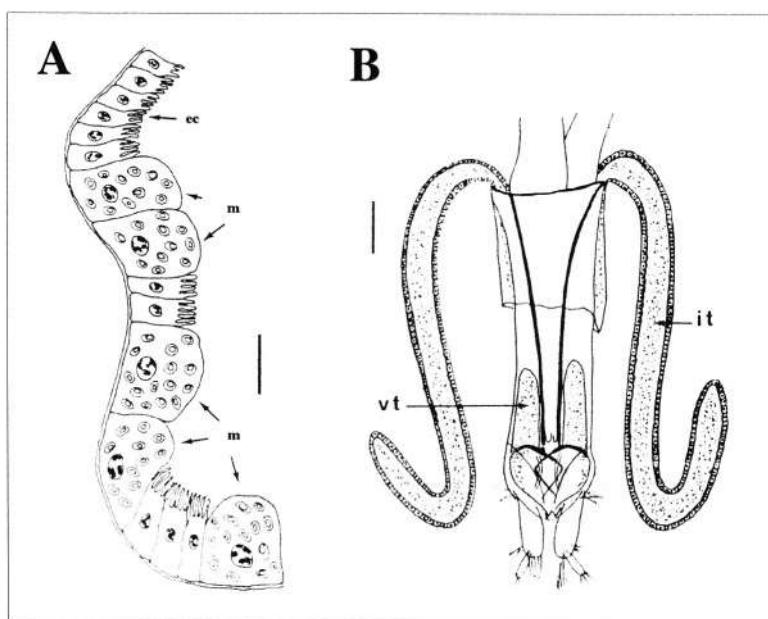


Figure 5. A: Schematic representation of the gut wall in *Stegobium paniceum*. m: mycetocyte, ec: non infected epithelial cells. Bar = 10 µm (from Buchner, 1965, with permission of Annals Soc. Entomol. Fr.). B: Egg-laying apparatus of *Stegobium striatum*. it: intersegmental tubules filled with symbiotic yeasts, vt: vaginal pockets. Bar = 100 µm (from Buchner, 1965, with permission of Annals Soc. Entomol. Fr.).

4.4.3. Bacteria as endocytobiontes

As shown in Figure 6, endocytobiotic bacteria of insects can be divided into five major groups: (1) the flavobacteria-bacteroid group which includes the cockroach and termite endocytobiontes; (2) the spiroplasma with only one representative (the endocytobionte of

the psyllid *Antonina crawii*); (3) the α -proteobacteria, a very homogeneous monophyletic group corresponding to the genus *Wolbachia* (Werren, 1997); (4) the β -proteobacteria (mealybug endocytobiontes); (5) the highly diversified polyphyletic group of γ -proteobacteria. From another point of view, Nardon and Grenier (1993) distinguished two main types of endocytobiontes. The first group is represented by the α -proteobacterium *Wolbachia* (associated symbiont), and the second is composed of integrated symbionts.

Associated bacteria: *Wolbachia* and others. There is now a huge bibliography concerning *Wolbachia* (see in the present book). *Wolbachia* has been found not only in numerous insects (whose number is increasing), but also in other arthropods and invertebrates (like nematodes). Symbiotic (or parasitic) status of *Wolbachia* is still controversial mainly because it is not completely "domesticated" by the host. Furthermore, the precise location in the host or in the infected cells is not clearly established. As an example, in the coleopteran *Callosobruchus sinensis*, *Wolbachia* has been detected by PCR from all tissues and organs: fat body, gut, muscle, ovary, wing, leg, head and antenna (Kondo *et al.*, 1999). In this insect, all tested individuals from 6 different populations of central Japan were infected. However, it is not always the case and very often, all the populations are not infected, and, in an infected population, all the individuals are not contaminated. This is the case in the weevil *Sitophilus oryzae* (Heddi *et al.*, 1999). Maillet (1971) studied several species of Auchenorrhynques (Homoptera) harboring Rickettsiae (α group of *Wolbachia*). He described that the bacteria may be free in the cytoplasm or included in vacuoles. They are located in the cytoplasm or in the nucleus, and also all tissues could be infected. In *Sitophilus oryzae*, *Wolbachia*, in some strains, cohabits with the principal endocytobionte within the bacteriocytes. This phenomenon is also found in tsetse fly *Glossina*, where *Wolbachia*, in some strains, cohabits with two other endocytobiontes: *Wigglesworthia* and *Sodalis* (Chenge *et al.*, 2000).

In *Glossina*, we observe different situations concerning their location: restricted to reproductive tissues in *G. brevipalpis* and *G. morsitans*, but also present in somatic tissues in *G. austeni*. The presence of *Wolbachia* in different populations of *Trichogramma* is highly variable (Pintureau *et al.*, 2000). This diversity is perhaps the consequence of an evolutive coadaptation. In infected *Trichogramma* (Hymenoptera), *Wolbachia* are concentrated at the posterior pole of the egg and are dispersed by embryonic movements. Finally they are present at a high density in the ovaries at the end of larval growth. The green rice leafhopper *Nephrotettix cincticeps* harbors three types of endocytobiontes: A and B types are located in bacteriomes and the bacteriocytes of ovarian pedicels, while *Rickettsia* has been found in almost all tissues, and principally in the nucleus (Mitsuhashi and Kono, 1975). The bamboo Pseudococcid *Antonina crawii* also harbors three types of endocytobiontes (Fukatsu and Nikoh, 2000). Two are identified as β and γ proteobacteria, and are found in the bacteriome. The third is very close to *Spiroplasma*. It is never in the bacteriome, but in various other tissues.

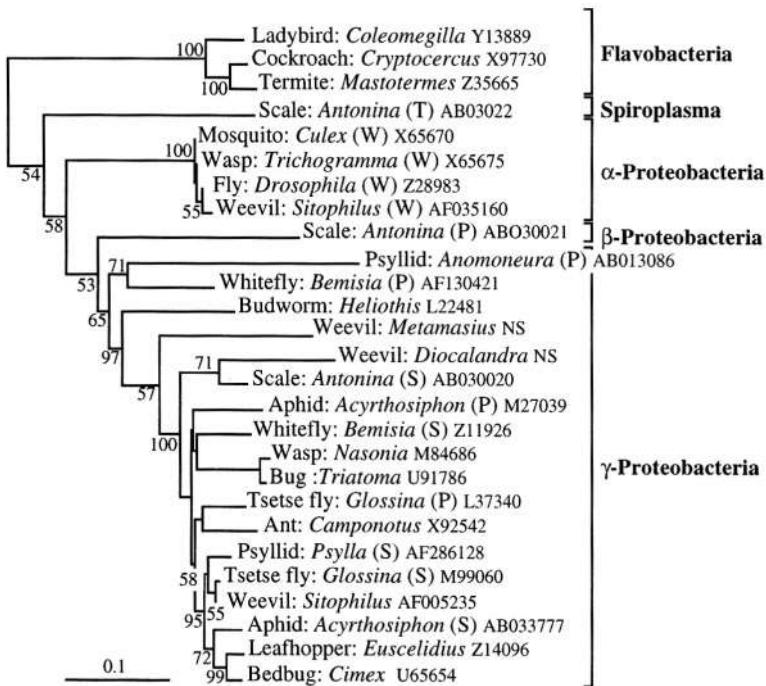


Figure 6. Neighbour-Joining phylogenetic tree of some insect endocytobionts based on 16S rDNA gene sequences and built with the distance of Galtier and Gouy (1995) accounting for unequal sequence base compositions. Endocytobiontes are presented with their host common name, followed by the host genus name and the Genbank accession number. P, S, T: primary, secondary and tertiary endocytobiontes. W: endocytobiontes of the *Wolbachia* group. NS: non submitted sequences (Lefevre, personnal communication).

To summarize, the location of these associated endocytobiontes is not precise (except perhaps in eggs for some of them), they do not induce the formation of special cells or devices and they are not always in all populations. Furthermore, in an infected population, they are not always found in all individuals. This indicates that they are not obligate and their influence on the fitness of their hosts, most of the time, is negligible. That is the reason why *Wolbachia*, according to the above authors, may be considered either as a parasite or an endocytobionte.

Integrated endocytobiontes: general characteristics. As seen above, most of the bacterial endocytobiontes are Gram-negative. Among exceptions in insects are the symbiotic bacteria of the human body louse *Pediculus humanus* (Eberle and McLean, 1983), and those of the *Ambrosia* beetle *Xyleborus ferrugineus* (Peleg and Norris, 1972) that have been described to be Gram-positive. We have proposed the term "integrated endocytobiontes" for such cytobiontes, since they are always present in the populations of a given species (in contrast to *Wolbachia*) and are completely dependent on the host for their growth. They are not cultivable *in vitro* (Nardon and Grenier 1993) and are obligate for the host. As rare exceptions, there are weevils *Sitophilus oryzae*, *S. zeamais* and *S. granarius* (Nardon, 1973 – Nardon and Grenier, 1988), in which natural and experimental populations can be devoid of symbiontes. The host is greatly affected but

are able to survive and to reproduce under certain conditions (Nardon, 1973). The comparison of symbiotic and aposymbiotic insects allows the study of morphological and physiological effects of the endocytobiontes. In the case of symbiose-free *Sitophilus oryzae*, the weevils have a smaller size and a lighter color.

The morphological aspect of endocytobiontes varies between the species, but this is not obligatory. For instance, bacteria of the weevils *Sitophilus oryzae* and *S. granarius* are very similar, being more or less long bacilli. But we may have a diversification according to geographical strains. In a strain of *S. oryzae* from Benin, symbiotic bacteria are always long ($10\text{-}30\text{ }\mu\text{m}$) as compared with another strain from Guadeloupe that do not exceed $10\text{-}15\text{ }\mu\text{m}$. Another example of variation is found in the whitefly *Bemisia tabaci* (Costa *et al.*, 1995). Insects from Florida, Arizona, and Hawaï (B biotype) have two morphological types of bacteria inside bacteriocytes (coccoid C1 and P, pleomorphic), while insects of A biotype from Arizona and Mexico contain three distinct morphological types: P, C1 and C2. The authors suggested a possible coevolution between the host and its endocytobiontes. The size of endocytobiontes may also change according to the physiological state: in the coleopteran *Oryzaephilus surinamensis*, symbiotic bacteria are the longest in the pupa ($60\text{-}70\text{ }\mu\text{m}$) and appear as short bacilli ($3\text{ to }6\text{ }\mu\text{m}$) in the ovaries of adult beetles (in Nardon and Grenier, 1989). Finally,, two close species like *S. oryzae* and *S. zeamais* (sibling species) are only easily distinguished by the morphology of their endocytobiontes, bacilliform in the first, and curved or spirals in the second [figure 7]. Concerning *S. zeamais* where the bacteria are so variable, the question arises: "Are these different forms the expression of a pleomorphy or the manifestation of the existence of several genetical types?" So far, in *S. zeamais*, only two types of endocytobiontes have been recorded (Dash, 1975 – Campbell *et al.*, 1992), but it is not known which type corresponds to which morphology. All insect endocytobiontes are not always flexuous bacilli. In aphids they are roundish, $3\text{-}6\text{ }\mu\text{m}$ in diameter, but in nurse cells of *Metamasis hemipterus* Y forms may be seen (Nardon *et al.*, 1985). It seems that these endocytobiontes are larger than their free-living relatives (Enterobacteriaceae for weevils and aphids). *E. coli* is generally $0.4\text{-}0.7\text{ }\mu\text{m}$ wide and $1.0\text{-}3.0\text{ }\mu\text{m}$ long, while the *S. oryzae* bacilli are $0.3\text{-}0.5\text{ }\mu\text{m}$ wide and $4.0\text{-}30.0\text{ }\mu\text{m}$ long (Nardon, 1971).

Ultrastructural studies show that most of the symbiotic proteobacteria are typically Gram-negative. Nevertheless, particular structures can be found. For instance, in the aphid *Rhopalosiphum padi*, the endocytobiontes contain paracrystalline components (Akhtar and Emben, 1994). In Homoptera various other bodies can be seen inside the endocytobiontes, like the electron-dense inclusions in the cytoplasm of the "t" symbionts in *Euscelis* or *Helochora* (Hook and Griffis, 1980 – Körner, 1978).

As described by Buchner (1965), the host evidently controls the density of its endocytobiontes. This has been demonstrated in the weevil *Sitophilus oryzae* (Nardon *et al.*, 1998), but the physiological mechanism is not known. One possibility is the destruction by lysosomes of supernumerary bacteria. This is sustained by lyse pictures (myelin-like structures) in bacteriocytes of insects and other invertebrates, such as *Sitophilus* (Nardon, 1971), aphids (Hinde, 1971a), or the whitefly *Bemisia tabaci* (Costa *et al.*, 1993).

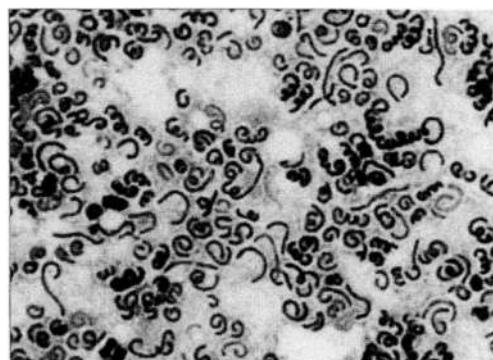


Figure 7. Endocytobiontes of *Sitophilus zeamais*. Squash of bacteriome. Methylene Blue. Bar = 10 µm (photograph by P. Nardon with permission of *Annals Soc. Entomol. Fr.*).

Location of integrated endocytobiontes in bacteriocytes. In insects, endocytobiontes are almost exclusively located in the cytoplasm of bacteriocytes. Nevertheless, some exceptions are known. In two termite species collected in the wild, *Kalotermes flavicollis* and *Reticulotermes lucifugus*, symbiotic bacteria were found both in the nucleus and the cytoplasm of female germ cells (Grandi *et al.*, 1997). We do not know if these observations can be extended to the whole species.

Endocytobiontes are generally included in host cell vacuoles (symbiosomes). Usually only one bacterium is inside a vacuole, but, occasionally, two or more symbiontes are seen in the same envelope, probably arising from recent divisions (Hinde, 1971b). How the endocytobiontes penetrate into bacteriocytes is not yet known, and a phagocytic process is generally believed to occur for the formation of symbiosomes. But the reality appears more complex, and from observations of several authors (Louis, 1980 – Hinde, 1971 – Daniel and Brooks, 1972 – Chang and Musgrave, 1972, 1973 – Grinyer and Musgrave, 1966 – Louis *et al.*, 1976), we can distinguish three kinds of host/endocytobionte interface: the most widespread is the symbosome formation. Sometimes bacteria lie free in the cytoplasm (and in nucleus) of the host cell. This is the case in the weevils *Sitophilus* (Nardon, 1971) and other Rhynchophorinae (Nardon *et al.*, 1985), and in the glossine midgut (Hübner and Davey, 1974). The “companion symbiontes” in the leafhopper *Helochara communis*, the “RC” symbiontes of *Cimex lectularius* are also among endocytobiontes not included inside vacuoles. Sometimes, a free endocytobionte is surrounded by a halo in fixed preparations (Nardon, 1971). This halo is not a true capsule and is interpreted as a reaction of the host cell (Louis, 1980). When an insect harbors several types of endocytobiontes (plurisymbiosis), as in *Cimex lectularius*, the mode of inclusion in the cell appears symbionte-specific since in the same host cell coexist the primary endocytobionte in symbiosomes and the secondary endocytobionte which is free in the cytoplasm (Chang, 1975; Hypsa and Aksoy, 1997). But in other cases the host can influence the location of symbiotic bacteria: the endocytobionte of *Glossina* would be free in the midgut cells, but encapsulated in the

ovaries (Hübner and Davey, 1974). The “t” symbiotes of *Euscelis plebejus* are free only in bacteriocytes (Louis *et al.*, 1976), not in the embryo (Körner, 1976).

In certain insects, like pseudococcines or *Pediculus*, endocytobiotes always form microcolonies inside large chambers. These “enclaves” are filled with a proteinaceous and mucous substance (Louis, 1967). Such “Mucous enclaves, as simple vacuoles, can be considered as a protection for bacteria in some circumstances, since they are destroyed when they escape in haemolymph, as in aphids (Hinde, 1971a) or in *Pseudococcus adonidum* (Louis, 1980).

It is to be noted that endocytobiotes are not always intracellular. In the weevil *Sitophilus*, they are always in the germ cells, except at the beginning of embryogenesis, when they are in the egg. In other insects, where the germinal line is not infected permanently, those bacteria must leave bacteriocytes to penetrate the ovaries. During this step, endocytobiotes are extracellular (for more details see reviews: Nardon, 1988; Nardon and Nardon, 1998).

Bacteriocytes, bacteriomes: structure and location. The host cells harboring bacterial endocytobiotes, bacteriocytes, are either dispersed in the body, especially in adipose tissue, or in intestine, or grouped to form bacteriomes. In the carpenter ants *Camponotus* bacteriocytes are interspersed between epithelial cells of the midgut. They are filled with rod-shaped Gram-negative **γ-proteobacteria**, lying free in the cytoplasm. They are also present in the ovaries (Shröder *et al.*, 1996). As for all insect endocytobioses, bacteria have no influence on the ovocyte morphology. On the contrary, bacteriocytes always appear as specialized and modified cells. Concerning the ant bacteriocytes they differ from normal epithelial cells by the absence of microvilli at their apical part. In cockroaches, bacteriocytes are disseminated among the fat body. They are relatively large, 256-512 ploid cells (Richards and Brooks, 1958; Sacchi and Grigolo, 1989). All aphids have an obligatory association with microorganisms, with the exception of phylloxerines. The endocytobiotes are generally bacteria. Yeasts have only been found in some cerataphidini, like *Astegopterix styraci* (Fukatsu and Ishikawa, 1992; Fukatsu *et al.*, 1994). The bacterial endocytobiotes are always within vacuoles. The bacteriocytes, singly or in small aggregates, are found beneath the intestine, within the abdomen of adult aphid, and around the gut in developing embryos. These bacteriocytes are huge cells, reaching 40-60 μm in diameter, and polyploid. They possess all cell organelles, and also vesicles and granular bodies. Microtubules and microfilaments have also been identified (Akhtar and Van Emden, 1994) in *Rhopalosiphum padi*. The cytoplasm is very rich in ribosomes and also contains a great number of vesicles which lie close to the symbosome membranes (Houk and Griffiths, 1980). In the pea aphid *Acyrthosiphon pisum* and in some other species, two different endocytobiotes are present (Unterman *et al.*, 1989). The most important is the cocciform primary symbiote (P), located inside typical bacteriocytes. The secondary symbiote (S) is smaller and bacilliform. It is located in cells which generally surround the bacteriocytes (Fukatsu *et al.*, 1998).

Many other insects are plurisymbiotic, permanently or occasionally. Furthermore the bacteriocytes, most of the time, are compacted to form symbiotic organs: the bacteriomes. The location of these bacteriomes seems to be under the control of the insect, since they are generally found in the same place for different species of a same genus or family, or sub-family. For instance, all the Rhynchophorinae examined so far, except *Sitophilus linearis* which is asymbiotic, possess a compact larval bacteriome at

the junction foregut/midgut (Nardon *et al.*, 1985; Nardon and Nardon, 1998). During metamorphosis, bacteriocytes migrate and invade the anterior mesenteric caeca of the adult, where they stay for about three weeks. Afterwards they disappear, so that in weevil more than three weeks old, the only symbionts in the female are those contained in oocytes, trophocytes and apical bacteriomes of the ovaries (Nardon, 1971; Nardon and Grenier, 1988). Endocytobiosis often differs between larvae and adults. The bacteriome must be considered as a new organelle, dependent on the physiology of the host, as other organelles, also remodulated during metamorphosis.

The bacteriomes are single as in *Sitophilus*, or paired. As in numerous species with one or two compact larval bacteriomes, the *Sitophilus* bacteriome is limited by a sheath of small flattened cells, and is composed of polypliod bacteriocytes, rich in ribosomes, and of small accessory cells. It possesses numerous tracheae. In *Rhizopertha dominica* and other Bostrichidae, the structure of paired bacteriomes is a little different since the center is occupied by a multinucleated syncitium. In *Lycus linearis*, paired bacteriomes lie close to the lateral lobes of the fat body, between midgut and gonads. It is formed of a total of 7 - 12 syncitia (50 to 100 μm) with irregular nuclei centrally arranged, and surrounded by 8 - 14 smaller ones with round nuclei. The syncitia are embedded in epithelial cells, and each type contains one endocytobiont (Gambetta, 1927). It would be interesting to verify this point with modern techniques.

In Homoptera, numerous types of plurisymbiosis are described. In psyllids the bacteriomes, deeply penetrated by tracheoles, lie between gonads and the alimentary canal. It is 500 to 700 μm long in *Psylla pyricola* (Chang and Musgrave, 1969). It is composed of a central syncitium, of peripheric bacteriocytes and of a cellular covering sheath. Two types of endocytobionts have been described in *Anomoneura mori*: X and Y (Waku and Endo, 1987), now identified as γ -proteobacteria, but belonging to different lineages (Fukatsu and Nikoh, 1998). The primary (P) symbionts are in the bacteriocytes, while the secondary (S) symbionts are in the syncitial region of the bacteriome (Spaulding and Von Dahlen, 1998; Thao *et al.*, 2000).

It is not possible, here, to present all aspects of endocytobiosis. Some insects, like *Laodelphax striatellus*, harbor both bacteria and yeasts (Noda *et al.*, 1979). According to Buchner (1965), 55% of the cicadas would be disymbiotic, 30.5% trisymbiotic, 4.2% tetrasymbiotic, 1.5% pentasymbiotic, 0.5% hexasymbiotic, with only 5.4% monosymbiotic and 2.5% without symbiont. Concerning the location of bacteriomes five main types may be distinguished: gut caeca, midgut, compact organs associated with the midgut, fat body and malpighian tubules.

5. Discussion and conclusion

De Bary (1879), in his definition of symbiosis, was already convinced that all degrees of intimacy existed between associated partners, and notably between mutualism and parasitism (see in Nardon and Grenier, 1993). It is sometimes difficult to trace a frontier, and we have seen that the bacterium like *Wolbachia*, may be considered either as a parasite or a symbiont, according to the host species and the author. One remarkable feature is that *Wolbachia* seems to have no morphological influence on the host cell. In his book, Combes (1995) proposed to replace the De Bary concept of symbiosis by the new concept of "durable interactions", in order to avoid some

confusion about the quality (useful or not) of the association. This is certainly a good idea from a genetic point of view, but difficult to assume physiologically. Symbiosis is not simply an interaction, but also an innovative mechanism (Margulis and Fester, 1991). In fact, it appears that this new concept is essentially adopted by the parasitologists. Why? Probably because the neoDarwinism puts forward the notion of conflict, more familiar to parasitologists than to symbiologists, whose approach is quite different, with the notion of complementarity between the partners. Even if we can observe a continuum between parasitism and mutualism, it is nonetheless evident that the two types of associations can be easily distinguished. The example of bacteria infecting *Amoeba proteus* is demonstrative since the same endocytobiontes pathogenic in a first step, became symbiotic after only 200 generations, and since the two situations were perfectly distinguishable. We have proposed (Nardon and Grenier, 1993) the concept of "symbiocosm" which represents the new biological entity created by symbiosis, and submitted to natural selection. The notion of symbiocosm supposes that different genomes interacting (nuclear, mitochondria] and / or plastidial, symbiotic) have reached an equilibrium. During the phase of adaptation, in some associations, the morphology of the partners are not, or seldom modified (contrarily to physiological ways): an example is the association *Hydra* / *Chlorella*. But, generally, in all types of symbiosis, almost all partners in the symbiocosm, have been modified for some morphological features. For instance, bacteriocytes and mycetocytes are hypertrophied and polyploid cells in all cases. In the squid, appendages disappear when symbiosis begins and the *Vibrio* loses its flagella. In *Rhizobium* legume symbiosis, the two partners are modified, and exchanges of signals are almost completely discovered. In this example it is the bacterium that directly induces the nodule formation. In *Sitophilus oryzae*, the larval bacteriome and apical bacteriomes of adult female ovaries disappear in aposymbiotic (=deprived of symbiontes) insects. This suggests that a signal from the symbiotic bacteria trigger the differentiation of bacteriomes. But we do not know if such a situation is general. It does not seem, since in *Oryzaephilus surinamensis* bacteriomes are also formed in the absence of bacteria through 25 generations (Koch. 1967)

The modification of the partner morphology can be spectacular and lead to the formation of complex structures, or symbiotic organs: galls, trophosome, luminous organs, lichens, mycangia... Some of these inycangia disappear in the absence of symbiontes. But such an inducing power is not always easy to verify. It is possible that in certain symbiosis, and especially in ectosymbiosis, some structures would be more ancient than the association and secondarily exploited. That could be the case in the ant/plant symbiosis, where domatia are not induced by ants. Rahat (1990) also evokes the concept of preadaptation to explain the association of algae with *Hydra*. It appears to us that the phenomenon of symbiosis is more frequent in invertebrates than in vertebrates. It allows surviving in various conditions, by complementarity. On the contrary, in the more complex vertebrates the parasitism appears more frequent than symbiosis, perhaps in relation to a better system of defence. These morphological structures of symbiosis are a good illustration of what R. Dawkins (1982) termed as the "extended phenotype", that is the possibility for a gene to be expressed outside the organism. These genes which control the symbiotic phenotype are necessary for the maintenance of the symbiocosm (symbiotic gene). They also create new structures. But we have tried to show that innovations and transformations have common

characteristics in all types of symbiosis. The biodiversity of symbiosis mainly corresponds to the biodiversity of the partners.

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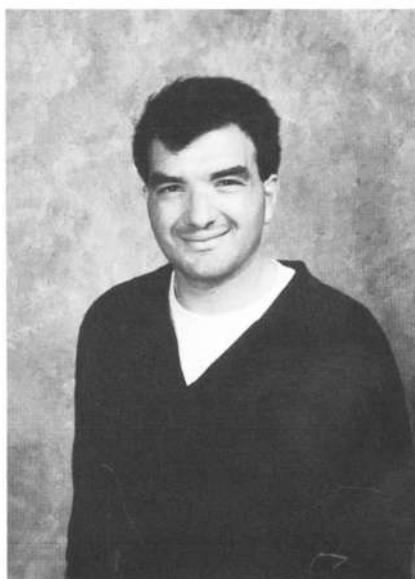
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SYMBIOSSES AND THEIR CONSEQUENCES FOR COMMUNITY AND APPLIED ECOLOGY

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1. Introduction

Symbiosis researchers normally focus on intimate relationships between and among species, and they seek to identify the “sign” of those interactions, the positive or negative effects of participating in the interaction for hosts and symbionts. They often do so in isolated, controlled laboratory settings, using methods and insights from physiology and biochemistry. Population and community ecologists also study interactions between and among species, evaluate the sign of those interactions in terms of positive and negative effects on individuals and populations, and subsequently determine how such interactions structure ecological communities through food chains, food webs, and ultimately interaction webs. They also focus on the relative strength of those interactions and on their temporal and spatial variability, often by means of manipulative, replicated, and controlled field experiments.

So why do ecologists and biologists who study symbioses, who apparently have so much in common, rarely talk to one another, seldom read the same journals, and infrequently attend the same conferences? Ecologists, on the one hand, are charged with unraveling the interactions among organisms in nature, and increasingly with applying that knowledge to conservation and restoration efforts in an increasingly degraded natural world. They also are asked to contribute data based insights to human problems such as maximizing crop yields or fisheries production and managing pests in agricultural and aquacultural systems. Many of these basic and applied questions depend on a detailed understanding of the biology of symbionts and the species they interact with. Symbiologists, on the other hand, understand in great detail some of the tightest and strongest linkages among species, many of which are integral parts of the ecosystems that these field ecologists seek to understand. They often study impacts at a physiological or biochemical level of hosts and symbionts on each other.

Where do symbiotic species interactions fit into the larger context of population and community ecology? Here I will use the symbiotic system I am most familiar with, that between temperate sea anemones and their endosymbiotic algae, as a starting point to explore some of what I will argue is underexploited “common ground” between the two groups. I will then review some familiar and unfamiliar examples of symbiotic interactions, with a particular emphasis on what we know about impacts of symbioses at the level of the ecological community, and on issues in applied ecology and conservation.

In this chapter, “community ecology” refers to the dynamic analysis of a “greater or lesser number of populations” of different species interacting with one another (Townsend *et al.* 2000) under field conditions. If ecologists pay more attention to symbioses, they will have a clearer view of the structure and function of the communities they study. Moreover, if symbiologists pay more attention to ecology, they will be more confident in their conclusions’ applicability to the real world.

2. Case Study: A Field Ecological Approach to a Temperate Algal-Invertebrate Symbiosis

Sea anemones in the genus *Anthopleura* are unique among cnidarians in that they harbor two very different species of algal symbionts, zooxanthellae (*Symbiodinium* spp.) and zoochlorellae (*Chlorella*-like chlorophyte algae unidentified below the phylum level). This dual symbiosis is much more than just a natural history novelty. In fact, *A. elegantissima* is the most abundant intertidal sea anemone on rocky shores from Alaska to Baja California, Mexico, in both sheltered and exposed habitats (Kozloff 1983, Ricketts *et al.* 1985). It is a major occupier of space in the mid-intertidal zone (Dayton 1971), a significant predator on zooplankton and benthic invertebrates (Sebens 1981), and, as a host for endosymbiotic algae, it contributes to primary productivity on a par with macroalgae (Fitt *et al.* 1982). It reproduces clonally by means of binary fission, and also sexually by free-spawning gametes that form feeding planula larvae (Sebens 1982; Siebert 1974), generating the potential for either horizontal or vertical transmission of endosymbiotic algae. I have studied this symbiosis primarily using the methods of field ecology, and by collaborating with laboratory-based algal physiologists.

2.1. POPULATION BIOLOGY OF SYMBIONTS IN ANTHOPLEURA HOSTS

Symbiologists who focus on the details of intimate interactions between pairs of species under conditions of laboratory isolation would do well to consider their favorite systems more fully, as distinct populations against a dynamic backdrop of interacting species. The symbiosis between *A. elegantissima* and its two algal symbionts varies tremendously on temporal and spatial scales; it is not a static, constant interaction throughout the geographic range of the species involved, nor is it even constant on a spatial scale of meters or a temporal scale of weeks to months. Work on this symbiotic system in the field has yielded insights not immediately evident from lab work alone. For example, quantitative census data and reciprocal transplant experiments along a gradient from full sunlight to deep darkness within an intertidal cave system suggest that anemones interact with one symbiont, then a mix of the two symbionts, then the other symbiont, and finally are totally aposymbiotic at the darkest reaches of a cave. The nature of the symbiosis varies dramatically on a spatial scale of tens of meters, and reciprocally transplanted anemones in the field can even switch symbionts on a temporal scale of months (Secord 1995; D. Secord and G. Muller-Parker, in prep).

The interaction also varies on a biogeographic scale, with latitudinal replacement of symbiont species from Washington State to Mexico (LaJeunesse and Trench 2000; Secord and Augustine 2000). Further, field measurements of light and temperature regimes, coupled with physiological performance data from the lab, suggest that the sign and specificity of many symbiotic interactions may vary with physical conditions

seasonally and even diurnally (e.g. Engebretson and Muller-Parker 1999; see also Douglas and Smith 1989; Saunders and Muller-Parker 1997). Related host species, such as the large solitary *A. xanthogrammica*, may also harbor either zooxanthellae or zoothorellae (or both), and the distribution of the symbionts varies with physical conditions set not only by geography, but also by microhabitat (i.e. intertidal height) and even host body size (Bates 2000; McFadden *et al.* 1997; O'Brien 1980; O'Brien and Wyttenbach 1980; Secord and Augustine 2000). Finally, animal behavior can strongly (and reciprocally) mediate the field distribution and performance of both hosts and symbionts: *A. elegantissima* displays phototaxis determined by the identity and density of the symbionts it harbors (Pearse 1974; M. Bracken, B. Seavey, and D. Secord, unpub. data). Only with a field perspective can symbiologists hope to gain a more complete understanding of the highly variable ecological context within which hosts and symbionts evolve and interact.

Finally, a potentially fruitful way to view *Anthopleura* spp. and their symbiotic algae (and perhaps many other symbiotic interactions, e.g. Clayton *et al.* 1992) is through the patch dynamics and metapopulation models used by a broad spectrum of conservation biologists, epidemiologists, and ecologists. In such models, each anemone is an individual patch of habitat containing discrete populations of at least two symbiotic algal species (and perhaps two or more clonal genotypes of each alga!). In facultatively symbiotic, clonal *A. elegantissima*, polyps (patches) may be occupied by one alga, by both algae, or be unoccupied, in the case of aposymbiotic anemones. Each anemone (i. e. patch) may then be thought to contain asexual population(s) of zooxanthellae, zoothorellae, or both, and thus by extension, a population of anemones harbors metapopulations of algae. Symbiont populations may be dispersed among host patches in the feces of predators on anemones, by transport of putative free-living (e. g. flagellated) stages, or by binary fission of host anemones, which (along with sexual recruitment) is also a mechanism of new patch formation.

Just as patches change periodically in their occupancy status in other ecological scenarios, host anemone patches may lose or regain their algal populations, due to extrinsic (e. g. temperature or UV light) or intrinsic (e. g. changes in host physiology) factors (White and Pickett 1985). Furthermore, these are clonal anemones and so patches may differ in their quality with host genotype, particularly if anemones vary genetically in their affinity for symbionts. Patches may also change in other measures of quality, such as their size as anemones grow or shrink, or their physical location (affecting many possible microhabitat parameters) as host anemones walk about on rocky shores and “select” their own habitat. Many of these variables may be incorporated into models of the population biology of interacting species (e.g. that of May and Nowak 1994) that may in turn be parameterized by means of well-designed field observations and manipulative experiments.

2.2. FROM SYMBIONT POPULATIONS TO COMMUNITY IMPACTS

Building up from this population-level approach to interaction strengths (*sensu* Paine 1980, 1992) of host and symbiont species, biologists will be able to better quantify effects of symbiotic interactions on communities and even ecosystems. For example, what are the community consequences of the fact that symbionts of *Anthopleura elegantissima* locally contribute to primary productivity at a level comparable to that of macroalgae on a per-area basis (Fitt *et al.* 1982)? We know little about the answer, since photosynthate in

this case is not transmitted through food webs via species that are especially abundant, tractable, or traditional subjects of benthic field experiments in ecology. A standard approach to communities, the search for “top-down” effects imposed in this case by sea anemones’ predators, would require data on species that are not especially easy to enclose, exclude, or otherwise manipulate. Algal “bottom-up” effects may be even more difficult to trace through food chains.

For example, intertidal sculpins may be major predators on alga-filled anemone tentacles, but are difficult to exclude or enclose in the field (but see Augustine and Muller-Parker 1998 for suggestive laboratory data). Furthermore, for field samples, it may be impossible to identify anemone tissue in analyses of sculpin gut contents (C. Pfister, pers. comm.). Pandalid shrimp, another putative predator on anemones, pose a similar problem (G. Jensen, pers. comm.). Benthic predators on *Anthopleura*, such as the sea star *Dermasterias imbricata* and the sea slug *Aeolidia papillosa*, are rarely abundant or predictable enough in time and space to be attractive subjects for field manipulations (but see MacFarland and Muller-Parker 1993). However, as will be shown below, there is ample and increasing precedent for manipulative field experiments involving symbiotic species.

If we are to understand the roles of symbiotic partners in interaction webs, we shall need to be more creative about the design of manipulations in natural habitats. At present, we would be hard pressed to answer quite straightforward ecological questions, such as whether the fish, shrimp, and starfish predators on symbiotic anemones above are acting as herbivores (digesting symbiotic algae) or as carnivores (consuming host anemone tissue) in a simple food web.

3. Consequences of Symbiotic Interactions at the Population and Community Levels

In this discussion I consider symbiosis as encompassing all intimate, sustained interactions between or among dissimilar species, independent of the sign of the interaction(s). Sign here refers to the relative costs and benefits of symbiosis for the partners, frequently expressed as (++) interactions (mutualisms) in which both species benefit, (+-) interactions (the special case of predation known as parasitism) in which one species benefits and one is harmed from the association, and (+0) interactions (commensalism) in which one species benefits and the other is neither helped nor harmed by the interaction. The latter case of commensalism is particularly difficult to detect or measure, given the challenge of documenting and quantifying the *absence of an effect* in ecology or any other complex science.

We simply do not often know all of the proper currencies by which to measure benefit or harm, let alone the ways in which benefit and harm vary along temporal or spatial gradients. Indeed, the more we study classical symbioses that are, say, parasitic or mutualistic according to conventional wisdom, the more we discover that the sign of intimate symbiotic interactions is a moving target. Interaction sign varies widely when we move from pairwise analysis of symbioses in the laboratory, to the complex matrix of species in field situations, subject to ever-changing environments. In fact, this may be one of the best reasons to study symbioses using the methods and theory of ecology, necessarily coupled with the more traditional reductionist and laboratory approaches to symbioses. Only then will we know how insights from physiological or biochemical analysis play out in the real world, and how our understanding of the community structure

of aquatic and terrestrial habitats changes when we consider smaller, hidden, but perhaps critically important symbiotic species. The potential centrality of symbionts in ecological interaction webs was perhaps stated most clearly by the ecologist R. T. Paine (known for manipulative field experiments involving tractable, macroscopic organisms) when he said that there “should be an admonition [in food web analysis] not to exclude the taxonomically awkward soil and meiofaunal organisms, picoplankton, fungi, and other decomposers, and their little known interactions” (Paine 1996).

Community effects of symbiosis may frequently operate indirectly. For example, bioluminescent bacteria in pelagic fishes may have negative effects on the predators of their hosts, by making the fishes less visible from below on moonlit nights. In terrestrial systems, *Wolbachia* bacteria that alter the sex ratios and mating patterns, and therefore demography, of their host insects, could thereby indirectly regulate populations of their hosts' predators (e.g. Stouthamer and Werren 1993). Perhaps with greater ecological impact, primary production by zooxanthellae in tropical marine systems is mediated by coral and other invertebrate hosts as it “trickles up” through food webs. As such, zooxanthellae on coral reefs may interact strongly enough to merit description as keystone species, or at least “critical species” -- or, if dinophyte symbionts actually constitute a complex of sibling taxa (Rowan and Powers 1991; Rowan *et al.* 1997), then they might collectively be considered a “critical guild”. Community-level (or landscape, *sensu* Rowan *et al.* 1997) effects of their removal would probably be profound, and well out of proportion to their biomass. Absence of this critical group would remove both primary production and the source of reef architecture, since coral calcification is enhanced by the activities of symbionts.

Removal of zooxanthellae would have major bottom-up effects, and disturbance to them on coral reefs (e. g. due to global temperature increase, pollution, or greater UV light flux) could have consequences for local or regional diversity as profound as those of better known critical species whose effects are top-down, such as sea stars and sea otters in the temperate northeast Pacific. Emerging statistical tools such as path analysis may prove extremely useful in elucidating the community consequences of symbiotic interactions (see Wootton 1994), especially when combined with innovative field approaches (e.g. Wipfli and Merritt 1994; Wootton 1994).

Obviously, some symbioses form the basis of entire ecosystem types by conferring “novel metabolic capabilities” upon organisms (Douglas 1994). There is already an ample literature on coral reefs and their zooxanthellae, termites and their gut symbionts, legumes and their N-fixing bacterial root nodules, and eukaryotic cells and their organelles. Instead, in what follows I will focus on interactions that are labile in ecological time, and subject to investigation using the established research methods of population and community ecology.

3.1. EVIDENCE FOR COMMUNITY EFFECTS OF TERRESTRIAL SYMBIOSES

Terrestrial symbioses are classic examples of species interactions that to most ecologists are “out of sight, out of mind.” They represent, however, some of the clearest documented examples of how intimate symbiotic interactions, regardless of sign, may help determine the structure of ecological communities. The best-known category of terrestrial symbionts is mycorrhizal fungi, which inhabit the roots of the majority of land plant species, including many plants of importance as agricultural crops or as weedy

pests. Endophytic fungi, a less-well-known category of land-plant symbionts, also have effects beyond the pair of species directly involved.

As for intimate interactions on land involving animal species, I will not discuss the extremely well known interactions of plants and their pollinators (arguably not a true symbiosis owing to its temporally fleeting nature), nor will I discuss the ecological roles of insect parasitoids, though the latter clearly regulate insect populations, as a large literature already exists in ecological entomology. There are many symbioses on land that fall into the categories of natural history novelties (e.g. birds that pick insects off of mammals, epiphytic orchids) or public health issues (e.g. hantavirus, Lyme Disease, helminth parasites). Information (especially quantitative data based on experimental data) on the community-level consequences of such symbioses is often sorely lacking.

3.1.1. Evidence for Community Effects of Mycorrhizal Symbioses

Community ecologists typically ask questions about topics such as the effects of natural or anthropogenic disturbance on local or regional biodiversity, the causes and consequences of varying levels of diversity through time and space, the centrality of competitors versus consumers in determining community structure, the relative importance of top-down and bottom-up effects in communities, and the relative interaction strengths of species in communities. Mycorrhizal fungal symbioses comprise perhaps the best-studied set of examples of how symbioses play into the above questions and processes. This is in spite of general neglect by ecologists of the impacts of such symbioses beyond the individual species involved (Knapp *et al.* 1998) and even unfounded assumptions about the sign of mycorrhizal symbioses when they are studied in a community context (Francis and Read 1995).

Among the best long-term datasets on the role of mycorrhizae in communities comes from the native grasslands of central North America, where up to 20 species of mycorrhizal fungi may be associated with tallgrass prairie roots in a single small sampling site (Knapp *et al.* 1998). At the well-studied Konza Prairie research site, symbiotic fungi are just as important as more frequently-studied determinants of plant community structure such as disturbance regime (mowing and fire), topography, competition, and herbivory. They may also significantly interact with other biotic factors, as when interplant hyphal connections mediate interspecific competition among host plant species.

The effects of mycorrhizae on individual species, and thus on plant community structure, also vary across the full spectrum from mutually positive to parasitic interactions. For example, C4 grasses may perform better in the presence of mycorrhizal fungal spores in the soil, while other grasses and forbs may be so inhibited. The Long-Term Ecological Research (LTER) site at Konza Prairie in Kansas has also produced ample evidence of spatial, seasonal, and interannual variation in the relative importance of fungal symbionts in determining plant community structure. Symbiotic interactions have indeed proven amenable to field manipulation in the form of factorial experiments (e.g. fungal suppression by benomyl application in selected plots), generating critical insights lacking in many studies of plant community ecology (Knapp *et al.* 1998).

Experimental removal or suppression of mycorrhizae has produced numerous changes at the community level. For example, fungicidal reduction in soil mycorrhizae significantly reduced the abundance of obligately mycotrophic grasses in favor of grass species, normally competitively subordinate, that are only facultatively mycotrophic (Hartnett and Wilson 1999). Long-term application of fungicides at the Konza Prairie site also produced changes in the abundance and biomass of soil bacteria and fungivorous

and carnivorous nematodes. More research is necessary to determine effects on the aboveground plant community of these belowground changes (Smith *et al.* 2000). Similarly, long-term fungicide applications at sites in Europe have not only favored certain plant species, but reduction of mycorrhizae significantly interacted with nutrient enhancement (through experimental phosphate enrichment) in determining dominant plant species (Smilauer and Smilauerova 2000). Additionally, there is increasing evidence that differential plant *dependency* on mycorrhizae may be a critical determinant of plant community composition (van der Heijden *et al.* 1998).

Mycorrhizae interact with the physical and biotic environments in determining community structure in a wide range of habitats. For example, in arid Namibian grasslands, moisture gradients and stability of dune soils determined fungal community composition (Jacobson 1997). Indeed, mycorrhizae seem to be more common in arid environments than was previously thought, but there have been very few field studies on the community consequences of their presence (McGee 1986). Ectomycorrhizal fungal succession may proceed differently following physical disturbance such as wildfire (Visser 1995). Additionally, there is increasing evidence of mycorrhizae acting via indirect biological interactions in communities. For example, they may mediate the outcomes of competitive interactions among plant species (e.g. Smith *et al.* 1999). In turn, grazing soil fauna may influence the development of symbioses between mycorrhizal fungi and plant roots (Setala 1995).

Mycorrhizae may influence – either positively or negatively – plants’ resistance to mammalian herbivory (Moore *et al.* 1991). Small fungivorous marsupials may even regulate the extent to which the symbiotic relationship achieves importance in *Eucalyptus* forests (Johnson 1995; Johnson 1996). Finally, physical factors (patch formation) and biological ones (succession) may interact in a way that is indirectly mediated by mycorrhizae: fungal dispersal by small mammals may be the limiting factor in determining the rate at which conifers invade meadows formed by successional beaver ponds (Terwilliger and Pastor 1999).

3.1.2. Evidence for Community Effects of Fungal Endophytic Symbioses

Like mycorrhizae, fungal endophytes are very common symbionts of terrestrial plants whose ecological effects are not well understood. Rare extant data indicate that the interaction is not merely between plants and these fungi that inhabit their host tissues (perhaps causing direct harm), but one in which fungi *indirectly* aid the host when a third partner is considered. This third species may be an herbivore that feeds on fungally-affected plants, and whose herbivory is deterred by fungal infection (Clay 1996). Experimental evidence from the lab and the field suggests that plant competitive hierarchies may be reversed by differential herbivory. This differential herbivory may in turn be conferred upon plants by grasses’ fungal endophytes that produce distasteful or toxic alkaloids (Clay 1996). The role of symbiosis should be investigated more as a potential mediator of other well-documented competitive hierarchies (which may in turn be modified by top-down interactions) in terrestrial as well as aquatic habitats. For example, undescribed symbiotic interactions may help to explain geographic variation in the interaction strengths of certain keystone species.

3.1.3. Terrestrial Symbioses Involving Animals

Terrestrial animals may frequently be involved in cryptic, parasitic, pathogenic, or otherwise unappreciated symbiotic roles in community interaction webs. Soil organisms,

as part of what plant ecologists call “belowground food webs”, may be neglected compared with larger, more experimentally tractable aboveground organisms and parts of organisms (Strong *et al.* 1996). A telling example involves several trophic levels of small, cryptic, symbiotic soil-dwelling organisms whose interactions may determine important properties of overall community structure. Lupines are a central feature of Northern California coastal shrub habitats. They are preyed upon belowground by the larvae of ghost moths, which in turn are victimized by a previously undescribed species of nematode that transmits a pathogenic bacterium. Yet another layer of community complexity is added by a nematophagous fungus that acts as a consumer on the nematodes. Two of the links in this chain – fungi killing nematodes and ghost moth larvae killing lupines – may inflict up to 100% mortality in field populations. Depending on the population dynamics of each of these intimate players, lupines may be either very abundant or nearly absent at particular locales. This is an excellent example of the neglected primacy of the small in determining the face of a community and its spatial and temporal dynamics (Strong *et al.* 1996).

There are a number of other ways in which symbionts of or on animals may influence species interactions or community composition, beyond simple pairwise interactions between the immediate partners. For example, bloodsucking terrestrial arthropods may dramatically regulate populations of their avian or mammalian hosts, with strong potential for community consequences (Dye 1992). Parasitic flatworms “cause a ubiquitous and constant drain on the energetic resources of most free-living organisms,” with significant effects on the reproduction, survival, behavior, and other properties of interacting host populations (Dobson *et al.* 1992; Kearn 1998). Finally, microbial symbionts of insects may allow insect herbivores to exploit chemically defended or infected plants that would otherwise be unsuitable prey (Down 1991). Given the enormous attention paid by empirical and theoretical ecologists to insect herbivory in terrestrial communities, such symbioses should be considered potentially very important in basic and applied community ecology.

3.2. EVIDENCE FOR COMMUNITY EFFECTS OF MARINE SYMBIOSSES

Several excellent volumes on coral reef community ecology already exist, addressing the community and ecosystem consequences of coral-zooxanthella symbioses (see Karlson 1999; Birkeland 1997; Sorokin 1993; Dubinsky 1990). In this section I will address a few less-familiar marine symbioses, including those occurring on coral reefs, as epibionts on mangrove roots, and behavioral symbioses.

On some coral reefs in the Indian and Pacific Oceans, a true mutualistic symbiosis among macrofaunal species may produce clear differences in community structure. Xanthid crabs and alpheid shrimp spend most of their time living among the branches of pocilloporid and acroporid corals, and protect the structure-forming corals from predation by the starfish *Acanthaster planci* (Glynn 1987). Since *Acanthaster* is a keystone predator on coral reefs, differential protection of coral species by shrimps and crabs produces differential mortality and colony size reduction of corals. For example, during a starfish outbreak in the Gulf of Oman in 1978-1980, nearly all acroporid corals were killed, leaving a pocilloporid-dominated reef that was protected from starfish predation by guard-symbiotic crustacean species (Glynn 1987). A wide variety of other animal symbioses are documented on coral reefs, but consequences beyond the population pairs directly involved are little known (Castro 1988). A similar protective symbiosis occurs in

temperate coral communities off the coast of North Carolina. The crab *Mithrax forceps* grazes fouling invertebrates and algae off the surfaces of the coral *Oculina arbuscula*. Manipulative field experiments have revealed that this is a true behavioral-trophic mutualism, with corals providing crabs with protection from its predators as well as with energy from coral mucus. Since many other species depend on habitat formed by structure-creating but competitively inferior species such as *Oculina*, this is another example of symbiotic interactions contributing importantly to marine community structure (Stachowicz and Hay 1999).

Finally, epibiotic interactions may significantly affect the structure of marine communities. Mangrove roots immersed in brackish water host a wide variety of epiphytic plants and animals, including several species of sponges. In Belize mangrove forests, these sponges significantly enhance nutrient uptake by mangrove tree roots by promoting rootlet growth. Not unlike some terrestrial interactions between plants and mycorrhizal fungi, faster growth of mangroves, facilitated by symbiotic sponges, may allow habitat-forming mangroves to exist in otherwise marginal habitats (Ellison *et al.* 1996).

3.3. ECOLOGICAL THEORY AND SYMBIOSES

Ecologists have paid increasing attention to the phenomenon of mutualism in recent years (e.g. Boucher 1985, Kawanabe *et al.* 1993; Townsend *et al.* 2000). They have begun to shed the unfounded assumption that positive interactions among species are unimportant in nature simply because they tend to become mathematically unstable when modeled as pairwise Lotka-Volterra dynamics (Douglas 1994). Indeed, when enmeshed in community interactions, mutualisms' population dynamics are no longer locally destabilizing. When incorporated into an even slightly larger (than pairwise) community matrix of four species, model results are consistent with empirical observations of the ubiquity of both facultative and obligate mutualisms (Ringel *et al.* 1996). This result even allows for variation in the sign of symbioses.

Additionally, an increasing number of meta-analyses of symbiosis types such as epibioses is emerging. For example, a survey of over 2000 associations found that most epibionts also occur on non-living substrates, leading to the generalization that obligate, host specific epibionts are relatively rare in nature (Wahl and Mark 1999). Theoreticians are also turning to life history theory of both hosts and parasites to explain the importance of parasitism at the level of the ecological community (Thomas *et al.* 2000). Finally, empiricists and theoreticians are increasingly recognizing the "mutualism-parasitism continuum" along which most symbioses function in the real world (e.g. Johnson *et al.* 1997).

4. Applications of Symbiosis Research to Conservation Biology and Applied Ecology

Symbiotic interactions may play a significant, underappreciated role in applied ecology. Most applied ecologists and conservation biologists are familiar with such ecologically important symbiotic interactions as those involving zooxanthellae and reef-building corals, those between mycorrhizal fungi and land plants, those involving disease-causing microbes, and those that comprise lichens, important indicators of air quality. But fewer

ecologists really consider the richness of data on symbioses in understanding the structure of communities and in applied and conservation biology. For example, biological control of pest organisms often focuses on intimate (if not strictly symbiotic) interactions involving organisms as diverse as parasitoid insects and pathogenic fungi. The focus of researchers in applied agroecology is often on host specificity testing, yet these biologists seldom come from backgrounds in basic symbiosis research (Secord and Kareiva 1996; Secord 1998).

The sign, specificity, and intimacy of species interactions in applied ecology are widespread themes in marine as well as terrestrial systems. For example, parasitic barnacles have been proposed as a controversial biological control agent of an introduced green crab, *Carcinus maneus*, on the west coast of North America, and a rich mixture of field and lab data on host specificity, symbiont life cycles, and the potential for the evolution of host shifts will be critical in evaluating the environmental safety of releasing an organism that could potentially sterilize native crabs as well (Lafferty and Kuris 1996)! Below I briefly review a few applied biological topics for which a fuller consideration of the biology of symbiosis might be helpful.

4.1. SYMBIOSES AND AGROECOLOGY

Symbionts play a wide variety of roles in agricultural ecosystems, greater recognition of which may increase agricultural yields, improve sustainable agriculture, and contribute to programs of integrated pest management (IPM), including biological control. Indeed, agricultural ecologists have probably recognized the critical positive and negative roles of symbiotic interactions more than any other group of applied biologists. For example, increasing attention is being paid to the *diversity* of arbuscular mycorrhizal fungi in agricultural fields, their roles in crop plant nutrient uptake, and farming methods that will maximize their contribution to sustainable agriculture (Douds and Millner 1999). Applications of fungicide to control fungal crop pests may inadvertently alter belowground community structure of mycorrhizae and other soil microbes (Smith *et al.* 2000). Fire, mowing or harvesting regime, and fertilization may also alter mycorrhizal diversity and distribution, and therefore the structure of agroecosystems (Eom *et al.* 1999). Bacteria introduced for biological control may substantially alter bacterial communities on crop plants' roots, with unknown consequences for soil community ecology, as well as policy implications for release of genetically modified bacterial biocontrol agents (Gilbert *et al.* 1993).

Symbioses may also be valuable in agricultural pest control. Numerous studies indicate that mycorrhizae may reduce plants' susceptibility to disease, especially when conditions are ripe for opportunistic pathogens that might attack crop plants. Increased research on incorporating mycorrhizae into IPM plans may lead to increased yields and reduced crop mortality (Linderman and Pfleger 1994). Indeed, Linderman (1994) argues that in areas where economic margins are thin for farmers, increased recognition of the roles of mycorrhizae in agroecosystem food webs, and simple changes in farming practices based on that recognition, may make all the difference in the economic viability of farms. Using natural mycorrhizal communities in pest control may be an "environmentally friendly" component of IPM (Duchesne 1994), perhaps especially in comparison with classical biological control (Secord and Kareiva 1996). Sustainable farming enterprises that involve mixes of animal and plant products should heed data indicating that cattle grazing may locally alter mycorrhizal diversity (Knapp *et al.* 1998).

Finally, parasitic or pathogenic fungi may even be used in biocontrol of insects, especially when fungi are host specific on insect taxa that attack crop plants (Whisler 1979).

4.2. SYMBIOSES, BIODIVERSITY CONSERVATION, AND GLOBAL CHANGE

Small, cryptic organisms, many or most of them symbionts of various kinds, comprise an enormous component of global biodiversity (Wilson 1992; Townsend *et al.* 2000). In particular, keystone species are known in a wide variety of terrestrial and marine habitats to have structural effects on communities well out of proportion to their abundance. Such consumers may operate singly, diffusely as guilds of similar species, or not at all (Allison *et al.* 1996). Symbioses should increasingly be considered as *potential* keystones, either as parasitic consumers, or as keystone mutualists (e.g. Lanner 1996). Pathogens may also set off chain reactions of ecological interactions that set communities on a course towards entirely new stable states (Done *et al.* 1996).

Symbioses should also be incorporated, wherever biologically appropriate, into plans to conserve Earth's major habitat and ecosystem types (see Kinzie 1999 for a coral reef example), just as non-symbiotic mutualisms such as pollinator-plant interactions are now beginning to be (Buchmann and Nabhan 1996). Cryptic endosymbionts may play enormous roles in biodiverse communities as consumers, pathogens, or mediators of competitive interactions (e.g. Bentis *et al.* 2000), and should be treated seriously in management plans until demonstrated to be unimportant. Similarly, symbiotic interactions should be explicitly incorporated into plans for ecological restoration of degraded communities (e.g. essentially no data on marine symbioses are typically collected or considered in the aftermath of major environmental catastrophes such as oil spills; Paine *et al.* 1996). Finally, symbioses should be included in models of the biotic consequences of global climate change (e.g. Rillig and Allen 1999). For example, overall community structure and composition may change over relatively short time scales with changes in the physical environment (Sagarin *et al.* 1999), and, coupled with anthropogenic disturbances such as overfishing, may decrease or increase the prevalence of behavioral symbioses or endosymbioses (e.g. Parker and Dixon 1998).

Symbiotic organisms are frequently ignored even at an alpha taxonomic level, and assumptions about their expendability from ecological communities are almost certainly unfounded. As we engage in conservation triage more and more frequently, however, functionally significant symbionts are perhaps at greatest risk, given their lower profile compared with "charismatic megafauna". To the extent that they have structurally important roles in communities, however, we ignore symbionts at the peril of all organisms and habitats of conservation concern.

4.3. SYMBIOSES AND BIOLOGICAL INVASIONS

Biological invasions are now regarded as among the world's greatest threats to biodiversity and sustainability, as evidenced by the recent (1999) creation of a new scholarly journal devoted to them. Symbiotic interactions are a key part of the emerging science of invasion biology in several ways. For example, intact symbiotic partnerships may be limiting steps in successful anthropogenic invasion of some species. Absence of natural enemies, including symbiotic species, is one component of the success of many nuisance invaders. Finally, symbionts may themselves be invasive, affecting native communities as alien parasites or pathogens.

Plant invasions in particular feature myriad dependencies on mutualistic interactions, many of which are symbiotic (see Richardson *et al.* 2000 for an excellent recent review). In marine systems, native or introduced parasitic castrating barnacles may infect their crab hosts in different ways (Hines *et al.* 1997). Native egg-parasitic nemerteans may also switch from native to introduced crab hosts (Torchin *et al.* 1996). Experimental data have shown that fungal endophytic symbionts dramatically increased the competitive success of damaging invasive fescue grass in the eastern United States (Clay and Holah 1999). Similarly, as mediators of plant competitive interactions, mycorrhizal symbionts may favor invasive over native plants in the grasslands of western North America (Marler *et al.* 1999). Interestingly, the ascidian *Molgula manhattensis* seems to require its cyclically reestablished, eukaryotic and prokaryotic endosymbionts in its native range, but not in San Francisco Bay, where it is an invasive exotic species (Saffo 1991).

Finally, in Mediterranean subtidal habitats dominated by the invasive toxic alga *Caulerpa taxifolia*, secondary metabolites may be responsible for the recent disappearance of digenetic parasites of the predatory fish *Syphodus ocellatus* (Bartoli and Boudouresque 1997). Biological invasions represent an unprecedented scrambling and homogenization of Earth's biota, and symbiotic interactions will likely prove critically important in the identity, prevention, and control of exotic species introductions.

5. Conclusions

Species interactions are frequently variable in time and space, and symbiotic relationships are certainly no exception. Empirically derived rules of host specificity and interspecific interactions may apply locally under some conditions, but we must exercise care when we extrapolate results across species' ranges or when local conditions change. In the words of the ecologist Larry Slobodkin (1987), "the study of symbiosis should remain elegant natural history, combined with the full spectrum of modern biology at all levels." The level of community ecology has historically been somewhat lacking! An interdisciplinary mix of approaches and insights will be necessary as conservation biologists, population and community ecologists, resource managers, epidemiologists, and symbiosis researchers tackle basic and applied problems in habitats as diverse as old growth forests, coral reefs, and agroecosystems. A marriage of the data and methods of field ecology with laboratory analysis of the physiological and biochemical details of symbiosis, and increasing interactions among community ecologists and symbiologists, will nudge biological science and its applications farther along this necessary path.

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II. Origin & Evolution

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WHAT'S IN A TREE?

Does Horizontal Gene Transfer Determine Microbial Taxonomy?

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1. Introduction

Among eukaryotes, symbiosis can lead to the acquisition of new traits not originally seen in a lineage. Luminescent fish, nitrogen-fixing plants, and respiring eukaryotes have traits that are attributed to the symbiotic acquisition of innovations which evolved in prokaryotes (Margulis, 1993, Margulis and Fester, 1991). Among prokaryotes, acquisitions of evolutionary innovations also occur, but they occur primarily as a result of horizontal gene transfer rather than cyclical or permanent symbioses. As a result of direct uptake of naked DNA, transduction or conjugation, prokaryotes can acquire stretches of DNA that either confer new phenotypic traits, or that replace versions of already existing genes with similar genes from a different organism. Evidence of such transfers can be detected in phylogenetic analyses of individual genes, which show up as conflicts in the branching topology when trees constructed from two different genes are compared (Gogarten, *et al.*, 1996, Olendzenski and Gogarten, 1999). In both symbioses and horizontal gene transfer, the assumed tree-like nature of evolution is transformed to a reticulate, or net-like pattern (Hilario and Gogarten, 1993).

The role of horizontal gene transfer (HGT), has long been recognized in the spread of antibiotic resistance and selfish genes and in the formation of pathogenicity islands, (Lawrence, 1999). The formation of operons has been attributed to HGT and through this process is suggested to be a major force in structuring prokaryotic genomes (Lawrence and Roth, 1996). In addition to genes under sporadic selection, HGT affects house keeping genes (e.g., the archaeal-type ATP synthase transferred to Deinococcaceae and Spirochetes (Hilario and Gogarten, 1993) and functions involved in information processing (e.g., the archaeal type lysyl-tRNA synthase in *Rickettsia* and Spirochetes (Ibba *et al.*, 1997) and the archaeal type prolyl tRNA synthase in *Thermus* and *Deinococcus*, representatives of the Bacteria (Gogarten *et al.*, 1999, Olendzenski *et al.*, 2000)). The widespread occurrence of HGT has been amply documented through the analyses of microbial genomes that have been completely sequenced (e.g., Bult *et al.*, 1996, Deckert *et al.*, 1998, Koonin *et al.*, 1997, Nelson *et al.*, 1999).

Nevertheless, the sheer amount of HGT suggested from comparative genome analyses came as a surprise to most microbiologists.

Today, the fact that HGT occurs is no longer disputed; however, the impact of HGT on microbial evolution, in particular on our ability to reconstruct organismal evolutionary history, remains controversial. The question addressed here is: how much does horizontal gene transfer impact our ability to reconstruct the evolutionary history of organisms from molecular data? With regards to the role of HGT in shaping bacterial phylogeny, we propose the possibility that HGT frequency, rather than vertical inheritance, is the main determinant of taxonomic units in prokaryotes.

2. Are 16S Ribosomal RNA Phylogenies a Result of Vertical Inheritance or Horizontal Transfer?

Since the acceptance of Darwin's idea of natural selection, the goal of taxonomy has been to develop a natural system for the classification of organisms, i.e., one that is based on shared ancestry. A simplified view of the evolutionary process considers the generation of offspring with variation and selection as the main ingredients of biological evolution. The selection process often is described as survival of the fittest. While there are many well studied instances of fitness increase through collaboration (e.g., lichen, kefir, and the nucleated cell of eukaryotes are all the result of symbiosis (Margulis, 1993)) the traditional view of evolution is based on vertical inheritance, i.e., the passing on of genetic information from parent to offspring, and competition/selection of individuals within a population.

The ability to easily sequence DNA, and the enormous increase of available molecular data has dramatically changed our view of microbial evolution. Comparison of 16SrRNA sequences successfully provides a method for relating all organisms to each other, and has given us a model for microbial taxonomy. Using animal and plant evolution as a paradigm, microbial evolution has been assumed to be a process determined primarily by vertical inheritance. This view of evolution is based on gene trees constructed from markers thought to have never or rarely undergone horizontal transfer. Inter-species horizontal gene transfer is regarded as an important but rare exception. Under this vertical inheritance model of evolution, a natural bacterial classification reflecting shared ancestry and based on the majority of vertically inherited genes emerges after weeding out a few horizontal transfers which occur primarily between closely related species.

This conservative model, in which taxonomy is based on vertical inheritance represents one extreme in a continuum of current views (e.g., Doolittle, 1999, Woese, 2000). Under this model, we can assume that most molecular phylogenies reflect organismal phylogeny. There is some HGT between less related species, but these events are easily detected as conflicts with the majority consensus of "conserved" genes. If reality is close to this model dominated by vertical inheritance, then interdomain and interkingdom HGT events provide an excellent means of correlating the evolution in the different parts of the tree of life (Figure 1). However the available data is equally compatible with a more extreme view. Under the proposed model, it is the frequency of HGT which determines "relatedness", i.e., Bacteria are more similar to other Bacteria, and are recognized as Bacteria, because they more frequently exchange

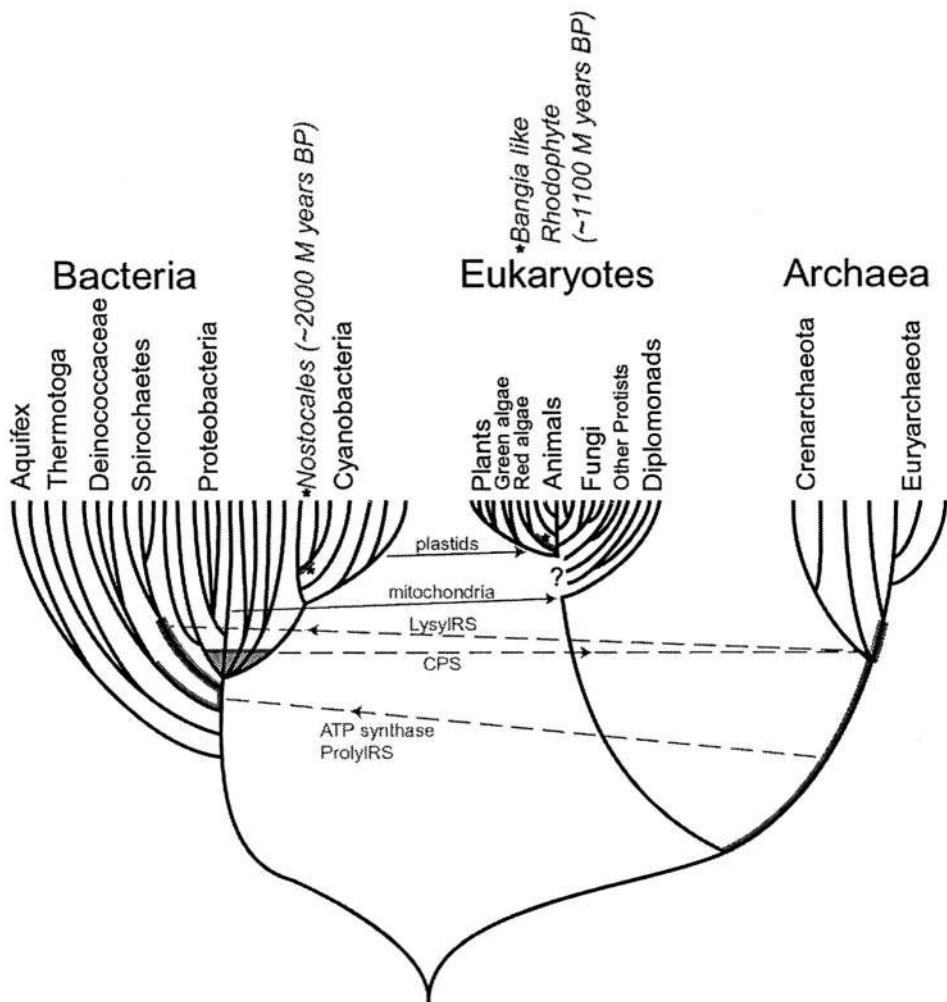


Figure 1. Horizontal gene transfer against a background of primarily vertically inherited genes. The tree depicted represents a consensus view of the three domains. Individual gene trees show that transfers have occurred from the Archaea (Lysyl tRNA synthase (Ibba, et al., 1997)) or the ancestors of the Archaea (ATP synthase (Hilario and Gogarten, 1993), prolyl tRNA synthase (Gogarten, et al., 1999, Olendzenski, et al., in press)) to certain lineages in the Bacteria. Genes from the Bacteria (carbamoyl phosphate synthase (CPS) (Gogarten, et al., 1998)) have also been transferred to the Archaea. Additionally, the plastids and mitochondria of Eukaryotes have evolved from bacterial symbionts. Phylogenetic positions and dates for Bangia-like rhodophytes (Butterfield, et al., 1990) and the akinete forming cyanobacteria (Golubic, et al., 1995 and A.Knoll, pers. communication) are given to provide a correlation with the early fossil record. The branch lengths in this tree do not reflect time, rather they were chosen to approximate the number of substitution events in conserved protein coding genes. One explanation for the long basal branches might be higher substitution rates during early evolution. Gene transfer events can be used to correlate events in the different portions of the tree, and they provide information as to the sequence of evolutionary events. For example, bacterial CPS genes present in the Euryarchaeota but not in the Crenarchaeota suggest that this transfer postdates the divergence of these two archaeal lineages.

genes with other Bacteria than with less related groups (e.g. Archaea). The same statement would be true for other taxonomic categories: e.g., alpha-purple bacteria are more similar to other alpha-purple bacteria because they more frequently exchange genes with other alpha-purple bacteria than with beta, gamma or delta-purple bacteria. Under this extreme interpretation the recognized taxonomic categories would be created exclusively through a gradient in the frequency of HGT.

A crucial parameter is the steepness of the gradient with which HGT-frequency drops when considering less “related” species. If HGT-frequency occurs on a shallow declining gradient from more closely related species to less closely related species, the present taxonomic categories (including the domain level) could be exclusively determined by horizontal transfer frequency. Levels of transfer would decrease among more distantly related lineages, with fewest occurring between the domains. The degree of relatedness could be defined as the degree of similarity between two genomes, as the distance in a genome content tree, or as the distance in a 16SrRNA phylogeny – however, under the assumptions of a HGT frequency model, more “relatedness” does not correlate with more recently shared ancestry, but with higher HGT frequency. At first sight this definition might seem circular; however, the point is that relatedness as defined by the usual operational measures might be due exclusively to higher rates of HGT.

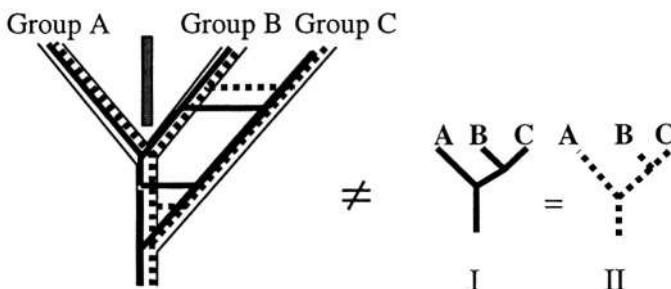


Figure 2. How horizontal gene transfer (HGT) may influence our ability to reconstruct organismal evolution. The gray and the dotted line represent two gene trees. The solid line represents organismal evolution (species tree). In this illustration Group A is a more recently branching taxonomic unit; however, after its separation from Group B, it occupies a niche, or develops a physiology that drastically reduces the frequency with which genes are shared with group B and C (gray bar). In contrast, Groups B and C continue to exchange genes. As a result, the molecular phylogenies of the separate gene trees (I and II) differ from the organismal phylogeny. According to this scenario, a taxonomy that is based on molecular data reflects HGT frequency. In particular the deep branching lineage in the gene trees is the one that less frequently participates in HGT, not one that evolved earlier.

Surprisingly, the rather heretical point of view depicted in Figure 2 cannot be easily discounted given the current data. To the contrary, it makes some interesting predictions that appear to agree with microbial taxonomy. Figure 2 depicts how the consequences of extreme HGT in the evolutionary history of organisms can lead to gene trees that do not truly reflect organismal phylogeny. Figure 2 details two

successive speciation events, the more recent one, between Groups A and B, giving rise to a lineage (A) that adapts to an ecological niche that prevents frequent HGT with the other lineages. As a result of HGT ongoing among the other lineages, Groups B and C will become more similar to one another, and the more isolated lineage (A) will be recovered as the deepest branching lineage in most molecular phylogenies (e.g. gene trees I and II). This isolation with respect to HGT could either be caused by an environment that is less conducive to HGT, or by physiological or genetic isolating mechanisms that evolve within the organisms. An example of the former could be seen in the adaptation to extreme thermophily: in a hot environment, naked DNA would be less stable and therefore transformation would be much less frequent. In a thermal environment, HGT could still occur via transfection and conjugation, but these mechanisms appear to be most effective only for close relatives. Is it a coincidence that the deep branching lineages in both the bacterial and the archaeal domain are occupied by extreme thermophiles? Could the Thermotogales be Gram positive Bacteria that adapted to an extremely hot environment, where the more recent inventions made in the bacterial domain were not available to them by HGT and thus did not reach them, and where the only genes available were mostly from extremely thermophilic Archaea? These occurrences would cause an organism such as Thermotoga to contain many Archaeal genes and to branch at the base of molecular phylogenies, as has been observed (Nelson, et al., 1999).

An example of an internal barrier toward HGT would be the invention, or adaptation, of transcription or translation features that make the machinery of the cell incompatible with incoming DNA. Could it be that the Archaea form a distinct, and in molecular phylogenies, deep branching lineage because they invented a different approach to transcription? With the discovery of the many differences between Archaea and Bacteria the idea that Archaea might only be a derived group of Bacteria had been rejected (Woese and Gupta, 1981), however, the idea that HGT-frequency might be the cause of the recognizable features used in taxonomy resurrects this possibility. The evolution of the RNA polymerase functioning in mitochondria provides an example of how drastic replacements in the transcription machinery can occur (Cermakian, et al., 1997, Rousvoal, et al., 1998, Schinkel and Tabak, 1989).

The hypothesis that taxonomy might be based exclusively on HGT-frequency and not on vertical inheritance makes predictions about the physiology and ecology of deep branching lineages. Clearly, the same characteristics are also compatible with the interpretation that microbial taxonomy is a natural systematic system, i.e. one based on shared ancestry. Under a vertical inheritance model of taxonomy, particular traits – e.g., living in isolated environments, having a different transcription machinery – are assumed to reflect historical facts: the last common ancestor was an extreme thermophile (e.g., Pace, 1991) or the environments on early earth selected for thermophilic organisms (e.g., Gogarten-Boekels, et al., 1995). The last common ancestor is also assumed to have had a less well established transcription machinery than modern day prokaryotes; therefore the two lineages descending from the common ancestor (Bacteria and Archaea/Eukaryotes) evolved a different machinery for regulation of DNA directed RNA synthesis (Marsh, et al., 1994). However, while both taxonomic models are compatible with the distribution of phenotypes on the tree of life, it is noteworthy that the model based on vertical inheritance would also be compatible

with the converse observations (e.g., if deep branching lineages were found to be living in the same environment as the majority of other microorganisms), whereas the HGT based taxonomic model, which predicts that deep branches contain organisms that live in environments that isolate them from HGT, would be falsified through finding different properties of the deep branching lineages (e.g. that they did not live in environments with inherent DNA instability or that they did not have incompatible transcription machinery). However, this slight predictive advantage is clearly insufficient to decide where between the two extreme scenarios actual microbial evolution occurs and has occurred in the past.

A strong argument presented against an important role of HGT in determining microbial taxonomy was the finding that phylogeny based on gene content were surprisingly similar to the phylogeny of rRNA (Fitz-Gibbon and House, 1999, Snel *et al.*, 1999, Tekaia *et al.*, 1999). Could it be that some genes, and in particular ribosomal RNA operons, were less prone to HGT? However, an alternative explanation for the congruence between gene content and ribosomal RNA phylogenies is that both the genome and the ribosomal RNA are mosaics which are formed through HGT. In both cases the similarity of both types of trees might mainly reflect HGT frequency and not vertical inheritance.

3. Genome and Gene Mosaicism

The horizontal transfer of genes and their complete uninterrupted incorporation into the genome of the recipient, lead to a mosaic genome where different parts of the genome reflect different histories. The potential difference between a molecular or gene tree and the organismal evolution has been widely recognized; the latter is often assumed to be net-like or reticulate (e.g., Gogarten, 1995, Hilario and Gogarten, 1993). However, genes are not immutable units of inheritance, and even at the gene level, evolution can be reticulate. If multiple identical or similar copies of a gene are present in the same cell, gene conversion events can tend to make the two copies of the gene more similar to one another (Gogarten and Olendzenski, 1999). These conversion tracts, i.e., the stretches of genomic information that are copied from one gene to another, are usually in the range of only a few hundred nucleotides (Betran *et al.*, 1997, Sweetser *et al.*, 1994, Yang and Waldman, 1997) - much smaller than the lengths of a typical gene. The result is that genes do not evolve as a whole, but rather, different parts of genes can have different histories.

Many instances of apparently independent and parallel gene duplication events (for example in interferon and chaperonin evolution) might be due to gene conversion events that result in copies within one lineage being more similar to one another than to the orthologues in the different lines of decent (Archibald *et al.*, 1999, Gogarten and Olendzenski, 1999, Gogarten *et al.*, 1992). Only in a few cases does other evidence provide support for gene conversion events. For example, the less conserved promoter sequences of the different interferon genes retain their functional specificity even though the protein coding parts of the genes are largely homogenized (Sick *et al.*, 1998). In Archaea a partial gene conversion event has been detected for one of the chaperonin subunits (Archibald *et al.*, 1999). Additionally, Goodman and

collaborators have been able to map conversion tracts in globin evolution (Fitch and Goodman, 1991).

The frequency of recombination between a gene obtained by HGT and a homologue already present in the genome depends on several factors. These include the presence of at least one sequence that allows homologous recombination, the time over which the homologues coexist in the same cell; and the number of copies of each of the genes in the genome. Transfer between closely related organisms will often result only in a couple of hundred nucleotides being replaced in the genome of the recipient because restriction enzymes chop down the incoming DNA and similarities in sequences between donor and recipient organisms provide many possible recombination points (McKane and Milkman, 1995). When considering populations within a species or closely related species, intra-gene mosaicism can be expected to be the rule rather than the exception. The impact of this recombination between nearly identical gene versions on the reconstruction of gene trees, however, will be rather limited because the parts that form the mosaic have nearly identical histories. The recombination between genes from different genera, families or phyla will have a more dramatic impact on phylogenetic reconstruction.

4. HGT and Mosaicism of RNA genes

Protein coding genes are mainly under selection for the amino acid sequence they encode. Because of the redundancy of the genetic code, two sequences can diverge in their nucleotide sequence but retain the same encoded function. If a protein coding gene is transferred between divergent organisms, the new copy either will replace the old gene, or both might be retained because the two homologues have acquired some different functionality. Recombination between the two copies, while detected in a few instances, is expected to be a rare event because of the divergence in such protein encoding sequences.

In contrast, ribosomal RNAs function on the RNA level. Some parts of rRNA sequences are universally conserved. This high level of conservation at the nucleotide sequence level is the reason that environmental PCR is so successful for rRNA sequences. These conserved regions also might allow for homologous recombination between different copies of the rRNA coding genes. Other factors that are expected to favor recombination of rRNA genes are the presence of multiple copies per genome; the compatibility *in vitro* and *in vivo* of rRNA genes from one organism with the cellular machinery of another; and the frequency with which the translation machinery is a target of antibiotics.

One of the justifications for using rRNA genes as markers for microbial taxonomy is that they were believed to not undergo horizontal gene transfer (Woese and Fox, 1977). Due to their central role in translation and the complexity of the ribosome, it was traditionally thought that the likelihood of an organism being able to use an rRNA from another organism was very low. However, recent studies have shown that rRNA genes *can be* transferred (replacing the native complement) in the lab, and *have been* transferred in nature (e.g., (Asai *et al.*, 1999, Yap *et al.*, 1999). Furthermore, Asai *et al.* (1999) managed to substitute a domain of yeast 28SrRNA for a domain of *E. coli* 23S rRNA without drastic effects on viability.

Although gene conversion tends to homogenize the different members of a gene family, several cases of divergent rRNA encoding operons present in the same genome have been reported. For the present discussion the most striking is the case of *Thermomonospora chromogena* (Yap, et al., 1999). The rRNA operons present in this organism provide a snapshot of homogenization in progress. Of the 5 operons sequenced in this actinomycete, 4 are very similar to each other, whereas the fifth is more similar to the rRNA operons from *Microbispora bispora* (Wang, et al., 1997).

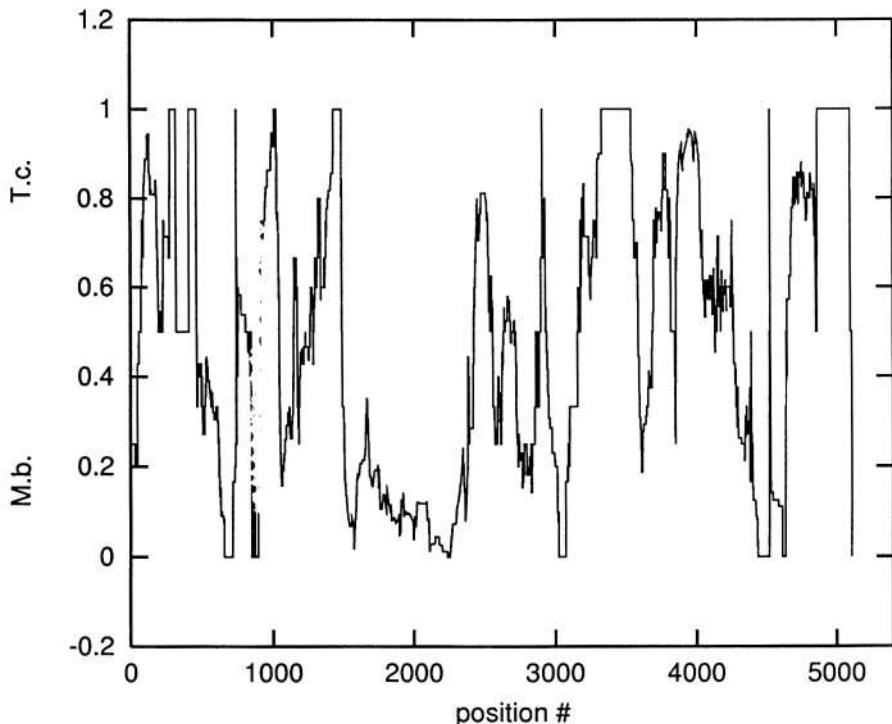


Figure 3. Comparison of rRNA operons from *Thermomonospora chromogena* with those from *Microbispora bispora*. The sequences analyzed are from Wang, et al., 1997, and Yap, et al., 1999. The graph depicts the relative identity between the one "atypical" *T. chromogena* rRNA operon with the remaining five *T. chromogena* rRNA operons and with the four from *M. bispora*. The ordinate gives the relative similarity of the atypical rRNA operon for a sliding window of 90 nucleotides. Relative similarity is calculated as the proportion of nucleotide differences between the atypical operon and other *T. chromogena* operons to the total number of observed differences between the atypical operon and all operons in the dataset. Relative similarity values above 0.5 indicate that the atypical *T. chromogena* rRNA operon is more similar to the remaining *T. chromogena* rRNA operons while values below 0.5 indicate that the atypical *T. chromogena* rRNA operon is more similar to *M. bispora* operons. For identical regions the value of the relative similarity is set to 0.5. While on the whole the atypical *T. chromogena* operon is more similar to the *M. bispora* operon, this atypical operon clearly represents a mosaic formed from frequent recombination events between the different rRNA operons present in *T. chromogena*.

Analyses by Yap et al. indicate that this operon is still expressed, and that while overall more similar to the *Microbispora bispora* operons, gene conversion events between the other *Thermomonospora chromogena* operons and the one transferred from *Microbispora bispora* have resulted in a mosaic rRNA operon (Figure 3). With additional gene conversion events occurring over evolutionary time, this mosaicism can be expected to become more and more fine-grained until it is no longer recognizable. Despite the difficulty in detecting these events in the majority of organisms, these findings show that rRNA encoding genes are not immune to HGT, and that after HGT has occurred, gene conversion events can produce mosaic rRNA genes.

5. Congruence Between Gene Content Trees and rRNA Phylogenies: Evidence for Genome Mosaicism?

Whole genome phylogenies (Figure 4) agree with traditional rRNA phylogenies to a surprising degree (Fitz-Gibbon and House, 1999, Snel *et al.*, 1999, Tekaia *et al.*, 1999). Indeed, a strong argument against an important role of HGT in determining microbial taxonomy was the finding that phylogeny based on gene content were surprisingly similar to the phylogeny of rRNA (Fitz-Gibbon and House, 1999, Snel *et al.*, 1999, Tekaia *et al.*, 1999). The main argument is that some genes, and in particular ribosomal RNA operons form an immutable core portion of a genome that are less prone to HGT. However, an alternative explanation for the congruence between gene content and ribosomal RNA phylogenies is that BOTH the genome and the ribosomal RNA are mosaics which are formed through HGT. Therefore the congruence between gene content based phylogenies and rRNA phylogenies could be due to the mosaic nature of both the genome and the rRNA. If this mosaicism is generated by the same process, HGT with a graded frequency distribution, the resulting phylogenies are expected to be similar. In this case, the similarity of both types of trees might mainly reflect HGT frequency and not vertical inheritance.

The original analyses showing congruence between genome content and rRNA phylogeny were preformed using a limited number of genomes available at the time (e.g. Figure 4, left). Note that the Crenarchaeote is the most deeply branching among the Archaea while *Thermotoga* and *Aquifex* are the deepest branching Bacteria. It should be noted however, that as more genomes are added, the agreement between rRNA phylogenies becomes less. For example, in Figure 4, right, this genome content tree, constructed using the majority of complete genomes currently available, shows *Halobacterium* as the deepest archaeal branch. This result is interesting, given that the other Archaea sequenced are all thermophiles, and would be expected to be able to exchange genes with each other, whereas *Halobacterium* is excluded from these environments. Rather it lives in an environment where it would be more likely to encounter bacterial species. Clearly as more genome data becomes available, our views may change, but for the moment, genome content trees and their overall similarity to rRNA phylogenies are compatible with a model of evolution dominated by HGT rather than vertical inheritance. Additionally, genome content trees are not robustly ‘tree-like’. The discrepancy between the matrix of pairwise distances and the distances in the tree is relatively large, indicating that the resulting tree does not fit the data very

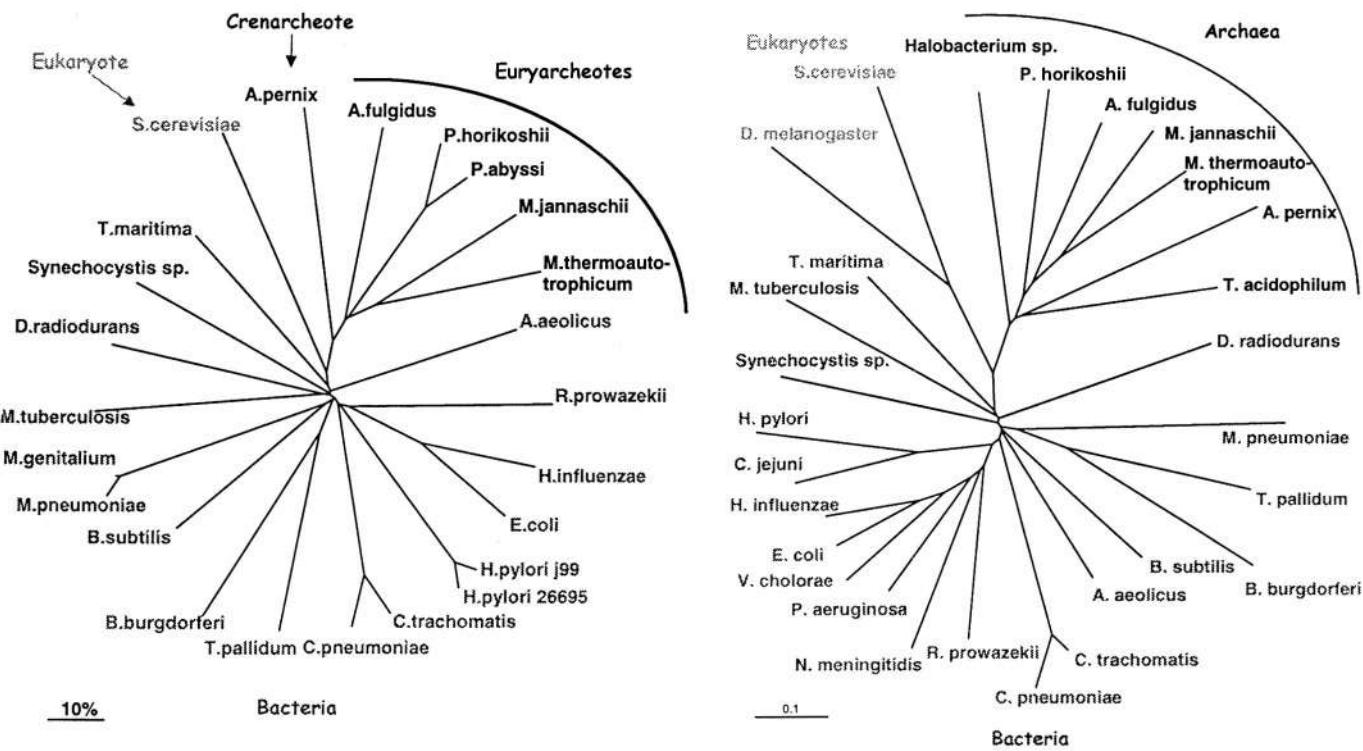


Figure 4. Genome content trees. The tree on the left was calculated from a distance matrix based on the asymmetric distance measure (100 minus the percent of shared genes). Genes were considered as shared, when their BLAST score had an E-value below 10^{-4} . The distance matrix was calculated using Robert L. Charlebois' NeuroGadgets site at <http://ngi.bio.uottawa.ca/>. The tree on the right was constructed using 28 complete genomes from the Microbial Genomes Page @ NCBI. Every genome was BLASTed against every other genome with an E-value cutoff of 10^{-4} . The ratio of the number of ORFs which had a BLAST match to the total number of ORFs in a query genome was calculated and the distance between two genomes was defined as 1.0 minus that ratio. All distances were compiled into a distance matrix and the tree was reconstructed using the FITCH program from the PHYLIP package (<http://evolution.genetics.washington.edu/phylip.html>). Compare also the analyses of (Teklaia, et al., 1999, Snel, et al., 1999, Fitz-Gibbon and House, 1999).

well. The branching patterns depicted are very deep, and the overall topology is approaching that of a star more than a bifurcating tree. This could be a function of the fact that the genomes compared are very distantly related, but it also may be another indication that genome evolution is not tree-like, but rather fits a more reticulate pattern.

Although the existence of HGT in nature is very well established, the effect that it may have on our ability to reconstruct microbial taxonomy remains controversial. We have presented an extreme view of how HGT affects molecular phylogenies and how it can skew our interpretation of taxonomy. To date, the available data is compatible with a view of evolution dominated by vertical inheritance or horizontal gene transfer. The truth may lie somewhere in between both of these extremes. In any case, it is important to understand what processes are shaping the molecular phylogenies we use as indicators of microbial taxonomy and evolution, and to adjust our interpretations to take into account all possibilities.

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THE COMMONALITY OF CYANOBACTERIAL ENDOSYMBIOSES DOES NOT SUPPORT THE ENDOSYMBIOTIC THEORY FOR ORIGIN OF EUKARYOTIC ORGANELLES

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1. Introduction

The cyanobacteria (blue-green algae) are found in nearly all ecological niches. They grow in hot springs water, fresh water, and the oceans. They are also found extensively in the soil. They are a major part of biological mats and ancient and modern stromatolites. According to Desikachary (1959) there are about 2,000 species. They are indeed ubiquitous. The cyanobacteria were one of the first groups of organisms on Earth (Schopf 1999) and probably were the main contributors to the production of oxygen in our atmosphere.

It is, however, the purpose of this chapter to look at blue-greens, which have developed an endosymbiotic relationship with another organism. It is assumed that these relationships are mutualistic, where each organism derives a benefit from the association. It is difficult to determine this with certainty in all cases, and some of these associations may actually be parasitic. Parasites are very common in the biological world, and some report that there are more species of parasites than there are of free living organisms (Windsor 1998). Some, however, say that parasitism is a form of endosymbiosis. It is the intent in this chapter to summarize the cyanobacterial endosymbiotic systems and then to see how this scenario fits into evolutionary theory and the biological world. It is not the intent in this chapter to provide great detail in regard to these endosymbionts as this in one chapter, and Rai (1990) has produced a very good multi-authored book of 253 pages on this topic. Chapters on this topic are also found in the various books on blue-greens such as the Blue-Green Algae (Fogg *et al.* 1973). Older volumes such as the one edited by Henry (1966) provide a good basis of understanding symbiosis. More recent volumes such as the one edited by Goff (1983) and Ahmadjian and Paracer (1986) provide excellent coverage of the topic. The volume by Smith and Douglas (1987) also provides very good coverage. In this chapter of cyanobacterial endosymbiosis, I will present only sketchy information on the variety of types because as I have mentioned the details are available from a number of different sources. I will try to bring in work we have done which is mainly unpublished.

2. Cyanobacterial Endosymbioses

2.1. LICHENS

These are symbiotic relationships between fungi and algae including the cyanobacteria. There are about 15,000 to 20,000 known species of lichens with about 520 having both a blue-green and a green component (Rai 1990). About 1,600 species of lichen have a blue-green photobiont. The fungi are Ascomycetes, Basidiomycetes and Fungi Imperfection, and a wide variety of blue-greens. Ahmadjian and Paracer (1986) provide a list of blue-green species. The fungal component rarely penetrates into an associated blue-green. Instead the alga is found in the capsular material or just in certain layers of the lichen.

The number of associations in the lichen is, as pointed out above, very large. The number of papers published on lichen-blue-green-symbiosis is also very large. Some of the papers using electron microscopy are very good. However, many are not very informative. Fixation in many cases is so poor little can be ascertained in the blue-green. In other papers such low magnification is used that no details can be observed. The investigators would have been as well off if they had used only a light microscope.

It is not the intent of this brief review to cover the topic in depth. This has been done many times and in a very fine fashion. The chapter by Ahmadjian (1982) is very well illustrated, as is his earlier book (Ahmadjian 1967). Adams (2000) provides a very nice chapter on symbiotic interactions in the book, *The Ecology of Cyanobacteria*. Schenk (1992) in Chapter 22 in the Prokaryotes discusses in excellent detail Cyanobacterial symbioses. His table 2 provides a good summary of many blue-green symbioses.

We (Spector and Jensen 1977) have investigated the ultrastructure of the lichen *Leptogium cyanescens* from nature and the blue-green in culture. Scanning electron microscopy showed that the isidia break at the point of contact with the thallus. This suggests a possible dehiscence mechanism. The isidia contain algal and fungal cells interdispersed in an amorphous matrix. The ultrastructure of the lichenized and cultured *Nostoc commune* was compared. A tubular array was found in the cells of the phycobiont. Cultured cells contained the tubular array, paracrystalline structures, and membrane-bounded inclusions.

Many others have investigated the fine structure of the blue-green phycobiont of lichens as mentioned above. This brief review will mention only a few studies. Boissière *et al.* (1987) cultured the blue-green from 4 *Peltigera* isolates and found they were all *Nostoc punctiforme*. The isolate from *Collema tenax* was *Nostoc commune*. Koriem and Ahmadjian (1986) isolated and grew in culture the *Nostoc* from *Peltigera canina*, *P. rufescens*, and *P. spuria*. In culture the blue-green had a thicker gelatinous sheath, few cyanophycin granules and polyhedral bodies. They found more polyglucoside granules and more thylakoid whorls than in the blue-green in the lichens. Bergman and Hällbom (1982) in a similar study of the isolated *Nostoc* from *Peltigera canina* found a similar organization. They did, however, report akinetes in the blue-green for the first time. In the aquatic lichen *Hydrothyria venosa* the *Nostoc sphaericum* cells had no plastoglobuli in the summer but many in the winter (Jacobs and Ahmadjian 1973). They also observed polyphosphate bodies in the blue-green.

We can see from the above that again a specific fungus and a specific blue-green form the interaction to produce the lichen. Dick and Stewart (1980) show fimbriae on the blue-green in the lichen *Peltigera canina*. They suggest they may be involved in motility and specificity to establish the association.

The lichen *Peltigera aphthosa* has the green alga *Coccomyxa* in the main thallus (Rai *et al.* 1981). However, a *Nostoc* is located in superficial pockets called cephalodia. It also fixes nitrogen.

2.2. CYCADS

Cycads are part of the gymnosperm group of plants. There are about 150 cycad species, and at least half have been shown to produce coraloid roots with the associated blue-greens, generally *Nostoc* but sometimes *Anabaena*, *Calothrix* or perhaps some other nitrogen fixing cyanobacterium.

The blue-greens are found in a zone in the outer part of the root cortex of the coraloid roots. They occupy mucilage-filled intracellular cavities (Smith and Douglas 1987). There are nine genera of cycads and about 90 species of which only 30 contain cyanobacteria (Smith and Douglas 1987). Costa *et al.* (1999) has shown the *Nostoc* diversity in several cycad species. In a single coraloid root it is all the same strain, but a single plant may have different strains of *Nostoc* in different coraloid roots. Grabbelar discusses the cycad-cyanobacterium symbiosis in detail in the chapter in the book by Rao and Rodriguez-Barueco (1993). The *Handbook of Symbiotic Cyanobacteria* by Rai (1990) also has a chapter on this association by Lindblad and Bergman.

The ultrastructure of the coraloid roots blue-green association has been investigated many times. In many studies there seemed to be no difference in the blue-green grown naturally or in the coraloid roots. In some cases differences were observed. Caiola (1980) found many of the *Nostoc* cells had many cyanophycin granules. Grille (1975) found that the blue-green in the coraloid roots of *Dioon edule* had many plastoglobules, many cyanophycin granules, and thylakoids parallel to the cell wall. The organism in culture had no plastoglobules or cyanophycin granules and the thylakoids had various orientations.

In *Encephalartos arenarius* a *Nostoc* sp. has many plate filament arrays (Joubert *et al.* 1989). They are also found in the blue-green in *E. transvenosus* and *E. woodii* but not in the blue-green in *E. arenarius* (Joubert *et al.* 1989). In the *Nostoc* sp. in *E. transvenosus* a rod-like inclusion was also observed. None of the blue-greens has phycobilisomes. The plate filament array has the same morphology as the one described by Jensen and Bowen (1970) in *Nostoc pruniforme*. The *Nostoc* in *E. transvenosus* also had gas vesicles. The *Anabaena* in *Cycas revoluta* has many cyanophycin granules in the winter but few in the summer. The blue-green also had many polyphosphate bodies (Obukowicz 1981).

The blue-green in the coraloid roots of *Encephalartos altisteinii* had many cyanophycin granules and a crystalline body (Caiola 1975). The crystalline body has the same morphology as the bodies found in several blue-greens (Jensen 1985).

The ultrastructure of the blue-green in the various cycads seems to be quite variable. Most have numerous cyanophycin granules, but this may depend on the time of year the organisms were fixed. Most have the same inclusions as free living blue-

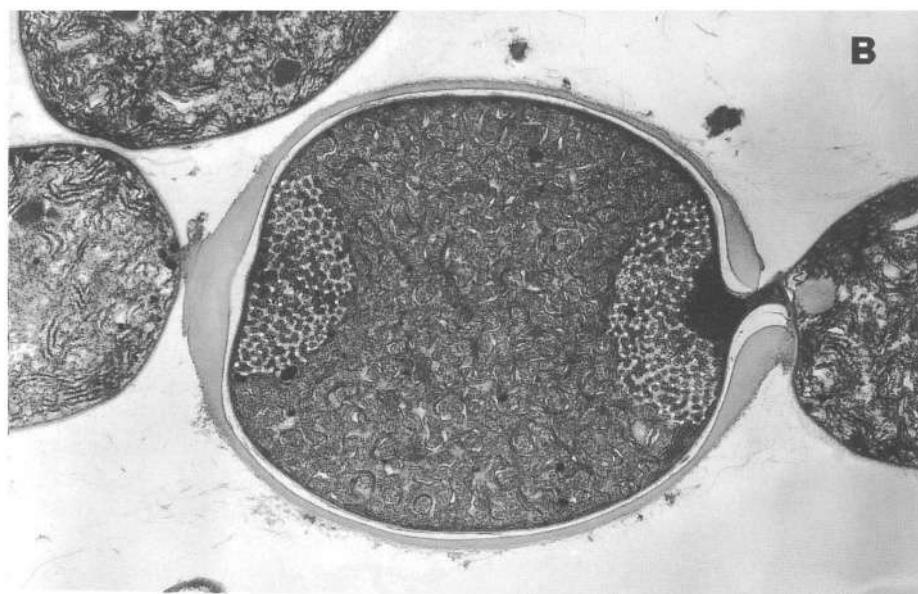
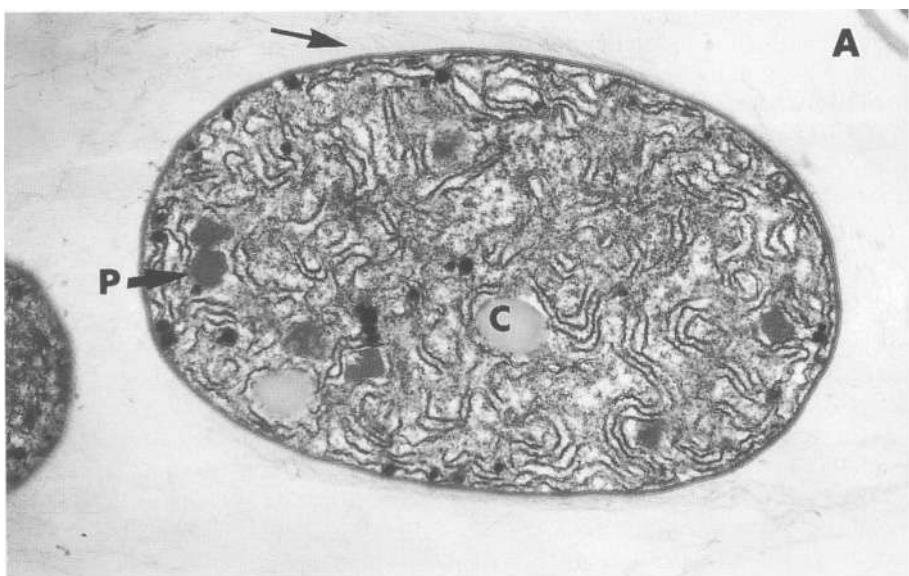


Fig. 1. (A) Thin section of *Anabaena azollae* vegetative cell. Visible in the cell are polyhedral bodies (P), cyanophycin granules (C), thylakoids and lipid bodies (small electron dense). On the surface of the cell (arrows) can be seen what appear to be fimbriae external to the cell wall. 20,000 x. (B) Thin section of a heterocyst in *Anabaena azollae*. The thickened cell wall can be seen and the thylakoid arrangement adjacent to the pore. 18,000 x

greens. The lack of reports on polyphosphate bodies is probably due to the methods employed. Epoxy resins do not penetrate the bodies, so they tend to fall out of the sections leaving a hole. Few workers recognize this and probably ignore the blue-green cells with holes.

The infection process of the blue-green to enter the coralloid roots has been much studied. Ahern and Staff (1994) present a very nice description of how the blue-green gets established in the cycad *Macrozamia communis*. It is not a simple process.

2.3. GUNNERA

The genus Gunnera, an angiosperm, has about 40 species, all of which have symbionts identified as *Nostoc punctiforme* (Smith and Douglas 1987). In this case the blue-green is located within the cells but has the host plasma membrane around it, like a phagocytotic vesicle (Fogg *et al.* 1973).

Events in establishing the endosymbiosis are well described by Johansson and Bergman (1992). Part of the process is that motile hormogonia move to mucilage secreting stem glands. Johansson and Bergman (1994) also present very nice electron micrographs of fimbriae on the *Nostoc* strains. This is probably part of the recognition phenomenon. *Nostoc punctiforme* seems to be the blue-green, which forms the association (Bergman *et al.* 1992). At the ultrastructural level the free living blue-green has the same morphology as the infective form. These authors provide a very good review of this endosymbiosis.

2.4. AZOLLA

Azolla is a genus of aquatic heterosporous ferns (Goff 1983). Generally 6 species are recognized. The leaves of *Azolla* species develop a cavity, and filaments of *Anabaena* become associated with each developing leaf in the area of the forming cavity. Peters and Calvert (1983) present details of the development of the Azolla ferns in relation to the *Anabaena*. Duckett *et al.* (1975) have studied the ultrastructure of *Anabaena azollae* and the multicellular hairs in the leaf cavities, which contain the blue-green. The blue-green has basically the same structure as free living *Anabaena* species (Duckett *et al.* 1975). It has also been shown by electron microscopy that *Azolla* (Nierzwicki-Bauer *et al.* 1989) produces the inner envelope that confines the blue-green in *Azolla*. The envelope keeps the blue-green in association with *Azolla* hair cells. Kobiler has reported a lectin from *Anabaena azollae*, and this is probably the recognition system, which allows the specific infection in *Azolla* (Kobiler *et al.* 1982). Bergman *et al.* (1992) provides a fine review of the *Azolla-Anabaena* symbiosis.

2.5. MARINE SPONGES

Thirty-eight genera belonging to Calcarea and Desmospongia have blue-greens located in cells or between cells (Rai 1990). In this case the blue-green may shield the sponge tissue from damage by high light, contribute to the organic nitrogen needs of the sponge, and/or provide fixed carbon to the host. *Oscillatoria spongiae* is an endosymbiont in the sponge *Dysidea herbacea*. The blue-green has the usual inclusions but also has a spheroid body. The bodies have a darker central region from

which radiate a large number of rod-shaped arms. They have been called stellar bodies (Berthold *et al.* 1982). This inclusion has not been observed in any other blue-green. The sponge *Verongia aerophoba* has an extracellular blue-green (chroococcales); it is often phagocytosed and digested by the sponge. The blue-green is unusual in that it has a single thylakoid arranged in a spiral of one or two turns (Vacelet 1971). Deep in the sponge tissue the thylakoid has two to four turns. In the sponge *Ircinia variabilis*, *Aphanocapea feldmanni* is found in the mesophyll or inside cells while *A. rasaigellae* is found extracellularly or in cavities of the mesophyll.

We can see that cyanobacterial sponge associations are quite common with a variety of blue-greens being the endosymbiont.

2.6. LIVERWORT AND MOSSES

Nostoc sp. is found in two genera of liverwort *Anthoceros cavicularia* and *Blasia*. The blue-green occurs in mucilage filled cavities of the sporophyte. Gorelova *et al.* (1996) investigated the ultrastructure of the blue-green in the moss *Blasia pusilla* both in-situ and in culture. They report a high degree of heteromorphism is manifested in both cases. In the *Nostoc* sp. in *Anthoceros punctatus* again in-situ and in culture a 4 to 5 fold increase in heterocysts was found in-situ (Enderlin and Meeks 1983). It is interesting that the *Nostoc* sp. in *Blasia pusilla* possess a vesicular crystalloid (Baulina and Gorelova 1996), which is morphologically identical to those reported by Jensen and Baxter (1981; Jensen 1985). Rodgers and Stewart (1977) in an extensive study of the blue-green *Blasia pusilla*- *Anthoceros punctatus* symbiosis found *Nostoc* colonies in mucilaginous cavities on the undersurface of the gametophyte. The blue-green *Nostoc sphaericum*, when free living, has 3-6% of its cells as heterocysts. However, in the association with *Blasia* it increased to 30% and in *Anthoceros* it was 43%. They also showed that nitrogen fixation benefits the host and provides a better environment for the blue-green. Stewart *et al.* (1983) provides a good review of the *Blasia Anthoceros* endosymbiosis. They present very nice electron micrographs showing fimbriae on the blue-greens. This again is probably the recognition system to establish the association.

Nostoc sp. are also reported to occupy water-filled non-photosynthetic hyaline cells of *Sphagnum lindbergii* and *Sphagnum riparium* (Smith and Douglas 1987). In England a *Sphagnum* sp. also sometimes contains the blue-green *Haplosiphon* sp. (Granhall and Hofsten 1976).

We can see that the blue-greens have developed many associations with liverworts and mosses in their evolutionary history. The motile hormogonia of the blue-greens and most likely the recognition system through fimbriae has evolved to establish this association.

2.7. DIATOMS

Several diatoms have been shown to have blue-greens as endosymbionts. The diatom *Rhizosolenia styliformis* has *Richelia intracellularis* (Smith and Douglas 1987). The diatom *Rhopalodia gibba* also has a unicellular blue-green in its cytoplasm (Smith and Douglas 1987). *Rhopalodia gibba* has what appears to be nitrogen fixing blue-green in its cytoplasm (Floener and Bothe 1980). The diatoms *Hemiaulus haukii* and *H.*



Fig. 2 *Nostoc* sp. from the liverwort *Blasia*. Some of the cells had tabular thylakoids (T). 51,000 x

membranaceus have the blue-green *Richelia* in about 80% of their cells. Again the blue-green fixes nitrogen, which is shared by the host.

An interesting association is found in *Solenicola setigera*, a heterotrophic protist found only with the centric chain forming diatom *Leptocyliners mediterraneus*. The diatom has *Synechococcus* in large numbers embedded in the matrix covering the cells (Buck and Bentham 1998). This association should be examined more thoroughly.

2.8. ASCIDIANS

Ascidians are colonial animals, which are sessile filter feeders that inhabit the coastal environments of warm seas. In about 20 species of Ascidians Prochloron has been reported (Smith and Douglas 1987). Prochloron is a blue-green like organism, which live either within the tunicine matrix or in the common excretory tubules. The blue-green has most of the features of other cyanobacteria but lack phycobilins and phycobilisomes (Swift 1989). They also have both chlorophyll a and b, as do other free-living prochlorophytes. Swift (1989) and Cox (1986) provide good fine structure observations on *Prochloron*. They have in addition to regular blue-green inclusions those, which occur in a few isolates including gas vesicles and paracrystalline arrays. Thinh *et al.* (1985) in another study at the electron microscope level report again paracrystalline inclusions (rods), crystalline inclusions and a vesicular crystalloid (my interpretation). Similar inclusions have been reported in other free living blue-greens, which have chlorophyll a only (Jensen 1996).

Other prochlorophytes, which are free living, have been reported. Golecki and Jürgens (1989) studied the ultrastructure of *Prochlorothrix hollandica* and reported the usual blue-green inclusions including gas vesicles. No phycobilisomes were observed.

Prochlorococcus is a marine blue-green with both chlorophyll a and b (Partensky *et al.* 1999). Some of these isolates have phycoerythrin. The cells are very small being only 0.5 to 0.7 μm across. They are globally significant produces.

It would appear that chlorophyll a and b containing blue-greens are yet another adaptation to occupy niches on Earth. The prochlorophytes are cyanobacteria. They are not links to any groups.

2.9. ECHIRUOID WORMS

In the worms *Ikedosoma gogoshimense* and *Bonellia fuliginosa* blue-greens have been reported in the subepidermal connective tissue (Rai 1990). Little else is known about the relationship.

2.10. OTHER ENDOSYMBIOSSES

Geosiphon pyriforme is a fungus with a true endosymbiotic blue-green inside the cells, probably in a symbiosome (Schüßler *et al.* 1996). The true nature of the relationship has not been thoroughly studied. Schüßler *et al.* (1994) also reports that, in addition to the blue-green, a bacterium is present in the cytoplasm of the fungus.

With the present definition of endosymbiosis we must also accept that some mats, crusts and biofilms are cyanobacterial-endosymbioses. The Antarctic microbial mat described by Vincent *et al.* (1993) has blue-greens as its main component. Also woven

into the mat are other bacteria like *Thiocapsa*, *Chlorobiaceae* and *Chloroflexacae*. On the other hand a microbial mat in a temperate climate in a saline-alkaline lake (Goodenough Lake, British Columbia) had again blue-greens as the main component but also purple bacteria and many other bacteria (Schultze-Lam *et al.* 1996). In another study of microbial mats Caumette *et al.* (1994) in the hypersaline ponds of Mediterranean blue-greens in the salterns occupied the most dominant layer. Also present were purple sulfur-oxidizing bacteria. Ward *et al.* (1998) reviews the microbial biodiversity of hot springs cyanobacterial mat communities. Cyanobacteria are generally the dominant bacterium, but a wide variety of other bacteria make up the mat.

In desert crusts cyanobacteria exopolysaccharides are the structural basis (Mazor *et al.* 1996). They are found on surfaces of soil throughout the world from the tropics to polar regions and arid deserts.

With our present definition of endosymbiosis we could also consider the mix of blue-greens, diatoms and green algae reported in Aldabra, Western Indian Ocean as endosymbiosis (Braithwaite *et al.* 1989).

Another large group of suspected cyanobacterial endosymbionts are the organisms containing cyanelles. The most widely studied is *Cyanophora paradoxa* and *Cyanidium caldarium*. *Cyanophora paradoxa* cyanelles DNA has recently been studied, and it was found to be 130 kb, which is similar to a plastid (Schlichting and Bothe 1993). A blue-green has a tenfold larger genome. *Cyanidium caldarium* has been investigated extensively by Seckbach and associates (Seckbach and Frederick 1981, and papers cited therein). At present it appears that the organism is a red alga with a cyanelle.

Glaucozystis nostochinearum has also been much studied. Again the true nature of the cyanelle is not known. The cyanelle does appear to have some blue-green inclusions but also some other inclusions such as granular deposits at the end of the cyanelle, which have not been reported in blue-greens (Hall and Claus 1967; Robinson and Preston 1971). The protists *Cyanophora biloba* and *C. paradoxa* also have cyanelles which are reported to be between a blue-green and a chloroplast (Kugrens *et al.* 1999).

Endolithic cyanobacteria are well known and common. *Plectonema terebrans* bore into calcareous rhodophytes and become established in cell walls of live and dead organisms (Ghirardelli 1998). This phenomenon occurs in the lower intertidal zone in the Gulf of Trieste (Northern Adriatic Sea, Italy). Kies (1980) provides an excellent review of cyanelles or endocyanosomes including several not mentioned here.

All endo protozoan and algal systems examined so far for symbiotic algae or cyanobacteria are never situated free in the host cytoplasm (Reisser 1986). They are always in perialgal vacuoles. When it is occasionally reported that they are in the cytoplasm, we should look closely at the quality of the electron microscopy in the study.

3. Summary

In science we must use what data we have in any area to try to understand the topic. On a set of information we should build our theories. If we go too far beyond the data just to make a good sounding story or make some look innovative, we will have switched from science to Aristotle's type of reasoning to arrive at "truth" in an area. It is useful to run "thought experiments," but they must remain in the realm of the data that is available on a topic. We must remember that we generally do poorly in predicting the future. No one can accurately predict the weather even for a year ahead. In science we should be cautious when we go far beyond solid data. Beyond the data generally becomes mere speculation. In this regard, where is the endosymbiont theory? It is certainly imaginative, elegant and really neat. However, if we want to stay in the scientific arena we must insist that we look at the data in this regard from all points of view. We must consider if it is possible that a phagocytotic event by an unknown organism could have occurred. The proposed five phagocytotic events must be considered, and again we must accept and work around the little data we have. To speculate on events that seem to have occurred about 1.2 billion years ago may just be that, speculation. However, let's ask a few questions and look at the data we have. Can any present day prokaryotic carry out phagocytosis or pinocytosis. The answer based on the data is **no**. Prokaryotes generally have a cell wall so this scenario is not possible. Jensen (1996) removed the cell wall, using lysozyme, from a variety of prokaryotes and found none would take up colloidal gold. On the other hand numerous protozoa readily took up the colloidal gold and, of course, other items. We have little data on this topic, but we must construct theories only on the basis of existing data. Uptake of anything by present day prokaryotes does not occur.

Another approach is to compare DNA sequences from mitochondria and plastids with various prokaryotes. This is a difficult task. A blue-green has a genome of about 2.7×10^6 bp (Chen and Widger 1993), while a plastid has a genome of about 120-140 kbp (Howe *et al.* 1998). This means that a plastid has only about 10% of the DNA that a blue-green has. We must keep in mind that blue-greens and chloroplasts both carry out oxygenic photosynthesis using chlorophyll a, as does a chloroplast. A few cyanobacteria also have chlorophyll b, as do many eukaryotic photosynthetic organisms. We should and do find a lot of similarity between a blue-green and a chloroplast. They both do the same thing in this regard. However, with only 10% of the amount of DNA in a plastid compared with a blue-green this is not good solid data. I feel we can draw very few conclusions on the basis of this data. We can only say with confidence that a blue-green has the code for everything needed for oxygenic photosynthesis and a chloroplast has some of the code for these biochemical pathways. We should not push our data too far beyond what it says. In spite of the overwhelming claims by the molecular map scientists, I do not see the data supporting any theory.

We have also probably gotten it wrong in regard to extant species being ancient. The fate of all species we must conclude is extinction. This holds for all species whether they are dinosaurs or microbes. When we look at the data, this can be easily seen. All of the dinosaurs are only in the fossil record as are mastodons and wooly mammoths. It is easy to accept that for large organisms this is their fate. The question we wish to pursue is whether this holds for smaller organisms in the eukaryotic world

and does it hold for the prokaryotic kind of cellular organization. It will be my thesis that if the fate of a large organism is extinction the same should hold true for smaller organisms including single celled organisms and also the bacteria. All organisms which are extant today did not exist very far back into history, just as all the dinosaurs are gone I propose that all of the single celled forms including the prokaryotes of that period are gone into only the fossil record.

Let's take a closer look at this view by way of the data. As I have said above the recognition of this is no problem in regard to large organisms, which are well preserved in the fossil record. We could try to argue that for single celled eukaryotes this scenario did not occur. Why should a free living protozoa become extinct when we can see no real threat to its continued existence, that is food source, proper habitat and reproduction? When we look at the data we see that this is not the case. If we look at the protozoa in the Foraminifers we see that we have numerous fossil species, which may resemble but are not the same as extant species. Caron (1985) looked at Cretaceous planktonic Foraminifers as zonal marker species. He shows the marker species go about 10 million years, some much less. For another part of the Cretaceous most marker species become extinct in about 8 million years. One, however, was extant for 35 million years. If we look at another eukaryotic single celled group we see the same scenario. The diatoms, which are a very successful group today, have left because of their silicon dioxide walls, a good fossil record. Barron (1985) looked at diatoms in the late Cenozoic, Miocene to Halocene. The longest a species survived was about 17 million years with most being extinct in about 5 million years. We can see the fossil record is the source of the ecology rule "the fate of a species is extinction."

If we now look at prokaryotic cells should we expect a different scenario? I propose no. Cells are cells whether prokaryotic or eukaryotic. The bacteria species in the past were in the same kind of ecological habitat, as were the now fossil foraminifers and diatoms. There was no logical reason for them to become extinct. The diatoms only need nutrients and water but still they did not survive. We accept that the foraminifers and diatoms, which are extant, came into being as new species not more than about 10 million years ago. We should, based on this data, accept that all extant bacteria are new species also that did not exist several millions of years ago. The same forces have been acting on bacteria that acted on the other fossil species. Bacteria have four means of exchanging genetic material: conjugation, transduction, transformation and through plasmids. Eukaryotic cells have only one means of exchanging genetic information, which is of course meiosis. The above points to the fact that bacteria are probably more genetically variable than eukaryotic cells. This variation is what natural selection operates on. This means that new species will come into existence at a much more rapid rate than in eukaryotic cells, which must rely on meiosis and random mutations, which provide the variation for natural selection. The bacterial fossil record shows only the shape of the organism (body plan). This kind of record gives us no clue to what the organism was capable of carrying out, we can only speculate.

It is certainly a logical progression of thought based on the data we have to accept that all life forms today on Earth are of fairly recent origin. Even so called ancient species that are extant today are really not that ancient but of fairly recent origin. Raup (1991) discusses the fact that what appear to be living fossils is probably not correct

but based on a subjective impression. He further states that species are temporary with 10 million-year survival unusually long; most plant and animal species survive only 4 million years. Based on the data we have it is safe to say that none of the species we see today existed a hundred million years ago. When we compare organisms by whatever means, morphology, 16S RNAs, DNA, etc. we must keep this in mind. We must keep this in mind also when we program computers. It will give back only what we have instructed it to do. We can make many "trees", but are they valid?

We can see that the blue-greens have established many endosymbiotic associations. They have been shown to be a part of lichens from the lower Devonian, which is about 400 million years ago (Taylor *et al.* 1997). In spite of this long association no fungal cell or inclusion has become a part of a blue-green, and no cyanobacteria have become incorporated into a fungal cell. The same applies to all of the blue-green endosymbioses. If the endosymbiont thesis for the origin of plastids were correct, this would certainly have occurred in a number of organisms with which the blue-greens have established an association. For the origin of the eukaryotic cell we must accept that Darwin got it right by using the data available at his time that species originate by natural selection. Based on the data we have assumed that cells, whether prokaryotic or eukaryotic and their inclusions, evolve by natural selection. When a cell inclusion is varied probably due to a genetic change this set of inclusions is now in competition with the non-changed set of inclusions. A series of such changes could lead to the equivalent of a new species but in this case a new kind of cell. This is most likely the way a eukaryotic cell came into being, by gradual change in a prokaryotic cell following the Rule of Natural Selection until we had the new entity, the eukaryotic cell. How do we get new species? Darwin provides the data that it is slow and gradual until at some point we have a new species and the old species is now probably part of the fossil record. Nakamura's (1998) endomembrane theory for the origin of the eukaryotic cell should certainly be given serious consideration because it is based on data. Intracytoplasmic membrane structures are a regular domain in bacteria (Pinevich 1997). There is no reason why they would not be a part of prokaryotic cells that existed billions of years ago. Thus by variations in the membrane organization in the cell, if a variation made the cell more competitive and able to reproduce faster than the cell without this variation it would be selected for. Jensen (1993) has observed in certain extant blue-greens a variety of membranes and membrane limited structure. This same selection process could occur, so over time natural selection in some bacterial line produced the first eukaryotic cell. Jensen (1989) has also suggested that the chloroplast, nucleus and mitochondria arose by an endomembrane event. In extant blue-greens a large number of inclusion bodies (organelles) have been described (Jensen 1985). Again there is no reason to not suppose that in ancient blue-green species a variety of organelles were produced. If one of these, or more, made the organism more competitive that cell line would be selected for. Over time with slow change and intracellular natural selection the eukaryotic cell could have arisen (Jensen 1994; Jensen 1999).

We can see from the above that when we look at only the data we have and extrapolate from it we must accept that all species living today are of recent origin. Cyanobacteria have and probably will continue to form endosymbioses with many different organisms. That is what they are and we cannot claim this is proof of the endosymbiotic theory for the origin of the eukaryotic cell. Slow gradual change by

natural selection produces new species. A new species exists for only about 10 million years. The eukaryotic cell most likely came into being by the same process, only in this case it was intracellular natural selection.

4. Acknowledgments

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NON SYMBIOTIC ORIGIN OF LOCOMOTORY ORGANELLES

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1. Origin of organelles

Organelles are all the conspicuous structures of cells, starting from the most conspicuous ones, i.e., those visible at the light microscope. Some of them are basically *inactive* from a metabolic standpoint, such as the cell walls or many inclusions which range from sulfur beads to polysaccharide deposits: usually they only have a peripheral turnover due to apposition or removal of their constituent material. Other organelles are *active* on metabolic ground: usually they have chemical reactions beyond those involved in their mere growth or reduction.

Active organelles are mainly made up of protein complexes or protein-lipid membranes. *Protein organelles* frequently have some association with a membrane; reciprocally, *membrane organelles* also include proteins like any cell membrane and the space it surrounds. For the origin of protein organelles, which may also include chromosomes and ribosomes, no symbiotic (or cytobiotic) hypothesis has been put forward. This kind of origin has only been hypothesized for some membrane organelles of eukaryotes. Regardless of the duration, intensity and other characteristics of the symbiosis, a common beginning that is expected by all those hypotheses consists of a close proximity of the plasma membranes of the cells involved, at least in a narrow zone. The close proximity does not mean a true contact between the plasma membranes because of the presence, in the prokaryotic partners of the symbioses, of the cell wall (except for very few species) and frequently, in addition, the outer membrane.

The possible symbiotic origin of the most conspicuous organelle of the living world, i.e., the nucleus, is a highly controversial matter (Li, 1999; Martin, 1999; Rizzotti, 2000). For the other widespread organelles of eukaryotes, i.e., the endoplasmic reticulum, the dictyosomes, and most vesicles and vacuoles, nobody thinks any more of a symbiotic origin. Among widespread albeit less conspicuous organelles, the peroxisomes are no longer supposed to have had such an origin (Erdmann and Kunau, 1997; South and Gould, 1999), and among less widespread ones, the hydrogenosomes are believed to have had it (Müller and Martin, 1999). For the very widespread organelles mitochondria and plastids, the conclusions on their symbiotic origins are virtually "unassailable" (Palmer, 2000).

This means that analogous conclusions regarding other organelles remain fairly "assailable". In particular, the cilium (or eukaryotic flagellum or undulipodium) mainly

involves protein structures and could be defined as a protein organelle; however, it is wrapped inside the plasma membrane, except at its base, so some scholars could regard it as a membrane organelle. This latter view is strongly claimed in the symbiotic hypothesis on its origin which has been put forward by Margulis (1967, as Sagan; 1993, 1996) and supported by others (Szathmáry, 1987; Maynard Smith and Szathmáry, 1995). In contrast to this exogenous proposal, the endogenous proposal has been asserted by Pickett-Heaps (1974), Cavalier-Smith (1978, 1992), Mignot (1996), and the author (1993, 1995, 2000).

2. Locomotory organelles

Locomotion of a cell cannot consist of either simple variations of shape and size or simple rotations and vibrations of the cell itself. We may assume that locomotion implies a translocation that is at least of the order of the major diameter of the cell and occurs within a reasonably short time. Moreover, locomotion is an active process and thus excludes thermal (Brownian) motion or any kind of passive transport. As a consequence, locomotory organelles are part of the metabolically active ones.

Various locomotory mechanisms are displayed by prokaryotes, the most understood among them being the propulsion due to the flagellum. This protein organelle has a rotary motor at the level of the plasma membrane and rotates in both senses (Lloyd et al., 1999; Macnab, 1999). Perhaps the best choice in defining the clockwise (CW) or counterclockwise (CCW) senses, is to view the rotor from the inner part of the cell, i.e., from the proximal position toward the distal one. Actually, this is the convention adopted to describe the rotational symmetries of the cilium: the reversal of this convention would face fierce opposition by any scholar involved in the study of these complicated structures. For the sake of easy communication in science, the same convention could be adopted both for the machines of the mechanical technology and for all geometrical and physical rotational phenomena regarding structures which lie at the peripheries of the cells, so that the vectorial reference to the cell body with respect to the external medium has no ambiguity. As the extracytoplasmic portion of the flagellum is rigid, helical and left-handed, when it rotates CW it propels the cell like a ship propellor in a "run", as it lets the opposite pole of the bacterium go forward into the liquid medium; at least, this happens in the simplest case of a single polar flagellum. When many flagella inserted all around the bacterial surface rotate CW, they gather together with a global effect which is directionally similar to that caused by a single polar flagellum. In contrast, when they all rotate CCW, they remain separate and cause a so-called "tumble", during which the cell turns in a random fashion: thus, in this case they do not act as locomotory organelles.

As regards the origin of the flagellum, its rotary motor may have derived from another rotary motor, i.e., the one that works in the catalytic complex of the ATPase (Rizzotti, 1998, 2000). It is even possible that all cellular rotary motors have a single ancestral complex.

The other locomotory mechanisms of prokaryotes are less widespread than that of the flagellum. One is due to the extracellular projections termed pili and has been

described in some eubacteria endowed with the outer membrane (Kaiser, 2000). The pilus adheres with its distal end to a solid object, be it the substrate or another cell; then it shortens by depolymerization at its proximal end at the level of the plasma membrane. In some examples, the elongated cell rises perpendicular to the substrate, falls on the other side, emits pili at the opposite pole, and repeats the shortening of its pili. Other locomotory mechanisms include the slow gliding of other non-flagellated bacteria apparently due to polysaccharide secretions (Hoiczyk and Baumeister, 1998; Youderian, 1998; Martínez et al., 1999) and the still unexplained rapid shift coupled with rotation of some cyanobacteria (Brahamsha, 1999). The slow translocation inside eukaryotic cells of the bacteria *Listeria monocytogenes* and *Shigella flexneri* (Loisel et al., 1999) can also be regarded as active locomotion. This is achieved by inducing polymerization of microfilaments behind them (Cameron et al., 2001) and perhaps also slipping along microfilaments (Kuo and McGrath, 2000). All these mechanisms are thought to involve protein organelles and no symbiotic origin has been hypothesized for them.

In eukaryotic cells, locomotion occurs by means of crawling, gliding or beating of cilia. All of these mechanisms strongly depend on a well-developed [endo]cytoskeleton (the different cell walls can be considered as exocytoskeletons). Crawling basically consists of transient modifications of the cell shape owing to growth and reduction of the components of the cytoskeleton that change their length more quickly, i.e., the actin microfilaments. The cell translocates in a liquid or gaseous medium relative to a solid or semisolid substrate to which it can adhere. The leading edge of the cell protrudes and adheres to the surface of the substrate, while its trailing edge deadheres and is pulled forward. The leading edge is frequently made up of protrusions (pseudopodes) that can be slender (filopodes) or broad (lamellipodes). The adhesion to the substrate of the plasma membrane of the leading edge is due to suitable proteins. The membrane at the leading edge advances because it is pushed forward by the growth of microfilaments due to addition to their plus (growing) ends of single actin molecules thanks to a thermal-ratchet mechanism, but myosin may also play some role (Anderson and Cross, 2000). Myosin is certainly needed for the traction of the trailing edge and thus the whole cell, and its action is coupled with depolymerization of microfilaments at their minus ends. In any case, microfilament-dependent crawling is considered as the most primitive locomotory mechanism of eukaryotes because it relies on the most primitive component of the cytoskeleton and is related to another primitive function of eukaryotes, i.e., endocytosis.

A sort of crawling is also possible by means of other cytoskeletal fibers without any involvement of motor proteins (such as myosin). This alternative solution is only known to occur in the sperms of nematods (round worms), in which bundles of filaments of the major sperm protein replace actin microfilaments (Italiano et al, 2001). Gliding, with the deposition of trails, has been described in apicomplexans (Sibley et al., 1998), which include many parasites of humans. Nobody seems to invoke a symbiotic event for the origin of the various mechanisms of crawling and gliding in eukaryotes.

In contrast to pseudopodes, the cilia are permanent cellular protrusions and are based on tubulin microtubules. They allow the cells to shift in a liquid medium by means of complex movements based on active local bending. This bending gives rise to either lateral beating that proceeds on a plane along the shaft of the cilium (like the waves of a

whip) or more complicated helical movements in the three dimensions. There are many examples in animals of cilia that are superficial but immobile or are on epithelia of internal organs; of course, neither of them have any locomotory function.

As regards the origin of the cilium, the symbiotic hypothesis resorts to the merger between a spirochete and an archaeobacterium (Margulis, 1996; Margulis et al., 2000). Necessary conditions for this hypothesis are, first, that the spirochetes truly locomote and, second, that their putative locomotory mechanism is likely to have been passed on to eukaryotes.

3. Do Spirochetes locomote?

Spirochetes are very peculiar bacteria in that they are thin (down to $0.1\text{ }\mu\text{m}$), long (up to $500\text{ }\mu\text{m}$) and helical in shape. Their helix is usually right-handed (sometimes left-handed: see Barbour and Hayes, 1986) and kept in shape by the cell wall that lies on the outer surface of the plasma membrane. The wall is loosely wrapped within the pleated outer membrane (scheme in Cox et al., 1992) that in most species is reinforced by some kind of coat on its outer surface to form a sheath. The space between the plasma and the outer membranes is termed periplasmic and is partially occupied by the wall.

Spirochetes are believed to locomote by means of their flagella. These, unlike those of the other flagellated bacteria, are periplasmic in the sense that they are inserted in the plasma membrane near the poles of the long cell, extend toward the middle of the cell within the periplasmic fissure between the wall and the outer membrane, and are often collected into a ribbon-like structure. They rotate both CW and CCW like in other flagellated bacteria, and their rotation causes various movements, but as to whether they can really propel the cell remains an open question (Holt, 1978) because, in any case, their action is carried out in the periplasm and thus is very different from that of the usual flagella.

There are two possible simple ways for periplasmic flagella to propel the cell. One is that of acting as friction wheels between the outer surface of the cell wall and the inner surface of the outer membrane (Berg, 1976; Figure 1, left). In this way, the cytoplasm and the outer membrane rotate in opposite senses, provided all flagella of the ribbon rotate in the same sense (this implies that those inserted at one pole rotate CW and those inserted at the other pole rotate CCW). If the flagella of the opposite poles do not rotate according to this coordinated way, no translocation occurs. As the two poles are usually equal, the direction of translocation is determined by the rotation sense of flagella. Provided this mechanism really works, the cell advances into a viscous liquid like an usual right-handed screw does into the wood. The flexible outer membrane adapts to the helical shape of the cell wall, and its opposite rotation sense could help the progression of the screw-shaped cell.

How sound and general this first mechanism could be is unclear. If the flagella are gathered in a helical ribbon, as usual, there is friction between the cell wall and the outer membrane all around the cell except where there is the ribbon of flagella. Unfortunately, in the ribbon, the flagella themselves encounter friction against each other, therefore braking their rotation. Moreover, the individual flagella are also helical in shape, so that

their action as friction wheels is expected to be rather inefficient because their contacts with the other two cellular structures only occur at the turns of the flagella themselves and not throughout their length. To sum up, this mechanism is favored by the helical shape of the cytoplasm and disfavored by the helical shape of the flagella. In addition, if spirochetes have a permanent longitudinal groove (Hollande and Gharagozlou, 1967; Margulis and Schwartz, 1998) due to a junction between the plasma membrane and the outer membrane (Bayer, 1991), there is no possibility for the two cellular structures to rotate relative to each other.

The other simple mechanism relies precisely on the helical shape of the flagella: actually, in spirochetes they seem to be rigid left-handed helices like those of the other flagellated bacteria (Charon et al., 1992). If each turn of a helical flagellum pushes against the outer membrane, it creates a small protrusion on the outer surface of it (Figure 1, right) which migrates along the cell. If the cytoplasm is cylindrical and a flagellum rotates CW at one pole, the series of small surface protrusions due to its left-handed helix migrate toward the middle of the cell. If at the second pole the opposite happens, the cell receives a weak thrust that could let it advance with its first pole into the viscous environment.

This mechanism, unlike the previous one, is favored by the helical shape of the flagella and disfavored by the helical shape of the cell because the turns of the cell wall determine a friction against the medium, unless the movements of the flagella give it some additional help. Also in this idea, the same rotation senses of the flagella inserted at the two poles prevent any progression. In contrast, no problem is posed to this mechanism by the possible longitudinal junction of the plasma and outer membranes.

In any case, both mechanisms are unsatisfactory, so the intriguing problem of the possible locomotion of spirochetes cannot be considered as solved. Maybe they do display “complex movements—rotation, torsion, flexion, and quivering” (Margulis and Schwartz, 1998, p. 74), but do not locomote. If they truly locomote, they probably do so thanks to other mechanisms, and different mechanisms or combinations of them are perhaps valid for different species (Canale-Parola, 1978; Charon et al., 1992). The only certainty is that the flagella have a role in their movements, but at each pole the flagella can be either steady, or rotating CW, or rotating CCW; the two poles together can adopt nine possible combinations. In order to rapidly choose the most suitable combination in a particular environmental and physiological situation, the bodies of spirochetes may be run by signals going from pole to pole (we cannot even exclude that their plasma membranes are excitable like that of nervous fibers of animals); alternatively, the usual overlapping of their flagellar ends in the middle of the cell may be a way to communicate the state of a pole to the other one.

A related problem is how the peculiar cellular arrangement of spirochetes appeared during evolution. One possibility is that it arose very early (Margulis et al., 2000), probably deriving from that of bacteria endowed with the outer membrane and external flagella. Natural selection favored their arrangement because, when their flagella lost the capacity to cross the outer membrane, their propelling action in the bodies of water became more efficient than those of the other bacteria, for unknown reasons. No intermediate steps in the evolution of periplasmic flagella from external ones (with their respective movements and locomotory effects) have been proposed.

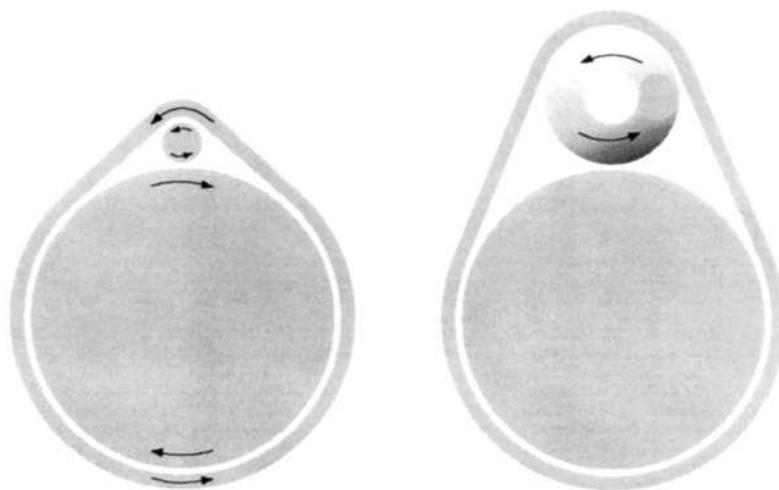


Figure 1. Alternative simple (yet unsatisfactory) mechanisms for periplasmic flagella to propel spirochetes (cross section). For the sake of simplicity, the right-handed helical shape of the cell is ignored. Arrows indicate sense of rotation. Left: cytoplasm and outer membrane are induced to rotate in opposite senses by rotating flagellum. Right: small protrusions of the outer membrane, due to the turns of the left-handed helical flagellum, move along the cell. In both cases, the cell advances toward the back of the page.

An alternative possibility is that spirochetes have been selected as late and very specialized exploiters of liquid substrates which were rich in organics and thus more viscous than conventional bodies of water. High viscosity affects both molecular diffusion and mechanical action. Diffusion of organic nutrients and metabolic discards is increased by elongation and thinning of the cell, but this reduces the locomotory efficiency of the external flagella. At some moment, the flagella lost the capacity to cross the outer membrane and became periplasmic. In this way they were no longer in direct contact with the medium and thus could rotate almost normally although the medium was much more viscous (Canale-Parola, 1978). Moreover, their rotation favored diffusion across both the plasma and the outer membranes (Rizzotti, 2000).

In a long and thin cytoplasm there is also the problem of favoring diffusion of RNA molecules and proteins, and the flagellar shaking of the cell could fulfill this requirement too. Actually, an "average" spirochete 20 μm long and 0.2 μm wide could bear a single genome copy 1 μm long. Such a situation is particularly probable in *Borrelia*, where the linear main chromosome and the linear plasmids are present with the same copy number per cell, and each genome must be present with at least one copy in each of the two cells resulting from the transversal fission. As a matter of fact, the genome is supposed to be present at the division site in analogy with the mitotic segregation of eukaryotic cells (Hinnebush and Barbour, 1992). In this idea, the flagella of spirochetes might have lost any true locomotory function. In any case, the aid to diffusion could be at the evolutionary origin of the flagellum in general, and this function could still be important in bacteria with normal external flagella (Rizzotti, 2000).

As regards the screw shape of the cytoplasm, it can be related to some still unclear locomotory mechanism. Alternatively, it can increase diffusion following flagellar rotation because helical flagella shake helical cytoplasms much more than if both structures were straight. Moreover, when spirochetes quiver inside organic mats or between parasitized cells, the outer side of the turns of their helical cell pushes against the more solid components of their environment, whereas the inner of the turns remains in contact with the more fluid components; this liquid microenvironment along the axis of the helix also ensures more efficient diffusion.

In any case, spirochetes are sometimes regarded as so efficient in locomotory performances that they are also presumed to move large unicellular eukaryotes, such as *Myxotricha paradoxa*, along the hindgut of termites and wood-eating cockroaches (Margulis, 1993), like a tow-boat pushing a barge along a river. Even if spirochetes locomote, if they are attached with one pole all around the unicellular eukaryote, not in a preferential place, and stir all together, they could at most compress or depress the eukaryotic cell. They could propel it only if they either are attached in preferential areas, or with preferential tilts, or move quickly in a region and slowly in the opposite one. But in these cases, their contribution to a shift of *Myxotricha* could be effective even if they simply tremble, in the absence of any true locomotory activity. So, the possible propulsion of *Myxotricha* does not demonstrate that spirochetes locomote.

In any case, the ectosymbiosis of spirochetes with *Myxotricha*, which is endowed with its own cilia, may have a completely different explanation. The spirochetes, instead of pushing *Myxotricha*, may exploit its autonomous ciliary locomotion to remain in the gut instead of being expelled from it. In other words, spirochetes are likely not to be the tow-boats of passive barges, but the fouling of barges endowed with their own engines and propellers. Their generic trembling can also increase diffusion of the molecules in which *Myxotricha* is interested.

Spirochetes also participate in a different ectosymbiosis, i.e., that of *Thiodendron*, that is found in bodies of water instead of animal guts. *Thiodendron* is a consortium that only includes prokaryotes. It is unclear whether spirochetes are supposed to have any locomotory function in it (Margulis et al., 2000); if so, the conclusions are the same as for *Myxotricha*.

4. Non symbiotic origin of the cilium

Even if spirochetes are able to propel other cells beyond themselves, they may have nothing to do with the cilium all the same. Many points have already been raised against a derivation from a spirochete of the cilium, as this has nothing in common with flagella, including periplasmic ones, at either the structural or the functional levels (Cavalier-Smith, 1992; Rizzotti, 1995, 2000).

In particular, there is no reason to hypothesize any role of spirochetes in the origin of the microtubular cytoskeleton (to be true, of any component of the cytoskeleton); even more so, there is no reason to hypothesize any role of spirochetes in the origin of any specialized portion of the microtubular cytoskeleton, such as the microtubular axial scaffold of the cilium, as is expected by the supporters of the exogenous hypothesis

(Szathmáry, 1987; Margulis, 1993). Actually, after the complete sequences of the genomes of two spirochete species have been determined, i.e., those of *Borrelia burgdorferi* (Fraser et al., 1997) and *Treponema pallidum* (Fraser et al., 1998), no evidence emerges in favor of such a role because spirochetes simply lack any tubulin gene. The only gene related to tubulins, and present with a single copy in both genomes, is that of the protein FtsZ (Löwe and Amos, 1998), which gathers below the plasma membrane at the furrow between two dividing cells. However, this same gene is present in almost all prokaryotes (Glass et al., 2000), so that there is no need to disturb the spirochetes in particular. Moreover, a similar gene is supposed to have been present in the common ancestor of all present living things, so there is no reason either to involve a symbiotic event to acquire it, because every ancient lineage already had it. In addition, the motive force that is presumed to help the separation of the two dividing cells is not due to any motor protein that moves along the putative linear structures of FtsZ, as dynein does along the microtubules including those of the cilium, but is probably due to some structural transition of FtsZ itself (Lu et al., 2000).

As regards the remainder of the 200 genes of the cilium which were expected to have homologues in spirochete genomes (Margulis et al., 1990), no trace has been found of them. Moreover, although both complete genomes belong to species which are pathogenic for humans, they are quite distant from the point of view of their general organization (one genome is linear and one is circular) as well as from that of their genes (Paster et al., 1991; Lafay et al., 1999), so it is really improbable that future complete genomes of spirochetes will upset our conclusion.

The alternative proposal regarding the origin of the cilium, i.e., the endogenous one, is exposed in the most detailed way in the peduncle hypothesis (Rizzotti, 1995, 2000). The main advantage of the peduncle hypothesis is that it provides a rationale for the 9 + 2 geometry of the axial scaffold of the cilium, which constitutes its most striking feature and receives no explanation in alternative hypotheses. The reason for thinking of a peduncle instead of any generic extroversion is that a peduncle needs a stronger axial skeleton than any free extroversion does, so it takes more selective advantage of optimizing its skeleton. Moreover, a peduncle is more likely to need a preferential swinging plane, and this could select its swinging possibilities by means of the geometry of the axial skeleton. The 9 + 2 geometry is precisely explained by the advantage of a preferential swinging plane (Rizzotti, 1995, 2000). In this hypothesis, the peduncle stage of the cilium explains its origin, but is only a transient, albeit important, stage, so that no record of it is expected to be present in the extant biosphere. Actually, in spite of frequent polemic requests of extant “missing links” (Behe, 1996), it is well-known that primitive and intermediate stages of organs (and organelles) are frequently lost forever during evolution.

5. Summary

Locomotory organelles are widespread among both prokaryotes and eukaryotes, but only for the cilium (i.e., the eukaryotic flagellum) a symbiotic origin is supported at present (in particular by Margulis in Chapman et al., 2000, and Margulis et al., 2000). In

this view, the cilium is regarded as originated from the slender cell of a spirochete that established an external symbiosis with an eukaryotic ancestor; the symbiosis was followed by the fusion of one of the poles of the spirochete with the plasma membrane of the partner cell.

However, the idea that spirochetes truly locomote is questioned because their flagella, unlike those of the other prokaryotes, rotate within the periplasm between the cell wall (just outside the plasma membrane) and the outer membrane. This implies that the simplest mechanisms that can be supposed to propel the spirochetes seem to be ineffective. Also in the examples of external symbioses between unicellular eukaryotes and spirochetes there is no evidence of any locomotory role of the latter. As regards the expected origin from spirochetes of the machinery of the cilium, the complete genome sequences of two spirochete species do not provide any evidence of such an origin.

The alternative view, according to which the cilium derived from a specialization of the microtubular component of the eukaryotic cytoskeleton (Cavalier-Smith, 1992; Mignot, 1996; Rizzotti, 1995, 2000), does not meet any apparent difficulty. In particular, the hypothesis of the origin of the cilium from a cellular peduncle (Rizzotti, 1995, 2000) also explains the striking 9 + 2 geometry of its microtubular array.

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THE CYANELLE (MUROPLAST) OF *CYANOPHORA PARADOXA*: A PARADIGM FOR ENDOSYMBIOTIC ORGANELLE EVOLUTION

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1. Introduction

A number of reviews on the molecular biology of *Cyanophora paradoxa* and the cyanelle genome (Löffelhardt *et al.*, 1997ab) and on protein translocation into and within cyanelles (Schwartzbach *et al.*, 1998; Löffelhardt *et al.*, 1998, 1999) are available. After briefly rephrasing what is known, we emphasize new data and trends about the unique phenomenon among eukaryotic cells of an organelle surrounded by a prokaryotic wall. Such "fossils" that presumably mimic an early stage in organelle evolution are not known for other plastid types and are also absent among mitochondria. The term "cyanelle" is not entirely correct because the transition from endosymbiont to organelle has certainly taken place but we will keep this name for historical reasons. After 20 years of research, the plastid research community has become familiar with the term. Other names which emphasize the plastid nature of the cyanelle are "muroplast" and "cyanoplast" (Schenk, 1994). The former addresses the unique organelle wall (Figure 1) whereas the latter focuses on ancestry and would also be applicable to the plastid of e.g. *Cyanidium caldarium*.

2. Cyanelle Genes

The nucleotide sequence of the cyanelle genome was completed in 1995 (Stirewalt *et al.*, 1995). Cyanelles are primitive plastids, a name which embraces the plastids from glaucocystophytes and red algae and the secondary plastids derived from red algal endosymbionts. In addition, "primitive" plastids from ancient green algae have recently been added to the list (Turmel *et al.*, 1999; Lemieux *et al.*, 2000). The cyanelle genome includes more than 70 genes that are not found in the advanced chloroplasts from higher plants. Gene number in *C. paradoxa* muroplasts is not as high as the number found in the plastids of *Porphyra purpurea* (Reith and Munholland, 1995) but it is similar to that from *Odontella sinensis* (Kowallik *et al.*, 1995), *Guillardia theta* (Douglas and Penny, 1999), or *C. caldarium* (Glöckner *et al.*, 2000). Analysis of the genomes present in primitive plastids and the comparison of gene number and nature

with the characteristics found in green algae and higher plants has considerably improved our understanding of plastid evolution (Martin *et al.*, 1998).



Figure 1. Immunodecoration of the cyanelle envelope using antisera directed against *E. coli* peptidoglycan. The septum is shown to literally cleave the photosynthetic apparatus (carboxysome and thylakoids) and the genetic system (the cyanelle nucleoid surrounding the carboxysome) into two halves.

Our understanding of plastid gene complement and operon structure owes much to comparisons with the entirely sequenced genome of *Synechocystis* sp. PCC6803 (furtheron called *Synechocystis*). Open reading frames encoding proteins of unknown function that are shared by at least two plastid genomes or are shared by any plastid genome and the *Synechocystis* genome are called *ycf*'s. At present 86 *ycf*'s are known. The numbering proceeds and *ycf*'s whose functions become known are eventually removed from the list. A survey of *ycf*'s, shared by the cyanelle genome is given in table 1. Most of the 24 genes have a counterpart in the genomes of other primitive plastids. The functions of approximately 40 of 192 genes (Figure 2) were unknown when the cyanelle genome was last reviewed (Löffelhardt *et al.*, 1997ab). We will discuss the progress now.

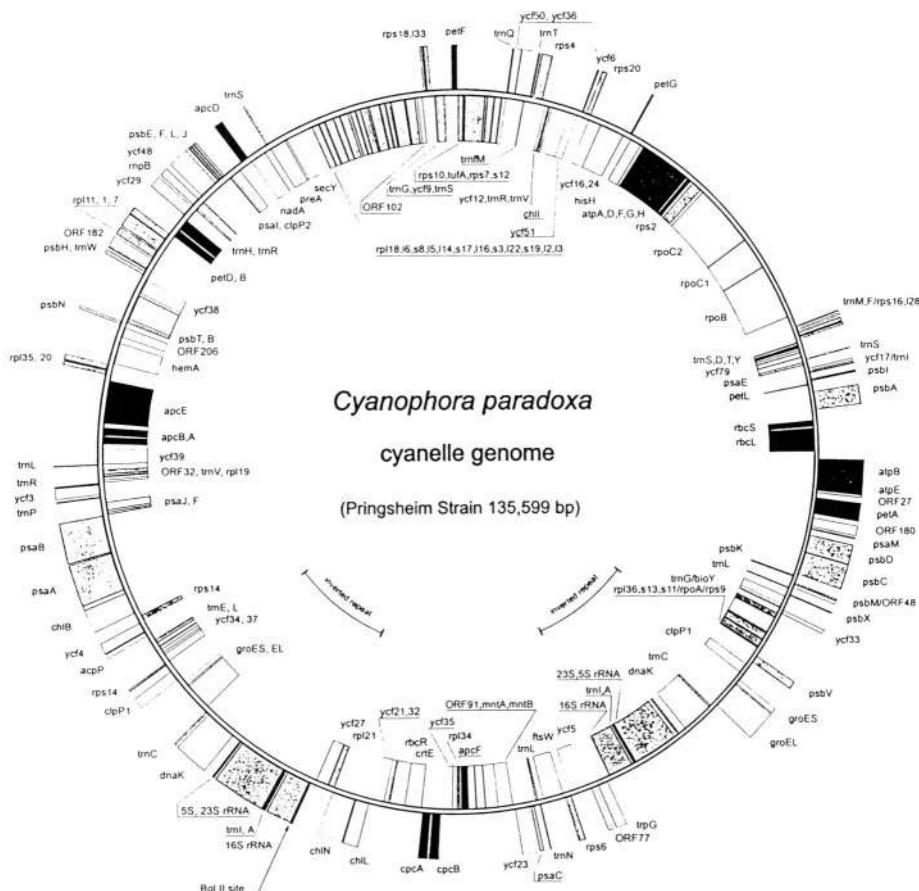


Figure 2. Map of the cyanelle genome of *Cyanophora paradoxa* 555 UTEX (Pringsheim isolate). Transcription of the genes on the outer circle is clockwise.

2.1. GENE IDENTIFICATION AND FUNCTIONAL DESCRIPTION

2.1.1. Protein-coding Genes

In all cases described below a (putative) function was assigned to homologs of cyanelle genes in other organisms. The cyanelle-encoded prenyltransferase CrtE (geranylgeranyl diphosphate synthase) is involved in carotenoid biosynthesis. The higher prenyltransferase, PreA, very likely participates in the biosynthesis of the plastoquinone side

TABLE 1. *ycf*'s on the cyanelle genome of *C. paradoxa*. In all cases a homolog exists in the genome of *Synechocystis* (*Syn*) which is indicated when no plastid homologs are known. Taken from the compilation by Stoebe *et al.*, 1998.

Gene	Shared by higher plant chloroplasts and primitive plastids ¹	Shared by primitive plastids only	Probable function, Comments
<i>ycf3</i>	+	+	n.k. <i>ycf4</i> + + n.k.
<i>ycf6</i>	+	+	assembly/stability of cyt <i>b/f</i> complex ²
<i>ycf9</i>	+	+	assembly/stability of PSII ³ core antenna
<i>ycf12</i>	+	+	not present in angiosperms
<i>ycf16</i>	-	+	ABC-transporter
<i>ycf17</i>	-	+	ELIP superfamily
<i>ycf21</i>	-	+	n.k.
<i>ycf23</i>	-	+	n.k.
<i>ycf24</i>	-	+	ABC-transporter
<i>ycf27</i>	-	+	regulatory component of sensory system
<i>ycf29</i>	-	+	regulatory component of sensory system
<i>ycf32</i>	-	+	n.k.
<i>ycf33</i>	-	+	n.k.
<i>ycf34</i>	-	+	n.k.
<i>ycf35</i>	-	+	n.k.
<i>ycf36</i>	-	+	n.k.
<i>ycf37</i>	-	+	n.k.
<i>ycf38</i>	-	+	n.k.
<i>ycf39</i>	-	+	n.k.
<i>ycf48</i>	-	-	ORF333 ⁴ , <i>Syn slr2034</i> ; PSII assembly/stability
<i>ycf49</i>	-	+	n.k.
<i>ycf50</i>	-	-	ORF108, <i>Syn slr2073</i>
<i>ycf51</i>	-	-	ORF163, <i>Syn sll1702</i>
<i>ycf79</i>	-	+	annotated as 13 kDa protein in PSII

n.k. - not known

¹*P. purpurea*, *G. theta*, *O. sinensis*, *C. caldarium*; ²Proposed name: *petN* (Hager *et al.*, 1999)

³Ruf *et al.* (2000); ⁴Homolog of *hcf136* from *Arabidopsis thaliana*.

chain (*trans* additions). The latter shows high identity scores to octaprenyl diphosphate synthase from *E. coli* and to nonaprenyl diphosphate synthase from *Synechocystis*. The cyanobacterial gene complemented the lethal *ispB* mutation in *E. coli* (Okada *et al.*, 1997). Two nuclear genes from *A. thaliana* show high sequence similarity to the cyanelle and cyanobacterial transferases. Both nuclear genes include N-terminal extensions of 60 to 70 amino acids, which seem to encode the transit peptides for chloroplast import. A previously assumed function for PreA in peptidoglycan biosynthesis, i.e. a role as an undekaprenyl diphosphate synthase (*cis* additions to farnesyl diphosphate) is less likely because the corresponding gene from *E. coli* has recently been characterized. This gene shows only limited sequence similarity to *preA* (Shimizu *et al.*, 1998). Furthermore, the *preA* gene is also found on the genomes of *P. purpurea* and *C. caldarium* plastids that are devoid of an organelle wall.

Orf333 (*ycf48*; Meurer *et al.*, 1998) was shown to correspond to the (nuclear) *hcf136* gene from *A. thaliana*. The phenotype of the plant mutant indicates a function for the gene product, which was localized to the thylakoid lumen, in photosystem II stability or assembly. Interestingly, the cyanelle and cyanobacterial precursors possess signal sequences devoid of the "twin arginin" motif, which is present in the bipartite presequence of plant genes (Meurer *et al.*, 1998).

The *ycf27* gene product shows similarity to OmpR-type DNA-binding response regulator proteins. Two homologs, *rpaA* and *rpaB* (more closely related) are present in the *Synechocystis* genome (Ashby and Mullineaux, 1998). Mutants of *rpaA* show a preference for energy transfer from phycobilisomes to photosystem II, whereas *rpaB* mutants distribute more electrons to photosystem I. *ycf27* is found in cyanobacterial genomes and is present only in the genomes of plastids with phycobiliprotein antennae. A role for *rpaA* and *rpaB* in the long-term adaptation of light harvesting in these organisms is therefore likely. A second, nucleus-encoded component, a counterpart to RpaA, might exist in the algal systems. Another putative transcriptional regulator gene, *ycf30*, has been named *rbcR* (Stoebe *et al.*, 1998), implying a function in the regulation of Rubisco (ribulose bisphosphate carboxylase/oxygenase) synthesis. It remains to be seen if this classification will hold: identity scores of *ycf30* towards a *lysR*-like gene (*sll0998*) from *Synechocystis* are much higher than against authentic *rbcR* genes specifying regulators of Rubisco operon transcription.

A reading frame formerly termed *orf188* has been renamed after it was shown to exhibit high identity scores towards the cyanobacterial *bioY* gene specifying biotin synthase. Similarly, *orfs244* and 299 are now tentatively named *mntA* and *mntB*, based on their similarity to manganese transport proteins. Additional data on a manganese transporter in the cyanobacterial inner envelope membrane (Bartsevich and Pakrasi, 1999) indicated that manganese is a likely substrate for the corresponding cyanelle gene products but the transport of metal ions other than Mn^{2+} cannot be excluded. Sequence conservation among *Synechocystis* and primitive plastids is even higher for another pair of ABC-transporter subunits (ATP-binding subunit and integral membrane protein), also encoded by clustered genes: *ycf16* and *ycf24*. Here the substrate is still unknown (Table 1).

The number of protein genes unique to the cyanelle genome has now been reduced by the inclusion of ORFs present in *Nephroselmis olivacea*, *G. theta* and *C. caldarium* (see the comparison of Stoebe *et al.*, 1998). Cyanelle-specific are *bioY*, *crtE*, *groES*, *hemA*, *hisH*, *mntA*, *mntB*, *nadA*, *ycf48*, *ycf50*, and *ycf51*.

2.1.2. Cyanelle-encoded Stable RNAs

rnpB: The function of this gene in forming the RNA moiety of cyanelle RNase P has been corroborated. A functional enzyme could be functionally reconstituted from *in vitro* synthesized (from the cyanelle coding region) P RNA and P protein from *Synechocystis* but not from *E. coli* (Pascual and Vioque, 1999). Site directed mutagenesis that converted a loop into the conserved sequence found in bacteria resulted in an inactive RNA. This result is not unexpected because cyanelle RNase P RNA and protein are more related to the cyanobacterial than to the *E. coli* genes. Furthermore, the influence of cyanelle P protein on cyanelle P RNA structure (which is not catalytically active *per se*) seems to be more pronounced than among the bacterial counterparts. This view is also supported by enzymatic and chemical accessibility

experiments on cyanelle P RNA in the holoenzyme which behaves in this respect more like the eukaryotic complex (Cordier and Schön, 1999). Taken together, cyanelle RNase P seems to represent an evolutionary intermediate between the bacterial enzyme with a catalytic RNA and the chloroplast protein functioning without an RNA component (Schön, 1999).

Recent analyses corroborated the ancient nature of the sole intron present in the cyanelle genome, that in the *trnL^{UAA}* gene (Besendahl *et al.*, 2000). This intron is found in plant and most green algal plastid genomes, but is missing from the genomes of rhodophytes and cryptomonads. No evidence has yet been obtained for a cyanelle-encoded signal recognition particle (SRP) RNA (Packer and Howe, 2000) but candidate genes are present on the plastid genomes of *P. purpurea* and *O. sinensis*. Should a 4.5S SRP RNA be substantiated for primitive plastids, this would be in contrast to higher plant chloroplasts. This would entail the prospect of considerable sequence divergence of a cyanelle-encoded 4.5S RNA which might not be identified through primary and secondary structure comparison, or of RNA import into cyanelles.

3. The Nuclear Genome

3.1. NUCLEAR GENES FOR CYANELLE POLYPEPTIDES

At the cDNA level, 12 coding regions, precursors for 10 proteins, are known at present. Their most interesting features are the transit sequences that are compiled in figure 3. Southern-type hybridizations point to single copy genes in most cases. Two genes were identified by PCR, for Rieske Fe-S protein and Atpl. Remarkable is the presence of Atpl in the nuclear genome because *atpI* had been considered ubiquitous in the genomes of all photosynthetic plastids investigated. The only exception are cyanelles (Stoebe *et al.*, 1998). Light stimulation of expression is likely for proteins of the photosynthetic apparatus, but has been demonstrated thus far only for cytochrome *c*₆ (Steiner *et al.*, 2000).

3.2. GENES FOR COMPONENTS OF THE CYTOSOLIC TRANSLATION APPARATUS

Until recently, only two components of the *Cyanophora* cytoplasmic translation apparatus were known at the cDNA level: ribosomal protein L13a (Steiner *et al.*, 1997) and elongation factor 1α (C. Hink and W. Löffelhardt, unpublished). This number has increased considerably. Transcripts of 80S ribosomal protein genes and translation factors obviously are quite abundant in *C. paradoxa* mRNA. They are well represented in cDNA libraries. Many of the genes compiled here emerged as false positives in a variety of screens, e. g. in complementation experiments of mutants in peptidoglycan biosynthesis or with antibodies directed against soluble muroplast proteins. Sequence information exists for L39a, L21e and eIF-5α containing the unusual amino acid hypusine (C. Hink-Schauer and W. Löffelhardt, unpublished) as well as for S11, S12, S21, and L17 (A. Nickol and H. Schenk, unpublished) and for L6, L31, and the 60S subunit acidic ribosomal protein P2 (A. Nickol, N. Müller and H. Schenk, unpublished).

Figure 3. Transit sequences of the precursors to nucleus-encoded cyanelle proteins.

atpi - ATPase subunit CF₀-IV (J. Steiner and W. Löffelhardt, unpublished), petj - cytochrome *c*₆ (Steiner *et al.*, 2000), psad - photosystem I subunit D (A. Nickol and H. Schenk, personal communication), fnr - ferredoxin-NADP⁺ oxidoreductase (Jakowitzsch *et al.*, 1993), gapa - glyceraldehyde-3-phosphate dehydrogenase (H. Brinkmann, personal communication), rieske - Rieske Fe-S protein (J. Steiner and W. Löffelhardt, unpublished), ald - aldolase (Nickol *et al.*, 2000), tket - transketolase (Y. Ma and W. Löffelhardt, unpublished), apcc - phycobilisome core linker ApC (J. Steiner and W. Löffelhardt, unpublished).

3.3. OTHER NUCLEAR GENES

ATP citrate lyase (ACL, Ma *et al.*, submitted), was originally obtained as a cDNA clone upon library screening with polyclonal antisera directed against soluble cyanelle proteins. The clone was first considered to be 5'-truncated, since it lacked a transit sequence and the encoded protein would be much smaller than the 110 to 120 kDa ACL polypeptides known from metazoa. In addition, higher plant ACL was reported to be a chloroplast enzyme. Attempts to extend the length of the clone by 5' RACE met little success and, in contrast to FNR, the ACL polypeptide obtained by *in vitro* transcription/translation was not imported by isolated cyanelles. However, recently it was reported that in the fungus, *Sordaria macrospora*, the genes *ac11* and *ac12* encode two polypeptides with homology to the N- and C-terminal parts of the animal ATP citrate lyase polypeptide (Nowrousian *et al.*, 2000). This also applies for other fungi and even for *A. thaliana*. Sequence conservation is very high thus there is no doubt about the function of the plant genes and, interestingly, there are no indications for transit sequences. The *C. paradoxa* clone appears to be full-length, specifying the larger subunit (*ac11*) which contains the catalytic sites. A genomic clone was isolated that corresponded to the ACL cDNA with 11 introns of 53 to 65 bp in length with canonical GT/AG borders and a putative branch point consensus sequence of 5'-CTGAC-3' interspersed. A determination of the subcellular distribution of ACL activity in *C. paradoxa* showed an exclusively cytoplasmic localization for this enzyme. A proposed function for cytosolic ACL in the alga (and, most likely, also in plants) could be in chain elongation of preformed fatty acids rather than in their *de novo* biosynthesis, which is confined to the plastids. The fortuitous detection of a cDNA with an unexplainable, seemingly non-functional, structure resulted in a revision of our views about metabolic pathways.

The cytoskeletal proteins actin (Bhattacharya and Weber, 1997) and α - and β -tubulin (Keeling *et al.*, 1999) were sequenced with the aim of phylogenetic analysis. The actin gene is the only other gene of *C. paradoxa* characterized at the genome level and its introns show features similar to those of *ac11* (Bhattacharya and Weber, 1997). A calmodulin-binding kinesin-like protein (KLP, 133 kDa) was identified in *C. paradoxa* using heterologous antibodies and a Calmodulin-Sepharose affinity column. A partial cDNA sequence has been obtained (Abdel-Ghany *et al.*, 2000). Based on phylogenetic analysis, the *C. paradoxa* calmodulin-binding KLP definitely groups with the corresponding coding regions from higher plants indicating that these motor proteins evolved before the divergence of plants and animals.

4. Characterization of Cyanelle Proteins

Cytochrome *c*₆ (Steiner *et al.*, 2000) has been purified to homogeneity and characterized by N-terminal sequencing and mass spectrometric determination of the molecular weight of the holoenzyme and peptides generated through enzymatic or chemical cleavage. Antibodies were raised in order to investigate the sub-organellar distribution of the protein. Preliminary data indicate a periplasmic location for a considerable fraction of this cytochrome. This raises two questions: how is the protein transported to

the periplasm, in addition to its known location in the thylakoid lumen, and what is the function of a periplasmic cytochrome *c*₆ in *C. paradoxa*. In cyanobacteria, the Sec translocase was shown to reside in both the inner envelope and thylakoid membranes (Nakai *et al.*, 1994). The periplasmic localization of a *c*₆-type cytochrome involved in respiratory electron transport was also documented (Serrano *et al.*, 1990). Experiments are under way to verify this for cyanelles too.

In contrast to higher plants where the cytosolic and chloroplast aldolases are of class I, both are class II metalloenzymes in *C. paradoxa*. Recently the Calvin cycle (cyanelle) enzyme was shown to be bifunctional for FbP and SubP (Flechner *et al.*, 1999).

In the course of a biochemical comparison of soluble inorganic pyrophosphatases (sPPases) from cyanobacteria and microalgae, the enzyme of *C. paradoxa* was isolated and characterized (Gómez *et al.*, 1998). Starting from intact cells or from isolated cyanelles a protein of apparent MW 34 kDa was obtained, the N-terminal amino acid sequences being identical in both. The enzyme activity was found almost exclusively in the cyanelle fraction, suggesting sPPase as a cyanelle protein which resembles other eukaryotic enzymes in size, subunit composition (monomeric) and N-terminal sequence. Under this assumption, the cyanelle enzyme would be divergent from the cyanobacterial sPPases (hexamers of 20 kDa subunits). One possible explanation is that the endosymbiont gene was lost and that the host cell gene product was targeted to the organelle.

NADH- and NADPH-dependent glutathione reductase activities appeared to be absent from *C. paradoxa* (A. Serrano, personal communication) which might be a reason for the long observed sensitivity of *C. paradoxa* to high light.

A DNA polymerase has been characterized. It resembles eukaryotic γ -type enzymes, i.e. with structural similarity to chloroplast polymerases rather than those reported from cyanobacteria (White *et al.*, 1997). A photosystem I preparation from *C. paradoxa* (Koike *et al.*, 2000) allowed for the analysis of amino-terminal sequences of the cyanelle-encoded PsaB, PsaC, PsaE, and PsaF and their comparison with sequences in the databases. Complete cleavage of the N-terminal methionine was observed for PsaB and PsaC and partial cleavage for PsaE. The predicted signal peptidase cleavage site (Löffelhardt *et al.*, 1997a) for pre-PsaF (...DVRA↓DVGAL...) was corroborated. Two other bands were identified by N-terminal sequencing as nucleus-encoded subunits. In the case of PsaD the cleavage site of the respective precursor could be determined by comparison with the cDNA sequence (A. Nickol and H. Schenk, personal communication; acc. no. AJ132477):

...FIVRA↓EEEEA...

For PsaL no cDNA has yet been isolated but the now available partial sequence will be useful for primer design. LHCI proteins belong to the Cab superfamily. They are present in all plastid types investigated, including chlorophyll *b*-less rhodoplasts (Durnford *et al.*, 1999). No immunological detection of LHCI proteins has been possible in cyanelles using antisera directed against LHCI proteins from *Porphyridium cruentum* and *Chlamydomonas reinhardtii*, respectively (Koike *et al.*, 2000) suggesting a loss of the respective genes (in addition to the chlorophyll *a/b* antenna) in the glaucocystophyte lineage. Three small proteins with apparent molecular weights of 4.4,

4.1, and 3.7 kDa were identified through their N-terminal sequences as the cyanelle-encoded PsaJ, PsaI, and PsaM proteins, respectively (Shibata *et al.*, 1998). In the case of PsaJ, the N-terminal methionine appeared to be processed.

5. The Peptidoglycan Wall

The cyanelle sacculus (Figure 1) is not a "rudimentary wall" but rather thicker and more highly crosslinked than that of *E. coli*. In contrast, the number of layers (2-3) are reduced with respect to the wall of the ancestral cyanobacterium (5-7). Also, the cyanobacterial high molecular weight, covalently bound carbohydrate is missing. Another adaptation to the predicament of an organelle wall is the reduction of the negative net charge per peptide side chain (-2) through amidation of the C-1 carboxy group of the D-isoglutamyl moiety with N-acetylputrescine (Pfanzagl *et al.*, 1996a). This modification, which is unusual in the eubacterial kingdom, was not encountered in peptidoglycan from the cyanobacterium *Synechocystis* sp. PCC6714, but in the cyanelle walls from two other glaucocystophyte algae, *Glaucocystis nostochinearum* and *Cyanoptche gloeocystis* (Pfanzagl *et al.*, 1996b) and thus can be considered typical for this "eukaryotic" peptidoglycan. A cyanelle envelope membrane preparation enriched for inner membranes catalyzed the undecaprenol phosphate and UDP-N-acetylmuramylpentapeptide-dependent incorporation of labeled UDP-N-acetylglucosamine and labelled putrescine, respectively, into substituted disaccharide pentapeptide (Pfanzagl and Löffelhardt, 1999). This occurs most probably at the stage of lipid II and is completed through subsequent acetylation with acetyl CoA, also catalyzed by a membrane-bound enzyme. The biosynthetic pathway of cyanelle peptidoglycan thus is quite similar to that known in *E. coli*, with respect to compartmentation into soluble (i.e. cytosolic or stromal), membrane-bound, and periplasmic steps.

Upon completion of the cyanelle genome sequence it seemed surprising that only one potential gene (*ftsW*) for an enzyme of peptidoglycan biosynthesis was present. This indicated that more than 30 enzymes had to be present as nuclear genes and all would have to be imported into the muroplasts. Thus, this prokaryotic structure *par excellence* is constructed with the aid of "eukaryotic" enzymes. The function of FtsW is not yet certain in *E. coli*: a possible role as "flippase" is discussed (J. Ayala, personal communication), i.e. in shifting the disaccharide pentapeptide building block from the cytoplasmic side to the periplasmic side of the inner envelope membrane. The function of genes with similarity to *ftsW* and *ftsI* (penicillin-binding protein 3) in the plastid genomes from the (ancient) green algae *N. olivacea* and *Mesostigma viride* (Turmel *et al.*, 1999; Lemieux *et al.*, 2000) is equally not clear. In the absence of any evidence for peptidoglycan walls around the respective chloroplasts the genes might be considered orphan genes, relics of abandoned prokaryotic biosynthesis pathways, possibly with altered functions. A similar explanation could apply with respect to genes for enzymes involved in the biosynthesis of lipopolysaccharide, another constituent of prokaryotic cell envelopes. Such genes were recently identified in the plastome of *C. caldarium* (Glöckner *et al.*, 2000). The activity of a β -lactamase has not been detected in cyanelles (Claus *et al.*, 2000) which is in accordance with the observed sensitivity of *C. paradoxus* to β -lactam antibiotics (Berenguer *et al.*, 1987).

6. Phylogenetic Position of *C. paradoxa* and its Cyanelles

C. paradoxa has often been included in phylogenetic analyses. Depending on the sequences and algorithms that were chosen for analysis, cyanelles were variably placed close to cyanobacteria, rhodoplasts and even chloroplasts. Apart from the lack of sufficient sequence information in earlier years, this lack of resolution simply reflects the "bridge nature" of cyanelles (Löffelhardt *et al.*, 1997a). More recent examples are now available. Cyanelle PsaA and PsAB cluster with cyanobacteria, ChlL with cyanobacteria and red algae, and PetB with plants (Xiong *et al.*, 1998). The analysis of EF-Tu sequences place *C. paradoxa* at the base of the plastid phylogenetic tree (Ishida *et al.*, 1997). A combined phylogenetic analysis of 46 protein genes common to 10 completely sequenced plastid genomes established cyanelles as the closest relatives of cyanobacteria and as the first branch after the singular primary endosymbiotic event (Martin *et al.*, 1998). The muroplasts of *C. paradoxa* also formed the base of a plastid tree based on seven concatenated plastid-encoded protein sequences (Zhang *et al.*, 1999). When trees were constructed based on the cytoplasmic glyceraldehyde-3-phosphate dehydrogenase (GapC) or the chloroplast counterpart (GapA), *C. paradoxa* branches before the divergence of rhodophytes and chlorophytes (R. Cerff, personal communication). This was also the outcome of a phylogenetic analysis based on the *trnL^{UAA}* intron sequences (Besendahl *et al.*, 2000). A singular primary endosymbiotic event should also become evident from phylogenies that compare nuclear and mitochondrial genes. In this scenario, a sister group relationship must emerge between glaucocystophytes, chlorophytes, and rhodophytes, which are all derived from the same "host cell". An 18S rRNA-based phylogenetic analysis placed *C. paradoxa* and two other glaucocystophytes as a sister group to cryptomonads (Bhattacharya *et al.*, 1995). Although *C. paradoxa* once was classified as a cryptomonad alga (Wasemann *et al.*, 1987) this assignment clearly was not satisfactory, and indeed was not supported by phylogenetic trees based on actin (Bhattacharya and Weber, 1997), Hsp70 (Rensing *et al.*, 1997), or α - and β -tubulin sequences (Keeling *et al.*, 1999). In the case of α -tubulin a sister group relationship between *C. paradoxa* and red algae became apparent. The PsaD tree shows *C. paradoxa* at the base together with cyanobacteria and red algae (plastid-encoded sequences) but the branching order relative to the chlorophytes cannot be resolved (A. Nickol, N. Müller and H. Schenk, personal communication). The untypical nucleus-encoded cyanelle aldolase grouped with the cyanobacterial branch of the class II subtype B1 (Nickol *et al.*, 2000). 80S ribosomal proteins are promising traits and have been used (Hink-Schauer, 1999; Müller, 2000; Nickol 2000) but, at present, the respective trees suffer from the fact that the sample size of plant and algal sequences is too small. In an analysis of six concatenated nuclear genes (α - and β -tubulin, actin, Hsp70, elongation factor 1 α , and the VatB subunit of the vacuolar ATPase) a conclusive result was obtained (Moreira *et al.*, 2000). Green algae (and plants), red algae and glaucocystophytes (*C. paradoxa*) placed as each others closest relatives, although the branching order was uncertain and the statistical support not very strong. A phylogenetic comparison of concatenated mitochondrial genes from the recently completed mitochondrial genome of *C. paradoxa* with orthologs from red and green algae arrived at the same conclusion (G. Burger and F. Lang, personal communication). It has been postulated that the cyanobacterial ancestor of chloroplasts possessed

chlorophyll *b* and phycobiliproteins (Bryant, 1992; Löffelhardt *et al.*, 1997b). This is now supported by data from *Prochlorococcus marinus*, an extant example for a union of the two light harvesting systems (Hess *et al.*, 1999). In addition, new evidence suggests a common evolutionary origin of chlorophyll *a* oxygenases (enzymes involved in the biosynthesis of chlorophyll *b*) from prochlorophytes and chlorophytes (Tomitani *et al.*, 1999).

7. Cell Biology of *C. paradoxa*

7.1. CHARACTERIZATION OF INDIVIDUAL MEMBRANES

Plasma membrane vesicles of *C. paradoxa* were purified and characterized (Heimann *et al.*, 1997). Sterol (40%) and diacylglycerol (43%) were the major lipid components. Several glycoproteins, containing fucose, galactose and mannose as the predominant sugars, were identified among the more than 20 proteins that could be resolved.

Cyanelle envelope membranes were separated from thylakoid membranes by flotation centrifugation (Pfanzagl and Löffelhardt, 1999). They were used for studies of peptidoglycan biosynthesis (see below). In addition, heterologous antibodies directed against Toc75, Tic 55, and Tic 110 from pea and the Toc75 and Tic55 homologs from *Synechocystis* (obtained from J. So11) were used for immunological detection. Signals were obtained at 78 kDa and 55 kDa, respectively, for the *Synechocystis* Toc75 and Tic55 antisera alone (B. Pfanzagl, unpublished).

7.2. PROTEIN IMPORT INTO CYANELLES

Recent estimates about the protein complexity of *Arabidopsis* chloroplasts assume 2,000 to 2,500 proteins (Abdallah *et al.*, 2000; vanWijk, 2000). The protein complement of the muroplast might be somewhat smaller considering that *trans*- and *cis*-splicing and RNA editing are absent. Also, the number of cyanelle-encoded proteins exceeds that for *Arabidopsis* by approximately 50% (Stirevalt *et al.*, 1995). This leaves around 1,500 cytosolically synthesized precursor proteins that have to be imported across two membranes and the peptidoglycan wall inherited from a cyanobacterial ancestor that is unique to muroplasts and without precedence among eukaryotic organelles. Protein translocation across the plasma membrane, wall, and eventually outer membrane of bacteria is largely an export process. Thus one might envisage the chloroplast protein import apparatus as an eukaryotic achievement, that developed after the primary endosymbiotic event. Such a view does, however, not exclude the recruitment of suitable cyanobacterial envelope membrane proteins for the gradually evolving protein translocation machinery.

Chloroplast protein import has been studied for 25 years with significant progress especially in the last five years (for reviews see Keegstra and Cline, 1999; Chen *et al.*, 2000; Schleiff and So11, 2000). Nucleus-encoded chloroplast proteins targeted to the stroma, the thylakoid system, or the inner envelope membrane in general are synthesized in the cytosol with a N-terminal extension of 35 to 85 aa, the chloroplast transit peptide or stroma-targeting peptide. This presequence is necessary and sufficient for *in vitro* import into isolated chloroplasts (mostly studied with pea or spinach). Its

characteristics are: a hydrophobic N-terminus but overall a rather hydrophilic nature with a high amount of serine and threonine residues, a positive net charge, and a cleavage site consensus for the stromal processing protease (SPP; Gavel and von Heijne, 1990). Several components of the cooperating translocons in the outer (Toc) and inner (Tic) envelope membranes were identified at the protein and gene level. Two of those, Toc75 and Tic55, appeared to have homologs on the respective membranes of *Synechocystis*. GTP is required (two of the putative receptors, Toc160 and Toc34 are G-proteins) and ATP, the latter at varying concentrations and for many processes (cytosolic, inter-envelope-membrane space, stromal) during import. In addition, phosphorylation of transit peptides and Toc34 was shown to be important (Shveshnikova *et al.*, 2000). At low ATP concentrations or at 0°C only binding is observed but no translocation occurs.

An important question was if cyanelles surrounded by their wall would require transit sequences different from those present in plants and green algae. Euglenoids and chromophytic algae, organisms whose plastids are surrounded by more than two membranes do indeed use different plastid targeting signals (Schwartzbach *et al.*, 1998). Upon inspection of 12 transit sequences of precursors to cyanelle proteins from *C. paradoxa* (Figure 3) grouping is quite apparent with stroma-targeting peptides from plants. A weak consensus sequence at the N-terminus (MAFVxxPV) is not found with plants and the content of negatively charged amino acids is higher in the *Cyanophora* presequences. However, the positive net charge, overall amino acid composition and hydropathy plots resemble the plant presequences.

It was expected that the structural integrity of cyanelles surrounded by a wall would make them an ideal system for *in vitro* protein import, where protease treatment of isolated organelles after incubation is required to remove externally attached precursor and thus demonstrate protection of the internalized mature protein. However, the "intactness" of isolated cyanelles, as judged from phase contrast microscopy, does not entail that their outer membranes remain intact during the isolation procedures (Giddings *et al.*, 1983). Isolated cyanelles are not suitable for protein import, unless great care is taken to avoid even a mild osmotic shock during breakage of the cells which has to be done in a blender under strictly hypertonic conditions (Schwartzbach *et al.*, 1998; Steiner *et al.*, 2000). With these precautions, otherwise following conditions equivalent to chloroplast import, efficient translocation and processing of the precursors for **f**erredoxin-NADP⁺ reductase (FNR, Jakowitsch *et al.*, 1996), cytochrome *c*₆ (Steiner *et al.*, 2000) and transketolase (Y. Ma, unpublished) could be demonstrated.

Precursor proteins from *C. paradoxa* performed equally well during heterologous *in vitro* import experiments into isolated pea chloroplasts. In contrast, precursors to plant chloroplast polypeptides proved disappointing in reciprocal heterologous import experiments: preFNR from *Mesembryanthemum crystallinum* entered isolated cyanelles with low efficiency (Jakowitsch *et al.*, 1996), and five other higher plant precursors showed no import at all (Löffelhardt *et al.*, 1998). The reason for this behavior is not known. The first *Cyanophora* presequences characterized showed a positive net charge not higher than +3, whereas chloroplast stroma-targeting peptides range from +6 to +10. We hypothesized that electrostatic interaction of highly positive transit sequences with the negatively charged cyanelle wall might impede the import into cyanelles and that a constraint for moderate positive net charges in transit peptides from *C. paradoxa* might exist (Löffelhardt *et al.*, 1998, 1999). The subsequent detection of transit peptides with

net charges of +6 to +9 (Figure 3) make this hypothesis less likely. It cannot be excluded at present that certain hitherto unrecognized subtle features of transit peptide (or mature protein) in precursors from higher plants are incompatible with (efficient) import into isolated cyanelles. The cytosolic guidance complex (May and Sol1, 2000) demonstrated in higher plants (phosphorylated precursor/14-3-3 protein/Hsp70) might not exist during cyanelle import and, thus, the Toe-associated phosphatase postulated in plants for the essential dephosphorylation step might be lacking, too. Alternatively, the multiple receptors Toc160, Toc132, and Toc120 reported for *A. thaliana* (Bauer *et al.*, 2000) might be a later development and primitive plastids could be devoid of the "quantitative" receptor Toc160. Thus the precursors to Cab, Rubisco small subunit, OE23, ferredoxin, and plastocyanin, all of which are not imported into cyanelles *in vivo*, might be unable to interact with their appropriate receptor(s). Among the *Cyanophora* precursors, pre-cytochrome *c*₆ is not imported *in vivo* into pea chloroplasts but, as FNR and transketolase, obviously can interact with the higher plant receptors or use a bypass as shown for pea chloroplasts where the cytosolic domain of Toc160 had been proteolytically removed (Chen *et al.*, 2000). Our data indicate functions and composition of the cyanelle protein import complexes that are basically similar to those present in chloroplasts (and presumably also in rhodoplasts). However, it is also obvious that the cyanelle (and likely the protoplastid) machineries should be considered prototypes that underwent modifications after the loss of the organelle wall. In this context, the antibody cross-reaction of cyanelle Toc and Tic components with counterparts from cyanobacteria but not with those from chloroplasts may be a significant sign.

7.3. CONSERVATIVE SORTING

Nucleus-encoded plant chloroplast proteins of the thylakoid lumen or extrinsic thylakoid membrane proteins with their N-terminus and bulk hydrophilic domains in the lumen are synthesized in the cytosol with a bipartite presequence consisting of a N-terminal transit sequence followed by a signal sequence for thylakoid translocation. The latter was viewed as the preexisting cyanobacterial targeting signal to which a "eukaryotic" targeting sequence was added after the endosymbiotic event and transfer of the respective gene to the nucleus (Smeekens *et al.*, 1990). This implied the conservation of prokaryotic preprotein translocases by plastids. The first indication of such a translocon in the thylakoid membrane came from the identification of a *secY* gene on the genome of cyanelles (and other primitive plastids) and from the complementation of a thermosensitive *E. coli* mutant by *secY* from *C. paradoxa*. Since then, four different prokaryotic pathways/translocons have been established in higher plant chloroplasts (for reviews see Keegstra and Cline, 1999; Woolhead *et al.*, 2000) for translocation across or integration into the thylakoid membrane. Each system is responsible for the transport of a certain subset of luminal/membrane proteins. The ATP-dependant, azide-inhibited SecY/E/A pathway accepts unfolded (apo)protein precursors for e.g. plastocyanin, PsaD, and OE33. The ΔpH-dependant Tat pathway, which is blocked by nigericin, succeeds in transporting folded precursors whose signal sequences carry a characteristic "twin arginine" motif preceding the hydrophobic core (OE23, OE17, polyphenol oxidase). The posttranslational SRP pathway is thought to be reserved for thylakoid integration of Cab protein family members. The fourth

("spontaneous" or "unassisted") pathway mediates the insertion of selected membrane proteins, such as AtpG, PsbX, and PsbW.

As with envelope import the complement of precursors (or intermediates) translocated across the thylakoid membrane is somewhat different for chloroplasts and cyanelles. In *C. paradoxa* cytochrome *c*₆ (instead of plastocyanin) performs the electron transport between the luminal regions of the *b*_{6/f} complex and photosystem I and the extrinsic photosystem II proteins PsbP (OE23) and PsbQ (OE17) are replaced by the unrelated proteins PsbV (**cytochrome c₅₅₀**) and PsbU, respectively. Furthermore, with respect to the thylakoid integration candidates, Cab antennae are absent from cyanelles and AtpG is cyanelle-encoded and thus lacks the bipartite presequence of the chloroplast counterpart.

The presequence of the cytochrome *c*₆ precursor is bipartite (Figure 3, with the putative SPP cleavage site after aa 37: **VRM↓S**). When labeled protein is offered, the majority of the precursor was internalized into isolated cyanelles during a three minute incubation. Low ATP (in the dark) or low temperature allowed binding only (Steiner *et al.*, 2000). Under normal import conditions no intermediate (mature protein plus signal peptide) accumulated, indicating that envelope translocation was the rate-limiting step. Conservative sorting operated also in a heterologous *in vitro* system (spinach or pea chloroplasts): mature cytochrome *c*₆ appeared in the thylakoid lumen after two processing steps. Sensitivity of cyanelle thylakoid import towards sodium azide (as shown by accumulation of intermediate in the stroma) but not towards nigericin pointed towards the Sec pathway. This was corroborated by competition experiments in the heterologous system: the Sec passenger OE33 inhibited thylakoid translocation of i-cytochrome *c*₆ whereas the ΔpH passenger OE23 had no effect (J. Steiner, unpublished). Cyanelle and cyanobacterial thylakoid vesicles are not suitable for import experiments (in contrast to those from pea chloroplasts). They can only be obtained in a structurally compromised form and are leaky which precludes protease protection of the internalized mature polypeptide (Schwartzbach *et al.*, 1998). Thus, the FNR transit sequence was engineered to the N-terminus of the cyanelle-encoded pre-cytochrome *c₅₅₀* and the import was performed with isolated cyanelles. The mature protein appeared in the thylakoid lumen after two processing steps. Considering the energy requirements and the effects of azide/nigericin upon thylakoid translocation, the Sec pathway is also operative (T. Köcher and J. Steiner, unpublished). Heterologous import and sorting of this composite precursor to the thylakoid lumen of spinach chloroplasts was also observed. The replacement of PsbV by PsbP in chloroplasts is accompanied by a change in the translocon used. Owing to the lack of a twin-arginine motif in the signal sequence, the cyanelle-encoded Hcf136 homolog from *C. paradoxa* (see above) can be predicted to use the Sec pathway for thylakoid translocation, in contrast to the precursor from *A. thaliana* which is a passenger of the Tat translocon (Hynds *et al.*, 2000). Thus far, no thylakoid (lumen) protein (precursor) with a twin arginine motif in the signal sequence is known for *C. paradoxa* and this also applies for *Synechocystis*. However, in a recent paper on proteomics of the periplasm of this cyanobacterium at least 10 candidates for the Tat pathway (via the corresponding translocon in the inner envelope membrane) were found (Fulda *et al.*, 2000).

Chloroplast Rieske Fe-S protein is a candidate Tat passenger (R.B. Klösgen, personal communication) based on the energy requirements for thylakoid insertion,

although there is no twin arginine in the uncleaved N-terminal signal anchor sequence. This could be justified because the apoprotein most likely attains its Fe-S cluster in the stroma (A. Seidler, personal communication) and because the twin arginine is present in the cyanobacterial orthologs. In this case, the *C. paradoxa* counterpart seems to position with the higher plant proteins. Experiments are under way to investigate whether the cyanelle Rieske Fe-S protein emerges as the first Tat passenger targeted to the lumen of cyanelle thylakoids. The current opinion is that the posttranslational SRP pathway in chloroplasts is reserved for Cab proteins and thus should be absent from cyanelles and rhodoplasts (N. Hoffman, personal communication). We challenge this hypothesis and postulate that thylakoid integration of the nucleus-encoded AtPI is SRP-mediated. From preliminary sequence data (J.M. Steiner, unpublished) we infer the presence of three trans-membrane domains in the mature protein for interaction with SRP54 and the presence of an interspersed hydrophilic region with similarity to the "L18" domain of Cab which has been shown to bind to SRP43 (DeLille *et al.*, 2000).

We consider conservative sorting a key feature of organelles derived from prokaryotic endosymbionts. The prototype nature of the cyanelle envelope Toc and Tic translocons might also apply for conservative sorting. Future research is directed towards the possibility of a dual localization of translocases of the Sec, Tat, and SRP pathways in cyanelle thylakoid and inner envelope membranes.

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THE SYNTROPHY HYPOTHESIS FOR THE ORIGIN OF EUKARYOTES

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1. Introduction

The last three decades of the twentieth century will be remembered as historical times for evolutionary biology, and most particularly, microbiology. In 1977, applying Zuckerkandl and Pauling's idea that biological macromolecules accumulate evolutionary information (Zuckerkandl and Pauling, 1965), Woese and Fox published a phylogenetic tree of organisms using the universally conserved 16/18S ribosomal RNA (rRNA) as molecular marker. This event was doubly revolutionary. First, it opened up the possibility of reconstructing an actual phylogeny of all living organisms, including prokaryotes, for which taxonomy and evolution had long been disconnected. And second, it led to the recognition of the Archaea (archaeabacteria) as a third lineage or domain of organisms (Woese and Fox, 1977; Woese *et al.*, 1990). Archaea were, in terms of rRNA sequence, as distantly related to eukaryotes as they were to (eu)bacteria, and their distinctness was supported by a number of other features, such as the presence of exclusive ether-linked lipids of particular stereochemistry (Kates, 1993).

The discovery of the Archaea complicated the question of the origin of eukaryotes. Until then, two theoretical possibilities were conceivable. Either eukaryotes were ancestral and prokaryotes derived from them by a reductive process (Reanney, 1974) or eukaryotes derived from simpler prokaryotes by increasing complexity, a widespread idea that was already implicit in the phylogeny proposed by Haeckel one century earlier (Haeckel, 1866). But now, the prokaryotic world was profoundly split in two lineages, Bacteria and Archaea, apparently equally distant from eukaryotes. Where did eukaryotes come from?

1.1. MOLECULAR PHYLOGENY AND CONTROVERSIES ON THE TREE OF LIFE

A possible answer to the above question could be deduced from the work of several authors who, using conserved pairs of ancestrally duplicated (paralogous) genes, had placed the root of the tree of life on the bacterial branch (Gogarten *et al.*, 1989; Iwabe *et al.*, 1989). This implied that archaea and eukaryotes share a more recent common ancestor. That eukaryotes arose from a common stem leading also to archaea as sister group is still the most popular hypothesis to explain eukaryotic origins. This idea has been modernized to accomodate the existence of horizontally acquired information, which is deduced from whole genome comparisons (Doolittle, 1998; Woese, 2000).

However, the use of some proteins as phylogenetic markers and, more recently, comparison of entire genome sequences revealed important contradictions with the universal rRNA-based tree. Eukaryotic informational genes, i.e. those related to replication, transcription and translation, are archaea-like, whereas eukaryotic operational genes, i.e. those mainly related to metabolism and transport, are typically more akin to, specifically, Gram negative bacterial counterparts (Brown and Doolittle, 1997). In order to overcome such contradictions, chimeric models began to appear, stating that eukaryotes are the outcome of the merging of archaeal and bacterial lineages (reviewed in Gupta and Golding, 1996; Lopez-García and Moreira, 1999). From these, symbiosis-based models are the most evolutionary and biologically consistent. Margulis' serial endosymbiotic theory proposed that the endosymbioses originating mitochondria (derived from α -proteobacteria) and chloroplasts (from cyanobacteria) had been predated by a primordial symbiosis between spirochetes, providing motility, and *Thermoplasma*-like wall-less thermoacidophilic archaea (Margulis, 1996). Other, metabolic, advantages for this symbiosis based on sulfur metabolism have been incorporated to this model only very recently (Margulis *et al.*, 2000).

Among the first metabolic symbiosis models for the origin of eukaryotes is that of Searcy, who proposed that sulfur transfer between *Thermoplasma*-like archaea and sulfur-dependent proteobacteria triggered eukaryogenesis (Searcy, 1992; Searcy *et al.*, 1978). The sulfur-dependent proteobacteria would have originated mitochondria and, in so doing, they would have produced eukaryotes. The idea that the origin of mitochondria concurs with the origin of eukaryotes has been also exploited in the hydrogen hypothesis by Martin and Müller (Martin and Müller, 1998) but, here, the metabolic product exchanged is hydrogen, between fermentative anaerobic α -proteobacteria (future mitochondria) and hydrogen-consuming strict anaerobic archaea, presumably methanogens.

1.2. SYMBIOSIS IN EVOLUTION.

Symbiosis has an immense explanatory potential in biological evolution, both from empirical and theoretical viewpoints.

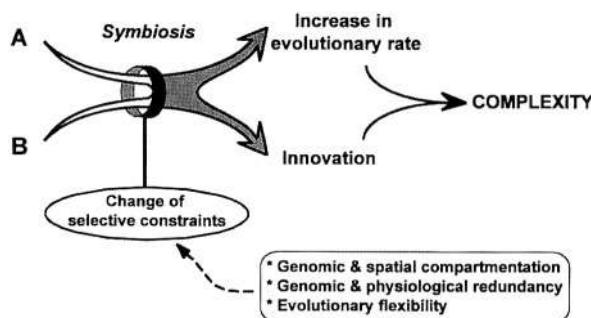


Figure 1. Selective and evolutionary consequences of the establishment of a symbiosis.

When two organisms, A and B, establish a symbiotic association, a change in the selective constraints is necessarily generated (Figure 1). Simultaneously, mechanisms to overcome the new constraints result from the genomic and spatial compartmentation, genomic and physiological redundancy and evolutionary flexibility derived from the symbiosis (Smith and Szathmary, 1995). All this represents an extraordinary increase in evolvability (Kirschner and Gerhart, 1998). Two consequences follow that can allow a considerable increase in complexity: an increase in evolutionary rate, and the capacity to create new features, i. e. to innovate (Margulis and Fester, 1993). Paradigmatic in this sense is the example of lichens as complex and idiosyncratic products of symbiosis between cyanobacteria or algae and fungi.

Symbiosis between bacteria and archaea could, therefore, well explain the mixed heritage of eukaryotic genes through a pathway of acquisition of new properties and increasing complexity. However, although previous metabolic symbiosis models explain why a putative eukaryogenetic symbiosis might have been established, they fail to account for major eukaryotic features such as the cytoskeleton, or the origin of the nucleus (and the selective force to create it).

2. Building up a Model for Eukaryogenesis

In order to elaborate a feasible pathway for eukaryotic evolution, we started from two undisputable facts. On the one hand, eukaryotic genomes predominantly have archaeal-like informational genes and bacterial-like operational genes (Ribeiro and Golding, 1998; Rivera *et al.*, 1998). On the other hand, symbiosis has played a major role in eukaryotic evolution as attested by mitochondria and chloroplasts. Not only their acquisition was key to an extraordinary ecological and evolutionary success but, in the case of mitochondria, their evolution might have been simultaneous to the origin of eukaryotes themselves, since all eukaryotes known today appear to have harboured mitochondria once (Germot *et al.*, 1996; Horner *et al.*, 1996; Roger *et al.*, 1996; Roger *et al.*, 1998). A tempting idea began to shape: Could eukaryotes have evolved from a symbiotic event between archaea and bacteria? Looking for an answer, we first asked whether such symbioses exist in nature.

2.1. SYMBIOSES BETWEEN ARCHAEA AND BACTERIA

The only known contemporary type of archaeal-bacterial symbiosis is established in anaerobic environments between methanogenic archaea and a limited variety of bacteria, usually sulfate-reducing **δ -proteobacteria**, and is based on interspecies hydrogen transfer (Fenchel and Finlay, 1995). In sulfate-rich sediments, sulfate-respirers are efficient competitors of methanogens, since they require hydrogen for sulfate reduction, and methanogens, for methanogenesis. However, when the environment is depleted in sulfate, these organisms ferment, liberating hydrogen which is then taken up by methanogens. The bacteria depend on methanogens to remove hydrogen, since the fermentation process is only possible when the partial pressure of hydrogen is extremely low (Fenchel and Finlay, 1995). Many of these symbioses are obligatory, which has sometimes led to the missclassification of symbionts as a single organism, as was the

case of "Methanobacillus omelianskii", shown to be formed by two dissimilar species in close contact (Bryant *et al.*, 1967). Cells develop extensive membrane contact to favour gas exchange and avoid loss of volatile hydrogen, and very stable (often obligatory) consortia are generated (Fenchel and Finlay, 1995).

This metabolic symbiosis, or syntropy ("feeding together"), based on bacteria-to-archaea hydrogen transfer is widespread in anaerobic regions of our planet, from forest soils to freshwater and oceanic sediments (Fenchel and Finlay, 1995), and it may have been even more ubiquitous on early Earth (see below). Could an ancestral symbiosis of this type have initiated eukaryogenesis? A possible answer to it could come from shared specific characters of eukaryotes and the prokaryotic partners involved in those symbioses.

2.2. EUKARYOTIC TRAITS IN METHANOGENS AND δ -PROTEOBACTERIA

Methanogens, being archaea, share a core of informational genes with eukaryotes (Brown and Doolittle, 1997) (Ribeiro and Golding, 1998). However, this can equally favor a symbiotic origin of eukaryotes involving a methanogenic partner, and the classical model based on a specific archaeal-eukaryotic sisterhood. More telling could be the striking occurrence of some specific characters exclusive to eukaryotes and certain methanogenic archaea. Perhaps the most remarkable of these is the presence of histones and nucleosomes. Archaeal histones are *bona-fide* homologs of eukaryotic H4 (and H3 in less degree) histones, which are considered among the most conserved proteins (Grayling *et al.*, 1996; Pereira and Reeve, 1998). Archaeal histones are present in early branching Euryarchaeota, but are absent in the other archaeal kingdom, the Crenarchaeota (or "eocytes") (Kawarabayasi *et al.*, 1999), and also in some mid-branching euryarchaeota, notably in *Thermoplasma* species (Ruepp *et al.*, 2000). *Thermoplasma* spp. possess instead HTa, a DNA binding protein homologous to the bacterial HU protein of probable horizontal acquisition (DeLange *et al.*, 1981; Grayling *et al.*, 1994). Histone genes are present in the tip-branching euryarchaeote *Halobacterium salinarium* (Ng *et al.*, 2000), and they appear to exist also in *Methanosarcina* spp. (information available at the Archaeal Histone Database: <http://www.biosci.ohio-state.edu/~microbio/Archaealhistones/index.html>). However, in the latter, the precise role and significance of histones is yet to be established, since these organisms are known to use MC1 proteins to bind and compact their DNA (Laine *et al.*, 1991). Bacterial HU, HTa, MC1, and also the small DNA binding proteins of the Sso/Sac7 family existing in Sulfobolales (crenarchaeota) act in a similar way: they intercalate into the double helix inducing different degrees of bending and compaction (Gao *et al.*, 1998; Robinson *et al.*, 1998). However, archaeal histones act in a radically different way. They are not only sequence homologs to their H3-H4 eukaryal counterparts, but they form structures homologous to the H4-H3 eukaryotic tetramer, that function in a similar manner: they induce DNA wrapping around a central protein core (Pereira and Reeve, 1998). Furthermore, both tetramers are able to undergo similar dynamics, since they can flip from right to left-handedness, thus accomodating positive or negative DNA supercoils, depending on parameters such as salt concentration or temperature (Musgrave *et al.*, 2000). All this, together with a comparable complement of DNA topoisomerases that are exclusively relaxing (Lopez-Garcia, 1999; Moreira and

Lopez-Garcia, 1998) suggest that eukaryotes and histone-containing moderately thermophilic-mesophilic methanogens share a similar basis for genome structure and function (Bendich and Drlica, 2000) with, presumably, a common origin. The presence of similar polar lipids (glycosylated phosphatidylinositol) (Nishihara *et al.*, 1992), headgroups (phosphoserine and phosphoethanolamine) (Koga *et al.*, 1993), and the apparent occurrence of specific metabolic pathways in lipid metabolism (Yamaghisi *et al.*, 1996) in methanogens, but not in other archaea, appear to reinforce this idea.

Regarding the bacterial partner, we drew our attention to myxobacteria, which are Proteobacteria of the δ subdivision, whose ancestor was likely sulfate-reducing (Woese, 1987). Myxobacteria share striking similarities with eukaryotes as well. These bacteria have complex life cycles and a highly developed social behaviour. Cells are able to communicate and establish organized movements. They differentiate and form reproductive structures that are multicellular (Reichenbach and Dworkin, 1992). Common to eukaryotes and myxobacteria are, among other features, many components of regulatory processes, such as serine and threonine kinases, calmodulin-like proteins or the existence of a phosphatidyl-inositol cycle possibly involving G-proteins (reviewed in Dworkin, 1996). Amazingly, their ability to secrete various hydrolytic enzymes and antibiotics (many against eukaryotes), the production of melanin and the complexity of their life cycles and reproductive structures led to their classification as fungi for nearly a century (Reichenbach and Dworkin, 1992).

3. The Syntrophy Hypothesis

The syntropy hypothesis proposes that eukaryotes evolved from a metabolic symbiotic association mediated by interspecies hydrogen transfer between a δ -proteobacterium, in particular an ancestral sulfate-reducing myxobacterium, and a moderately thermophilic or mesophilic methanogenic archaeon endowed with histones and nucleosomes. This ancestral symbiosis took place in environments where bacterial fermentation was favoured over sulfate-respiration, as is the case for today's **methanogen- δ -proteobacterial** symbioses. In our model, the acquisition of mitochondria from α -proteobacteria is an independent event, and we propose that the α -proteobacterial ancestors of mitochondria had the ability to oxidize methane (Lopez-García and Moreira, 1999; Moreira and Lopez-García, 1998).

This hypothetical evolutionary pathway is depicted in Figure 2, and briefly detailed below.

3.1. MORPHOLOGICAL AND STRUCTURAL EVOLUTION: THE NUCLEUS

The starting point in our evolutionary model is the establishment of well-integrated syntrophic consortia where methanogens were completely surrounded by δ -proteobacterial cells. Membranes would be in close contact to favour gas and metabolite exchange, as it happens in present-day consortia (Fenchel and Finlay, 1995). As the symbiosis became obligatory, further membrane extrusions and invaginations to increase and optimize surface contact would have been developed. Methanogenic cell

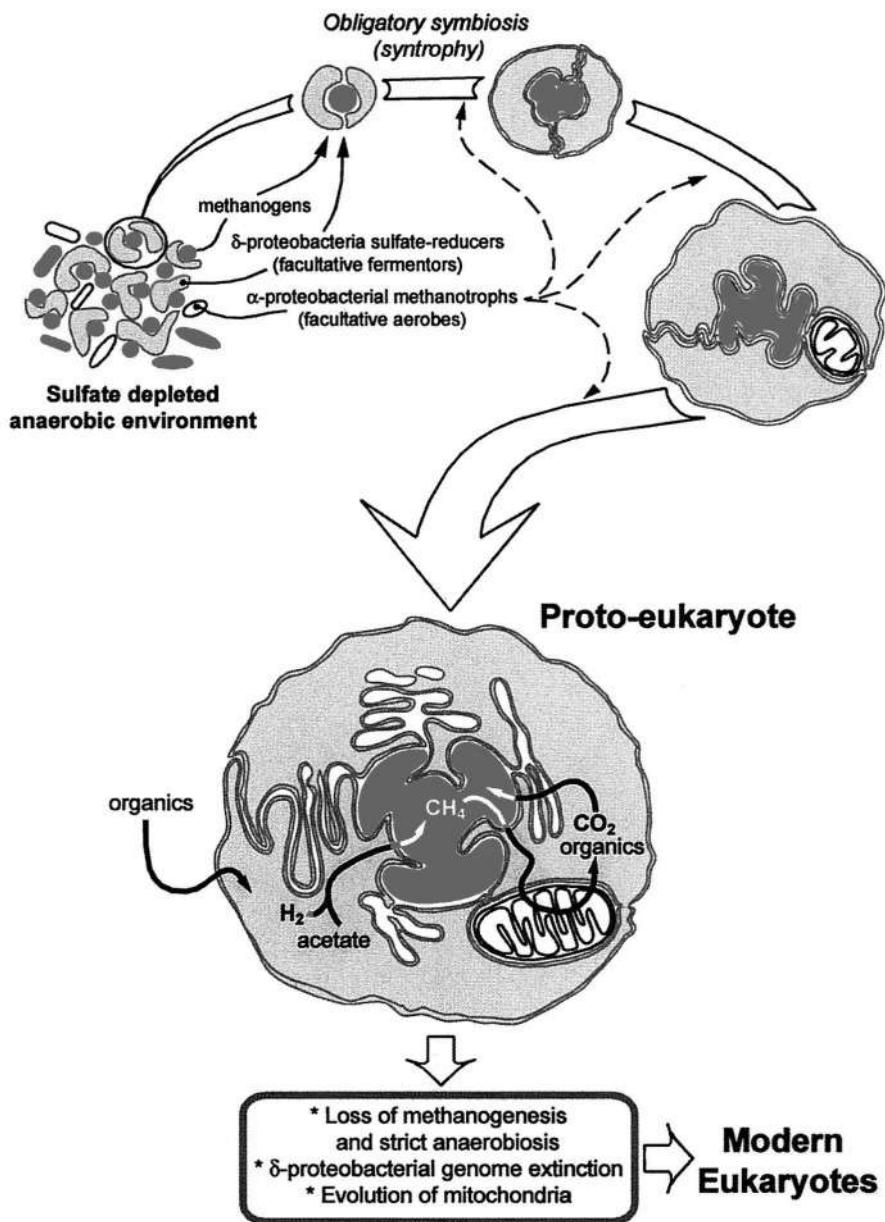


Figure 2. The syntropy hypothesis: A model for the evolution of eukaryotes from a H_2 -mediated metabolic symbiotic consortium of δ -proteobacteria and methanogens. Mitochondria derived from α -proteobacterial methanotrophs acquired at early stages of eukaryotic evolution.

surface would be thus entirely and intimately coupled to bacterial membranes (possibly from one or two bacterial cells between which intercytoplasmatic bridges eventually appeared). At a certain point, archaeal membranes would have been lost. Archaeal ether-linked lipids would have disappeared, while bacterial ester-lipids would have prevailed. However, useful membrane components, and their synthetic pathways, would have been retained. Bacterial membranes would be essential because of their adaptation to acquire organics for fermentation from the environment. Archaeal lipids would become redundant and no longer advantageous, since a buffered anaerobic environment would be already provided for the methanogen by the surrounding bacteria, and metabolites could circulate more easily through bacterial membranes and membrane pores.

At an advanced stage, a proto-nuclear region would be defined by membranes with different invaginations and extrusions (the future endoplasmic reticulum), that would contain the old archaeal cytoplasm. Future nuclear pores would have been originated at this point by recruiting and organizing transmembrane proteins in order to allow traffic of macromolecules or even higher-order structures such as ribosomal particles. In this sense, the whole eukaryotic nucleolus could be viewed today as a remnant of the ancestral archaeal cytoplasm. Ribosomal proteins and RNAs are precisely folded and assembled in today's eukaryotic nucleolus to produce ribosomal subunits that are subsequently exported to the cytoplasm, where complete ribosomes are finally built up. During the nucleolar phase of ribosomal assembly, a number of guide RNAs and specific proteins are required. Highly conserved homologs of small nucleolar RNAs (snoRNAs) and nucleolar proteins, such as fibrillarin, Nop56/58, Nop10p and Gar1p, have been found in archaea (Omer *et al.*, 2000; Watanabe and Gray, 2000). Therefore, eukaryotes appear to have inherited also the archaeal way of assembling ribosomes. In our model, large pores would have developed to allow ribosomal subunit export to the metabolically active bacterial-derived cytoplasm.

Other typical eukaryotic features such as the cytoskeleton and phagotrophy, would have appeared as a consequence of symbiotic innovation. During the long-term obligate syntrophy, a great membrane plasticity would have been developed, a prerequisite for phagotrophy. Cytoskeletal proteins to facilitate and stabilize morphological changes would be required. These proteins would be possibly recruited from pre-existing proteins. A change in the selective constraints, and the existence of duplicated copies of equivalent genes in the syntrophic consortium, could have facilitated the acquisition of novel functions best fitted for the new situation. An example could be the case of the prokaryotic cell division protein FtsZ, a tubulin homolog essential for bacterial mitosis (Faguy and Doolittle, 1998). Euryarchaeota are known to have at least 2 copies of an archaeal version of FtsZ, whereas, intriguingly, crenarchaeota lack FtsZ together with other associated genes involved in replication (Bernander, 2000). At least one of the euryarchaeal FtsZ copies may have speeded up its evolutionary rate in the consortium originating tubulin. The cytoskeleton, that would have evolved originally with a mitotic function to allow efficient chromosome segregation, would have extended its functions to other cellular requirements. Thus, phagotrophy, an essential property for the consortium when it finally lost methanogenesis and depended on the capture of organic matter, would have evolved in parallel to the cytoskeleton that sustained short-term cell-shape changes.

3.2. GENOME EVOLUTION

We propose a pathway of genome evolution that resulted in final **δ -proteobacterial** genome extinction in favour of a centralized proto-eukaryotic genome (Moreira and Lopez-Garcia, 1998). Bidirectional archaeal-bacterial gene flow would have occurred but, for some reason (similar to that applicable to the analogous organelle genome evolution process), the archaeal genome would have become stabilized as recipient genome. Briefly, genes arriving in the archaeal genome would have met a new genetic environment and would have had to adapt. Many genes would have been lost, but others would have acquired regulatory signals and, eventually, targeting sequences to trace the products back to the bacterial cytoplasm. Redundant genes could either get lost, replace archaeal counterparts or speed up their evolutionary rate often resulting in new functions. Once the first bacterial genes were settled in the archaeal genome, the symbiosis became irreversible. The process would have been accompanied by an increase in genome size, and genome partition.

3.3. METABOLIC EVOLUTION AND THE ORIGIN OF MITOCHONDRIA

The primary syntrophy would have been established in anaerobic sulfate-depleted environments by a fermentative (potentially sulfate-reducing) **δ -proteobacterium** liberating hydrogen, that would be immediately taken up by a hydrogen consuming methanogen. Together with hydrogen, other useful metabolites would have been transferred, such as acetate (many methanogens are acetoclastic). As a whole, the syntrophic consortium would have a variety of metabolic capabilities contributed by the bacterial partner which was able to ferment a great diversity of organic compounds. It would begin to play the ecological role of an organotrophic scavenger. As the consortium evolved and gene transfer towards the archaeal genome, including metabolic pathway genes, occurred, the proto-eukaryotic genome would have gained control of the consortium metabolism. Less efficient methanogenesis would finally have been lost in favour of a much more efficient heterotrophy, especially after the acquisition of mitochondria. The proto-eukaryotic nucleus, i.e. the old archaeal cytoplasm, originally a metabolic compartment, would have become a true nucleus with exclusive genome management tasks.

Undoubtedly, the acquisition of mitochondria was a crucial event that consolidated the heterotrophic metabolism. Future mitochondria would have been acquired at some point during the evolution of the proto-eukaryotic consortium. We propose that mitochondrial ancestors were facultatively anaerobic **α -proteobacteria** able to oxidize methane and to establish a permanent symbiotic association with the already existing consortium. Indeed, the existence of anaerobic consortia composed by sulfate-reducers, methanogens and methanotrophs has been described (Hoehler *et al.*, 1995). **α -proteobacterial** methanotrophs would recycle methane in the syntrophic consortium, liberating useful compounds for the methanogen such as CO₂ and organics, and speeding up methanogenesis by acting as CH₄ sink (Figure 2). After methanogenesis be lost, the need for strict anaerobiosis would disappear, and the shift to the much more

efficient aerobic respiration would easily be selected, especially on a planet with increasing oxygen atmospheric levels.

For us, **α -proteobacterial** methanotrophs are appealing alternatives as ancestors of mitochondria compared to highly derived *Rickettsia*-like organisms, considered by some authors as the closest relatives to mitochondria (Gray *et al.*, 1999). Methane-oxidizing **α -proteobacteria** belong to class II methanotrophs, which possess a complete Krebs cycle and generate internal membranes parallel to the plasmic membrane that do invaginate (Hanson and Hanson, 1996). They are often found as endosymbionts in the cytoplasm of eukaryotes or even other bacteria (Hanson and Hanson, 1996; Larkin and Henk, 1996). To date, all isolated species of methanotrophic bacteria are aerobic, and use O_2 as electron acceptor for the oxidation of CH_4 to formaldehyde. This has traditionally raised doubts about the actual existence of anaerobic methanotrophy. However, strict anaerobic methanotrophic consortia depending on SO_4^{2-} composed by methanotrophic archaea (presumably methanogens operating in reverse) and sulfate-reducing **δ -proteobacteria** from deep-sea methane hydrates have very recently been characterized (Boetius *et al.*, 2000). Also recent data suggest that NO_3^- can be used by methanotrophic bacteria for methane oxidation under anaerobic or microaerophilic conditions (Ren *et al.*, 2000). These findings make plausible the hypothesis that the ancestors of mitochondria were facultative anaerobic methanotrophic bacteria (**α -proteobacteria**), using alternative electron acceptors in the absence of oxygen.

Under the syntropy hypothesis, eukaryotic metabolism would thus derive from **δ** - and **α -proteobacteria** that contributed the major heterotrophic pathways to gain carbon and energy, including oxygen respiration.

4. Testing the Syntropy Hypothesis

Can the syntropy hypothesis be tested? The most honest answer to this question is that at least its plausibility can, by testing some of the hypothesis' predictions. Data from different disciplines should converge towards the construction of a coherent history.

4.1. COMPARATIVE GENOMICS AND MOLECULAR PHYLOGENY

The syntropy model predicts that eukaryotic genomes should contain genes from methanogenic archaeal, **δ -proteobacterial** and **α -proteobacterial** (mitochondrial) origins. Although the phylogenetic signal of many genes may have been lost with time, comparative genomics and molecular phylogeny may help to validate or invalidate our proposal. In fact, there are already some data that would favour the syntropy model over some others. For instance, it became clear that eukaryotic genomes are not more similar to crenarchaeal genomes after comparison with the *Aeropyrum pernix* genome sequence (Faguy and Doolittle, 1999; Kawarabayasi *et al.*, 1999). "Eocyte" models appear now old-fashioned. On the contrary, the existence of many genes in common with euryarchaeota, including FtsZ and some replicative proteins (Bernander, 2000), and histones, favours the idea of eukaryotes deriving from this archaeal branch. Also some genes, such as the conserved RPL10, RPS12, and SecY, appear to be more related to

their euryarchaeotal than to their crenarchaeotal counterparts (Brown and Doolittle, 1997).

At present, few genome sequences of δ -proteobacteria are available. It would be most interesting to have myxobacterial genome sequences, since these bacteria have striking similarities to eukaryotes mostly in signaling components (Dworkin, 1996). A limiting factor for myxobacterial genomes projects up to date has been that their genome sizes are among the largest in bacteria, up to 12 Mbp (Chen *et al.*, 1990). An interesting insight could come, however, from the phylogenetic analysis of pyruvate ferredoxin oxidoreductase, an enzyme found in some anaerobic protists and bacteria. The eukaryotic enzyme seems to have a monophyletic origin and, although some horizontal transfers can be detected among bacteria, α -proteobacterial sequences appear distant from the eukaryotic counterparts. From the available sequences, the closest are found in δ -proteobacteria (*Desulfovibrio* spp.) (Horner *et al.*, 1999); and data not shown).

Also, the determination of more α -proteobacterial genome sequences should help to identify the closest relatives to mitochondria. Traditionally, *Rickettsia*-like organisms are considered as these organelles' ancestors (Gray *et al.*, 1999). However, *Rickettsia* spp. are parasitic and display long branches in phylogenetic trees, as mitochondria do, which are prone to generate long-branch attraction artefacts. Therefore, their sisterhood is likely artefactual, and the actual α -proteobacterial ancestors of mitochondria are yet to be unequivocally determined (Lopez-García and Moreira, 1999).

4.2. MICROBIAL ECOLOGY

Symbiotic associations appear to be dominant in anaerobic environments (Bernhard *et al.*, 2000). A detailed analysis of how present-day anaerobic consortia form and become obligatory, the genes that are transferred and how, will be certainly very informative. Molecular ecology techniques, allowing the identification of environmental phylotypes without cultivation can contribute to understand many anaerobic environments traditionally poorly studied. Looking for possible relatives of our proposed partners, especially sulfate-reducing myxobacteria, or facultative methanotrophic α -proteobacteria, in native consortia may reveal interesting surprises, as well as studying the diversity of anaerobic eukaryotes, a field for which there is an increasing interest. Obtaining phylogenetically informative gene sequences from selected environments is now a major objective of molecular evolutionists with the hope to fill evolutionary gaps in trees based on cultivated species.

In addition to modern molecular ecology techniques, classical approaches are clearly needed. For instance, in the late 1970s, several strains of methanotrophic yeasts were isolated and characterized, and there was some suggestion that methane oxidation could be carried out in mitochondria (Wolf *et al.*, 1980; Wolf and Hanson, 1979). Unfortunately, nobody has pursued these investigations, and the strains became lost. The search for methane-oxydizing eukaryotes, and the study of the subcellular localization of this activity, could help to determine whether methane oxidation is indeed a remnant activity of ancestral mitochondria.

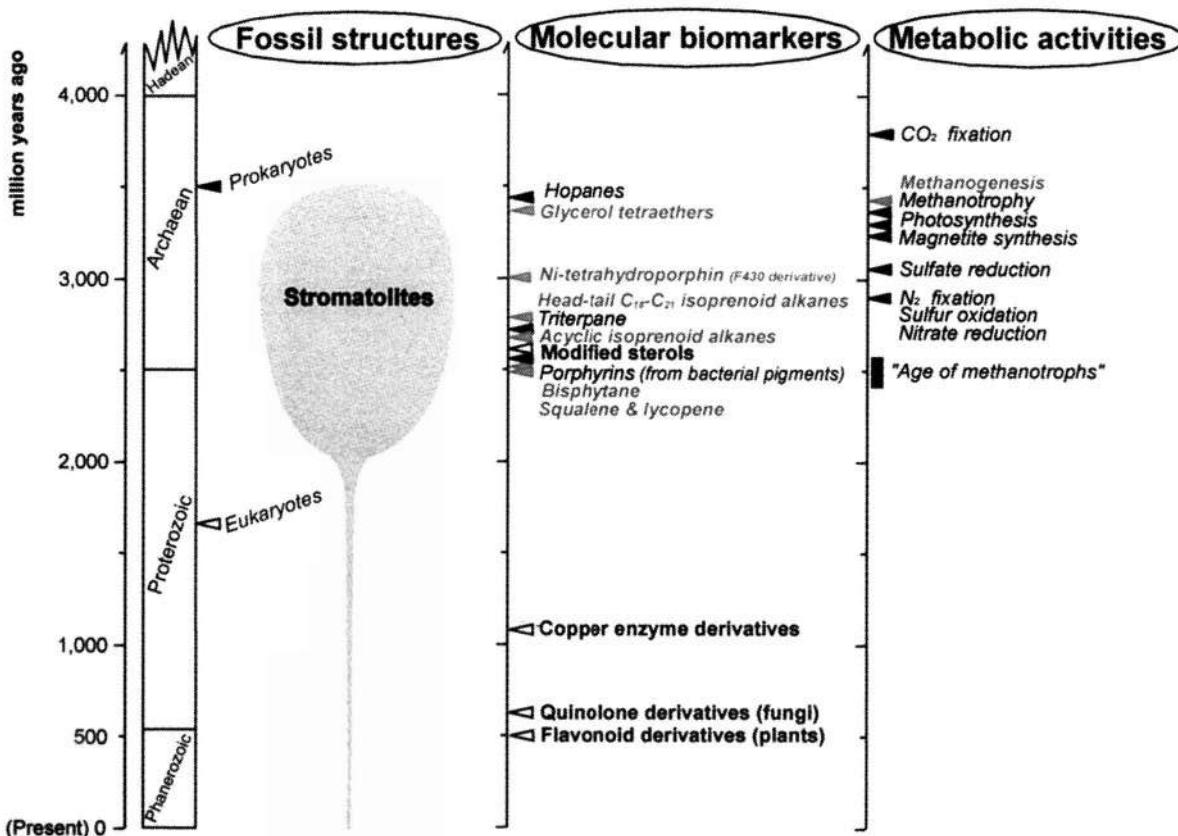


Figure 3. The Precambrian fossil record. The earliest occurrence of fossil structures, molecular biomarkers and traces of metabolic activities is indicated for bacteria (black italics), archaea (grey italics), and eukaryotes (black bold).

4.3. GEOLOGY AND MICROPALAEONTOLOGY

The kind of environment and the type of syntrophy we propose could be as old as the first living organisms. During the last decades information on microfossils has accumulated (Knoll, 1990; Knoll, 1999); (Schopf, 2000). The fossil record does not only encompass fossil structures, but also molecules (usually lipids) and isotopic traces of biological activities (see Figure 3). The first prokaryotic microfossils detected were living in laminated communities (stromatolites), and can be traced back to 3.5-3.8 billion years ago, together with prokaryotic molecular markers (bacterial hopanes and archaeal lipids), and traces of prokaryotic metabolic processes (Knoll, 1999; Schopf, 2000). Eukaryotic fossils and the correspondent molecular markers are much more recent. The latest news may place eukaryotes at around 2.1 billion years ago based on sterane traces of possible eukaryotic origin (however, note that some prokaryotes, notably methanotrophs also synthesize sterols, from which steranes derive) (Brocks and Logan, 1999). Interestingly, traces for methanogenesis, methanotrophy and sulfate reduction co-existed since very early in microbial stromatolites (Figure 3). The development of micropaleontological techniques allowing a deeper study of single microfossils should bring new valuable information. Solid micropaleontological data must provide a historical framework coherent with any evolutionary model.

5. Concluding Remarks

The syntropy hypothesis proposes an evolutionary pathway for the origin of eukaryotes based primarily on a symbiotic event between methanogenic archaea and facultative fermentative-sulfate reducing δ -proteobacteria (ancestral myxobacteria). The mitochondrial symbiosis is an independent event involving methane-oxidizing α -proteobacteria. An outcome of the model is that information-processing systems are of (eury)archaeal origin, whereas metabolic, social and developmental functions are of bacterial origin. A major difference with other chimeric models that could also explain those similarities is that, for the first time a selective force for the origin of the eukaryotic nucleus is advanced: *metabolic compartmentalisation*. That is, the nucleus could have originated not to isolate the genetic material from the cytoplasm, as is generally believed, but to allow the coexistence of two inter-dependent metabolic pathways in the protoeukaryotic cell. The cytoskeleton and other eukaryotic properties are products of symbiotic innovation. The primary symbiosis leading to eukaryotes took place in microbial communities thriving in the widespread anaerobic environments that characterized the Archaean earth.

6. Acknowledgements

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7. References

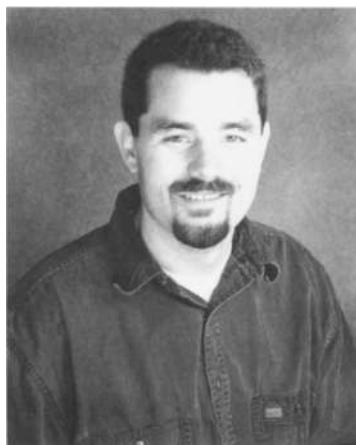
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Biodata of **David L. Valentine** contributor of “*Thermodynamic Ecology of Hydrogen-based Syntrophy*”.

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THERMODYNAMIC ECOLOGY OF HYDROGEN-BASED SYNTROPHY

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1. Introduction

Life is abundant on the surface of the Earth where incident radiation drives the production of oxygen and biomass through the process of photosynthesis. Most of the organisms we are familiar with live near the surface and are either photosynthetic themselves, or live off the oxygen and biomass produced by photosynthesis. When organic material is trapped away from light and oxygen, anaerobic microorganisms take over the role of primary consumers. In doing so, these communities play an important ecological role by converting complex organic material back to its basic constituents, a process termed remineralization. This chapter focuses on the close microbial associations that allow for metabolism of organic material in anoxic, microbially-dominated environments. Particular emphasis is placed on the thermodynamic associations that drive tightly coupled community metabolism in environments where organic matter consumption is coupled to methane production.

Syntrophy is defined as a nutritional situation in which multiple organisms must function together in order to consume a single substrate. Syntrophic associations are essential for the complete remineralization of organic material (Fig. 1), particularly in environments where organic matter decomposition is coupled to methane production (i.e., methanic environments). Complex molecules (carbohydrates, proteins, fats) are first broken down into more simple molecules (sugars, peptides, fatty acids) by the action of extracellular enzymes. These molecules are subsequently converted to smaller organic molecules (fatty acids, amino acids, H_2 , and CO_2) by fermentation. The small organic molecules are converted mainly to H_2 , CO_2 , and acetate by syntrophic fermentation. Small organic molecules and H_2 are subsequently used for methane production by methanogens. The consumption of small organic molecules is driven thermodynamically by the low ambient concentration of H_2 and acetate, which are rapidly converted to methane. The close coupling of H_2 production and consumption in methanic environments is referred to as interspecies H_2 transfer (Wolin, 1982). This process is based firmly on thermodynamic principles and acts to control the pathways of organic-matter remineralization in many anoxic environments; the term ‘thermodynamic ecology’ is used here to describe this situation.

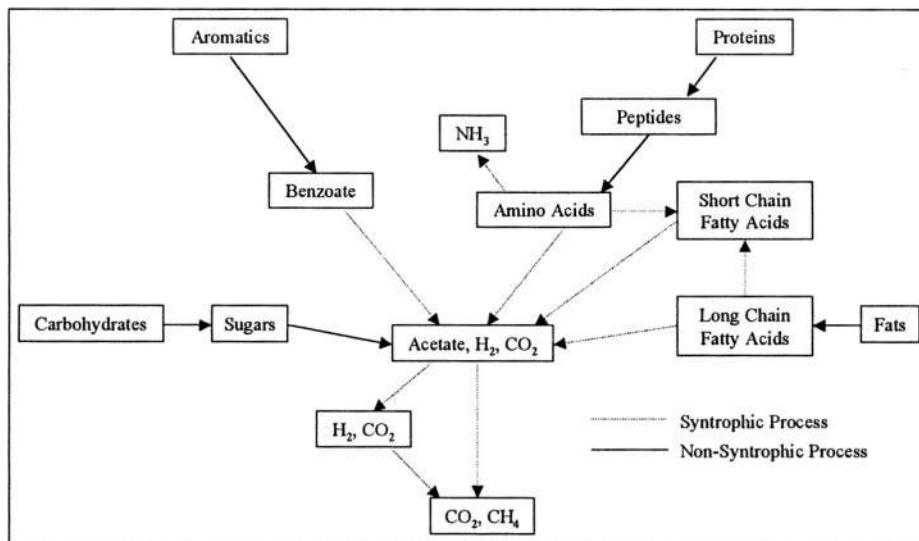


Figure 1. Pathways of organic matter remineralization in methanic environments

2. Thermodynamics of life at low energy

Starvation is a condition in which an organism is unable to acquire sufficient energy to perform basic metabolic functions. Microorganisms have two essential requirements to avoid starvation. First, they must be supplied a sufficient quantity of substrate (i.e., the flux through the catabolic pathway) to maintain their metabolism. Second, they must be able to conserve energy generated from substrate catabolism. In aerobic environments organisms can conserve sufficient energy by oxidizing reduced compounds with molecular oxygen, and more frequently face an insufficient supply of reduced compounds. In anaerobic environments there is often an abundance of reduced compounds, but organisms are limited by the ability to conserve energy from consumption of the available substrates. Table 1 represents important thermodynamic differences between metabolism in aerobic versus methanic environments.

Anaerobic microbial communities are adapted to conserve very small quantities of energy during metabolism because so little energy is available from their substrates. The minimum quantity of energy an organism can conserve during metabolism is the amount of energy required to extrude one ion (usually H^+) across a charged membrane (Mitchell, 1966). This value is thought to be equivalent to 1/3 of the free energy required to convert phosphate and ADP to ATP under physiological conditions, approximately 20 kJ mol^{-1} , and is referred to as the biological energy quantum. There are no known mechanisms to conserve lesser quantities of energy. Organisms involved in syntrophic associations typically produce ATP during catabolism (often coupled to the production of organic acids), but they frequently reinvest energy in the

form of a chemiosmotic potential. A typical energetic scheme for a syntroph might involve production of one ATP per round of catabolism, conversion of that ATP to a chemiosmotic potential, and the use of the chemiosmotic energy to drive H_2 production. This general method of energy conservation allows syntrophs to live off the difference between ATP produced and chemiosmotic potential utilized, generally resulting in the net conservation of 1/3 ATP per round of catabolism (Schink, 1990).

Table 1. Thermodynamics of aerobic and methanogenic metabolism

Substrate		Aerobic Metabolism		Methanogenic Metabolism	
	Products		$\Delta G^\circ (\text{kJ mol}^{-1})$	Products	$\Delta G^\circ (\text{kJ mol}^{-1})$
Sugars	$\text{C}_6\text{H}_{12}\text{O}_6$	$6\text{CO}_2 + 6\text{H}_2\text{O}$	-2870	$3\text{CO}_2 + 3\text{CH}_4$	-418
Proteins	$\text{C}_5\text{H}_{11}\text{NO}_2$	$5\text{CO}_2 + 5\text{H}_2\text{O} + \text{NO}_3^-$	-2950	$2\text{CO}_2 + 3\text{CH}_4 + \text{NH}_3$	-136
Fats	$\text{C}_{10}\text{H}_{20}\text{O}_2$	$10\text{CO}_2 + 10\text{H}_2\text{O}$	-6010	$3\text{CO}_2 + 7\text{CH}_4$	-288

Because there is only minimal energy available for metabolism in anaerobic environments, and because several species often share the available energy, many organisms live at or near the biological energy quantum. That is, the net energy conservation for many organisms in anaerobic environments is the extrusion of one ion per round of catabolism. This generalization holds true for the terminal electron-accepting processes of methanogenesis and sulfate reduction (Conrad and Wetter, 1990; Hoehler *et al.*, 1998), as well as for most syntrophic fermentations (Schink, 1990; Seitz *et al.*, 1990; Scholten and Conrad, 2000). This prevalence of low-energy yields during anaerobic metabolism provides a predictive capability for understanding complex community metabolism in anoxic environments.

3. Microbial Syntrophy

Numerous syntrophic associations contribute to the remineralization of organic material in anoxic environments. Because of the complexities of anaerobic microbial communities, the variety of substrates consumed, the slow rates of syntrophic processes, and the difficulties inherent in studying metabolism in natural environments, our knowledge of these processes relies heavily upon available isolates and studies of waste digestors. Our knowledge of syntrophic associations in nature is even more limited. This section considers the mechanisms, bioenergetics, and broader environmental significance of various syntrophic associations. Table 2 gives several environmentally important reactions involved in syntrophic associations and interspecies H_2 transfer. Figure 2 shows the influence of H_2 concentration and temperature on the thermodynamics of several key reactions.

3.1. TERMINAL ELECTRON-ACCEPTING PROCESSES

Terminal electron-accepting processes are forms of dissimilatory metabolism that can be coupled to remineralization of organic material. These include the following

Table 2. Reactions important to syntrophic associations

Compound Class	Reaction	ΔG°	$\Delta G^{\circ a}$	Eq.
H₂-Producing Reactions				
Organic Acids				
Acetate	$\text{CH}_3\text{COO}^- + \text{H}^+ + 2\text{H}_2\text{O} \rightarrow 2\text{CO}_2 + 4\text{H}_2$	+94.9	-10.2	1
Propionate	$\text{CH}_3\text{CH}_2\text{COO}^- + 2\text{H}_2\text{O} \rightarrow \text{CH}_3\text{COO}^- + \text{CO}_2 + 3\text{H}_2$	+76	-13.6	2
Butyrate	$\text{CH}_3\text{CH}_2\text{CH}_2\text{COO}^- + 2\text{H}_2\text{O} \rightarrow 2\text{CH}_3\text{COO}^- + \text{H}^+ + 2\text{H}_2$	+48.3	-25.9	3
Glycolate	$\text{CH}_2(\text{OH})\text{COO}^- + \text{H}^+ + \text{H}_2\text{O} \rightarrow 2\text{CO}_2 + 3\text{H}_2$	+19.3	-58.9	4
Amino Acids				
Alanine	$\text{CH}_3\text{CH}(\text{NH}_3^+)\text{COO}^- + 2\text{H}_2\text{O} \rightarrow \text{CH}_3\text{COO}^- + \text{CO}_2 + \text{NH}_4^+ + 2\text{H}_2$	+2.7	-75.5	5
R-	$\text{R}-\text{CH}(\text{NH}_3^+)\text{COO}^- + 2\text{H}_2\text{O} \rightarrow \text{R}-\text{COO}^- + \text{CO}_2 + \text{NH}_4^+ + 2\text{H}_2$	N/A	N/A	8
Alcohols				
Ethanol	$\text{CH}_3\text{CH}_2\text{OH} + \text{H}_2\text{O} \rightarrow \text{CH}_3\text{COO}^- + \text{H}^+ + 2\text{H}_2$	+9.6	-47.5	6
Aromatics				
Benzoate	$\text{C}_7\text{H}_5\text{O}_2^- + 6\text{H}_2\text{O} \rightarrow 3\text{CH}_3\text{COO}^- + 2\text{H}^+ + \text{CO}_2 + 3\text{H}_2$	+49.5	-74.4	7
Hydrocarbons				
Methane	$2\text{CH}_4 + 2\text{H}_2\text{O} \rightarrow \text{CH}_3\text{COOH} + 4\text{H}_2$	+166.6	+35.3	9
Methane	$\text{CH}_4 + 2\text{H}_2\text{O} \rightarrow \text{CO}_2 + 4\text{H}_2$	+131	+12.8	10
Terminal Reactions				
Methanogenesis				
H ₂ /CO ₂	$4\text{H}_2 + \text{CO}_2 \rightarrow \text{CH}_4 + 2\text{H}_2\text{O}$	-131	-12.8	11
Acetate	$\text{CH}_3\text{COO}^- + \text{H}^+ \rightarrow \text{CH}_4 + \text{CO}_2$	-35.9	-22.8	12
Sulfate Reduction				
H ₂	$4\text{H}_2 + \text{SO}_4^{2-} + \text{H}^+ \rightarrow \text{HS}^- + 4\text{H}_2\text{O}$	-152.3	-38.1	13
Acetate	$\text{CH}_3\text{COO}^- + 2\text{H}^+ + \text{SO}_4^{2-} \rightarrow 2\text{CO}_2 + \text{HS}^- + 2\text{H}_2\text{O}$	-57.4	-48.3	14
Acetogenesis				
H ₂ /CO ₂	$4\text{H}_2 + 2\text{CO}_2 \rightarrow \text{CH}_3\text{COO}^- + \text{H}^+ + 2\text{H}_2\text{O}$	-94.9	+10.2	15

* H₂ assumed to be 1 Pa, CO₂ assumed to be 2 × 10⁴ Pa, CH₄ assumed to be 10⁵ Pa, all other aqueous reactants and products assumed to be 1 mM. Otherwise standard conditions are assumed for all calculations.

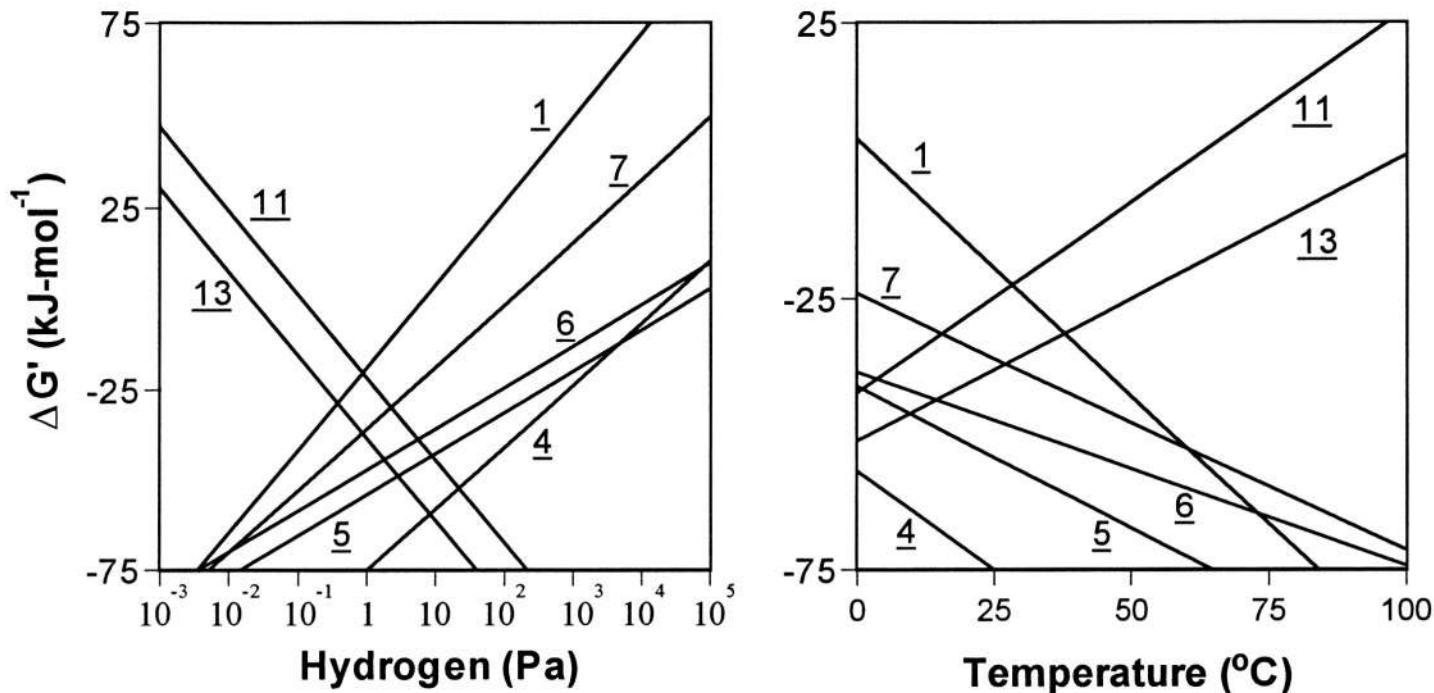


Figure 2. The influence of hydrogen partial pressure and temperature on the free energy yields for reactions listed in Table 1. The temperature dependence was determined assuming $H_2 = 1.0$ Pa, and the H_2 dependence was determined assuming 25°C. Besides H_2 and temperature, standard thermodynamic conditions were assumed for all calculations.

anaerobic processes in order of their thermodynamic yields: NO_3^- reduction, Fe(III) reduction, Mn(IV) reduction, SO_4^{2-} reduction, and methanogenesis (Berner, 1980; Cord-Ruwisch *et al.*, 1988; Hoehler *et al.*, 1998; Lovely and Klug, 1983; Lovley and Phillips, 1987). Hydrogen is an important intermediate in anaerobic environments, and the competition for reduced compounds is clearly indicated by the ambient H_2 concentration (Cord-Ruwisch, Steitz and Conrad, 1988; Hoehler *et al.*, 1998; Lovley and Goodwin, 1988). Competition for H_2 between the various terminal electron-accepting processes ensures a low ambient H_2 concentration and serves as the driving force behind interspecies H_2 transfer. Competition between methanogens and sulfate-reducing bacteria has been studied most extensively (Cord-Ruwisch *et al.*, 1988; Hoehler *et al.*, 1998; Lovely and Klug, 1983). Sulfate-reducing bacteria are able to maintain ambient H_2 at such a low level that methanogens are unable to conserve energy from methanogenesis with H_2 and CO_2 . As shown in Fig. 2, these H_2 levels allow many syntrophic processes to proceed with sufficient energetic yields.

Hydrogen-based syntrophy is known to be a key process in methanic environments (Schink, 1997), and may also be important in sulfidic environments (environments where organic matter decomposition is coupled to the reduction of sulfate). However, the prevalence of interspecies H_2 transfer coupled to the reduction of NO_3^- , Fe(III), and Mn(IV) is not well established and may not be of environmental significance. These alternative forms of respiration generally conserve large quantities of biologically useful energy. Consequently, the responsible organisms tend to have a broad substrate range, and compete more successfully for substrates than syntrophs. Additionally, the H_2 concentrations in such settings are so low that only small fluxes of H_2 are possible in syntrophic couplings.

3.2. ORGANIC ACIDS, ALCOHOLS, AND AROMATICS

Organic acids are key intermediates in the anaerobic degradation of organic material. Short-chain (2-5 carbons) organic acids are the primary products of fermentation and are produced from a variety of compounds including long chain fatty acids, amino acids, sugars, aromatics, and alcohols (Fig. 1). Molecules more reduced than organic acids are also produced during fermentation, but in **low- H_2** environments it is likely that organic acids are the primary products as their production can be directly coupled to ATP production. Propionate and butyrate are key organic acids produced by the process of fermentation (Koch *et al.*, 1983; Krylova *et al.*, 1997; McInerney, 1988; McInerney *et al.*, 1981). Syntrophic associations responsible for the consumption of propionate and butyrate are tightly coupled from a thermodynamic perspective. Acetate, CO_2 , and H_2 are the major products of propionate and butyrate oxidation, and this metabolism has been studied extensively (see Schink, 1997). The energy available for these transformations is minimal (Table 2, Fig. 2), and the stringent thermodynamic coupling leads to exceptionally slow growth rates in syntrophic cultures. Recent reports indicate that propionate degradation may proceed with an energetic yield below the commonly accepted biological energy quantum of $\sim 20 \text{ kJ mol}^{-1}$ (Scholten and Conrad, 2000). Such reports raise fundamental questions about our understanding of the biological energy quantum.

The resolution of the ethanol-degrading “*Methanobacillus omelianskii*” as a coculture of a methanogen and an ethanol oxidizer (Bryant *et al.*, 1967) was the first report of interspecies H₂ transfer. Despite the historical importance of this discovery and the prevalence of this metabolism in laboratory cultures (Bryant *et al.*, 1977; Reddy *et al.*, 1972; Schink, 1985; Valentine *et al.*, 2000b), syntrophic degradation of alcohols has not been shown to be of broad environmental significance. The primary reason is that many fermenting organisms can produce either an organic acid or the corresponding alcohol during growth. In low-H₂ environments it is advantageous for an organism to produce the acid as more energy can be conserved. In environments with high levels of H₂ an organism would need to invest energy to produce H₂ from an alcohol, and instead simply produces the alcohol. Because of the bioenergetics, alcohol fermentation tends to occur in ‘transient’ environments where fermentation is the dominant form of metabolism and is not coupled to a terminal electron-accepting process. Organic acid production is likely the dominant form of metabolism in more ‘stable’ anoxic environments.

Aromatic moieties are important biologically as components of amino acids, natural products, and lignins, and are also major components of pollutants which are degraded by microbes in the environment. Aromatic rings are exceptionally stable, and microbial consumption is limited by the high activation energy required for dearomatization. In methanic environments aromatic compounds are generally converted first to benzoate by side chain removal (Heider and Fuchs, 1997). Benzoate is the central intermediate in the degradation of aromatic compounds in methanic environments (Harwood *et al.*, 1999). As shown in Table 2 (Eq. 7) benzoate is converted syntrophically to acetate, H₂, and CO₂. The nature of the syntrophic association which allows for benzoate degradation is unusual in that both H₂ and acetate are key intermediates, and the minimum thermodynamic yield is higher than for other syntrophs (Schocke and Schink, 1997; Schocke and Schink, 1998). Energy conservation during syntrophic benzoate fermentation is also unusual and apparently involves the coupling of pyrophosphate cleavage to the generation of a chemiosmotic potential during benzoate activation (Schocke and Schink, 1999). The mechanism and energetics of dearomatization are not yet completely understood.

3.3. AMINO ACIDS

Proteins are important components of biomass in all ecosystems, and thus their degradation is important in the global carbon cycle. In methanic ecosystems, proteins are hydrolyzed to peptides and amino acids, which are eventually degraded to NH₄⁺, CO₂ and CH₄. Individual amino acids are generally degraded by either fermentation, reductive deamination, or by syntrophic oxidation via interspecies H₂ transfer (Barker, 1981; McInerney, 1988). Several amino acids are known to be degraded via syntrophic oxidation, including glycine, serine, leucine, isoleucine, valine, alanine, glutamate, aspartate, and methionine (Baena *et al.*, 1998; Baena *et al.*, 1999; Barker, 1981; Fardeau *et al.*, 1997; McInerney, 1988; Stams and Hansen, 1984; Zindel *et al.*, 1988). Syntrophic oxidation is likely to be important on a global scale because of the

predominance of low H_2 environments, such as aquatic (freshwater and oceanic) sediments, marshes, and waste digesters.

The specific pathways of amino acid degradation have not been determined for most anoxic environments. In environments with low H_2 and amino acid concentrations, syntrophic oxidation may be favored over a classical Stickland-type fermentation. However, due to the number of possible reactions between amino acids, the variations in amino acid abundance, and the similar catabolic yields for different reactions, it is difficult to predict which pathways may dominate. Several different species are capable of consuming amino acids by way of a syntrophic associations, and a substantial fraction of amino acids are likely also degraded in this fashion in natural settings.

3.4. THE SPECIAL CASE OF ACETATE

Methanogenesis in many environments proceeds through the aceticlastic pathway in which the carbon-carbon bond of acetate is cleaved to yield methane and CO_2 . In some environments this process must compete with the syntrophic fermentation of acetate to H_2/CO_2 coupled with H_2/CO_2 methanogenesis, a process referred to as non-aceticlastic methanogenesis from acetate (Petersen and Ahring, 1991; Schnurer, Schink and Svensson, 1996; Zinder and Koch, 1984). Thermodynamically, syntrophic acetate degradation is favored by high temperature (Fig. 2), and the process was originally thought to occur only in high temperature environments. Isolation of the thermophilic '*reversibacter*' further bolstered this dogma (Lee and Zinder, 1988). However, a mesophilic bacterium has since been isolated that is capable of growth on acetate in syntrophic association with a methanogen, broadening the temperature range within which this process is known to occur (Schnurer *et al.*, 1996).

Evidence collected from numerous marine sediments indicates that methanogenesis from H_2/CO_2 is the primary pathway for methane production in many cold marine sediments (Whiticar, 1999; Whiticar, Faber and Schoell, 1986). Acetate is certainly produced by fermentation in such environments, which raises an important question: what is the fate of acetate in cold methanic marine sediments? Surprisingly, there are hints that acetate is converted to methane through H_2/CO_2 , a process yielding little or no energy at such low temperatures (Hoehler, 1998; Shaw *et al.*, 1984). Because so little energy is available for the conversion of acetate to CO_2 and H_2 , it is possible that the interconversion of acetate and CO_2/H_2 is performed at equilibrium (analogously to the **formate/H₂** interconversion) to produce H_2 (the preferred substrate); however, the impetus for the conversion is unclear.

3.5. ANAEROBIC METHANE OXIDATION

Anaerobic methane oxidation is a microbially-mediated process of global significance. This process occurs in anoxic environments where both sulfate and methane are present (Alperin and Reeburgh, 1984; Hoehler and Alperin, 1996; Valentine and Reeburgh, 2000). Biogeochemical studies of anoxic marine sediments (Hoehler *et al.*, 1994) led to the hypothesis that anaerobic methane oxidation is performed by a

consortium of SRB (Sulfate-Reducing Bacteria) and methanogens, and that the mechanism involves interspecies H₂ transfer (Fig. 3, top panel). Several pure and mixed cultures of methanogens were screened for methane oxidation ability under appropriate conditions, but methane oxidation was not observed (Valentine et al., 2000a), indicating that the process is not performed by all methanogens. Studies of archaeal biomarkers and microbial community structure (Hinrichs *et al.*, 1999) have refined the consortium hypothesis and identified certain Archaea (not necessarily methanogens) as the primary consumers of methane in marine methane seeps. The more recent study of Boetius *et al.* (2000) has microscopically identified a putative methane-oxidizing consortium in methane-hydrate bearing sediments, revealing both the specific species and physical associations involved (Fig. 3).

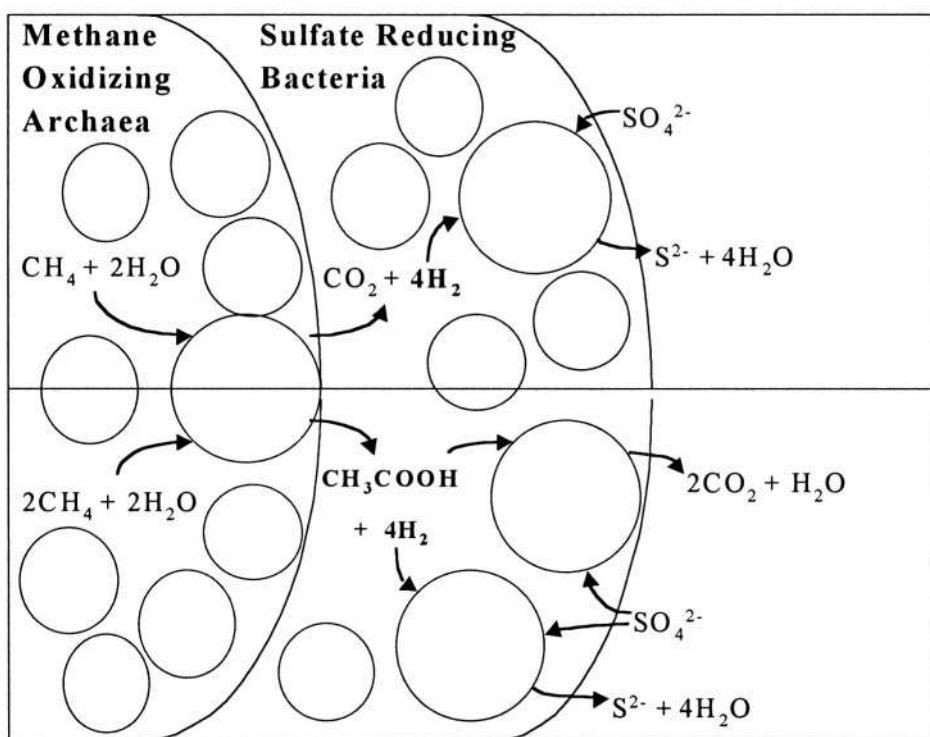


Figure 3. Two proposed mechanisms for anaerobic methane oxidation

The combined evidence for a archaeal-SRB consortium (Hinrichs *et al.*, 1999; Hoehler *et al.*, 1994) led Valentine and Reeburgh (2000) to consider possible mechanisms for anaerobic methane oxidation. A hypothesis was presented involving interspecies transfer of both H₂ and acetic acid (Fig. 3, bottom panel). This new hypothesis is consistent with previous observations, allows for a greater energy yield

for the organisms involved, and better explains the presence of isotopically-depleted bacterial lipids in methane oxidizing sediments. Future laboratory and environmental studies will undoubtedly yield additional information about the syntrophic mechanism of anaerobic methane oxidation.

3.6. SECRET LIVES FOR SULFATE REDUCERS?

Sulfate-reducing bacteria are so named for their ability to utilize sulfate to oxidize reduced compounds including H_2 and organic material. However, some SRB are able to alter their metabolism and can act as syntrophic partners for methanogens when sulfate becomes depleted (Bryant *et al.*, 1977). Some organisms classified as syntrophs are capable of utilizing oxidized sulfur compounds, and are phylogenetically related to SRB (Harmsen *et al.*, 1995; Schollen and Conrad, 2000). These observations have blurred some distinctions between SRB and syntrophs. Perhaps these observations are best understood in terms of anoxic marine sediments. Sulfate is abundant in the oceans, and sulfate reduction is the dominant microbial process in most anoxic marine sediments. However, sulfate becomes depleted in many marine sediments, and gives way to methanogenesis. What happens to the populations of SRB when a sulfidic sediment becomes methanic? One possibility is that the SRB continue to consume organic material, but instead of coupling their metabolism to sulfate reduction, they produce H_2 in a syntrophic association with methanogens. From an evolutionary perspective it follows that some SRB may have specialized in syntrophic associations and led to the obligately-syntrophic bacteria.

3.7. H_2 OR FORMATE?

From a chemical perspective H_2 is the simplest possible molecule. From the biological perspective H_2 is the most fundamental electron carrier as it is generated by the reduction of protons, which are ubiquitous in aqueous solution. Many of the examples given in this chapter deal with interspecies hydrogen transfer, but there is evidence for the involvement of other molecules in analogous processes; the more general term interspecies electron transfer more accurately describes situations where molecules besides H_2 act as interspecies electron carriers.

Formate (HCOO^-) is thought to be an important agent in interspecies electron transfer (Boone *et al.*, 1989; Goodwin *et al.*, 1991; Thiele and Zeikus, 1988). Formate can equilibrate with H_2 in a readily reversible reaction catalyzed by formate dehydrogenase (Eq. 16).



The aqueous concentration of formate is much higher than H_2 at equilibrium, and models indicate that interspecies formate transfer can transport reducing equivalents more effectively than H_2 at intracellular distances greater than ca. 10 μm (Boone *et al.*, 1989). However, the preferred method of interspecies electron transfer seems to vary

depending on the environment, and either one or both mechanisms may be active in any giving setting.

4. The importance and limitations of hydrogen-based syntrophy

Low H_2 levels are characteristic of most anoxic environments, and known pathways of remineralization are well adapted to this situation. It is likely that there are numerous other processes involving interspecies H_2 transfer that have yet to be characterized. Advances in molecular ecology and biogeochemistry are leading to a further understanding of anaerobic microbial community metabolism. The importance of syntrophic associations in known environments coupled with the near-ubiquity of low H_2 levels in anoxic environments and the prevalence of anoxic environments on Earth all point toward the importance of thermodynamic ecology in the biogeochemistry of Earth.

There are numerous outstanding questions related to thermodynamic ecology and H_2 -based syntrophy. Future studies are likely to yield further information about the fundamental bioenergetic limits of life, the biochemistry of syntrophic metabolism, the mechanism and prevalence of anaerobic methane oxidation, the importance of interspecies formate transfer, the importance of syntrophic associations in sulfidic environments, the fate of acetate in marine environments, syntrophic associations in a variety of different environments, the importance of syntrophic associations in evolution, and the possibility of microbial life elsewhere in the Universe.

The principle of thermodynamic ecology holds true for many ‘stable’ anaerobic environments where intermediate metabolism is coupled to some terminal electron-accepting process. Some environmental conditions cause changes that alter the microbial community metabolism for a given environment, and cause deviations from the principles outlined here. Examples of such conditions include the presence of toxic substances, a heterogeneous supply of oxidants to the system, and transient environmental changes. One important example is the buildup of acetate after major environmental changes. This process has been observed after flooding of wetlands, and after seasonal climatic shifts in coastal marine sediments. Acetate buildup is generally caused by a population imbalance between acetate producers and consumers, where the population of consumers develops slowly relative to the producers. However, this condition is generally transient, and the community metabolism will tend toward a thermodynamically-determined steady state once the populations stabilize. Many, but not all, such examples can be explained from the perspective of thermodynamic ecology. Thermodynamic ecology is a principle meant to aid our understanding of anaerobic microbial communities. There are limitations to the application of this principle, and such limitations should be used to further our understanding of anaerobic microbial communities and their important role in the global carbon cycle.

5. Conclusion

The energy available for metabolism in many anoxic ecosystems is so minimal that organisms are forced to live at the fundamental energetic threshold for life. Because organisms in these environments are energy limited, and because the amount of energy generated during catabolism hardly exceeds the biological energy quantum, competition for reducing equivalents (mainly H_2) is based firmly on the bioenergetic yield of catabolism. Competition between H_2 consumers gives rise to a cooperative thermodynamic situation, allowing H_2 producers to convert most organic material to CO_2 , acetate, and H_2 . Hydrogen production is dependent on constant H_2 consumption without which H_2 production is thermodynamically inhibited; this syntrophic process is termed interspecies H_2 transfer. The term thermodynamic ecology is used here to describe the overriding principle governing microbial competition and cooperation in relevant anoxic ecosystems. The principle of thermodynamic ecology is environmentally important and can be applied to numerous anoxic environments to understand the pathways of organic-matter remineralization.

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NUTRITIONAL SYNTROPHIES AND CONSORTIA AS MODELS FOR THE ORIGIN OF MITOCHONDRIA

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1. Introduction

The purpose of this review is to consider microbial syntrophies as models for the origin of mitochondria. Numerous examples of syntrophies are known – some are communities of non-attached cells while others are colonies of tightly attached cells. What factors affect the evolution of such associations? Why do some become increasingly intimate, even intracellular, while others do not?

1.1. DEFINITIONS

“Syntrophy” is a nutritional term. As defined in most textbooks, it involves the transfer of compounds between species such that both species benefit, and neither grows well in the absence of the other. An example of syntropy is a mixed culture of a sulfur-reducing species and a sulfide-oxidizing species. If the sulfur is available in limited amounts, neither species can grow unless the other species is present to recycle the sulfur back to its initial oxidation state. Thus, each species is dependent on the other, and such associations can be remarkably stable. A particular sulfur-based example is described later; see section “2.2. RECYCLE”.

“Symbiosis” and “consortium” are structural terms that refer to different species living together in intimate contact. “Consortium” is generally used for bacterial associations that have a discrete, stereotyped appearance and are so compact that they may be mistaken for single organisms. An example is “*Chlorochromatium aggregatum*”, described later and illustrated in Figure 3.

Thus, there can be syntrophies that are not consortia, and vice versa. For example, methanogenic syntrophies may be loosely associated species that have no intimate contact, and thus are not symbioses. Similarly, symbioses exist that are not nutritionally based, such as those that facilitate defenses against predators. Nonetheless, many associations are both symbiotic (intimate) and syntrophic (involving mutualistic nutrient exchange).

1.2. THE MITOCHONDRIAL NUTRIENT-EXCHANGE PATTERN

Modern eukaryotic cells divide metabolism between the cytoplasm and mitochondria in a manner reminiscent of a syntrophic relationship. Carbohydrates are metabolized by the cytoplasm to pyruvate and reducing equivalents (NADH), which are then passed to the mitochondrion. In the absence of mitochondrial activity, such as during a period of anoxia, toxic products such as lactic acid or ethanol accumulate, and the cell produces less ATP. In more normal, aerobic circumstances metabolic products flow from the cytoplasm to the mitochondria so that there is mutual benefit both cellular components, consistent with the concept of a syntrophic association.

There is general agreement that mitochondria originated from symbiotic α -**Proteobacteria**. The conventional "Serial Endosymbiosis Theory" assumes that in the initial symbiosis metabolic functions were partitioned between the host and symbiont in a pattern similar to that in modern eukaryotic cells (Margulis, 1993; Gray *et al.*, 1999). Thus, the ancient host contained the glycolytic and the pentose phosphate pathways, while the pre-mitochondrial symbiont contained the Krebs Cycle and oxidative phosphorylation.

However, the metabolic arrangement described above has not been productive for modeling the pre-mitochondrial symbiosis. Proteobacteria are versatile organisms that often can use sugars directly, and need not depend on other species to provide fermentation products to them. Furthermore, it is generally true that fermentative organisms grow more slowly than aerobes, and that modern Archaea, the putative co-descendants of the nucleocytoplasm, grow particularly slowly. Thus, in mixed cultures the aerobic Proteobacteria simply overgrow the fermentative species, which disappear from the culture. To obtain a stable co-culture, other types syntrophic nutrient exchanges should be considered, especially those that involve inorganic compounds.

2. Nutrient-exchange patterns

2.1. FLOW-THROUGH

Methanogenic communities, such as those found in fresh water sediments (Brock *et al.*, 1984), are examples of "Flow-through Syntropy." (See Figure 1.) Typically, polysaccharides such as cellulose enter the community and are first metabolized by primary and secondary fermenter species, releasing compounds such as organic acids, CO_2 , and H_2 . These products are taken up by the methanogen, and CH_4 is released. The carbon atoms pass through only once. The important feature of this community is interspecies H_2 exchange, described in section "3.1. HYDROGEN".

In addition to consortia that are entirely prokaryotic, there also are associations of methanogens with protozoa. These include ciliates and amoeboflagellates that contain endosymbiotic methanogenic bacteria (Brock *et al.*, 1984; Fenchel, 1996). The protozoan hosts produce H_2 , which is then consumed by the methanogen, so that these associations function in a way that is identical to the prokaryotic flow-through community described above.

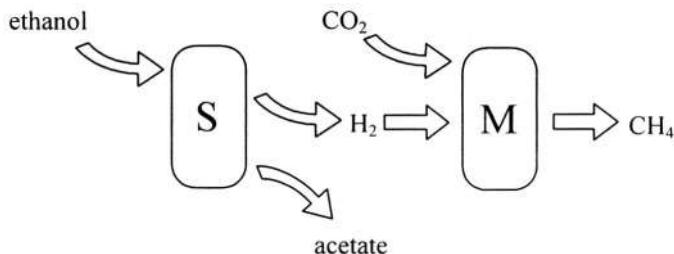


Figure 1. "Flow-through" nutrient exchange pattern illustrated by *"Methanobacillus omelianskii"*, a mixed-species culture. Species "S" ferments ethanol to produce H_2 and species "M" uses H_2 to make methane. Since H_2 accumulation is inhibitory to species "S", it cannot grow well unless species "M" is present. See Lengeler (1999).

2.2. RECYCLE

A second category of syntrophy is characterized by nutrient recycling. The archetypal example is that described in 1967 by van Gemerden (reviewed by Pfennig, 1980). Cultures of *Desulfovibrio sp.* and *Chromatium vinosum* were combined in conditions that were anoxic and provided with formate, a small amount of SO_4^{2-} and light for photosynthesis. *Desulfovibrio* oxidized the formate to HCO_3^- while reducing SO_4^{2-} to HS^- . Both products were consumed by *Chromatium*, which used the HS^- as an electron donor for photosynthesis and oxidized it back to SO_4^{2-} . Total growth (= cell mass) was proportional to amount of formate provided, and not to the amount of SO_4^{2-} . Increasing the SO_4^{2-} concentration over 15 μM resulted in only slightly increased yield. Thus, although SO_4^{2-} was required by one species and HS^- by the other, each was needed in only a catalytic amount because it was regenerated by the other species. Sulfur exchange was rapid, completing a cycle every 15 min, compared to a cellular doubling time was about 5 h.

Another example of syntrophic co-culture was provided by Wolfe and Pfennig (1977) and illustrated in Figure 2. In this experiment the heterotroph *Sulfurospirillum deleyianum* (Schumacher *et al.*, 1992) reduced elemental sulfur (S^0) to H_2S while the photosynthetic green bacterium *Chlorobium sp.* oxidized it back to S^0 . This cycle involving elemental sulfur is of particular interest because it may resemble that which occurred during the origin of mitochondria. (See later.)

In these examples it can be seen that two species are mutually dependent, as required by the definition of syntropy. In separate pure cultures, either organism would quickly convert all the sulfur into a form it could not use, and then stop growing. Together, the two species each regenerate the sulfur for the other, so that a small amount of sulfur can serve as a catalyst for substantial growth.

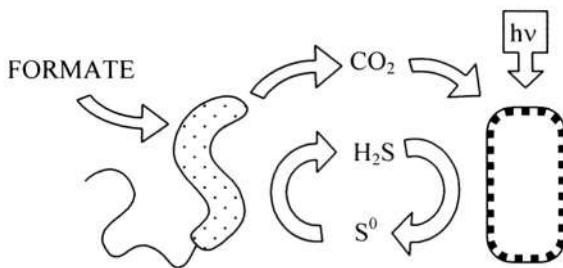


Figure 2. "Recycle" nutrient exchange pattern. The first species is *Sulfurospirillum deleyianum*, which reduces elemental sulfur (S^0) to H_2S . The second species, *Chlorobium sp.*, uses H_2S as a photosynthetic electron donor to reduce CO_2 . Although sulfur is available in limited amounts, each species can grow well because the sulfur is rapidly recycled. A small amount of sulfur has a catalytic effect on overall growth. (Wolfe and Pfennig, 1977)

Other compounds. Most examples of microbial syntrophies involve H_2 or sulfur, but other compounds can be exchanged equally well. Some examples are listed in Table 1, including hypothetical possibilities that have not been observed.

Although many compounds in Table 1 should be familiar, a few deserve special comment. (1) Oxygen (O_2) is included because it can be important in anaerobic situations where symbiotic green algae supply O_2 to their animal hosts. (2) Quinones are known to shuttle electrons between cells and extracellular insoluble substrates. Thus, it is easy to imagine that they may shuttle electrons between cells equally well. (3) Free manganese and iron are hypothetical candidates for syntrophic electron shuttles, and are discussed more below.

Every element that can be oxidized and reduced by living organisms potentially might appear in Table 1, in conformity with the ecological generalization that no resource is left unexploited. Indeed, the entire biosphere is a syntropy involving redox cycles of oxygen, carbon, hydrogen, and nitrogen.

Discussion of selected elements follows below.

3. Specific nutrients

In order to be useful in syntrophic exchange, a compound should be transferable between cells and able to react either on the cell surface or by entering the cytoplasm. Thus, compounds such as ATP and NADH are poor candidates for exchange because they cannot be used on the external cell surface and are seldom transported through membranes. (Mitochondrial ATP transport is an exception.) Instead, nutrients exchanged in syntrophies are typically small molecules, stable to hydrolysis, and substrates for oxidation- and reduction-type reactions. In essence, they function as electron carriers between species.

TABLE I Nutrients that may be involved in syntrophic exchange between species.

Compound	Example	Couple	Standard Redox Potential (pH 7)	
			E_0' (mV)	Ref.
Formate	Methanogenic communities	$\text{CO}_2/\text{HCOO}^-$	-430	[1]
Carbohydrates	Algal symbioses in animals and lichen	$\text{CO}_2/[\text{CH}_2\text{O}]$	-430	[3]
H_2	Methanogenic communities	H^+/H_2	-414	[3]
Pyruvate, fatty acids	Eukaryotic mitochondria	$\text{CO}_2/\text{pyruvate}$ $\text{CO}_2/\text{palmitate}$	-370* -245*	[2]
Methane	Methanotrophic communities	CO_2/CH_4	-244	[3]
Sulfur	Consortia; laboratory syntrophies	S^0/HS^- $\text{SO}_4^{2-}/\text{HS}^-$	-240 -218	[3]
Acetate	Methanogenic communities	$\text{CO}_2/\text{CH}_3\text{COO}^-$	-240	[1]
Quinones	Hypothetical	Q/QH_2	-100 to +350	
Cytochrome c	Laboratory syntropy	cyt c ox/red cyt c ₁ ox/red cyt c ₃ ox/red	+167 +230 -290	[4] [1] [1]
Iron	Ecosystem	$\text{Fe(OH)}_3/\text{FeCO}_3$ $\text{FeOOH}/\text{Fe}^{2+}$	+200 +150	[4] [3]
Nitrogen	Ecosystem	$\text{NO}_3^-/\text{NH}_4^+$ NO_3^-/N_2	+363 +751	[3]
Manganese	Ecosystem	$\text{MnO}_2/\text{Mn}^{2+}$	+390	[3]
O_2	Algae symbioses in animals and lichen	$\text{O}_2/\text{H}_2\text{O}$	+810	[3]

*By calculation from thermodynamic data.

References: [1] Brock (1991). [2] Lehninger (1975). [3] Lengeler *et al.* (1999). [4] Seeliger *et al.* (1998).

3.1. HYDROGEN (H_2) AND FORMATE

A standard example of syntropy described in many microbiology textbooks (e.g.- Brock, 1991; Lengeler *et al.*, 1999) is that of a prokaryotic methanogenic community. Methanogenesis takes place in 2 or 3 stages, where the initial stages occur in fermentative species that produce acetate, CO_2 , and H_2 . The thermodynamic free energy (ΔG) driving the fermentation reactions forward is not great, so that if the H_2 is produced and not removed the reactions come to a stop. The H_2 can be removed up by methanogens, which use it to produce methane according to the reaction: $4 \text{ H}_2 + \text{CO}_2 \rightarrow \text{CH}_4 + 2 \text{ H}_2\text{O}$. Thus, both types of organism depend upon H_2 exchange. The

fermenters cannot grow unless H_2 is removed, and the methanogens cannot grow unless H_2 is provided. Consequently, methanogenic associations are remarkably stable.

Although some methanogenic communities involve mixed species of non-attached cells, others are composed of cells that are tightly associated. For example, eukaryotic methanogenic associations often involve endosymbiosis. Fenchel (1996) reported that freshwater anaerobic ciliates commonly produce methane and contain methanogenic endosymbionts. In such associations the eukaryotic cytoplasm evidently carries out primary fermentation, providing H_2 and a carbon compound to the prokaryotic symbiont (Fenchel and Finlay, 1992).

There are numerous variations on the methanogenic scheme. For example, formate or acetate may be transferred between species instead of H_2 and CO_2 (Schink, 1992). Organic ions differ from dissolved gases in that they do not diffuse so freely through cell membranes. Thus, ions can be trapped and selectively exchanged between partners without being shared with the surrounding organisms. That could have consequences for the evolution of intimate associations, as described later below.

3.2. SULFUR

Compared to carbon, sulfur is characterized as a large, soft atom that often reacts without a catalyst (Widdel and Hansen, 1992). Although the Standard Reduction Potentials (E_\circ') for both CH_4 and H_2S are similar, methanogenic enzymes require special cofactors and are very sensitive to inactivation by O_2 . In contrast, H_2S production occurs evidently without need for special factors and does not necessarily require anoxic conditions. (See below.)

Sulfur syntrophies are common and well documented, including examples of both non-attached cells and tightly associated consortia.

“Consortia” occur worldwide, and many have been described (for reviews, see Pfennig, 1980; 1989; and Lengeler *et al.*, 1999). Although the first was described nearly 100 years ago (Lauterborn, 1906), consortia continue today to be objects of considerable fascination (Abella *et al.*, 1998; Overmann *et al.*, 1998). Typically, each consortium is microscopic and consists of a central large heterotrophic bacterium surrounded by one or two dozen small sulfide-oxidizing photosynthetic bacteria (Figure 3). Because the metabolism of the central bacterium has not been studied in isolation, the details of the association are uncertain. But presumably, since the photosynthetic bacteria are species that consume H_2S , the large central cell is a sulfidogen. Since the consortia are reported to trap and accumulate sulfur, evidently the oxidized sulfur from the peripheral cells can be recycled back to the central cell to be used again. See section “5.5. NUTRIENT ACCUMULATION”.

The first consortium to be described was *“Chlorochromatium aggregatum”* (Lauterborn, 1906), which was rediscovered and described synonymously a few years later in a complete paper by Bruder (1913). Bruder’s paper is the source of part of our Figure 3, and includes first use of the term “Konsortium” in its present meaning. The photosynthetic cells in the consortium are green sulfur bacteria related to *Chlorobium*, and of particular interest because they deposit extracellular S^0 (Pfennig, 1980), which

could be a factor in the development of tightly associated assemblages of cells. (See "5.4. INSOLUBLE NUTRIENTS".)

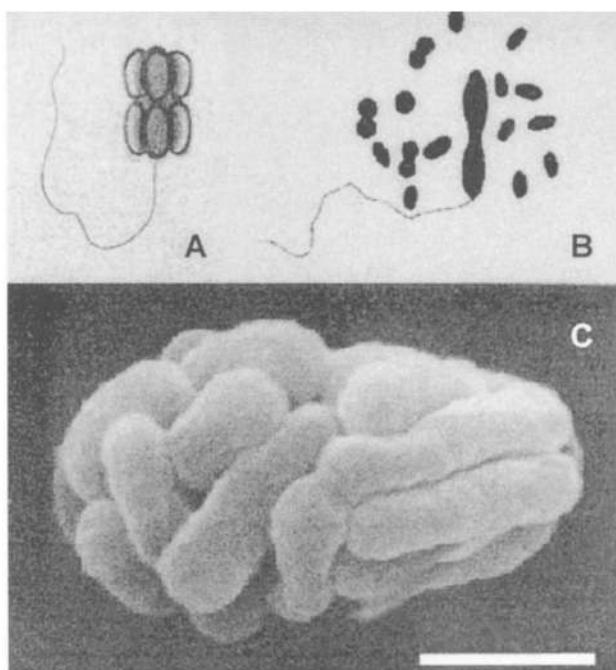


Fig. 3. Drawings and electron micrograph of the bacterial consortium "*Chlorochromatium aggregatum*" from Buder (1913). A- undisturbed consortium B- squashed and dispersed C- scanning electron micrograph from Croome and Tyler (1984). Bar = 1 μm .

A final example of a sulfur-based association is eukaryotic (Fenchel and Bernard, 1993; Fenchel, 1996). *Strombidium purpureum* is an anaerobic ciliated protozoan that contains symbiotic purple sulfur bacteria similar to those put forward as the free-living organisms most closely related to mitochondria (Dickerson, 1980; Yang *et al.*, 1985). Purple sulfur bacteria can be versatile organisms that perform sulfide-dependent photosynthesis in the light and aerobic respiration in the dark. The authors conjecture that, like others in its group, *S. purpureum* was originally totally anaerobic, but by acquiring the purple symbiont it acquired the capacity to be microaerophilic in the dark. Such a scenario is particularly interesting because it replicates the hypothetical first steps that occurred during the ancient origin of mitochondria.

3.3. IRON AND CYTOCHROME C

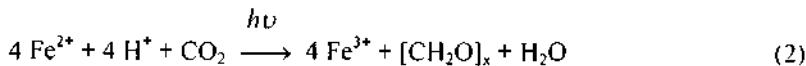
Iron is abundant in the environment and has a reduction potential that is useful for cellular respiration (Lovley *et al.*, 1997). Nonetheless, no syntrophies based on free iron have been reported. Two problems with iron can be recognized: (1) Fe^{3+} is insoluble; for example, the solubility product for Fe(OH)_3 is 10^{-39} M⁴ (Bard, 1966). (2) Ferrous iron in the presence of O_2 produces hydroxyl radical (OH^\cdot), which is one of the most toxic of all free radicals (Halliwell and Gutteridge, 1985).



Nonetheless, it has been suggested that respiration on Fe^{3+} is a primitive trait for all living cells (Vargas *et al.*, 1998). All of the bacteria near the base of the universal tree of life that were tested could respire on Fe^{3+} . No eukaryote was tested, but since they branch from near the base of the tree, by generalization they might also be able to reduce Fe^{3+} .

In an unpublished experiment we tested the soil amoeba *Acanthamoeba castellanii* for capacity to use insoluble Fe_2O_3 . In preliminary measurements, in anoxic conditions at 30°C, the cells produced 4 nmol Fe^{2+} min⁻¹ (g protein)⁻¹. That compares to thermophilic bacteria that reduced Fe^{3+} about 100x faster (Vargas *et al.*, 1998). But considering that the bacteria were measured at 90°C and were provided with soluble, chelated Fe^{3+} , *A. castellanii* did not do badly.

If one species produces Fe^{2+} , for syntrophic exchange to occur the second species must be able to oxidize it back to Fe^{3+} . That has been reported for certain purple sulfur bacteria, which use Fe^{2+} as an electron donor for photosynthesis (Widdel *et al.*, 1993; Dobbin *et al.*, 1996). For example:



where $[\text{CH}_2\text{O}]_x$ is a generic representation for organic matter. At neutral pH the Fe^{3+} is expected to precipitate as hydroxide.

Thus, iron potentially is able to substitute for sulfur in the syntrophic “Recycle” scheme shown in Figure 2. An interesting extension of such a concept is to use iron chelated by protein, as described next below.

Cytochrome c. Cytochrome *c* may be thought of as chelated, soluble iron. The iron atom is buried within the protein molecule and bound at all 6 ligand positions, making it unreactive with O_2 and H_2O_2 . The only known substrate (and product) of cytochrome *c* is an electron. The problems associated with free elemental iron are avoided; in the form of cytochrome *c* the iron is soluble and there is no production of OH^\cdot radicals.

The bacterium *Geobacter sulfurreducens* secretes 2 types cytochrome *c* that function as extracellular electron carriers between the cell and remote insoluble substrates such as Fe(OH)_3 , S^0 , or MnO_2 (Seeliger *et al.*, 1998; Lloyd *et al.*, 1999). The extracellular cytochrome *c* also can function as an electron shuttle between cells, as described next.

In model laboratory cultures, *G. sulfurreducens* was combined with *Wolinella succinogenes* (Cord-Ruwisch *et al.*, 1998; Seeliger *et al.*, 1998). *G. sulfurreducens* oxidized acetate and reduced the cytochrome *c*. *W. succinogenes* oxidized the reduced cytochrome, and NO_3^- was the final electron acceptor. The cytochrome *c* cycled rapidly between the species, functioning as an electron shuttle in an excellent example of Recycle Syntrophy.

In modern mitochondria, cytochrome *c* is the only member of the electron transport chain that is not an integral membrane component. Instead, it is weakly bound on the outer surface of the mitochondrial inner membrane, and can be easily dissociated by physiological buffers (Rasmussen and Rasmussen, 1997). This location is most easily understood as a vestige of its ancient function as a soluble, extracellular electron carrier. Might cytochrome *c* have functioned as a diffusible electron carrier between host and symbiont in the ancient pre-mitochondrial symbiosis? If so, then mitochondria could have originated from an iron-based syntropy.

3.4. NITROGEN

Nitrogen is a potential candidate for syntrophic exchange, and both NO_3^- -producing and NO_3^- -consuming bacteria are ubiquitous in nature. Some mitochondria can use NO_3^- in place of O_2 as a terminal respiratory electron acceptor (Finlay *et al.*, 1983). In addition, *Paracoccus denitrificans*, the archetypical NO_3^- consumer, has been suggested as a good model for the ancestor of mitochondria (John and Whatley, 1975).

However, for a pre-mitochondrial syntrophic symbiosis based upon NO_3^- , the host cytoplasm would have to be a NO_3^- producer, is not known to occur. Instead, the cytoplasm excretes reduced forms of nitrogen such as NH_4^+ and urea. Thus, a syntropy based upon nitrogen exchange seems unlikely to have been important in mitochondrial origins.

3.5. QUINONES

Soluble quinones have been shown to function as extracellular electron shuttles between bacterial cells and insoluble substrates such as humic acid (Newman and Kolter, 2000). By conjecture, they should be able to function equally well also between two cells. Nonetheless, no examples of quinone-based syntropy are known at present.

4. Mixed-species associations – general considerations

Some syntrophies are loose co-cultures of species not in contact with each other, whereas others involve cells that are intimately attached. Since intimate integration is a hallmark of the mitochondrial symbiosis, a discussion of the factors that bring it about is central to understanding the origin of mitochondria.

4.1. WHY HAVE MULTI-SPECIES COMMUNITIES?

Why not combine all metabolic functions into one cell? Such an organism would: (1) eliminate redundancies between organisms, such as in the genetic apparatus, (2) achieve ultimate proximity between metabolic pathways, such as between electron-producing and electron-consuming reactions, and (3) prevent losses of nutrients that unavoidably occur during transfers between cells.

And yet mixed-species communities are common in nature. Nutrients generally pass through multi-species food chains, and may be recycled locally. The explanation for why that can not be done more efficiently within a single organism is not always obvious. For example, there are species of SO_4^{2-} reducing bacteria that combine both organic fermentation and SO_4^{2-} reduction in one cell, and one might expect such cells to colonize and coordinate their growth more easily than associations of mixed species. Nonetheless, multi-species communities dominate in nature. Typically, these are composed of several specialist species, such as specialized fermenters and specialized SO_4^{2-} reducers (Lengeler *et al.*, 1999). The explanation for the prevalence of mixed-species communities probably includes several aspects.

Epidemiological mechanisms. Homogeneous dense populations of organisms are vulnerable to predation and disease. At low population densities the prey species can survive by becoming “fugitive species”, meaning that each species lives at low density and effectively hides from its specific predators. In such a situation a specialized predator also is limited to low density because of prey scarcity. In a microbial community, it is protozoa and viruses that are the predators. Thus, predation and infection maintain the prey species at low density, so that resources and space are not fully exploited, and allowing for multiple species in each niche. That may account for at least some of the population diversity that is observed in microbial communities.

Compartmentation. Communities may gain an advantage when individuals have specialized roles. By analogy with human society, cultures can be more productive when some individuals are specialized to be farmers and others are bankers, compared to a society where each individual is self-reliant and trades nothing with others.

In eukaryotic cells the division of the cytoplasm into multiple compartments could be a manifestation of the same principle. Calculations can show that when enzymes and substrates for specific pathways are concentrated into separate, specialized compartments, metabolism can run more rapidly. For prokaryotic cells, syntrophic association can be one way to obtain multiple specialized compartmentation.

4.2. REPRODUCTION RESULTS IN LESS-MIXED ASSEMBLAGES

Cell division results in colonies of identical cells. For example, imagine that in a methanogenic community the primary fermenter species are interspersed between the methanogens so as to optimize H_2 transfer between them. At the location of each initial cell reproduction will soon produce colonies of identical daughter cells. Interspecies H_2 transfer will then become more difficult, since it will be between colonies of cells

instead of between adjacent individual cells, and the overall growth rates of the 2 species will suffer (Schink, 1992).

Such an outcome is obviously selected against, so that successful mixed associations have evolved mechanisms that effectively prevent clumping of identical daughter cells. One mechanisms may be filamentous growth, which pushes daughters out into a linear array. But in most cases the mechanisms that avoid clumping are not known.

Below is a discussion of some of the evolutionary forces that may tend to select for close cellular contacts between species.

4.3. COMPETITION FOR SUBSTRATE

Nutrients are often scarce in nature, and competition for them can be intense. In such a case it is advantageous to be close to the source. An example can be found in the methanogenic community of the sheep rumen (Stumm, 1982). After fasting overnight, 65% of the ciliated protozoa in the rumen were covered with attached methanogenic bacteria. When the sheep were fed, the number of covered ciliates dropped to 25%. Attachment of the methanogens was shown to correlate with low H_2 concentrations in the rumen. Since the bacteria obtain H_2 from the ciliates, by being attached they can obtain more than bacteria that are not attached. Immediately after the sheep are given a meal, H_2 production exceeds the demand so that there is little competition for H_2 , and then the bacteria detach.

Another example is found in anaerobic marine mud, where H_2 is produced by primary fermenters such as anaerobic protozoa. Hydrogen consumers include methanogens and SO_4^{2-} -reducing bacteria. Since reduction of SO_4^{2-} occurs more easily than methanogenic metabolism, the SO_4^{2-} reducing bacteria take up most the H_2 and the free-living methanogens can not compete (Schink, 1992). Nonetheless, methane production can occur in this environment, and that is by symbiotic bacteria that are inside the protozoa. By being intracellular, the methanogens are closest to the source of the H_2 , and only in that way can they survive the competition from the extracellular SO_4^{2-} reducers (Fenchel and Finlay, 1995).

4.4. SHORT DIFFUSION DISTANCE

Nutrient transfer by diffusion is expected to proceed more rapidly over shorter distances. A well-studied, economically important example occurs in macroscopic flocs of mixed species that appear in sewage treatment plants (Thiele *et al.*, 1988). The flocs consist of diverse bacterial species embedded in a secreted extracellular matrix. Although not necessarily in direct cellular contact, within the floc the H_2 producers and consumers are closer to each other than they would be if randomly dispersed in the bulk fluid, and therefore H_2 transfer is faster. The explanation can be found in the mathematics of diffusion: when distances are 100x less, the concentration gradient is 100x greater, and the rate of diffusion is 100x faster (Schink and Thauer, 1988). Experiments indicate that of the H_2 generated within each floc, more than 90% is consumed in the same floc (Conrad *et al.*, 1985).

A eukaryotic illustration of the principle of proximity occurs in anaerobic ciliates (Fenchel and Finlay, 1992 & 1995). These cells engulf particulate food, and are in

danger of being eaten themselves by predators. Thus, both for acquiring food and to avoid predation, there are advantages to being large. In their cytoplasm, the ciliates produce H_2 that can cause metabolic inhibition if it accumulates. There are at least 2 options for disposing of the H_2 : (1) diffusion out through the cell surface, and (2) feeding it to intracellular bacteria. Diffusion out through the cell surface is adequate for smaller cells, but larger protozoa have too great a distance between the cell interior and the surface. For example, in the amoeba *Pelomyxa palustris* the distance can be up to several mm (van Bruggen *et al.*, 1983). But, by having endosymbiotic methanogens dispersed throughout the cytoplasm, cells such as *P. palustris* prevent H_2 buildup, even the largest cells.

4.5. INSOLUBLE NUTRIENTS

When an insoluble compound is passed between cells, direct cell contact may be the best way. Sulfur and iron each have insoluble forms, such as S^0 , Fe(OH)_3 , FeCO_3 , and FeS . These all occur biologically, and are potential candidates for syntrophic nutrient exchange. For example, purple sulfur bacteria oxidize Fe^{2+} to Fe^{3+} that precipitates as Fe(OH)_3 , and green sulfur bacteria excrete globules of insoluble S^0 .

In the laboratory, green sulfur bacteria easily form syntrophic relationships with a variety S^0 -reducing bacteria (Biebl and Pfennig, 1978). In nature, green sulfur bacteria seem to be narrowly adapted and inefficient organisms (as Biebl and Pfennig suggest), but nonetheless are successful because their excretion of extracellular S^0 is highly conducive to the formation of productive symbiotic relationships.

4.6. NUTRIENT ACCUMULATION

In some cases growth may be limited by a nutrient that is in short supply. But by forming interspecific associations, the nutrient can be recycled and accumulated. An example involves the consortium "*Pelochromatium roseum*", which is very similar to the consortium shown in Figure 3. The photosynthetic member of the consortium uses sulfide as an electron-donor, and when analyzed in pure cultures the rate of photosynthesis was limited by the availability of H_2S . In the consortium, the rate of photosynthesis is higher than can be explained by the amount of sulfide available in the medium (Overmann *et al.*, 1998). Together, these observations suggest that sulfur is recycled and concentrated within each consortium, enabling the observed rate of photosynthesis.

Compared to cells that are not closely associated, recycling can occur between attached cells more rapidly and with the less loss. Therefore, recycling nutrients between species can be a selective pressure for the evolution of symbiotic associations.

4.7. MICROENVIRONMENTS

Endosymbionts may live in special, protected environments. For example, methanogenic metabolism is exceptionally sensitive to O_2 , and inhibited by the slightest traces of air. Nonetheless, protozoa with methanogenic symbionts continue to produce methane even in partially aerobic conditions (Fenchel and Finlay, 1992). Evidently, the

host cells provide an **O₂-free** environment in which the methanogenic bacteria can continue to function (van Bruggen *et al.*, 1983; Fenchel, 1996). Interestingly, this is the reverse of Martin and Müller's hypothesis for the origin of mitochondria, in which an hypothetical methanogen is placed around the outside of a potentially aerobic Eubacterium. See Section 5.4 below.

5. Origin of Mitochondria

5.1. THE PREMITOCHONDRIAL SYMBIONT

The most likely scenario for the origin of mitochondria is that they originated once, and from within the **α -Proteobacteria** (Gray *et al.*, 1999). All modern eukaryotic cells are presumed to be descendants from that event. Amitochondriate eukaryotes ("Archaezoa") were once thought to be relicts of premitochondrial eukaryotes. However, they are known now to contain mitochondria-related DNA sequences, suggesting that mitochondria were once present but were lost after some of their genes had been transferred to the nucleus.

Disagreements remain about whether the mitochondrial ancestor was primarily *Rickettsia*-like or *Rhodospirillum*-like (Gray, 1998; Andersson and Kurland, 1999). Each organism suggests a different scenario for mitochondrial origin: a *Rickettsia*-like ancestor suggests that mitochondria originated by infection, whereas a *Rhodospirillum*-like ancestor suggests origin by syntrophic symbiosis.

5.2. THE HOST CYTOPLASM

A more difficult question concerns the nature of the organism(s) that was the host for the pre-mitochondrion, and became the eukaryotic nucleus and cytoplasm. Although sequence data indicate that the genetic apparatus is primarily Archaeal in origin including replication, transcription, and translation, most metabolic enzymes are eubacterial in origin. One explanation could be that the nucleo-cytoplasm was a product of an Archaea-Bacterial fusion before mitochondria became involved (Zillig *et. al.*, 1989; Gupta and Golding, 1996; Moreira and López-García, 1998). Alternatively, the ancestral symbiosis was simply between an Archaeal cell and the bacterial pre-mitochondrion, followed by takeover of most Archaeal metabolic functions by eubacterial enzymes presumably because they were more efficient (Searcy *et al.*, 1981).

In the presently-accepted evolutionary trees of life, such as that based upon 16S rRNA sequences, eukaryotes branch off from below the diversification of Archaea. Thus, extant organisms such as *Thermoplasma acidophilum* might be cited as models for the ancestor of eukaryotic cytoplasm, but are certainly derived organisms that have changed from the ancient ancestor. It is unlikely that any extant organism is identical to the ancient ancestor of nucleocytoplasm. That is a point that has been lost on many evolutionary theoreticians.

Nonetheless, one can reconstruct what the ancestor might have been like by assembling consensus features from various organisms near the base of the Archaeal tree. Thus, low-branching Archaea are anaerobes that respire on minerals such as

elemental sulfur and Fe^{3+} (Achenbach-Richter *et al.*, 1987; Vargas *et al.*, 1998), suggesting that to be a plausible phenotype also for the primitive nucleocytoplasm.

5.3. ORIGIN BY INFECTION

As an alternative to mitochondrial origin by mutualistic symbiosis, mitochondria might have originated from a pathogenic infection (Jeon, 1991; Jeon, 1995; also Amann *et al.*, 1991). In Jeon's laboratory model a bacterial infection of *Amoeba proteus* has been followed for several years. Initially the infection was pathogenic, but gradually became less so, until now the amoeba and bacteria cannot be separated. The nature of dependence is not fully understood, but since the bacteria are completely inside the amoeba there must be nutrient flow like that from host to parasite. It might be argued that this is not far removed from the syntrophic "Flow-through" nutrient pattern, except that the initial infection was detrimental to the host.

Obstacles to such a scheme include: (1) Since the initial interaction was pathogenic, the host was at a competitive disadvantage *vis-à-vis* uninfected amoebas. Initially, the infected amoebas were nursed along in Jeon's laboratory, but in nature would have probably perished. (2) If the infected amoebas survive, they will be under selective pressure to evolve defense mechanisms rather than greater integration with the pathogen. And (3) there are no clear examples of similar pathogenic infections evolving into mutualistic symbioses. Instead, symbiotic associations such as those involving green algae were probably mutualistic from the beginning, in contrast to pathogenic infections where the species are immediately in an adversarial relationship.

Instead of being an active infectious agent, it is possible to argue that the pre-mitochondrial bacterium was a passive prey that was endocytosed by an amoeba, but somehow not digested. Such a scenario suggests that the mitochondrial outer membrane originated from the old endocytic vesicle, in contrast to the mitochondrial outer membrane originating from the bacterial outer membrane. Since there is some evidence for each type of membrane origin, too much evolution may have occurred by now to permit a simple answer.

5.4. METHANOGENIC SYNTROPHY

Since methanogens are ubiquitous symbionts in anaerobic protozoa (Fenchel and Finlay, 1995), there is no doubt that such associations can be successful. Whether or not a methanogenic symbiosis evolved into the mitochondrion remains to be resolved.

Two hypotheses for mitochondrial origin involving methanogens have been proposed. (1) The Martin-Müller (1998) hypothesis asserts that mitochondria evolved from α -Proteobacteria that excreted H_2 , and that the host cell that was a methanogen. (2) The Moriera-López-García (1998) hypothesis postulates that the nucleocytoplasm evolved from a syntrophy between a methanogen and a δ -Proteobacterium (not α -), which were bound together in methane-producing flocs. The δ -Proteobacteria were outermost, and gave rise to the cytoplasm, while the methanogens were interior and evolved into the nucleus. Later in a second symbiotic event the nucleocytoplasm engulfed a methane-consuming α -Proteobacterium, which became the mitochondrion.

Both conjectures above have been criticized for incompatibility with molecular sequence information (Andersson and Kurland, 1999). The enigma is more evident when considering the universal tree of life. According to 16S rRNA evolutionary trees, eukaryotes branched from the base of the Archaeal Domain, and not from within the methanogenic subgroup of Archaea. (That contrasts with mitochondria, which map to a specific location on the tree within the α -Proteobacteria.) Whereas basal Archaea are sulfur- and iron-reducers (Achenbach-Richter *et al.*, 1987; Woese, 1987; Vargas *et al.*, 1998), methanogens are highly specialized, and contain unique methanogenic enzymes and cofactors (DiMarco *et al.*, 1990; Whitman *et al.*, 1992). No trace of those has been found in any eukaryote.

Methanogenic metabolism is exceptionally sensitive to O_2 , and is inhibited by the slightest trace of air (Whitman *et al.*, 1992). In contrast, eukaryotic cytoplasm probably never was totally anaerobic (Biagini and Bernard, 2000). For example, eukaryotic membranes contain *b*-type cytochromes that directly use O_2 . Similar *b*-type cytochromes evidently occur in Archaea (Searcy and Whatley, 1982). Thus, there are several reasons to doubt that the ancestors of eukaryotic cells included a methanogen.

5.5. SULFIDE SYNTROPHY

According to the Sulfide Hypothesis for the origin of mitochondria, the ancestor of the cytoplasm reduced S^0 to H_2S and pre-mitochondrial symbionts oxidized it back to S^0 (Searcy, 1992). This hypothesis shares some features with the methanogen hypotheses. In each, electrons are carried by diffusible inorganic molecules (H_2 or H_2S). The similarity is even greater when one considers that most methanogens are capable of S^0 reduction, and prefer sulfidogenesis to methanogenesis when S^0 is available (Stetter and Gaag, 1983).

Although green sulfur bacteria seem to be particularly good candidates for the development of sulfur-based syntrophies, according to molecular sequence data it was the purple bacteria that gave rise to mitochondria. Purple bacteria are metabolically more versatile than are green sulfur bacteria, so that they can use organic molecules such as acetate as electron donors for photosynthesis, and in the dark some purple bacteria can respire on O_2 . Evidently, it was such versatility that preadapted purple bacteria to evolve into mitochondria.

There are enzymatic activities in eukaryotic cytoplasm that are consistent with sulfidogenic ancestry. For example, red blood cell cytoplasm reduces S^0 to H_2S in quantities that correspond to 3 moles H_2S per mole glucose consumed (Searcy and Lee, 1998). Unexpectedly, H_2S production occurs equally well in air as in anoxic conditions, in contrast to the intolerance for O_2 shown by methanogenesis.

When eukaryotes were surveyed for anoxic S^0 reduction, it was found in all organisms tested, including all 4 Kingdoms of eukaryotes (Searcy *et al.*, 1999). Sulfur reduction is a metabolic activity shared also with basal archaeabacteria, but not with all bacteria (Stetter and Gaag, 1983).

A contrary detail is that H_2S is a deadly poison to most animals, which is explained by its inhibition of mitochondrial cytochrome oxidase. In contrast, cytoplasmic proteins apparently are little affected by H_2S , since they already are in a reduced state and maintained that way by glutathione. Red blood cells incubated with S^0 accumulated 25

mM H₂S, suggesting that cytoplasmic metabolic activity continued even in high concentrations of H₂S (Searcy and Lee, 1998). There are other examples of eukaryotes functioning in high H₂S concentrations, such as protozoa (Fenchel and Finlay, 1995) and the worm *Halicryptus spinulosus* (Oeschger and Vetter, 1992). *H. spinulosus*, when exposed to H₂S, simply goes without mitochondrial function and lives on fermentative metabolism. These observations indicate that H₂S has little effect on the cytoplasm, consistent with a sulfide-adapted ancestry for that component of eukaryotic cells.

Although high concentrations of H₂S inhibit mitochondria, at lower concentrations (<5 μM) mitochondria are avid sulfide-consumers. Chicken mitochondria, chosen because chickens are not sulfide-adapted organisms, nonetheless oxidized H₂S by respiratory reactions coupled to ATP synthesis (Yong and Searcy, 2001). In 5 μM to 10 μM H₂S respiration became uncoupled from ATP synthesis, and above that range respiration was inhibited. These observations parallel those on the closest bacterial relatives of mitochondria, erroneously called the “Purple Non-Sulfur Bacteria”, which similarly can use low concentrations of H₂S but are inhibited by higher concentrations of H₂S. Historically, early researchers used too much H₂S when studying both mitochondria and purple sulfur bacteria, and observed only the inhibition.

The sulfide model is a “Recycle” nutrient scheme in which the sulfur turns over rapidly. Elemental sulfur (S⁰) or polysulfide (HSS_nH) is likely to be the oxidized form that is transferred to the cytoplasm because most eukaryotic cells can not reduce SO₄²⁻ (Searcy, 1992). The insoluble nature of S⁰ could have helped select for intimate associations between species, as discussed earlier.

5.6. IRON SYNTROPHY

Iron is an alternative to sulfur for syntrophic exchanges. Archaeal species that map near the base of the evolutionary tree are reported to all reduce Fe³⁺ in addition to reducing S⁰ (Vargas *et al.*, 1998). Although found higher in the Archaeal tree, *Thermoplasma acidophilum* is an avid reducer of Fe₂O₃, magnetite, and other insoluble forms of Fe³⁺ (Searcy, unpublished). If direct membrane contact is involved, this could help explain the loss of the bacterial cell wall and origin of a well-developed cytoskeleton, both definitive features of eukaryotic cells (Searcy and Hixon, 1991).

In order to complete a “Recycle” exchange pattern with iron, Fe²⁺ should be oxidized by the ancestral mitochondria. Although iron oxidation has not been studied in eukaryotes, it has been reported in photosynthetic purple α -Proteobacteria (Dobbin *et al.*, 1996). Thus, by conjecture the host cytoplasm might have been a Fe³⁺-reducer and the symbiont an Fe³⁺-oxidizer. Modern eukaryotes should be tested for vestiges of those activities.

Cytochrome c. Problems with free iron include its insolubility and OH[•] radical production. Chelating the iron, as discussed earlier, can solve both difficulties, and cytochrome c is a type of biologically chelated iron. Thus, the possibility that cytochrome c, rather than free iron, was exchanged in the pre-mitochondrial symbiosis can not be discounted.

6. Conclusion

Mutualistic symbioses are arrangements in which both partners benefit, often by exchanging nutrients. The exchanged compounds function as electron shuttles, and potentially include any compound or element that can be oxidized and reduced by living cells. Typically, they have oxidation potentials that fall within the range -500 mV to +800 mV. Several elements are not currently known to be involved in interspecific electron shuttles, such as iron, manganese, and nitrogen, but syntrophies involving them might be found in the future.

Exchanges of nutrients occur in 2 patterns: “Flow-through” and “Recycle”. Of these, only the “Recycle” pattern is able to trap and accumulate nutrients. Exchanges of insoluble materials such as S^0 or $Fe(OH)_3$ might also occur, and be facilitated by the formation of intimate cell-to-cell contacts. That could lead to symbiotic associations, and be a factor in promoting the origin of mitochondria.

The relationship of mitochondria to modern eukaryotic cytoplasm is that of a “Flow-through” nutrient pattern involving organic acids. Nonetheless, that was not necessarily the entire situation during the evolutionary period of pre-mitochondrial symbiosis. Instead, mitochondria evolved from a syntrophy in which inorganic compounds were exchanged, including compounds of sulfur and possibly iron. The prokaryotic relatives of modern mitochondria (purple sulfur bacteria) and of the eukaryotic nucleocytoplasm (Archaea) have enzymatic activities consistent with either sulfur or iron exchange. Modern eukaryotic cells also have enzymatic activities for sulfur reduction in the cytoplasm and sulfide oxidation in the mitochondria. By hypothesis, those activities could be the vestigial remnants of the origin of mitochondria from an ancient sulfur syntrophy.

TABLE 2. Summary of nutrient exchange patterns, including with mitochondria.

Reduced nutrients	Nutrient flow pattern	Tolerance to O ₂	Activity in eukaryotic cytoplasm	Activity in mitochondria
H ₂ (including methanogenesis)	Flow-through	Strictly anoxic	None	In anaerobic protozoa mitochondria (=hydrogenosomes) export H ₂ .
H ₂ S	Recycle or flow-through	Anoxic or aerobic	HS ⁻ is produced	HS ⁻ is oxidized and coupled to ATP synthesis.
Organic acids, pyruvate, fatty acids	Flow-through	Anoxic or aerobic	Organic acids are produced.	Organic acids are imported and oxidized with coupling to ATP synthesis.

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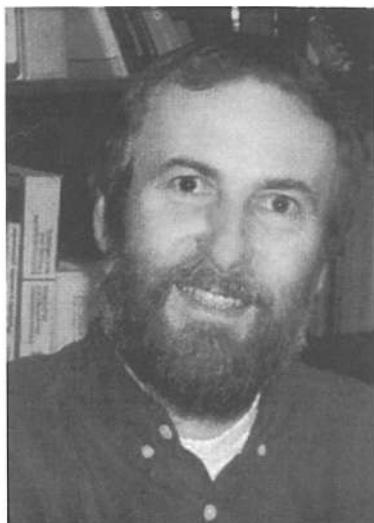
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REVERSION OF ENDOSYMBIOSIS?

The Case of Bleaching in Euglena¹

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1. Two Dimensions of Endosymbiosis: Synthesis and Its Reversion

The endosymbiotic hypothesis for the origin of eukaryotic cells implies two contradictory processes. Mereschkowsky nearly a century ago hypothesised not only the endosymbiotic origin of chloroplasts by the equation, Animal plus Chloroplasts (Cyanobacteria) = Plants, but also the reverse, Plants minus Chloroplasts = Animals (Mereschkowsky, 1910). This anticipates organisms that through a loss of chloroplasts differentiate into secondary heterotrophs. The first equation has been transformed into the now widely accepted endosymbiotic theory but until recently, the second equation was only speculative. Studies of bleaching phenomenon in the mixotrophic flagellate *E. gracilis* have demonstrated that treatment with xenobiotics results in irreversible elimination of functional chloroplasts. Bleaching can be thus considered as a process supporting Mereschkowsky's second equation.

1.1. ORGANELLAR REGRESSIONS

According to the endosymbiotic theory, each organelle traces its descent to a free-living bacterial ancestor that entered into an endosymbiotic relationship with a host cell. Evolution of the endosymbiont to an organelle (chloroplast, mitochondria) involved gene losses or transfers from the endosymbiont genome(s) to the host nucleus. In the case of plastid acquisition by secondary endosymbiosis, the process whereby a protist engulfs a eukaryotic alga, genomic extinction of the nucleus has happened repeatedly. What are the rules and limits of reductive evolution of endosymbionts? Why have organelles retained genomes? Studies on reductive evolution have the potential to answer these questions.

1.1.1. Reductive evolution of plastids

Comparative studies of the completely sequenced plastid genomes have shown that

¹ This article was initiated at University of Nebraska (Lincoln) by visiting Professor Juraj Krajcovic.

individual genes have been independently lost many times in different lineages and that the flux of genes from the plastid to the nucleus is an ongoing process (Herrmann, 1997, 1999; Martin and Herrmann, 1998; Martin *et al.* 1998). A reduced but functional plastid genome is retained in nonphotosynthetic holoparasitic flowering plants, *e.g.* *Conopholis*, *Cuscuta*, *Epifagus*, *Lathraea*, *Orobanche* (Wolfe *et al.* 1992; Wolfe and de Pamphilis, 1997), some heterotrophic algae, *e.g.* *Astasia*, *Prototheca*, *Polytoma*, *Polytomella* (Bodyl 1996; Hachtel 1996, 1998), and in protozoan parasites of the phylum *Apicomplexa*, *e.g.* *Plasmodium*, *Toxoplasma*, *Eimeria* (Köhler *et al.* 1997; McFadden *et al.* 1997; Denny *et al.* 1998). Sister-group relationships between photosynthetic and non-photosynthetic dinoflagellates also suggest a photosynthetic history of extant heterotrophs (Schnepp, 1992). Cavalier-Smith (1999) argues that the last common ancestor of all Alveolata (the superphylum which includes dinoflagellates, apicomplexans and ciliates) was photosynthetic.

The colourless heterotrophic flagellate *Astasia* has lost half of its plastid genome compared to its photosynthetic counterpart *Euglena* (Siemeister *et al.* 1989; Gockel *et al.* 1994; Gockel and Hachtel, 2000). The reduction of plastid genome size in nonphotosynthetic plastids is mainly due to the loss of photosynthetic genes which are under relaxed functional constraints, and most probably results from highly specific deletion events within the original chloroplast genome of their ancestral photosynthetic relatives. The high conservation of an extranuclear genetic apparatus does not appear to be plausible if it is not needed for the synthesis of some gene product(s) that are indispensable for cell growth and metabolism.

Why have rudimentary plastids been retained? It is well documented in a wide range of plant tissues that nonphotosynthetic plastids are important sites for the biosynthesis of starch, fatty acids, heme, and for the assimilation of nitrogen into amino acids (for review see Neuhaus and Ernes, 2000). These plastid pathways are homologous to cyanobacterial ones. Plastid-containing cells have become dependent on these pathways for certain metabolic products that are exported from the organelle to the surrounding cytosol. This dependency makes plastids indispensable, even in non-photosynthetic plants and algae (Delwiche and Palmer, 1997; Waller *et al.* 1998). It is not clear whether the endosymbiont pathways replaced pre-existing host cell pathways or whether they were adopted as novel ones. According to Feierabend (1992), genes in residual plastid genomes do not necessarily have to play specific biosynthetic or metabolic functions, but may be associated with regulatory processes which make plastid loss impossible.

1.1.2. Reductive evolution of mitochondria

A parallel to chloroplast loss in *E. gracilis* during bleaching is mitochondrial regression connected with the petite syndrome in the yeast *Saccharomyces cerevisiae* (for review see Contamine and Picard, 2000). Molecular data indicate that the common ancestor of all extant eukaryotes contained mitochondria. The primitive taxa that lack mitochondria, collectively called Archezoa, lost the original organelle or modified it into a hydrogenosome (Keeling, 1998). Comparative studies of protist mitochondrial genomes suggest that as found for chloroplasts, individual mitochondrial genes have been independently lost many times in different lineages with the flux of genes from the mitochondrion to the nucleus being an ongoing process (Gray *et al.* 1999).

Genome evolution of intracellular bacteria resembles the evolution of organelles in

many ways. Similar features include reduced genome size, biased nucleotide base composition, fast sequence evolution, frequent gene rearrangements and pseudogene formation connected with the loss of certain functional categories of genes, including DNA repair genes, which affect mutational patterns. Evolution of the genomes of intracellular bacteria appears to be an example of the principal of population genetics known as Muller's ratchet which predicts that recurrent bottlenecks and low recombination rates will lead to the progressive accumulation of deleterious mutations in asexually propagated genomes with small population sizes. The reductive evolutionary processes acting on genomes of intracellular bacteria are likely the same processes that have shaped the structures of organellar genomes (for review see Andersson and Kurland, 1998; Andersson and Andersson, 1999; Blanchard and Lynch, 2000; Moran and Werneburg, 2000).

2. The origin of *Euglena* Chloroplasts

It is generally agreed that chloroplasts have a monophyletic origin resulting from a primary endosymbiotic association between a heterotrophic eukaryote and a photosynthetic prokaryote (Herrmann, 1997, 1999; Kowallik, 1997; Martin *et al.* 1998; Cavalier-Smith, 1999; Gray, 1999). The cyanobacteria, rhodoplasts and chloroplasts of higher plants and green algae subsequently evolved from this singular endosymbiotic event with the two chloroplast envelope membranes evolving from the cell membrane of the endosymbiont (for review see Cavalier-Smith, 1999). *Euglena* is one of a group of organisms containing complex chloroplasts; chloroplasts which have three or 4 envelope membranes. *Euglena* and dinoflagellate plastids have three envelope membranes (Dodge, 1975; Gibbs, 1978, 1981a; Dubertret and Lefort-Tran, 1982; Cavalier-Smith, 1999, 2000) while apicomplexan, cryptomonad, chlorarachniophyte, brown algal, diatom and other chromophyte plastids have 4 envelope membranes (Gibbs, 1981a,b; Häuber *et al.* 1994; McFadden *et al.* 1997; Waller *et al.* 1998). Complex chloroplasts arose through multiple secondary endosymbiotic associations between a heterotrophic or possibly phototrophic photosynthetic (Häuber *et al.* 1994) eukaryotic host and a photosynthetic eukaryotic endosymbiont.

2.1. PHYLOGENETIC IDENTIFICATION OF THE ANCESTRAL EUGLENOIDS

The euglenoid flagellates represent one of the earliest divergences among the eukaryotes. The evolutionary relationship between euglenoids and the Kinetoplastida, one of their putative sister groups, is supported by morphological data (Kivic and Walne, 1984), by nuclear Small Subunit (SSU) rDNA alignments (Schlegel, 1991; Van de Peer *et al.* 1993), and by the addition of a spliced leader sequence at the 5' end of the nuclear pre-messenger RNAs through a trans-splicing mechanism (Tessier *et al.* 1991). The most recent phylogenies obtained with mitochondrial gene sequences (Inagaki *et al.* 1997; Tessier *et al.* 1997; Yasuhira and Simpson, 1997) as well as with some nuclear encoded proteins (Hashimoto *et al.* 1994; Henze *et al.* 1995) also places *Euglena* and *Trypanosoma* far apart from other eukaryotes; these protists represent the earliest mitochondrion containing organisms.

Most euglenoid phylogenies are based on morphological characters. A recent study

based on nuclear SSU rDNA sequences (Linton *et al.* 1999) shows that the euglenoids form a distinct monophyletic clade. The phagotrophic members diverged prior to the phototrophic and osmotrophic members. This suggests that *Euglena* evolved through secondary endosymbiosis between a phagotrophic ancestral trypanosome and a eukaryotic alga.

2.2. PHYLOGENETIC ORIGIN OF THE *Euglena* CHLOROPLASTS

The ancestor of euglenoid chloroplasts which have envelopes of three membranes is one of the most attractive problems in chloroplast evolution. Gibbs (1978) suggested that the chloroplasts of *Euglena* may have evolved from symbiotic green algae. Since then several different hypotheses have been proposed to explain the origin of *Euglena* chloroplasts mostly anticipating their secondary green algal origin (for review see Cavalier-Smith, 1999, 2000). The availability of several complete chloroplast genome sequences has provided insight into this question. Rooted topologies inferred from the concatenated and aligned sequences of proteins common to the sequenced chloroplast genomes with cyanobacteria as an outgroup provide an overview of genomic changes during chloroplast evolution, and serve to identify ancient and more recent gene losses (Martin *et al.* 1998; Turmel *et al.* 1999, see also Herrmann, 1997, 1999; Kowallik, 1997; Cavalier-Smith, 1999, 2000; Gray, 1999). In phylogenetic studies based on these datasets, *Euglena* chloroplasts appear as a sister group to higher land plants (Martin *et al.* 1998). The *E. gracilis* chloroplast genome is unequivocally nested within green algae when the chloroplast genome sequence of two green algae, *Chlorella vulgaris* and the prasinophyte *Nephroselmis olivacea*, is included in the analysis (Turmel *et al.* 1999). This agrees with the results of gene-cluster analysis of chloroplast genomes (Stoebe and Kowallik, 1999) and suggests that *Euglena* chloroplasts are of green algal origin.

2.3. BIOCHEMICAL EVIDENCE THAT *Euglena* ACQUIRED PLASTIDS THROUGH SECONDARY ENDOSYMBIOSIS.

The majority of *Euglena* chloroplast proteins are encoded in the nucleus and synthesized on cytoplasmic ribosomes (for review see Schwartzbach 1990). In contrast to the direct import from the cytoplasm of nuclear encoded proteins of plastids such as those of higher plants and green algae that evolved from the singular primary endosymbiotic event, *Euglena* chloroplast proteins are synthesized on membrane bound cytoplasmic ribosomes (Kishore and Schwartzbach, 1992a) and transported in vesicles as integral membrane proteins from the ER to the Golgi apparatus prior to chloroplast localization (Sulli and Schwartzbach, 1996; Battacharya and Medlin, 1998). *Euglena* chloroplast protein presequences are approximately 140 amino acids in size and have a common structure (Sulli *et al.* 1999). The presequence N-terminus contains a signal peptide that is cleaved during precursor insertion into canine microsomes and a hydrophobic domain approximately 60 amino acids from the signal peptidase cleavage site anchors the protein in the microsomal membrane (Kishore *et al.* 1993; Sulli *et al.* 1999). The presequence remaining after signal peptide cleavage directs the import of the *Euglena* precursor into pea chloroplasts identifying this presequence region as a functional transit peptide (S. Schwartzbach unpublished). *Euglena* thylakoid proteins

contain an additional thylakoid targeting signal within the transit peptide at the presequence mature protein junction (Vacula *et al.* 1999).

Vacuolar protein precursors contain signal peptides and are transported in vesicles from the Golgi apparatus to the vacuole (Neuhaus and Rogers, 1998). If *Euglena* evolved from a phagotrophic trypanosome that engulfed a eukaryotic algae (Gibbs, 1978, 1981a,b; Neuhaus and Rogers, 1998; Linton *et al.* 1999), the endosymbiotic algae would have evolved into a chloroplast within a phagocytic vacuole. Signal peptides are among the most ancient targeting signals and both the host and endosymbiont probably used this system for vacuolar targeting. The endosymbiont most likely had transferred some chloroplast genes to its nucleus and utilized a transit peptide to return the encoded proteins to the chloroplast. Reduction of the endosymbiont to a chloroplast involved gene transfer from the endosymbiont nuclear and chloroplast genomes to the host nucleus. Genes transferred from the endosymbiont nucleus probably contained a transit peptide. The addition of a signal peptide and a vacuolar targeting signal to the transit peptide containing presequence provided the mechanism to return proteins to the plastid evolving within the phagocytic vacuole. The targeting signals in *Euglena* chloroplast protein presequences are consistent with the proposal that the outermost of the three *Euglena* envelope membranes is derived from the host's phagocytic vacuole membrane with the other 2 envelope membranes being derived from the chloroplast envelope of the photosynthetic eukaryotic endosymbiont (Melkonian, 1996). Eventually, the phagocytic vacuolar targeting signal evolved into a more specific Golgi to chloroplast targeting sequence with the endosymbiont's transit peptide dependent import system being utilized to transfer the protein into the chloroplast. Taken together, the domain structure of *Euglena* chloroplast protein sequences, the ER to Golgi to chloroplast transport pathway and the three membrane envelope provide strong biochemical evidence for acquisition of the *Euglena* chloroplast by secondary endosymbiosis.

3. Bleaching phenomenon in *Euglena*

The chloroplast system of the unicellular photosynthetic flagellate *Euglena gracilis* is particularly sensitive to various chemical and physical factors which induce irreversible loss of chloroplasts and depletion of chloroplast DNA (ctDNA) in a process called bleaching (for review see Mego, 1968; Schiff and Epstein, 1968; Gillham, 1978; Ebringer, 1978; Kempner, 1982; Ebringer and Krajčovič, 1986, 1994). The antichloroplastic bleaching activity of various agents is macroscopically manifested by permanent loss of capability of cells to form green colonies. Bleached mutants lack chlorophyll as well as detectable phototransformable protochlorophyll(ide) (Parthier and Neumann, 1977; Bingham and Schiff, 1979; Osafune and Schiff, 1980a, 1983). Some bleached mutants contain carotenoids while carotenoids are lacking in other mutants indicative of phenotypic variations found among the bleached mutants (Parthier and Neumann, 1977; Bingham and Schiff, 1979; Fong and Schiff, 1979). Bleaching in *Euglena* enables us to follow the process of morphological, functional and genetic degradation of chloroplasts and to see how far the reduction can go. Is it possible to eliminate the chloroplast completely or are there limits to chloroplast loss and if so why?

Bleaching in *Euglena* is firmly connected with the colourless flagellate *Astasia longa*

which is virtually indistinguishable by light microscopy from artificially bleached mutants of green *E. gracilis*. The loss of chloroplasts and stigma was assumed by Pringsheim and Hovasse (1948) to be a change that enables a transition from *E. gracilis* to *A. longa*. They thought that *A. longa* is only an apochlorotic race of *E. gracilis*. Pringsheim (1963) has forwarded the idea that algae without chlorophyll are descendants of algae containing chlorophyll. The view that *Astasia* is most probably a natural colourless variant of *Euglena*, that *A. longa* is identical to bleached *E. gracilis*, was for a long time accepted by various authors. The finding that the *Astasia* plastome has been reduced to half the size, 73,345 bp (Gockel and Hachtel, 2000), of the plastome of its photosynthetic counterpart *Euglena*, 143,172 bp (Hallick *et al.* 1993) provided support for this view. Studies appearing in the 60s and 70s that revealed biochemical, physiological and structural differences between the bleached strains of *E. gracilis* and *A. longa* (Blum *et al.* 1965; Rogers *et al.* 1972; Kivic and Vesl, 1974a,b) argued against the simplistic view that *A. longa* was simply a bleached *Euglena*. More recent molecular studies of chloroplast genome loss during bleaching clearly refute this simplistic view and suggest that *Euglena* and *Astasia* shared a common ancestor whose genome was reduced during evolution of *Astasia* by a process fundamentally different from bleaching.

3.1. BLEACHING AGENTS

Spontaneous bleaching in *E. gracilis* was observed as early as 1912 by Ternetz (1912), and it was later reported by several authors. In 1948 Provasoli *et al.* (1948) reported that streptomycin induced mass transformation of green *Euglena* to the permanent bleached state. Streptomycin was thus the first defined agent showing that there was a method for controlled bleaching of all cells in a population (Provasoli *et al.* 1948; Jírovec, 1949). This discovery stimulated considerable interest in searching for new bleaching agents as well as for initiating studies on the mechanism of induced plastid loss. In 1952 Pringsheim and Pringsheim (1952) reported that exposure of *Euglena* cells to elevated temperatures (between 32°C and 34°C) resulted in irreversible bleaching of these cells. In 1959 Lyman, Schiff, and Epstein (1959) characterized the ultraviolet irradiation bleaching process. Initial experiments with streptomycin, heat and U.V. light bleaching were later extended in an attempt to explain how these agents acted (for review see Gillham, 1978). A series of kinetic and pedigree studies attempted to determine the number of bleaching agent sensitive targets per cell and whether plastids were diluted out during bleaching (DeDeken-Grenson, 1959; Lyman *et al.* 1961; Gibor and Granick, 1962; Grenson, 1964; Hill *et al.* 1966; Uzzo and Lyman, 1969; Nicolas *et al.* 1997).

In 1961 Ebringer reported that erythromycin and some other macrolidic antibiotics induce irreversible loss of chloroplasts in *E. gracilis* (Ebringer, 1961, 1962) further stimulating investigations of chemically induced bleaching. It was eventually shown that practically all inhibitors of bacterial protein- and DNA-synthesis affected chloroplasts in *E. gracilis* (Ebringer *et al.* 1969a; Ebringer, 1972, 1978; Ebringer and Foltínová, 1976) inducing transformation of green cells to white ones without any loss of cell viability. However, because the permanent elimination of functional chloroplasts resulted in a complete loss of photosynthetic activity, white mutants only grow on organotrophic media. Chloramphenicol is an exception among these antibiotics. Although it inhibits protein synthesis in chloroplasts (Eisenstadt and Brawerman,

1964), no permanent loss of chloroplasts is induced under standard bleaching conditions. This anomaly has been shown to be due to photosynthetic reduction and thus detoxification of chloramphenicol (Vaisberg *et al.* 1996). Reductive detoxification explains the two reports that chloramphenicol causes permanent bleaching, but only under certain conditions (Miyoshi and Tsubo, 1969; Marčenko, 1974).

Several drugs, primarily streptomycin, chloramphenicol, cycloheximide and nalidixic acid have been studied so extensively and their mode of action has been so well established that they have developed into research tools for *Euglena* (for review see Gillham, 1978; Ebringer, 1978; see also Hashimoto and Murakami, 1982; Heizmann *et al.* 1982).

In addition to antibacterial drugs, U.V. light, elevated temperature, high pressure, antihistamines, and o-methylthreonine, there is a broad group of chemical substances, so called mutagens and/or carcinogens which are also able to bleach irreversibly *E. gracilis*. A large body of information has been accumulated about the action of mutagens and carcinogens especially about the action of nitrosoguanidine and nitrofurans (for review see Mego, 1968; Ebringer, 1978, 1990; Kempner, 1982; Ebringer and Krajčovič, 1986, 1994). Since *Euglena* seems to be a convenient model for screening compounds for mutagenic and carcinogenic activity, this topic will be discussed separately (see Section 4).

3.2. ULTRASTRUCTURAL CHANGES

There are profound ultrastructural differences between wild type green *E. gracilis* (as described *e.g.* in Buetow, 1968; Leedale, 1982) and bleached cells obtained by treatment with xenobiotics and other bleaching agents. Massive changes in cell ultrastructure during bleaching are primarily associated with chloroplast destruction and elimination although some antibacterial agents also affect mitochondria.

3.2.1. Effect on chloroplasts.

The reversal of endosymbiosis through bleaching results in diverse ultrastructural changes with different bleached mutants displaying different changes. In green *E. gracilis* cells exposed to various bleaching agents in liquid medium, most notably quinolone drugs and mutagens such as nitrosoguanidine and furylfuramid, both damaged and normal chloroplasts are found with the percentage of aberrant organelles being proportional to the length of treatment (Polónyi *et al.* 1987, 1990). A reduction of thylakoid number was observed. In some cells, short ring like thylakoids were found. In the terminal part of some chloroplasts, swollen spaces having a light matrix filled with fine microfibrillar material were seen. The cytoplasm and nucleus were not altered in these cells which still contained chlorophyll but damaged mitochondria were often found (Polónyi *et al.* 1990; Ebringer *et al.* 1993).

When white clones were analyzed after plating on agar, the majority of the cells obtained from a single clone did not contain chloroplast remnants. A few exceptional cells had proplastid-like figures with several three-laminar membranes twisted around a granular matrix. The cytoplasm of these white cells contained small vacuoles, paramylon grains and a number of mitochondria. New cytoplasmic membranous structures were seen especially after the treatment with furylfuramid (Polónyi *et al.* 1987). The cytoplasm was filled with membranes, lamellar bodies or thylakoid-like

figures and great bags of secondary lysosomes containing partly digested organelles (mitochondria, vesicles of different content and diameter). Empty vacuoles with small pieces of very dense material at their border were sometimes seen (Polónyi *et al.* 1990).

The stable bleached mutant **W₃BUL** contains a plastid remnant (Heizmann *et al.* 1976; Parthier and Neumann, 1977; Osafune and Schiff, 1980a; Osafune *et al.* 1987) that exists in the dark as a thin, irregular, circular leaflet of narrow stroma devoid of ribosomes. Light exposure produces a limited expansion of the stroma with the formation of a vacuole and prolamellar body (Osafune and Schiff, 1980a; Osafune *et al.* 1987). **W₃BUL** contains the thylakoid membrane specific sulfolipid (Saidha and Schiff, 1989) and sulfolipid synthesis is induced (Saidha and Schiff, 1989) by light exposure, **W₁₀BSmL** differs from **W₃BUL** in that it lacks detectable carotenoids (Fong and Schiff, 1979), sulfolipid (Saidha and Schiff, 1989), a stigma (Osafune and Schiff, 1980b) and an identifiable plastid remnant (Osafune and Schiff, 1983).

The absence of the stigma and paraflagellar swelling is the main feature distinguishing *A. longa*, the “naturally occurring” bleached mutant of *Euglena* from bleached *Euglena* produced in the laboratory (Kivic and Vesk, 1974b). Kivic and Vesk (1974b) felt that the other reported ultrastructural differences between *Astasia* and bleached *Euglena* (Blum *et al.* 1965; Rogers *et al.* 1972) were not greater than those found between different bleached *Euglena* mutants produced in the laboratory. The presence of plastid like structures in *A. longa* containing a circular 73 kbp ptDNA has not been shown unambiguously (Kivic and Vesk, 1974a) although Shashidhara and Smith (1991) localized porphobilinogen deaminase to a membranous structure in *A. longa*. Hachtel (1996) recently identified vesicular structures in *Astasia* as rudimentary plastids.

3.2.3. Cup-like mitochondria formation.

Antibacterially active chemicals, quinolones and coumarins, inhibitors of bacterial DNA gyrase subunit *gyrA* and *gyrB*, respectively, are very potent bleaching agents (Krajčovič *et al.* 1989, 1990). In addition to chloroplast loss, quinolone antibacterial drugs and coumermycin **A₁** also affect *Euglena* mitochondria (Polónyi *et al.* 1990, 1998; Ebringer *et al.* 1993). Damaged mitochondria were large and swollen with a flocculent matrix. Cristae of vesicular form were localized to the periphery of damaged mitochondria, close to the inner mitochondrial membrane, producing an empty cristae free space inside the organelle (Ebringer *et al.* 1993). The most striking features of these damaged mitochondria were small cavities usually localized in the mitochondrial center and communicating with the surrounding cell cytoplasm. Cup-like mitochondria (in sections ring-like, O-shaped) were the most frequent mitochondrial abnormalities produced by the antibacterial drug ofloxacin. As many as 45% of the mitochondria were damaged. At least two populations of O-shape mitochondria were observed. Some mitochondria had cavities filled with cytoplasmic fragments or other membranous structures. Other mitochondria had ring-like structures with electron-lucent cavities filled with fine granular or fibrillar structure (Ebringer *et al.* 1993). In contrast to the irreversible elimination of chloroplasts after cultivation in ofloxacin-free media, the mitochondria regained their normal morphology indicating the mitochondrial changes are reversible (Polónyi *et al.* 1990; Ebringer *et al.* 1993).

In at least one case, the mitochondrial alterations produced by bleaching agents do not appear to be reversible. The N-succinimid derivative of ofloxacin produced a

bleached mutant of *E. gracilis*, WZOfIL, with permanently altered mitochondria. Some mitochondria are about 5-8 μm long and wide having an unusual shape. Others are giant disfigured mitochondria with unusual shapes. Giant swollen oval shaped megamitochondria and some very long (50-60 μm) mitochondria are also seen in addition to normal mitochondria (Polónyi *et al.* 1998). In contrast to the loss of the plastid, amitochondrial cells are never found as expected for an obligate aerobe like *Euglena*.

3.3. CHLOROPLAST GENOME DEGRADATION AND REARRANGEMENT

The unique feature of *Euglena* is that a large number of chemical and environmental agents produce permanently white cells in a process called bleaching while having no effect on cell growth and viability (for review see Mego, 1968; Schiff and Epstein, 1968; Schiff *et al.* 1971, 1980; Ebringer, 1972, 1978, 1990; Gillham, 1978; Kempner, 1982; Ebringer and Krajčovič, 1986, 1994). All bleaching agents produce permanent changes in the chloroplast genome (for review see Hallick and Buetow, 1989). An intact chloroplast genome is undetectable in bleached mutants (Edelman *et al.* 1965; Chelm *et al.* 1977). Fragments of the chloroplast genome have been found in some bleached mutants with different mutants retaining different genomic segments (Heizmann *et al.* 1981, 1982). Portions of the chloroplast genome, most notably the rRNA cistrons appear to be amplified during the bleaching process. Estimates of the amount of rRNA cistrons present in bleached mutants range from 30-100 copies per cell with other fragments present at 2-6 copies per cell depending on the mutant analyzed (Hussein *et al.* 1982). The amount of rearranged DNA found in bleached mutants is 100-1000 fold less than the amount in wild type cells (Heizmann *et al.* 1982; Hussein *et al.* 1982).

Recent work suggests that some bleaching agents do not produce chloroplast gene rearrangements at least during the early stages of the bleaching process. Treatment with the quinolone, ofloxacin, and the mutagen, nitrosoguanidine, resulted in a massive degradation of *Euglena* chloroplast DNA (Krajčovič *et al.* 1999). At the level of detection by ethidium bromide staining of agarose gels, restriction digests of chloroplast DNA extracted from plastids isolated from bleaching cells produced smears indicative of DNA degradation rather than the unique band patterns obtained with intact chloroplast DNA. Southern blotting however detected restriction fragments of the same size as found in wild type cells with the DNA amount of the fragments decreasing as bleaching progressed indicating chloroplast DNA is degraded during bleaching. Southern blotting failed to detect chloroplast genome rearrangements during bleaching (Krajčovič *et al.* 1999).

A complete loss of chloroplast DNA has also been observed during heat bleaching. Conkling *et al.* (1993) found a delayed but complete loss of chloroplast DNA in cells bleached by growth at 34°C. On the other hand, Krajčovič *et al.* (1999) detected the expected restriction fragments as well as some novel restriction fragments indicative of genome rearrangements on Southern blots of chloroplast DNA extracted from plastids of cells grown at 34°C. The discrepancy between these two studies may result from the increased sensitivity obtained with Southern blots of DNA extracted directly from the bleaching plastids rather than from whole cells. Alternatively, this difference may reflect the varied phenotypes of the stabilized bleached mutants which are independent of the bleaching agent used. The studies of chemical and temperature induced bleaching

indicate that although some chloroplast DNA may be present in bleached mutants, these mutants clearly lack a complete functional chloroplast genome. Studies of the bleaching process can thus provide information on the reversion of endosymbiosis that has occurred a number of times over the course of evolution.

*3.3.1. Plastid genome in the colourless Euglenoid *Astasia longa*.*

Astasia, a close relative of *Euglena* has retained approximately 50% of the chloroplast genome of its photosynthetic counterpart *Euglena* (Hallick *et al.* 1993; Gockel and Hachtel, 2000). The *Astasia* and *Euglena* chloroplast genomes share extensive structural and nucleotide sequence similarities providing further evidence of a close evolutionary relationship between *Astasia* and *Euglena*. The size reduction of ptDNA of *Astasia* is primarily associated with the loss of photosynthetic genes indicative of highly specific deletions of the ancestral *Astasia* plastid genome. In addition to the extensive deletions, a number of sequence rearrangements are found when the *Astasia* and *Euglena* genomes are compared (Gockel *et al.* 1994; Gockel and Hachtel, 2000). In comparison to the *E. gracilis* chloroplast genome, the *Astasia* genome lacks genes for components of photosystem I, the cytochrome b6/f-complex and the ATP-synthase. The *Astasia* genome contains all of the genes for components of the plastid transcriptional and translational apparatus together with the large subunit of Rubisco. It is implausible that these genes and the plastid are retained in *Astasia* without the plastid providing functions indispensable for cell growth and viability. The *rbcL* gene is retained in the plastid genome of a number of nonphotosynthetic higher plants where its function is unknown (Wolfe and dePamphilis, 1997). Siemeister and Hachtel (1989) proposed that the *Astasia* plastid *rbcL* gene may be required for Rubisco activity associated with glycine and serine production through the photorespiratory cycle. Whether the *Astasia* plastid is retained because the *rbcL* gene is required or whether the product of one of the unassigned plastid open reading frames is required for growth and viability remains to be determined (Gockel *et al.* 1994; Hachtel, 1998; Gockel and Hachtel, 2000).

Bleached mutants of *Euglena* lack most if not all of the plastid genome. The *Astasia* plastid has retained approximately 50 % of the plastid genome. This indicates that in contrast to *Astasia*, the plastid genome of *Euglena* does not encode a gene required for cell growth and viability. This difference strongly suggests that although *Euglena* and *Astasia* share a common ancestor, the evolution of a stable and functional *A. longa* plastome was fundamentally different from the process of induced bleaching which has been extensively studied in *Euglena* (Bodyl, 1996).

3.4. TRANSCRIPTIONAL ACTIVITY IN BLEACHING PLASTIDS

Changes in steady-state levels of chloroplasts transcripts and in transcriptional activity in bleaching plastids have been most extensively studied in *E. gracilis* during heat-bleaching. It has been shown that along with the changes in chlorophyll accumulation, one of the earliest indicators of heat-bleaching at the molecular level is a decreased rate of protein synthesis (Ortiz and Kutner, 1990; Ortiz *et al.* 1992). Brandt and Wiessner (1997) reported that DNA-dependent RNA polymerases of *E. gracilis* have different temperature optima which could explain heat bleaching. Thomas and Ortiz (1995) however reported that transcription of *psbB* and *psbC* genes remains essentially unchanged during heat bleaching. The levels for the mature transcripts for CP47 and

CP43, the chlorophyll *a* binding apoproteins of photosystem II encoded by the *psbB* and *psbC* genes, decline sharply very early during heat-bleaching. The reduction in the synthesis rate of specific chloroplast proteins during heat bleaching appears to result from changes in mRNA levels due to posttranscriptional events such as a reduction in mRNA stability rather than from a reduction in gene transcription rates.

In *Astasia*, the minimal plastid genome is transcribed. Transcripts for the plastid encoded genes *tufA*, *rbcL*, a number of ribosomal protein genes, the 23S and 16S rRNA genes, and three unassigned open reading frames have been detected in *Astasia* (Gockel *et al.* 1994; Gockel and Hachtel, 2000). Immunoblot analysis has detected the gene product of *rbcL* as the expected a 53 kDa polypeptide. Detection of plastid encoded proteins clearly indicates that the translational machinery within the *Astasia* plastid is functional as expected if an *Astasia* plastid encoded protein is required for cell growth and viability.

3.5. EXPRESSION IN BLEACHED MUTANTS OF NUCLEAR ENCODED GENES FOR CHLOROPLAST PROTEINS

A large number of genes were transferred from the endosymbiont genome to the host cell nucleus during chloroplast evolution (Martin *et al.* 1998; Herrmann, 1999). The result of this gene transfer is that many components of multiprotein complexes are encoded by both the nuclear and the chloroplast genome. The coordination of nuclear and organelle genome expression by light ensures the orderly assembly of these complexes. In the case of *Euglena*, the expression of nuclear genes encoding chloroplast proteins is regulated by light acting through a chloroplast localized photoreceptor, protochlorophyll(ide) and one or more nonchloroplast photoreceptors (for review see Schwartzbach, 1990). In addition to regulating expression of genes encoding chloroplast proteins, these photoreceptors also regulate the expression of nuclear genes encoding mitochondrial, microbody and cytoplasmic proteins testifying to the total integration of the endosymbiont into cellular metabolism.

The light harvesting chlorophyll *a/b* binding protein of photosystem II is a major chloroplast protein encoded by the nuclear genome and its regulation typifies that of light induced proteins. Light exposure increases the rate of synthesis of LHCPII by as much as 100 fold (Rikin and Schwartzbach, 1989a) while producing approximately a two fold increase in the steady state level of LHCPII mRNA (Rikin and Schwartzbach, 1989a; Kishore and Schwartzbach, 1992b). The fraction of LHCPII mRNA present on polysomes in dark grown resting *Euglena* and cells exposed to light are similar (Kishore and Schwartzbach, 1992b) clearly demonstrating that LHCPII synthesis is controlled by light at the level of translational elongation rather than transcription. Two dimensional gel electrophoresis found that the rate of synthesis of over 200 proteins increased after 24 h of light exposure while no change was detected in the levels of the 250 most abundant translatable poly(A)⁺ RNAs (Monroy *et al.* 1987) confirming that photoregulation of nuclear encoded protein synthesis occurs primarily at the translational rather than the transcriptional level. Studies of nuclear gene expression in bleached *Euglena* provides a unique opportunity to study the extent to which expression of genes transferred from the chloroplast to the nucleus is dependent upon the presence of a functional chloroplast genome.

3.5.1 Transcripts for nuclear encoded chloroplast proteins in bleached mutants.

Transcripts for the nuclear encoded chloroplast proteins, Rubisco SSU, LHCPII and cytochrome c-553 are present in all bleached mutants studied (Kishore and Schwartzbach, 1992b; Krajčovič *et al.* 1999). LHCPII mRNA levels in dark grown cells of the bleached mutant **W₃BUL** are significantly lower than in dark grown wild type cells (Kishore and Schwartzbach, 1992b). Light exposure increases LHCPII mRNA levels in **W₃BUL** to the level found in dark grown wild type cells. On the other hand, LHCPII mRNA levels in dark grown resting cells of the bleached mutant **W₁₀BSmL** are the same as in dark grown resting cells and they are not increased by light exposure (Kishore and Schwartzbach, 1992b). LHCPII mRNA is associated with polysomes in both **W₃BUL** and **W₁₀BSmL** indicating that the gene produces a functional mRNA (Kishore and Schwartzbach, 1992a).

Cytochrome c-553 (*petJ*) mRNA was present in the ofloxacin-bleached mutant WZOflL (Vacula *et al.* 2000). Dark grown cells of WZOflL had the same cytochrome c-553 level as wild type cells. Light exposure transiently increased cytochrome c-553 mRNA levels and then they declined to levels below those found in dark grown wild type cells. This contrasts with the absence of light induced changes in cytochrome c-553 mRNA levels in wild type *Euglena* (Krajčovič *et al.* 1999).

In colourless *Astasia* transcripts of the nuclear genes encoding the chloroplast localized photosynthetic proteins Rubisco SSU, LHCPII, cytochrome c-553, the oxygen-evolving enhancer protein 1 and hydroxymethylbilane synthase were undetectable (Vacula *et al.* 2000). In contrast to the reversion of endosymbiosis produced in the laboratory by treating wild type green *Euglena* with bleaching agents, the reversion of endosymbiosis that occurred during the evolution of *Astasia* resulted in the retention of a large number of chloroplast genes and a loss of the ability to transcribe nuclear genes encoding chloroplast proteins. Whether these genes are still present in the nuclear genome of *Astasia* remains to be determined.

3.5.2 Nuclear encoded chloroplast proteins in bleached mutants.

The synthesis of *Euglena* chloroplast proteins is primarily regulated at the level of translational elongation rather than transcription (Monroy *et al.* 1987; Kishore and Schwartzbach, 1992a). As a rule, chloroplast enzymes as measured by enzyme activity are present in the bleached mutant **W₃BUL** at levels comparable to those found in dark grown wild type cells and absent from the bleached mutant **W₁₀SmL** (for review see Schwartzbach, 1990). Some but not all of these enzymes are induced by exposing **W₃BUL** to light. Two dimensional gel electrophoresis was used to directly measure protein levels in **W₃BUL** and **W₁₀SmL**. At least 7 proteins found in **W₃BUL** were undetectable in **W₁₀BSmL** (Monroy and Schwartzbach, 1983). The amounts of 12 polypeptides increased upon exposure of **W₃BUL** to light. Although 5 of these proteins are found in **W₁₀SmL**, their levels are unchanged by light exposure.

Pulse labeling with ³⁵S-sulfate has been used to directly measure the rate of synthesis of LHCPII in the bleached mutants **W₃BUL** and **W₁₀SmL**. LHCPII synthesis was undetectable in both mutants even though both mutants contain LHCPII mRNA which is associated with polysomes (Schiff *et al.* 1991; Kishore and Schwartzbach, 1992a,b). The LHCPII precursor is extremely large and has a half life of 20 min which is the time required to transport the protein from its site of synthesis in the cytoplasm to the Golgi apparatus and then to the chloroplast (Rikin and Schwartzbach, 1988; Sulli and

Schwartzbach, 1995). The failure to detect synthesis is not due to rapid turnover in the absence of chloroplast localization. Since the synthesis of chloroplast proteins including LHCPII is regulated at the level of translational elongation (Kishore and Schwartzbach, 1992a), the absence of LHCPII synthesis in the bleached mutants indicates that a chloroplast signal is required to release the translational block.

Taken together, the studies of RNA and protein levels in bleached mutants indicates that chloroplast loss results in a loss of the ability to express some but not all nuclear genes for chloroplast proteins. What is surprising is that different mutants have lost the ability to express different proteins. In some cases the message is produced but translation is blocked while in other cases it is the photoresponse that is lost. Reversion of endosymbiosis does not produce a common loss of function highlighting the complexity of the interactions between the endosymbiont (organelle) and nuclear genomes.

4. *Euglena* as a model for detection and testing genotoxic effect of xenobiotics

Euglena gracilis with its wide latitude of growth conditions, well established composition, structure, biochemistry and chloroplast genome sequence is a very attractive organism to study biological effects of various chemical and physical factors. *Euglena* can be grown over the pH range 3.0-9.0 mixotrophically with an organic carbon source in the light, heterotrophically with an organic carbon source in the dark or autotrophically with CO₂ and light as the sole source of carbon and energy. *Euglena* can also be maintained for prolonged periods under non-dividing conditions on a resting medium lacking a utilizable carbon source for growth. Environmental conditions such as light intensity and temperature can be varied allowing effects of hyperthermia and heat shock to be readily determined. There are several reviews of the effects of a variety of compounds on *Euglena* growth (e.g. Mego, 1968; Schiff and Epstein, 1968; Schiff *et al.* 1971, 1980; Ebringer, 1972, 1978, 1990; Gillham, 1978; Kempner, 1982; Ebringer and Krajčovič, 1986, 1994). Because of large range of permissible growth conditions, Kempner (1982) reviewing stimulation and inhibition of metabolism and growth of *E. gracilis* called this flagellate a protozoan chameleon.

The chloroplast transcription, translation and DNA replication machinery is prokaryotic in nature having evolved from a cyanobacterial ancestor. Antibacterial agents will specifically interfere with chloroplast gene expression while having no effect on cell growth and viability. *Euglena* can thus be considered a sensitive model organism for studies of the antibacterial biological effects of xenobiotics, especially for detection of genetically active substances (mutagens/antimutagens). The utility of *Euglena* as a model organism is based on the preferential and selective influence of xenobiotics on the chloroplast genetic apparatus resulting in bleaching, the elimination of the functional chloroplasts from the cells, which represents the reversal of endosymbiosis.

4.1.1. Chemical structure and biological activity relationship.

Numerous studies have shown that bleaching can be used to determine the relationship between chemical structure and biological activity of new drug derivatives. Streptomycin and dihydrostreptomycin are effective bleaching agents in *Euglena*

(Ebringer *et al.* 1969b, 1970). Treatment of streptomycin with hydroxylamine forms streptomycinoxime which does not have antibacterial activity while the antibacterial activity of dihydrostreptomycin is unaffected by hydroxylamine treatment (Ebringer *et al.* 1967b). Studies of streptomycin and dihydrostreptomycin binding to *Euglena* chloroplast ribosomes demonstrated that hydroxylamine reversed streptomycin binding and established that bleaching was the result of the selective inhibition of protein synthesis on chloroplast ribosomes (Schwartzbach and Schiff, 1974).

A study of 22 derivatives of oleandomycin and erythromycin found a significant correlation between the effects of these derivatives on *E. gracilis* and on bacterial infection *in vivo* (Celmer and Ebringer, 1967). Similar correlations between structure and biological activity were demonstrated for derivatives of lincomycin (Ebringer and Foltínová 1976) and mitomycin (Ebringer *et al.* 1969a), quinolones and coumarins (Krajčovič *et al.* 1989, 1990; Krajčovič and Ebringer, 1990). Quinolones (nalidixic acid, oxolinic acid, ofloxacin, ciprofloxacin, norfloxacin, pefloxacin, enoxacin, cinoxacin) and coumarins (coumermycin A₁, novobiocin, chlorobiocin) are DNA gyrase inhibitors. The DNA gyrase inhibitors are potent bleaching agents in *E. gracilis*. As found for the inhibitors of bacterial protein synthesis, there is a significant correlation between the bleaching activity and antibacterial activity of the analyzed DNA gyrase inhibitors (Krajčovič *et al.* 1989). In contrast to the inhibitors of protein synthesis, DNA gyrase inhibitors produce both a hereditary loss of chloroplasts and a non-lethal alteration of mitochondria (Polónyi *et al.* 1990; Ebringer *et al.* 1993). Taken together, these studies establish that due to the prokaryotic nature of the plastid DNA replication, transcription and protein synthesis machinery, bleaching in *E. gracilis* can be used as a simple model for the rapid preliminary evaluation of the toxicity of antibacterial drugs (Ebringer, 1972, 1978, 1990; Fasulo *et al.* 1981).

4.1.2. Bleaching as a rapid assay for mutagenic compounds.

Nitrofurans are identified as mutagens by the Ames assay, phage-induction assay and the DNA-repair assay. Nitrofurans are effective bleaching agents in *Euglena* (Ebringer *et al.* 1967a, 1976, 1982; McCalla, 1965; McCalla and Reuvers, 1970). Only those furan derivatives having the nitro-group in position 5 of the furan ring were found to be active in the standard mutagenicity tests. If the nitro-group was in the position 4 instead of 5, the mutagenic activity was greatly reduced or eliminated. On the basis of these results, it is generally assumed that the 5-nitrofuran moiety itself is responsible for mutagenic and carcinogenic activities of these compounds. The only nitrofurans with bleaching activity were those with the nitro-group in position 5 establishing that chloroplast loss in *Euglena* can be used as a simple and rapid mutagenicity assay.

On the basis of the correlation between *Euglena* bleaching and the official mutagenicity tests requested by the Organization for Economic Co-Operation and Development (OECD) a nitrofuran food preservative (5-nitro-2-furylacrylic acid) was banned in Czechoslovakia. This compound is structurally similar to furylfuramide (AF-2), which was used in Japan as a food preservative until 1974 when it was found to be carcinogenic (Kada, 1974). 5-nitro-2-furylacrylic acid was originally introduced into Czechoslovakia and the Soviet Union as a wine stabilizer. It was proposed for use as a preservative of non-alcoholic beverages replacing sorbic acid. This compound bleached *E. gracilis*, induced sister chromatid exchanges in V79 cells, induced lambda phage in a lysogenic culture of *Escherichia coli*, transformed V79b cells *in vitro* and induced

mutations in *Salmonella typhimurium* and *Escherichia coli* clearly establishing the mutagenicity of this compound (Siekel *et al.* 1987; Ebringer *et al.* 1988). Similar correlations were made between bleaching effectiveness and genotoxicity as well as embryotoxic effects of Nitrovin, a 5-nitrofuran derivative used in many countries as a feed additive (Chreňo and Ebringer, 1984; Ebringer *et al.* 1987; Chreňo *et al.* 1988).

4.1.3. Euglena - a model organism for the study of antimutagenesis; screening of antimutagens.

During the study of bleaching by genotoxic agents it was found that bleaching of *E. gracilis* can also be used to study antimutagenesis. The classical antimutagens, selenium, vitamin C, tocopherol, butylated hydroxyanisol prevented bleaching by known mutagens and carcinogens (Fotínová *et al.* 1994; Ebringer *et al.* 1996a,b, 1997; Križková *et al.* 1996). These antimutagens as well as tetracycline antibiotics also prevent bleaching by fluoroquinolones. After treatment with chemical and physical mutagens, natural dietary inhibitors of mutagenesis, lignin, suberin and flavonoids, significantly reduced the level of bleached mutants (Belicová *et al.* 1999, 2000; Ebringer *et al.* 1999; Križková *et al.* 1999, 2000a,b). These results suggest that bleaching in *E. gracilis* can be used as a general screening method for naturally occurring or synthetic antimutagens.

4.1.5. Plastids as drug targets.

Identification of bleaching agents has taken on additional significance with the discovery of a residual plastid genome contained within the apicoplast of protozoan parasites of the phylum *Apicomplexa* (Köhler *et al.* 1997; McFadden *et al.* 1997; Denny *et al.* 1998). Organisms within this phylum such as *Plasmodium* and *Toxoplasma*, are major disease causing agents. Quinolones are potent bleaching agents in *Euglena* (Krajčovič *et al.* 1989, 1990; Krajčovič and Ebringer, 1990). The replication of the apicoplast genome in *T. gondii* tachyzoids is specifically inhibited by the quinolone ciprofloxacin, and this inhibition blocks parasite replication (Fichera and Ross, 1997). New quinolones also induce cleavage of 35-kbp plastid genome in the apicoplast of the marlaria parasite *P. falciparum* (Weissig *et al.* 1997). In contrast to the *Euglena* plastid, the apicoplast is required for parasite growth and viability. The plastid like metabolic machinery of the apicoplast thus make this organelle a promising target for therapeutic agents and drug development since agents affecting the apicoplast should be lethal to the parasite while having no lethality for the infected eukaryotic host cells. Prokaryotic metabolic pathways in the relict plastid of apicomplexan parasites make this organelle a promising new parasite-specific target for therapeutic agents and for drug development (Fichera and Ross, 1997; McFadden and Ross, 1999). A wide range of well-characterized drugs and herbicides are suspected of blocking the activity of the apicomplexan plastids (McFadden and Ross, 1999). Bleaching in *Euglena* could provide a rapid screening method for these agents allowing the identification of those agents that specifically inhibiting plastid and thus apicoplast replication while having no effect on eukaryotic metabolic activity.

5. Conclusions

Chloroplasts were acquired through establishment of an endosymbiotic relationship between a heterotrophic eukaryotic host and a photosynthetic prokaryote or in the case of organisms having complex chloroplasts such as *Euglena*, a eukaryotic symbiont. The reduction of the symbiont to a chloroplast involved transfer of many symbiont genes to the host cell nucleus and integration of the symbiont's replication and metabolism into that of the host cell. The most obvious evidence of this metabolic integration is the regulation of chloroplast development by light. In all known photosynthetic organisms except the Euglenoids, the endosymbiosis can not be reversed. Chloroplast elimination is a lethal event indicating that the chloroplast performs some metabolic function required for growth and viability. What this function is remains unknown. The dependence of cell viability on the chloroplast is most likely due to loss from the host nucleus of genes encoding proteins whose function was duplicated by proteins encoded by the chloroplast gene. Alternatively, the less likely possibility is that the chloroplast acquired a new function from the host nucleus. In either case, the symbiotic relationship is stabilized by the host's dependence on the chloroplast.

A large variety of environmental and chemical agents bleach *Euglena* reversing endosymbiosis. Bleaching is not due to the absence of metabolic integration between the host and chloroplast genomes. Chloroplast development is controlled in *Euglena* by light and even after elimination of the chloroplast genome, nuclear genes for chloroplast components continue to be photoregulated. This indicates that during the evolution of *Euglena*, the host did not lose vital functions that must now be performed by the plastid. *Astasia* shares a common ancestor with *Euglena* but it has lost approximately half of its plastid genome most notably the genes encoding components of the photosynthetic apparatus. Molecular studies of the bleaching process indicate environmental and chemical bleaching agents rapidly eliminate most if not all of the plastid genome. This strongly suggests that the evolution of *Astasia* from the ancestor shared with *Euglena* represents an example of a true reversal of endosymbiosis through the gradual loss of plastid genes either through elimination or transfer to the nucleus. Whether this is a continuing process or whether the genome has become stabilized through the necessity for the chloroplast to synthesize a protein required for cell growth and viability remains to be determined.

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SYMBIOSOMES

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1. Introduction

In almost all-intracellular symbioses the smaller symbiont is surrounded by a symbiosome consisting of a membrane of host origin and a space between this membrane and the outer surface of the symbiont. The symbiosome space is the immediate environment of such symbionts, and since the symbiosome membrane surrounds them completely, it can potentially control the movement of all materials entering and leaving the symbionts. In other words, the symbiosome plays a crucial role in the physiology and biochemistry of intracellular symbioses, yet very little is known of the composition and functions of either the symbiosome membrane or the symbiosome space. Associations between legumes and nitrogen-fixing bacteria (rhizobia - species of *Rhizobium*, *Azorhizobium*, *Bradyrhizobium* and *Sinorhizobium*) are the exception, as there has been a considerable amount of research on the symbiosomes in this symbiosis. The legume-*Rhizobium* symbioses have been reviewed extensively (Brewin, 1991; Udvardi and Day, 1997; Whitehead and Day, 1997). Until recently, the presence of the symbiosome has largely been ignored in papers on the physiology of other symbioses.

Several terms have been used in the literature to describe symbiosomes (as defined above); for example, the symbiosome spaces in legume root nodules are usually called "peribacteroid spaces", and the symbiosome membranes are known as "peribacteroid membranes". In other associations, terms such as "symbiont-containing vacuole (or vesicle)" and "parasitophorous vacuole" have been used (Roth *et al.*, 1988). Roth *et al.* (1988) argue that "endosome" should be used when it is not clear whether the intracellular organism is food, a parasite, a commensal or a mutualistic symbiont, and that "symbiosome" should be reserved for mutualistic associations. However, the term "symbiosis" was originally coined to describe all close, persistent associations between members of different species, in which case all endosomes, except food vacuoles, can be covered by the term "symbiosome". Roth *et al.* (1988) also suggest that "symbiosome" should be used only when the enclosed symbionts are capable of dividing, presumably because this indicates that the association is, indeed, a persistent one. In this chapter we will use the term "symbiosome" when discussing persistent associations, though it is not always clear that they are all mutualistic (Hinde, 1987). Some authors explicitly include the symbiont as part of the symbiosome (e.g., Schüssler *et al.*, 1996; Wakefield and Kempf, 1999), while most do not specify whether they

regard it as part of the compartment or as a separate entity. We will treat it as a separate entity.

Symbiosomes around intracellular microsymbionts form closed compartments, and their membranes are modified during establishment of the symbiosis (see below). There are, however, a number of symbiotic associations between fungi and plants in which parts of the fungus are within plant cells, while the rest extends into the extracellular space or even, in mycorrhizae, into the soil. These biotrophic fungal associations may be pathogenic (e.g., rusts and mildews) or mutualistic (e.g., mycorrhizal associations). In these associations the fungus is also separated from the plant cytoplasm by a membrane derived from the plant plasma membrane, but it is not clear whether these membranes are modified in the same way as those surrounding intracellular symbionts. In spite of this, Parniske (2000) has used the term "symbiosome" for the membranes around symbionts of both types. These two types of symbiosomes have much in common, in that they separate symbionts from the cytoplasm of their hosts and have the potential to control fluxes of nutrients into and out of the symbionts (Parniske 2000). On the other hand, Smith and Smith (1990) argue that the many differences between symbioses make the use of a single term misleading, and suggest that the terms "extrahaustorial membrane" and "periarbuscular membrane" be retained. Since these biotrophic fungal associations have been reviewed in detail (Smith and Smith, 1990; Harrison, 1999; Heath and Skalamera, 1997; Parniske, 2000), and differ in many ways from intracellular symbioses, we will not review them again here.

Roth *et al.* (1988) describe the symbiosome as a "compartment". If the symbiosome is to be regarded as a functional compartment of the host cell, it is necessary to establish that its membrane is capable of actively transporting some molecules and preventing the passage of others. These properties have been demonstrated in the symbiosomes of legume root nodules, which are impermeable to sugars, glutamate, oxoglutarate and pyruvate, but which actively transport succinate, malate and fumarate inwards (Day *et al.*, 1989). Active transport occurs across the symbiosome membrane in the symbiosis between the anemone *Anemonia viridis* and its symbiotic dinoflagellates ("zooxanthellae") (Rands *et al.*, 1993). It is likely that all symbiosomes are true intracellular compartments, which play an active role in interactions between host and symbiont. For such symbioses, it will not be possible to describe the functioning of the symbiosis fully until we understand the nature of the symbiosome. In addition, since chloroplasts and mitochondria are widely held to have evolved from endosymbiotic prokaryotes, their outer envelopes are likely to have been derived from a symbiosome. Studies of symbiosomes may, therefore, shed light on the evolution and function of the outer membranes of organelles.

2. Symbiosome Structure and Composition

2.1 THE ORIGIN AND DEVELOPMENT OF SYMBIOSOMES

The mechanisms by which functional symbiosomes develop, and the timing of major events in development, are unknown for most symbioses; despite much work, they are still not completely understood even for legume-*Rhizobium* associations. Symbionts may be recognized before phagocytosis, causing the process of internalization of the phagosome to be modified and leading directly to the formation of a functional

symbosome. Alternatively, the prevention of lysosomal activity, or some other event after internalization of the symbiont in a phagosome, may trigger the development of the symbosome by modification of the phagosome.

So far all the evidence suggests that symbosomes originate from phagocytic vesicles, but rapidly change their composition. They are modified to prevent the digestion of the symbiont by the host, and may also be modified for specialized functions related to the symbiosis. In some cases this modification may be a host response to recognition of the symbiont, and involves addition of extra, host-derived components to the symbosome membrane. The modifications may also include the addition of symbiont-derived components to the membrane. There is very little information about the contents of the symbosome space in any symbiosis.

Intracellular symbionts originally enter host cells by endocytosis (phagocytosis), but in many associations they are then passed from host cell to host cell as the association grows. Initial entry by phagocytosis implies that the symbosome is originally derived from the phagocytic vacuole. However, once the symbiosis is established, the amount of this membrane increases. For example, when symbionts divide, either the daughter cells remain within a single symbosome, or the symbosome also divides, keeping the number of symbionts per symbosome constant (Roth *et al.*, 1988). Either way, membrane must be added to the symbosome system to keep pace with increasing numbers of symbionts. When host cells divide, both daughter cells normally receive symbionts; since these always seem to be inside symbosomes, it is assumed that the symbosomes are also divided between the daughter cells, although there are no published studies of this process. It has been estimated that the area of the symbosome membrane is about $21,500\mu\text{m}^2/\text{nodule}$ cell in soy root nodules, where the surface area of the host cell's plasma membrane (from which it originates) is about $2,800\mu\text{m}^2/\text{cell}$ (Roth and Stacey, 1989). In addition, the formation of the infection thread in these nodule cells requires another $600\mu\text{m}^2$ of membrane (Roth and Stacey, 1989). Estimates of up to 100:1 have been made for the ratio of the area of symbosome membrane to the plasma membrane area in other legumes (Roth and Stacey, 1989).

2.1.1. Avoidance of host defenses

When particles are phagocytized, the plasmalemma of the phagocytic cell gives rise to the membrane of the phagosome. Once the particle has been internalized, lysosomes normally fuse with the phagosome membrane, releasing enzymes, which break down the contents of the phagosome. Intracellular pathogens and symbionts must avoid being destroyed by lysosomal hydrolyses, and indeed by other host defenses. Parasites that do this include the protists *Toxoplasma gondii* and *Leishmania* spp., and the bacteria *Brucella* spp., *Salmonella* spp., *Shigella* spp., *Mycobacterium tuberculosis*, *Chlamydia psittaci* and *Legionella pneumophila* (Hall and Joiner, 1991; Kim *et al.*, 1994; LeVier *et al.*, 2000; Viprey *et al.*, 1998). There seem to be several mechanisms for avoiding digestion. For example, microorganisms are often killed by toxic oxygen species generated by the respiratory burst that accompanies ligation of a phagocytic receptor. To avoid this, *Leishmania* spp. use a special receptor, which does not trigger a respiratory burst (Hall and Joiner, 1991). Within host cells, *Leishmania* spp. are enclosed in phagolysosomes that do contain acid hydrolases; however, the *Leishmania* survive these conditions and seem to be adapted to them, having a higher metabolic rate in acidic media (Hall and Joiner, 1991). Within the phagolysosome *Leishmania* secretes

a protease that degrades lysosomal hydrolases, and may also be protected from the hydrolases by a lipophosphoglycan in its surface coat (Hall and Joiner, 1991). Virulent stages of *Trypanosoma cruzi* disrupt the membrane of the phagolysosome within two hours of entering a phagocytic cell, and then enter the cytoplasm, thus escaping lysis (Hall and Joiner, 1991). *Toxoplasma gondii* reside within a specialized symbiosome that is deficient in host glycoproteins and does not fuse with lysosomes; however, if *T. gondii* are coated with antibody before infection, the vacuole acidifies and lysosomal fusion occurs normally (Hall and Joiner, 1991). Intracellular bacterial pathogens of mammals (e.g., *Salmonella*, *Shigella*) and of plants (e.g., *Erwinia*, *Pseudomonas*) secrete virulence proteins, encoded by a cluster of genes that comprise the bacterial type III secretion system, which establish pathogenesis during infection. LeVier *et al.* (2000) found that *Brucella abortus*, which lives in a modified phagosome in mammalian cells, requires the gene *bacA* for virulence; *Rhizobium meliloti* also requires this gene to establish itself in symbiosis.

A similar range of mechanisms for avoiding host defenses probably exists in mutualistic and commensal symbioses, but the processes by which symbionts are recognized and avoid digestion are largely unknown. However, there are some studies on *Amoeba proteus*, symbiotic *Chlorella* spp. in *Paramecium* and *Hydra*, and in symbioses between legumes and rhizobia, as outlined in the following paragraphs.

The *Amoeba proteus* symbiosis started when strain D, a laboratory culture of the amoeba, became infected with bacteria accidentally; although these bacteria were initially pathogenic, over several years the amoebae and the bacteria became dependent on each other (Jeon, 1992). The dependent strain of *A. proteus* (strain xD) has about 42,000 bacteria per cell, enclosed in symbiosomes. Normal D strain amoebae can be infected with freshly isolated bacteria in the laboratory; the bacteria are taken up by phagocytosis, but lysosome-phagosome fusion does not occur (Jeon, 1992). Lipopolysaccharides derived from the bacteria are incorporated into the symbiosome membrane, where they are located on the side facing the cytoplasm of the amoeba (Choi and Jeon, 1992). If antibodies to this lipopolysaccharide are injected into the amoeba, the inhibition of lysosomal fusion is removed (Jeon, 1992). Dead bacteria are taken up by the amoebae, but their presence does not prevent fusion of lysosomes with the phagosomes (Jeon, 1992). As well as the lipopolysaccharide, the symbiosome membrane contains a 96-kDa protein which is not found in any other component of the amoeba, but is present in the bacteria; this protein and the lipopolysaccharide can be detected in the symbiosome membrane within 7 days of infection, although they are not detectable 3 days after infection (Jeon, 1992; Kim *et al.*, 1994).

The green unicellular alga *Chlorella* is common as a symbiont in freshwater protists (e.g., the ciliate *Paramecium*) and invertebrates (e.g., sponges, *Hydra*). In both *Paramecium* (Karakashian and Rudzinska, 1981) and *Hydra* (Hohman *et al.*, 1982), when living *Chlorella* are phagocytized, lysosomes fail to fuse with the symbiosome. When heat-killed *Chlorella* are taken in, lysosomes do fuse with the phagosomes and the algae are digested. For example, when *Hydra* were fed either living or heat-killed *Chlorella*, only 3% of phagosomes containing live *Chlorella* showed acid phosphatase activity 1 h and 5 h after feeding. After 1h, 50% of phagosomes containing only dead *Chlorella* showed acid phosphatase activity, and after 5h this figure reached 75%. In addition, electron microscopy was used to show that heat-killed *Chlorella* were digested after phagocytosis, while live ones persisted and showed no signs of digestion (Hohman *et al.*, 1982). The signals that mediate these reactions are not known; however.

lysosomes did fuse with phagosomes containing *Chlorella* that had been pretreated with the polycationic compound polylysine, and these algae did not persist in the host cell. Polyanionic polypeptides did not prevent the algae from persisting in the host cells (Hohman *et al.*, 1982). Polycations may mask surface features of the algae that are involved in the prevention of lysosome fusion, or they may alter the production of a metabolite that controls phagosome-lysosome fusion (Hohman *et al.*, 1982). Similarly, if the algae were treated with DCMU before being fed to the *Hydra* and the *Hydra* were kept in the dark after feeding to prevent photosynthesis, approximately 50% disappeared from the host cells within 24 h. In controls in which the algae were photosynthesizing the numbers in the host cells were approximately constant over 24h (Hohman *et al.*, 1982).

During the establishment of symbioses between legumes and rhizobia, the bacteria secrete a number of proteins which are essential for effective nodulation (Brewin, 1991; Viprey *et al.*, 1998). In some rhizobia, these include products of the type III secretion system, which are probably involved in lowering host defenses, as they do in pathogenic bacteria. Thus these proteins may play a role in determining host specificity (Viprey *et al.*, 1998). Given that the type III secretion system plays a role in infections by intracellular pathogens of animals and plants and by rhizobia, it may also turn out to be important in other associations of prokaryotes with eukaryotes. A number of plant proteins, called nodulins, which are produced or up-regulated in response to infection with rhizobia, also help control the infection process and the differentiation of effective, nitrogen-fixing root nodules (Brewin, 1991; Whitehead and Day, 1997).

2.1.2. Sources of the symbiosome membrane

Both free-living and symbiotic dinoflagellates are bounded by an amphiesma that consists of four or more layers of membrane (Rands *et al.*, 1993; Taylor, 1968; Trautman *et al.*, submitted; Trench and Blank, 1987; Wakefield *et al.* 2000) and may also include cellulose and sporopollenin (Markell *et al.*, 1992; Morrill and Loeblich, 1981; Taylor, 1968); the number of layers may vary markedly, even within a single species. In intracellular symbioses between dinoflagellates and invertebrates, electron microscopy suggests the presence of a symbiosome membrane and a symbiosome space around the symbionts (Gates and Muscatine, 1992; Rands *et al.*, 1993). Because of the multiple membranes of the amphiesma, it has been difficult to be certain whether there really is a host-derived membrane present around the algae in the host cell. Rands *et al.* (1993) proposed that there were several layers of symbiosome membranes around the algae in the anemone *Anemonia viridis*. However, Wakefield and Kempf (1999), used antibodies to symbiosome membrane proteins to show that, although there are multiple layers of membrane around the zooxanthellae in the anemone *Aiptasia pallida*, only the outermost layer is of host origin. It has been proposed that the reason for the variation in the number of layers of membrane around symbiotic zooxanthellae in their hosts is that, like free-living dinoflagellates, they may shed their thecae at intervals (Wakefield *et al.*, 2000). The nature of the amphiesma also means that it is impossible to determine whether zooxanthellae that have been isolated from their hosts still have a symbiosome around them simply by counting the membranes. Recently we have used fluorescent probes to stain the symbiosome membrane in three species of Cnidaria with zooxanthellae in their endoderm cells (Trautman *et al.*, submitted). We have been able to stain the symbiosome membrane specifically, with a dye, which does not stain the cytoplasm, plasma membranes or other membranes of either the host endoderm cell or

the algae. The plasma membranes of zooxanthellae that have been cultured, free of the host, for some generations, and are, therefore, free of symbiosomes, do not stain either. In suspensions of zooxanthellae freshly isolated from their hosts, the surfaces of most cells stain, but when the algae are stripped of all host material by repeated passage through a hypodermic needle, staining is abolished. Algae stripped in this way still photosynthesize normally. These results indicate that the dye used is specific for the symbiosome membrane. Since nothing except the symbiosome membrane stains in either the host cells or the symbiotic algae, it appears that the host membrane(s) are modified during formation of the symbiosome, and that the symbiosome membrane has at least one unique component. This technique will allow us to compare the physiology of isolated zooxanthellae with and without symbiosomes, as well as isolating samples of the symbiosome membranes themselves. For the first time, it will be possible to study the role of the symbiosome in the physiology of zooxanthellae.

The soil fungus *Geosiphon pyriforme* is the only true fungus known to have intracellular symbiotic cyanobacteria; it forms a specific association with *Nostoc punctiforme*. The symbiosomes in the fungal cells consist of a symbiosome membrane and a symbiosome space (Schüssler *et al.*, 1996). Electron microscopy showed the presence of a dark-staining amorphous layer between the symbiosome membrane and the walls of the cyanobacteria. This layer does not look the same as the slime layer of free-living *Nostoc* when fixed chemically for electron microscopy, but it does give a slight staining response with the periodic acid-thiocarbohydrazide-silver proteinate method for visualizing polysaccharides (Schüssler *et al.*, 1996). It appears to contain chitin but no detectable cell wall components derived from the *Nostoc* (Schüssler *et al.*, 1996). It is believed that the symbiosome membrane is derived from the fungal plasmalemma, which invaginates to take up the symbionts. This was confirmed by labelling with lectins. The results suggested that the symbiosome contains chitin, sialic acid, and mannose and/or glucose, which are also found in the hyphal wall of the fungus; but it lacks other major hyphal wall components, including N-acetyl galactosamine, galactose and fucose (Schüssler *et al.*, 1996). The cyanobacteria did not label with any of the lectins used, and so presumably the fungus was the source of all these components of the symbiosome. These data also suggest that the symbiosome membrane is modified; it incorporates fewer components of the cell wall of the fungus than the plasma membrane does (Schüssler *et al.*, 1996).

Plants may show a response to infection that resembles that of *G. pyriforme*. Many plants deposit cell wall components in the symbiosome space when infected with intracellular microorganisms or with biotrophic fungi (Parniske, 2000; Schüssler *et al.*, 1996). In arbuscular mycorrhizae there are no fungal compounds in the symbiosome (Schüssler *et al.*, 1996). Taken together, these data suggest that in plant-fungus associations, only the host can lay down cell wall material in the symbiosome, whether it is the plant or the fungus which is the host (Schüssler *et al.*, 1996).

The only case in which there have been detailed studies of the development of the symbiosome, and of the genes involved in it, are the nitrogen fixing associations of rhizobia with legume root nodules (reviewed by Brewin, 1991). Briefly, rhizobia enter nodule cells via an infection thread, formed by invagination and extension of the plasma membrane. The bacteria are then released into symbiosomes in the host cytoplasm. Golgi and endoplasmic reticulum vesicles are abundant near the end of the infection thread, where bacteria move from the thread into symbiosomes (Parniske, 2000; Roth and Stacey, 1989). While the bacteria are still enclosed within the infection thread they

lose their polysaccharide capsules and the ends of the symbiosome membranes form, apparently *de novo* rather than from vesicles or from the infection thread, close to their outer membranes (Roth and Stacey, 1989). Addition of membrane from the infection thread, the endoplasmic reticulum and the Golgi apparatus completes the formation of the symbiosome membranes (Roth and Stacey, 1989; Whitehead and Day, 1997). Phosphotungstic acid-chromic acid (PACA) stains plasma membrane but not endoplasmic reticulum, nuclear envelope or other internal membranes of plant cells. In nodules, PACA stains parts of both newly forming and mature symbiosome membranes, suggesting that some parts are derived from plasma membrane and some from other sources. Parts of the bacteroid outer membranes also stain with PACA. Thus at all stages of its development the symbiosome membrane consists of a mosaic of areas derived from the infection thread/plasma membrane, the Golgi and the endoplasmic reticulum vesicles, and probably of newly synthesized membrane as well (Parniske, 2000; Roth and Stacey, 1989). In the area where bacteroids are released from the infection thread into symbiosomes, lysosomal vesicles form and break down the walls and membranes of the infection thread; some of the lysosomes fuse with the symbiosomes (Parniske, 2000; Roth and Stacey, 1989). Subsequent work has shown that, while most of the proteins in the pea symbiosome membrane are of plant origin, at least one is synthesized by the *Rhizobium* bacteroids (Simonsen and Rosendahl, 1999). The fact that some plant-synthesized proteins are also found in the bacteroid membranes (Simonsen and Rosendahl, 1999) may account for the observation that parts of the bacteroid outer membrane stain with PACA (Roth and Stacey, 1989). Unlike symbiosomes in plant-fungus associations, legume symbiosomes have neither plant nor symbiont (bacterial) cell wall material in the symbiosome space (Brewin, 1991).

The symbiosomes of legumes pass through four developmental phases – initiation of the symbiosome, proliferation, maturity (during which the nodules fix nitrogen) and degradation, when the nodule senesces (Brewin, 1991; Udvardi and Day, 1997; Whitehead and Day, 1997). Structure, composition and function of the symbiosomes all change with phase of development. This developmental sequence can be disrupted by mutations, in either the bacteria or the legume host, which disrupt signal pathways or interfere directly with membrane synthesis (reviewed by Whitehead and Day, 1997). During degradation the bacteroids lyse, and this may happen earlier in development if they are prevented from fixing nitrogen; this supports the idea (reviewed by Brewin, 1991) that the symbiosome is a modified lysosomal compartment in which lysis of the bacteroids can occur if the pH drops low enough. While the bacteroids are fixing nitrogen, they counteract the tendency of the ATPase to acidify the symbiosome space, by their uptake of malate and succinate and by the release of ammonia (Brewin, 1991).

The pattern of proteins in legume symbiosome membranes changes during development of the nodule, and these changes appear to be controlled by signals from the rhizobia (Udvardi *et al.*, 1991). The pattern of lipids also changes during development (Hernandez and Cooke, 1996). In pea nodules, the total phospholipid content is similar in all the membranes, but the ratio of phosphatidylcholine to phosphatidylethanolamine is about 2:1 in the symbiosomes, the symbiosome membranes and the plant microsomal fraction, but about 1:2 in the plant plasma membrane. The overall sterol composition (and to some degree the fatty acid content) of the symbiosome membrane is similar to that of the host microsomal fraction, rather than to that of the host plasma membrane (Hernandez and Cooke, 1996). Comparisons with free-living bacteria suggest that the bacteroids do not contribute to the lipid content

of the symbiosome membrane (Hernandez and Cooke, 1996). The differences found suggest that the symbiosome membrane in pea and lupin nodules is probably more fluid than that of the host plasma membranes, which may aid in symbiosome development and alter the activities of membrane proteins (Hernandez and Cooke, 1996; Whitehead and Day, 1997).

The examples given above suggest that, in a wide range of associations, the symbiosome membrane commonly shares some properties of both the host plasma membrane and other host membrane systems, but often incorporates symbiont-derived material as well. Thus it is clearly differentiated from all other membrane systems of the host cell. These data, and the symbionts' ability to avoid host defenses, clearly show that symbiosomes are not simply non-lytic phagosomes or extensions of the plasma membrane.

2.2 FUNCTIONS OF SYMBIOSOMES

As indicated above, the symbiosome membrane is unique in its composition, and in some, if not all, cases is a mosaic of host membranes of various types, sometimes with symbiont-derived components as well. What is known of the activities of the components of symbiosome membranes?

ATPases have been demonstrated in the symbiosome membranes of a number of associations, but only legume symbiosomes have been studied in detail. It is interesting that among fungal-plant associations, the symbiosome membranes in mutualistic symbioses have ATPases, but those in parasitic associations do not (Smith and Smith, 1990). The lack of a symbiosome ATPase may be a mechanism for ensuring that the host cannot reabsorb nutrients from the host-parasite interface (Smith and Smith, 1990). In mycorrhizae and various intracellular symbiotic associations, the occurrence of ATPases on both host (symbiosome) and symbiont membrane is consistent with the bi-directional transport that is characteristic of mutualistic intracellular symbioses.

2.2.1. *Symbiosomes in legume-rhizobial associations*

In legume nodule cells, the symbiosomes have active **H⁺-ATPases**, which are associated with transport systems (Day *et al.*, 1989). Their activity leads to accumulation of protons in the symbiosome space (Smith and Smith, 1990; Whitehead *et al.*, 1998a) and the establishment of a membrane potential (positive inside) (Whitehead and Day, 1997). The symbiosome ATPases are similar to the P-type ATPases of plasma membranes, which have a low optimal pH, are stimulated by cations including NH₄⁺ and inhibited by vanadate (Christiansen *et al.*, 1995; Rojas-Ojeda *et al.*, 1998; Whitehead and Day, 1997). Soybean symbiosomes also have pyrophosphatase and protein kinase activity (Whitehead and Day, 1997). The calcium-dependent protein kinases in symbiosome membranes are involved in the regulation of channel activity (Lee *et al.*, 1995; Weaver *et al.*, 1991) and may have other regulatory roles.

Symbiosomes clearly have a role in the exchange of nutrients between symbiont and host, and they could be the main site of regulation of these exchanges (Price *et al.*, 1987; Udvardi and Day, 1997; Whitehead and Day, 1997). In legumes, the plant supplies organic carbon to the bacteroids, and the bacteroids release fixed nitrogen, mainly or entirely in the form of ammonia. While symbiosomes from various species of legume can take up many compounds, they all take up dicarboxylic acids, particularly succinate and malate, most rapidly (Day *et al.*, 1989; Price *et al.*, 1987; Whitehead and

Day, 1997). There is a dicarboxylate transporter in the symbiosome membrane (Price *et al.*, 1987; Udvardi and Day, 1997; Whitehead and Day, 1997), and the rates of uptake are consistent with the measured rates of nitrogen fixation (Udvardi and Day, 1997; Whitehead and Day, 1997). Fixed nitrogen leaves the bacteroid as ammonia but is protonated in the symbiosome space, which is acidic (Whitehead and Day, 1997). In symbiosis, the ammonium transporter of the bacteroids is repressed, so they cannot take up the resulting NH_4^+ . The symbiosome membrane has a voltage-gated cation channel which transports NH_4^+ (and other monovalent cations) from the symbiosome to the plant cytosol (Whitehead *et al.*, 1998a). As this channel is inwardly rectified, NH_4^+ cannot move back into the symbiosome (Whitehead *et al.*, 1998a). The relative activities of the proton pumps and other transporters regulate the pH of the symbiosome space (Whitehead and Day, 1997; Whitehead *et al.*, 1998a), which itself affects the rate of nitrogen fixation and the viability of the bacteroids (Brewin, 1991). Several mechanisms, by which amino acids could be supplied to the host directly by the bacteroids, or dicarboxylic acids could be exchanged for amino acids from the bacteroids, or organic nitrogen compounds could be supplied to immature bacteria, have been proposed (Udvardi and Day, 1997; Whitehead and Day, 1997; Whitehead *et al.*, 1998b). While isolated bacteroids have the necessary amino acid transporters, such transporters have not been found on the symbiosome membranes of legumes; movement of amino acids across the symbiosome membrane occurs extremely slowly by diffusion (Udvardi and Day, 1997; Whitehead *et al.*, 1998b). Nevertheless, diffusion of amino acids through the symbiosome membrane may be sufficient to supply supplementary organic nitrogen to the bacteroids, since they have amino acid transporters with high affinities for several amino acids (Whitehead *et al.*, 1998b). This may be particularly important during differentiation of the bacteroids, before they start to fix nitrogen (Udvardi and Day, 1997).

The symbiosis-specific protein, Nodulin 26, is located in the symbiosome membrane. Its sequence is similar to several intrinsic membrane proteins that form ion or water channels (Lee *et al.*, 1995; Weaver *et al.*, 1990; Whitehead and Day, 1997). In artificial membranes Nodulin 26 forms channels which are slightly more permeable to cations than to anions (Weaver *et al.*, 1994) and which act as both water and glycerol channels (Dean *et al.*, 1999). *In hospite* this protein may be involved in osmoregulation of the symbiosome (Whitehead and Day, 1997). Several other nodulins appear to be targeted to the symbiosome membrane (Whitehead and Day, 1997; Winzer *et al.*, 1999).

There is also evidence of other channels and carriers on legume symbiosome membranes. For example, bacteroids require phosphate, which probably uses an anion transporter that also allows a number of other anions to enter the symbiosome (Udvardi and Day, 1997). There is probably a Ca^{2+} transporter, as the symbiosome space contains considerable amounts of Ca^{2+} (Andreev *et al.*, 1999). The bacteroids have a large requirement for iron, for the synthesis of nitrogenase and cytochromes. Iron enters the symbiosome as ferric citrate (and possibly also as ferrous ions); much is stored in the symbiosome space, bound to bacterial siderophores (Moreau *et al.*, 1995; Udvardi and Day, 1997). Isolated symbiosomes from soybean have a transporter that appears to direct the plant hormone indole-3-acetic acid (IAA) towards the bacteroids (Rosendahl and Jochimsen, 1995). IAA has an as yet undefined role in the development of root nodules (Rosendahl and Jochimsen, 1995).

2.2.2. Symbiosomes in algal-invertebrate associations

Rands *et al.* (1993) used electron microscopy and cytochemistry to investigate the symbiosome of the anemone *Anemonia viridis*. They showed that both the symbiosome membranes and the cell membranes of the symbiotic alga, *Symbiodinium* sp., have ATPase activity. The ATPase on the algal membranes is less sensitive to the external concentration of Mg²⁺ than the one on the symbiosomes. This may be due either to the ATPases having different properties, or to the availability of endogenous Mg²⁺ in the algal cells (Rands *et al.*, 1993). In this association, the symbiosome also contains at least two types of phosphatase, one apparently derived from the algal cells and one from the host cells. The algal cytoplasm and membranes, and the symbiosome membranes, have β -glycerophosphate-dependent phosphatase activity. The host cell cytoplasm, and the symbiosomes, have an α -naphthylphosphate-dependent phosphatase activity; this is absent from the symbiotic algae (Rands *et al.*, 1993). The symbiosome spaces are not markedly acidic in either the *A. viridis/Symbiodinium* association (Rands *et al.*, 1993) or the *Hydra/Chlorella* symbiosis (Rands *et al.*, 1992). The presence of the ATPase in the algal and symbiosome membranes of *Anemonia viridis* shows that both these membranes are capable of active transport, and thus of controlling fluxes of nutrients into the algae (Rands *et al.*, 1993) and also from the algae to the host. The presence of the phosphatases in the algal and symbiosome membranes in the same association has important implications for understanding the control of fluxes of inorganic phosphorus to symbiotic algae. It had earlier been proposed that the fluxes of inorganic nutrients from the host's endoderm cells into the algae are controlled simply by the rate at which they are used by the algae, and the resulting concentration gradient favoring their movement into the algae (the diffusion-depletion hypothesis - D'Elia, 1977; D'Elia *et al.*, 1983). In contrast, Jackson *et al.* (1989) and Jackson and Yellowlees (1990) isolated phosphatases from the symbiotic algae of the coral *Acropora formosa*; they proposed that phosphate esters from the host cell enter the symbiosome, where they are hydrolyzed by phosphatases released into the symbiosome space by the algae. The phosphate ions produced by hydrolysis could then enter the algae, perhaps with the aid of a phosphate carrier (Jackson and Yellowlees, 1990). Rands *et al.* (1993) argued that their results support the hypothesis of Jackson and Yellowlees (1990) and refute the diffusion-depletion hypothesis. They also argued that their results imply that the symbiosome membrane is impermeable to phosphate ions. The phosphatases and phosphate carrier in the symbiotic algae of *A. formosa* are inhibited by phosphate at the concentrations normally found in cnidarian cells (Jackson *et al.*, 1989; Jackson and Yellowlees, 1990), and hence could not be detected cytochemically if the symbiosome membrane were freely permeable to phosphate (Rands *et al.*, 1993). This argument relies on the assumption that the enzymes and carriers are similar in *Symbiodinium* from *Acropora formosa* and those from *Anemonia viridis*. The diffusion-depletion hypothesis for phosphate requires that the symbiosome membrane be permeable to phosphate. The other crucial nutrient for symbiotic algae is nitrogen, and the pH of the symbiosome space is potentially very important in controlling the movement of ammonia from host cell to alga. The cytochemical method used by Rands *et al.* (1992, 1993) shows that the symbiosome space is not strongly acidic, but does not rule out mild acidity. Ammonia can diffuse through biological membranes, but ammonium ions cannot. If the symbiosome space is more acidic than the host cytoplasm, ammonia may diffuse into it, be converted to ammonium ions, and be trapped within the symbiosome (Rands *et al.*,

1993). From there it could be actively transported across the algal cell membrane. The importance of this mechanism will depend on the pH of the symbiosome space.

2.2.3. Symbiosomes of Amoeba proteus

In the *Amoeba proteus*-bacterial symbiosis, actin produced by the amoeba accumulates in the symbiosome and is attached to the surfaces of the bacteria; however, its function is not known (Jeon, 1992). The bacteria synthesize and release a 29-kD protein, which is exported to the host's cytoplasm through the symbiosome membrane (Pak and Jeon, 1996). This protein appears to be necessary for the survival of infected *A. proteus* (Park and Jeon, 1990).

In summary, the functions so far demonstrated for symbiosomes include protection of the symbiont from host defenses; the control of nutrient fluxes between host and symbiont and *vice versa*; the regulation of the symbiont's environment, particularly of pH and electrochemical gradients; and the storage of iron and calcium. Other proposed functions are in the recognition processes which ensure the maintenance of specificity in symbiosis, the control of other aspects of the environment of the symbiont (e.g., osmolarity, ionic composition, oxygen content), regulation of hormone concentrations and the storage of other inorganic or organic compounds.

3. Future Studies

Clearly in legume root nodules, the symbiosome plays several vital roles in the metabolism and regulation of the association. Based on the small amount of work done on them to date, similar roles seem likely in symbioses between invertebrates and dinoflagellates and, by inference, in other symbioses. Nothing is known of the signals, which pass between the symbionts in most associations, including algal-invertebrate symbioses, in the course of establishing the association. In legume nodules, there is a very complex set of signals involved in both the development of the nodules and the differentiation of the bacteroids. These suggest a long period of co-evolution. However, even within the legume species which can be infected with rhizobia, there is some diversity in the composition (and, therefore, probably in the function) of the symbiosome membrane. There are bound to be major differences among symbiosomes when the whole range of intracellular symbiotic associations is considered.

In order to understand intracellular symbioses properly, it is essential to understand the environment in which the symbiont actually lives – that is, the environment within the symbiosome. To do this, we need to understand its composition in detail, as well as the functions of the various enzymes and transporters in the symbiosome membrane. It will also be of great interest to compare the signaling systems and the degrees of genetic integration between the symbionts in a range of associations.

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THE ABSENCE OF NITROGEN-FIXING ORGANELLES DUE TO TIMING OF THE NITROGEN CRISIS

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1. Introduction

In many ecosystems, as well as for many individual plants, nitrogen is the limiting nutrient (Vitousek *et al.*, 1977; Falkowski, 1997; Tyrrell, 1999). This may be surprising because 80% of the atmosphere is composed of N_2 . However, to be useable by plants nitrogen must be "fixed" into either nitrate or ammonia. Nitrogen fixation is an energetically expensive process requiring about 16 moles of ATP per mole of N_2 fixed (Lehninger, 1975; Phillips, 1980). Currently only certain prokaryotic organisms are capable of performing nitrogen fixation and they do so with a group of closely related enzymes, nitrogenase, genetically encoded with a small set of genes known as the *nif* genes (Postgate, 1987).

Given that nitrogen fixation is performed only by prokaryotes at high metabolic costs and that many, if not most, plants are limited in their growth rate by the availability of fixed nitrogen, then it would seem plausible that nitrogen-fixing bacteria would be incorporated in the eukaryotic cell as organelles. This would allow nitrogen fixation to have direct access to the energy supplies of the cell in exchange for providing fixed nitrogen to the cell. However, there are no nitrogen-fixing organelles. Instead plants have evolved numerous other methods of obtaining fixed nitrogen. These include the development of specialized symbioses with free-living bacteria (Postgate, 1987), root nodules in which nitrogen-fixing bacteria are incorporated as intracellular endosymbionts (Postgate, 1987; Szczyglowski *et al.*, 1998), and the presence of endophytic nitrogen-fixing bacteria in plant tissues (Stoltzfus *et al.*, 1997; Chaintreuil *et al.*, 2000). Remarkably, some plants have developed mechanical systems that trap small insects as a source of nitrogen (Chandler and Andersen, 1976; Gutschick, 1981; Karlsson and Carlsson, 1984; Hanslin and Karlsson, 1996; Schulze *et al.*, 1997). Indeed the famous Venus flytrap, *Diana muscipula*, is thought to have developed its elaborate fly-catching mechanism as a means of obtaining fixed nitrogen. If nitrogen is so universally in shortage and plants have apparently spent considerable evolutionary and metabolic effort to acquire nitrogen, it is curious that nitrogen-fixing organelles do not exist.

The question of nitrogen-fixing organelles is relevant to studies of microbial evolution and to the economic issues of plant nutrition. Along these lines, Postgate

(1977) suggested the artificial development of nitrogen-fixing organelles in plants as a way of boosting plant productivity.

In this paper we consider the puzzle of the absence of nitrogen-fixing organelles in light of phylogenetic relationships recently determined for the *nif* genes (Young, 1992; Fani *et al.*, 2000) and models for abiotic sources of nitrogen on the early Earth (Navarro-González *et al.*, 2001). We propose that nitrogen fixation arose after the period of organelle development that resulted in mitochondria and chloroplasts. By the time nitrogen-fixing bacteria were present, organelle formation was no longer possible.

2. The Lack of Nitrogen-Fixing Organelles

A possible explanation for the lack of nitrogen-fixing organelles is some fundamental incompatibility that prevented nitrogen-fixing bacteria from becoming organelles, even if they were present at the time for the formation of the eukaryotic cell. Allen and Raven (1996) have summarized the arguments against this position, the most compelling of which seems to be that mitochondria and plastid organelles developed from ancestors that were closely related phylogenetically to nitrogen-fixing strains.

As another possible explanation for the absence of nitrogen-fixing organelles, it has been pointed out (Smith and Douglas, 1987; Douglas 1994) that if the need for nitrogen fixation arose after plants were already large and morphologically complex, then the natural location for the bacterial association would be in parts of the plants (e.g., roots) far from the germ cells. In this case, the formation of true organelles that are inherited directly would be difficult.

Allen and Raven (1996) have proposed a genetic explanation for the absence of N₂ fixing organelles. They point out that the formation of organelles results when most of the genes of the endosymbiont bacteria are transferred to the host cell. They posit that the formation of oxidative radicals associated with the energetic processes of the organelle functions cause high mutation rates in the organelle genome. Any genes transferred from the organelle to the host cell benefit from a reduction in mutation rate. Only the genes directly related to the energy transduction processes of the organelle (and the genes needed for gene expression) would be retained in the organelle genome. They argue that since low O₂ levels, and hence low levels of oxidative radicals, must be maintained for nitrogen fixation, then high mutation rates would not be a problem and thus the impetus for gene transfer from any endosymbiont N₂ fixing bacteria to the host cell would be minimal and the endosymbiont would not develop into an organelle.

3. Was Nitrogen Fixation Too Late for Eukaryogenesis?

It is widely thought that the eukaryotic cell arose by the endosymbiotic association of prokaryotes (Margulis, 1970, 1981). It is not clearly known when these endosymbiotic events occurred and over how long a period. The paleontological record suggests that the origin of the eukaryotic cell occurred earlier than 1.5 Gyr ago and maybe before 2.1 Gyr ago (Chela-Flores, 1998; Knoll, 1992) but Brocks *et al.*, (1999) reported the presence of steranes -- a compound associated with eukaryotes -- in sediments that are 2.7 Gyr old. There are arguments that favor the main endosymbiotic events occurring

soon after the divergence of the eukaryotic lineage (Katz, 1999). There is clear evidence of gene transfer between eukaryotes, bacteria, and Archaea (Zillig, 1991; Golding and Gupta, 1995; Brown and Doolittle, 1997) and this may have continued over extended periods of time depending on the nature of the transfer (Katz, 1999). However, it may be that there was only one point at which endosymbiosis leading to organelles occurred and there is no indication of more than one origin of the eukaryotic cell type; the mitochondria derive from a single lineage within the alpha subdivision of the Proteobacteria (Gray *et al.*, 1999). Thus, it might be argued that at some point in Earth history a prokaryote became capable of incorporating some of its fellow prokaryotes as organelles and did so to construct the eukaryotic cell and that, for whatever reason, the opportunity for such a union has not arisen again.

Thus the organelles in the eukaryotic cell would be limited by the capabilities that were available in free-living bacteria at the time. Metabolic functions that arose later that were of need to the eukaryotes would have to be incorporated in other, possibly *ad hoc*, ways.

The timing of the origin of nitrogen fixation in prokaryotes is uncertain. Recent phylogenetic studies indicate that nitrogen fixation is widespread among both bacteria and Archaea. One explanation for this is that nitrogen fixation was present with the common ancestor (Young, 1992; Fani *et al.*, 2000). Nitrogen isotope ratios in ancient materials are also consistent with an early origin (before 3.5 Gyr ago) for biological nitrogen fixation (Beaumont and Robert, 1999). The presence of heterocysts in 1.5 Gyr old fossils (Golubic *et al.*, 1995) suggests nitrogen fixation may have predated the rise of higher plants. The sensitivity of the enzyme to O₂ is a possible indication that it arose before atmospheric oxygen began to rise 2.2 Gyr ago (Kasting, 1993). While all indicating an early development of nitrogen fixation, none of the observations just listed provides conclusive evidence of the timing of nitrogen fixation.

Several authors have considered when and under what conditions biological nitrogen fixation arose. Because it is energetically expensive it is clear that biological nitrogen fixation would have only been developed in response to a shortage of available fixed nitrogen. There have been three suggested possibilities for what caused this biological crisis in nitrogen availability. Many authors (Cloud, 1968; Walker, 1977; Raven and Yin, 1998) have argued that immediately after the origin of life, organisms would have depleted any initial reservoir of fixed nitrogen and abiotic organic material, and thereby faced a shortage of organic energy and fixed nitrogen. In this view, the crisis that prompted the development of biological nitrogen fixation followed soon after the origin of life. Toward the other extreme, Mancinelli and McKay (1988) suggested that only with the development of higher plants on land did the net primary productivity increase to such an extent that abiotic sources of fixed nitrogen were inadequate to meet the biological demand. In this case the origin of biological nitrogen fixation came well after the origin of the eukaryotic cell and after the origin of higher plants. Navarro-González *et al.* (2001) have pointed out that even if the early biosphere was able to survive on abiotic sources of fixed nitrogen after the origin of life, these sources probably declined significantly due to the reduction in atmospheric CO₂ with time. The rate of fixation of nitrogen by lightning in atmospheres of CO₂ and N₂ decreases steeply when the CO₂ mixing ratio falls below 0.3 (Navarro-González *et al.*, 2001). This reduction in abiotic nitrogen fixation would have created a demand for biological fixation. In this case the development of biological nitrogen fixation occurred well after the origin of life, but

well before the origin of higher plants. Figure 1 shows a timeline with the key events in evolution and the timing of the three possible crises in the availability of fixed nitrogen.

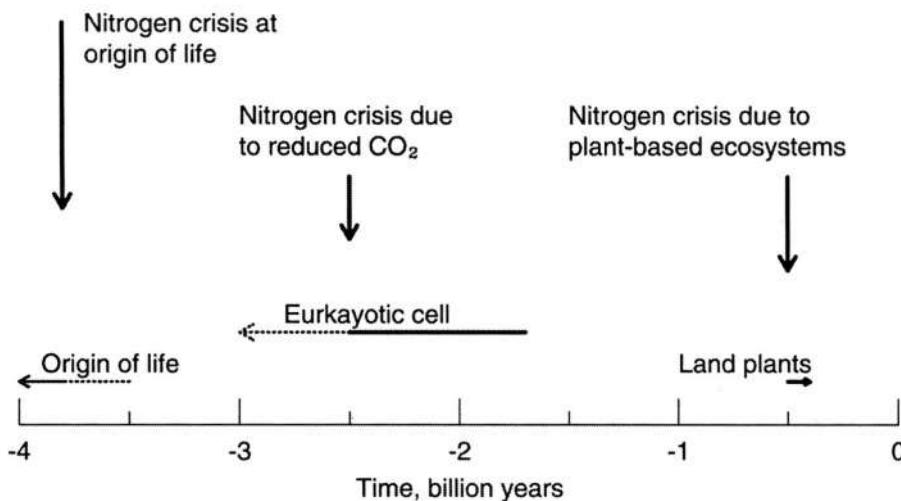


Figure 1. Some key events in the history of life compared to the three different proposals for the origin of biological nitrogen fixation. If the crisis that prompted the development of biological nitrogen fixation occurred after the origin of the eukaryotic cell, this could explain why there are no nitrogen-fixing organelles.

Of the three proposals for the timing of the origin of biological nitrogen fixation the latter two have the possibility that the eukaryotic cell had already reached its evolutionary maturity before the advent of microorganisms specializing in nitrogen fixation. Clearly, if nitrogen fixation arose only with the development of higher plant ecosystems then eukaryogenesis was over at that time. If nitrogen fixation arose in response to declining CO₂ levels then the timing of this development relative to the origin of eukaryotes is not clear since the precise dates for both of these events are uncertain. However, based on the CO₂ paleosol measurements of Rye *et al.* (1995) it can be inferred that the nitrogen crisis suggested by Navarro-González *et al.* (2001) would have occurred about 2.2 Gyr ago. While there are uncertainties in the timing of the origin of the eukaryotic cell, the biomarker data place this much earlier than 2.2 Gyr ago and thus before the origin of nitrogen fixation as postulated by Navarro-González *et al.* (2001).

4. Other Endosymbioses

Another indication arguing for the difficulty of recent organelle formation is the presence of symbiotic methane and sulfur bacteria in marine invertebrates. The tubeworms at the deep-sea vents, *Riftia pachyptila*, derive their energy from the oxidation of H₂S by intracellular bacteria (Cavanaugh *et al.*, 1981; Childress and Fisher, 1992; Fisher *et al.*, 1988). There are similarly other types of marine invertebrates with intracellular chemoautotrophic and methanotrophic symbionts (Fisher, 1990; 1996; 1998). While the presence of the bacteria is essential to the animal, these symbionts are incorporated from free-living populations and have not co-evolved with their hosts

(Feldman *et al.*, 1997; Lane and Nelson, 1997; Millikan *et al.* 1999). Clearly, these associations, which are similar to the uptake of nitrogen-fixing bacteria as endosymbionts, arose after the origin of the eukaryotic cell and after the development of tissue multicellularity. Although once incorporated within the host cell the endosymbionts degenerate morphologically and genetically, there is no organelle formation apparent.

An extreme example that illustrates the barriers for modern organelle development from endosymbionts is the case of *Buchnera*, an endosymbiont that provides essential amino acids to its aphid host (Douglas, 1997; Andersson, 2000). The symbiosis is mutually obligatory and appears to have been co-evolutionary for at least 200 Myr. There has been extensive transfer of essential genes from the bacteria to the host cell (Douglas, 1997; Andersson, 2000) which would appear to suggest a case of an organelle in formation (Andersson, 2000). However, this pseudo-organelle, unlike mitochondria and chloroplasts, is not present in every cell of the host organism and it is passed to offspring in special tissues.

5. Conclusion

The absence of organelles that perform nitrogen fixation is puzzling given the widespread need for fixed nitrogen and the evolutionary and metabolic cost to plants of acquiring fixed nitrogen. Nitrogen fixation has been incorporated into eukaryotes via symbiotic, endosymbiotic, and endophytic bacteria but not as true organelles like mitochondria and chloroplasts. We have considered possible explanations for the absence of nitrogen-fixing organelles in eukaryotes. We suggest that the absence of nitrogen-fixing organelles in eukaryotes resulted from the relative timing of the origin of the eukaryotic cell and the origin of biological nitrogen fixation; that is microorganisms that could fix nitrogen were not present at the time of eukaryogenesis. Similarly, methane and sulfur endosymbiotic bacteria in marine invertebrates are also not incorporated as organelles, further supporting the hypothesis that organelle incorporation occurred readily only in the initial states of the formation of the eukaryotic cell. Even in the endosymbiont *Buchnera*, which has many of the genetic features of a true organelle of the aphid host cells, is still restricted to only a certain tissues of the host indicating that organelle development does not occur in differentiated multicellular organisms. One implication of this suggestion is the possibility of inducing endosymbiosis and thereby creating a true endosymbiotic nitrogen-fixing organelle may be an alternative to gene insertion for developing nitrogen fixation capabilities in higher plants (Postgate, 1977).

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NUCLEUS SYMBIOSIS HYPOTHESIS: *Formation of Eukaryotic Cell Nuclei by the Symbiosis of Archaea in Bacteria*

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1. Introduction: The Eukaryotic Cells

The appearance of prokaryotic cells took only less than one billion years after the formation of the Earth. However, the appearance of eukaryotic cells required more than two billion years (Han and Runnegar, 1992; Knoll, 1992; Feng *et al.*, 1997; Vellai and Vida, 1999). The three groups, archaeabacteria (Archaea), eubacteria (Bacteria) and Eukarya, now comprise all living things (Woese and Fox, 1977; Woese *et al.*, 1990; Brown and Doolittle, 1997; Ribeiro and Golding, 1998; Rivera *et al.* 1998). The following are the main advantages of eukaryotic cells over prokaryotic cells. They have a much larger genome as well as cell size, which allows for more complex structures with various characteristics; polyploidicity of their chromosomes, which protects their DNA functions against damage by UV light and natural negative mutations, and also resistance to several antibiotics (Amils *et al.*, 1989). The appearance of eukaryotic cells with these characteristics can be considered an essential key step in the subsequent evolution of life.

2. Mechanism of the Formation of Eukaryotic Cells

Much work has been carried out on this mechanism (Carlile, 1982; Iwabe *et al.*, 1989; Langer *et al.*, 1995; Brown and Doolittle, 1997; Vellai *et al.*, 1998; Doolittle, 1998; Martin and Müller, 1998; Gupta, 1998; Vellai and Vida, 1999; Philippe and Forterre, 1999; Doolittle, 1999; Martin, 1999; Andrade *et al.*, 1999; Gupta, 2000). Phylogenetic tree analyses, including analyses of the 16S rRNA, have revealed the chimeric genome structure of eukaryotic cells originating from Archaea and Bacteria (Woese and Fox, 1977; Iwabe *et al.*, 1989; Woese *et al.*, 1990; Gupta, 1998; Ribeiro and Golding, 1998; Andrade *et al.*, 1999). There are two scenarios for this formation: the divergence of eukaryotic cells from Archaea followed by the subsequent transfer of the Bacterial genes (Ribeiro and Golding, 1998; Yamagishi *et al.*, 1998; Martin, 1999; Andrade *et al.*, 1999), and the symbiosis of Archaea in Bacteria forming cell nuclei (Lake, 1988; Lake and Rivera, 1994; Moreira and López-García, 1998). However, there has been no conclusive evidence supporting either of these scenarios.

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Recently complete genomic data, including bacterial data, has been accumulating (Pennisi, 1998; Doolittle, 1998). To analyze the mechanism of the formation of eukaryotic cells, we have analyzed the origins of yeast genes (ORFs; Open Reading Frames) from individual archaeabacterium and eubacterium (Horiike *et al.*, 2001). ORFs from sixteen unicellular organisms whose whole genome DNA sequences have been clarified were used including *Saccharomyces cerevisiae*, six kinds of Archaea and nine kinds of Bacteria, excluding parasitic bacteria. Yeast ORFs (total 3,508 ORFs), classified into 43 categories (or sub-categories) (MIPS, 2000), were analyzed. Two sub-categories of mitochondrial organization and transport, along with ORFs noted as "mitochondrial" as well as those related to the tricarboxylic acid cycle and electron transport system were separated from the 43 categories (or sub-categories).

Using Gapped BLAST (Altschul *et al.*, 1997), yeast ORFs with the highest degree of similarity to each bacterial total ORF was detected. Conversely, bacterial ORFs (in each bacterium) with the highest degree of similarity to the yeast ORFs were also detected. Finally (orthologous) ORF pairs with the highest degree of similarity between one another were computed. This operation was done for the detection of orthologous ORFs (Tatusov *et al.* 1997) and to avoid the effect of gene duplication after the divergence of yeast from each bacterium. In each functional category (or sub-category), yeast orthologous ORFs were counted as hit numbers in the category (or sub-category) to each bacterium ORF.

The threshold of the similarity (E-value) was set at intervals of 5 from 5 to 185 as $-\log E$. Hit numbers of ORFs at each E-value were calculated for each bacterium. Two t-tests (First; for the hit numbers at each E value. Second; for the first t-test in the range of E values containing more than 5 ORFs in any bacterium) were carried out for the Archaeal group and Bacterial group. Thus, the homology of each ORF group in yeast to Archaeal ORFs or to Bacterial ORFs was judged statistically. This newly developed method was named "Homology Hit Analysis". As a result, the yeast ORF groups related to the sub-categories of meiosis, DNA synthesis and replication, cell cycle control and mitosis, rRNA transcription, mRNA transcription, ribosomal proteins, translation (initiation, elongation and termination), organization of endoplasmic reticulum, and nuclear organization showed homology to Archaeal ORFs rather than to Bacterial ORFs. On the other hand, the yeast ORF groups related to amino-acid metabolism, nitrogen and sulfur metabolism, nucleotide metabolism, C-compound and carbohydrate metabolism, metabolism of vitamins (cofactors and prosthetic groups), energy, cellular import, stress response, detoxification and ionic homeostasis showed more homology to Bacterial ORFs. The origins of the "mitochondrial related ORFs" as Bacterial (Yang *et al.*, 1985) was confirmed by this analysis. Finally, the origins of 20 yeast ORF groups as being of Archaeal or Bacterial origin were determined. Most ORF groups judged as being of Archaeal origin are related to the nucleus. Translation occurs on the endoplasmic reticulum and in the cytoplasm. However this is closely related to transcription. Endoplasmic reticulum is contiguous to the nuclear membrane. Therefore it is possible to regard the translation and the endoplasmic reticulum as being nuclear related. On the other hand, most of the ORF groups judged as being of Bacterial origin are related to the cell cytoplasm. A conceptual scheme is shown in Figure 1.

In the above analysis, after the detection of orthologous ORFs in yeast total ORFs to each bacterium, each orthologous ORF was classified into a category (or sub-category). Another analysis was also carried out. Before the detection of orthologous ORFs, each

yeast ORF was classified into a category (or sub-category). Then the Homology Hit Analysis for each category (or sub-category) was carried out. By this analysis, two categories, the assembly of protein complexes and DNA repair were newly judged to be of Archaeal origin, while five categories, lipid, (fatty-acid and isoprenoid) metabolism, protein folding and stabilization, signal transduction, organization of the plasma membrane and organization of the cytoplasm, were newly judged to be of Bacterial origins. On the other hand, the origins of two categories (meiosis and cellular import, which were determined by other analyses) were not judged (unpublished results).

3. Nucleus Symbiosis Hypothesis; Mechanism for the Formation of the Mosaic Structure of the Eukaryotic Genome Derived from Archaea and Bacteria

There are three possibilities for the appearance of the mosaic structure: gene transfer by mitochondrial symbiosis (Margulis, 1970; Margulis *et al.*, 2000), the accumulation of small-scale lateral gene transfer (Aravind *et al.*, 1998; Jain *et al.*, 1999), and massive gene transfer by nuclear symbiosis.

Concerning mitochondrial symbiosis, ORFs related to the mitochondria were removed and analyzed separately. As the proportion of mitochondrial ORFs to other ORFs is small, their influence may be negligible.

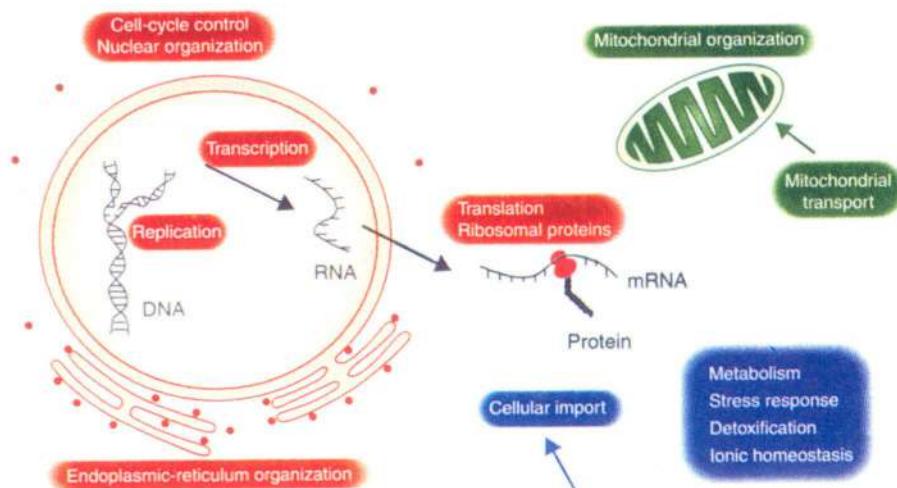


Figure 1. Schematic figure of an eukaryotic cell showing the origin of each subcellular organ and function determined by Homology Hit Analysis. Yeast ORF groups originating from Archaeal ORFs or Bacterial ORFs are shown in red or blue, respectively. Mitochondrial related ORFs, originating from Bacterial ORFs, are shown in green. (Reprinted by permission from Nature Cell Biology [see Horiike *et al.*, 2001] copyright (2001) Macmillan Magazines Ltd.)

The second possibility is the accumulation of small-scale lateral gene transfer. On the assumption that yeast (eukaryotic) cells originated from a common Archaeal (or Bacterial) ancestor, all gene groups that seem to be of Bacterial (or Archaeal) derivation must have been introduced by lateral gene transfer. However, the frequency of lateral gene transfer to yeast from Archaea or Bacteria may not differ by very much. Therefore, it is not likely that many ORF groups containing many ORFs were replaced by the accumulation of small-scale lateral gene transfer.

Finally, the possibility of the symbiosis of an Archaeal cell in a Bacterial cell, forming cell nucleus, remains. During symbiosis, most ORFs related to the Archaeal metabolic system was replaced by ORFs of Bacterial derivation.

We have obtained a strong evidence for the "Nucleus Symbiosis Hypothesis" by a newly developed method using large numbers of ORFs. In addition to this evidence, the following evidence should be considered. Examples are the presence of introns (Kjems and Garrett, 1988), histon homologues related to H3-H4 (Grayling *et al.*, 1994; Starich *et al.*, 1996; Pereira *et al.*, 1997; Reeve *et al.*, 1997; Zlatanova, 1997) and resistance to several antibiotics (Amils *et al.*, 1989) in the Archaeal bacteria, and also actin-related protein genes in Bacteria (Bork *et al.*, 1992; Doolittle, 1998; Löwe and Amos, 1998). Other evidence can be found by re-considering previous and future data from the viewpoint of nuclear symbiosis.

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III. Bacteria, Cyanobacteria & Algae

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PHOTOTROPHIC CONSORTIA: A TIGHT COOPERATION BETWEEN NON-RELATED EUBACTERIA

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Introduction

Many studies in microbial ecology rely on physiological measurements of pure laboratory cultures. This approach has provided important insights into the physiological capabilities of different bacteria. However, microscopic observations in environmental samples together with some mathematical calculations challenge the concept of a largely independent metabolism of single bacterial species under natural conditions. The physiology of some bacteria changes significantly when they reach high cell densities. In the case of quorum sensing, the excretion of autoinducer molecules signals high cell density and triggers light production, expression of virulence factors, swarming (Fuqua and Greenberg, 1998), or prevents cell aggregation (Puskas *et al.*, 1997). Similarly, myxobacteria exhibit complex social interactions. When deprived of nutrients they enter into a complex developmental cycle that results in the formation of a multicellular fruiting body which contains myxospores (Reichenbach, 1985).

Very commonly, daughter cells remain connected after division, thereby forming coenobia, net-like microcolonies or mycelia. These types of aggregates have been described for a wide variety of physiologically and phylogenetically diverse bacteria (Hirsch, 1984). In some bacterial species, aggregation of previously separated cells has been observed and may be mediated by strain-specific surface structures (Overmann and Pfennig, 1992; Stove-Poindexter, 1964). Aggregate formation in planktonic bacteria changes their sedimentation rate (Overmann and Pfennig 1992). Such an aggregation of the cells of one single bacterial species can substantially alter the biogeochemical cycles of a whole ecosystem (Overmann *et al.*, 1996; Overmann *et al.*, 1999).

Compared to the numerous well documented cases of cell-cell-interactions in monospecific bacterial associations, much less is known of the occurrence and significance of structured associations between different types of bacteria. However, evidence for the occurrence of such heterogeneous assemblages is increasing. Microcolonies, aggregates or biofilms on solid substrates which are composed of morphologically different bacteria have been frequently observed (Alldredge and Youngbluth, 1985; Eichler and Pfennig 1990; Overmann *et al.*, 1996; Seitz *et al.*, 1993; van Gemerden *et al.*, 1989, Weise and Rheinheimer, 1978). Coaggregation of genetically distinct bacteria is especially well studied for dental plaque, where strains belonging to 18 different genera form multiple interactions (Kolenbrander and London, 1993). Evidence has accumulated for the existence of 10 - 100 µm-scale patches of free-

living bacterial cells (Krembs et al. 1998). This would indicate that, under natural conditions, a selective pressure exists for the formation of close microbial associations.

Often, bacterial cocultures of unknown composition which are capable of certain degradation reactions are referred to as “consortia”. However, the term consortia should be reserved for those cases of symbiosis in which two or more microorganisms form an organized morphological structure and maintain a permanent cell-to-cell contact (Hirsch, 1984; Schink, 1991; Trüper and Pfennig, 1971).

Consortia may represent the extreme case of mutual interdependence of different bacteria and could be the result of coevolution of phylogenetically non-related bacterial species. The present discussion focusses on phototrophic consortia, especially because of the recent progress which has been made towards an understanding of their physiology and phylogeny.

1. Bacterial consortia known to date

Table 1 provides a compilation of all bacterial consortia *sensu strictu* which have been described to date. The different types are arranged according to their habitats.

1.1. PHOTOTROPHIC CONSORTIA

Phototrophic consortia were first discovered by Lauterborn at the beginning of the last century (Lauterborn, 1906). They contain green sulfur bacteria which are arranged with colorless partner bacteria in three different ways. To date, a total of nine different phototrophic consortia can be distinguished (Fig. 1A-G). Most have been repeatedly observed in freshwater habitats.

Seven of the morphotypes are spindle-shaped and motile. A colorless central rod-shaped bacterium is surrounded by 13 - 69 green- or brown-colored epibionts. In “*Chlorochromatium aggregatum*”, the colorless bacterium is surrounded by green-colored rod-shaped bacteria while brown epibionts are found in “*Pelochromatium roseum*” (Fig. 1A,E). Phototrophic consortia of the type “*Chlorochromatium glebulum*” are bent and contain gas-vacuolated green epibionts (Fig. 1B), “*Chlorochromatium magnum*” is composed of a straight central colorless bacterium carrying about 40 green-colored and gas-vacuolated epibionts. The epibiont cover of “*Pelochromatium roseoviride*” consists of an inner layer of brown-colored cells and an outer one of green-colored bacteria (Fig. 1C). Recently, two previously unknown types of phototrophic consortia were described. “*Chlorochromatium lunatum*” and “*Pelochromatium selenoides*” (Fig. 1D) carry half-moon-shaped green or brown epibionts, respectively, and thus can be distinguished from the other phototrophic consortia which contain rod-shaped green sulfur bacteria.

Two additional non-flagellated morphotypes of the phototrophic consortia exhibit a different arrangement of the two bacterial partners. Bacterial cells are arranged in a sheath-like fashion in “*Chloroplana vacuolata*”, in which long slender colorless rods alternating with chains of rod-shaped green sulfur bacteria (Fig. 1G). Both types of bacteria contain gas vesicles and are immotile. Large aggregates of “*C. vacuolata*” may contain up to 400 cells of green sulfur bacteria.

TABLE 1. Naturally occurring bacterial consortia

Consortium*	bacterial partners	References
Chemocline of freshwater lakes		
" <i>Chlorochromatium aggregatum</i> "	Green-colored green sulfur bacteria around central motile colorless bacterium	Lauterborn, 1906 Fröstl and Overmann, 2000
" <i>Chlorochromatium glebulum</i> "	Bent consortium with green-colored green sulfur bacteria with gas vesicles around central motile colorless bacterium	Skuja, 1957 Fröstl and Overmann, 2000
" <i>Chlorochromatium magnum</i> "	~ 40 green-colored green sulfur bacteria with gas vesicles around central motile colorless bacterium	Fröstl and Overmann, 2000
" <i>Chlorochromatium lunatum</i> "	Green-colored half-moon-shaped green sulfur bacteria around central motile colorless bacterium	Abella et al., 1998
" <i>Pelochromatium roseum</i> "	Brown-colored green sulfur bacteria around central motile colorless bacterium	Lauterborn, 1913 Tuschak et al., 1999
" <i>Pelochromatium roseo-viride</i> "	Outer layer of green-colored green sulfur bacteria and inner layer of brown-colored green sulfur bacteria around central motile colorless bacterium	Gorlenko and Kusnezov, 1972
" <i>Pelochromatium selenoides</i> "	Brown-colored half-moon-shaped green sulfur bacteria around central motile colorless bacterium	Abella et al., 1998
" <i>Chloroplana vacuolata</i> "	Green-colored green sulfur bacteria with gas vesicles alternating with colorless, gas-vacuolated bacteria	Dubinina and Kuznetzov, 1976
N.n.	Small chemotrophic bacteria attached to heterocyst cells of <i>Anabaena azollae</i> , <i>A. spiroides</i> , <i>A. oscillarioides</i>	Paerl and Kellar, 1978
N.n.	<i>Desulfocapsa thiozymogenes</i> , purple sulfur bacteria	Tonolla et al., 2000

TABLE 1 (continued)

Freshwater mud samples

" <i>Cylindrogloea bacterifera</i> "	Green-colored green sulfur bacteria around central chain of colorless bacteria with thick capsules	Perfiliev, 1914 Skuja, 1957
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Marine sediments, in Peruvian upwelling region

N.n.	<i>Thioploca</i> sp., <i>Desulfonema</i> sp.	Jørgensen and Gallardo, 1999
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Methane hydrate-rich marine sediments, Cascadia convergent margin, Oregon

N.n.	Archaea of the order Methanosciricales surrounded by sulfate-reducing bacteria of the <i>Desulfosarcina/Desulfococcus</i> -group	Boetius et al., 2000
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Hindgut of the termite *Reticulitermes flavipes*

N.n.	Rod-shaped Gram-negative bacteria oriented longitudinally along central trichome of endospore-containing bacteria	Breznak and Pankratz, 1977
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N.n.	Methanogenic archaea oriented longitudinally along central trichome of endospore-containing bacteria; consortium attached to hindgut wall	Leadbetter and Breznak, 1996
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Nitrifying activated sludge, flocs

N.n.	<i>Nitrobacter</i> spp. in small microcolonies in contact with larger cell clusters of <i>Nitrosomonas</i> spp.	Mobarry et al., 1996
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Dental plaque

Corn-cob formations	<i>Bacterionema matruchotii</i> , spherical streptococci	Jones, 1972; Mouton et al., 1977
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* N.n., no name. Scientific names of consortia are without standing in nomenclature and hence given in quotation marks.

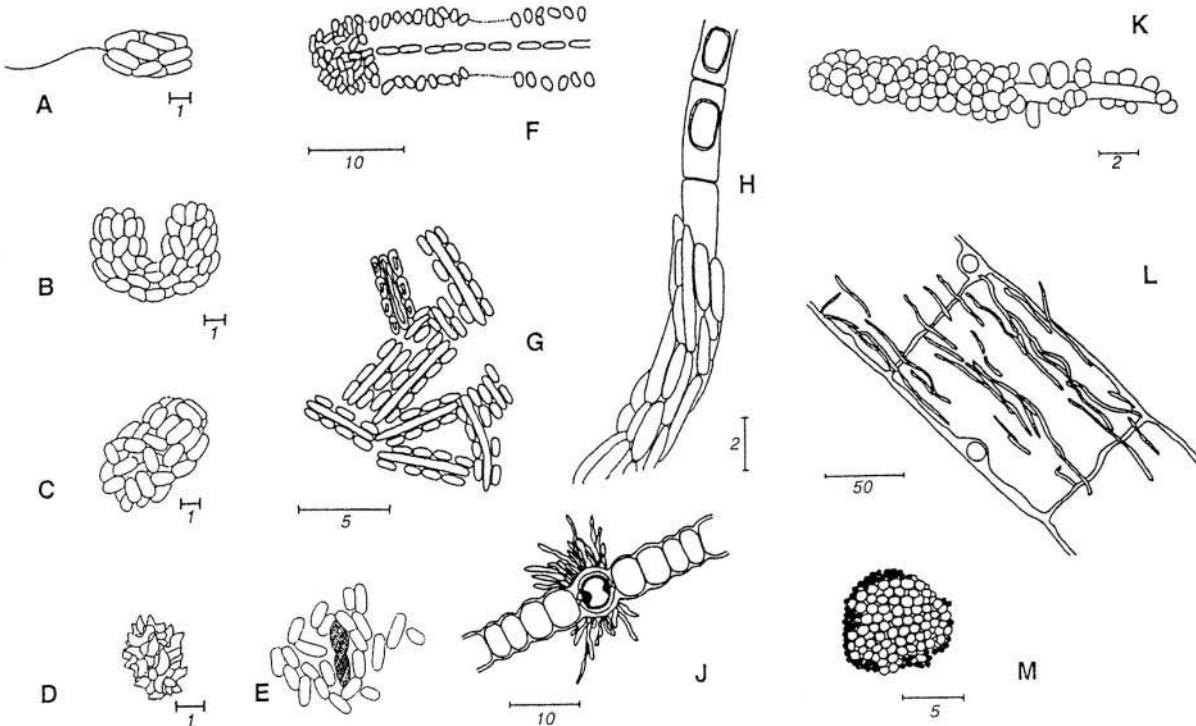


Figure 1. A. Morphotype of *"Chlorochromatium aggregatum"* and *"Pelochromatium roseum"*. B. *"Chlorochromatium glebulum"*. C. Morphology of *"Chlorochromatium magnum"* and *"Pelochromatium roseo-viride"*. D. Morphotype of *"Chlorochromatium lunatum"* and *"Pelochromatium selenoides"*. E. *"Chlorochromatium aggregatum"* after disaggregation, the colorless central bacterium is visible. F. *"Cylindroglloeaa bacterifera"*. Longitudinal transect on the right shows the central rod-shaped bacteria. G. *"Chloroplana vacuolata"*. Gas vacuolation of colorless filaments and rod-shaped green sulfur bacteria is depicted for only a few cells. H. Consortium from the hindgut microbial community of the termite *Reticulitermes flavipes*. Upper portion shows central chain of rod-shaped bacteria containing endospores. J. *Anabaena* sp. filament containing one heterocyst cell covered by chemotrophic bacteria. K. Corn-cob formation from dental plaque. L. *Thioploca* sp. covered with filamentous sulfate-reducing bacteria *Desulfonema* sp. M. Novel archaeal-bacterial consortia in which a central aggregate of archaeal cells (hollow) is surrounded by a few layers of sulfate-reducing bacteria (filled). Values on all bars are in μm .

"*Cylindrogloea bacterifera*" is an association which was observed only twice (Perfiliev, 1914; Skuja, 1957). It consists of a central chain of colorless bacteria embedded in a 7 to 8 μm -thick cylindrical mucilagous sheet which is covered with a large number of gas-vacuolated green sulfur bacteria (Fig. 1F).

It has to be kept in mind that all binary names of phototrophic consortia are without standing in nomenclature since the consortia consist of two different bacteria. Consequently all names above have to be given in quotation marks.

Based on the presence of chlorosomes in the epibiont cells, it was concluded that the latter belong to the green sulfur bacteria (Caldwell and Tiedje, 1974). Applying a highly specific oligonucleotide probe for fluorescent *in situ* hybridization (FISH), the phylogenetic affiliation of the epibionts with the division of green sulfur bacteria could be verified (Tuschak et al., 1999). However, it came as a surprise when FISH demonstrated that the central rod-shaped bacterium in several types of phototrophic consortia is a member of the β -proteobacteria (see below).

Recently, aggregates consisting of small-celled purple sulfur bacteria and sulfate-reducing bacteria were detected in the chemocline of a meromictic alpine lake (Tonolla et al., 2000). Based on the analyses of 16S rRNA gene sequences, the purple sulfur bacteria are affiliated with *Amoebobacter purpureus* and *Lamprocystis roseopersicina*, the sulfate-reducing bacteria belong to the species *Desulfocapsa thiozymogenes*.

1.2. CONSORTIA CONTAINING CYANOBACTERIA

Bacterial consortia involving oxygenic phototrophs have been observed in the natural bacterioplankton of lakes as well as in laboratory cultures. In this type of consortium, numerous small heterotrophic bacteria are specifically attached to the heterocysts of the cyanobacteria *Anabaena azollae*, *A. spiroides*, or *A. oscillarioides*, while the vegetative cells of the cyanobacterial filament are almost devoid of epibionts (Paerl and Kellar, 1978) (Fig. 1J).

1.3. CONSORTIA CONTAINING COLORLESS SULFUR BACTERIA

The sheaths of large marine *Thioploca* filaments are densely covered by filamentous sulfate-reducing bacteria of the genus *Desulfonema* (Fig. 1L). *Thioploca* migrates within its own sheaths, takes up nitrate in the surface layers of the marine sediments and stores it in large central vacuoles. Sulfide, on the opposite, is taken up in deeper, anoxic sediment layers and oxidized using the stored nitrate as electron acceptor. Since sulfide concentrations in the deeper sediment layers are low, it has been suggested that the association with the sulfide-producing *Desulfonema* is of advantage to *Thioploca* (Jørgensen and Gallardo, 1999). The close association of the two bacteria indicates that a rapid recycling of sulfur compounds occurs. The chemolithoautotrophic *Thioploca* may supply organic carbon for *Desulfonema*. In addition, *Thioploca* may form and excrete reduced sulfur compounds which are preferred over sulfate as electron-accepting substrate. Similar to associations described for *Desulfovibrio desulfuricans* and a marine *Thiobacillus thioparus* strain (van den Ende et al., 1997), an exchange of reduced sulfur compounds would lead to increased cell yields of the sulfate-reducing *Desulfonema*.

1.4. CONSORTIA CONTAINING NITRIFYING BACTERIA

Fluorescent *in situ* hybridization of bacteria in nitrifying activated sludge revealed that the nitrite-oxidizing *Nitrobacter* spp. occurred in small microcolonies which were frequently in contact with larger cell clusters of the ammonia-oxidizing *Nitrosomonas* spp. (Mobarry et al., 1996). Thus, both types of bacteria tend to grow in closely associated aggregates, with nitrite representing the intermediate exchanged. Here the advantage of consortia formation most likely is that nitrite, which is toxic to the ammonia oxidizer, is removed by the *Nitrobacter* spp.

1.5. CONSORTIA CONTAINING METHANOGENIC BACTERIA

Interestingly, bacterial consortia which are structurally similar to phototrophic consortia have been described from an entirely different ecosystem, namely the hindgut of the termite *Reticulitermes flavipes*. The epibionts exhibit the bluegreen fluorescence characteristic of coenzyme F₄₂₀ and accordingly were identified as members of the methanogenic archaea (Leadbetter and Breznak, 1996). The 1.5 μm -wide bacterial cells of the central trichome contain endospores. The association itself is attached to the gut wall of the termite host. The overall morphology is very similar to that of the consortium depicted in Fig. 1H.

Less well defined consortia form in sewage sludge digestors and are composed of different species, including for example syntrophic propionate-oxidizing bacteria and hydrogen-consuming methanotrophs. The bacteria are embedded within an exopolymer matrix (Harmsen et al., 1996). These flocs usually have dimensions of several 100 μm to a few mm. The existence of similar consortia in lakes sediments has been inferred from kinetic measurements of H₂-turnover (Conrad et al., 1985; 1986).

The bacterial consortia detected most recently are composed of methanogenic archaea and sulfate-reducing bacteria. In these structured associations, up to 100 archaeal cells are surrounded by one or two cell layers of sulfate-reducing bacteria (Boetius et al., 2000). As demonstrated by fluorescent *in situ* hybridization, the archaea are affiliated with the order Methanosarcinales whereas the surrounding sulfate-reducing bacteria belong to the *Desulfosarcina/Desulfococcus* group (Fig. 1L).

1.6. OTHER CONSORTIA

Electron microscopic investigations of the hindgut microbial community of the termite *Reticulitermes flavipes* revealed the presence of an intimate physical association between thin rod-shaped Gram-negative epibionts with tapered ends and a chain of thicker rods (Fig. 1H) (Breznak and Pankratz, 1977). The epibionts are aligned longitudinally along the central trichome. Cells of the trichome are engulfed in a continuous wall layer, have a Gram-positive cell wall structure, and contain endospores. The attachment of the epibionts to the central trichomes is mediated by fibrous holdfast material.

The dense bacterial layer at the enamel surface of teeth, the dental plaque, harbors morphologically conspicuous aggregates which are composed of two different types of bacteria. These so-called corn-cob formations (Jones, 1972) consist of central rod-

shaped bacteria which are surrounded by spherical cells. One study (Mouton et al., 1977) used micromanipulation to dissect these formations. Subsequently, the rod-shaped bacterium was identified as *Bacterionema matruchotii* and the outer cells as spherical streptococci. Such associations have also been generated in the laboratory between *B. matruchotii* and *Streptococcus sanguis* (Lancy et al. 1980).

Since the description of the above consortia to a large extent depends on mere microscopical observations, they do not include associations between morphologically similar, but physiologically and phylogenetically different bacteria. It is to be expected that many more types of consortia exist in various environments and that the ecological significance of consortia formation is even higher than deduced from the few known cases. As an example, consortia of unknown composition which are morphologically similar to those of the termite hindgut were recently discovered within tufts of *Thiothrix* from a marine pond near Woods Hole, Ma, USA (J. Overmann, unpublished). However, most of the consortia listed in the preceding paragraphs have only been described from complex natural samples but could not be grown in laboratory cultures. Therefore the physiological capabilities of the bacteria forming the associations have remained entirely obscure; however, the phototrophic consortium "*Chlorochromatium aggregatum*" represents the most notable exception. The presence of regularly structured associations of two different types of bacteria in various and very different environments indicates that a selective pressure for consortia formation exists under different ecological conditions. In order to understand the principle advantage of consortia formation, physiological investigations of intact consortia and their isolated bacterial components are needed.

2. Selective advantage of consortia formation

2.1. PHYSICAL EFFECTS OF CELL-TO-CELL DISTANCE

In their natural environment, planktonic bacteria reach total cell numbers of 10^6 ml^{-1} , while sediments and soils harbor bacteria at densities of 10^9 ml^{-1} and 10^{11} cm^{-3} , respectively (Fægri et al., 1977; Whitman et al., 1998). Similarly, the microbial communities found in the rumen of cattle and sheep, the human colon, or microbial mats contain $10^{10}\text{-}10^{11} \text{ bacteria cm}^{-3}$ (Whitman et al., 1998). At these increasing densities, and assuming a homogenous distribution, the average cell-to-cell distance would amount to 112, 10, and $\sim 1 \mu\text{m}$. Over such distances, molecular diffusion of small molecules like hydrogen, carbon substrates or signalling compounds (diffusion coefficients D in the order of $1.5 \cdot 10^{-9} \text{ m}^2 \cdot \text{s}^{-1}$; Broecker and Peng, 1974) takes 4.2 seconds in pelagic waters, 0.03 seconds in sediments, and 0.0003 seconds in soils, as calculated from

$$T = \frac{L^2}{2 \cdot D} \quad (1)$$

(with T, time of diffusion and L, mean diffusive path of a molecule; Hirsch 1984).

Steep concentration gradients of intermediates exist only in the immediate vicinity of the cells producing them. Bacterial cells which are 112, 10, or $2 \mu\text{m}$ apart receive

0.008, 0.01, or 25% of the flux of metabolites compared to a cell at a distance of 1 μm (Boone et al., 1989). As a consequence of the laws of diffusion, a substrate source at a large distance cannot be found by chemotaxis but merely by chance. Even if they sense the substrate gradient around another cell, bacteria probably are not able to respond fast enough to remain near a substrate source 2 – 2.5 μm in diameter (Jackson, 1987). Consequently, a permanent association of bacteria with different, but complementary, metabolism is of selective advantage especially in dilute bacterioplankton associations.

2.2. PHYSICAL EFFECTS OF THE NUMBER OF EPIBIONTS

Many of the consortia described in the preceding section contain only one layer of epibionts. Therefore it is of interest whether such a single layer of cells at maximum physiological activity is in principle capable of counteracting the diffusion of substances into the center of the consortium. According to the formula for two-dimensional diffusion (Koch, 1990), the resulting concentration gradient ΔC across the epibiont layer can be calculated from the dimensions of the consortium (r_o and r_i , outer and inner radius of the epibiont layer; w = cell length, if cells are oriented longitudinally along the central bacterium), and the flux of the substance J (in $\text{mol} \cdot \text{s}^{-1}$, calculated from cell specific consumption rate for the compound in question and the number of cells arranged around the central bacterium):

$$\Delta C = J \cdot \frac{1}{2\pi D w} \cdot \ln \frac{r_o}{r_i} \quad (2)$$

2.3. CAN EPIBIONTS OFFER PROTECTION AGAINST HIGH AMBIENT CONCENTRATIONS OF O_2 OR H_2 ?

It has been suggested that the epibionts associated with cyanobacteria shield the heterocysts from high ambient O_2 concentrations during periods of high photosynthetic rates. This conclusion was based on several observations. The N_2 -fixation by the cyanobacterial partner increases in the presence of the epibiotic chemotrophic bacteria. As judged from the zones of reduction of two different tetrazolium dyes, the redox potential at the surface of heterocysts is poised between +490 and +50 mV (Paerl, 1978). Consequently, it has been proposed that epibionts remove molecular oxygen, which leads to microzones of oxygen depletion < 3 μm in diameter, and prevents nitrogenase inhibition during periods of high ambient oxygen concentrations (Paerl and Kellar, 1978).

However, this mechanism must be questioned since molecular diffusion of O_2 can only be counteracted by bacterial metabolism if the size of an aggregate exceeds a certain diameter (Ploug et al., 1997). It can be calculated that an aggregate tightly packed with bacteria which respire at a maximum rate (500 nmol $\text{O}_2 \text{ mg} \cdot \text{protein}^{-1} \cdot \text{min}^{-1}$) has an anoxic center only if the aggregate diameter exceeds 100 μm . Investigation of oxygen profiles in bacterial flocs from activated sludge demonstrated that even at a larger diameter (> 400 μm) and at excess organic carbon substrate concentrations, most of the aggregates were not anoxic in their center (Schramm et al., 1999). Respiration protects anaerobic bacteria in the rather unstructured purple microbial aggregates which

occur in salt marshes (Seitz et al., 1993). These latter aggregates are $\geq 600 \mu\text{m}$ in diameter.

The ecological significance of cell density on oxygen penetration has been especially thoroughly studied for monospecific accumulations of the chemolithotrophic sulfur-oxidizing bacteria *Beggiatoa* sp. (Jørgensen, 1982) and *Thiovulum majus* (Fenchel and Glud, 1998). Special adaptations like chemotaxis and the formation of stable cell veils enable these bacteria to position themselves exactly at the oxic/anoxic interface. It is only due to the high cell densities which are maintained over a large, **50 – 100 μm -thick**, layer that these bacteria can effectively prevent an abiotic reaction of their two substrates, sulfide and molecular oxygen. All the dimensions mentioned above are significantly larger than the diameter of heterocysts covered with chemotrophic bacteria (**6 - 10 μm**). Because of physical constraints, the respiration capacity of epibionts simply is not sufficient to create anoxia under air saturation in cyanobacterial consortia.

Under anoxic conditions, degradation of ethanol, propionate and butyrate by H_2 -producing fermentative bacteria often can only proceed in the presence of H_2 -consuming methanogenic or sulfate-reducing bacteria, i.e. under conditions of interspecies hydrogen transfer. In granular sludge, microniches with decreased partial pressure of H_2 are provided inside aggregates which consist of hundreds to thousands of cells. This raises the question, whether also a monolayer of methanogenic cells like that in the methanogenic consortia of the termite hindgut could provide protection against the relatively high ambient H_2 concentrations ($\geq 1000 \text{ Pa}$; Ebert and Brune, 1997), and thereby create a microniche for the fermentation of volatile fatty acids. Methanogenic bacteria isolated from the termite hindgut form methane at rates of up to $7.55 \cdot 10^{-10} \mu\text{mol} \cdot \text{h}^{-1}$ (Leadbetter and Breznak, 1996), which corresponds to a consumption of $30.2 \cdot 10^{-10} \mu\text{mol H}_2 \cdot \text{h}^{-1}$. The central filament is surrounded by about eight epibionts (cell length x width = $1.4 \times 0.4 \mu\text{m}$). With the diffusion coefficient for H_2 of $D = 3.81 \cdot 10^{-9} \text{ m}^2 \cdot \text{s}^{-1}$ (Ebert and Brune, 1997), the methanogens can maintain a concentration gradient of $\Delta C \approx 85.6 \text{ nM}$ across the epibiont layer (equation (2)), corresponding to a difference in partial pressure of 10.6 Pa.

If the epibionts are assumed to provide a metabolic barrier to the diffusion of hydrogen in the center of the consortium, they should lower the $p\text{H}_2$ to values $p\text{H}_2^{\text{crit}}$ which allow ethanol, propionate and butyrate fermentation to precede. $p\text{H}_2^{\text{crit}}$ for these three reactions can be calculated following the approach of Conrad et al. (1986), since concentrations for most of the substrates and products are known or can be estimated (acetate, 71.4 mM; lactate, $\leq 3.7 \text{ mM}$; ethanol, propionate, butyrate all $\leq 4 \text{ mM}$; $p\text{H}_2 = 1000 \text{ Pa}$ at the gut wall, $T = 20^\circ\text{C}$; $\text{pH} = 7.0$; $[\text{HCO}_3^-] = 4 \text{ mM}$; Breznak and Brune, 1994; Brune et al., 1995; Conrad et al., 1986; Ebert and Brune, 1997; Tholen et al., 1997). As an example, the fermentation of butyrate



under conditions in the termite hindgut yields a Gibbs free energy of

$$\Delta G = +88.2 \text{ kJ} \cdot \text{mol}^{-1} + RT \ln \frac{[\text{acetate}]^2 [p\text{H}_2]^2 [\text{H}^+]}{[\text{butyrate}]} = +27.2 \text{ kJ} \cdot \text{mol}^{-1} \quad (4)$$

Based on these values, pH_2^{crit} can be calculated from

$$\log pH_2^{crit} = \log pH_2 - \frac{(20 + \Delta G)}{nRT \cdot 2.303} \quad (5)$$

Data for the standard Gibbs free energy were taken from Conrad et al. (1986) and a minimum free energy quantum for ATP formation of ~ 20 kJ. (mol H₂)⁻¹ (Schink, 1991) was assumed. The resulting values have to be regarded as maximum estimates and are 47, 0.4, and 0.062 Pa for ethanol, propionate and butyrate fermentation, respectively. This means that the partial pressure of hydrogen in the termite hindgut would have to be decreased by the methanogenic epibiont layer from 1000 to 0.062 Pa in order to permit growth of a butyrate-fermenting bacterium in the center of the consortium. The actual decrease which is physiologically possible is (by 10.6 Pa, see above) from 1000 to 989 Pa. Obviously, interspecies hydrogen-transfer is not the selective factor for consortia formation in the termite hindgut.

In conclusion, just a single layer of epibiont cells cannot offer an efficient protection against high ambient concentrations of rapidly diffusing molecules like O₂ or H₂. Hence a principle difference must exist between regularly arranged types of consortia (Fig. 1) and the bigger cell aggregates with more random cell arrangement (flocs from granular sludge). It has to be postulated that in the former group, compounds which are not present at significant concentrations in the environments are exchanged between the symbiotic partners.

2.4. INTERPRETATION OF CONSORTIA FORMATION

The existence of an anoxic zone around the heterocysts is unlikely. Alternatively, the selective advantage for the cyanobacterium may be a supply of growth factors formed by the chemotrophic bacteria like, e.g., vitamins (Paerl, 1982). When evaluating the advantage of consortium formation it also has to be kept in mind that cyanobacteria and their epibionts may have an antagonistic interaction in that they compete for phosphorus, iron and trace metals (Paerl, 1982). Microautoradiography demonstrated that the chemotrophic bacteria associated with *Anabaena* heterocysts incorporate a variety of organic compounds, including the amino acids serine, alanine, glycine, as well as glucose (Paerl, 1978). Since such compounds are excreted by cyanobacteria (Paerl, 1982), it was suggested that the epibionts benefit from utilizing cyanobacterial excretion products (Paerl and Kellar, 1978). Colonization experiments in lake water samples demonstrated that motile bacteria often move towards the heterocysts and become firmly attached and immotile.

Probably a syntrophic cooperation exists at least in some of the consortia. The term "syntrophy" describes those cooperations in which both partners depend entirely on each other to perform a metabolic activity not possible for the isolated partners, and in which mutual dependence cannot be overcome by simply adding a cosubstrate or any type of nutrient (Schink, 1991). However, a syntropy based on interspecies H₂ transfer as it was suggested for methanogenic consortia of the *Reticulitermes flavipes* hindgut (Leadbetter and Breznak, 1996) appears rather unlikely (see 2.3).

Because the archaeal-bacterial consortia of methanogenic and sulfate-reducing bacteria (Fig. 1M) were exclusively found in sediments containing methane hydrates, it has been proposed that anaerobic methane oxidation occurs within these associations. During anaerobic oxidation, methane is oxidized with sulfate as terminal electron acceptor, yielding carbonate and sulfide as reaction products (Boetius et al., 2000). Methane oxidation is believed to be catalyzed by the methanogens as a reversal of methane formation, and is only possible in the presence of the sulfate-reducing partner bacteria scavenging intermediates such as H₂ or acetate. ¹³C signatures of lipids of the sulfate-reducing partner bacteria indicate that acetate may be formed by a bimolecular reaction from methane and is subsequently used as a carbon source by the sulfate-reducing bacteria (Boetius et al., 2000). Based on the calculations described above, it has to be postulated that partial pressures of H₂ in the sediments surrounding the consortia are very low.

With two exceptions (*Chlorochromatium aggregatum*, *Anabaena heterocyst* consortia), none of the consortia known to date have been cultured in the laboratory. As a consequence, information on the type of physiological interaction, the degree of mutual interdependence, the mechanisms of cell-to-cell contact, and the coevolution of the non-related bacteria is scarce. Therefore a model system for detailed investigation of the highly structured type of consortia is urgently needed. Phototrophic consortia may represent such a model system.

3. New insights into the physiology and phylogeny of consortia: Phototrophic consortia as model systems

The physiology of the bacteria which constitute phototrophic consortia and the type of their interaction is still unknown. All strains of green sulfur bacteria isolated to date are obligate anoxygenic photolithotrophs which use sulfide, elemental sulfur, and – in some instances – thiosulfate as electron donor. In the presence of sulfide and CO₂, a limited number of organic carbon compounds (acetate, propionate, pyruvate) may be photoassimilated (Overmann, 2000). Since electron transport is stimulated by sulfide (Fröstl and Overmann, 1998) and intact consortia oxidize exogenous sulfide in the light (J. Glaeser and J. Overmann, unpublished results), sulfide indeed seems to serve as photosynthetic electron donor of the epibionts similar to free-living strains of green sulfur bacteria. In analogy to cocultures of green sulfur bacteria with sulfur- or sulfate-reducing bacteria, it had been proposed that a syntrophic sulfur cycle exists in phototrophic consortia and is the physiological basis of the symbiosis (Pfennig, 1980). In the natural habitat of phototrophic consortia, ambient sulfide concentrations are usually low. Hence a recycling of reduced sulfur would be advantageous and ensure a continuous supply of the anoxygenic photosynthetic epibionts of consortia with a suitable electron-donating substrate. Unexpectedly, several lines of evidence suggest that this model of an internal sulfur cycle is not true for phototrophic consortia.

Until recently, all attempts to enrich phototrophic consortia in the laboratory have been unsuccessful and none of the consortium members are in culture. The isolation of a green sulfur bacterium from “*Chlorochromatium aggregatum*” was reported by Mechsner (1957), but the strain was lost before detailed studies could be made. More

recently, chemotaxis experiments with "*Chlorochromatium aggregatum*" from a eutrophic freshwater lake revealed that this phototrophic consortium responds rapidly to 2-oxoglutarate. Using mineral media supplemented with this carbon substrate, a stable enrichment culture could be established for the first time since the discovery of phototrophic consortia (Fröstl and Overmann, 1998). Growth of intact consortia was observed exclusively in the simultaneous presence of 2-oxoglutarate and light. In addition, fluorescent *in situ* hybridization demonstrated that the central rod-shaped bacterium in several morphotypes of phototrophic consortia is a member of the **β-proteobacteria**, rather than the **δ-proteobacteria** to which the sulfur- and sulfate-reducing bacteria belong (Fröstl and Overmann, 2000). Finally, a recently established enrichment culture of "*Pelochromatium roseum*" depends on exogenous sulfide for growth (J. Glaeser and J. Overmann, unpublished results). Taken together, these results are not congruent with the existence of an internal sulfur cycle in the consortia.

Nevertheless, a close interaction clearly exists between the epibionts and the central rod of phototrophic consortia. The number of epibionts per consortium exhibit a non-random frequency distribution with a distinct maximum, indicating a coordinated cell division of all epibionts (Overmann et al., 1998). Also, the central bacterium in many specimen of consortia is in a later stage of cell division. Therefore, the chemotrophic bacterium must be capable of growth and division while in association with the phototrophic epibiont. Obviously, the cell cycles of all epibionts and the central rod are synchronized. Further evidence for a close interaction of both types of bacteria comes from physiological experiments. When intact consortia are suddenly illuminated with light of high intensity they exhibit a sudden change in the direction of movement ("Schreckbewegung"; Buder, 1914). "*Chlorochromatium aggregatum*" reverses the direction of movement when entering the dark (scotophobic response) (Fröstl and Overmann, 1998). Only the central colorless, hence blind, bacterium is monopolarly flagellated (Overmann et al., 1998). The photoreceptor of the scotophobic response exhibits an action spectrum identical to the absorption spectrum of the bacteriochlorophyll of the epibiont. Therefore, a rapid interspecies signal transfer has to be postulated between the non-motile, light-sensing epibionts and the motile, colorless bacterium.

The highly coordinated cell division in phototrophic consortia ensures that their spatial arrangement is always maintained during multiplication of the cells. Phototrophic consortia thus appear to use a highly evolved mechanism for maintaining the spatial arrangement of the partner bacteria and thus far represent the most specialized of all known consortia. The tight cooperation between non-related eubacteria in the phototrophic consortia raises the question whether this symbiosis is the result of a long coevolution of the two bacterial partners.

Using a newly developed set of PCR primers, 16S rRNA gene fragments of the green sulfur bacterial epibionts of consortia were selectively amplified by PCR and subsequently separated by denaturing gradient gel electrophoresis (Overmann et al., 1999). When a chemocline bacterial community in which "*Pelochromatium roseum*" dominated was analyzed with this method, none of the 16S rRNA gene sequences of green sulfur bacteria matched those available in the database (Overmann et al., 1999). This indicates that the epibionts of "*P. roseum*" represent a new and previously unknown phylotype. The phylogenetic study of the epibionts of phototrophic consortia

was recently extended to other lakes in Germany, Spain and the USA, representing habitats which are thousands of kilometers apart. Single intact phototrophic consortia were mechanically separated from other bacteria by micromanipulation, and their 16S rRNA gene sequences analyzed. Although the consortia observed in the oxic/anoxic interface of these lakes appear morphologically identical in the light microscope, almost each population had a single and distinct 16S rRNA gene sequence (Fröstl and Overmann, 2000; Glaeser and Overmann, unpubl. results). From these results it has to be concluded that (1) phototrophic consortia do not form by chance (otherwise more than one phylotype should have been detected among the epibionts in one lake), and (2) that the biodiversity of green sulfur bacteria which form stable associations with non-related chemotrophic bacteria is almost as great as for free-living species of green sulfur bacteria. Possibly, phototrophic consortia with phylogenetically distinct epibionts occupy geographically confined areas.

The picture which begins to emerge from this limited dataset is that the specialized associations evolved concomitantly with the radiation of green sulfur bacteria, and have an evolutionary history almost as long as all other green sulfur bacteria. To date, phototrophic consortia thus represent the most specialized and possibly even the oldest bacterial-bacterial interactions. It also appears likely, that at least this special type of bacterial symbiosis differs from the current paradigm of hydrogen or sulfur syntrophy and is based on some other, so far neglected, physiological interaction. In this sense, phototrophic consortia represent a very useful model for further studies of bacterial symbioses.

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STRUCTURE AND PHYLOGENY OF CYANOPHORA SPECIES

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1. Introduction

This chapter reviews the structure of *Cyanophora*, an unusual protistan genus from the Division Glaucoctophyta. *Cyanophora* is a colorless freshwater microflagellate whose cells contain blue-green chloroplasts, earlier called cyanelles (Pascher 1929). These plastidic organelles, also called “cyanoplasts” (Schenk 1990) or muroplasts (Schenk 1994), have characteristics of both cyanobacteria and true chloroplasts (Delwiche, Kuhsel, and Palmer 1995, Bhattacharya and Medlin 1996), and they have been considered to be an evolutionary intermediate between an endosymbiont and a chloroplast (Kies and Kremer 1986, 1989). Similarities of cyanoplasts and cyanobacteria include a lysozyme sensitive cell wall (Schenk 1970, White, Bailey, Clinton, Gordon, and Heinhorst 1997), the presence of a peptidoglycan (murein) cell wall remnant (Aitken and Stanier 1979, Pfanzagl and Loeffelhardt 1999), a concentric arrangement of unstacked thylakoids (Hall and Claus 1963, Kugrens, Clay, Lee and Meyer 1999), only chlorophyll a, and light harvesting phycobilisomes (Trench and Ronzio 1978, Kugrens et al. 1999). While cyanoplasts are in general relatively similar in their structure, host cell morphology varies considerably and includes unicellular flagellates, unicellular non-flagellates, and planktonic or attached colonies (Kies and Kremer 1989). Reproduction occurs by zoospores, by mitosis, and by autospores, but sexual reproduction is unknown.

The most studied genus in this division is *Cyanophora*, which currently contains *Cyanophora paradoxa*, *C. biloba* Kugrens, Clay, Lee & Meyer and *C. tetricyanea* Korschikoff (1941). Whether *C. tetricyanea* represents a separate species remains to be determined. *C. paradoxa* was discovered in dirty mud puddles in the Ukraine (Korschikoff 1924), and currently there are two strains of *C. paradoxa* that differ slightly in cyanellar genome size: strain LB555UTEX with a size of 127 kb and the Kies strain 1555 of 138 kb (Loeffelhardt, Mucke, Crouse and Bohnert 1983). Differences in cyanoplast genome restriction patterns and in the structure of nuclear rDNA units between the two strains could possibly justify renaming the two isolates as separate species (Loeffelhardt and Bohnert 1994). In Colorado *C. paradoxa* often forms visible blooms in Quincy Reservoir in Denver and in Pond B-1 at the Department of Energy’s Rocky Flats Plant near Broomfield during April and May (personal observation). Thus it can be an important constituent of the phytoplankton populations in some Denver area reservoirs. *C. biloba* was isolated from a bloom in an ephemeral alpine pond during

August 1996, at an elevation of 3397 m (Kugrens et al. 1999), and a culture has been deposited in the UTEX Culture Collection of Algae.

Detailed ultrastructural studies have been conducted on *C. paradoxa* and *C. biloba* and their cyanoplasts (Hall and Claus 1963, Mignot, Joyon and Pringsheim 1969, Trench, Pool, Logan and Engelland 1978, Kies and Kremer 1986, 1989, Kugrens et al. 1999). In addition, considerable biochemical and molecular data exist on the *C. paradoxa* cyanoplast and host cytoplasmic components (Floener and Bothe 1982, Trench and Ronzio 1978, Betsche, Schaller and Melkonian 1991, Schlichting and Bothe 1993, Bhattacharya, Helmchen, Bibeau, and Melkonian 1995, Helmchen, Bhattacharya, and Melkonian 1995). Kies and Kremer (1989) provided an excellent overview of the Glaucocystophyta in a review article; however, additional information has been published on *Cyanophora* since that time. It is the intent of this chapter to provide structural descriptions for the two species of *Cyanophora* and to compare ultrastructural data with molecular data to determine which characters might be useful in phylogenetic considerations.

2. *Cyanophora paradoxa* Cell Structure

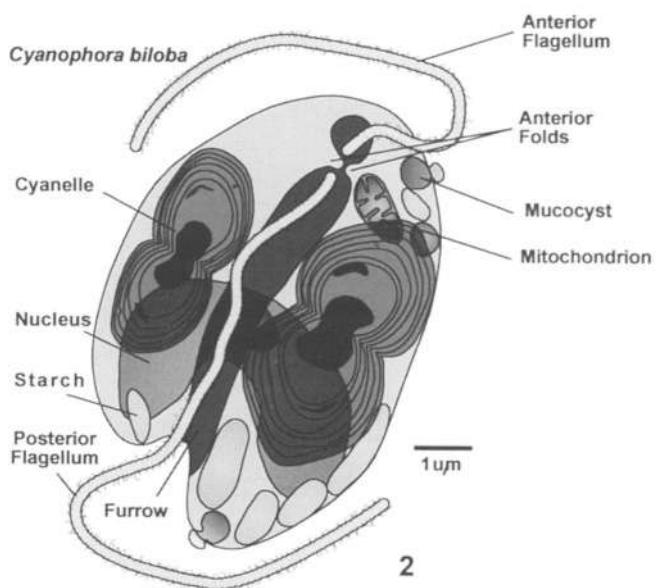
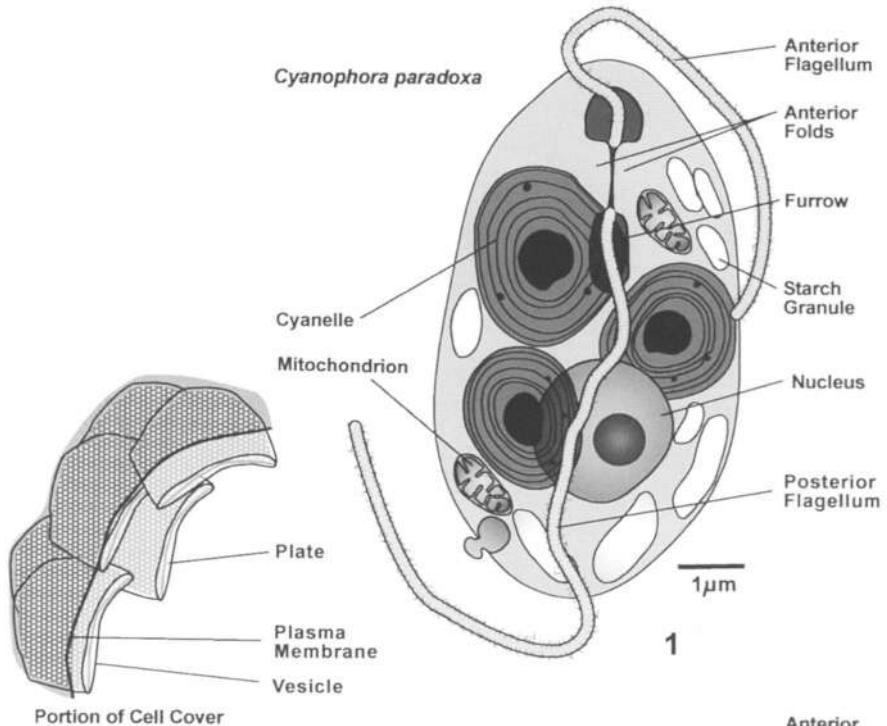
2.1. LIGHT MICROSCOPY

C. paradoxa is an oval shaped cell about 15 μm long and 9 μm wide. It has two subapically inserted flagella, one directed anteriorly while the other is directed posteriorly. The 1-3 μm coccoid to oval cyanoplasts vary in number, but usually range from 4 to 6, although sometimes as few as 1 or as many as 8, are found in the cytoplasm (Hall and Claus 1963, Kies and Kremer 1989). Starch grains are distributed throughout the cytoplasm (Mignot et al. 1969 and Trench et al. 1978).

2.2 SCANNING ELECTRON MICROSCOPY OF *C. PARADOXA*

In addition to confirming that cells are obovate, SEM of *C. paradoxa* shows that they have a shallow ventral furrow in the anterior one third of the cell and two large anterior folds that extend over the insertion points of both flagella (Figs. 1, 3, 4). The furrow extends from the anterior end of the cell, and it is approximately 2.5 – 4.0 μm in length. These anterior folds can either be separated or they may touch each other (Fig. 3). The folds are approximately 1.5-2.0 μm in length. Flagella are inserted subapically with one flagellum oriented anteriorly, whereas the second one is oriented posteriorly. Both flagella bear fine, non-tubular hairs. A portion of the posterior or trailing flagellum appears to reside in the shallow depression formed by the ventral furrow (Figs. 1, 3, 4). Both flagella bear numerous, solid fibrillar hairs (Fig. 1).

Fig. 1. Diagram of *Cyanophora paradoxa* showing cellular features. Fig. 2. Diagram of *Cyanophora biloba* showing cellular features.



2.3 INTERNAL CELL STRUCTURE

Freeze-fractured replicas of cells reveal an array of irregularly shaped plate areas. (Fig. 6) These plate areas may overlap, imparting a shingle-like appearance, and there may be as many as four plates that overlap (Figs. 5, 8).

A transverse section of the cell (Fig. 5) shows that the cell covering consists of thin, flattened, membranous plate vesicles located just underneath the plasma membrane. The partial overlap of plate vesicles is evident, and there may be as many as four plates that overlap. Thin lines are visible in some vesicles (Fig. 5), and these probably represent plates. Microtubules also are present beneath some of the plate vesicles. Each plate is approximately 10 nm thick. The thickness of the plates may be affected by fixation and/or dehydration since they do not fill the vesicle completely, although freeze-fracture replicas also suggest that the plates are thin. Furthermore, the plates appear angular and crystalline (Kugrens et al. 1999).

2.4. CYANOPLAST STRUCTURE

Cyanoplasts vary in number and they are oval (Fig. 8), although sometimes cyanoplasts with constrictions are observed, probably representing dividing cyanoplasts. Each cyanoplast has two enveloping membranes, the thylakoids are concentrically arranged, and phycobilisomes are attached to the thylakoids. A prominent central body is located in the center of the cyanoplast, and it may represent a carboxysome. Cell wall remnants are not easily visualized in sectioned material until the cyanoplast divides, and the wall is seen in the division furrow.

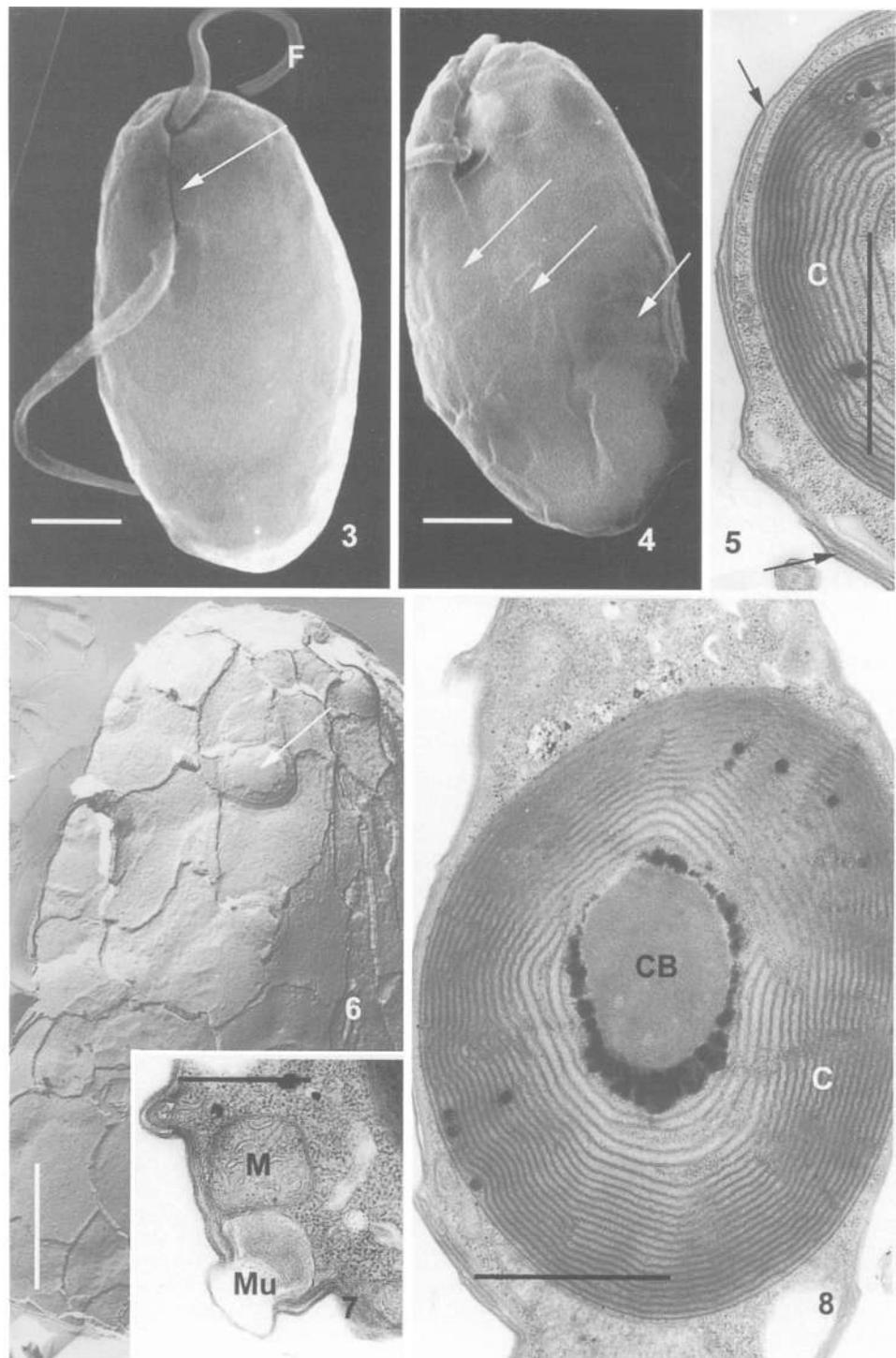
Mucocysts are membrane bound vesicles that protrude beyond the plasma membrane. Such mucocysts contain fibrillar and crystalline materials and are visible in freeze-fractured and sectioned cells (Fig. 7).

3. *C. biloba* Cell Structure

3.1 LIGHT MICROSCOPY

Cells are 10 – 15 μm long and 6 – 9 μm wide and 3 – 4 μm deep. The most unique feature of cells is the presence of two cyanoplasts, with one cyanoplast occurring along each side of the cell, imparting a bilobed appearance to the cells. Each cyanoplast is constricted in the center, and each is oriented parallel to the cell's longitudinal axis. Two

Figs. 3-8 are electron micrographs of *C. paradoxa*. Fig. 3. Scanning electron micrograph showing touching furrow folds. Fig. 4. SEM showing surface relief of some plates (arrows). Fig. 5. Section of a cell showing overlapping plates as part of the cell covering (arrows). Note the concentric single thylakoids in the cyanoplast (C). Fig. 6. Freeze-fracture replica of a cell indicating the various shapes and sizes of the plates. A mucocyst (Mu) is present (arrow). Fig. 7. Portion of a cell showing the typical shape and contents of a mucocyst. A mitochondrion (M) is adjacent to the mucocyst. Scale bar is 0.5 μm . Fig. 8. Cyanoplast (C) with its characteristic concentric thylakoid structure and cell body (CB, carboxysome). Figs. 3 and 6 from Kugrens et al. (1999). Scale bars = 10 μm unless otherwise indicated in the legends.



subapically inserted flagella originate near the anterior end of the furrow. One flagellum is directed toward the anterior whereas the other is directed toward the posterior. Starch granules occur in the cytoplasm.

3.2 SCANNING ELECTRON MICROSCOPY OF *C. BILOBA*

Cells have two large longitudinal lobes that are separated by a deep and wide central furrow (Fig. 9). Two subapically inserted flagella originate near the anterior end of the furrow. The flagella are oriented in opposite directions and the posterior one partially follows the furrow. The insertion point of the anteriorly directed flagellum is flanked by two relatively small anterior folds (Fig. 9). Both flagella are covered uniformly with thin, fibrillar hairs (Kugrens et al. 1999), thus the flagellar ornamentation is similar to *C. paradoxa*. (Kies 1979, Thompson 1973)

3.3 INTERNAL STRUCTURE OF *C. BILOBA*

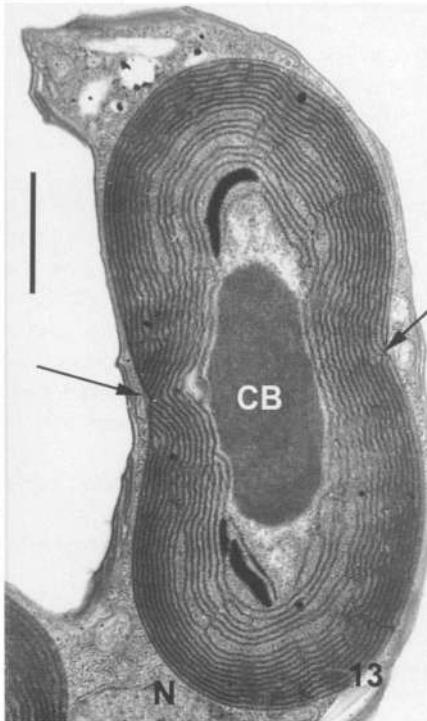
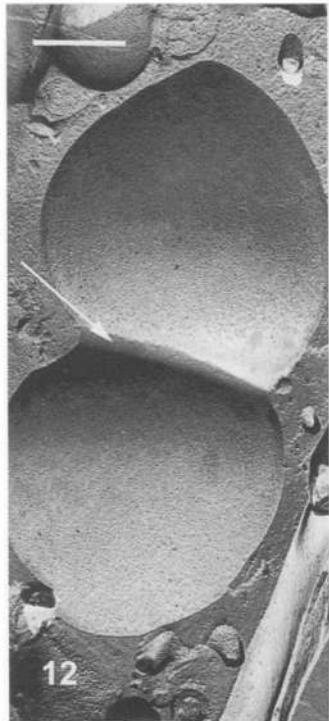
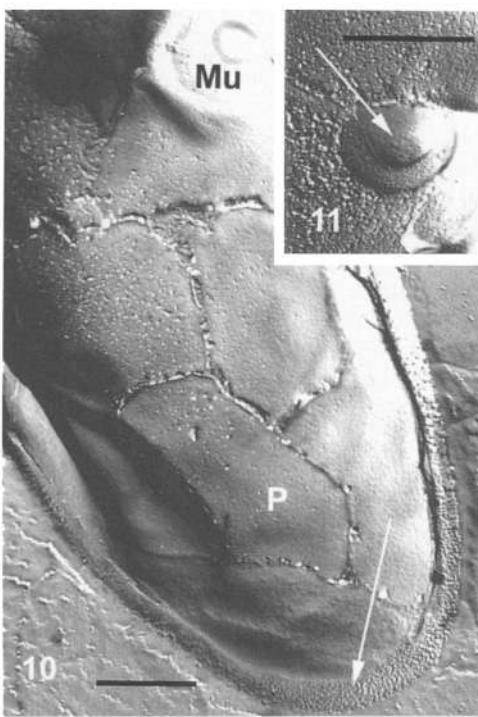
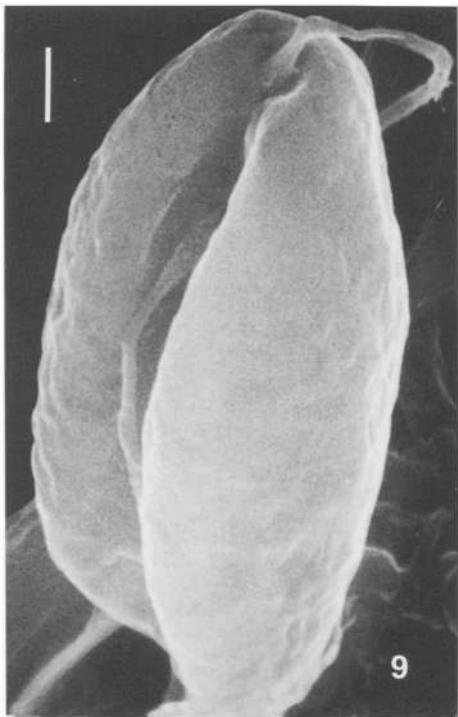
The cell is covered by cell plate vesicles that are irregular in shape, generally range 3-4 μm in length and width, and often overlap (Figs. 10, 11). Freeze etch of the plate vesicles demonstrated that the plates are crystalline, based on subunits being arranged in a repeating pattern (Kugrens et al. 1999). The plates are more angular than the vesicles in which they occur, although this may be a function of the fracture rather than the actual shape.

In sectioned cells the complex nature of the cell covering can be visualized. It consists of the plasma membrane, bearing short fibrillar material, and flattened, overlapping vesicles beneath the plasma membrane (Fig. 10). These vesicles contain granular material and often are seen as thin lines in side view (Fig. 14). These thin contents are approximately 10 nm thick but are difficult to visualize. Furthermore, the flattened vesicles are appressed to the plasma membrane (Fig. 14). Microtubules subtend some of these plate vesicles.

Mucocysts appear as elevated areas among the plates (Figs. 10, 11), but there aren't any distinct openings within the plates for the mucocysts. Instead, mucocysts between plate borders where they could extrude their contents through these discontinuities in the cell covering. A crystalline, light-staining material partially fills the mucocyst. The mucocysts of *C. biloba* have the same structure as described for *C. paradoxa* (Kies 1979, Kugrens et al. 1999).

Each cell contains two cyanoplasts, a posterior nucleus (Fig. 13), peripheral mucocysts, and cytoplasmic starch positioned in the peripheral cytoplasm (Fig. 14). One cyanoplast is located in each lobe.

Figs. 9-14 are electron micrographs of *C. biloba*. Fig. 9. SEM of a typical cell. Fig. 10. Freeze-fracture replica of a lobe showing the surface mucilage (arrows) and plates (P) that comprise the cell covering. A mucocyst (Mu) is also present. Fig. 11. Freeze-fracture replica of a mucocyst with an outer protruding region. Fig. 12. Freeze-fracture replica of a lobe showing the surface of a cyanoplast and its characteristic constriction in the mid-region. Fig. 13. Section of a cell through one of the lobes, showing the general shape of a cyanoplast. Arrows indicate the constricted region, with a cell body (CB, carboxysome) in the center. Fig. 14. Section of a cell showing an overlap of plate vesicles (arrow) and a starch grain (S) in the peripheral cytoplasm. Figs. 9 and 12 from Kugrens et al. (1999). Scale bars = 10 μm unless otherwise indicated in the legends.



3.4 CYANOPLAST ULTRASTRUCTURE OF *C. BILOBA*

Each cyanoplast has a central constriction (Figs. 12, 13). Longitudinal sections through the cyanoplast reveal concentric unappressed thylakoid membranes (Figs. 13, 14), and elongate, dark regions near the innermost thylakoid membranes that may represent thylakoid membrane reserves (Fig. 13). The cyanoplast thylakoids are single (Figs. 13, 14), and phycobilisomes are randomly distributed over the stromal side of the thylakoid membranes. A dense central body (Fig. 13) may be a protein body reserve, a pyrenoid precursor, or a carboxysome. Dark staining lipid bodies are scattered between some of the thylakoid membranes. A two-layered cyanoplast wall is most apparent in the constricted region. This remnant of a cell wall also has been reported for *C. paradoxa*. (Kies 1979).

Released cyanoplasts remain intact for several weeks or more and retain their shapes and constrictions until they degenerate. Thylakoids also remain distinct during this time.

4. Phylogeny of *Cyanophora*

4.1 EUKARYOTIC HOST CELL COMPARISONS BETWEEN *C. PARADOXA* AND *C. BILOBA*

The two species that have been examined differ in cell shape, furrow length, number and structure of cyanoplasts, and plate sizes. The cells of *C. paradoxa* are obovate and have relatively larger anterior folds that flank or cover the insertion points of both flagella (Figs. 1, 3-5). Conversely, cells of *C. biloba*, have a bilobed shape and have small folds covering the insertion points of the flagella (Fig. 2, 10). A shallow furrow that does not extend the full length of the cell is present in *C. paradoxa*, whereas *C. biloba* possesses a deep furrow that runs the full length of the cell, and is flanked by the two lobes (Figs. 3 and 9).

Cells in both species have flattened cortical vesicles (Mignot *et al.* 1969) that have a shingle-like arrangement (Trench *et al.* 1978), with plates inside these vesicles. The plates of *C. paradoxa* are 0.5-3 μm in size, they have a thickness of about 10 nm, and they are barely detectable in cross section (Fig. 8). The plates in cortical vesicles of *C. biloba* are larger, measuring 3-4 μm in length and width. While the plate vesicles in *C. paradoxa* overlap considerably, the plate vesicles in *C. biloba* generally touch without overlapping. Vesicle and plate biogenesis are not known for either species; however, they presumably are produced by the endoplasmic reticulum or the Golgi apparatus.

4.2 CYANOPLAST COMPARISONS BETWEEN *C. PARADOXA* AND *C. BILOBA*

The cyanoplasts of *C. paradoxa* and *C. biloba* have two structural differences. In *C. paradoxa*, there are some granules that are lighter staining and slightly larger than the lipid bodies in the cyanoplasts (Hall and Claus 1963, Kugrens *et al.* 1999). These are not present in *C. biloba*. Second, the cyanoplasts of *C. biloba* have persistent, deep constrictions which probably represent incomplete cleavage furrows. Such a furrow was

observed frequently by Hall and Claus (1963) in *C. paradoxa* cyanoplasts which precedes host cell division. However, this furrow is a transitory structure in the cyanoplasts of *C. paradoxa*, but it is a persistent feature of *C. biloba*, lasting until cell division commences. Finally, *C. paradoxa* has a variable number of cyanoplasts (Hall and Claus 1963), but it can have as few as one or as many as eight (Trench *et al.* 1978). Non-dividing cells of *C. biloba* invariably possess two cyanoplasts per cell.

4.3 ENDOSYMBIOSIS

The concept that chloroplasts evolved from the endosymbiosis of a cyanobacterium was first proposed by Mereschkowsky (1905). Pascher (1914) coined the term cyanelle for putative cyanobacterial endosymbionts, and the association between the cyanelle and heterotrophic host was called a cyanome. It was later discovered that chloroplasts (Ris 1961, Ris and Plaut 1962) and mitochondria (Nass and Nass 1963 a, b) contain their own DNA. This ultimately led to the Serial Endosymbiosis Theory or SET (Goksoy 1967, Margulis 1970, Taylor 1974) which described the bacterial origins of mitochondria and chloroplasts. SET proposes that plastids and mitochondria were derived from prokaryotes that were engulfed by eukaryotes and, subsequently, established themselves as endosymbionts. This symbiotic relationship became more and more obligate by gene transfer from the endosymbiont to the host (Schenk 1994).

By providing more efficient energy production, the symbionts (protomitochondrion and protoplastid) imparted a selective advantage to the ancestral, energetically inefficient host cell that lived in an increasingly aerobic environment. Over time, most of the endosymbiont's genetic information was transferred to the host nucleus, and the former endosymbiont became an organelle (Gray 1984, Cavalier-Smith and Lee 1985).

Several lines of evidence strongly support the endosymbiotic origin of chloroplasts. The supporting biochemical, physiological and morphological data led Gray and Doolittle (1982) to conclude that there is no doubt that plastids evolved from photosynthetic prokaryotes. These observations include the sensitivity of chloroplasts to prokaryotic, but not eukaryotic, protein inhibitors (e.g. chloramphenicol), the presence of prokaryotic 70S ribosomes in chloroplasts, the presence of a single circular DNA molecule in chloroplasts, and the fact that the inner chloroplast envelope is high in the phospholipid cardiolipin found elsewhere only in mitochondria and bacteria. The outer chloroplast membrane, however, does not possess cardiolipin.

Molecular data also strongly support SET. For example, sequence studies employing the small (Bonen and Doolittle 1975) and large ribosomal subunit genes (Phillips and Carr 1981), the amino acid sequences of several proteins (Schwartz and Dayhoff 1981) and DNA-RNA hybridization comparisons between chloroplasts, nuclei, and cyanobacteria (Pigott and Carr 1972) all display a strong similarity between chloroplasts and free-living cyanobacteria, and little similarity between chloroplasts and the surrounding host cell.

Phylogenetic inferences regarding an autotrophic protist is complicated, given that both plastids and mitochondria are of apparent bacterial ancestry. In this case, the components that make up the *Cyanophora* chimaera - the host, the cyanoplasts and the mitochondria - are probably descended from three divergent evolutionary lines. The phylogenies of the host and the cyanoplasts are discussed briefly.

4.4 EUKARYOTE PHYLOGENY

From a molecular perspective, recent information on actin gene introns (Bhattacharya and Klaus 1997) and on maximum likelihood analyses (Bhattacharya *et al.* 1995) using 18S ssrRNA data suggest that the eukaryotic components of *Cyanophora paradoxa*, *Glaucoctysis nostochinearum*, and *Gloeochaete wittrockiana*, form a distinct lineage. This tree topology also suggests that cryptomonads may be a sister group to the Glaucoctystophytes. However, bootstrap values from neighbor-joining and weighted maximum parsimony analyses on the same data set were reported as 59 and 68, respectively. These relatively low values indicate weak branch support and, therefore, a cryptomonad/glaucoctystophyte association seems tenuous. Furthermore, tubulin gene analyses in *Cyanophora* and the cryptomonads *Guillardia theta* and *Goniomonas truncata* (Keeling, Deane, Hink-Schauer, Douglas, Maier, and McFadden 1999) also do not support a phylogenetic relationship between the cryptomonads and *Cyanophora*.

Cytological support for a cryptomonad/glaucoctystophyte relationship also is limited (Kugrens 1999, Clay, Kugrens and Lee 1999). Although cryptomonads and glaucoctystophytes possess plates as integral parts of their cell coverings, the plates in cryptomonads are never located inside vesicles. Second, both possess extrusomes (Kugrens *et al.* 1999), however, the mucocysts in glaucoctystophytes and the ejectisomes in cryptomonads differ in structure and function and therefore are not homologous. Third, bipartite tubular hairs adorn at least one flagellum in cryptomonads (Kugrens and Lee 1991) but glaucoctystophyte flagella only bear non-tubular hairs. Finally, cryptomonads have a characteristic transition region consisting of two transverse septa (Kugrens and Lee 1991). The uppermost septum occurs in the vicinity of the origin of the central axonemal MT's, and the lower septum has a central thickening (Taylor and Lee 1971, Kugrens and Lee 1991). Conversely, the flagellar transition region of *Cyanophora* appears to be simple, and a single transverse partition and an invagination of the plasma membrane just above the cell are the major structural features (Mignot *et al.* 1969). In conclusion, platycristate mitochondria are the only structural feature that cryptomonads and *Cyanophora* share. These collective cytological data suggest that a cryptomonad/glaucoctystophyte relationship is tenuous at best.

One of the primary cytological characteristics that is used in protist comparisons is the cell covering. The additional information provided on this elaborate cell covering (Kugrens *et al.* 1999) suggests a possible phylogenetic relationship of *Cyanophora* to two other protists. Superficially, the *Cyanophora* cell covering appears to be structurally similar to the dinoflagellate amphiesma. An amphiesma consists of flattened vesicles, termed alveoli, which contain polysaccharide plates and are associated with subpellicular microtubules. They vary in structural complexity and it is those of intermediate complexity that most resemble *Cyanophora*. For instance, plate overlap which is common in *Cyanophora*, is also present in some gonyaulacoid and peridinoid amphiesmas (Taylor 1987). In dinoflagellates the plate junctions, called sutures (Dodge and Crawford 1970, Tomas and Cox 1973, Dodge 1987) are similar in appearance to the plate boundaries seen in *C. biloba*. Finally, some sub-pellicular microtubules, which are integral parts of amphiesmas, also were found underneath the cell covering of both *Cyanophora* species. While structural evidence of the cell covering suggests a *Cyanophora*/dinoflagellate relationship, the primitive mitosis (Pickett-Heaps 1972, Trench, Pool, Logan and Engelland 1978) and the type of nucleus

found in the dinoflagellates (Dodge 1966, Kubai and Ris 1969, Ris and Kubai 1974, Triemer 1982) probably rules this group out as a close relative of *Cyanophora*.

Another protist that displays a possible structural affinity to *Cyanophora* may be the colorless, protalveolate, *Colponema* (Mignot and Brugerolle 1975). *Colponema* lacks cyanoplasts, but both have subapical flagellar insertions and both have an anteriorly directed flagellum and a posteriorly directed flagellum. The posterior flagellum of both organisms lies in a ventral furrow. Also, the flagella in both genera have fibrillar hairs. The flagellar transition regions of both protists possess thick transverse partitions near the cell level. Perhaps the most important structural commonality between the two are the presence of flattened cortical vesicles (alveoli) that underlie the cell membrane. Cortical alveoli are common to *Colponema*, ciliates, apicomplexans, dinoflagellates, and possibly glaucocystophytes. Furthermore, several researchers have proposed that ciliates and dinoflagellates evolved independently from a *Colponema*-like protalveolate, and *Cyanophora* may also be derived from a similar type of organism. Whether the alveoli in *Colponema* contain plates needs to be reexamined since the single study of *Colponema* (Mignot and Brugerolle 1975) did not show the presence of plates inside the vesicles, as in fact, most studies on *Cyanophora* have not. Despite the similarities between *Cyanophora* and *Colponema* they differ in several features. The posterior flagellum of *Colponema* differs from *Cyanophora* by the presence of wings and absence of hairs. *Cyanophora* has mucocysts, while *Colponema* possesses extrusive organelles in the form of toxicysts. *Colponema* also possesses tubular mitochondrial cristae whereas *Cyanophora* has flat cristae that also occur in cryptomonads, red algae, prasinophytes and higher plants, but not in other protozoa. While this is a compelling hypothesis, many details are lacking in the description of *Colponema*. Therefore, more investigations, including molecular studies, are needed for *Colponema* to test the *Cyanophora/Colponema*-like ancestry hypothesis.

4.5 CYANOPLAST PHYLOGENY

Molecular data on cyanoplast phylogeny indicate that the cyanoplast of *Cyanophora* may be an evolutionary intermediate between cyanobacteria and true chloroplasts. Similarities between these two structures include the presence of a reduced peptidoglycan cell wall (Schenk 1970, Aitken and Stanier 1979, Kraus, Gotz and Loeffelhardt 1990, Pfanzagl, Zenker, Pittenauer, Allmaier, Martinez-Torrecuadrada, Schmid, Pedro and Loeffelhardt 1996, Zenker, Pittenauer, Pfanzagl, Loeffelhardt and Allmaier 1996, Kies and Kremer 1986, 1989, Pfanzagl and Loeffelhardt 1999), a concentric arrangement of unstacked thylakoids (Hall and Claus 1963, Kugrens et al. 1999), and the presence of chlorophyll *a* and phycobilisomes (Chapman 1966, Trench and Ronzio 1978, Kies 1979, Kies and Kremer 1986, 1989, Kugrens et al. 1999). Just as in rhodoplasts, cyanoplasts have retained the ability to code for the small subunits of the carbon dioxide fixing enzyme ribulose-1,5-bisphosphate carboxylase/oxygenase (Rubisco), whereas chloroplasts have transferred this gene to the cell nucleus. Heterologous hybridization studies on the genes of the large and small subunits of Rubisco reside in the cyanoplast (Heinhorst and Shively 1983).

In cells with chloroplasts, genes that code for ribosomal proteins (Bayer and Schenk 1989, Bryant and Stirewalt 1990, Michalowski, Pfanzagl, Loeffelhardt and Bohnert 1990), genes that code for the electron transport chain protein ferredoxin I (Neumann-

Spallart, Brandtner, Kraus, Jakowitsch, Bayer, Maier, Schenk and Loeffelhardt 1990), and genes that code for NAD⁺ biosynthetic pathway enzyme quinolinate synthetase (Michalowski, Flachmann, Loeffelhardt and Bohnert 1991) have been transferred to the nucleus. However, in *Cyanophora*, these genes have been retained in the cyanellar DNA and provide the cyanoplast with more autonomy than a chloroplast. Nonetheless, there are enough characteristics which imply that the cyanoplast is a true chloroplast. These include a similar pathway for nitrate reduction (Floener, Danneberg and Bothe 1982), the lack of NADPH-dependent respiration (Floener and Bothe 1982), a genome size of about 130 kbp, which is about 10% the size of the cyanobacterial genome (Breiteneder, Seiser, Loeffelhardt, Michalowski and Bohnert 1988, Herdman and Stanier 1977), and the presence of nuclear encoded proteins in the cyanoplast (Bayer and Shrenk 1986, Burnap and Trench 1989, Bayer *et al.* 1990). The latter observation implies that a sophisticated N-terminal signal sequence import mechanism has evolved which targets proteins translated in the cytoplasm to the chloroplast. Finally, cyanoplasts cannot live outside the host cell, which also is similar to chloroplasts (Trench *et al.* 1978).

5. Conclusions

Phylogenetic analyses from gene mapping and sequence studies place the cyanoplast somewhere between cyanobacteria and chloroplasts (Lambert, Bryant, Stirewalt, Dubbs, Stevens and Porter 1985, Kraus, Gotz and Loeffelhardt 1990, Valentin and Zetsche 1990, Morden, Delwiche, Kuhsel, and Palmer 1992 and Evrard, Weil and Kuntz 1990). However, *Cyanophora* continues to be an enigma among protists. Although it now appears that the cyanoplast represents an independent line towards the evolution of a true chloroplast, the origins of the eukaryotic component are still uncertain, as indicated by the contrasting structural and molecular evidence.

6. References

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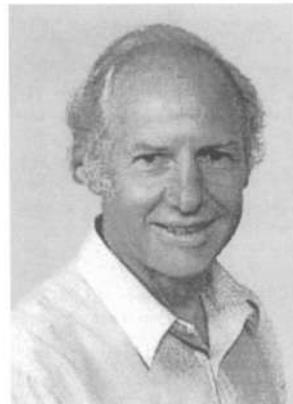
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THE MICROENVIRONMENT AND PHOTOSYNTHETIC PERFORMANCE OF *PROCHLORON* SP. IN SYMBIOSIS WITH DIDEHMNID ASCIDIANS

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1. Introduction

Prochloron spp. (with the type species initially called *Synechocystis didemni* and later renamed *Prochloron didemni* (Lewin 1977)) are oxygenic photosynthetic prokaryotes, i.e. oxyphotobacteria, living in symbiosis almost exclusively with didemnid ascidians in tropical waters (see Lewin and Cheng 1989 for a comprehensive overview). The presence of phototrophic microorganisms (Maurice 1888; Smith 1935) and oxygen production in ascidians (Tokioka 1942) has been known for a long time. However, it was the discovery of *Prochloron* (Lewin 1975; Newcomb and Pugh 1975), and especially its, among prokaryotes unique, pigment composition with chlorophylls *a* and *b* but absence of phycobilins (Lewin and Withers 1975), that triggered more intensive studies of the symbiosis in didemnid ascidians. Thereby, also other prokaryotic symbionts, like cyanobacteria (Lafargue and Duclaux 1979; Larkum *et al.* 1987) and the conspicuous chl *d* containing oxyphotobacterium, *Acaryochloris marina* (Miyashita *et al.* 1996), were discovered in didemnid ascidians. Furthermore, two different free-living prochlorophytes were found (Burger-Wiersma *et al.* 1986; Chisholm *et al.* 1988).

Part of the great interest in *Prochloron* is due to the implications of its photosynthetic apparatus and ultrastructure for our view of the evolution of oxygenic photosynthesis in eukaryotes. Initially the possession of chl *b* and appressed thylakoids led to the suggestion that *Prochloron* was descended from a prokaryotic oxyphotobacterium which gave rise to the green plastids of algae and higher plants (and inspired the name *Prochloron* as well as the general term prochlorophyte (see Lewin 1984). This claim was strengthened by the discovery of the free-living prochlorophytes. Later it was shown that chl *b* was bound to a protein, which is unrelated to the Cab protein from plastids of green algae and higher plants (La Roche *et al.* 1996). Furthermore, SSU-rRNA analysis has established that the three known prochlorophytes all lie within the cyanobacterial radiation, but each on a different branch (Turner 1997). Thus the idea of a direct connection between prochlorophytes and green plastids can no longer be entertained. Nevertheless, the similar pigment composition and structural similarity (such as the presence of appressed thylakoids) makes a comparison of the photosynthetic properties between prochlorophytes and green plastids of considerable

interest, since the prochlorophytes lack the phycobilisome system that typifies cyanobacteria, and gives cyanobacteria photosynthetic characteristics rather different to green plastids (see below).

Despite intensive studies of *Prochloron* cells and of intact ascidians with *Prochloron*, all attempts to cultivate *Prochloron* have failed so far, and a report of partial isolation success (Patterson and Withers 1982) remains unconfirmed. However, literally nothing is known about the microenvironmental conditions that *Prochloron* experiences in its ascidian hosts, and this may in part explain failure to obtain isolates. In this chapter we review recent studies on the ecophysiology of *Prochloron* with emphasis on the microenvironmental characteristics that one can deduce from such studies or, in some cases, directly measure in intact *Prochloron*-ascidian associations.

2. Distribution and photosynthetic characteristics of *Prochloron*

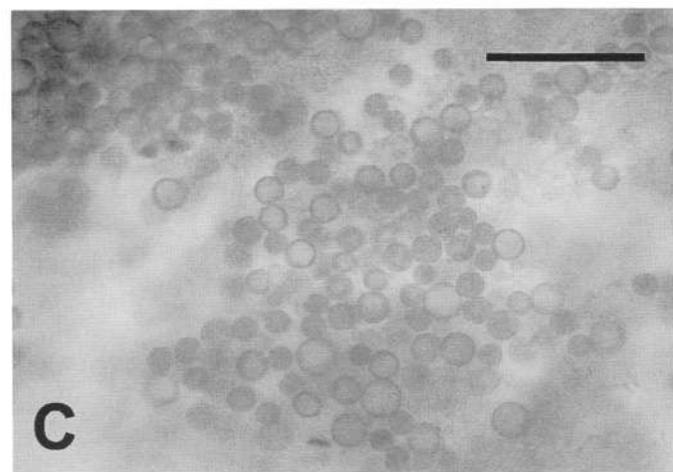
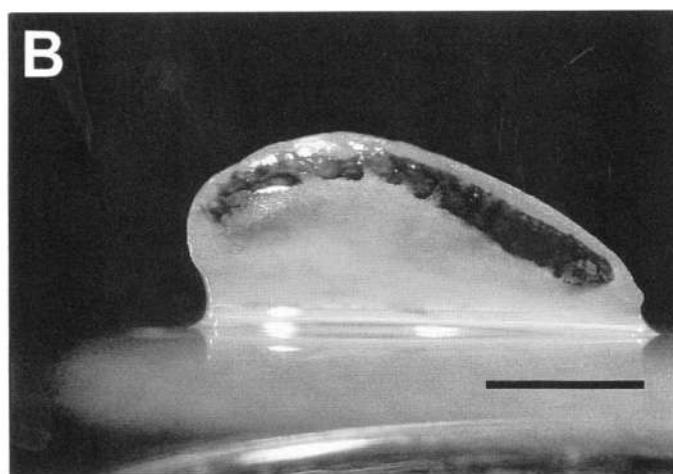
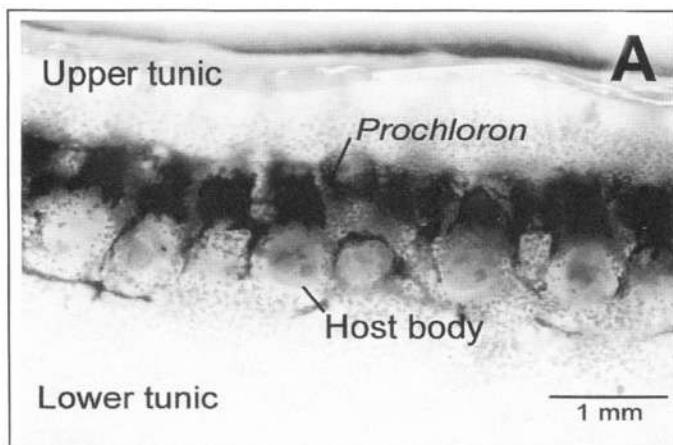
2.1 DISTRIBUTION, CARBON AND NITROGEN TRANSFER IN THE HOST

2.1.1 Distribution in the host

Prochloron cells line the common cloacae or are embedded in folds of the gelatinous test matrix of colonial didemnid ascidians (Smith 1935; Pardy and Royce 1992) (Fig. 1A,B). *Prochloron* has also forms biofilms on other ascidians (Lewin 1975), sponges (Parry 1986), and holothurians (Cheng and Lewin 1984). In most cases *Prochloron* occurs embedded in a mucilaginous matrix extracellular to the host cells (see e.g. Thinh and Griffiths 1977, Fig. 1C), but intracellular occurrence in phagocytes within the ascidian test has also been reported (Hirose *et al.* 1996, 1998; Lewin and Cheng 1989).

The test is composed of a transparent or slightly opaque gelatinous (in some species more fibrous or even cartilaginous) matrix of protein and cellulose-like carbohydrates, and with a thin tough cuticle composed almost entirely of protein (Goodbody 1974). In many, but not all, didemnids the test also contains calcareous spiculospheres. The test is interwoven by blood vessels, microfibrils and host cells like vanadocytes and amoebocytes. Host cells in the test contain highly acidic ($\text{pH} < 2-3$) vacuoles of sulfuric acid, and can contain high concentrations of reduced organo-metallic complexes, especially with vanadium and iron (Carlisle 1968). A survey of Stoecker (1980) showed that didemnid ascidians from Bermuda especially accumulated iron and less vanadium, but if this is a general property of didemnids is to our knowledge unknown. *Prochloron* can also accumulate high levels of phenolic compounds (Barclay *et al.* 1987). Acidity and high concentrations of reduced metal compounds and phenolics help protect ascidians against overgrowth and grazing (Stoecker 1980). The test is thus a light transparent living tissue providing a structured and protected environment with a large internal surface area for colonization by *Prochloron*.

Figure 1. *Prochloron* and its distribution in tissues of didemnid ascidians. A. Thin section through the ascidian *Lissoclinum patella*, showing the presence of green *Prochloron* cells in the cloacal cavities under the upper tunic/test of the ascidian (modified from Dionisio-Sese *et al.* 1997, with permission of Springer Verlag and Dr. Tadashi Muruyama). B. Vertical cut through *Diplosoma virens*. *Prochloron* cause the green coloration below the transparent upper tunic/test. C. *Prochloron* cells from *Diplosoma virens* embedded in mucous slime matrix. Note the characteristic peripheral arrangement of the thylakoids. The physical dimension is indicated by scale bars in each picture, i.e. 1 mm in panel A and B, and 0.1 mm in panel C. 



2.1.2 Carbon transfer to the host

Several studies (since the first report by Tokioka 1942) have demonstrated net oxygen production from illuminated intact ascidians with *Prochloron* (Thinh and Griffiths 1977; Pardy 1984; Olson 1986; Alberte 1987; Griffiths and Thinh 1987). Ratios of net oxygen evolution to total respiration range from ~0.6 to ~9 (reviewed in Alberte 1989). The presence of photosynthetic *Prochloron* can significantly enhance the host respiration (Pardy 1984) and the growth rate of the ascidian host in light (Olson 1986). Engulfment of *Prochloron* by host amoebocytes has been suggested as a way of, albeit slow, carbon transfer (Cox 1983). A fast transfer of photosynthates to the host has been demonstrated (Pardy and Lewin 1981; Griffiths and Thinh 1983) involving solute exchange of a range of early products of photosynthesis (Kremer *et al.* 1982). The mechanisms of the fast exchange remain unsolved, but the transfer of photosynthate from *Prochloron* to the host has been estimated to contribute up to ~60% of the hosts carbon demand (Alberte 1987). However, the contribution of *Prochloron* to the carbon demand of their host's differs among different species (Koike and Suzuki 1996). In ascidians like *Didemnum molle*, the carbon demand cannot be covered by the symbionts and must be supplemented by external carbon uptake of the host, while in *Lissoclinum voeltzkowi* the host's carbon demand can be fully met by *Prochloron* (Koike *et al.* 1993).

2.1.3 Nitrogen exchange and fixation

Ammonium is the major nitrogenous waste product of the ascidian host (Goodbody 1974) and is effectively taken up by *Prochloron* (Parry 1985). Whether the symbionts are nitrogen limited or not is, however, still a matter of debate (see Alberte 1989). A recent report indicates that nitrogen is efficiently recycled within the *Prochloron*-ascidian association (Koike *et al.* 1993). Nitrogen fixation in light was reported in *Lissoclinum patella* (Paerl 1984) but only in intact *Prochloron*/ascidian associations, and neither isolated *Prochloron* cells nor several other ascidians showed significant nitrogenase activity. Another study showed **N₂-fixing** activity in encrusting *Prochloron*/ascidian associations, which was, however, not associated with *Prochloron* itself (Odintsov 1991). In contrast, stable nitrogen isotope signatures of isolated *Prochloron* cells were interpreted as evidence of facultative N₂ fixation by *Prochloron* (Kline and Lewin 1999).

It was speculated (Paerl 1984), that N₂ fixation in light was due to a low oxygen microenvironment of *Prochloron* inside the host effectuated by intense host respiration as well as strong oxygen binding by vanadium-sulfuric acid complexes in the vanadocytes of the host. The role of vanadocytes was, however, disputed by Parry (1985). Aerobic N₂ fixing cyanobacteria have been described (Bergman *et al.* 1997), but the exact mechanisms protecting nitrogenase activity under aerobic conditions remain to be identified. Further studies of nitrogen turnover combined with measurements of the oxygen conditions within didemnid ascidians are needed.

2.2 THE PHOTOSYNTHETIC APPARATUS OF PROCHLORON

While there is still much to be discovered about the photosynthetic machinery of prochlorophytes, the evidence so far indicates that it is similar in many ways to that of

thylakoids of green plastids. The thylakoids in *Prochloron* lie in the cytoplasm near the periphery of the cells (Fig. 1C), forming compact undulating parallel layers and often surrounding a central clear region with the nucleoid and various cytoplasmic inclusions (Cox 1986; Swift 1989). Christen *et al.* (1999) obtained highly active thylakoids from *Prochloron didemni*. Further fractionation after passage through a *Yeda* press indicated that no fraction was enriched in PSII (in contradiction to higher plant thylakoids), indicating a fairly homogenous distribution of PSI and PSII in the thylakoid membrane. **P680⁺** reduction kinetics indicated that the reactions were typical for both cyanobacteria and higher plants, suggesting that there is high conservation of the water-oxidizing complex in all the known organisms that perform oxygenic photosynthesis. This agrees with the evidence, from immunolabeling with antibodies directed against *Prochlorothrix hollandica* antenna protein, showing that the light-harvesting complexes are fairly homogeneously arranged in that prochlorophyte (Bullerjahn *et al.* 1990). D2 protein of PSII and PSI has also been shown to be homogeneously distributed (Lichtlé *et al.* 1995). Nevertheless different regions of thylakoids have been observed, based on freeze-fracture particle distribution (corresponding to appressed and non-appressed regions of thylakoids) (Miller *et al.* 1988). Also, recent evidence has shown that in some prochlorophytes at least (*Prochlorococcus*) there are small differences in the structural components of PSI and in the light-harvesting arrangements of PSI (Van der Staay *et al.* 1999; Garczarek 2000).

The light-harvesting antenna of *Prochloron* appears to be similar to that of other prochlorophytes (La Roche *et al.* 1996). The ca 35 kDa Pcb protein is related to the isiA protein of some cyanobacteria and more to the CP43 and CP47 (psbC/D) products. In *Prochlorothrix hollandica* three Pcb's (A, B & C) have been found; however in *Prochloron* only one Pcb gene has been found (allied to PcbA/B), along with an isiA protein (La Roche *et al.* 1996). In *Prochloron* the Pcb protein binds [3,8-divinyl]-protochlorophyllide (Mg-2, 4-divinyl phaeoporphyrin A₅ monomethyl ester), as well as chl's *a* and *b*, in a light-harvesting capacity (Larkum *et al.* 1994; Helfrich *et al.* 1999).

The existence of state transitions in *Prochloron* is not well established. Whether Pcb moves between the photosystems and thereby redistributes energy between PSI and PSII is not fully established, although evidence to that effect was presented (Post *et al.* 1993); the evidence could be interpreted as the dissociation of Pcb from PSII without its re-association with PSI. Schuster *et al.* (1985) established that the light-harvesting protein in *Prochloron* is permanently phosphorylated in the light. This makes unlikely the system of redistribution found in green algae and higher plants, where control is effected by the redox state of plastoquinone. Furthermore it is not known whether Pcb is actually associated with PSI. Hiller *et al.* (1985) found chl *b* associated with PSI but in the absence of the 35 kDa lhc (Pcb). Thus it is possible that chl *b* is attached to one or more of the core proteins. In *P. hollandica*, van der Staay and Staehelin (1994) found that de-phosphorylation of Pcb (probably PcbC) occurs in the dark, but after much longer periods than in green plastids. They also found evidence for some heterogeneity of PSI and PSII on a microscale.

Thus, there are clear differences between the structure of thylakoids between prochlorophytes and green plastids. Nevertheless, the existence of an intrinsic light harvesting protein in prochlorophytes, in contrast to the extrinsic system in cyanobacteria (based on phycobilisomes), may mean that the photosynthetic reactions

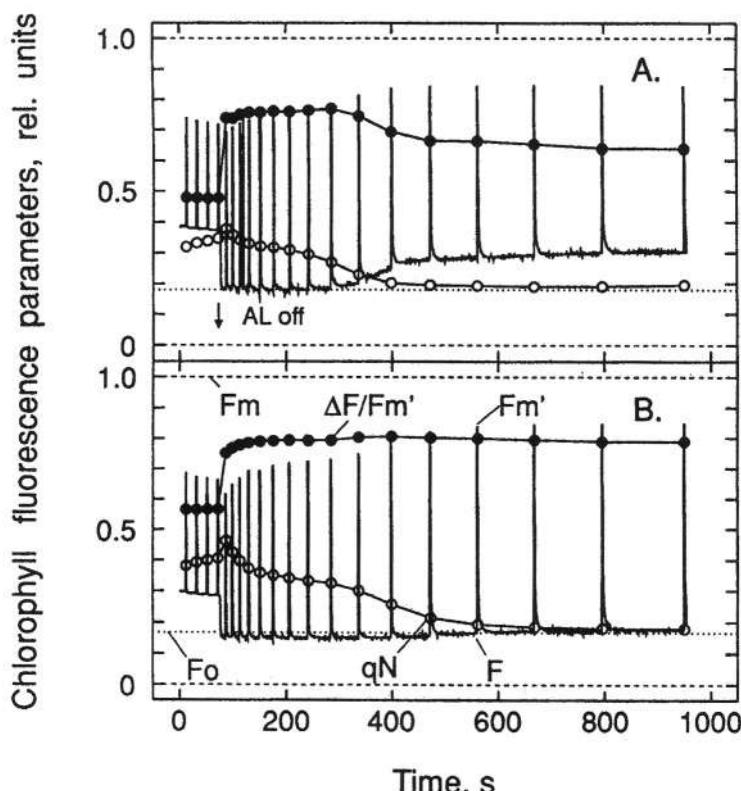


Figure 2. Light-dark relaxation kinetics of fluorescence yield and on-line calculated fluorescence parameters of *Prochloron* in *Lissoclinum patella*. Actinic light (of $170 \mu\text{mol photons m}^{-2}\text{s}^{-1}$) was turned off as indicated (AL off). A. Following AL off, the sample was fully darkened. B. Simultaneously with AL off, continuous far-red light at an intensity of 10 W m^{-2} was applied. The notations of the characteristic fluorescence levels and of on-line calculated fluorescence parameters are given in panel B. Two different samples were used in the experiments of Figs. 3A and B. (from Schreiber *et al.* 1997, with permission of the publisher).

of prochlorophytes show similarities to those of green plastids, where the Cab light-harvesting protein is also intrinsic. In terms of chlorophyll fluorescence the photosynthetic characteristics of *Prochloron* cells are similar to those of higher plants (high PSII yields of ~ 0.8 , and rapidly entrained non-photochemical quenching) and markedly different from those found in cyanobacteria (Schreiber *et al.* 1997). However, there is evidence for the operation of chlororespiration; i.e. that endogenous reductants feed electrons into an intersystem electron transport chain, probably at the plastoquinone level, driven by a cytoplasmic NADPH/NADP reductase. In the dark, oxygen concentration controls the reduction level, keeping PSII acceptors more reduced under low O_2 (Schreiber *et al.* 1997).

Figure 2 (from Schreiber *et al.* 1997) shows the chlorophyll fluorescence kinetics of *Prochloron* cells in hospite following a light-dark shift (after 5 min in the light). Only a small part of non-photochemical quenching (qN) relaxes in the first minute after darkening (in contrast to typical responses of green leaves), and the much slower relaxation of qN is closely followed by an increase in steady-state fluorescence yield, F. These results led to the conclusion that chlororespiration is active in thylakoids, but only while oxygen is present in the tissue, and as soon as O₂ becomes depleted (within ~5 min) F rises (Fig. 2A). However, when far-red light is used as background light to activate PSI the plastoquinone pool is no longer over-reduced at low oxygen concentration. These results are consistent with recent observations that the *Prochloron* containing part of the ascidian goes anaerobic after ~10 min (see Section 3.1).

2.3 IRRADIANCE EFFECTS ON PROCHLORON PHOTOSYNTHESIS

Didemnid ascidians with *Prochloron* inhabit a wide range of irradiance regimes. Some encrusting forms like *Lissoclinum voeltzkowi* thrive on surfaces exposed to high irradiance environments, while species like *Diplosoma similis* and *Lissoclinum punctatum* are confined to shaded environments. Other didemnids like *Lissoclinum patella* and the motile *Diplosoma virens* can inhabit both low and high irradiance environments. *Lissoclinum voeltzkowi* and *D. virens* even survive periods of air-exposure during low tide. In high irradiance environments, UV-radiation and photooxidation are primary stress factors for *Prochloron* photosynthesis, while shaded environments call for efficient use of available irradiance and minimization of self-shading. Especially, species inhabiting varying light regimes exhibit a phenotypic plasticity in their response to light and optimization of symbiont photosynthesis (Alberte 1989).

2.3.1 Photosynthesis and respiration as a function of irradiance

Isolated *Prochloron* cells exhibit high rates of photosynthesis, which can be even higher than rates in free-living cyanobacteria and microalgae (e.g. Critchly and Andrews 1984; Alberte *et al.* 1986; Griffiths and Thinh 1987). Ratios of net photosynthesis to respiration are >5 in high light adapted *Prochloron* cells and can be as high as 16 in cells isolated from shade-adapted colonies (Alberte 1989). Respiration rates of *Prochloron* are higher than in most free-living photosynthetic microorganisms (Alberte *et al.* 1986)), and are ~10 times higher in high-light adapted cells than in cells isolated from shade adapted ascidians. The high respiration results in high compensation irradiances when net photosynthesis is plotted vs. irradiance for isolated *Prochloron* cells (Fig. 3A; Alberte *et al.* 1986). Besides lower respiration and photosynthesis rates as well as lower compensation irradiance, *Prochloron* cells from shade adapted ascidians exhibited a lower chl *a*/chl *b* ratio, and larger PSI and PSII size than in high light adapted cells. Cells from high light adapted ascidians show no photoinhibition even at 2000 µmol photons m⁻² s⁻¹, while *Prochloron* from shade adapted ascidians are inhibited at higher irradiances (Fig. 3A; Alberte *et al.* 1986). In a more recent study Dionisio-Sese *et al.* (2001) found slight photoinhibition of isolated *Prochloron* cells above 1500 µmol photons m⁻² s⁻¹.

Intact *Prochloron/ascidian* associations, whether high or low light adapted, generally show no photoinhibition up to the highest ambient irradiances encountered in their habitat ($\sim 2500 \mu\text{mol photons m}^{-2} \text{s}^{-1}$) (Fig. 3B; Alberte *et al.* 1987). Apparently, the ascidian host is able to protect its symbionts against excessive irradiance (see also 2.3.2 and 3.2). However, significantly higher compensation irradiances and lower photosynthetic rates (per unit chl) are observed in intact *Prochloron/ascidian* associations as compared to isolated symbionts (Alberte *et al.* 1986, 1987; Griffiths and Thinh 1987). Alberte (1987) attributed the lower rate to self-shading effects in the densely packed *Prochloron* containing zone of the ascidians, while Griffiths and Thin (1987) speculated that host restriction of symbiont photosynthesis may control symbiont proliferation to match the growth rates of the host.

We speculate that other factors may also limit *Prochloron* photosynthesis *in hospite*. While isolated *Prochloron* are investigated in free suspension, the symbionts are densely packed and imbedded in a mucous matrix (or intracellular) within the ascidian, which will affect the light microenvironment and solute transport via diffusion. Like in other photosynthetic biofilms (e.g. Kühl *et al.* 1996) steep light gradients and build-up of high pH and O_2 levels within the *Prochloron* layer may take place in light, while supply of e.g. inorganic carbon for photosynthesis becomes limited by diffusion. We have recently obtained first experimental evidence that such microenvironmental conditions are indeed present in intact *Prochloron/ascidian* associations (see 3.1 and 3.2).

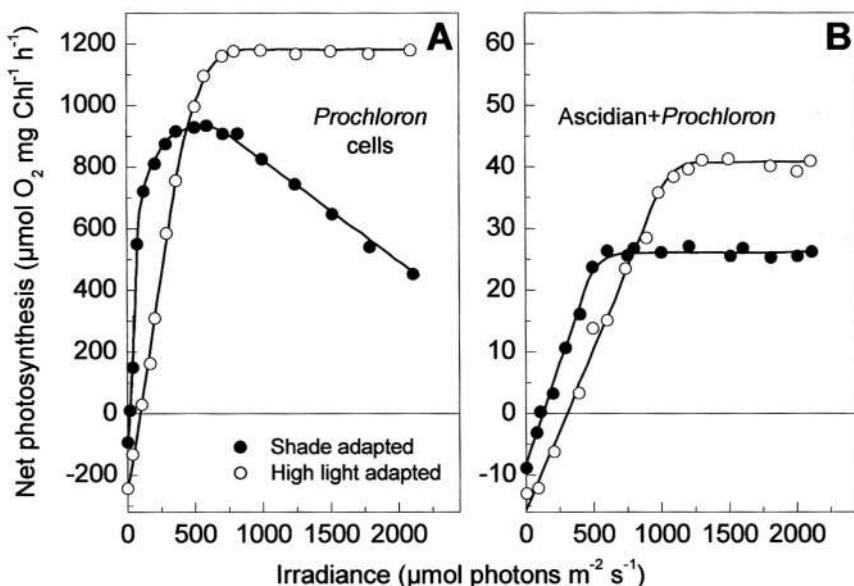


Figure 3. Photosynthesis vs. irradiance of high light (○) and low light (●) adapted *Prochloron* cells (A) as well as of intact *Prochloron*-ascidian associations (B) in *Lissoclinum patella*. (Redrawn from Alberte *et al.* 1986, 1987, with permission of the publisher).

2.3.2. Protection against UV-radiation and photooxidation

Ecophysiological studies indicate that the ascidian host provides protection against excessive radiation (Dionisio-Sese *et al.* 1997, 2001; Alberte *et al.* 1987), and *Prochloron* most probably has adapted to the light regime inside the host tissue. Isolated *Prochloron* cells from *Lissoclinum patella* were inhibited by excessive UV-B radiation, whereas cells in intact ascidians showed no photoinhibition by UV-B (Dionisio-Sese *et al.* 1997, 2001). The same authors demonstrated that the outer test is strongly UV absorbing (Fig. 4) and contains water-soluble mycosporine-like amino acids, i.e. mycosporine-glycine, palythine, and shinorine, acting as UV sunscreens. Shinorine, albeit in low concentrations, was also found in *Prochloron* and in the basal tunic of ascidians. *In vivo* spectra of *Prochloron* exhibited a high absorbance below 360 nm (Thinh and Griffiths 1983), which may be due to presence of MAA's. It is not known whether the MAA's are of host or symbiont origin, neither is it known where the water-soluble MAA's are localized and retained in the test matrix.

High irradiance in combination with the high density of pigmented *Prochloron* in the ascidian tissue may lead to photosensitizing processes producing significant amounts of reactive oxygen species, i.e. superoxide radicals, hydrogen peroxide and hydroxyl radicals. The production of these highly reactive compounds is directly proportional to the oxygen level (Jamieson *et al.* 1986), which in photosynthetic systems normally increases with increasing irradiance. Ascidians with *Prochloron* show an intense oxygen production in light (Tokioka 1942, and several studies thereafter) indicating oxygen supersaturation within the symbiont containing ascidian tissue under high irradiance, which may cause photooxidation (see also 3.1).

Lesser and Stochaj (1990) studied protection against photooxidation in *Prochloron* sp. and *Lissoclinum patella* and demonstrated the presence of the antioxidant enzymes superoxide dismutase, ascorbate peroxidase and catalase. The protein activity was directly proportional to irradiance, while pigment content was inversely proportional to irradiance. Furthermore, the high respiration activity of *Prochloron* and its host, which increases with irradiance (Alberte 1986, 1987), may also help moderating local oxygen supersaturation. *Prochloron* within its ascidian host thus seems well protected against photooxidation as well as UV-damage, and this explains why intact *Prochloron*/ascidian associations generally exhibit no photoinhibition even at highest natural irradiances (Alberte 1987; Dionisio-Sese *et al.* 2001).

2.3.4 Regulation of *Prochloron* photosynthesis by host behavior

Another feature of some didemnid ascidians, which may modulate the photosynthesis of their symbionts, is the ability to move with phototaxis or to change the morphology of the colony. *Diplosoma virens* colonies can thus move several mm per day (Birkeland *et al.* 1981; Thinh *et al.* 1981). Furthermore, *Diplosoma virens* can expand its surface area by 60-70% during daytime, which is likely to optimize exposure of *Prochloron* to favorable light conditions (Ryland 1990). Interestingly, the expansion was not directly triggered by irradiance but exhibited a circadian rhythm. The growth pattern and motility of *Lissoclinum patella* was also found to provide optimal growth of its symbionts (Swift and Robertson 1991).

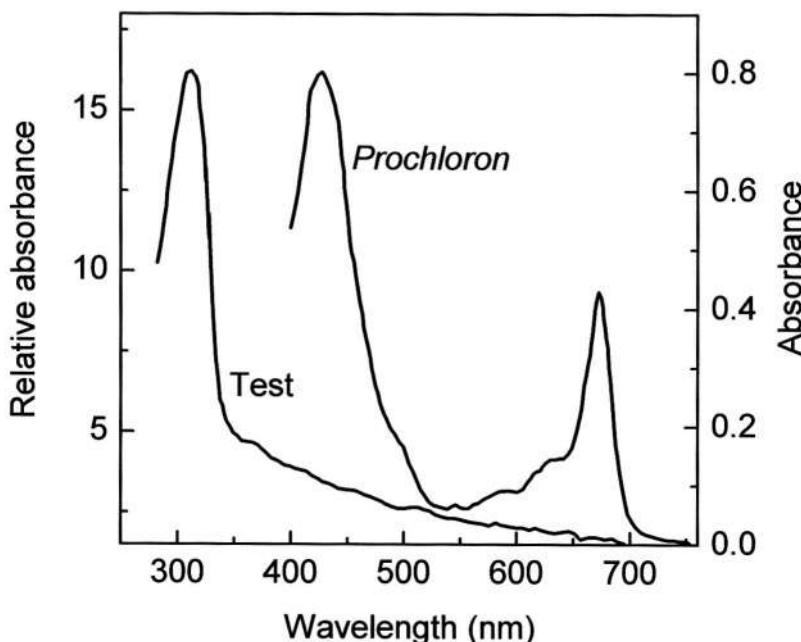


Figure 4. *In vivo* spectral absorbance of *Prochloron* cells in suspension, and relative absorbance of the outer test of *Lissoclinum patella* (the latter was redrawn from Dionisio-Sese *et al.* 1997, with permission of the publisher).

2.4 EFFECTS OF OTHER ENVIRONMENTAL VARIABLES

2.4.1 pH effects

Isolated *Prochloron* cells are photosynthetically competent from pH 6.8 to 9.5, with a peak in photosynthetic rate at the pH of seawater (~pH 8.0-8.2) (Dionisio-Sese *et al.* 2001). The observed peak was, however, rather broad and high rates of photosynthesis, i.e. >50% of maximum photosynthesis, were observed even at pH 9.5, which is significantly above ambient seawater pH. Apparently, *Prochloron* is well adapted to high pH levels, which may build up in the *Prochloron* containing zone of the ascidian during periods of intense photosynthetic carbon fixation. High pH may impose CO₂ limitation, which can, however, be alleviated by host respiration or by inorganic carbon concentrating mechanisms and the enzyme carbonic anhydrase present in *Prochloron* (Dionisio-Sese *et al.* 1993).

Acid release from vanadocytes and/or other host cells in response to strong physical disturbance of the ascidian host can lower the pH to <3.5, and this has lethal effects on *Prochloron* (Lewin and Cheng 1989; Thinh and Griffiths 1977). Consequently,

separation of viable *Prochloron* from the host tissue should always be done in buffered medium

2.4.2 Temperature effects

Maximal photosynthesis of both isolated *Prochloron* cells and intact ascidian/*Prochloron* associations is found at 28-35°C (Thinh and Griffiths 1977; Alberte *et al.* 1986; Dionisio-Sese *et al.* 2001). A steep decline in activity occurs above 40°C, which seems to be the upper temperature limit of *Prochloron*. Ambient temperatures experienced by didemnid ascidians with *Prochloron* typically range from 25-30°C, but even higher local temperatures may be experienced in shallow protected waters.

While *Prochloron* is well adapted to its ambient temperature range (and even to temperatures almost 10°C above it), it is very sensitive to decreasing temperature and photosynthetic activity ceases below 20°C. Alberte *et al.* (1986,1987) found that *Prochloron* cells and intact ascidian/*Prochloron* associations were twice as sensitive to temperature changes below ambient (Q_{10} of ~3.5) as compared to temperature changes above ambient temperature (Q_{10} of ~1.5). The temperature dependence of *Prochloron* respiration (Q_{10} of ~1.7) as well as the combined ascidian/*Prochloron* colony respiration (Q_{10} of ~2) was constant from 15-45°C. The low temperature sensitivity of *Prochloron* photosynthesis seems a prime factor limiting its distribution to tropical waters. We speculate that other photosynthetic symbionts of ascidians may exhibit the same temperature relation, which would explain the apparent absence of symbionts in didemnid ascidians of colder waters (e.g. Sanamyan 1999).

3. Microenvironment of *Prochloron*

Existing information on the distribution and photosynthetic performance of *Prochloron* (see above) indicates that the symbionts are growing under special microenvironmental conditions with respect to light, oxygen, pH, nutrients and inorganic carbon inside their ascidian hosts. Yet, the microenvironment of *Prochloron* remains virtually unstudied. Use of minimally invasive microsensor techniques (reviewed in Kühl and Revsbech 2001) has given new insights to microenvironmental controls in the symbioses of microalgae with corals (Kühl *et al.* 1995; De Beer *et al.* 2000, Salih *et al.* 2000), benthic foraminifera (Köhler-Rink and Kühl 2000), and sponges (Sand-Jensen and Pedersen 1994). Recently, we have used a similar approach to study *Prochloron*-ascidian associations (Kühl and Larkum, 2001). Here we present some preliminary examples of our microsensor data (Fig. 5), which give experimental evidence of some of the earlier mentioned speculations on the microenvironment of *Prochloron*.

3.1 OXYGEN MICROENVIRONMENT OF PROCHLORON

A characteristic feature of *Prochloron* is that it grows attached in a mucous polymer matrix lining the cloacae or external surfaces of its hosts (Lewin and Cheng 1989). The symbionts thus form a photosynthetic biofilm closely associated with the host tissue. In such biofilms very dynamic oxygen conditions are to be expected (Kühl *et al.* 1996).

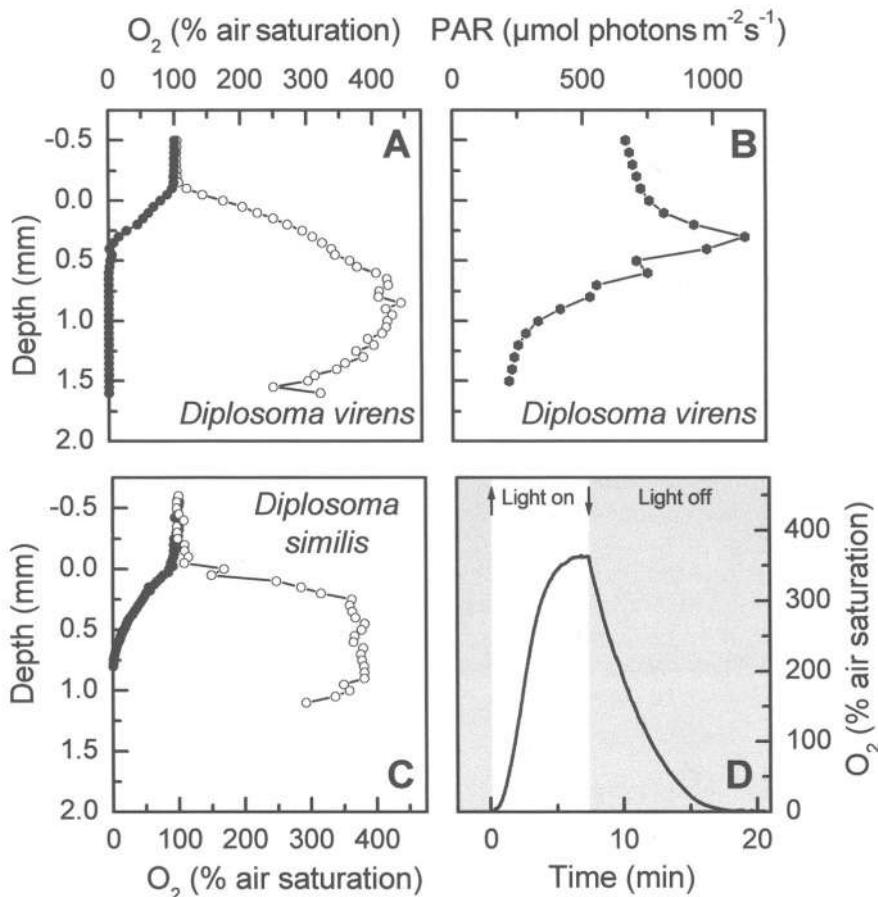


Figure 5. Oxygen microenvironment and light regime of *Prochloron* in hospite. Oxygen concentration profile measured with an electrochemical oxygen microsensor in *Diplosoma virens* (A) and *Diplosoma similis* (C) in light (○, $\sim 300 \mu\text{mol photons m}^{-2} \text{s}^{-1}$) and darkness (●). B. Depth distribution of PAR (●) in *Diplosoma virens*. D. Continuous measurement of O_2 concentration within the *Prochloron* containing zone of *Diplosoma virens* during an experimental light-dark shift. The outer test of the ascidians was 0.2–0.5 mm thick. The *Prochloron* containing zone below was ~ 0.5 –1 mm thick.

First microsensor measurements in *Diplosoma virens* and *Diplosoma similis* indeed showed a very dynamic change in internal oxygen levels as a function of irradiance (Fig. 5). In the light, oxygen levels in the ascidian show a pronounced supersaturation of up to 4 times the O_2 concentration in air saturated water. In darkness, strong oxygen depletion created anoxia in the ascidian below the outermost 0.5–1 mm of the test. Internal oxygen levels were determined by intensive *Prochloron* photosynthesis in light.

Only ~8 minutes after the start of illumination, the photosynthetic activity had changed the internal O_2 levels from anoxia to supersaturation (Fig. 5D). Conversely, a strong oxygen consumption by host and symbionts restored anoxia within ~10 minutes after onset of darkness.

Our data give the first experimental evidence that *Prochloron* lives in an environment exhibiting extreme variations in O_2 as a function of available irradiance for photosynthesis. While respiration is clearly O_2 limited in darkness, the O_2 supersaturation observed in light stimulates host respiration, which is in line with observations by Alberte *et al.* (1987). The accumulation and rapid depletion of O_2 measured within ascidians (exposed to flowing water), and the shape of steady state O_2 profiles indicate that rapid diffusion is a major mode of mass transfer between symbionts and host. Whether advective transport due to pumping of host cells and circulation of test fluids within the ascidian are of significance for internal O_2 levels remains to be investigated. Our data also indicate a potential for various anaerobic reactions like e.g. N_2 fixation within the ascidian tissue under low light or dark conditions (see 2.1.3). Even microaerophilic or anaerobic microbes may be able to proliferate within the ascidians, and an inventory of the bacterial diversity present within didemnid ascidians should be very interesting to undertake.

3.2 LIGHT REGIME OF PROCHLORON IN HOSPITE

A major conclusion of earlier studies was that photosynthesis-irradiance characteristics of *Prochloron* reflect adaptation to light regimes *in hospite* and not the irradiance incident on the ascidian surface (Alberte 1989). The optical properties of the ascidian test modify the internal light regime of *Prochloron in hospite*. The test can screen out UV radiation efficiently due to presence of MAA's (see 2.3.2), while PAR (400-700 nm) absorption in the semitransparent test matrix is very weak and 4-8 times lower than for UV-radiation (Fig. 4). Light attenuation in the test is due to both absorption and scattering, which leads to a diffuse light field. Measurements of PAR with a scalar irradiance microprobe (Kühl *et al.* 1997) showed the presence of a peak of PAR in the outer ~0.2-0.5 mm thick test of the ascidian *Diplosoma virens* followed by an exponential decrease of PAR in the *Prochloron* containing zone of the ascidian (Fig. 5B).

The apparent light trapping in the outer test is due to a high scattering to absorption ratio for visible light in combination with a higher refractive index of the test matrix as compared to the overlaying water (Kühl and Jørgensen 1994; Grunwald *et al.* unpublished data). Like in other multiple scattering tissues (Vogelmann *et al.* 1996; Motamedi *et al.* 1989) the light trapping results in an increased pathlength of photons per vertical depth interval transversed, which again enhances the probability of absorption. Therefore, even the presence of relatively low amounts of UV-screening compounds in the outer test can lead to efficient screening of UV-radiation, while PAR is propagating almost unaltered to the underlying *Prochloron*. Our data obtained in *Diplosoma virens* (Fig. 5B) indicate no major light limitation of *Prochloron* due to self-shading (see also 2.3.1)

In some ascidians like *Lissoclinum patella* the test is more optically dense and host pigments in the test may also alter the spectral characteristics of visible light transmitted

to *Prochloron*. Alberte (1989) reported the presence of compounds in the test with strong absorption of green and orange light. The same author hypothesized that the light regime of *Prochloron*, as determined by host pigmentation as well as the ambient light climate of the ascidian in their habitat, is more like terrestrial habitats putting a selective pressure for development of light-harvesting systems with chlorophyll *a* and *b*.

4. Summary and outlook

To isolate and maintain strains of *Prochloron* sp. remains a major task in the study of these fascinating oxygenic prokaryotic phototrophs. However, the microenvironmental conditions under which *Prochloron* sp. and other oxyphotobacteria thrive in symbiosis with didemnid ascidians, are just beginning to be investigated with microsensors and other techniques that allow studies of *Prochloron in hospite*. Such studies will help identify critical boundary conditions for future isolation attempts. The first data on the light and oxygen microenvironment of *Prochloron* within its ascidian host demonstrate that it shares many characteristics with photosynthetic biofilms. *Prochloron* lives in a biofilm within strong gradients of light (largely defined by the optical properties of the test matrix) and oxygen. Oxygen concentration *in hospite* is very dynamic, mainly as a function of irradiance, and O₂ levels change from supersaturation to anoxia or *vice versa* within a few minutes after light-dark or dark-light shifts. Given that *Prochloron* isolation attempts have been based on liquid cultures and suspended cells, it is not surprising that such attempts have failed. It is now time to consider isolation attempts, where the biofilm mode of *Prochloron* life and its physical-chemical microenvironment is acknowledged.

Other recent discoveries also may influence future *Prochloron* research. Another oxyphotobacterium, *Acaryochloris marina* (Miyashita *et al.* 1996) has been found in didemnid ascidians. *Acaryochloris* is using chl *d*, which absorbs maximally around 715 nm, as the major photopigment. *Prochloron* and *Acaryochloris* thus have rather complementary light absorption characteristics, which may allow them to co-exist in close proximity within the ascidian host. Preliminary observations (Kühl and Larkum unpublished results) of the spatial organization of *Prochloron* in the didemnid *Diplosoma virens* thus indicate that *Prochloron* is imbedded in a mucous matrix of small Chl *d* containing microorganisms. Further microscopic investigations are now underway to confirm this spatial organization. Cyanobacteria with special phycobilin pigmentation have also been found in didemnid ascidians (Cox *et al.* 1985; Larkum *et al.* 1987) but their interaction with the host and other symbionts remain unstudied. Interactions with oxyphotobacteria and other bacteria *in hospite* may therefore be another important aspect of *Prochloron* ecophysiology yet to be studied in detail.

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IV. Fungi Symbiosis

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SURVIVAL STRATEGIES IN ARBUSCULAR MYCORRHIZAL Symbionts

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1. Introduction

Arbuscular mycorrhizal (AM) symbionts are obligately biotrophic fungi (Zygomycetes), which establish mutualistic symbioses - arbuscular mycorrhizas - with the roots of about 80% of plant species belonging to all phyla of land plants. AM fungi play a major role in soil fertility and plant nutrition, since they are able to uptake nutrients from the soil and transfer them to the host plant by means of extraradical hyphae, which explore the surrounding environment and provide an extensive surface area for water and nutrient absorption (Smith and Read, 1997).

Moreover, transfer of carbon and phosphorus has been shown to occur between different plant species connected by a common mycorrhizal mycelium and a higher floristic diversity was found in experimental microcosms and in the field in the presence of AM fungi. These findings suggested that AM symbionts probably play an important role not only in nutrient uptake by plants but also in interplant nutrient transfer, representing fundamental factors for the exploitation and redistribution of resources within plant communities and for the establishment, survival and maintenance of plant community diversity (Francis and Read, 1984; Grime *et al.*, 1987; Read, 1998; Van der Heijden *et al.*, 1998).

AM fungi produce spores in the soil which are able to germinate and grow, in the absence of the host, in response to different edaphic and environmental conditions, but are unable to produce extensive mycelia and to complete their life cycle, without establishing a functional symbiosis with a host plant. This behaviour appears inconsistent in AM symbionts, which are obligate biotrophs, and could have represented a strong selective disadvantage. Notwithstanding, these organisms are regarded as fundamental to the colonization of land by plants (Pirozynski and Malloch, 1975; Pirozynski, 1981; Pirozynski and Dalpé, 1989), and both fossil records and DNA sequence data have shown that they had established mycorrhizal symbioses as early as 410-360 million years ago (Simon *et al.*, 1993; Remy *et al.* 1994; Phipps and Taylor, 1996).

The evolutionary success of AM fungi indicates that they must have evolved efficient survival strategies to compensate for the lack of regulation of spore germination. The wide host range and the consequent low host specificity of AM fungi undoubtedly represent an efficient strategy to increase the probability of individual germlings to contact host roots. However, this does not appear enough to explain why this ancestral group of organisms has survived the past 400 million years.

The aim of this chapter is to review recent developments which have contributed to the understanding of some evolutionary mechanisms conserved in these "living fossils" which have permitted the survival of individuals and populations.

2. Host recognition

Host-derived signals do not represent essential factors for spore germination of AM fungi, since they are able to germinate in axenic culture in the absence of the host (Godfrey, 1957; Mosse, 1959; Daniels and Trappe, 1980; Koske *et al.*, 1981a; Hepper, 1983; Tommerup, 1983; Mugnier and Mosse, 1987).

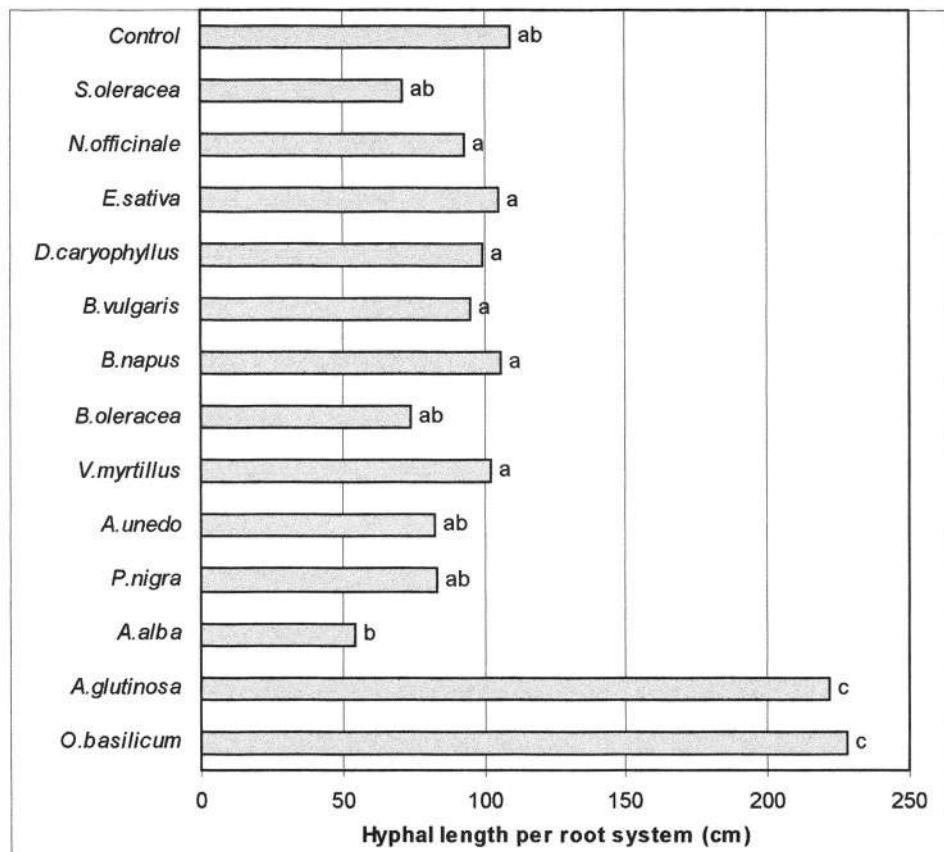


Figure 1. Hyphal growth of *Glomus mosseae* in the presence of roots of arbuscular mycorrhizal plants (*Aleuris glutinosa* and *Ocimum basilicum*), non-mycorrhizal plants (*Spinacia oleracea*, *Nasturtium officinale*, *Eruca sativa*, *Dianthus caryophyllus*, *Beta vulgaris*, *Brassica napus*, *Brassica oleracea*), plant hosts of non-arbuscular mycorrhizas (*Vaccinium myrtillus*, *Arbutus unedo*, *Pinus nigra*, *Abies alba*) and in the absence of plants (control) (from: Giovannetti *et al.*, 1994).

Although nothing is presently known of the molecular signals which relieve spore dormancy and activate the cell cycle, diverse environmental conditions triggering the initiation of germination processes in different genera and species of AM fungi have been identified, such as pH, temperature, moisture, nutrients and microorganisms (see Giovannetti and Gianinazzi-Pearson, 1994; Giovannetti, 2000). Host roots and host root exudates have been shown to affect hyphal growth depending on experimental conditions and on plant and AM fungal species (Fig. 1) (Graham, 1982; Azcón and Ocampo, 1984; Hepper, 1984; Elias and Safir, 1987; Bécard and Piché, 1989; Gianinazzi-Pearson *et al.*, 1989; Schreiner and Koide, 1993; Giovannetti *et al.*, 1993a; 1994; Tawaraya *et al.*, 1996; Ishii *et al.*, 1997).

By contrast, roots or root exudates of nonhost plants such as nonmycorrhizal species or plants hosting mycorrhizas other than the arbuscular type, do not produce any effect on spore germination and/or hyphal growth, showing that the growth-promoting effects are host-specific (Tester *et al.*, 1987; Glenn *et al.*, 1988; El-Atrash *et al.*, 1989; Gianinazzi-Pearson *et al.*, 1989; Avio *et al.*, 1990; Giovannetti *et al.*, 1993b; 1994; Giovannetti and Sbrana, 1998).

The best candidates for the role of signal molecules originating from host roots are flavonoids and phenolic compounds, which actually stimulate hyphal growth of germinated spores (Gianinazzi-Pearson *et al.*, 1989; Nair *et al.*, 1991; Siqueira *et al.*, 1991; Tsai and Phillips, 1991; Bécard *et al.*, 1992; Morandi *et al.*, 1992; Phillips and Tsai, 1992; Poulin *et al.*, 1993). Nevertheless it is still to be demonstrated whether they act as signals for the establishment of the symbiosis (Bécard *et al.*, 1995).

The first host-derived compounds described to act as host signals for AM fungi are represented by volatiles, which affect hyphal growth direction and are involved in chemotropism of hyphae towards host roots (Koske, 1982; Gemma and Koske, 1988; Koske and Gemma, 1992). Further studies reported increases in hyphal branching and proliferation in response to host roots and root exudates, which were often detected in the vicinity of the root system (Mosse and Hepper, 1975; Powell, 1976; van Nuffelen and Schenck, 1984; Carr *et al.*, 1985; Elias and Safir, 1987; Bécard and Fortin, 1988; Mosse, 1988; Bécard and Piché, 1989). Such qualitative observations represented the basis for detailed and quantitative investigations on differential hyphal morphogenesis elicited in AM fungal mycelium by signals associated with host roots, compared with nonhost roots (Giovannetti *et al.*, 1993b). By using an *in vivo* system and a physical barrier - a Millipore permeable membrane - separating the growing fungus from plant roots, it was possible to monitor hyphal growth before the establishment of the symbiosis and to detect extensive hyphal development and branching located in close association with the roots of host plants growing underneath the membrane. This morphogenetical effect involved dramatic changes in hyphal behaviour, since individual hyphae abandoned their linear growth pattern, regular branching and apical dominance, to give rise to a hyphal net with irregular branches, reduced inter-hyphal spacing and repeatedly altered directional growth (Fig. 2). When growing in the presence of different plant hosts, such as *Fragaria vesca*, *Helianthus annuus*, *Ocimum basilicum*, *Lycopersicon esculentum*, *Triticum aestivum*, the AM fungus *Glomus mosseae* produced an hyphal net whose density was 4-fold that of the control. On the contrary, nonhost plants, such as *Brassica napus*, *Eruca sativa*, *Dianthus caryophyllus*, *Lupinus albus*, did not elicit any morphogenetical event (Fig. 3).

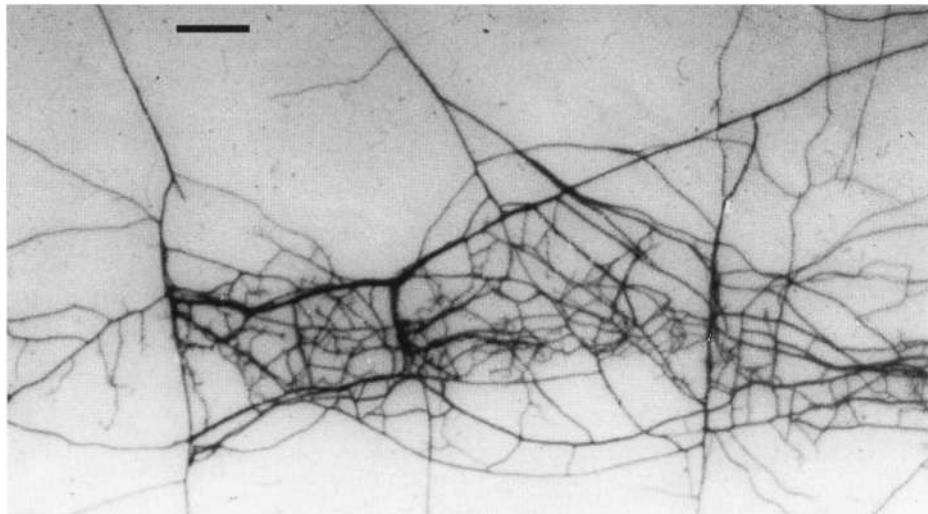


Figure 2. Light micrograph showing differential morphogenesis in *Glomus mosseae* hyphae, elicited by the roots of the host plant *Ocimum basilicum*, growing underneath a permeable millipore membrane. Scale bar, 150 μm .

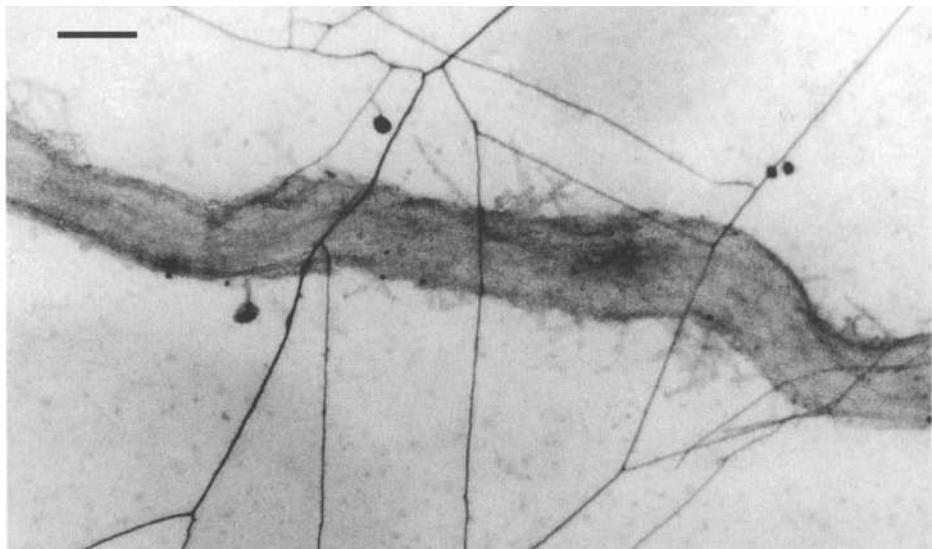


Figure 3. Light micrograph showing the absence of any differential morphogenesis in *Glomus mosseae* hyphae growing on the roots of a nonhost plant. Scale bar, 150 μm .

Time-course experiments showed that AM mycelium was able to change the elongation and branching pattern as early as 24 h after the beginning of the interaction between plant host and fungal symbiont (Giovannetti *et al.*, 1993b; Giovannetti, 1997).

The evidence that plant hosts of mycorrhizas other than the arbuscular type were unable to elicit any differential morphogenesis in the hyphae of AM fungi confirmed that host-derived signals represent the early cues to which AM fungi respond, and through which they are able to discriminate unambiguously hosts from nonhosts (Giovannetti *et al.*, 1994). The differential hyphal morphogenesis detected in *in vivo* experiments was successively described also in hyphae growing in the vicinity of host roots and root exudates *in vitro* (Giovannetti *et al.*, 1996; Nagahashi *et al.*, 1996; Nagahashi and Douds, 1999; Buee *et al.*, 2000) and in hyphae growing in the absence of the host on membranes bearing host root exudates (Logi, 1997).

Many challenges remain concerning the nature of the host-derived signals involved in fungal recognition responses. Studies carried out by using dialysis membranes allowed us to determine the molecular cut-off beyond which host root exudates were unable to pass, and showed that the signal molecules have a maximum molecular weight of 500 Da (Giovannetti *et al.*, 1996).

The morphogenetical changes occurring only in the presence of compounds released by host roots may have the function of producing hyphal structures capable of locating suitable sites for root infection, and represent the early developmental switch indicating hyphal commitment to the symbiotic status in AM fungi (Giovannetti, 1997; Giovannetti and Sbrana, 1998). This assumption is confirmed by the finding that infection structures - appressoria - were never formed on the root surface of nonhost plants which had not elicited any differential hyphal morphogenetical response. On the contrary, appressoria were formed as early as 36 h after the beginning of the plant-fungus interaction, in the presence of host roots (Fig. 4) (Giovannetti and Citernesi, 1993; Giovannetti, 1997).

It is interesting to note that even in the presence of host root exudates, thigmotropic stimuli in the form of nylon, silk, cellulose, polyamide or glass threads did not trigger appressorium differentiation (Giovannetti *et al.*, 1993a). Moreover, no appressoria were formed on the roots of a large number of nonhost plants (Powell, 1976; Malajczuk *et al.*, 1981; Tommerup, 1984; Glenn *et al.*, 1985, 1988; Bedmar and Ocampo, 1986; Avio *et al.*, 1990; Parra-Garcia *et al.*, 1992; Giovannetti *et al.*; 1993a, 1994). On the other hand, both differential hyphal morphogenesis and appressoria were elicited in the presence of the roots of mycorrhizal resistant plants identified among non nodulating(*nod⁻*) pea mutants, called "early *myc⁻* mutants", where mycorrhizal infection is hindered by plant defence responses consisting in thick cell wall appositions at the sites of appressoria formation (Fig. 5) (Gollotte *et al.*, 1993). This suggests that pea *myc⁻* mutants are not altered in genes producing compounds acting as signals for host recognition by AM fungal symbionts.

Since appressoria are the most significant evidence indicating the successful recognition of a potential host plant (Staples and Macko, 1980), the fact that they are not produced by hyphae of AM fungi. When challenged with the roots or root exudates of nonhost plants, is further evidence that these ancestral biotrophic symbionts have evolved a finely tuned regulation of infection structures differentiation, functional to their survival.

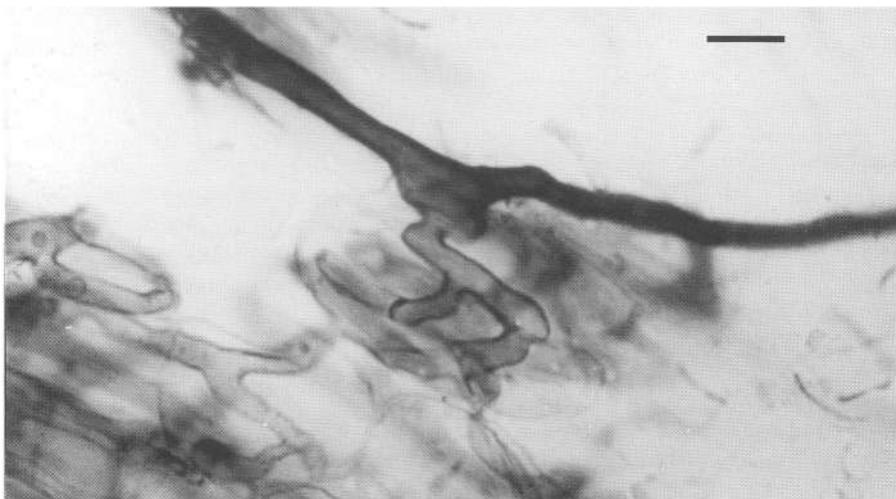


Figure 4. Light micrograph showing an appressorium (arrow) formed by *Glomus mosseae* hyphae on the root surface of a host plant. Scale bar, 20 μm .

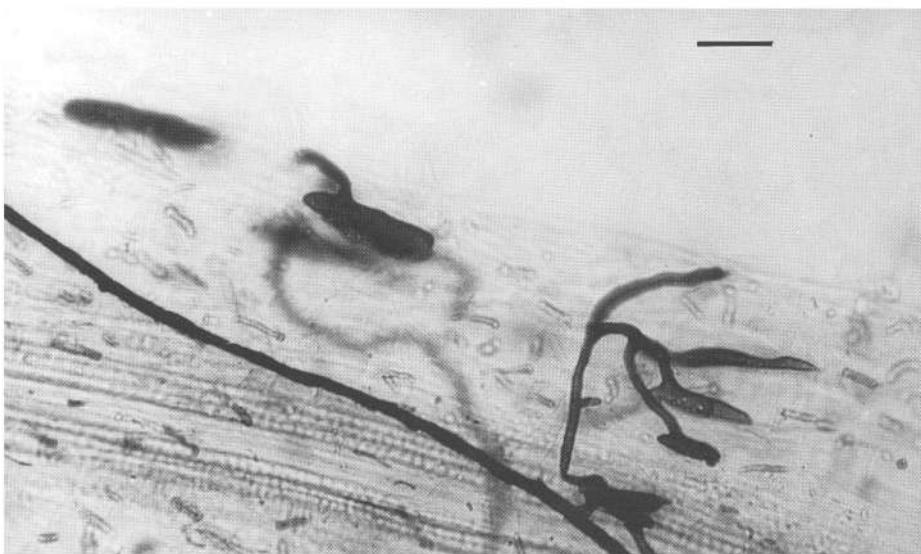


Figure 5. Light micrograph of appressoria formed on the root surface of a myc- mutant of *Pisum sativum* resistant to arbuscular mycorrhizal infection. Scale bar, 60 μm .

3. Programmed growth arrest in the absence of the host

It has already been mentioned that AM fungal spores are able to germinate in the absence of the host, but are unable to grow and to complete their life cycle without establishing a functional symbiosis with a host plant. Even when growing in the most suitable media, hyphal development is poor and germlings cease growth within 8-20 days of germination (Mosse, 1959; Hepper and Smith, 1976; Beilby and Kidby, 1980; Koske, 1981a; Giovannetti, 2000). During the growth period, germlings produce a coenocytic mycelium, whose total length is highly variable among different genera, species and individuals. For example, mycelial length in *G. caledonium* reached 30-50 mm, depending on the individual spore, after 10-15 days growth on water agar, and the mean growth rate of the mycelium during the early phase was $1.97 \pm 0.39 \mu\text{m}/\text{min}$ (Logi *et al.*, 1998). In *G. clarum* new hyphae extended up to 8 mm after 10 days incubation (Louis and Lim, 1988). In *Gigaspora rosea* hyphal length after 9 days growth was 42 mm (Giovannetti *et al.*, 2000), while in *G. margarita* it was 8 mm and 25 mm respectively, in two different experimental conditions (Becard and Piché, 1989; Gianinazzi-Pearson *et al.*, 1989).

Detailed developmental studies performed on living cells growing in appropriate microchambers and the use of time-lapse video microscopy, image analysis and epifluorescence microscopy, have provided information on the fundamental cell events leading to the arrest of hyphal growth in the absence of the host in different species of AM fungi belonging to the genera *Glomus* and *Gigaspora*.

Time-lapse and video microscopy studies showed that after a period of 5-10 days postgermination, the mycelium originating from germinating spores entered a state of developmental arrest, while cytoplasm, nuclei and cellular organelles were retracted from most peripheral hyphae and cross walls were produced, separating viable from empty mycelium (Fig. 6).

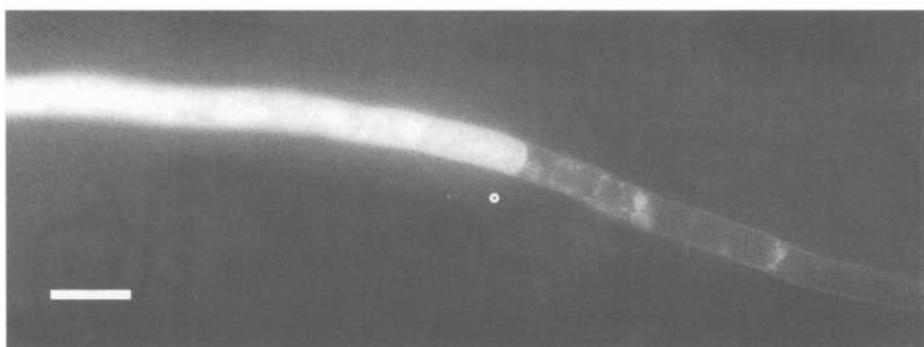


Figure 6. Epifluorescence microscopy of a hypha of *Glomus caledonium*, visualized by fluorescein diacetate staining, showing a segment completely devoid of protoplasm, separated from the living hyphal tract by the formation of a cross wall. Scale bar, 10 μm .

Observations of the dynamics of retraction-septa formation showed that withdrawal of protoplasm started from hyphal tips, with the development of apical vacuoles, which progressively extended in volume and number, leading to the formation of empty areas, which eventually became isolated from the protoplasm-containing hyphal segments by the formation of cross-walls (Logi *et al.*, 1998; Giovannetti *et al.*, 2000). No nuclei were observed in empty hyphal tips or tracts, while they were detected in viable hyphal segments, close to the retraction septa. The intense signal for cytoskeletal proteins detected in growing hyphae of *G. mosseae* and *G. caledonium* are consistent with the role of the cytoskeleton in facilitating the migration of nuclei and cellular organelles during either active growth or protoplasm reallocation (Åström *et al.*, 1994; Logi *et al.*, 1998).

Video microscopy was also used to record protoplasm withdrawal from hyphal tips and the formation of retraction septa. The average rate of empty segment formation between the 7th and the 19th day ranged from 0.1 ± 0.05 to $0.28 \pm 0.08 \mu\text{m}/\text{min}$ in *G. caledonium* and from 0.27 ± 0.13 to $0.89 \pm 0.2 \mu\text{m}/\text{min}$ in *G. rosea* (Logi *et al.*, 1998; Giovannetti *et al.*, 2000). The occurrence of an increasing proportion of empty hyphae in the soil in the absence of host plants had been previously reported (Tommerup, 1984). Both decreases in the amount of free sterols and increases in bound sterols were observed in *G. caledonium* germinated spores after 14-day growth in axenic culture, suggesting the occurrence of a senescence phase in the mycelium of AM fungi, as described for other fungal species (Beilby and Kidby, 1980; Elliott, 1977).

Daily measurements of the extension of protoplasm-containing and empty hyphae, showed that, after reaching the stationary phase, decreases in the length of protoplasm-containing mycelium were paralleled by increases of empty mycelium. Interestingly, some spores of *G. rosea* produced new germ tubes and showed increasing lengths of viable mycelium in the emerging hyphae corresponding to increasing lengths of empty hyphae in the old ones (Logi *et al.*, 1998; Giovannetti *et al.*, 2000).

These findings strongly suggest a mechanism involving control over allocation of spore reserves, through which spores germinating in the absence of the host undergo a process of resource reallocation, involving migration of nuclei and cellular organelles, which may be functional to maintaining the limited energy resources of germlings and enhancing their ability to survive in the absence of a carbon donor. This is consistent with other reports showing that spore reserves are never totally depleted when germlings cease growth (Hepper, 1979; Beilby Kidby, 1980; Koske, 1981) and with previous observations on the ability of fungal spores to germinate several times by producing successive germ tubes - up to ten in *G. margarita* over a period of 50 days (Mosse, 1959; Koske, 1981b).

Studies on long-term ability of AM fungal spores to retain infectivity when germinating in the absence of the host showed that even 4 months after germination spores of *G. caledonium* and *Acaulospora laevis* were still capable of developing infection structures and colonizing host roots (Tommerup, 1984b). This survival ability of spores was successively confirmed by studies on the viability of the mycelium, assessed by evaluating succinate dehydrogenase activity, which decreased over time in germinated spores of *G. caledonium*. Interestingly, metabolic activity was still detectable in hyphae proximal to the mother spore, 6 months after germination, and also infectivity was retained during the same period, allowing propagule long-term survival in the absence of the host (Logi *et al.*, 1998).

These data showed that after the spore had carried out a prolonged exploration of the surrounding environment and reached the phase of growth arrest, a variable length of mycelium with metabolic activity was maintained, and confirmed previous results showing that 26-day-old mycelium was resting, still viable and capable of renewed growth in response to host roots (Giovannetti *et al.*, 1996).

The wide occurrence of the phenomenon of protoplasm retraction, initiating at hyphal tips with the formation of successive retraction septa isolating empty, distal mycelium from viable mycelium proximal to the mother spore, suggests that this event may represent a strategic behaviour of AM fungi. When no host-derived signal from the surrounding environment is perceived by germinating spores, fungal hyphae undergo a programmed growth arrest, allowing long-term maintenance of viability and host infection capability.

Many questions remain concerning whether either a critical length or a critical age of hyphae may represent triggers for the onset of senescence, or whether there is a fixed pool of resources for germination, after whose consumption fungal growth is arrested. Whatever is the answer, the fact that mycelial development arrests within a few days after germination is evidence for the existence of an energy-saving mechanism operating when spores of the obligately biotrophic AM fungi germinate in the absence of a host.

4. Anastomosis formation with self-compatible mycelia

The use of fluorescence and video-enhanced high-powered microscopy has revealed fundamental biological processes occurring in living hyphae of AM fungi, such as movements of nuclei, organelles and vacuoles along hyphae developed from germinating spores (Bago *et al.*, 1998 a, b; Cole *et al.*, 1998; Logi *et al.*, 1998). In particular, a recent study on the structure of pre-symbiotic mycelium and on the dynamics of hyphal growth has shown that the mechanism allowing the formation of hyphal networks is represented by anastomosis, through which protoplasm flow between different hyphae is established. These findings evidenced that the fundamental mechanism operating in the formation of mycelial networks consists in self-recognition between compatible hyphae (Giovannetti *et al.*, 1999; Giovannetti and Sbrana, 2001).

For the first time the complete formation of a hyphal fusion in living hyphae of AM fungi was visualized over time, and it was shown that it was accomplished in 35 min after a hyphal tip showed directed growth towards another hypha. The establishment of protoplasmic continuity, the characteristic feature of true anastomoses, was evidenced by the detection of complete fusion of hyphal walls and bidirectional migration of a mass of particles between fused hyphae, which moved at the speed of **1.8±0.06 µm/s**. Epifluorescence microscopy revealed exchange of nuclei between hyphae of the same individual and of different individuals of the same isolate and many nuclei were detected in hyphal fusion bridges, confirming the complete compatibility of the anastomosing hyphae (Fig. 7).

Experimental pairings between different species of AM fungi showed that hyphae of individuals belonging to one species never formed anastomoses with hyphae of the other, revealing hyphal ability to discriminate self from other. For example, pairings between germinated spores of *G. mosseae* and *G. caledonium* produced 90 hyphal contacts and no fusions. When hyphae of *G. mosseae* and *G. caledonium* were

challenged with hyphae of a species belonging to a different genus, *G. rosea*, no fusions were detected over 140 and 232 contacts, respectively. During interspecific and intergeneric hyphal interactions the responses varied from no contact interferences, to contact responses such as formation of hyphal swellings or growth of one hypha along the other without anastomosis formation, confirming that AM fungi are able to discriminate self from non-self (Giovannetti *et al.*, 1999).

The ability to form intraspecific anastomoses may directly affect the fitness, viability and reproductive success of AMF. In fact, young mycelia produced by spores germinating in the absence of the host may plug into other mycelia as soon as germ tubes contact compatible hyphae, originating large networks and thus enhancing their chances to colonize host roots.

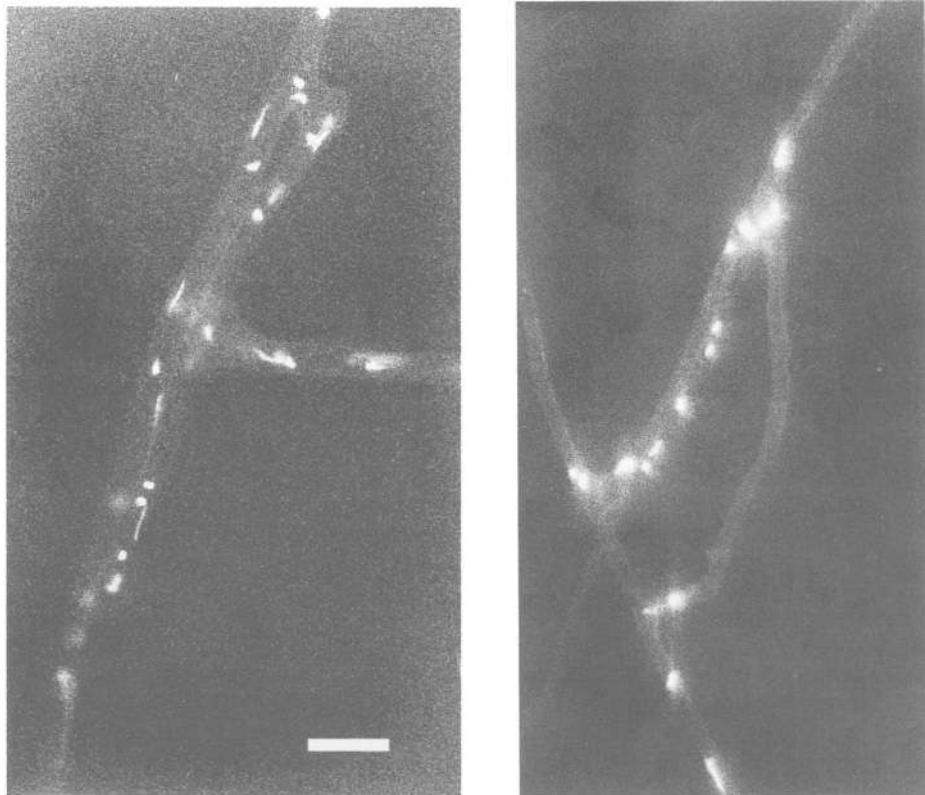


Figure 7. Epifluorescence microscopy of the nuclei detected in the hyphae and in the middle of the fusion bridge originated by two anastomosing hyphae of *Glomus caledonii*. Scale bar, 10 μm .

5. Concluding remarks

AM fungi are ancestral obligate symbionts, able to germinate, but unable to grow, in the absence of the host. Despite this, they have co-evolved with their host plants for about 400 million year. Many interesting hypotheses have been made on the evolutionary mechanisms which allowed these "living fossils" to survive, and the most important have been here discussed. The wide host range - 80% of land plants - ensures that germinating spores have a high probability to contact the roots of a host species, which are unambiguously recognised by AM fungi, which activate an "energy-saving" mechanism regulating the differentiation of infection structures. When no host-derived signals from the surrounding environment are perceived by germinating spores, fungal hyphae undergo a programmed growth arrest and resource reallocation, allowing long-term maintenance of viability and host-infection ability. The ability of hyphae originating from germinated spores to form anastomoses with self-compatible mycelia may provide a further survival strategy for obligately biotrophic AM fungi: the existence of large mycelial networks in soil means that young hyphae produced by spores germinating in the absence of the host may plug into the appropriate web as soon as the hyphal tip contacts a compatible mycelium. In my view, future research should focus on the role played by self-recognition between compatible mycelia in natural situations, where the wide host range of AM fungi and hyphal ability to form anastomosis may lead to the formation and establishment of indeterminate webs, from which young germlings growing far from their hosts could drain resources at an early stage, thus enhancing their chances of survival.

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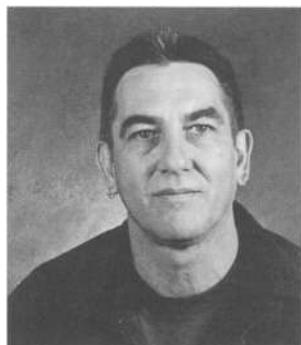
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THREE PART HARMONY – *ASCOPHYLLUM* AND ITS SYMBIONTS

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1. Introduction

Ascophyllum nodosum is among the most widespread and abundant species of seaweed in the cold temperate North Atlantic Ocean (Baardseth, 1970; Cousens, 1984; Åberg, 1990a). This is especially true in the waters of northeastern North America where vast stands of *A. nodosum* grow in the intertidal zones of the Bay of Fundy and coastal Nova Scotia (Cousens, 1984), and where commercial harvesting of this resource occurs (Sharp, 1986; DFO, 1998). Although some populations are subtidal (Peckol *et al.*, 1988), *A. nodosum* is most prominent in the intertidal zone where it grows in dense stands attached to rocky substrata. Because of its ecological importance, *A. nodosum* has been extensively studied in a variety of contexts including recruitment, growth, genetics and herbivory (Keser & Larson, 1984; Åberg, 1989, 1990b; Vadas *et al.*, 1990; Lazo *et al.*, 1994; Chapman, 1995). A further feature of *A. nodosum* is that it is among the longest-lived seaweeds (Baardseth, 1970). Based on the annual production of a new vesicle on growing axes, minimum frond ages can be determined. Accordingly, fronds commonly reach at least five years along the Atlantic coast of Nova Scotia and at least 20 years in the Bay of Fundy. Based on the combination of frond longevity and abundant biomass, it is not surprising that *A. nodosum* has evolved symbiotic interactions with other species.

The two primary symbionts associated with *A. nodosum* are the endophytic marine fungus, *Mycophycias* (formerly *Mycosphaerella*) *ascophylli*, and the epiphytic red alga, *Polysiphonia lanosa*. The former is an ascomycete that is always present, is found throughout the host plant, and is associated with no apparent pathology (Kohlmeyer & Kohlmeyer, 1972; Garbary & Gautam, 1989). The latter is an obligate epiphyte that is largely restricted to *A. nodosum* and which causes limited damage to its host as a consequence of rhizoid penetration (Rawlence & Taylor, 1972; Tian & Garbary, 1992). These species have undergone considerable coevolution resulting in extensive morphological, physiological and ecological interactions. As a tripartite symbiosis, the *A. nodosum*, *M. ascophylli*, *P. lanosa* is a model system for coevolutionary study. This may be the only marine system in which two photosynthetically independent organisms are linked by a fungus with which they interact for their individual and possibly mutual benefit. The objective of this review is to present an overview of the interactions in this tripartite symbiosis, and to show how the biological properties of the various components allow for mutualistic, commensal and parasitic interactions.

2. Taxonomic and nomenclatural account of primary organisms in symbiosis

2.1. *ASCOPHYLLUM NODOSUM* (LINNAEUS) LE JOLIS (Figs. 1, 2)

Basionym: *Fucus nodosus* Linnaeus

Classification: Phaeophyceae, Fucales, Fucaceae

Note: This monotypic genus is only found in the North Atlantic Ocean from New Jersey to Baffin Island, Greenland and Iceland in the western and north-central Atlantic and from Scandinavia to Portugal in the eastern Atlantic. Sequence studies of the internal transcribed spacer region of Fucaceae show that there is little molecular divergence within *A. nodosum*, and imply that *A. nodosum* is the sister group to all other Northern Hemisphere Fucaceae (Serrão *et al.*, 1999). This suggests that *A. nodosum* is a relatively old taxon (compared to *Fucus* spp.) that has had more time to evolve complex symbioses.

2.2. *POLYSIPHONIA LANOSA* (LINNAEUS) TANDY (Figs. 2, 3)

Basionym: *Fucus lanosus* Linnaeus

Important synonyms: *Vertebrata fastigiata* (Roth) Gray

Polysiphonia fastigiata (Roth) Greville

Classification: Rhodophyta, Ceramiales, Rhodomelaceae

Notes: *P. lanosa* is one of over 150 species of *Polysiphonia*. It is endemic to the North Atlantic Ocean, and has a distribution that largely overlaps that of its host, *A. nodosum*, but is not found at the extremes of its host's range. It is distinctive in being an obligate epiphyte and in having so many (ca. 25) pericentral cells that are uncorticated. On this basis some authors have classified *P. lanosa* in the monotypic genus *Vertebrata*, although there has been no phylogenetic analysis to support this conclusion. *P. lanosa* is also commonly found with the host specific, red algal parasite, *Choreocolax polysiphoniae* (Evans *et al.*, 1978).

2.3. *MYCOPHYCIAS ASCOPHYLLI* (COTTON) KOHLMAYER & VOLKMANN-KOHLMAYER (Figs. 4, 5)

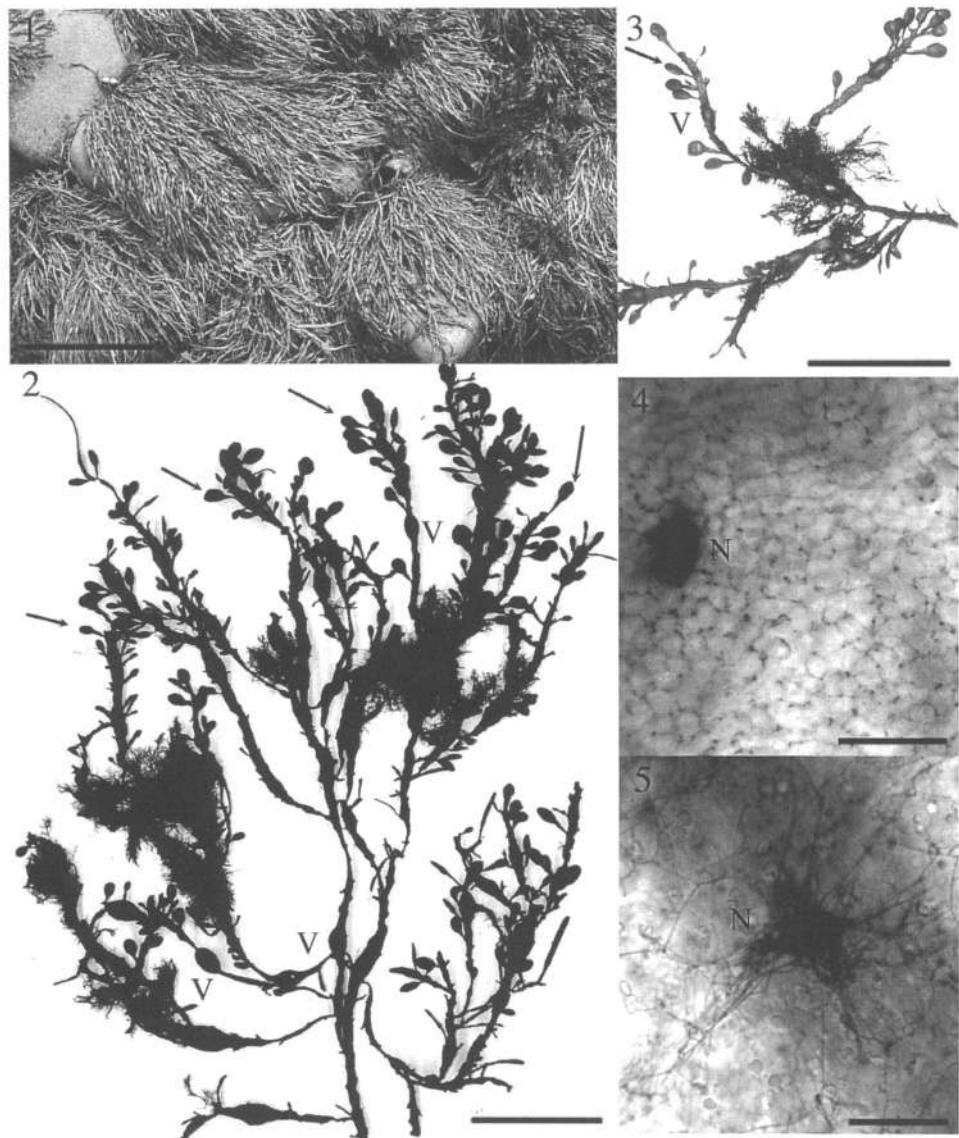
Basionym: *Mycosphaerella ascophylli* Cotton

Important synonyms: *Mycosphaerella velutiae* Sutherland

Septoria ascophylli Melnik et Petrov

Classification: Ascomycetes, Pyrenomycetes, Verrucariales

Notes: *M. ascophylli* is one of only two species ascribed to this genus. The second species, *M. apophlaeae*, is an obligate endophyte in the crustose red algal genus *Apophlaea* that is endemic to New Zealand. *M. apophlaeae* is known only from accounts of the taxonomy and morphology; none of the experimental work described here for *M. ascophylli* has been replicated with *M. apophlaeae*. *Septoria ascophylli* is likely the imperfect state of *M. ascophylli* (Kohlmeyer, 1968). Higgins (1984) provided an extensive account of *Mycophycias* (as *Mycosphaerella*). See Kohlmeyer & Volkmann-Kohlmeyer (1998) for a description of *Mycophycias* and a nomenclatural history of the species.



Figures 1-5. Ascophyllum nodosum and its symbionts. *Figure 1.* Plants in intertidal zone in Nova Scotia. Bar = 25 cm. *Figure 2.* Portion of large frond of *A. nodosum* from Kinvara, Ireland, with numerous immature receptacles (arrows), many floatation vesicles (V) and scattered clumps of epiphytic *Polysiphonia lanosa*. Bar = 10 cm. *Figure 3.* Portion of *A. nodosum* frond with large mass of *P. lanosa* with single receptacle (arrow) and vesicle (V) indicated. Bar = 10 cm. *Figure 4.* *Mycophycia ascophylli*. Extensive networks of hyphae and hyphal node (N) in epidermal and cortical layers of cleared tissue of *A. nodosum*. Bar = 25 μ m. *Figure 5.* *Mycophycia ascophylli*. Hyphal node (N) with numerous radiating arms of hyphae in cortex of *A. nodosum*. Bar = 25 μ m.

3. Interactions between *Ascophyllum* & *Mycophycias*

Mycophycias ascophylli was originally described from *A. nodosum* by Cotton (1909) (as *Mycosphaerella*). It has been variously interpreted as a parasite, a saprophyte, as forming a lichen-like association, or mycorrhiza-like association with one or another of its hosts *A. nodosum* or *Pelvetia canaliculata* (Webber, 1967; Kohlmeyer & Kohlmeyer, 1972). Extensive sampling has confirmed that all individuals of *A. nodosum* (and *P. canaliculata*) are infected, and that the infection is systemic in all parts of the host thallus (Kingham & Evans, 1986; Garbary & Gautam, 1989). Descriptive terms have included "lichenoid," and "primitive lichenization." The notion of this symbiosis being a lichen was dismissed because the alga undergoes sexual reproduction, and because the algal morphology dominates the association (Smith & Ramsbottom, 1915). A more modern definition of lichen includes the notion that the mycobiont is the exhabitant (Hawksworth, 1988). We concur that the *A. nodosum/M. ascophylli* association is not a lichen; however, it does seem to represent one extreme of a possible interaction between algae and fungi. Another fungal association involving the crustose brown alga, *Petroderma maculiforme*, was described as the lichen, *Verrucaria tavaresiae* Moe (1997). The growth of the mycobiont is much more extensive in *V. tavaresiae* than in *A. nodosum* and consistent with Hawksworth's definition.

Kohlmeyer & Kohlmeyer (1972) coined the term "mycophycobiosis" for algal/fungal systems in which there is "a permanent symbiotic association between a systemic marine fungus and a marine alga in which the habit of the alga dominates." We consider that the best analogy for the *A. nodosum/M. ascophylli* association is the mutualistic symbiosis between certain fungi (*Neotyphodium* spp.) and the grasses *Festuca* and *Lolium* that has been termed a "symbiotum" (Schardl *et al.*, 1991).

Elsewhere we highlight some new observations on the morphological interactions of the symbionts (Deckert & Garbary, in prep.). In this system there is a complex network of hyphae surrounding the base of every epidermal cell in which the hyphae grew in the cell junctions and formed polygonal (mostly hexagonal) arrays in the cortex. These arrays are connected to the host interior by radially directed hyphae that are later connected to longitudinally aligned hyphae, with much longer cells in the host medulla. Another conspicuous aspect of the fungal morphology is the occurrence of dense pseudoparenchymatous hyphal aggregations in the inner cortex of the host. These structures, termed 'hyphal nodes', consist of many, small irregular hyphae, and were connected to 5-8 hyphae radiating into the surrounding tissue. These structures may be interpreted as collection and/or distribution centers for metabolites.

Mycophycias ascophylli has been grown in culture independent of its host. Fries (1979) succeeded in growing the fungus apart from its host through hyphal culture, and was later able to germinate the ascospores (Fries, 1980). None of the cultures produced spores; however, antigens produced from the spore-derived cultures confirmed that the hyphal cultures were indeed *M. ascophylli* (Fries & Thoren-Tolling, 1978). Pedersén & Fries (1977) showed that cultures of *M. ascophylli* could produce a range of bromophenols. These compounds several of which diffused into the culture medium are potentially part of a mechanism to deter herbivores.

Fries (1988) later demonstrated a further complication in the *Mycophycias/Ascophyllum* symbiosis by taking segments of *A. nodosum* and treating them with antibiotics to produce branch segments with only the alga and the fungus. Without the associated bacteria (not identified), the hyphae penetrated through the wall of the *A. nodosum* and began to degrade its host. It would be of interest to know if the relevant bacteria were growing epiphytically or endophytically. This observation raises

the possibility that bacteria are important in regulating aspects of the symbiosis. Earlier, Chan & McManus (1969) had isolated a wide range of bacteria from *A. nodosum* and *P. lanosa*. They showed that a different bacterial flora was associated with the two algae. In addition, whereas the flora associated with *P. lanosa* was seasonally invariant and largely reflected that of the surrounding seawater, the flora associated with *A. nodosum* was seasonally variable, quite different from the surrounding seawater and produced many isolates of *Vibrio*.

Physiological studies have given some potential insights into the interactions of *A. nodosum* and *M. ascophylli*. In several studies exogenous ^{14}C -glucose and ^{14}C -mannitol were taken up by *P. canaliculata* and *A. nodosum*, but not by other Fucaceae. This correlation with the presence of *M. ascophylli* was strengthened in that when *P. canaliculata* was treated with the fungicide nystatin, uptake and metabolism of the two carbohydrates were inhibited (Kingham & Evans, 1986). There would seem to be considerable scope for further study of the metabolic and physiological interactions of the partners in the symbiotum.

The partners of the symbiotum are intimately linked with respect to reproductive ecology. In most (but not all) populations the pseudothecia are restricted to the host receptacles (Garbary & Gautam, 1989). In other populations, pseudothecia may also be found on vegetative apices. *Ascophyllum* has a limited reproductive season (4-6 weeks in Nova Scotia) in which the antheridia and oogonia mature on the unisexual individuals. This corresponds to the period of ascospore maturation and release from pseudothecia. Following gamete release, the receptacles are shed. The synchrony of ascocarp and host gamete maturation, even in populations where ascocarps are also found associated with vegetative apices of *A. nodosum*, suggests that the symbiotum is established by ascospores following zygote formation, and not by hyphae associating with oogonia in conceptico as was suggested by Webber (1967). Experimental support for this came from Garbary & MacDonald (1995) who described differential development of zygotes following infection with ascospores. These zygotes had a suite of morphological differences and grew faster than uninfected zygotes. These morphological changes were only induced when the ascospores were introduced within 48 h of fertilization.

Further evidence of mutualism in the symbiotum was presented by Garbary & London (1995). These authors showed that when zygotes were subjected to daily periods of desiccation (1, 2, 4, 8 h) that there was a significant increase in growth in infected plants desiccated for 2 h per day that did not occur in uninfected plants. This desiccation period also overcame the inhibition of rhizoid development that had been noted in infected, but non-desiccated plants.

4. Interactions between *Ascophyllum* and *Polysiphonia*

Polysiphonia lanosa may be considered a potential parasite whose negative impact is kept in check by the combined interactions of *A. nodosum* and *M. ascophylli*. These negative impacts might include: a) physical damage to host cells during penetration of rhizoids, b) opening of thallus to potential pathogens, c) weakening of thallus to result in additional breakage, d) blocking light penetration to host, e) nutrient absorption and f) attraction of herbivores. Although effect a) does occur (Rawlence & Taylor, 1970, 1972; Garbary and Deckert, in prep.), its effects are so localized (i.e., necrosis of a few cells resulting from chemical and physical injury), that the pathology has no apparent consequences to thallus vigor, even in heavily infested individuals. It seems that rhizoid

growth may be limited by a hypersensitive reaction in which the symbiotum transforms a number of host cells adjacent to each rhizoid. The hypersensitive reaction and any negative effect on *P. lanosa* may be mediated by the production of bromophenols that are known to be produced by *M. ascophylli* in pure culture (Pedersen & Fries, 1977).

There is no evidence that rhizoid penetration results in infection points for pathogens. Although *P. lanosa* is often associated with broken branches or damaged vesicles (Lobban & Baxter, 1983), there is no evidence that the epiphyte induces these effects. *P. lanosa* attaches in branch axils and on scar tissue because these areas have much slower epidermal peeling than undamaged or general areas of the *A. nodosum* surface. However, with the epiphyte *Elachista fucicola*, the massive tissue erosion (Garbary & Deckert, in prep.) and location of large epiphytic thalli commonly at the broken branch apices of *A. nodosum* (Garbary and Hageman, unpublished) suggests a greater parasitic interaction between these species.

Cousens (1985) argued that light is an important limiting factor in dense populations of *A. nodosum*. The consequent intraspecific competition may be partly mirrored by the interspecific competition with *P. lanosa* in that blocking of light and nutrients may diminish host tissue metabolism. However, biomass of *P. lanosa* is so limited relative to that of the host, that negative effects are unlikely, with the effects being highly localized. *A. nodosum* also grows in dense stands where self shading is a dominant feature, and only in rare cases of extremely high densities of *P. lanosa* would shading be increased significantly. The clumps of *P. lanosa* are an important habitat for crustaceans including gammarids and isopods (Pavia *et al.*, 1999). These herbivores feed on the *P. lanosa*, epiphytes on *A. nodosum* as well as tissue of *A. nodosum*. These isolated clumps of epiphytes are important refuges for the animals, and artificially defaunized clumps are recolonized in hours to days. Thus, *P. lanosa*, may indirectly be having a negative effect on the host. Alternatively the occurrence of occasional large clumps of *P. lanosa* may house sufficient animals that can graze the remaining surface and keep it epiphyte-free. This is one explanation for the highly contagious distribution of *P. lanosa* on individual fronds (Garbary *et al.*, 1991).

The dependence of *P. lanosa* on a photosynthetic host raised the question that there was a nutritional dependency based on a requirement for fixed carbon. In three separate studies three different results were obtained. Turner & Evans (1977) were unable to show transfer of fixed carbon from the host into *P. lanosa*. Harlin & Craigie (1975) showed some transfer, but the rates of transfer in either direction were no greater than could be accounted for by diffusion. Ciciotte & Thomas (1997) demonstrated that about 10% of the carbon fixed by either host could move into the other symbiont. Thus it appears that significant movement of carbon can occur, and that this can take place in either direction. Overall, these experiments demonstrate complex interactions between symbionts rather than providing an explanation for a dependency.

Studies of phosphate movement show similar variation. Penot *et al.* (1993) demonstrated long distance transport of ^{32}P in *A. nodosum* and its uptake by *P. lanosa*. What is of interest here is that the reverse did not occur. In earlier studies transfer rates were no greater than diffusion rates (Penot, 1974; Penot & Penot, 1974; Citharel, 1972). Both *A. nodosum* and *P. lanosa* have complex seasonal physiologies with respect to phosphorus (Chopin *et al.*, 1996), and this might account for discrepancies in phosphate mobilization described in the literature.

Polysiphonia lanosa is an obligate epiphyte and the occasional occurrences on *Fucus* spp. represent exceptional circumstances rather than an alternate strategy. *P. lanosa* clearly benefits from the association with *A. nodosum* in that it is provided with a substratum in a portion of the intertidal zone where it would be in an extremely large

light and nutrient shadow if it were to grow on the underlying rock substratum. Two questions need to be addressed: 1) what evidence is there that *P. lanosa* is an obligate epiphyte? and 2) why is there an apparent host specificity for *A. nodosum* when other fucoids seem to be available? In Nova Scotia where *P. lanosa* is widespread and often abundant, and several *Fucus* species (mostly *F. vesiculosus*) are present in the same habitat as *A. nodosum*, *P. lanosa* grows only on *A. nodosum*.

Spores of *P. lanosa* germinate and grow in unialgal culture. However, they do not assume normal morphology, and typically stop growing and senesce after about six weeks (Tian & Garbary, 1992). In experiments with branches excised from large vegetative plants, these also senesced more rapidly when not attached to a host relative to attached fragments (Tian & Garbary, 1992). In addition, when *P. lanosa* was surgically reattached to *A. nodosum* or to *F. vesiculosus* or *F. serratus* there was no difference in survival rates. Pearson & Evans (1991) demonstrated that rhizoid growth was stimulated by ultraviolet absorbing exudates from both *F. vesiculosus* and *A. nodosum*. These experiments showed equal survival or stimulation with both *A. nodosum* and *Fucus* species. The results of Pearson & Evans (1991) and Tian & Garbary (1992) confirm that *P. lanosa* is an obligate epiphyte, but provide no clues as to the specificity with *A. nodosum*.

The apparent host specificity of *P. lanosa* can be accounted for based on biophysical and ecological adaptations of the epiphyte to its environment. Pearson & Evans (1990) used a flow tank to show that shape differences in thalli of *A. nodosum* and *F. vesiculosus* led to random settlement of spores on the latter, and non-random as well as greater spore settlement on the former. The sites 'selected' by *P. lanosa* are preferentially those sites where it is normally attached in nature, i.e. axils of branches and pits caused by damage. Fucacean algae have several strategies for epiphyte removal, one of which is the shedding of the outer layer of the thallus wall at regular intervals (e.g., Filion-Myklebust & Norton, 1981). At points of damage and in branch axils, this shedding is much slower, thus giving epiphytes such as *P. lanosa* more time to grow through the epidermis and better anchor the thallus.

Reproductive phenology provides another ecological convergence that favors an association between these species (Garbary *et al.*, 1991). *A. nodosum* has a short window in which it matures and releases the gametes and then sheds the receptacles in which those gametes were formed. Hundreds of receptacles per erect frond thus make scar sites on the thallus surface which are suitable attachment sites for *P. lanosa*. *P. lanosa* typically sporulates in the several months subsequent to when the receptacles are shed, thus taking advantage of the production of new and suitable substrata.

These combined observations suggest that *P. lanosa* is restricted to *A. nodosum* as a host because of recruitment restrictions on other potential hosts (e.g., *F. vesiculosus*), not because of a biochemical dependency on *A. nodosum*.

5. Interactions between *Mycophycias* and *Polysiphonia*

Few ecological or morphological studies have implied interaction between *M. ascophylli* and *P. lanosa*. In addition, ultrastructural studies by Rawlence (1972) and Rawlence & Taylor (1972) did not uncover any interaction between the fungal hyphae and the rhizoids of the epiphyte. Although extensive degradation of host cells occurred in localized areas in advance of rhizoid tip growth, and in cells adjacent to the rhizoid, no comment was made about the potential involvement of *M. ascophylli* in this process. The only observation that suggested a possible interaction between *M. ascophylli* and *P.*

lanosa was the positive correlation between biomass of *P. lanosa* and density of pseudothecia on *A. nodosum* receptacles in transects through the intertidal zone in Nova Scotia (Garbary & Gautam, 1989). This correlation was considered somewhat spurious, and possibly reflected a similar position in the intertidal zone that was optimal for both species.

We have recently noted complex interactions between hyphae of *M. ascophylli* and rhizoids of *P. lanosa* (Garbary & Deckert, in prep.). Single hyphae are present on the rhizoid surface as well as masses of hyphae similar to the hyphal aggregations in the lower cortex of the host in tissues uninfected with *P. lanosa*. In addition, there is extensive penetration of the rhizoid cell wall by hyphae connected to the hyphal aggregations of the simple hyphae on the rhizoid surface. The functional significance of this association is unclear; however, it could be interpreted in several ways that are not mutually exclusive. First, the fungus may not be able to distinguish between the walls and intercellular spaces of its host and the rhizoid wall of *P. lanosa*. Secondly, this may be a parasitic interaction in which the fungus is attempting to withdraw nutrients from the rhizoid. Thirdly, it might be a unified response on the part of the symbiotum to restrict growth of the rhizoid, and limit its damage to the host.

In support of the latter argument is the observation of an apparent hypersensitive reaction in which the host symbiotum transforms a number of cells in the vicinity of the rhizoid. These transformed cells become darker in colour from a presumed increase in polyphenolics and become heavily infested with fungal hyphae (Garbary & Deckert, in prep.). Cause and effect relationships are unclear; however, this interaction seems equivalent to responses of terrestrial plants to infection by pathogens.

A fourth possibility can be hypothesized based on results of Penot *et al.* (1993) who described phosphorus transport from *A. nodosum* to *P. lanosa*. If this transfer was mediated by *M. ascophylli*, then this raises the spectre of the fungus acting like a mycorrhiza. What would complete this analogy is the regular transport of photosynthate into the fungus in excess of simple diffusion. Although this has not yet been demonstrated, it may be dependent upon life history stages of *P. lanosa* and source-sink interactions. For example, during development of reproductive organs it is unlikely that carbon would be massively transported to the rhizoid for possible transport to the fungus. This might explain the conflicting results of Harlin & Craigie (1975), Turner & Evans (1977) and Ciciotte & Thomas (1997).

6. Other Symbionts

Given the biomass and the surface area of *A. nodosum* it is not surprising that other algae and fungi have successfully colonized its surfaces or become endophytes or parasites. The multicellular algae involved are mostly generalized epiphytes, and include species of ectocarpoids (e.g., species of *Ectocarpus* and *Pilayella*) and *Polysiphonia* spp. other than *P. lanosa*. The abundance of the snail, *Littorina obtusata*, suggests that there is an epiphytic microflora that is being continually grazed. One microalga, *Navicula endophytica*, was described as an endophyte in the receptacles of *A. nodosum* and a related species, *N. fucicola*, was described in the frond apices of *Fucus vesiculosus* (Hasle, 1968; Taasen, 1972, 1975). The nature of the relationship between *N. endophytica* and *A. nodosum* is unknown, as is the mechanism of transmission from one host thallus to another.

Other than *P. lanosa* the most common epiphyte is the brown alga, *Elachista fucicola*. This species is an obligate epiphyte but it is not restricted to *A. nodosum*

(Fletcher, 1987). In Nova Scotia *E. fucicola* commonly occurs on both *A. nodosum* and *F. vesiculosus*. Two forms of the epiphyte seem to occur with different basal systems. In one form the epiphyte is superficial with penetrating rhizoids. In the other form there is an extensive basal system that penetrates the host (Garbary & Deckert, in prep.). The rhizoids break down the host cells in advance of the growing tips. This initially forms a narrow pocket that later expands into a conceptacle-like chamber that is plugged at the neck by the epiphyte. The host then redifferentiates an epidermal layer around the cavity chamber; however, the cavity is lined by a thick layer of gel-like material representing the remains of the cell wall material of the host. Hyphae of *M. ascophylli* remain in the chamber and become associated with the rhizoidal system of the *E. fucicola*, and even attach to the non-rhizoidal system of the epiphyte. Kingham & Evans (1986) reported a similar phenomenon in which hyphae that grew from within *A. nodosum* penetrated into the holdfast of epiphytic *Fucus* thalli.

Although *E. fucicola* may be independent photosynthetically and the basal system described for it above may represent only an attachment mechanism, this association does seem to represent a parasitic interaction. The cavities themselves represent real damage and may provide breakage sites. Indeed, large clumps of *E. fucicola* often terminate broken axes of *A. nodosum*, suggesting a parasitism. Cause and effect associated with this observation need to be investigated.

The ascomycetes *Orcadia ascophylli* and *Trailia ascophylli* are parasites described from species of *Ascophyllum*, *Pelvetia* and *Fucus*. Little is known about these taxa; however, Kohlmeyer & Kohlmeyer (1979) suggested that *O. ascophylli* was infecting previously damaged tissues, rather than being a primary parasite. The ascomycete *Orbilia marina* has been identified as a saprophyte associated with *A. nodosum* and other fucoids (Kohlmeyer & Kohlmeyer, 1979). More extensive analysis may reveal other fungi associated with *A. nodosum*.

7. Summary and Prospects

Ascophyllum nodosum and *M. ascophylli* clearly have a mutualistic symbiosis. The interaction between these species is highly unusual because both symbionts are obligate mutualists, but the endosymbiont has retained full sexual reproductive ability and horizontal transmission from host to host. In this sense the *A. nodosum/M. ascophylli* symbiosis differs from that found in the grass symbiotum. Thus for a portion of its life cycle, the endophyte is subjected to environmental conditions (and therefore evolutionary pressures) that may differ from the host. This situation has been proposed as being evolutionarily unstable, and runs counter to current theories of factors predisposing to mutualistic interaction (Herre *et al.*, 1999). Consequently, *A. nodosum* and *M. ascophylli*, may be a model system that can provide a greater understanding of the importance and function of sexual reproduction in mutualistic symbioses. The differential response of *M. ascophylli* to *P. lanosa* and *E. fucicola*, algae which differ in their affinities to *A. nodosum*, may also be a good model for the study of host specificity and host recognition mechanisms in fungi.

The intimate association of *P. lanosa* with the symbiotum allows scrutiny of the effects of third party interactions on the costs and benefits of the mutualism. *P. lanosa* shows a very limited parasitism with its host; however, the interaction is essentially benign (and therefore commensal). It is also possible that *A. nodosum* benefits from being epiphytized by *P. lanosa*, and further study is required to clarify this possibility. Morphological interactions between *M. ascophylli* and *P. lanosa* permit novel

interpretations of previously conflicting physiological data involving *A. nodosum*, and provide the basis for further experiments. Molecular studies should contribute to the ecology of dispersal in these symbionts, and to population and regional level differentiation. If one includes *E. fucicola* as a partner in a four way interaction, this could be a good model system to test Law's (1985) hypothesis that mutualistic environments have a tendency to undergo evolutionary improvements.

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Paola Bonfante

ARBUSCULAR MYCORRHIZAL FUNGI AND THEIR ENDOBACTERIA

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1. Mycorrhiza: a Symbiotic Life Style

The term "mycorrhiza" defines a symbiotic association established between a heterogeneous group of soil fungi and the roots of about 240,000 plant species including ferns, gymnosperms and angiosperms (Smith and Read, 1997). Mycorrhizas are successful in colonizing diverse environments, from alpine and boreal zones to tropical forests and grasslands: their ecological success (van der Heijden et al., 1998) reflects a high degree of diversity in the genetic and physiological abilities of the fungal endophytes. About 6,000 species in the Zygo-, Asco- and Basidiomycotina have been recorded as mycorrhizal, and the advent of molecular techniques is making this figure increasingly higher, also revealing a high degree of inter- and intraspecific genetic diversity (Kowalchuk, 1999).

Among the mycorrhizal types, arbuscular mycorrhiza (AM) is the most widespread, since it has been found in the roots of about 80% of plant species. Arbuscular mycorrhiza is defined by the taxonomic position of the fungal partner, which belongs to Glomales, and by the formation of a typical intracellular fungal structure (the arbuscule) inside the root cells. As in the other mycorrhizas, the fungus acts as a sink for organic molecules derived from the plant photosynthesis and as a source for minerals, mostly phosphates, assisting therefore the plant with the acquisition of nutrients and water (Smith and Read, 1997). As a result of these nutritional modifications AM fungi influence plant growth and protect the host from root diseases. They are currently considered as key organisms in agricultural programs based on a low input of chemicals (Bethenfalvay and Linderman, 1992).

2. AM Fungi are Ancient Organisms

AM fungi belong to the Glomales, a small order within the Zygomycetes (Morton et al., 1995). Their origin has been dated to 353-460 Mya on the basis of ribosomal sequences performed on isolates chosen as representatives of the three families

described in the Glomales: Gigasporaceae, Acaulosporaceae and Glomaceae (Simon et al., 1993). The phylogenetic tree shows that they had a long evolutionary history: *Glomus* is probably the oldest genus (ca. 460Mya), while *Acaulospora* and *Entrophospora* are more recent (250 to 110 Mya). Fossil reports show that tissues of Devonian plants such as *Rhynia* and *Aglaophyton* were colonized by fungal structures which resemble the infection structures produced by todays' AM fungi (Taylor et al., 1994). Since these first land plants had a poorly developed root system, their access to mineral nutrients would have been strongly improved by the presence of fungi able to take up elements from the soil. The close relationship existing between molecular phylogenesis and fossil reports, as well as the finding of a phosphate transporter expressed in the external hyphae of *G. versiforme*. (Harrison and van Buuren, 1995), lend support to the suggestion that AM fungi were instrumental in the colonization of land by ancient plants (Pirozynski & Malloch, 1975). Plants and fungi may therefore share a symbiotic life style since Devonian times, and this ancient association may have provided the genetical and structural background for the establishment of another important symbiosis, the *Rhizobium*/legume association (LaRue and Weeden, 1994).

3. The Biological Bases of the Interactions Between AM Fungi and Plants: a Short Summary

AM fungi constitute one of the most widespread microbial communities in the rhizosphere, where they undergo a complex morphogenesis both outside and inside the plant root (Fig. 1).

The fungal spores germinate in the soil and give rise to an extraradical mycelium able to perceive host-derived signals and to produce highly branched hyphae (Buee et al., 2000) that contact the host surface and differentiate appressoria (Giovannetti, 1998). Appressoria originate hyphae that initiate root infection and form inter- and intracellular hyphae, coils, highly branched arbuscules and, in some cases, vesicles. AM thus constitutes a complex system in which balanced development is achieved through regulation of fungal and host growth (Bonfante and Perotto, 1995).

Both the plant and the fungal genomes are responsible of the different colonization patterns described, and the analysis of plant mutants impaired in their symbiotic capabilities has demonstrated the genetic bases of the plant response to fungal colonization (see for example Gianinazzi-Pearson, 1996; Barker et al., 1998; Bonfante et al., 2000).

New technologies have allowed breakthroughs in our knowledge of the molecular basis of plant/fungal interactions (Harrison, 1999). Recent research on genomics, involving model plants like *Medicago truncatula*, has led to the development of about 100,000 expressed sequence tags (ESTs) from this legume plant (Downie and Bonfante, 2000). Techniques of subtractive hybridisation and analysis of differentially expressed sequences have allowed Gianinazzi-Pearson and her colleagues (2000) to draw a profile of the genes specifically expressed during the AM symbiosis.

Despite these recent achievements in our knowledge of the molecular basis of plant/fungal interactions, many aspects the biology of AM fungi - and in particular of their genome- are still obscure. This is mostly due to their biotrophic status, their

multinuclear condition, and an unexpected level of genetic variability (Hijri et al., 1999; Hosny et al., 1999; Lanfranco et al., 1999).

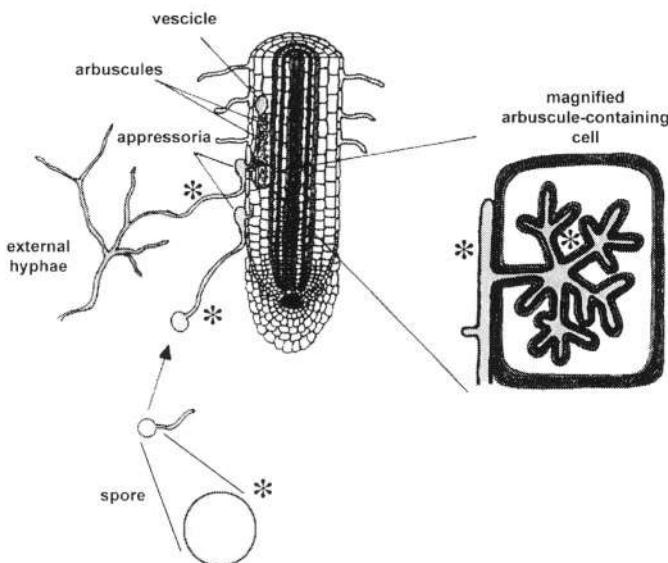


Fig.1. The scheme illustrates the most significant steps of the life cycle of AM fungi. An asterisk marks the fungal structures where endobacteria have been detected.

4. AM Fungi Harbor Bacteria in Their Cytoplasm

A peculiar feature of AM fungi is that they host intracellular structures very similar to bacteria (Fig. 2), called Bacteria-like Organisms (BLOs) and first described in the 1970s (Mosse, 1970; Scannerini and Bonfante, 1991 for a review). Ultrastructural observations clearly revealed their presence in many field collected fungal isolates. Further investigation on these BLOs, including the demonstration of their prokaryotic nature, was hampered for long time because of their inability to grow on plate. Only recently a combination of morphological observations (electron and confocal microscopy) and molecular analyses have allowed us to identify BLOs as true bacteria and to start unravelling their symbiotic relationship with AM fungi (Bianciotto et al., 1996).

Isolate BEG 34 of *Gigaspora margarita* contains a large number of BLOs which can be easily detected by staining with fluorescent dyes specific for bacteria and capable of distinguishing between live and dead bacteria. About 250,000 live bacteria were counted in a single spore, which is a large structure of about 200-220 μm . Ultrastructural observations performed on high-pressure freezing/freeze-substituted samples revealed a large number of rod-shaped BLOs in the vacuoles of germinating

spores, often associated with the abundant protein bodies (Bonfante et al., 1994). The bacteria feature a laminated wall and the cytoplasm is rich in ribosomes (Fig.3). Similar bacteria, some of them undergoing cell divisions, were found in both germinating and intraradical hyphae, as well as in the arbuscule trunks.

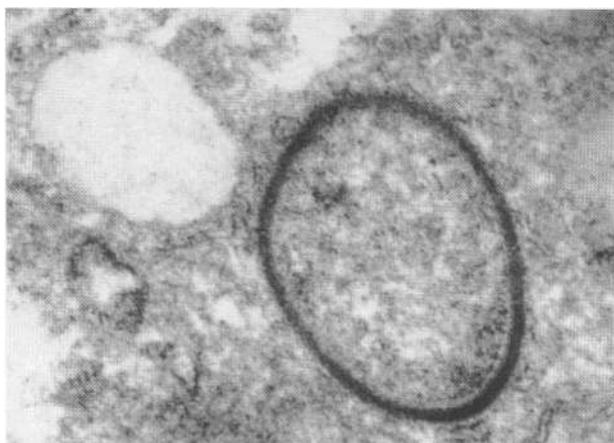


Fig.2 Transmission electron micrograph showing a BLO living in an intraradical hypha of *Glomus versiforme*.

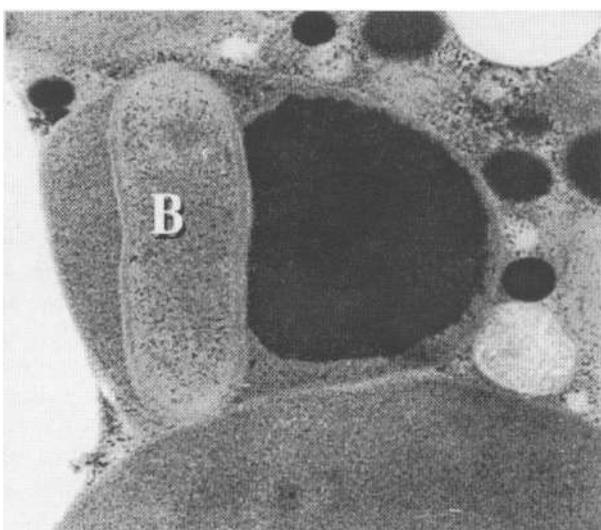


Fig.3 Transmission electron micrograph showing the endosymbiont Burkholderia living in the spore of *Gigaspora margarita*

Amplification of DNA extracted from spores of *G. margarita* BEG 34 with universal bacterial primers designed on the small subunit ribosomal gene (16S rDNA) produced a single DNA fragment, whose direct sequencing unambiguously showed that BLOs in *G. margarita* represent a homogeneous population of true bacteria. Comparison with other bacterial sequences indicated that they belong to the genus *Burkholderia*, a group belonging to the beta subdivision of the Proteobacteria (Bianciotto et al., 1996).

5. Detection, Identification and Phylogeny of Endobacteria in the Gigasporaceae

The detection of *Burkholderia* in the spores and intraradical hyphae of the BEG 34 isolate, originally from a New Zealand soil, raised the question whether the same bacteria are also harbored by other Glomales, and whether they can be found in isolates from different geographic areas. We focussed our attention on the Gigasporaceae, which comprise the genera *Gigaspora* and *Scutellospora*, because they provide a more amenable experimental system. In fact, bacterial DNA can be amplified also from Glomaceae (Bianciotto et al., 1996; Hosny et al., 1999a), but more detailed investigations are hampered by the low number of intracellular bacteria and by the strong microbial contamination of the spore surface (Bianciotto, unpublished observations).

Twelve fungal isolates collected from different geographic areas and belonging to six different species of Gigasporaceae were recently investigated with a combined morphological and molecular approach (Bianciotto et al., 2000). The fluorescent dye was first used to detect bacteria inside the spore cytoplasm, while 16S ribosomal genes were amplified by PCR using universal bacterial primers and primers specific for the endobacteria already identified in *G. margarita* BEG 34 (BLO primers). With the exception of the four isolates of *Gigaspora rosea*, bacteria could be visualized in the cytoplasm of all fungal isolates, and an amplified DNA fragment of the expected size was obtained with the universal bacterial primers. The BLO primers could further amplify DNA from seven out of eight isolates, belonging to five different species (Table I).

The number of species and isolates so far analyzed in the Gigasporaceae does not allow us to draw general conclusions on their presence in the whole family, which includes more than thirty different species according to Walker and Trappe (1995). However, our observations indicate some interesting features of the endobacteria/AM fungal association. In *Gigaspora*, where four out of the five species included in this genus were analysed (see the recent taxonomic revision of the genus *Gigaspora* in <http://invam.caf.wvu.edu/taxonomy.htm>) only *G. rosea* lacked the endobacteria. This finding demonstrates that endosymbionts are a common feature at least in *Gigaspora*.

Another interesting finding is that fungal isolates belonging to the same species but derived from distant geographic areas shared a similar situation in terms of bacterial number, shape and 16S rDNA sequence. By contrast, different species could present quite distinct features. *G. margarita* harbors an estimated number of 250,000 bacteria

per spore, whereas *G. rosea* shows none. *G. gigantea* contains numerous endosymbiotic bacteria, but they are different from those found in *G. margarita* and *G. decipiens* because of their round shape and because total spores DNA could not be amplified with the BLO primers.

In *Scutellospora*, both species investigated contained rod-shaped endobacteria that amplified with the BLO primers (Hosny et al., 1999a; Bianciotto et al., 2000), although some variability in the bacterial number was found in the two isolates of *S. persica*. Since the genus *Scutellospora* comprises almost thirty species, the analysis of a wider range of species is needed to elucidate how common are endobacteria in this genus.

AM fungi and bacteria interact at different levels of cellular integration, ranging from apparently loose association, through surface attachment, to intimate and obligatory endosymbiosis (Perotto and Bonfante, 1997). The simultaneous presence of bacteria outside and inside the hyphae requires therefore careful experimental procedures to ensure that PCR amplification is targeted to the endosymbiotic bacterial DNA and not to surface contaminating bacteria. To confirm the topological position of the fungal endobacteria, protocols for *in situ* hybridisation, coupled to the use of specific probes, were set up (Bianciotto et al., 2000). They successfully identified the bacterial cells inside the fungal cytoplasm of two isolates of *G. margarita*, mostly when they were grouped and close to the fungal lipid bodies present in the spores. These experiments provide a nice confirmation of the taxonomic identity of the endobacteria in the fungal cytoplasm.

To understand whether the bacteria living in the Gigasporaceae are closely related to each other, the 16S rDNA amplified from two isolates of *S. persica*, *S. castanea* and from one Brazilian isolate of *G. margarita* was sequenced and aligned with the closer bacterial sequences available in databases and with the sequence previously obtained from the BEG 34 isolate (Bianciotto et al., 2000). The *G. margarita* BEG 34 endosymbiont was originally classified as a sister group of *Burkholderia cepacia* (Bianciotto et al., 1996). However, a comparison of the 16S rDNA sequences has revealed a clear separation of *Burkholderia* into two branches, and a number of species previously assigned to this genus have been regrouped into the genus *Ralstonia* (Yabuuchi et al., 1995). This taxonomic rearrangement, as well as the identification of novel species of *Burkholderia*, has re-opened basic questions concerning the taxonomic position of the endosymbiotic bacteria of Gigasporaceae.

The neighbour-joining tree obtained from the alignment of the 16S rDNA outlined a strongly supported branch containing all endosymbiotic bacterial sequences so far obtained, thus indicating that endobacteria of Gigasporaceae are closely related. It also suggests that the closest relatives of these endobacteria are still inside *Burkholderia*, as they form a well supported branch nested in this genus and well separated from *Ralstonia*.

Table 1 AM fungal isolates investigated with morphological and molecular markers (Bianciotto et al., 2000).

Isolate	Origin	Isolate code [§]	Detection of bacteria	Amplification with BLO primers
<i>Gigaspora margarita</i> Becker & Hall	New Zealand	BEG 34	+	+
<i>Gigaspora margarita</i> Becker & Hall	U.S.A.	INVAM WV205A-5	+	+
<i>Gigaspora margarita</i> Becker & Hall	Brazil	Personal collection	+	+
<i>Gigaspora rosea</i> * Nicolson & Schenck	unknown	DAOM 194757	-	-
<i>Gigaspora rosea</i> Nicolson & Schenck	U.S.A.	BEG 9	-	-
<i>Gigaspora rosea</i> Nicolson & Schenck	U.S.A.	BEG 9	-	-
<i>Gigaspora rosea</i> Nicolson & Schenck	U.S.A. (Florida)	INVAM 185	-	-
<i>Gigaspora gigantea</i> Gедermann & Trappe	U.S.A	HC/F E30	+	-
<i>Gigaspora decipiens</i> Hall & Abbott	unknown	BEG 45	+	+
<i>Scutellospora persica</i> (Schench & Nicol.) Walker & Sanders	Porto Caleri (Rovigo) Italy	HC/F E28	+	+
<i>Scutellospora persica</i> (Schench & Nicol.) Walker & Sanders	Migliarino (Pisa) Italy	HC/F E09	+	+
<i>Scutellospora castanea</i> Walker	unknown	BEG 1	+	+

[§] BEG, European Bank of Glomales; DAOM, Department of Agriculture, Ottawa, Mycology, INVAM, International culture collection of arbuscular and Vesicular-Arbuscular Mycorrhizal Fungi; PC, Personal collection, HC/F, Herbarium Cryptogamicum Fungi, Department of Plant Biology, Turin, Italy

6. Some Hypotheses on the Establishment of Symbiosis Between AM Fungi and Their Endobacteria

Intracellular symbioses raise fascinating questions about the acquisition of the endosymbionts, their transmission and the evolution of partner adaptations (Margulies and Chapman, 1998). The presence of endobacteria, or BLOs, in all glomalean families suggests that the ability of AM fungi to establish this type of association appeared very early in evolution.

Margulies and Chapman (1998) discuss the importance of endosymbiosis as an evolutionary mechanism and distinguish between permanent and cyclical endosymbioses, the first remaining stable over time and the latter involving regular re-association events. In this respect, the type of relationship between AM fungi and their

endobacteria remains an open question that will be more properly addressed with the analysis of a wider range of species and isolates. However, the observations made so far in the Gigasporaceae suggest at least two possible and opposite scenarios.

Closely related endobacteria were found in isolates of *G. margarita* from distant geographic areas and formed a homogeneous bacterial population in the fungal spores. This situation, which reflects the establishment of a stable bacterial population in the fungal cytoplasm, may be the result of very rare events of bacterial acquisition during evolution, followed by a strictly vertical transmission of the endosymbionts (permanent symbiosis). The asexual reproduction typical of AM fungi and the coenocytic nature of the mycelium in the Zygomycetes (Sanders, 1999) are factors that could facilitate this type of transmission.

Morphological and molecular observations of *G. gigantea* indicate that its endobacteria are different from those of other species analysed. Sequencing of the rDNA from its endobacteria will elucidate whether failure to obtain an amplified fragment with the BLO primers is due to limited mismatches in the annealing primer region or to very diverging sequences. This latter case would be evidence of a separate endosymbiotic event, thus indicating that the frequency of bacterial acquisition during evolution could be relatively high. The situation in *G. rosea* would suggest that bacteria can be not only acquired during evolution, but also be lost by the AM fungus.

However, an alternative and opposite scenario can be also envisaged. The complex situation observed in the Gigasporaceae could be originated by more temporary but frequent associations of AM fungi with free-living soil bacteria (cyclical symbiosis), with different fungal species selecting their specific bacterial symbionts from the environment. Different species of free-living *Burkholderia* have been identified in the rhizosphere and the hyphosphere of AM fungi and may represent a reservoir of potential endosymbionts. The cell wall that surrounds the fungal hypha represents a physical constraint that makes endocytosis, and thus acquisition of bacteria from the environment, a very rare event in fungi. However, Zygomycetes may represent a special case among fungi since *Geosiphon pyriforme*, a Zygomycete closely related to Glomales (Gherig et al., 1996), is the only known case of a fungus able to establish cyclical endosymbiotic associations with cyanobacteria.

If endobacteria are acquired cyclically from the environment, their low level of genetic variation within individual fungal isolates and in different isolates of the same species would indicate a very high degree of specificity. Endosymbiotic interactions between plants and bacteria can be very selective, as demonstrated for example in the *Rhizobium*-legume symbiosis (Long, 1996). Prokaryotes can also interact very specifically with fungi, as recently demonstrated in the lichen symbiosis (Paulsrud and Lindblau, 1998). In lichens, the relationship established between fungi and nitrogen-fixing bacteria is not an endosymbiosis because the cyanobacteria, acquired from the environment during lichen formation, remain outside the fungal hyphae. However, analysis of the genetic polymorphism of *Nostoc* cyanobacteria in some lichen species has revealed a situation similar to the one observed for endobacteria in AM fungi. Cyanobacteria living within a single or in different thalli belonging to the same lichen species were found to be genetically very homogeneous (Paulsrud and Lindblau,

1998), likely as a result of specific recognition mechanisms, which allow only one or a few cyanobacterial strains to be accepted by one fungal species. Free-living relatives of the lichen-associated *Nostoc* strains were found by sequence analysis (Paulsrud and Lindblau, 1998). By contrast, no close relatives of the endosymbiotic bacteria of Gigasporaceae have been so far identified among the rDNA sequences of free-living *Burkholderia* deposited in gene banks. Attempts to isolate the endobacteria of *G. margarita* into culture have been so far unsuccessful (V. Bianciotto and E. Lumini, unpublished data), thus indicating that endosymbiotic *Burkholderia* have special growth requirements and may even be obligate biotrophs. In this latter case, the hypothesis of frequent acquisition from the soil as free-living bacteria would be more difficult to sustain.

A number of symbioses have been recently analyzed to investigate whether the phylogenetic pattern of the endosymbiont parallels the phylogeny of the host. Cospeciation, which offers evidence to coevolution has been demonstrated by Peek et al. (1998) for chemoautotrophic bacteria and deep sea clams by using the bacterial 16S rDNA sequence and the clam mitochondrial 16S rDNA sequence. It will be exciting to elucidate if cospeciation has occurred between AM fungi and their endobacteria by analysing a larger spectrum of endobacterial sequences.

7. What is the possible metabolic role for the endobacteria of AM fungi?

Symbiosis is a powerful source of physiological and structural novelty (Margulis, 1991), and all symbioses bring new functions into play. In particular, symbiotic associations of eukaryotes with bacteria usually involve the acquisition for the hosts of new pathways in nitrogen metabolisms: nitrogen-fixing bacteria in legumes or bacterial endosymbionts of insects are only some examples.

The specific role played by BLOs in AM fungi is still unknown, since our inability to grow them in pure culture prevents their functional analysis. The genus *Burkholderia* is characterized by a high metabolic versatility and includes members that may be pathogens on plants and humans, saprotrophs and free-living bacteria. *Burkholderia* isolated from tomato roots display biocontrol potential on pathogenic fungi and bacteria (Bevivino et al., 1994; Tipper et al., 1998).

To overcome the problems posed to a biochemical study of endobacterial functions, a more feasible genetic approach has been used to identify prokaryotic genes that may be related to specific biological processes or metabolic pathways.

Genes involved in the colonization process of eukaryotic cells and in the acquisition of nutrients were investigated by using a targeted approach. To isolate these genes, we screened a genomic library constructed with total DNA from spores of *G. margarita* (BEG 34), which was demonstrated to be representative of the bacterial genome (van Buuren et al., 1999).

As discussed previously, nothing is known about the molecular mechanisms that allow entry of endobacteria into the AM fungus, and about the control of colonization of the AM cytoplasm. A gene identified as a *vacB* gene was isolated from the genomic

library and characterized (Ruiz-Lozano and Bonfante, 2000a). Although VacB was first defined as a virulence factor, it is currently known to be an exoribonuclease RNase R involved in posttranscriptional processing of mRNAs. It enables production of the Vir proteins encoded by *virG*, *ipa*, region-3, region-4, and region-5 operons, and so modulates the ability of bacteria to adhere and to penetrate cells, and later spread for full virulence expression. Specific primers designed on the nucleotide sequence successfully amplified a PCR fragment of the expected size from DNA extracted from *G. margarita* and *S. persica* spores. No amplification was obtained from two isolates of *G. rosea*, which are devoid of intracellular bacteria. Endosymbiotic *Burkholderia* seem therefore to possess the molecular determinants required for the colonization of a eukaryotic cell. Interestingly, a corresponding DNA fragment could be amplified from some rhizospheric *Burkholderia* isolates (Ruiz-Lozano and Bonfante, 2000a).

AM fungi mostly assist their host plant by providing phosphate (Harrison 1999). To investigate whether *Burkholderia* can somehow influence phosphorus metabolism in AM, degenerated oligonucleotide primers were designed on conserved regions of a gene coding for a subunit of a bacterial phosphate transporter. When these primers were used to amplify total spore DNA from *G. margarita*, a DNA fragment was obtained, which was confirmed to correspond to the phosphate transporter subunit (Ruiz-Lozano and Bonfante, 1999). The insert was then used as a probe to screen the genomic library from *G. margarita*, leading to the isolation and characterization of the complete operon coding for a high-affinity phosphate transporter. The organization of the *Burkholderia* operon (e.g. gene order, direction of transcription) is the same as in *E. coli* and similar to many other bacteria, thus confirming the notion that this type of transporter is highly conserved. To demonstrate that the operon is contained in the genome of the intracellular *Burkholderia*, and to exclude the possibility that it may derive from surface spore contaminants, the same procedure already used for the *vacB* gene was followed. Specific primers were designed and used in PCR on DNA extracted from carefully surface sterilized spores. The primers successfully amplify fragments of the expected size in spores of *G. margarita* and *S. persica*, which contain related endobacteria (Bianciotto et al., 2000), whereas no amplification occurred on DNA from the related species *G. rosea*, which is devoid of intracellular bacteria (Ruiz-Lozano and Bonfante, 1999).

Since some free-living *Burkholderia* are known to fix nitrogen (Gillis et al., 1995), a strategy was developed to investigate whether *nif* genes are present also in the genome of endosymbiotic *Burkholderia*. Preliminary investigations based on the use of a *nifDK* probe from *Azospirillum brasiliense* have led to the identification of several positive clones during the screening of the genomic *G. margarita* library. The putative protein encoded by one of these clones showed a very high degree of sequence similarity (> 90%) with the NifD protein from different nitrogen fixing microorganisms. In addition, RT-PCR experiments performed with specific primers on the mRNA extracted from germinating spores of *G. margarita* indicate that the transcript is present at this developmental stage (Minardi et al., 2001). It will be exciting to get the complete sequence of these *nif* genes, to understand whether and when they are transcribed and whether a functional nitrogenase is produced.

The discovery in the genome of endosymbiotic *Burkholderia* of genes involved in some important metabolic functions opens of course a number of intriguing questions. The presence for example of a bacterial phosphate uptake system indicates that endobacteria may influence the phosphate flux that takes place between the AM fungus and the host plant. Since AM fungi possess a high affinity phosphate transporter that is active in the extraradical mycelium (Harrison and van Buuren, 1995), endobacteria may have direct access to this phosphorus source and they could use it for their own metabolism, thus reducing the phosphorus flux to the root. Physiological studies will be required to properly address this question.

The effects of intracellular bacteria on plant growth and nutrient uptake were investigated by comparing in pot experiments the growth effects of a mycorrhizal strain which contains endobacteria (*G. margarita*) with those of a taxonomically related strain lacking them (*G. rosea*). The results obtained on lettuce plants showed that *G. margarita* had a higher symbiotic efficiency in nutrient acquisition on poor soil. Moreover, plants colonized by *G. margarita* had a higher P and N content when compared with plants colonized by *G. rosea* or *Glomus versiforme* (Ruiz-Lozano and Bonfante, 2000b). Even if the experimental approach was surely not the optimal one because two different, though related, species were compared, the results suggest that the occurrence of endobacteria has no negative influence on the phosphorus flux to the root. On the contrary, the endosymbionts may lead indirectly to a positive effect on plant nutrition, at least in poor soil conditions.

8. Conclusions

Morphological and molecular evidence indicate that AM fungi have been successful in time and space thanks to a long co-evolution with their host plants. In addition to this well known interaction, they also associate with bacteria that reside in the fungal cytoplasm. The presence of bacterial endosymbionts is not a sporadic event, at least in the Gigasporaceae, where they have been found in many species. This endosymbiosis between bacteria and AM fungi adds a further level of complexity to arbuscular mycorrhiza because the cyclical AM symbiosis, according to Margulis and Chapman (1998), harbors a probably more permanent symbiosis with endobacteria.

Due to the large number of bacterial cells occurring in the extraradical as well as the intraradical fungal structures, endobacteria may influence not only the fungal host, but also indirectly the mycorrhizal plants. Like any endosymbiont, endobacteria represent a metabolic cost to the fungus, which must provide nutrients for their basic metabolism. However, the widespread occurrence of endosymbiotic bacteria among AM fungi suggests that they assist their host with some benefits, outweighing the balance and leading to an overall positive interaction.

9. Acknowledgement

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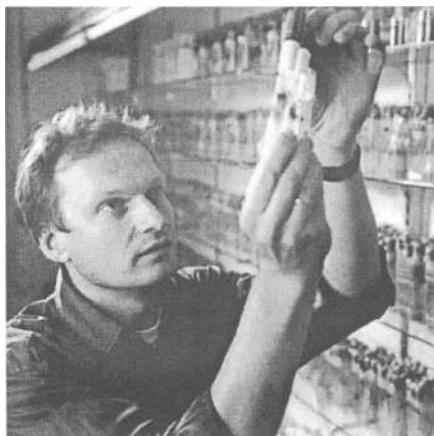
V. Lichens

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ORIGIN AND EVOLUTION OF GREEN LICHEN ALGAE*

Evolution of Green Lichen Algae

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1. Introduction

Lichens are the symbiotic phenotype of nutritionally specialised fungi (mycobionts) that derive carbon nutrition from algal and/or cyanobacterial cells (photobionts) located extracellularly within a matrix of fungal hyphae (Honegger 1991, Palmqvist et al. 1997). A central issue in lichen biology is the extent of diversity of both partners which form the various types of lichen associations. Approximately 13,500 lichen species have been named, corresponding to the diversity of the mycobiont (Hawksworth 1988, Kroken and Taylor 2000). Of these, 12,500 lichen species have a green alga as a photobiont and about 1,000 lichens species are associated with a cyanobacterium (Tschermark-Woess 1989, Kroken and Taylor 2000). Almost 40 genera of eukaryotic algae and cyanobacteria have been reported as lichen photobionts and their morphological diversity has been reviewed recently (Tschermark-Woess 1989, Büdel 1992, Gärtner 1992, Friedl and Büdel 1996). Only two genera of eukaryotic photobionts that are not green algae have thus far been reported (*Heterococcus*, *Xanthophyceae*, and *Petroderma*, *Phaeophyceae*: Tschermark-Woess 1989, Gärtner 1992). In this section, we describe the diversity and phylogenetic relationships of green algal photobionts as it is currently seen in phylogenetic analyses of nuclear-encoded ribosomal DNA (SSU and ITS rDNAs). rDNA sequences have been successfully used for inferring the phylogenetic relationships among the many lineages of green algae as well as to address questions below the level of genera including lichenized species (e.g., Friedl 1997, Marin and Melkonian 1999, Kroken and Taylor 2000, Helms et al. 2001, Friedl and O'Kelly, submitted). A large number of recent studies show lichen

* In memory of Professor Elisabeth Tschermark-Woess (1917-2001), a pioneer in the research of lichenized algae

fungal (e.g., DePriest and Been 1992, Gargas et al. 1995, Grube et al. 1996, Hibbet 1996, Takashima and Nakase 1997, Ito and Hirano 1999, Suh et al. 1999) and also lichen algal rDNA genes (Bhattacharya et al. 1996, Friedl et al. 2000) to be remarkably rich in group I introns. Therefore, the evolutionary history of rDNA introns from lichen associations will also be investigated here through phylogenetic analysis of intron and rDNA sequences.

2. Multiple origins of green lichen photobionts within the Viridiplantae

A phylogenetic analysis of SSU rDNA sequences from a small sample of green lichen photobionts and non-symbiotic representatives of all the major evolutionary lineages of the Viridiplantae (green plants) is shown in Fig. 1. This analysis clearly suggests that there are multiple origins of lichenization within the green algae. The Viridiplantae are divided into two major clades (Friedl 1997, Marin and Melkonian 1999): one clade contains the major green algal diversification representing the Chlorophyta (*sensu* Sluiman 1985) and the other unites a few green algae (charophytes) with the embryophytes representing the Streptophyta (*sensu* Bremer 1985). Green lichen photobionts, as currently sampled, are found only within the Chlorophyta. Within that clade, lichen photobionts are known from two phylogenetically distantly related lineages, the classes Trebouxiophyceae and Ulvophyceae (Fig. 1). The Trebouxiophyceae (Friedl 1995) appears to contain most known green algal symbionts. No lichen photobionts are known thus far from lineages representing the class Chlorophyceae and the prasinophytes. Within the Chlorophyta, the three lines Trebouxiophyceae, Chlorophyceae, and Ulvophyceae radiate nearly simultaneously from each other. In most analyses, the Trebouxiophyceae and Chlorophyceae form sister taxa with the Ulvophyceae as basal, but these relationships have proven difficult to establish with single-gene phylogenies (e.g., Fig. 1). The prasinophytes (green flagellates with scales on their cell and flagellar surfaces) form a heterogenous assemblage of lineages that arise at the base of the Chlorophyta (Nakayama et al. 1998). The prasinophyte, *Mesostigma viride*, is particularly interesting because it is basal to all charophytes and embryophytes (Fig. 1; Bhattacharya et al. 1998, Marin and Melkonian 1999).

The *Trentepohlia* strain used in Fig. 1 which has been isolated from a lichen is most closely related with the non-symbiotic *Cephaeluros parasiticus* which lives epiphytically on *Magnolia* leaves. *Trentepohlia* species form branched filaments in

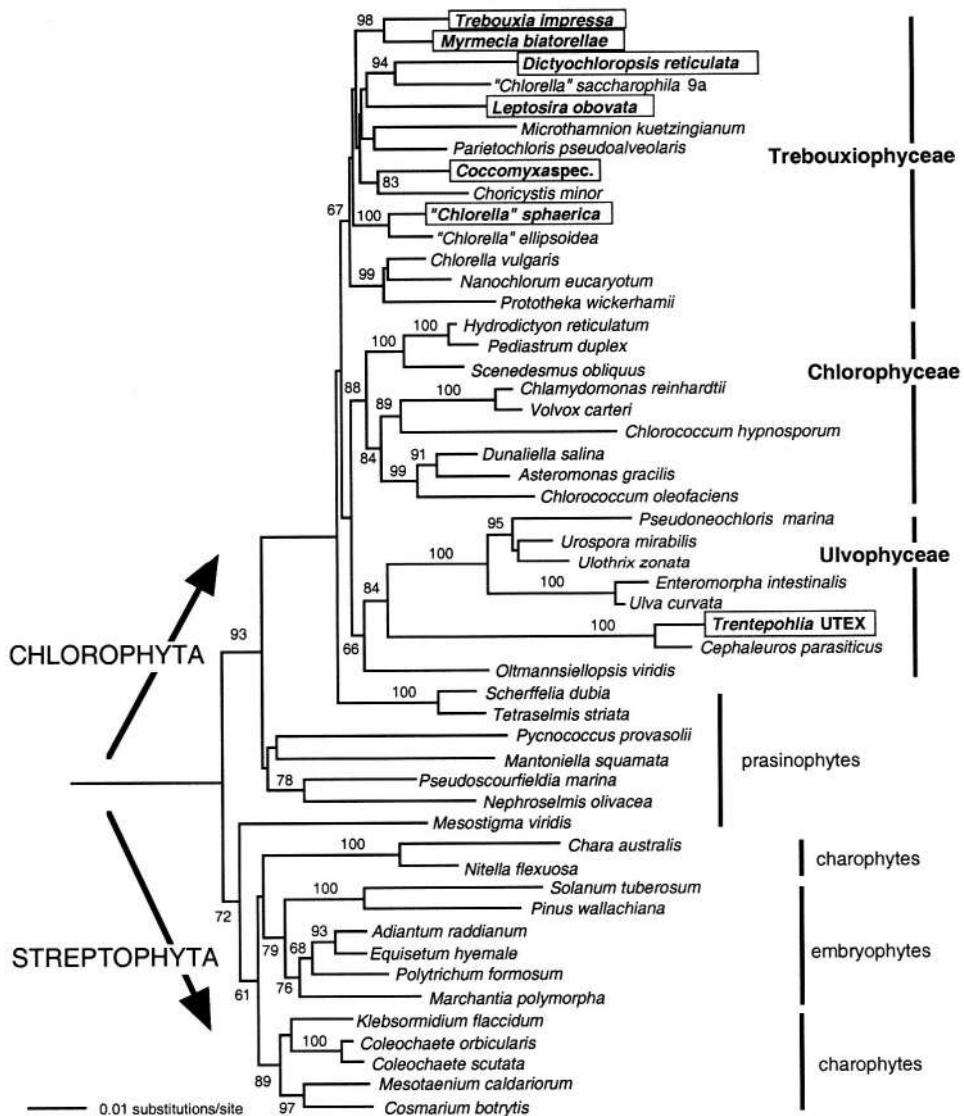


Fig. 1. LogDet tree of Viridiplantae based on SSU rDNA sequences that shows the multiple origins of green algal photobionts. Photobiont taxa are in boxes. A total of 1656 nt were considered. The bootstrap values result from the analysis of 1000 replicates (using PAUP* V4.0b8, Swofford 2001). The sequences used in this analysis may be directly accessed from the databases using the species names except the sequences for *Coccomyxa*, *Cephaleuros* and *Trentepohlia* which are unpublished.

culture and in the free-living state (where it occurs in terrestrial habitats as an epiphyte), whereas it is single-celled or of few cells per filament in the lichen symbiosis. *Cephaleuros* forms parenchymatous discs, but grows as filaments similar to those of *Trentepohlia* in culture. The sister taxa of *Trentepohlia* and *Cephaleuros* are not known yet. The common origin of *Trentepohlia* and *Cephaleuros* with other ulvophytes is corroborated by the counterclockwise orientation of basalbodies in the

zoospores (Chapman 1981, Roberts 1984), but the presence of a phragmoplast in *Cephaleuros* (Chapman and Henk 1986) is a feature typical of the Streptophyta. The phylogeny shown in Fig. 1 implies that a phragmoplast has evolved at least twice in the Viridiplantae. Whether a phragmoplast is developed within the nuclear envelope (as in *Cephaleuros*; Chapman and Henk 1986) or after the nuclear envelope has disappeared as in most streptophytes, may be a phylogenetically important distinguishing feature. Though robustly supported in Fig. 1, the exact position of *Trentepohlia/Cephaleuros* within the Chlorophyta needs to be further tested by a molecular systematic sequence analysis of additional putative ulvophytes; e.g., the Caulerpales and Siphonocladales (Floyd and O'Kelly 1990).

For other green lichen algae, the rDNA sequence studies reveal their phylogenetic position to be within the class Trebouxiophyceae (Figs 1,2). Most known members of this class exhibit a coccoid growth form and predominantly live in terrestrial habitats or occur in lichen symbioses. However, a few filamentous members (e.g., *Leptosira*, *Microthamnion*, and *Prasiola*) and even those from marine habitats (*Prasiola*) are known (Friedl and O'Kelly, submitted). The lichen-algal life style has arisen independently multiple times within the Trebouxiophyceae. Currently, five different lineages that contain lichen algae are resolved in 18S rDNA phylogenies (Fig. 2). The monophyletic origin of these lineages is well supported in bootstrap analyses, but their exact relationships within the class is ambiguous (Fig. 2). For the Trebouxiales (lineage 1 in Fig. 2), only coccoid members are known that form flagellated stages (zoospores) in culture. For many other lichenized trebouxiophytes outside of the Trebouxiales, no flagellated stages are known; i.e. the genera *Coccomyxa*, *Diplosphaera*, and *Elliptochloris*. *Trebouxia*, in its present circumscription, is paraphyletic with the genus *Myrmecia* which also contains lichenized species. "*T.*" *magna* appears to be phylogenetically more closely related with species of *Myrmecia* than with other *Trebouxia* species and this finding is corroborated by shared features of chloroplast morphology (Friedl and Rokitta 1997). No species or strains of *Trebouxia* are known that have been isolated from the free-living state, but for a few available culture strains of *Trebouxia*, their exact origin is not known and there is still an ongoing debate if *Trebouxia* is in fact a strictly lichenized genus or not. Within *Myrmecia* as well as within *Dictyochloropsis*, lichenized as well as non-symbiotic species and strains are known. *M. biatorellae* is most closely related with strains that have been isolated from soil (e.g., *M. israelensis*). The other four lineages within the Trebouxiophyceae that contain lichen symbionts (lineages 2-5 in Fig. 2) have not yet been formally named, but may represent different orders of that class. The coccoid *Dictyochloropsis reticulata* (within lineage 2 in Fig. 2) is most closely related to non-symbiotic coccoid green algae that previously were placed in the genus *Chlorella* (*Watanaabea reniformis*, "*C.*" *saccharophila*) which is known to be polyphyletic (Huss et al. 1999). *Leptosira obovata* (lineage 3 in Fig. 2) which may form filaments in culture has an independent position within the Trebouxiophyceae and its closest relatives are not yet known (Friedl 1996). In lineage four (Fig. 2), the monophyletic origin of the coccoid lichen photobionts, *Coccomyxa* sp. and *Elliptochloris bilobata* is well supported. Both species are sister taxa with the non-symbiotic *Choricystis minor* and *Nannochloris* sp. *C. minor*

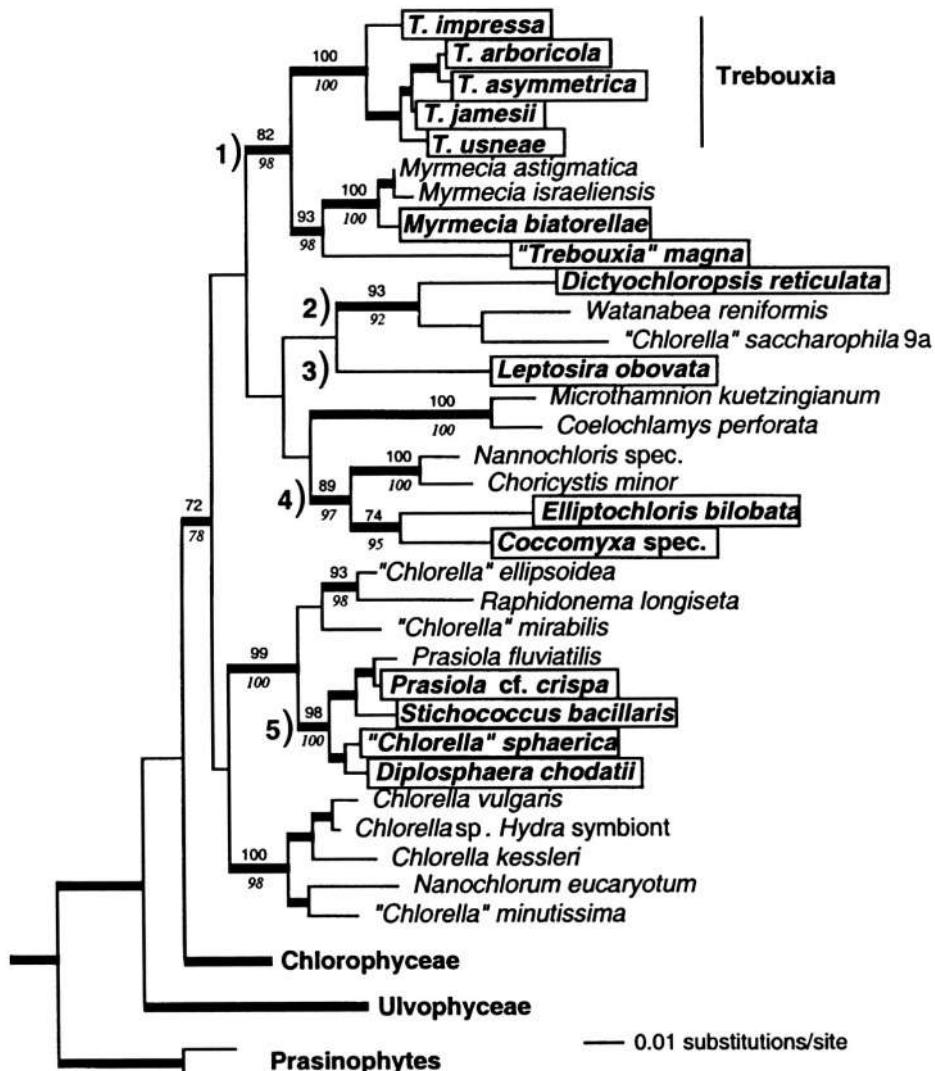


Fig. 2. Maximum likelihood tree showing the phylogenetic position of green algal photobionts (taxa in boxes) within the class Trebouxiophyceae. Numbers 1) to 5) refer to putatively independent multiple origins of lichenized taxa within the Trebouxiophyceae. A total of 1705 nt were considered. The bootstrap values above lineages result from the analysis of 1000 replicates using minimum evolution in connection with the "TrN+I+G" model of DNA substitution (Tamura and Nei 1993) which was chosen based on the likelihood ratio test statistic as implemented in the program MODELTEST 3.04 (Posada and Crandall 1998). The bootstrap values below lineages result from the analysis of 1000 replicates using maximum parsimony analyses with the sites weighted (rescaled consistency index [RC] over an interval of 1-1000, Bhattacharya and Medlin 1995). Only bootstrap values above 70% are shown. Thick lines indicate internal nodes which were defined by bootstrap support above 70% (of 1000 replicates) in minimum evolution.

and weighted maximum parsimony analyses and were also shared with the maximum likelihood tree. All tree searches were done using PAUP* V4.0b8 (Swofford 2001). The sequences used in this analysis may be directly accessed from the databases using the species names except the sequences for *Coccomyxa*, *Diplosphaera* and *Elliptochloris* which are unpublished.

is a frequent member of freshwater picoplankton (Krienitz et al. 1996). Whereas *Coccomyxa* is one of the most frequent lichen photobionts, *Elliptochloris* has only been rarely reported (Rambold et al. 1998). Another lineage with green lichen photobionts (5 in Fig. 2) is unique in that it consists of members of differing morphologies; i.e. coccoid and filamentous. The unicellular "*Chlorella*" *sphaerica* and *Diplosphaera chodatii* are most closely related to each other and they form a sister group to a clade consisting of filamentous genera. Green algae with a *Chlorella*-like appearance are taxonomically difficult to assign to a genus because *Chlorella* may be restricted to *Chlorella vulgaris* and its closest relatives (Huss et al. 1999). There may be more distantly related green algae with a "*Chlorella*"-like appearance occurring in lichen symbioses than previously has been assumed based on their simple morphology and small size. Whereas *Stichococcus* filaments are short and fragment easily, *Prasiola* develops multiseriate filaments that can even be arranged into flat thalli. *Stichococcus* is a common atomophytic or soil alga and only a few ascomycetes are known to form a lichen association with it (Tschermak-Woess 1989, Rambold et al. 1998). *Prasiola* is known as a photobiont only from an antarctic lichen (*Mastodia tessellata*), but most species occur as rock epiphytes on marine coasts or live atomophytically on tree bark where they prefer nitrogen-rich habitats. For other genera of green lichen photobionts; e.g., the coccoid *Pseudochlorella* and the filamentous *Dilabifilum*, the exact phylogenetic position has not yet been determined. However, preliminary SSU rDNA sequence analyses suggest that *Pseudochlorella* is a member of the Trebouxiophyceae and *Dilabifilum* belongs to the Ulvophyceae. The hypothesis that green lichen photobionts either belong to the Trebouxiophyceae (mostly coccoid forms) or the Ulvophyceae (filamentous forms) still needs to be tested with the inclusion of a larger sample of lichen algal genera in SSU rDNA sequence analyses. Thus far, the multiple independent origins of the lichen symbiosis life style within the Chlorophyta can be considered as proven. The sequence analyses also suggest that green lichen algae have their closest relatives among free-living terrestrial algae (e.g., epiphytes on bark or from soil) or taxa living in freshwater habitats. For many lichen photobionts, the same species is known to occur as both lichenized and free-living (e.g., *Trentepohlia*, *Dictyochloropsis*), but it has not yet been proven whether morphologically identical strains of both life styles are also conspecific.

3. Phylogeny within the genus *Trebouxia*, the most common lichen alga

Although lichen photobionts are quite diverse with respect to their phylogenetic positions within the Chlorophyta, the great majority of lichen photobionts are more narrowly related species and strains of the genus *Trebouxia*. To resolve their relationships, a more variable region of rDNA, the internal transcribed spacer regions

(ITS-1,2), has been studied. Phylogenetic analyses of ITS-1,2 sequences from *Trebouxia* consistently resolve four or five clades which are well separated from each other (Fig. 3; Friedl et al. 2000, Kroken and Taylor 2000, Helms et al. 2001). The monophyletic origin of each of these clades is well supported in bootstrap analyses.

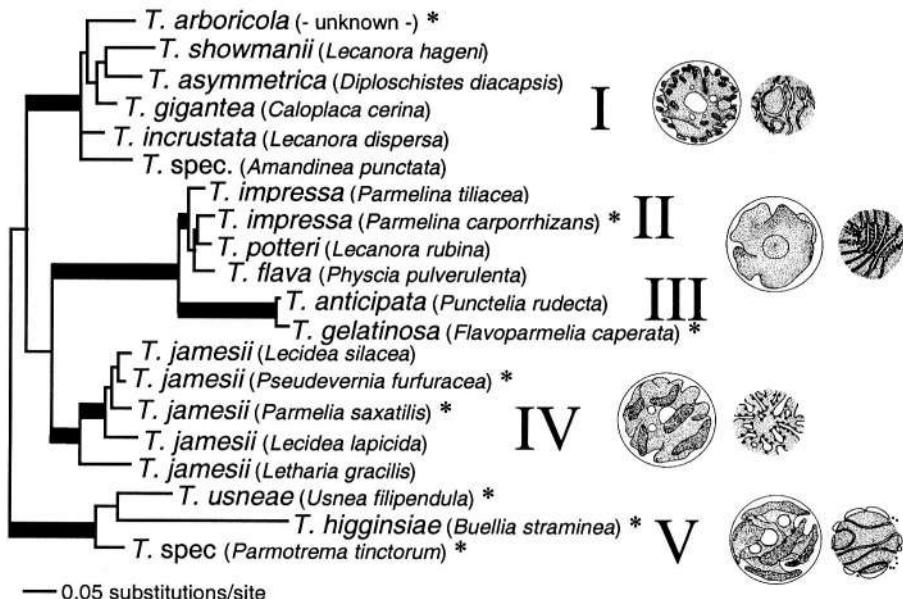


Fig. 3. Maximum likelihood phylogeny of ITS rDNA sequences from *Trebouxia*. Origin of the *Trebouxia* strains are given in brackets. An asterisk indicates the presence of an SSU rDNA intron at pos. 1512 (see text). Numbers I-V denote the five clades as resolved in previous analyses (Helms et al. 2001). Schematic drawings of chloroplast morphologies (left) and ultrastructural pyrenoid types (right) that are characteristic for *Trebouxia* spp. (Friedl and Rokitta 1997) are shown to the right of the clades. Thick lines mark those internal nodes that were supported in bootstrap tests using minimum evolution and weighted maximum parsimony in more than 70% of 2000 bootstrap replicates each and in 500 bootstrap replicates of maximum likelihood analyses using PAUP* V4.0b8 (Swofford 2001). For maximum likelihood and minimum evolution, the "GRT+G" model of DNA substitution (Rodríguez et al. 1990) was chosen based on the likelihood ratio test statistic as implemented in the program MODELTEST 3.04 (Posada and Crandall 1998). All sequences used in this analysis are publicly available from databases.

However, the interrelationships of these clades is largely unresolved. Each clade is defined by a characteristic chloroplast morphology and pyrenoid ultrastructural type (Fig. 3; Friedl and Rokitta 1997). The conserved morphological features of *Trebouxia* species may be correlated with their high degree of adaptation to the lichenized life-style and this phenotypic conservation may belie a significant genotypic diversity. In fact, each clade consists of several clearly separated lineages (Fig. 3). Within a morphologically defined species, several ITS variants may exist and these taxa may differ considerably in their ITS rDNA sequences (Helms et al. 2001). Regarding strains

for which cultures are available, many ITS variants have been found among taxa that are morphologically identical (Beck et al. 1998). For example, culture strains isolated

TABLE I. ITS sequences variants, their accession numbers, geographical origins and substrates within species of *Trebouxia*. The ITS sequence variants within a species of *Trebouxia* are identical.

<i>Trebouxia</i> species	sequence accession	origin (lichen species and locality)	substrate
<i>T. arboricola</i>	AJ293770	<i>Anaptychia ciliaris</i> (Germany, Munich)	tree bark
	Z68703	<i>Pleurosticta acetabulum</i> (Germany, Bayreuth)	tree bark
	AJ007387	<i>Xanthoria parietina</i> (Germany, Munich)	tree bark
<i>T. asymmetrica</i>	AJ249565	<i>Diploschistes diacapsis</i> (Spain, Catalunya)	sandstone
	AJ293784	<i>Buellia zoharyi</i> (Spain, Lanzarote)	soil
<i>T. impressa</i> (1)	AJ293785	<i>Dimelaena oreina</i> (Austria, Steiermark)	rock
	AJ249570	<i>Parmelina carporrhizans</i> (Switzerland, Bern)	tree bark
	AJ293778	<i>Physcia stellaris</i> (Germany, Munich)	tree bark
<i>T. impressa</i> (2)	AJ007388	<i>Parmelina tiliacea</i> (Germany, Bayreuth)	tree bark
	AJ318780	<i>Umbilicaria kappeni</i> (Antarctic, Rothera Point)	rock
<i>T. incrassata</i>	AJ293795	<i>Lecanora hagenii</i> (USA, Mass.)	glass
	AJ293791	<i>Rinodina atrocinerea</i> (Austria, Alps)	rock
<i>T. jamesii</i>	AF128270	<i>Lecidea silacea</i> (Austria, Salzburg)	rock
	AJ315854	<i>Umbilicaria antarctica</i> (Antarctic, Lagoon Island)	rock
	AJ315855	<i>Umbilicaria kappeni</i> (Antarctic, Rothera Point)	rock
<i>T. spec.</i>	AJ293780	<i>Amandinea punctata</i> (Italy, Elba)	coastal pebbles
	AJ293781	<i>Anaptychia runcinata</i> (Italy, Elba)	coastal rock

from the lichens *Lecidea silacea*, *Parmelia saxatilis*, and *Pseudevernia furfuracea* share identical morphological features which define *T. jamesii* (Fig. 3; Beck et al. 1998, Beck 1999). Species that are morphologically difficult to identify due to the paucity of morphological features can clearly be distinguished by ITS sequence indels. For example, the morphologically similar species, *T. asymmetrica*, *T. gigantea*, and *T. incrassata*, are separated by the presence/absence and differences within a sequence segment in the ITS-2 rDNA (Helms et al. 2001). An even greater resolution among closely related strains of *Trebouxia* from natural samples has been achieved using actin gene intron sequences (Kroken and Taylor 2000).

Identical ITS variants may be found in different lichens and localities. Table 1 lists cases in which the same ITS variant has been found in different lichens which were from different geographical origins and different habitats. From this compilation it becomes obvious that *Trebouxia* species and strains are not selective regarding their fungal partners. However, the fungal partners may be quite selective regarding their photobionts and different degrees of photobiont selectivity have been reported (Beck et al. 2001). For example, in foliose members of the ascomycete family *Physciaceae* only very closely related ITS variants (which all belong to one clade in the ITS phylogeny or a single species) have been found (Helms et al. 2001). In contrast, *Trebouxia* strains from different ITS clades have been found as the photobionts of the same species of *Umbilicaria* (Romeike et al., submitted). Low photobiont selectivity may be an

adaptive strategy to overcome situations where the most appropriate algal partner is not available (Romeike et al., submitted). In these situations, a lichen thallus may be formed with any strain of *Trebouxia* available in the habitat and this taxon may later be exchanged, apparently for a more suitable alga (Beck et al. 1998).

To identify strains and species of *Trebouxia*, a large variety of ITS rDNA sequences are publicly available and these can serve as a reference for newly determined sequences from natural lichen samples. Algal-specific PCR primers have been developed to determine ITS rDNA sequences of photobionts directly from lichen total DNA extractions and algal specific PCR has even worked with dry herbarium material (Kroken and Taylor 2000, Helms et al. 2001). ITS rDNA sequence comparisons are, therefore, a relatively fast and easy method to identify *Trebouxia* strains from a great number of lichen samples within a short period of time. This is often an important consideration in many studies on the taxonomy, biogeography, and population dynamics of lichens.

It is very likely that *Trebouxia* species have a world-wide distribution. The fungal partner likely protects the alga which enables the same strain of *Trebouxia* to occur in a broad range of habitats. For example, strains of *Trebouxia* have been detected in lichens from islands in Antarctica that are genetically identical (with regard to ITS regions) with those isolated from southern Germany or from Australia (Table 1; Romeike et al., submitted). Some strains of *Trebouxia* seem to be capable of tolerating high concentrations of iron and are heavy-metal tolerant (Beck 1999). A novel strain of *T. jamesii*, for instance, was found on an iron-rich and heavy-metal-containing substrate (Beck 1999). *Trebouxia* strains that form an independent lineage in the ITS rDNA phylogenies have also been reported in lichens from semiarid habitats on gypsum soil and volcanic rock in Spain. These strains are not known from any other lichens studied thus far (V. Souza-Egipsy, unpublished results).

4. Group I introns in lichen symbioses

Group I introns are short sequences (200-500 bp) of a conserved primary and secondary structure, and in the nucleus, are limited to rRNA genes. Lichenized green algae; i.e., members of the genus *Trebouxia*, were found to be particularly rich in group I introns which interrupt the SSU rRNA gene at the 1512 rDNA genic site (numbering based on the *Escherichia coli* gene). About one third of all *Trebouxia* strains and species studied thus far contain a 1512 intron in their SSU rDNA (Friedl, unpublished results). Recent studies (e.g., Gargas et al. 1995, Grube et al. 1996, Hibbet 1996, Suh et al. 1999, Bhattacharya et al. 2000) also show Euascomycetes, and in particular lichen fungal, rDNA genes to be remarkably rich in group I introns. Understanding how these introns have originated and their impact on the lichen symbiosis are central issues in lichen biology.

One central question regarding group I intron evolution in the lichen symbiosis is whether these introns are laterally transferred between the lichen symbionts. Clearly, showing the movement of introns between the symbionts would open the door to understanding whether genetic material, in general, is exchanged between mycobionts

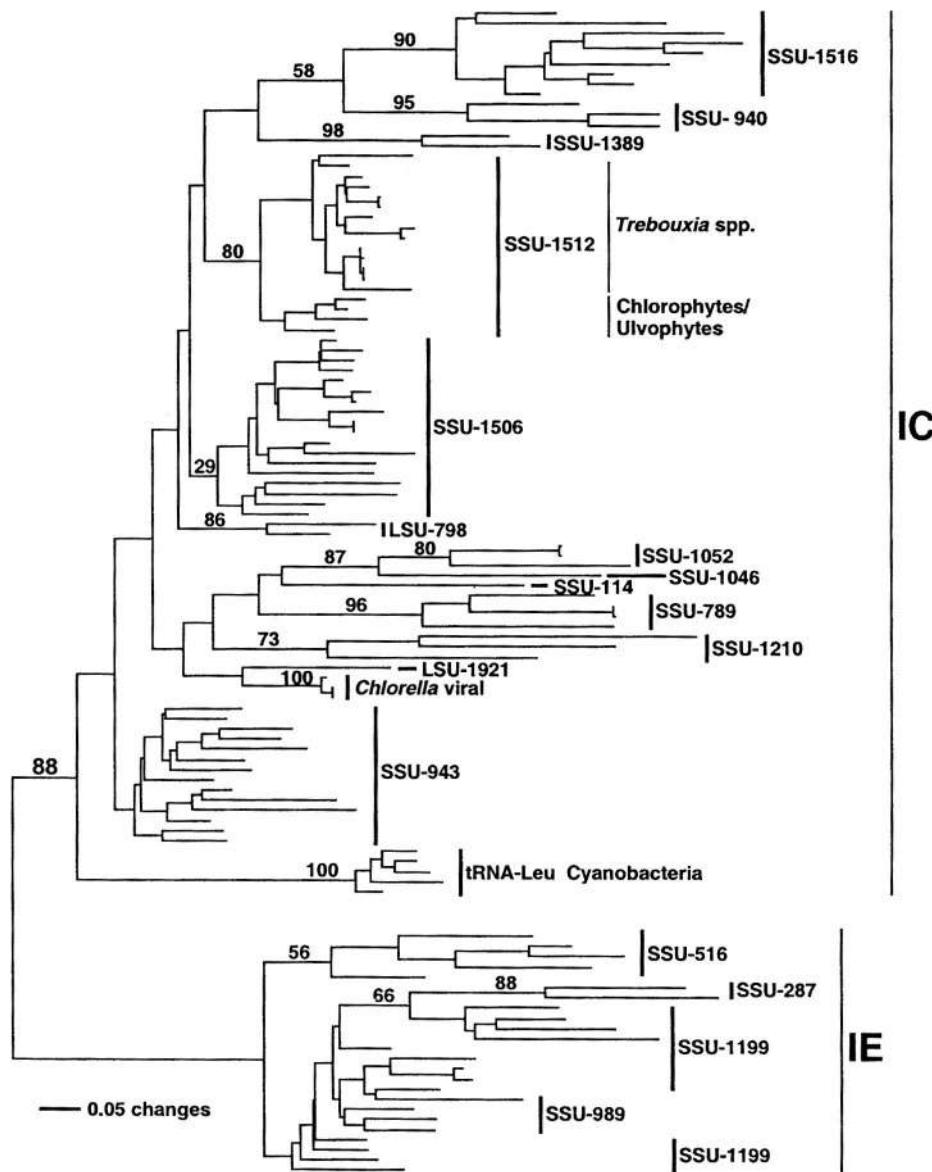


Fig. 4. Neighbour-joining tree of group I introns in lichen-fungi, the 1512 introns in *Trebouxia* and in other green algae, the cyanobacterial tRNA^{Leu} introns, and introns found in *Chlorella* viruses. A total of 144 nt were considered. The HKY-85 model (Hasegawa et al. 1985) was used to calculate the distance matrix. The bootstrap values result from the analysis of 1000 replicates (using PAUP* V4.0b8, Swofford 2001). The IC and IE intron groups (Suh et al. 1999) are shown.

and photobionts. To address this issue, we compiled a data base of fungal nuclear rRNA group I introns (i.e., Bhattacharya et al. 1996, 2001), aligned them using

secondary structure elements to identify conserved sequences, and did phylogenetic analyses to test the hypothesis that lichen fungal and lichen algal/cyanobacterial introns share a specific evolutionary relationship. A phylogeny inferred from 88 fungal (15 different rRNA sites), 18 green algal (SSU rRNA position 1512), and 5 cyanobacterial tRNA^{Leu(UAA)} group I introns is presented in Fig. 4. This tree shows clearly that no fungal intron shares a close evolutionary relationship with any of the *Trebouxia* 1512 introns or with the cyanobacterial tRNA^{Leu} introns (including introns in *Nostoc* spp., Paulsrud and Lindblad 1998, Besendahl et al. 2000) in our broad survey of lichenized and non-lichenized ascomycetes. The *Trebouxia* 1512 introns are, for example, more closely related to these introns in non-lichenized chlorophytes and ulvophytes than to mycobiont introns (Fig. 4). This suggests that none of the publicly available group I introns found in lichenized taxa have likely been transferred between the symbiont rRNA genes. The 1512 intron has, however, been laterally transferred among *Trebouxia* strains/species in the same or different lichens (see below), and group I introns are often transferred among lichen fungi (Bhattacharya et al. 1996, D. Bhattacharya, T. Friedl, and G. Helms, unpublished data). The lack of transfer of mobile introns between mycobionts and photobionts may be explained by the absence of a vector (e.g., a virus) that can co-infect both of these evolutionarily distantly related groups to mediate intron spread.

How might introns spread among rRNA genes? Group I intron mobility occurs at the DNA- or at the RNA-level. Group I introns in organellar, phage, eubacterial, and rarely in nuclear genomes (e.g., Haugen et al. 1999) may contain open reading frames (ORFs) which encode endonucleases to mediate their sequence-specific "homing" (DNA-level) into homologous, intronless coding regions (Dujon 1989). Intron spread at the RNA-level may occur through reverse-splicing, in which a free intron inserts into a homologous or a heterologous RNA by recognizing a short (4-6 nt) exon sequence at the 5' splice site that is complementary to the intron-encoded internal guide sequence (Woodson and Cech 1989, Cech et al. 1994). Reverse transcription of the intron-containing RNA followed by general recombination of the cDNA with the chromosomal copy of the gene can lead to intron lateral transfer (Cech 1985, Sharp 1985, Woodson and Cech 1989, Thompson and Herrin 1994, Zhang et al. 1995). The highly conserved RNA secondary structure of group I introns provides the active site for their own splicing reaction; i.e., many of the introns are self-splicing (Kruger et al. 1982, Jacquier 1996) and can also undergo autocatalytic splicing reversal (Woodson and Cech 1989). In vitro and bacterial expression analyses show, for example, that the *Tetrahymena thermophila* LSU rDNA group I intron can integrate, through reverse-splicing, into both homologous and heterologous sites in the LSU rRNA of *E. coli* (Woodson and Cech 1989; Roman and Woodson 1998). That the SSU rDNA introns in lichen symbionts do not encode an endonuclease ORF suggests that some fraction of these group I introns may be laterally transferred through reverse-splicing (Bhattacharya et al. 1996, Friedl et al. 2000).

At the level of phylogeny, lateral transfer of group I introns can be directly tested through the comparison of intron and host cell rRNA trees to identify putative cases of intron transposition (Bhattacharya et al. 1994, Bhattacharya et al. 1996, Holst-Jensen et al. 1999, Besendahl et al. 2000, Friedl et al. 2000). An analysis of the lichen alga

Trebouxia is shown in Fig. 5. This is a comparison of the ITS rDNA phylogeny of different *Trebouxia* strains and species and their SSU rDNA group I introns at position 1512. The four lineages of *Trebouxia* spp. as resolved in the ITS rDNA tree (Fig. 3) are also resolved in the intron tree (Fig. 4), but with three clear exceptions. The presence of *T. arboricola* SAG and *T. corticola/T. usneae* UTEX within the lineage formed by *T. gelatinosa* and *T. impressa* is inconsistent with the ITS tree. In addition, *T. jamesii* has multiple origins within the intron phylogeny as *T. jamesii* strains 01 and 132 are removed from strain 73 of that species (Fig. 5). The polyphyletic origin of *T. jamesii* and *T. arboricola*, as well as the position of *T. corticola/T. usneae* UTEX, is well supported in the bootstrap analyses in the intron phylogenies. These results suggest that the 1512 intron has been laterally transferred on three separate occasions in the studied *Trebouxia* spp. (transfers denoted by boxed taxon names in Fig. 5). Although vertically inherited in the common ancestor of *Trebouxia* spp. (Bhattacharya et al. 1996, Friedl et al. 2000), the 1512 intron has, thereafter, undergone multiple lateral transfers among these algae.

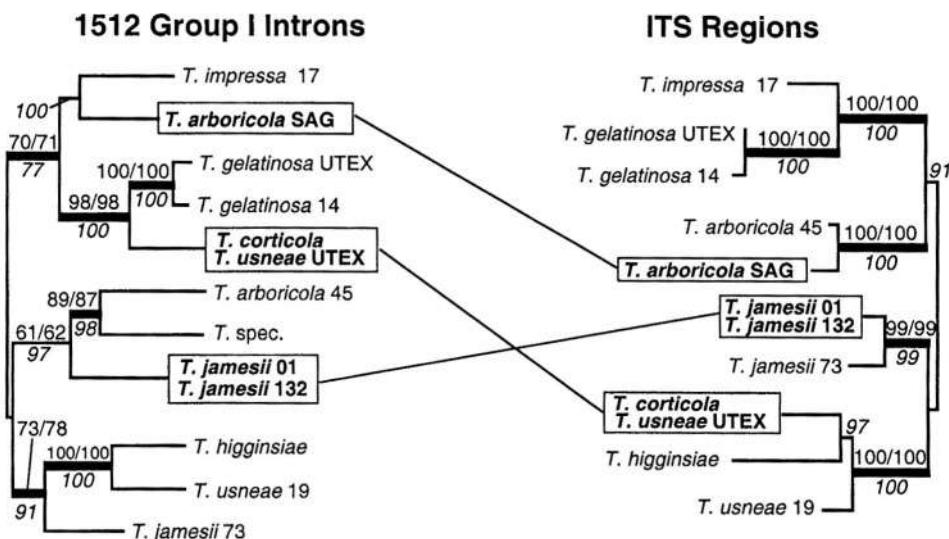


Fig. 5. Phylogeny of 1512 group I introns and ITS-1,2 rDNA sequences from *Trebouxia* spp. inferred from maximum parsimony analyses (midpoint-rooted). The ITS analysis included 432 aligned sequence positions whereas the intron analysis included 411 sequence positions. The numbers shown above the branches were inferred from a neighbor-joining bootstrap analysis using as input distance matrices calculated with the HKY-85 model (2000 replications, left of slash-mark) and from a LogDet bootstrap analysis (2000 replications, right of slash-mark). The bootstrap values shown below the branches were inferred from a maximum parsimony analysis (2000 replications). Bootstrap values were only recorded for those lineages that appear in > 50% of the replicates. The thick lines identify lineages that are highly supported in the bootstrap analyses. Where identical intron/ITS sequences for several taxa or strains were found, only one sequence was used for the analyses, the others were simply added to the figure. Boxed taxa are candidates for intron lateral transfers (see Friedl et al. 2000 for details).

Interestingly, analysis of the autocatalytic capacity of many of the 1512 introns showed that mobile introns were derived from lineages which could self-splice in vitro, whereas the non-mobile, vertically inherited introns often could not self-splice. This suggests that these introns spread through reverse-splicing and that this process may be dependent on the self-splicing capacity of group I introns (Roman and Woodson 1998, Friedl et al. 2000). In support of this scenario, mutations introduced in the *Tetrahymena* intron that shut-down or limit self-splicing (e.g., changing the G at position 264 to an A in the intron core) also inhibit reverse-splicing in bacteria (Roman and Woodson 1998). This suggests that an intron that splices poorly or is dependent on host cell splicing-mediation is unlikely to be a mobile element. Regarding the opportunities for intron lateral transfer among *Trebouxia* spp., the life history of lichens suggests that intron-containing and intron-less *Trebouxia* strains/species may co-exist within the same lichen thallus. Our previous work has documented the appearance of identical, intron-containing *Trebouxia* strains in different lichen fungi which suggests that the process of lichenization may facilitate the movement of introns and their host cells among different lichen partnerships (Friedl et al. 2000). We have also previously suggested (Bhattacharya et al. 1996) that the lateral transfer may be mediated by a viral vector during the early development of lichens. In this scenario, intron lateral transfer occurs during the time that different strains/species of *Trebouxia* inhabit a single lichen thallus and come in direct cell-to-cell contact (Friedl 1987, Ott 1987). Lichen maturation leads to the elimination of all but one strain of photobiont that then exclusively inhabits the lichen thallus. This scenario is consistent with previous observations of viral particles during transmission electron microscopic studies of lichen thalli (L. Goff, pers. comm.) and evidence for viral-mediated lateral transfer of group I introns in other algal cells (see Yamada et al. 1995, Bhattacharya et al. 1996, Nishida et al. 1998). Given the introduction of a foreign autocatalytic RNA into an algal cell, a process such as reverse-splicing could then lead to its integration into homologous sites in the rRNA of different *Trebouxia* spp. That 1512 group I intron mobility occurs only among *Trebouxia* photobionts (and not with the mycobiont, see Fig. 4) suggests that virus(es) that specifically infect *Trebouxia* may mediate the lateral transfer of 1512 introns into related strains. Our future analyses of lichen group I introns will test the reverse-splicing model of intron origin and address the implications of the unique lichen lifestyle in facilitating the spread of autocatalytic RNAs among genetically distinct photobionts and mycobionts.

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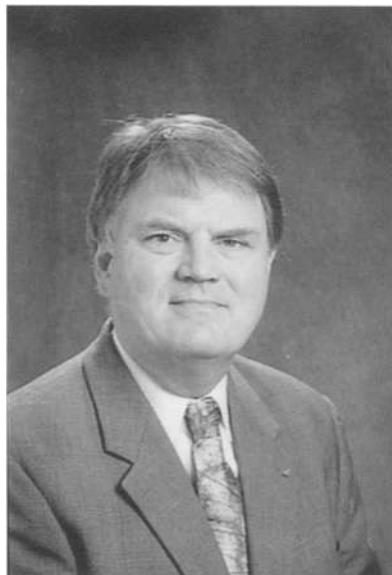
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LICHENIZATION OF THE TRENTEPOHliaLES

Complex Algae and Odd Relationships

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1. Introduction

The classic example of symbiosis is the lichen. And the type of symbiosis generally associated with lichens is mutualism. In fact, *The Facts on File Dictionary of Botany* defines mutualism as: "an intimate relationship between two or more living organisms that is beneficial to all the participants. A lichen is an example of an obligatory mutualism between an alga and a fungus, since neither can survive without the other."

If only it were so simple.

Perhaps this description is accurate, for the most part, when the algal component of the lichen, the photobiont (Greek *photos* = light, *bios* = life) is a small, noncomplex cyanobacterium or a unicellular green alga such as *Trebouxia*. Most lichenized algae fit this description. But more interesting than this type of straightforward symbiotic association between a single-celled photobiont and the fungal component of the lichen, the mycobiont (Greek *mykes* = fungus, *bios* = life), is the continuum of symbiotic relationships existing among lichenized members of the Trentepohliales (Ulvophyceae, Chlorophyta).

The trentepohlialean green algae are terrestrial, growing on rocks, soil, fences, statues, tree bark, and leaves. The group is considered subtropical to tropical, although it has been reported from caves in Belgium (Garbacki et al. 1999) and from Japan (Hirose and Yamagishi 1977) as well as houses in Ireland (M. Guiry, pers. com.). Lichenization of trentepohlialean algae is well known (Alexopoulos et al. 1996; Davis and Rands 1993; Tucker et al. 1991; Matthews et al. 1989; Chapman and Good 1983; Meier and Chapman 1983; Chapman 1976; Santesson 1952) and usually involves the genera *Cephaleuros* and *Trentepohlia*. Thirteen species of lichen collected in bottomland hardwood forests in Baton Rouge, Louisiana, were associated with eight families of loculoascomycete and discomycete fungi—all had trentepohliaaceous photobionts (Tucker et al 1991). Tucker (1981) in fact stated that 38 percent of 543 lichen species reported for subtropical Louisiana had trentepohliaaceous photobionts. Santesson (1952) reported the lichenized form of *Cephaleuros virescens* on 99 host species of vascular plants.

Like the free-living alga, lichenized trentepohlialean taxa are generally considered tropical to subtropical. At the Botarrama Trail in Costa Rica, Lücking (1999) reported 217 species of foliicolous lichens, representing 40 percent of the world's diversity. At his study site in this area, of 179 lichen species, the photobionts of four of the seven families he considered most important were trentepohlialean—the characteristic under story photobiont was *Phycopeltis*. Lichenized trentepohlialean taxa also have been reported, however, from temperate areas, including the North American west coast (Nash et al. 1987), Japan (Nakano and Ihda 1996; Harada 1996), a dimly-lit cave in Missouri (Davis et al. 1989), shaded overhangs in coastal Norway (Jorgensen and Tonsberg 1988), a suboceanic spruce forest in central Norway (Holien 1997), and the pre-Alps region of Italy (Tretiach and Modenesi 1999).

Lichenized trentepohlialean algae are interesting, because rather than being single-celled as are most lichen photobionts, these algae are branched filaments with specialized sexual and asexual reproductive cells and structures. Thus, the type of lichenization that occurs is different for each type of morphology.

2. *Trentepohlia* - The Namesake Genus

It is appropriate for this review of lichenized Trentepohliales to begin with *Trentepohlia*, from which the order gets its name. There are perhaps 27 species of *Trentepohlia* (López-Bautista 2000), and the basic taxonomy of the group is a challenge. In fact, the late Rufus H. Thompson, who was one of the world's greatest experts on the Trentepohliales, never dared to tackle this genus, although he did reduce the number of species when he erected the genus *Printzia* (Thompson and Wujek 1992).

Trentepohlia is a branched filamentous alga often growing as an orange felt on tree trunks, walls, or even house roofs. The orange color is caused by cytoplasmic accumulations of carotenoid pigments. The orange or yellow gold color is a feature of other Trentepohlialean genera, and most of the special features of *Trentepohlia* are shared by other genera in the order. Thus, an introduction to the special features of *Trentepohlia* will serve as a general introduction for the group.

The branched filamentous habit of *Trentepohlia* is the basic form for all of the taxa; however, in *Trentepohlia* the filaments grow loosely and do not form a tightly organized thallus with a specific shape. There is some heterotrichy, that is prostrate filaments growing off the substrate surface are somewhat distinct in form from the erect filaments. The differentiation of prostrate vis-à-vis erect filaments is more extensive in other genera such as *Phycopeltis* and *Cephaleuros*. In the latter, long, tapered, sterile trichomes and elaborate sporangiophores are striking features of the nonlichenized thalli.

In addition to the subaerial habitat and orange coloration, another key feature of the Trentepohliales is the production of morphologically differentiated sexual and asexual reproductive cells. The gametangia are larger and more globose than the rectangular vegetative cells and develop pores through which the gametes will be released. The enlargement of the gametangial cells is critically important in *Cephaleuros*, because the

thallus grows beneath the host plant cuticle. The enlargement of the gametangium eventually breaks through the cuticle, and the exit pore is formed at the break in the cuticle. Thus, when gametes are released they are outside of the leaf, not locked beneath the cuticle.

Perhaps the most distinctive characteristic of *Trentepohlia* and related genera is the formation of zoosporangia (see Figure 1) that abscise from their supporting stalk cell (via a multi-step process that includes a circumscisile tearing of wall material [Good and Chapman 1978]). The process of abscising (or, "popping off" from the stalk cell) seems to involve increased turgor pressure under moist conditions. This requirement for moisture generally ensures that the zoosporangia are released when there is adequate moisture for the quadriflagellate zoospores also to be released to start a new infection. Although the morphology of the sporangia-producing branches varies in each species, the basic morphology of the zoosporangia and their stalk cells and the abscission process are general features.

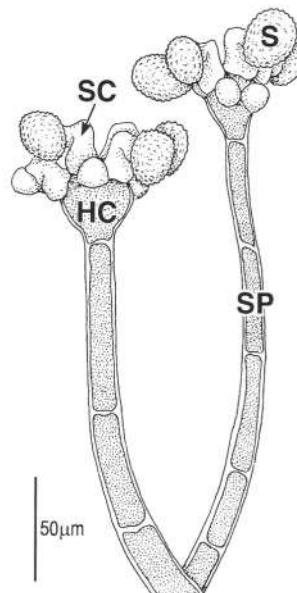


Figure 1. Sporangia of *Cephaleuros*: SP—sporangiophore; HC—head cell; SC—stalk cell; S—sporangium (after Thompson and Wujek 1997). Drawing by Mary Lee Eggart.

When lichenized, the *Trentepohlia* photobiont ceases production of gametangia and zoosporangia. In a study of nine specimens of *Pyrenula japonica*, Nakano and Ihda (1996) concluded that neither sessile nor stalked sporangia could be produced by the photobiont *Trentepohlia lagenifera* (identified through culturing techniques), because "their formation is obstructed by the compact fungal hyphae." Although the alga can sometimes be identified at least to genus by abundant carotenoids and long, branched filaments (Tretiach and Modenesi 1999), often the distinctive morphology of the photobionts is reduced or lost completely. Not only can the cell shape be atypical, but also sometimes there are no detectable filaments, and even the normal pigments can be

missing (Tucker et al. 1991). In such cases, the alga can be identified only by ultrastructural features.

Lücking (1999) suggested that in trentepohliaceous lichens, especially those with *Phycopeltis* as the photobiont, a highly reduced mycobiont could control the multicellular algal thallus. This is in contrast to lichens with *Trebouxia* as the photobiont, where each individual cell of the unicellular alga must be controlled by the mycobiont. According to Lücking, a reduced mycobiont thallus and increased algal thallus could result in a higher photosynthetic productivity for the lichen.

2.1 LICHENIZED TRENTEOPOHLIA

2.1.1 A Loose Association—*Coenogonium interplexum* (Gyalectaceae)

The filamentous lichen *Coenogonium interplexum* grows as a green to yellow-orange mat on the surface of trees or soil in subtropical to tropical climates. This lichen is interesting because, rather than taking its form from the mycobiont, the ascomycetous fungus *Coenogonium*, it resembles the photobiont. The photobiont genus, *Trentepohlia*, is considered the most diverse in form of the described trentepohlialean taxa, with branched, heterotrichous filaments growing in an erect rather than prostrate system. The morphologically diverse sporangia, which are borne terminally or on the terminal cell of a branched sporangiophore, are used in determining species. Gametangia are also terminal.

The photobiont and mycobiont of *Coenogonium interplexum* do not intertwine to form the single, stratified thallus generally associated with lichens (although Honegger [1992] stated that less than half of all lichens achieve a complex thallus). In fact, without a microscope the lichen can be distinguished from free-living *Trentepohlia* only by the presence of the yellow fungal apothecia.

In observations by light microscopy (Karling 1934, Uyenco 1965) and TEM (Meier and Chapman 1983), no haustoria were found in *Coenogonium interplexum*. Uyenco (1963, 1965) observed “loosely attached” fungal hyphae and concluded that an inhibition of branching in the alga was the only effect of lichenization. Ahmadjian (1993) considered that *Coenogonium* was not a true lichen, but rather a “lichen-like” association. Meier and Chapman (1983), however, argued that *Coenogonium interplexum* was a true lichen, based on TEM observations of “some modification of the photobiont in terms of inhibition of branching and distortions of cell shape” and modifications of the fungal wall, which “often appears thinner at points of contact with the alga.”

Should this loose association of algal and fungal filaments be considered a lichen? Perhaps not in the traditional sense of a lichen as a mutualistic symbiosis. There is no doubt that an intimate relationship exists between the mycobiont and the photobiont in *Coenogonium interplexum*, and therefore we would like to think there is some benefit to both the alga and the fungus.

The traditional thought on mutualism in lichens is that the alga benefits from the relationship by gaining a new habitat plus new sources of water and nutrients. The fungus gains new habitat plus photosynthates (Tapper 1981) or other nutrients from the photobiont. Most lichenologists consider that the biological fitness of both the algal and fungal components of macrolichens is enhanced by the symbiotic relationship (Lewis

1987, Smith and Douglas 1987). Some of the most common green algal photobionts, such as the unicellular *Trebouxia* spp., rarely occur except as lichen symbionts.

But, *Coenogonium interplexum* occurs in the same habitats where *Trentepohlia* thrives in the aposymbiotic state, so one wonders what benefits accrue to the lichenized alga? Although the biology of this lichen is still poorly understood, it could easily fit into that group of systems that Honegger (1992) stated “represent more or less mild forms of fungal parasitism on algal...hosts rather than mutualistic associations.”

2.1.2 Other trentepohliaceous lichens

Encephalograph elisae. In confirming *E. elisae* as a lichenized fungus with a trentepohlialean photobiont, it was noted that, “The photobiont cells are surrounded by appressed, branched hyphae formed by irregularly shaped cells, and are not penetrated by haustoria” (Tretiach and Modenesi 1999).

Chiadecton sanguineum. In *Chiadecton sanguineum*, in which *Trentepohlia* is the photobiont (Withrow and Ahmadjian 1983), the filamentous alga is normally broken up into short filaments and unicells by the mycobiont.

Arthonia, *Graphis*, and *Opegrapha*. Unlike the *Trentepohlia* cells in *Coenogonium interplexum* that are not penetrated by haustoria, the *Trentepohlia* photobionts in *Arthonia*, *Graphis*, and *Opegrapha* species were found to have haustorial penetration without an algal wall sheath in a light microscope study by Tschermak (1941).

Haustorial penetration of trentepohliaceous lichens. Ultrastructural studies by Matthews et al. (1989) and Tucker et al. (1991) showed that haustoria (sometimes two per algal cell) were present in four genera (see below) of Louisiana lichens with *Trentepohlia* as the photobiont. The photobiont in these four genera had homogeneous cross-wall structure, typical of *Trentepohlia*. Although *Cephaleuros* and *Strigula complanata* also have homogeneous cross-walls, they can be easily distinguished from *Trentepohlia* at the macroscopic level by the subcuticular position of thalli (Chapman 1976 and Tucker et al. 1991). In all cases, the algal wall appeared to invaginate at the point of contact of the invading haustorium and appeared thicker around the point of entry. The algal cell wall, in close proximity to the wall of the fungal cell, surrounded the haustorium. Tucker et al. (1991) did not find any of the anomalous structures, such as “wall apposition, collar or sheath around the haustorium, anomalous wall material at the point of haustorium entry” that were reported by Honegger (1986) and by Bracker and Littlefield (1973).

Tucker et al. (1991) suggested that the relationship between the mycobiont and photobiont in the studied genera might be “mutualistic when haustoria first invade healthy algal cells and then progress with time to a more parasitic state in which the algal cell deteriorates and dies.” Matthews et al. (1989) compared the observed progressive loss of algal wall to the “intra cellular condition” described by Honegger (1986) Algal cells that were still in good condition had intact organelles and membranes; especially the chloroplast thylakoids were still in compact arrays. The chloroplasts in degenerate algal cells had thylakoids that were less compact or less well organized (Matthews et al. 1989). Matthews et al. (1989) suggested, however, that

Honegger's terms 'intraparietal' and 'intracellular' in regard to lichen haustoria should be viewed as "stages along a continuum, rather than as alternatives." Matthews et al. (1989) concluded that in trentepohliaceous lichens, the algal cell walls invaginated or expanded around the invading fungal haustorium to an extent that most other host cells could not manage when attacked by parasitic fungi. The ability of the trentepohliaceous photobiont to tolerate the mycobiont "may reside at least in part in the growth potential of the algal cell wall" (Matthews et al. 1989).

The species of lichenized *Trentepohlia* studied by Matthews et al. (1989) and Tucker et al. (1991) included:

- *Anisomeridium tuckerae*—superficial crust; mycobiont hyphae observed to invade cork cells of the substrate; cellular, mycelial cortex overlying the algal layer; occasional branching of the haustorial tip; algal wall persists intact over the haustorial tip; invaded algal cells have both healthy and somewhat degraded algal cells among those containing haustoria;
- *Arthonia rubella*—superficial crust; mycobiont hyphae were observed to invade cork cells of the substrate; cellular, mycelial cortex overlying the algal layer; algal wall persists intact over the haustorial tip; invaded algal cells have both healthy and somewhat degraded algal cells among those containing haustoria;
- *Arthonia tumidula*—hyphae strictly intercellular in the host; superficial crust; cellular, mycelial cortex overlying the algal layer; presence of two haustoria in one algal cell; algal wall persists intact over the haustorial tip; invaded algal cells have both healthy and somewhat degraded algal cells among those containing haustoria;
- *Pyrenula anomala*—pyrenocarpous, endoperidermal; lichen thallus primitive, noncellular, amorphous type of cortex; hyphae strictly intercellular in the host; presence of two haustoria in one algal cell; algal wall appears thinner around the haustorial tip and shows some deterioration or loss of wall; invaded algal cells have both healthy and somewhat degraded algal cells among those containing haustoria;
- *Trypethelium ochroleucum*—pyrenocarpous, endoperidermal; lichen thallus primitive, noncellular, amorphous type of cortex; presence of two haustoria in one algal cell; algal wall persists intact over the haustorial tip; invaded algal cells have both healthy and somewhat degraded algal cells among those containing haustoria; and
- *T. tropicum*—pyrenocarpous, endoperidermal; lichen thallus primitive, noncellular, amorphous type of cortex; occasional branching of the haustorial tip; presence of two haustoria in one algal cell; algal wall persists intact over the haustorial tip; all algal cells containing haustoria appear degenerate.

3. Lichenized *Cephaleuros*

The morphology of *Cephaleuros* is interesting and more complex than that of *Trentepohlia*. *Cephaleuros* is an important, well-studied, obligate epiphyte, because it often grows subcuticularly and can be parasitic to the point of causing damage to the

leaves of commercially cultivated plants such as tea, coffee, and citrus (see review by Chapman and Good 1983). *Cephaeuros* is distributed worldwide. Mature thalli are often bright orange and “velvety” in appearance, the result of “abundant setae (also called sterile trichomes, hairs, or filaments) and fertile branches” (Chapman and Good 1983). These specialized branches, extending above the host cuticle, arise from a subcuticular, prostrate thallus. The prostrate filaments are branched. *Cephaeuros* has a number of specialized structures, mostly involved with asexual reproduction, and the heteromorphic sporophyte is reduced to a dwarf plant with a head cell, stalk cell, meiosporangia, and one or more suffultory cells (Chapman and Good 1983, Thompson and Wujek 1997). The distinct morphology of the filaments and reproductive structures is often obscured in these lichenized algae (Matthews et al. 1989).

3.1 *STRIGULA ELEGANS*—THE HAPLESS *CEPHALEUROS*

Strigula elegans is an obligately foliicolous lichen that can be a subcuticular epiphyte or parasite on the host plant. TEM studies of *Cephaeuros virescens* lichenized by *Strigula* showing penetration of the algal cells by fungal haustoria (Figure 2) suggest that the ascomycete mycobiont parasitizes the photobiont (Chapman 1976).

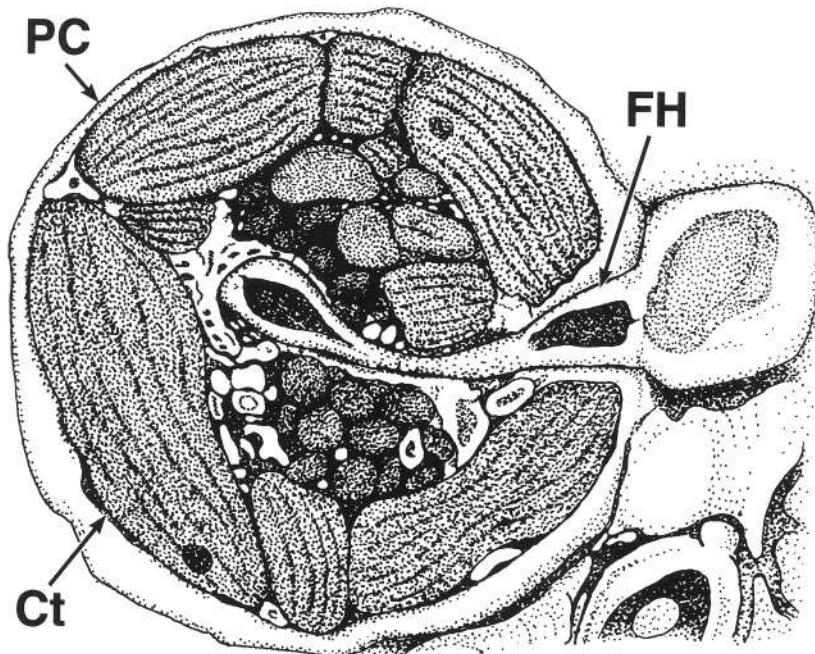


Figure 2. *Strigula elegans*. Penetration of *Cephaeuros* photobiont by fungal haustorium. Septum and remains of algal cell wall are visible along sides of haustorial wall. FH=fungal haustorium, Ct=chloroplast, PC=photobiont cell wall (drawn by Mary Lee Eggart from TEM approx. x14,300, Chapman 1976).

Lichenized and non-lichenized *C. virescens* occupy the same habitat. As with *Coenogonium*, in *S. elegans* the algal thallus is the habitat for the lichen. The alga and the fungus can be in close apposition to one another, or there can be intracellular penetration of the alga by the fungus. Tellingly, penetration by the fungal haustorium is not restricted to decaying or senescent cells, and “the fact that many parasitized cells are in poor morphological condition is a consequence of, rather than prerequisite for, penetration” (Chapman 1976). Haustorial penetration eventually destroys some photobiont cells. Clearly, *Cephaleuros* is not benefiting from the intimate relationship with its mycobiont partner (Chapman 1976, Chapman and Good 1983).

4. Lichenized *Phycopeltis*

Phycopeltis, although often epiphytic, is never parasitic, but rather the open filaments or pseudoparenchymatous thalli grow superficially on the leaves of a host plant or on other surfaces such as tree bark or rocks. The filaments are dichotomously branched, and the color can range from grass green if growing in a shaded, humid environment to deep yellow, orange-red, or copper-red if growing in higher light intensities (Chapman and Good 1983). The thalli do not produce sterile trichomes, and one can argue that the only heterotrichous thalli are the asexual thalli that produce stalk cells and zoosporangia that are “initiated by the production of a fertile trichomes from a single cell of the vegetative thallus” (Chapman and Good 1983). Thompson and Wujek (1997) described *Phycopeltis* as having an isomorphic alternation of generations.

Tucker et al. (1991) in a study of subtropical crustose lichens reported haustorial penetration of the *Phycopeltis* photobiont in species of *Anisomeridium*, *Graphis*, *Mazosia*, *Opegrapha*, *Porina*, and *Schismatomma*. Some photobiont cells in *Porina pulla* and *Graphis scripta* were penetrated by two haustoria. Although most of the algal cells invaded by haustoria showed at least some degeneration, most of the algal cells in *Porina pulla* did not show any evident damage.

5. Lichenized *Physolinum*

Davis et al. (1989; Davis and Rands 1993) reported finding in a dimly lit cave in central Missouri a branched filamentous association of *Physolinum monilia* and a mycobiont they described as characteristically ascomycetous. The single layer thallus consisted of the photobiont completely ensheathed by the mycobiont. Davis studied the microlichen periodically from 1984 to 1992. Initially, referring to Santesson (1952), Davis et al. (1989) declined to call the lichenized *Physolinum* a true lichen, because they did not observe ascospores and “associations without ascospores or ascocarps, and with hyaline fungus partners, cannot be termed lichens.”

The lichenized *Physolinum* most closely resembled the subtropical to tropical microlichen *Coenogonium moniliforme*; the photobiont of most other species of *Coenogonium* is *Trentepohlia* (see above). Davis and Rands (1993) concluded that the “cave association is unique and differs from all descriptions and diagrams of *C. moniliforme*,” based on the pattern of characteristic ensheathing cells, among other

things (Davis et al. 1989; Davis and Rands 1993). In 1993, Davis and Rands reported ascospores. In 1994, after studying the lichenized *Physolinum* exhaustively, both in the laboratory and from fresh specimens collected in all four seasons, Davis described the microlichens as new species, *Coenogonium missouriense*.

Where in the continuum of trentepohliaceous lichens does *Coenogonium missouriense* lie? SEM (Davis et al. 1989) and TEM evidence showed that haustorial contact with the photobiont was common (Davis and Rands 1993), a first for filamentous microlichens or lichenized algae. Although this contact may begin as shallow invaginations of both the fungal and algal walls into the chloroplast, Davis and Rands (1993) also reported that “the hyaline mycobiont cells extend haustoria bound by the fungus wall deeply into the photobiont chloroplasts,” and that once the haustoria penetrated the algal cell there was no evidence of the algal cell wall surrounding the haustoria. Based on examples of other trentepohliaceous lichens, changes in vegetative and reproductive morphology vis-à-vis the free-living *Physolinum* would be expected; however, other than smaller cell size, few differences in the photobiont were reported.

In describing the symbiotic interaction of the mycobiont and photobiont in this association, Davis et al. (1989) said “the single chloroplast that occupies most of the cell’s volume, the numerous, tightly packed thylakoids, and light focusing by ensheathing fungus cells may enable the organism to survive in a dimly lit environment.” In fact, Davis and Rand (1993) suggested that *Physolinum* might not be able to exist in the free-living state in the hostile environment of the cave. Thus, *Coenogonium missouriense* may represent the true mutualistic relationship in the continuum of types in trentepohliaceous lichens.

6. The Puzzle of Distribution

It is clear that many lichenized Trentepohliales do not gain a new habitat, and there is little or no information available to suggest other potential benefits to the photobiont (*Coenogonium missouriense* perhaps being a clear exception, i.e. a case where the photobiont could not survive as a free-living alga in the caves). In thinking about potential benefits to the enslaved photobionts, one can raise the possibility of enhanced distribution in the lichenized state. This topic cannot be discussed in much depth, because lichen propagules are not known for at least some if not all of the lichenized forms of Trentepohliales. Santesson (1952) suggested that “most foliicolous lichens depend on resymbiosis for successful establishment, since they disperse by ascospores or conidia.” Thus, one could say that lichenization does not result in more effective geographical dispersal for the alga, but there is at least one puzzling observation.

In Baton Rouge, Louisiana, and environs (a subtropical area of the USA), *Magnolia grandiflora* leaves colonized by *Cephaleuros virescens* invariably have younger and older thalli of various sizes distributed very patchily on the leaf surface. These thalli are often found lichenized later in the year. The sizes and distribution of the resulting lichen, *Strigula elegans*, reflect that of the original algae. Interestingly and puzzlingly, some leaves of *M. grandiflora* are virtually covered with small, uniformly sized thalli of *S. elegans*. It is difficult to believe that the host leaf had been simultaneously infected with so many young *C. virescens* thalli (such a situation is never encountered), and that

they all were lichenized at the same time. Also, such leaves often lack any large or small unlichenized *C. virescens* thalli. If one were forced to speculate, it certainly seems as if a shower of *S. elegans* propagules infected the *M. grandiflora* leaves.

This last puzzle simply highlights the clear need for further study and emphasizes that the lichenized Trentepohliales are a fascinating group of lichens. The fungal-algal interactions are indeed interesting and the preponderance of mychorrizal associations with vascular plants, together with the suggestion that fungal associations may have played an important role in the green plant conquest of the land (Redecker et al. 2000, Taylor et al. 1995) indicate we should indeed continue the investigation of fungal-plant interactions with vigor.

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TREBOUXIA: REFLECTIONS ON A PERPLEXING AND CONTROVERSIAL LICHEN PHOTOBIONT

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1. Introduction

Lichens are dominant in many parts of the world, both in terms of species diversity (arctic, Antarctic, deserts) and biomass (boreal forests). Over one-half of the estimated 15,000 species of lichens have the green, unicellular alga *Trebouxia* as their photobiont which makes it the most common and widely distributed alga in the world. Despite its ubiquitousness, *Trebouxia* remains poorly understood because of its symbiotic isolation in lichens.

I began my studies of *Trebouxia* in 1956 when I was a graduate student at Harvard University working under the direction of the late Ivan Mackenzie Lamb (a.k.a. Elke Mackenzie), Director of the Farlow Herbarium and Library. Lamb was a taxonomist who was putting together a monograph of the genus *Stereocaulon*, but he was also interested in lichen algae and resynthesis, interests that he passed on to me. Lamb himself was probably influenced by the eminent British lichenologist Annie Lorraine Smith with whom he worked at the British Museum of Natural History. Lamb taught me the lichen resynthesis techniques of earlier workers and also the micropipette method that the Finnish lichenologist, H. Waren used to separate photobionts from mycobionts. With this method, I isolated into axenic cultures the photobionts of different lichens and described new species of *Trebouxia* (Fig. 1, a-d) and this supported results of earlier investigators such as Chodat (1913), Waren (1918-1919) and Jaag (1929). My isolates were made from lichens chosen randomly and they included: *Trebouxia glomerata* (*Stereocaulon saxatile*, *St. pileatum*), *T. incrustata* (*Lecanora dispersa*), *T. potteri* (*Rhizoplaca chrysoleuca*), *T. erici* (*Cladonia cristatula*), *T. gelatinosa* (*Flavoparmelia caperata*), *T. impressa* (*Physcia stellaris*), *T. anticipata* (*Punctelia rudecta*), and *T. decolorans* (*Buellia punctata*, *Xanthoria parietina*). Algal taxonomy in the United States, at that time, was dominated by Harold C. Bold (University of Texas) and his students who did pioneering studies on green, unicellular soil algae such as *Chlorococcum*. The taxonomy of *Trebouxia* was not on anyone's priority list. At present, only about 30 species of *Trebouxia* have been identified but

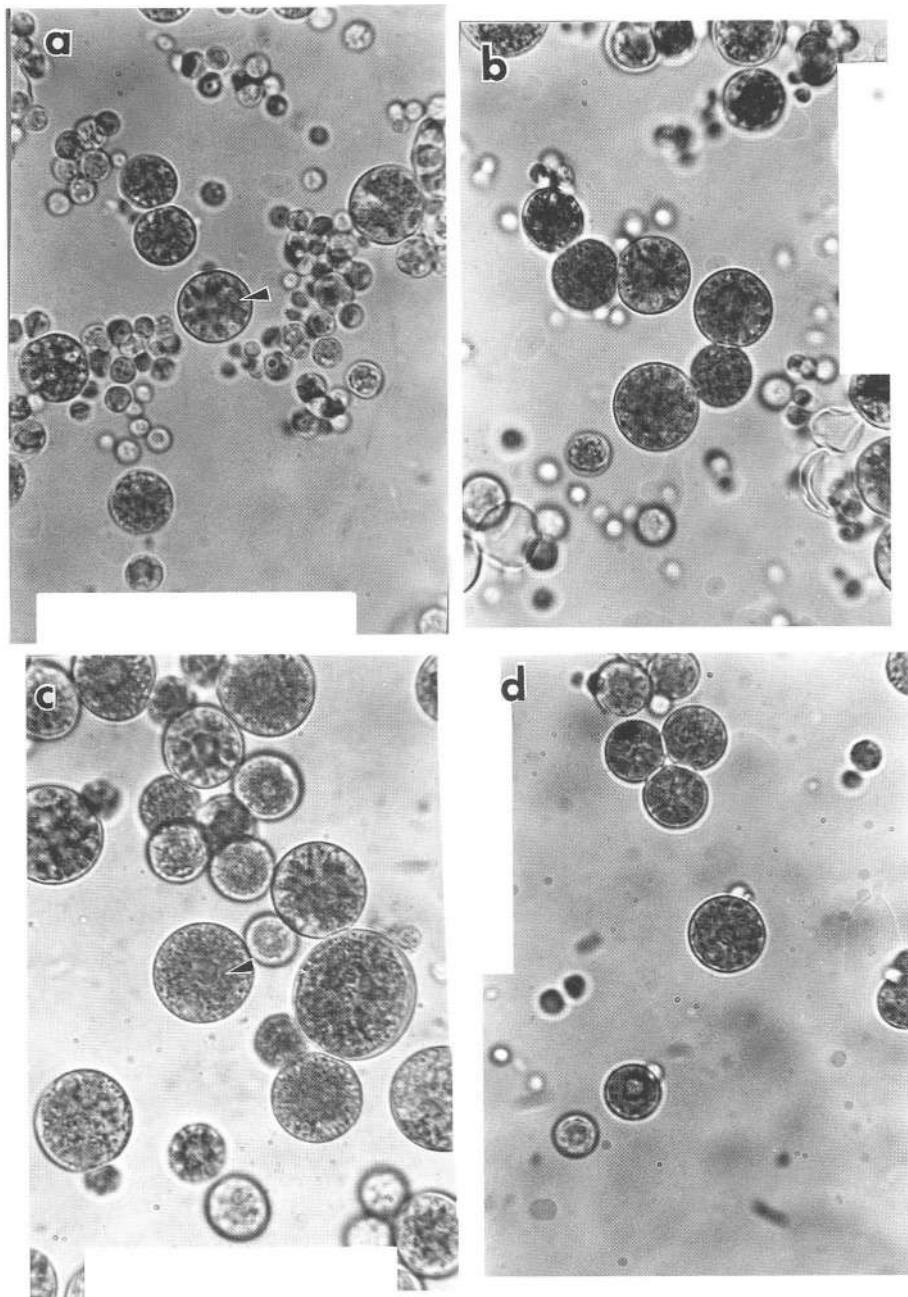


Figure 1. a-d. Cells of *Trebouxia* photobionts growing in axenic cultures isolated from the following lichens: a. *Cladonia bacillaris*; b. *Cladonia leporina*; c. *Porpidea albocaerulescens*; d. *Pertusaria* sp. Average mature vegetative cell diameter, 15 μm . Arrows indicate massive central chloroplast and pyrenoid.

more will be recognized as new molecular distinctions are made. Some workers have split *Trebouxia* into two genera, i.e. *Asterochloris*, to include photobionts of lichens that belong to the family Cladoniinae and *Trebouxia* for species which occur in lichens in the Lecanorineae (Rambold et al., 1998). *Trebouxia* is paraphyletic in rRNA phylogenies and is now part of a new class, Trebouxiophyceae, order, Trebouxiales, and family, Trebouxiaceae (Friedl, 1995).

2. The Photobiont *Trebouxia*: Sexuality, Specificity and Storage Deposits

During the course of my studies, the following questions arose: (a) Why are there so many species of *Trebouxia*, or so few depending on your perspective? (b) How did *Trebouxia* originate and does it occur free-living? (c) Does *Trebouxia* undergo sexual recombination? This last question I answered positively and described sexuality in fresh cultures of *Trebouxia impressa* as follows: "Sexual fusions were frequently seen between gametes of the same size which were indistinguishable from the zoospores. The resulting zygote, measuring 6.6 μm in diameter, was smooth-walled and possessed two separate, rounded chloroplasts; the fusion of the gametes took place almost immediately after the attached pair came to rest. The flagella disappeared very rapidly and the zygote developed in a normal vegetative manner" (Ahmadjian, 1959). If photobionts reproduce sexually in culture, then they most likely do so in a lichen, unless it is somehow suppressed. Gametes of different photobiont strains may escape from the thallus, fuse and form hybrids which then divide vegetatively and form micro-colonies that then could become lichenized by some of the many germlings of lichen ascospores. It should be emphasized that this has not been observed in nature, that is, there is no evidence that *Trebouxia* cells can escape from a thallus. At any rate, sexuality and escape from a thallus would not have a selective advantage for photobionts which are well adjusted to a symbiotic existence.

In an attempt to determine if some photobiont cells can survive the conditions of culture better than other cells, I undertook the following experiment with *Punctelia rudecta* (Ahmadjian, 1959). Twenty-eight algal cells were isolated from crushed isidia (26 single cells, and two groups of nine cells and two cells each). Each cell or group of cells had pieces of fungal hyphae attached to them, which had broken off when the isidia were fragmented. These hyphal pieces were visual proof that the algal cells were part of the photobiont population and were not living incidentally in the thallus. Before each cell was inoculated onto a nutrient agar slant it was examined under a microscope and its size, shape, and color were recorded and a sketch of the cell was made showing the amount and nature of the attached hyphae. After 4-5 weeks of incubation, six colonies were visible with a magnifying lens. These included five clones and the group of nine cells. The cells that formed colonies did not differ, outwardly at least, from ones which failed to develop. The advantage for lichens of having available different species of *Trebouxia* is not clear. One would assume that specific algae would pair with specific fungi, i.e. high specificity, but such is not the case (Beck et al., 1998). For example, *Trebouxia irregularis* has been found as a photobiont of over 30 species of

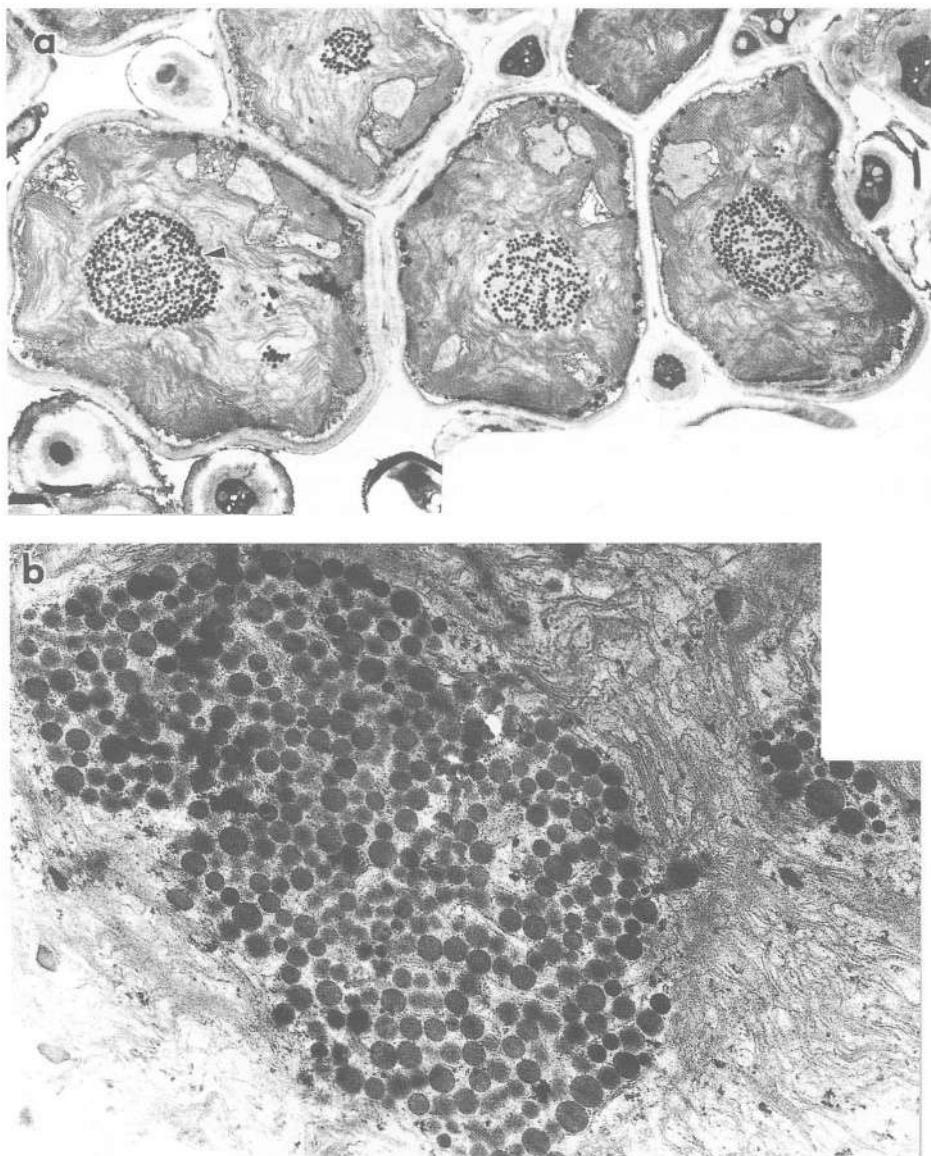


Figure 2, a,b. *Omphalora arizonica* natural lichen showing pyrenoids of photobiont cells which are filled with lipid globules (pyrenoglobules).

lichens: *Cetraria islandica*, *Cladonia mitis*, *C. rangiferina*, *C. bellidiflora*, *C. boryi*, *C. chlorophaeae*, *C. coccifera*, *C. deformis*, *C. furcata*, *C. pityrea*, *C. pleurota*, *C. pyxidata*, *C. squamosa*, *C. uncialis*, *C. verticillata*, *Diploschistes gypsaceus*, *Hypogymnia krogii*, *Parmelia stygia*, *Parmelia omphalodes*, *P. saxatilis*, *P. taylorensis*, *Parmeliopsis ambigua*, *P. hyperopta*, *Platismatia glauca*, *Porpidia albocaerulescens*, *Stereocaulon dactylophyllum*, *S. pileatum*, *S. saxatile*, *Xanthoria parietina*. These lichens grow in a wide variety of habitats and microclimates and morphologically they are very different. Why do such diverse lichens have the same photobiont? Conversely, why can one lichen have different species of *Trebouxia*? For example, *Xanthoria parietina* can associate with *Trebouxia arboricola*, *T. decolorans*, and *T. irregularis*. This situation is similar to that of ectomycorrhizal fungi where one tree can be a host to hundreds of different basidiomycetes.

Obviously, despite their differences many photobionts are similarly attuned to lichen mycobionts. One common trait shared by all *Trebouxia* photobionts is the presence of lipid rich gobules (pyrenoglobuli) in their pyrenoids. Pyrenoglobuli represent a vast storage depot (Fig. 2, a,b) but for what and for whom? They are probably used by the mycobiont for energy and water when they are respired. The source of these globules is not known. They may represent lipids that accumulate because of the mycobiont's inhibition of chloroplast lamellae synthesis of the photobiont.

3. Photobiont Relationships: Zoospores, Phototaxis and Heterotrophy

Clearly, the mycobiont rules the lichen association and the alga is relegated to a subservient role. Lichen fungi do not associate with common soil algae such as *Chlorococcum* and *Nautococcus*, which they parasitize and kill (Ahmadjian and Jacobs, 1981). Some soil coccoids (i.e. *Friedmannia israeliensis*) which are closely related to *Trebouxia* have been partially lichenized by the mycobiont of *Cladonia cristatella*.

Another perplexing aspect of *Trebouxia* is that they produce zoospores without a cell wall. I would think a cell wall would better protect the cells in lichens that grow in dry habitats. Naked zoospores, however, can change their shape quickly and thus can better squeeze through the network of fungal hyphae that makes up a lichen thallus. Zoosporangia are produced in thalli and motile zoospores are released (Slocum et al, 1980). These cells round up, form walls and become vegetative cells which increase the photobiont population of a thallus. Some species (*Trebouxia erici*) have eyespots (stigmas) which help to orient zoospores towards areas of high light intensity, presumably to allow the resulting vegetative cells optimum conditions for photosynthesis. This phototactic response of some zoospores, however, contradicts the photosensitivity of other *Trebouxiae*. For example, *Trebouxia decolorans* loses its pigmentation when it is exposed to light in laboratory cultures and yet it thrives in the lichen *Xanthoria parietina* which grows well in sunlight. In the lichen the algal cells are shaded by a covering of the orange compound parietin.

One observation which I made in my cultural studies was the preferential heterotrophy of the *Trebouxia* isolates. This characteristic was noted also by earlier

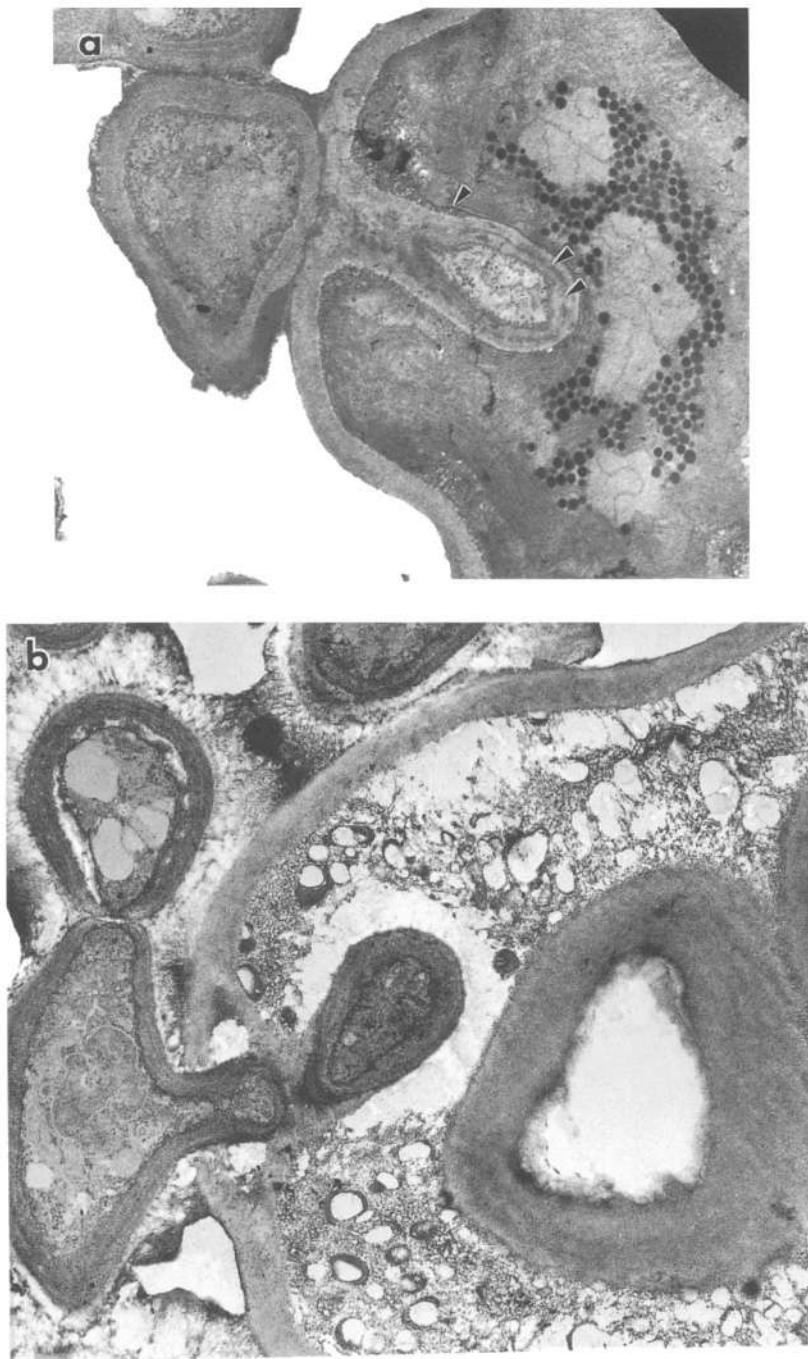


Figure 3, a,b. *Cladonia chlorophaea* natural lichen showing haustorial penetrations of *Trebouxia* cells. (a) haustorium has invaginated algal cell wall and plasma membrane (arrows); (b) haustoria within disintegrating algal cell, algal cell wall, 3 μm wide.

workers, as far back as Beijerinck (1890). All *Trebouxia* photobionts grow much faster if their culture medium contains glucose and an organic source of nitrogen such as asparagine or peptone, as compared to growth in a mineral medium with inorganic sources of nitrogen. Moreover, the isolates grow well in total darkness. What puzzled me was why an alga (*Trebouxia*) which in a lichen supposedly provides most of the nutrients it manufactures to a dominant mycobiont (the photobiont makes up only about 7% of the total thallus volume) grows so poorly in a mineral medium in the laboratory. Why can't it grow well independently apart from its fungal partner? One reason may be that photobionts in a lichen thallus live heterotrophically and they rely on the mycobiont for their nutrient needs. This concept is a complete turnaround from the usual view of lichens, namely, that the photobiont supplies food to the mycobiont. How does the mycobiont provide its photobiont with nutrients? Haustoria may be one means (Fig. 3a). Plant pathogenic fungi use haustoria to absorb food from the host cells. In a lichen relationship, however, haustoria may be feeding tubes which supply photobionts with nutrients that allow them to live as heterotrophs. This helps to explain why you don't see breakdown of photobiont cells in areas that are in contact with haustoria. In pathogenic fungi, haustoria are organs of destruction and signs of degeneration of host cells are visible. I feel that instead of being nutrient-absorbing organs, lichen haustoria are nutrient-providing organs. Where does the mycobiont obtain its nutrients to pass on to the photobiont? It does this through self-parasitism, i.e. by infecting and then assimilating nutrients from hyphae of its own thallus (Fig. 4) and by selectively harvesting older algal cells (Fig. 3b). Mycoparasitism among fungi in general is very common so it should be expected in lichen fungi. What happens to the photosynthetic products (photosynthates), such as ribitol, that are produced by the photobiont? Most of it (over 90%) is excreted out of the algal cells and absorbed by nearby mycobiont hyphae where it is converted to mannitol. *Trebouxia* cells are like little factories, churning out food for the mycobiont. Transforming a symbiont (in this case the photobiont) to address the nutritional needs of its host (the mycobiont) is not unusual -- it occurs in other symbioses, i.e. the *Agrobacterium*, *Rhizobium* and other nitrogen-fixing associations (Paracer and Ahmadjian, 2000).

4. Lichens as Fungal Farmers of Algae

According to Trevor Goward (1994) lichens are "fungi that have discovered agriculture." The comparison to agriculture is apt. The cultivation of fungi by leaf-cutter ants and termites using plant material has parallels with human agriculture. All agricultural ants grow their fungal crops asexually as clones. An ant species may farm a diversity of fungi and vice-versa, a single cultivar is grown by ants of distinct lineages (Paracer and Ahmadjian, 2000). Similarly, lichen fungi have evolved to cultivate *Trebouxia* and use them as a source of food.

Lichens are fungal farmers of algae. Given this view, the term photobiont seems inappropriate since "biонт" connotes an organism that has a specific mode of life and falsely implies some participatory role in a symbiotic association. In lichens, algae are

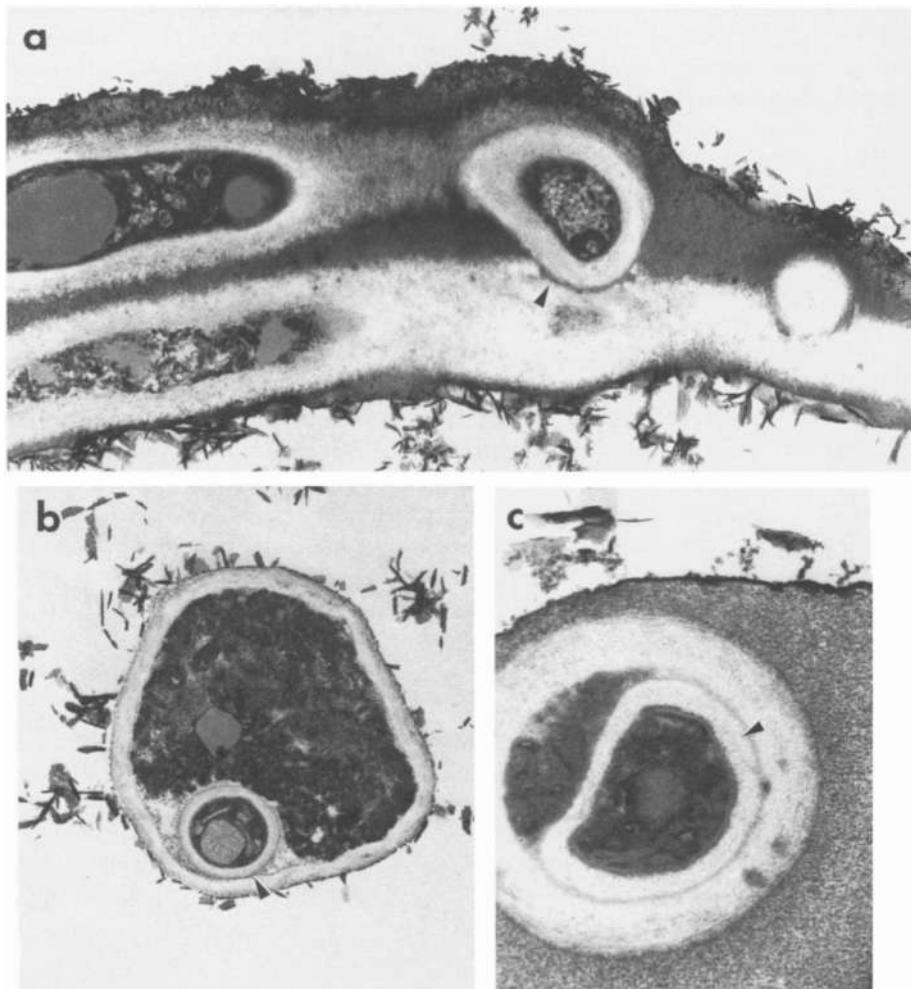


Figure 4. a,b,c. *Cladonia cristatella* mycobiont growing in nutrient medium showing penetrating hyphae (a) and intrahyphal (b,c, arrows).

comparable to heads of lettuce or similar products of agriculture, which are grown for consumption. Once again, we have come fullcircle in our thinking back to the original ideas of Simon Schwendener (1869) who believed that the algae were slaves that prepared the nutrients for themselves and their fungal masters.

5. Summary

Lichen fungi farm algae as a source of food. Many of these fungi cultivate *Trebouxia* as food cells by providing them with nutrients by means of haustoria. *Trebouxia* lives as a heterotroph inside a lichen thallus and is so dependent on the mycobiont that it cannot live independently- free-living *Trebouxia* do not exist. Most of the compounds produced photosynthetically by the photobiont are transported to the mycobiont where they are converted to products that are specific to the fungus.

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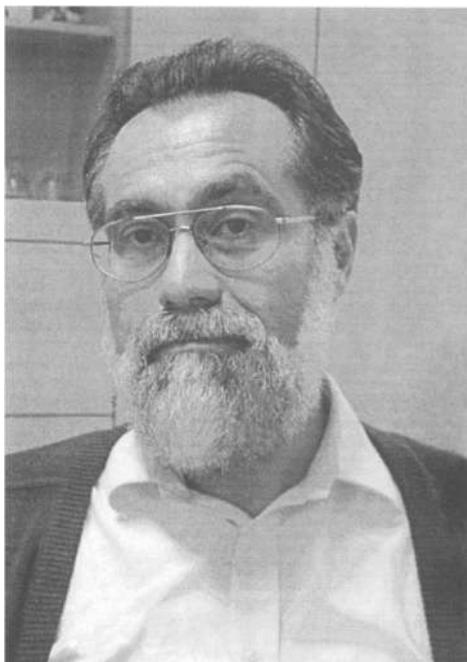
VI. Symbiosis in Plants

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ALGAE LIVING ON TREES

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1. Introduction

Algae form a most versatile group of pro- and eukaryotes performing aerobic photosynthesis. They thrive in most different habitats when sufficient light is available. Algae are successful competitors in both aquatic and terrestrial ecosystems and are also part of the aeroplankton.

Whereas research on aquatic algae has a long tradition and ecophysiology of marine and freshwater algae belongs to the standard canon of textbooks comparable data on aeroterrestrial algae are scarce. This is rather unexpected since, as daily experience shows, most of us are much more frequently in contact with aeroterrestrial algae than with aquatic specimen: We inhale them (there are about 300 - 500 algal cells in 1 m³ of air on a dry and sunny summer day), they may be found in ordinary house dust, they live on rubber seals of car windows, and, of course, as greenish crusts on all kinds of fences, plaster and roofs, greenhouse windows, as well as on rocks and as parts of soil crusts, and also on barks of bushes and trees. They are present even on leaves.

Most aeroterrestrial algae are experts for life at interfaces. An alga that wants to persist e.g., on a bark must be able to cope with immediate changes of light intensity, temperature, moisture, etc. It is exposed to UV, air pollution, and changing diurnal levels of ozone. Thus life of aeroterrestrial algae means a greater challenge than the more or less uniform conditions that are offered to aquatic algae. To understand the ecophysiological features of aeroterrestrial algae is one of the most interesting and promising fields in phycological research.

In the following chapter, I will concentrate mainly on eukaryotic algae living on various kinds of barks and will give a short overview on current knowledge of their ecophysiology. The main reason for this is that those algae may be considered as representative for aeroterrestrial algae that settle habitats exposed to the air. For those algae some interest of plant physiologists exists who want to learn more about adaptive strategies of algae. Another reason for my focus is the current discussion on the putative role of forests as sinks for carbon (Davidson and Hirsch 2001). One usually ignored point in this is the total lack of information on the photosynthetic productivity of aeroterrestrial algae in different forest ecosystems.

In this review, I deliberately exclude algae belonging to lichens which have their own scientific clientele and prominent place in phycological research as well as algae that are living as epi- or endobiotic partners mainly on and in leaves (for an overview see Chapman and Waters 1992).

2. Crust Forming Aeroterrestrial Algae Have to Cope with Special Environmental Conditions

Algal crusts can be observed on all types of barks. It is a yet strange but frequent observation that the design of algal crusts on two individual trees of same age and species growing at a distance of 2 m can be quite different. As a matter of fact, barks of trees and bushes form an enormously heterogeneous habitat which is designed by a lot of biotic and abiotic factors that create a plethora of ecological microniches. First and for all, there exist tremendous differences in bark topology: There are smooth bark types as e. g., in *Fagus* or *Platanus* and there are deeply sculptured ones as e. g., in *Tilia* or *Pinus*. Those differences can also occur between the younger and the older parts of one individual tree. What is more, some bark types offer a permanently available surface to settle on, as e. g., the older parts of a *Tilia* stem, whereas other types, such as of *Platanus* or *Betula*, offer a surface which consists of a patchwork of older and younger parts. Bark topology also influences the microclimatic conditions: Algae trying to settle on a smooth and even bark have to master conditions of irradiance, moisture, temperature and wind erosion that are quite different from conditions that algae meet in the deep wrinkles of a bark of e. g., *Pinus*, in which particles of dust and detritus may accumulate.

Besides topology of barks, there are other microclimatic factors such as amount and duration of light exposure, local temperature differences, direct or indirect exposure to wind abrasion and rainfall that might influence the settlement and growth of algae and may vary in a stand within a few meters.

Sometimes a clear vertical zonation can be observed: The lower part of a stem, up to about 1 m above the soil surface, shows a dark greenish color which is usually due to filamentous cyanobacteria whereas the upper parts are colored by the lighter green of chlorophycean and xanthophycean algae. The border line between those two areas is usually rather clearly marked. Cyanobacteria come in from the soil by capillary water which is sucked up predominantly by wrinkled bark types. It is a matter of discussion whether those algae should be classified as part of the typical bark inhabiting community of algae which lives in the "upper store". Those algae come in almost exclusively by air transportation and are part of the aerophytoplankton.

Other types of zonation are less common. On smooth types of bark as e. g., on *Fagus* the area of the stem surface where the rainwater runs down ("Stammabfluss") is often settled by a different kind of algae than other parts of the bark and thus probably reflects microhabitat differences in moisture and pH.

Biotic factors that might influence the algal population on a bark are less well understood. There may exist some intra- and interspecific competition between algal populations but any experimental data are lacking. To some extent, algae may also form a substrate for heterotrophs such as arthropodes or fungi as e.g., *Athelia* but this is also a rather speculative part of the scenario. During microscopic examinations of algal crusts on bark, fungal hyphae are frequently observed to be in contact with algae. It is tempting to speculate that these may be attempts at lichenization by fungi.

3. Crust Forming Aeroterrestrial Algae Mainly Belong to Chlorophycean and Xanthophycean Species

Probably the overwhelming part of the algal crusts on barks originates from cells that have been transported there by air as part of the aerophytoplankton. Accordingly, a great number of algal species could be isolated from various kinds of barks of different climatic and geographic locations. In principle they reflect the spectrum of aeroterrestrial algal species with a clear domination by unicellular Chlorophycean and Xanthophycean species (Gärtner 1994). There seems to exist some predominance of coccoid taxa such as of *Apatococcus lobatus*, *Stichococcus* sp. and *Chlorella* sp., and the term *Apatococcetum lobati* has been coined (Gärtner and Ingolic 1989). However, this should be handled with great care and without going into details one should bear in mind all the caveats which hold for the classical isolation and identification techniques applied in routine studies for screening aeroterrestrial algae.

In our studies on the influence of different kinds of air pollution to the species composition of aeroterrestrial algae on tree barks, any predominance of certain species could not be confirmed (Houben 1999). During those studies, we observed, however, a marked local patchiness in species distribution, which was independent of type of bark, sampling position on a tree or any stress by air pollutants. Within a range of a few centimeters we observed dramatic changes in dominating species. This is corroborated by physiological data as will be shown below (Fig. 2). We think that the number of different species as well as the size of a specific population predominantly depend on local biotic and abiotic parameters and may also be influenced by chaotic events. Those algae dominate which come in first and can exploit best the specific conditions of their new environment.

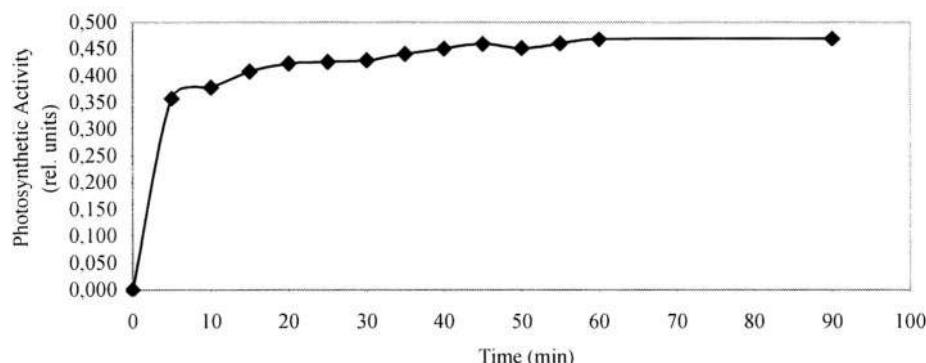
4. Special Ecophysiological Strategies of Adaptation of Crust Forming Aeroterrestrial Algae

Whereas our knowledge on the taxonomy of aeroterrestrial algae that live on tree barks still bases on a small but reliable data set, information on their ecophysiology does not exist or - at best – is rudimentary. It is interesting that as early as the 1920s *in situ* experiments on green algae living on various barks of trees were performed (Schmid 1927, Edlich 1936). These studies were initiated by the observation that algal covers are rather difficult to wet. As was discovered soon and repeatedly corroborated (Bertsch 1966, Ehresmann and Hatch 1975), survival and photosynthetic activity of aeroterrestrial algae on barks depend on the relative atmospheric humidity. An effective uptake of CO_2 usually is possible only at a relative humidity above 90%, whereas an imbibition has a negative effect on photosynthetic performance and survival. Thus algae seem to be remarkably adapted to their aerophilic way of life.

During our studies on the ecophysiology of aeroterrestrial algae we could corroborate those observations by *in situ* measurements: When a piece of bark is kept dry for about four weeks and then wetted by spraying in a closed chamber photosynthesis immediately starts and reaches its maximum activity within a few minutes (Fig. 1).

Experiments also show a different reaction of barks, which have been wetted by spraying water on them or, alternatively have been transferred to a water-saturated atmosphere. As is shown in Tab. 1, in general photosynthetic activity is higher when barks are transferred to a saturated atmosphere. This is demonstrated best with pieces of bark of *Tilia*: In *Tilia* liquid water has no effect at all. This shows an interesting parallel between the behavior of green algal covers on barks and of lichens with Chlorophycean partners (Lange *et al.*, 1986).

FIGURE 1: Activation of Photosynthetic Activity of a Chlorophycean Bark Crust after Increase in Relative Humidity



Both systems are able to endure long periods of low humidity and can react immediately by activating photosynthesis when sufficient relative humidity is present, however, water is effective only when present as vapor.

TABLE I. Net Photosynthetic Release and Uptake of Oxygen by Chlorophycean Crusts on Different Bark Types After Wetting by Liquid Water or by Increase of Relative Humidity

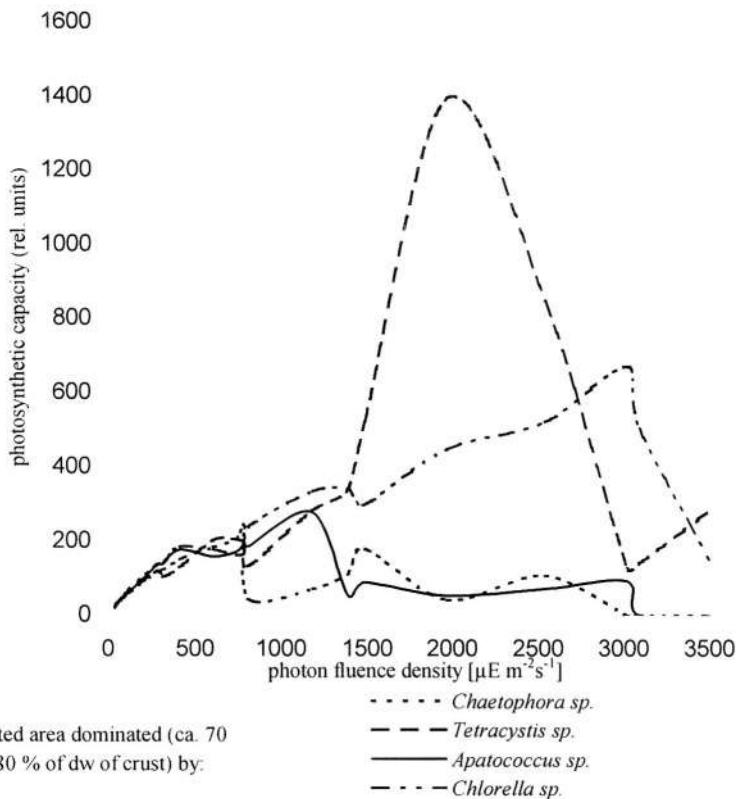
Bark of	wetting by liquid water	wetting by increase of relative humidity
<i>Platanus</i>	+ 4,10	+ 9,58
<i>Acer</i>	+ 4,87	+ 9,50
<i>Tilia</i>	- 3,80	+ 3,41

O₂: μMol g dw⁻¹ mg chlorophyll⁻¹ h⁻¹; photon fluence density: 100 μE m⁻² s⁻¹

Chlorophycean crusts on different bark types as e.g., on *Platanus*, *Acer*, and *Tilia* show a common but quantitatively different reaction in wetting experiments (Tab. 1). Obviously, different algal taxa can settle on the bark surfaces. Taxonomic studies confirm that different taxa of algae may grow on the same bark surface at a distance of a few centimeters. This taxonomic patching is reflected also by a physiological patchiness:

As is shown by Fig. 2, light saturation curves of different areas of *Platanus* bark that are dominated by different types of Chlorophycean algae are also different.

FIGURE 2. Lightsaturation Curves of Different Areas of Chlorophycean Crust on *Platanus* Bark



As could be shown elsewhere (Reisser and Houben 2001), physiological patchiness holds also for different adaptive reactions of aeroterrestrial algae isolated from tree barks to changing UV- and ozone regimes.

Another most important factor affecting the ecophysiology of Chlorophycean crusts on barks reveals the observation that those crusts show a net photosynthetic oxygen production at relatively low light intensities: As is shown in Tab. 1, algae release oxygen at photon fluence rates as low as $100 \mu\text{E m}^{-2} \text{s}^{-1}$. This is true also when considering that part of the amount of photosynthetic oxygen produced probably is needed for respiration of fungi as part of algal crusts. Our measurements on light intensities available for crusts located in the inner parts of the top of *Fagus* showed about $350 \mu\text{E m}^{-2} \text{s}^{-1}$, for those which are exposed to the sun light about $1500 \mu\text{E m}^{-2} \text{s}^{-1}$. Those data suggest that algal

crusts on barks are able to release oxygen under light conditions well below the photosynthetic compensation point of leaves and weeds. This is also shown by data of Fig. 2: Photosynthetic capacity of crust forming *Apatococcus* in situ reaches its maximum at about $1200 \mu\text{E m}^{-2} \text{s}^{-1}$. Maximum of *Chaetophora* is even lower (ca. $750 \mu\text{E m}^{-2} \text{s}^{-1}$), whereas maxima of *Tetracystis* and *Chlorella* are reached at much higher photon fluence densities. However, photosynthetic capacity of both taxa is comparable to the ones of *Apatococcus* and *Chaetophora* at lower photon fluence densities.

5. On The Role of Crust Forming Aeroterrestrial Algae in Photosynthetic CO₂-Fixation of Forest Ecosystems

Although data need corroboration by more measurements, they nevertheless allow a rough estimation on the role of algal crusts in deciduous forest ecosystems with regard to photosynthetic carbon fixation. Tab. 2 shows a comparison of relevant data of a "standard" specimen of *Fagus* and its algal crust.

Available data suggest that the net photosynthetic production of oxygen by algal crusts is approximately 2 % of the production by leaves per year. Calculating a 1 : 1 ratio of the amounts of released oxygen and fixed CO₂, and bearing in mind that this calculation implies some estimates and needs a broader experimental basis, it seems realistic to assume that photosynthetic CO₂-fixation by algal crusts in temperate deciduous forests most probably does not exceed 5 % of the fixation by trees themselves.

TABLE 2. Net Photosynthetic Release of Oxygen by *Fagus* and its Algal Crust

	<i>Fagus</i>	Algal Crust
Area available for photosynthesis (m ²)*	1 200	50
Chlorophyll (g)**	180	25
Chlorophyll (mg cm ⁻²)	0.015	0.05
release of Oxygen (l d ⁻¹)***	9 400	13
release of Oxygen (l a ⁻¹)***	1 692 000	4 781
release of Oxygen (l a ⁻¹ mg Chlorophyll ⁻¹)	9,4	0,19

* area of crust is estimated for a tree 15 m in height with a mean diameter of stem of 100 cm;

** from in situ measurements of Chlorophycean crust: 0.05 mg chlorophyll cm⁻², 20 µMol O₂ h⁻¹ mg chlorophyll⁻¹;

*** day (d) calculated as 12 h, year (a) calculated as 6 months for *Fagus* and 12 months for algal crust

Turner (1975) estimates from data of CO_2 -fixation by an artificial *Pleurococcus* layer, the uptake of about 0.08 liters CO_2 per hour by 50 mg of algal chlorophyll. Our measurements of the release of oxygen by a Chlorophycean crust on *Fagus* *in situ* (see Tab. 2) showed the production of about 0.022 liters per hour by 50 mg chlorophyll. This is about 27 % of Turner's data. Hence, for a further discussion on the productivity of algal crusts on barks, much more experimental data and predominantly *in situ* measurements for longer periods, are required.

Thus available data substantiate the assumption that algal crusts on tree barks probably play a minor part in the role of forests as CO_2 -sinks. However, crust forming aeroterrrestrial algae could well be the matter of choice when studying adaptive mechanisms in eukaryotic algae.

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CYCAD CORALLOID ROOTS HOUSING CYANOBACTERIA

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1. Introduction

Cycads are a very ancient group of Gymnosperms whose origin dates back to the Mesozoic period. They have dominated for a very long period, from about 300 to 70 million years ago. Their actual distribution extends over four continents: Africa, America, Asia and Australia, with about 10 genera and 190 species world-wide (Stevenson et al., 1990; Norstog and Nicholls, 1997). Only Europe does not have native Cycads. However, although native species have reduced and endangered habitats, the distribution of cultivated Cycads is increasing all over the world for ornamental purposes and scientific interest (Giddy, 1984). In fact many Cycads are very fine plants used to ornate gardens and parks both in pots as well as in ground. This appreciation led to the urgent need for strict conservation rules for the endangered species. Although *Cycas siamensis* and *C. media* are said to be regularly deciduous in nature and lose all their leaves before a new flush develops, Cycads are, palm-like evergreen plants, with a slow growth and a long life span. They have a columnar stem topped with a crown of leaves (Fig. 1). Many of them produce male or female cones, these latter maturing seeds usually grouped in the middle of the crown of leaves. Different morphology and ecology occurs in the different Cycad groups: in Africa there are mainly *Encephalartos*, whereas *Dioon* and *Microcycas* grow in Central America and *Macrozamia* and *Cycas* in Australia.

Many Cycads are toxic because their stem, leaves and seeds contain high amounts of cycasin alkaloids, macrozamin or methylazoxymethanol. Cycasin and macrozamin are harmful to the liver, whereas cycasin and methylazoxymetanol are neurotoxic and carcinogenic (Laqueror and Spatz, 1968; Lindblad et al., 1990). Because of these compounds many Cycads are responsible for pathological effects mainly among workers manipulating these plants. On the contrary, some people use Cycads stem and seeds as medical remedy against different symptoms such as high blood pressure, headaches, congestion, and pain in the bones (Van Wyk et al., 1997).

2. Cycad coralloid roots

Over 85% of Cycads form different types of roots: normal geotropic roots, non-coralloid aerial roots and apogeotropic roots (Pant and Das, 1990). These latter are swollen tips at the basis of normal secondary roots from where they sometimes spread in the ground but often go upwards (Ahern and Staff, 1994). These formations are characterized by

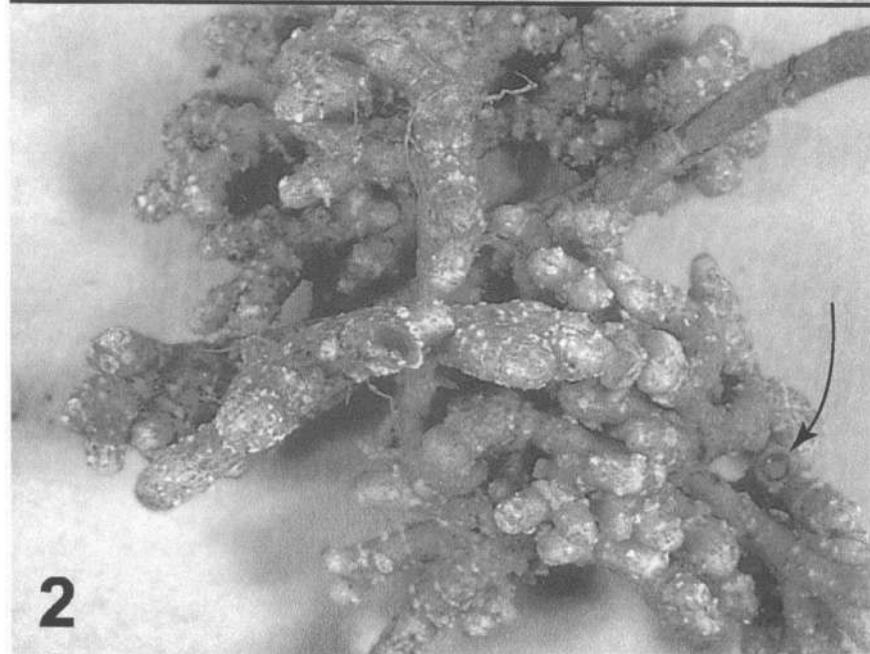


Figure 1. Collection of cycads at Naples Botanic Garden (Italy).

Figure 2. *Macraezamia* coralloid roots. Some coralloids transversally cut show the cyanobacterial zone as a green ring (→).

dichotomous branching, forming complex coral-like shapes, and are therefore named coralloid roots (Fig. 2). The origin and function of coralloid roots are still being debated.

Their development begins with the formation of young papillose roots named precoralloids; these then become mature coralloids and are invaded by cyanobacteria. Senescence is the last phase of coralloid roots, while new precoralloids originate from the basis of preexisting ones (Ahern and Staff, 1994). During coralloid development the meristematic region of the tip extends closer towards the root tip than usual. The peripheral root tip-cap cells deriving from it are not ultimately sloughed off but remain active and thus continue to cover the epidermal cells over the whole surface of the root by constituting the additional outer cortex. The original transformed rhizodermis becomes invaded by cyanobacteria, radial enlarging (Figs. 3 and 4) and taking on the characteristics of transfer cells. Among these cells, cyanobacteria grow, differentiate heterocysts and fix nitrogen. Intercellular spaces where cyanobacteria are localized are filled with mucilage.

If coralloid roots form independently from the presence of cyanobionts, why and how do the symbionts penetrate the host? The Cycad-cyanobacterial association, like other plant-cyanobacterial symbioses, is quite different from the typical symbiosis between Legumes and Rhizobia in that only rarely we find a specificity of symbiont to the host; the localization is mainly intercellular and the cyanobiont does not spread from the coralloid root to other parts of the host plant. As a result, many steps of this association, such as the molecular signals and the genes involved in the recognition between the partners, are very poorly understood. More knowledge is available on the physiology of the cyanobacterial zone such as nitrogen fixation and on the ways nitrogen compounds are utilized.

3. Cyanobionts

The cyanobacteria capable of associating with Cycads are mainly *Nostoc*, *Anabaena* or *Catothrix* (Grilli Caiola, 1996) (Figs. 5 and 6). Recent investigations indicate that *Nostoc* is the most frequent genus not only in Cycads but also in other symbioses, such as with *Anthoceros* and *Blasia*, *Azolla* and *Gunnera* (Grilli Caiola, 1992).

Cyanobionts can be isolated from Cycads and grown in cultures allowing to follow their life cycle and analyzing their identification. Identification of cyanobionts within Cycads is not easy because of the different aspects they show in coralloid roots (Figs 7-10) and in culture (Grilli Caiola, 1972b; Grilli Caiola, 1980). In addition, criteria for classifying these microorganisms are still not conclusive since they often vary according to the different authors, leading to the identification of numerous genera and species (Grilli Caiola, 1996).

Recently Ow et al. (1999) isolated *Nostoc* FUR 94201 from *Zamia furfuracea* and then used it to infect the native host so as to obtain coralloid with cyanobionts closely resembling the native ones. Infection was also obtained with *Nostoc* foreign to the genus, indicating that there is no specificity between the partners. The absence of specificity between the Cycad and *Nostoc* was already found by Grilli Caiola (1996) and Grobbelaar and Marshall (1993).

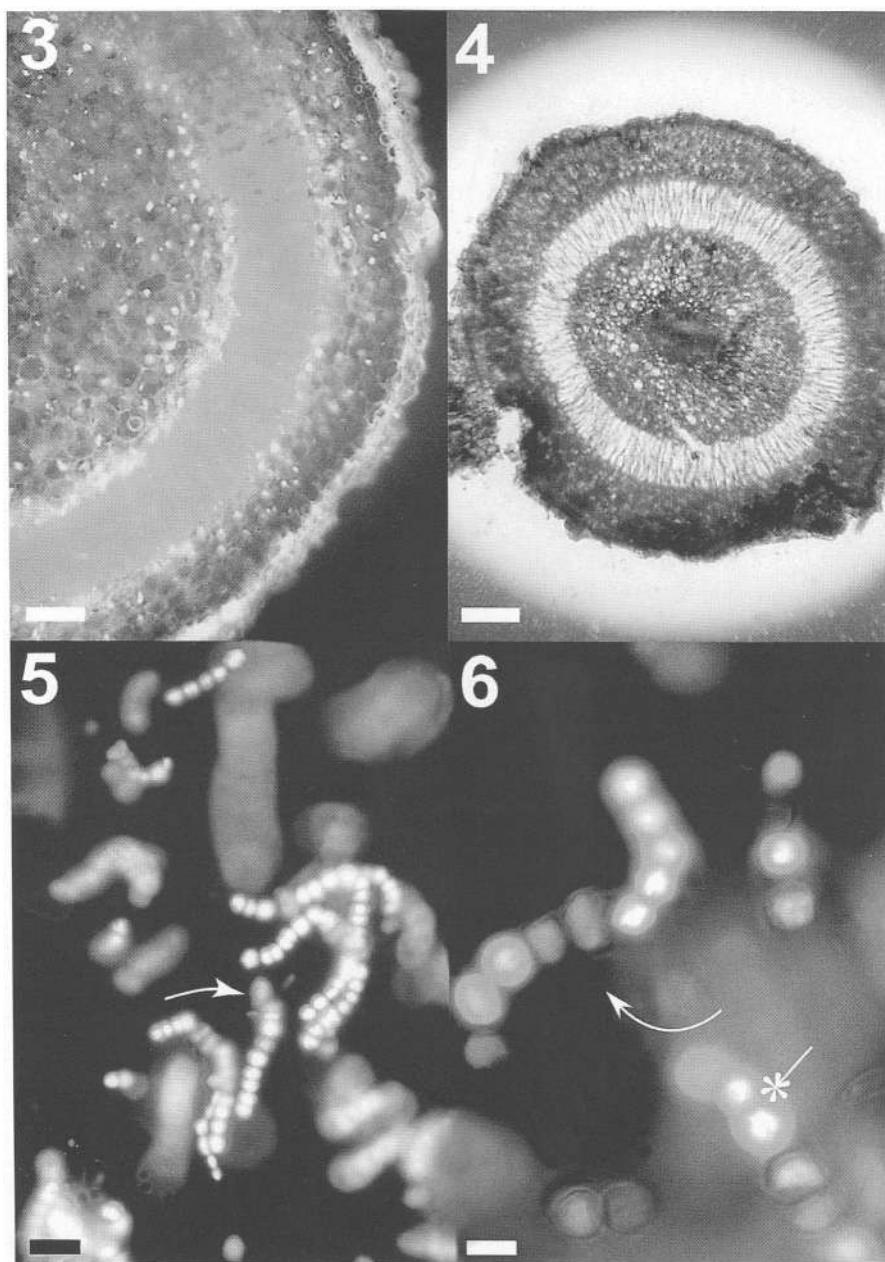


Figure 3. Transverse section of *Cycas revoluta* coraloid with cyanobacterial zone intensely red autofluorescent with exciting filter of 546 nm and a barrier filter of 580 nm. Bar = 0.5 mm. **Figure 4.** Transverse section of *Cycas revoluta* coraloid after iron treatment. Outer and inner cortex appear brown because of the presence of phenolic compounds, whereas cyanobacterial zone is unstained. Bar = 1 mm. **Figure 5.** Vegetative cells (◀) and single and double heterocysts (*) of cyanobionts *Cycas revoluta* after treatment. Bar = 20 μ m. **Figure 6.** Two filaments of cyanobionts of Fig. 5 with double heterocysts. Bar = 5 μ m

The reconstitution of a Cycad-cyanobacterial association permitted to satisfy not only Koch's postulates and the identification of cyanobionts, but also enabled the investigation of the ways in which recognition and penetration into the host take place (Ow et al., 1999).

4. Recognition and penetration of cyanobacteria into the host

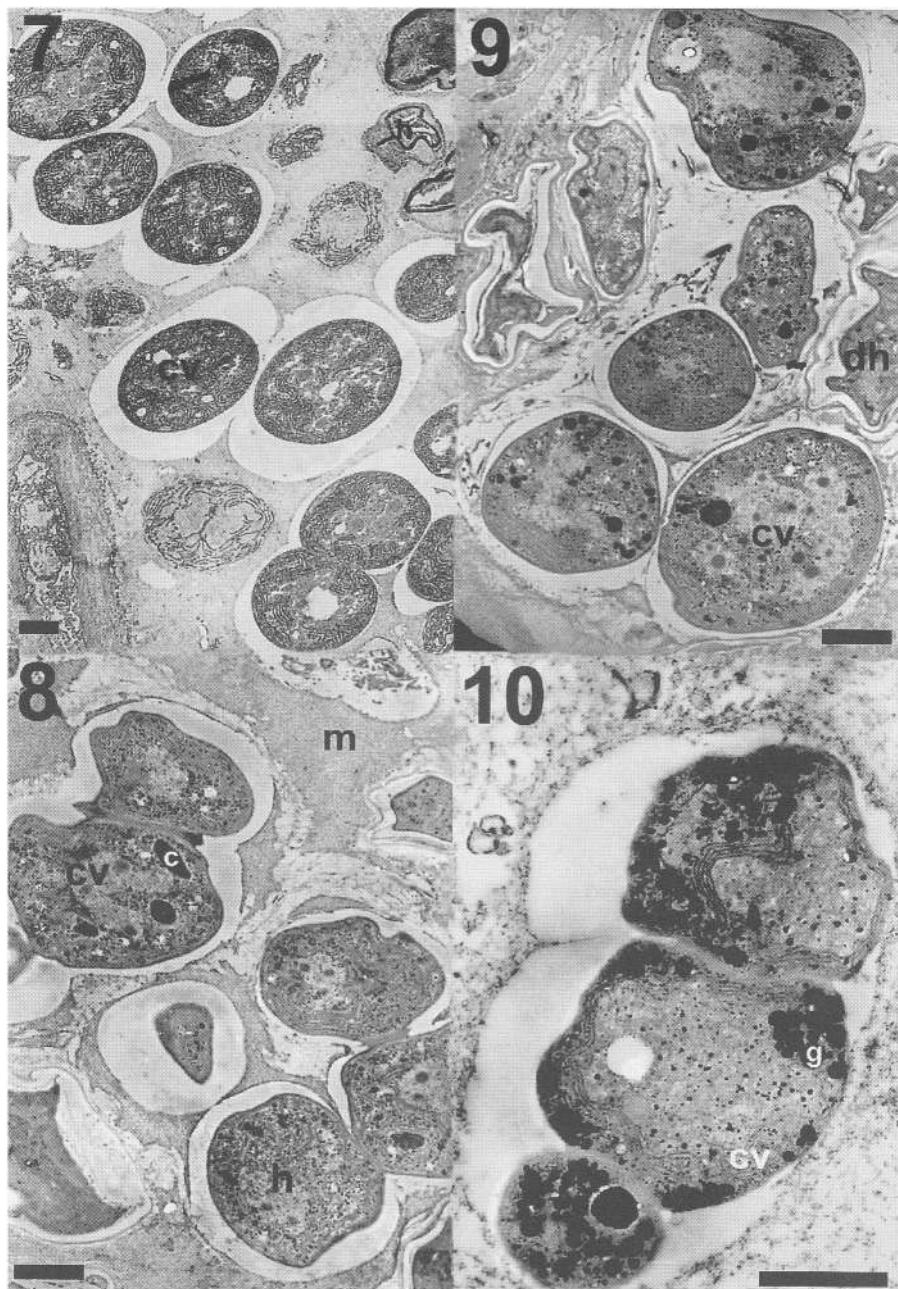
All coralloid roots infected by cyanobacteria have a pronounced layer of mucilaginous material where cyanobacteria occur as short hormogonial filaments. Hormogonia are the infective units of all competent *Nostoc* species involved plants symbiotic associations.

Hormogonia have also been observed to infect *Blasia*, *Anthoceros* and *Gunnera* (Tandeau de Marsac 1994; Watts et al., 1999).

Hormogonia are a type of motile *Nostoc* cells in short trichomes, without growth and whose cells are smaller than those of vegetative cells. In symbiotic strains, hormogonia are induced by activate genes of host by means of signal molecules inhibiting cell growth and heterocyst differentiation.

According to Nathaniels and Staff (1975), penetration of hormogonia into the coralloid roots of *Macrozamia communis* occurs via a break in the dermal layers and leading, through a continuous cortical channel, into the cyanobacterial zone. Intercellular migration of the cyanobionts is found to occur after dissolution of the host cell middle lamella, although host cortical cells are also destroyed near some of the cyanobionts. Intracellular migration is also suggested to be a likely pathway for the cyanobacteria as they proceed to the cyanobacterial zone. However in *Cycas revoluta* no intracellular migration or intracellular localization has been observed (Grilli, 1972a).

Infection of previously axenic roots provides an illustration of the idea supported by Milindasuta (1975) that cyanobacteria enter uninfected coralloid roots through the spaces between the cells of the rootcap, where at low frequency form breaks in the dermal layer and cortical channels beyond (Ow et al., 1999). In *Macrozamia communis* initial invasion involves penetration through the dermal layer at a point on the root axis distal from the tip. After entry through the dermal layer, the cyanobacteria migrate in all directions through the outer cortex to the cyanobacterial zone (Fig. 3). Here they migrate upwards, downwards and laterally, but not inwards the inner cortex (Staff and Aher, 1993). This could be due to the presence of abundant phenolic compounds in the cortical cells surrounding the cyanobacterial zone. Phenolic compounds in the Cycad coralloid roots have been cytochemically revealed by Obukowicz et al. (1981) and Grilli Caiola (1990a) (Fig. 4) by treatment with iron chloride. Dossaji et al. (1975) have chromatographically studied the actual distribution of biflavonoids in leaves of different groups of Cycads. These molecules could inhibit the penetration of other microorganisms into the cyanobacterial zone. Differently from other symbioses of plants with cyanobacteria (i.e. *Azolla-Anabaena*, *Gunnera-Nostoc*) in which bacteria are associated to cyanobacteria and may contribute to the lowering of oxygen concentration, thereby stimulating nitrogen fixation (Dhir et al., 1992; Grilli Caiola and Forni, 1998), bacteria do not appear associated in Cycad coralloids. However, taking into account that recent studies have identified phenolic compounds as for recognition signals in other symbiotic associations (such as Rhizobia and Legumes) (Dhir et al., 1992), the presence



Figures 7-9. Transmission electronmicrographs of cyanobacterial zone of *Cycas revoluta* coralloid.
7. Vegetative cells (cv) and some heterocysts (h); **8.** Vegetative cells (cv) with cyanophycin granules (c), heterocysts (h) and a dense mucilage (m) in the median zone of the coralloid. **9.** Vegetative cells (cv) and many degenerate heterocysts (dh) in the basal part of the coralloid.

Figure 10. Vegetative cells (cv) with lipid globules (g) in *Dioon* coralloid. All bars = 1 μ m.

of a very high amount of phenols in the outer cortex together with mucilage could represent a way of inducing hormogonia in *Nostoc* to enable their penetration into the host. It is significant that the cytochemical assay of phenolic compounds revealed a high positive response in the outer cortex and only a low response in the inner one (Fig. 4) (Obukowicz et al., 1981).

5. Cyanobacterial zone

The cyanobacterial zone shows an organization of transfer cells with invaginations localized sometimes only at the tangential wall. Such an organization should allow the transport of fixed nitrogen from the cyanobacterial zone towards the vascular tissue of the coralloid. The mucilage filling the intercellular space of cyanobacterial zone is mainly composed of polysaccharides whose composition changes according the distance from the growing tip. Sulfate and acid polysaccharide occur in the apical zone, whereas acid polysaccharides were mostly detected in the basal one (Grilli Caiola, 1990a). The cyanobacterial zone is the structural and physiological site of the Cycad-Cyanobacteria symbiosis. The cyanobionts inside the coralloid contain photosynthetic pigments as revealed by intense autofluorescence when observed by fluorescent microscopy (Fig. 3). However they behave as mixotrophic in terms of carbon source, whereas they perform nitrogen fixation in specialized cells, the heterocysts, whose frequency (i.e. percentage to vegetative cells) increases from the tip towards the coralloid basis (Fig. 5). The nitrogenase enzyme responsible for the reduction of molecular nitrogen is synthesized and protected in the heterocyst. Many double or multiple heterocysts occur in *Cycas revoluta* coralloids (Grilli, 1963) (Fig. 6). Such double heterocysts, however, do not occur in cyanobacteria from coralloids isolated and grown in culture. Nitrogenase activity is the capability of the enzyme to reduce molecular nitrogen which is usually expressed as acetylene reduction activity (ARA) because of the presence of acetylene which gives rise to ethylene. ARA values (nmol ethylene $h^{-1} \mu g Chl^{-1}$ or mg of dry weight) in the cyanobacterial zone are higher than in the isolated cyanobionts grown in culture (Grilli Caiola and Canini, 1994). Since nitrogenase activity is inhibited by high oxygen pressure and high ammonium concentration, both in symbiotic and in free living cells, different mechanisms to protect the enzyme have been adopted. This is demonstrated by measuring the correlations between ARA, oxygen concentration and ammonium content in the cyanobacterial zone of coralloid segments cut at different distances from the tip to the base. Measurements of the above parameters have been made *in situ* by microelectrodes to detect the presence of O_2 and ammonium and by means of immunogold labelling on cyanobionts to detect Fe-superoxide dismutase (Fe-SOD)(Grilli Caiola and Canini, 1994; Canini and Grilli Caiola, 1993) (Table 1).

Oxygen determination in the cyanobacterial zone of different segments of coralloid roots indicates that its pressure is quite constant along the axis of the coralloid, ranging from 0.18 atmospheres at the gonidial zone of apical segments to 0.17 atmospheres in the basal segments, amounting to 85% with respect to its concentration in the atmosphere.

The dissolved ammonium content in the apex is 2 mM, it doubles (4 mM) in the median zone reaching the highest value, and decreases at the basis at 3 mM. oxygen and ammonia determinations indicate that nitrogenase in symbiosis works in the presence of

high oxygen and ammonium concentrations in contrast with what occurs in free-living cyanobacteria. In this respect, the existence of an intense oxygen consumption has been hypothesized inside the heterocysts in order to produce electrons and ATP for nitrogen reduction. This process makes the heterocysts microaerobic, but it also produces toxic oxygen species such as superoxides which are destroyed by superoxide dismutase (SOD). In prokaryotes, superoxide dismutase contains mainly Fe or Mn or Fe-Mn, and the detection of Fe-SOD has been used to reveal the presence of superoxide. In the cyanobacterial zone of *Cycas revoluta* the localization of Fe-SOD by immunogold labeling technique showed a high content in the active heterocysts when compared to degenerating ones and to vegetative cells. Measurements of photosynthetic oxygen evolution have given very low values, although RuBisCo was present in normal amount. This indicates that oxygen consumption in the cyanobacterial zone is mainly due to the aerobic respiration which is higher in the active heterocysts than in the other cells. Also, calcium has been hypothesized to protect nitrogenase by acting as a regulator of envelope cohesion. Measurements of calcium ions in the cyanobacterial zone of *Cycas revoluta* carried out by means of microelectrodes, chlorotetracycline and electron spectroscopic imaging (ESI) and electron energy loss spectroscopy (EELS) analyses at transmission electron microscope (TEM) (Canini et al., 1994) indicated that the entire active heterocysts had calcium on their envelope, whereas degenerating heterocysts did not. Therefore, calcium alone or in combination with previously reported biochemical mechanisms, could protect nitrogenase inside the heterocysts.

TABLE I. Different values of heterocyst frequency (HF), nitrogenase activity (ARA), iron-superoxide dismutase (Fe-SOD) immuno labeling, ammonia and calcium content in the cyanobacterial zone of apical and basal segments of coralloid roots of *Cycas revoluta*.

	Apical	Basal
a. Heterocyst frequency (HF)	20	60
b. Nitrogenase activity (ARA)	1.1	0.2
c. Fe-SOD labeling v.c.	35	30
Fe-SOD labeling h.	50	20
d. Ammonia ion concentration (mM)	2	3
e. Calcium ion concentration (mM)	10^{-3}	1.2×10^{-2}

a. HF: percentage of heterocysts to vegetative cells;

b. ARA: nmol of C_2H_4 $\mu\text{g Chl}^{-1} \text{h}^{-1}$;

c. Fe-SOD labeling: number of gold particles per cell area (μm^2) after immunogold labeling at TEM in v.c. (vegetative cells) and h. (heterocysts);

d,e. Ammonia and calcium concentration (M) were measured by microelectrodes inside the cyanobacterial zone of coralloid roots.

A gradient of nitrogenous activity and ammonium content have been detected along the coralloid axis. Overall increased values of ARA and ammonium are found from the apex to the median zone; thereafter, all measured parameters decrease. This is because the heterocyst frequency increases from the apex towards the basis, reaching values around 40-70%, whereas most heterocysts from the median region are degenerated and inactive (Figs 7-10). This fact helps in further understanding the biology of coralloids.

As for the presence of high ammonium concentration and nitrogenous activity, a rapid transport of fixed nitrogen from the cyanobionts should exist in order to reduce its inhibiting effects. On this topic Pate et al. (1988) suggested the existence of some forms of apoplastic exchange between the partners, probably mediated through the highly distinctive columnar cells within the cyanobacterial zone. Interestingly, only in *Cycas* and *Bowenia* glutamine is transported in large amounts inside the coralloid towards the host plants whereas other Cycads, such as the Zamiaceae, usually transport both citrulline and glutamine. Since ammonium is virtually absent in the xylem and in tissue compartments, the primary route for assimilation of ammonium is likely to be via glutamine synthetase. Substantial levels of GS activity and specific GS immunolabeling found in heterocysts, as well as in vegetative cells of the cyanobionts of *C. revoluta*, suggest that glutamine might be exported from the endophyte either with or instead of ammonium (Rai et al., 1986).

In *Cycas revoluta* the symbiotic nitrogen-fixation involves a massive net synthesis of glutamine and no measurable synthesis or accumulation of citrulline by either symbiotic or non-symbiotic tissue. The symbiont has the capacity to assimilate the ammonia produced through nitrogen fixation, but in culture, unlike their free-living isolates, it lacks the ammonium transport system responsible for the import of ammonium via glutamine synthetase (Rai et al., 1986).

6. Significance of nitrogen fixation in cycads

The life time of coralloid roots varies according to the Cycad species being in many cases annual, perennial, or annual and perennial (Guo-fan et al., 1993)

Nitrogen fixation rates in *Cycas revoluta* coralloid roots were estimated to be sometimes higher than those measured in the isolates grown in culture, but similar to those obtained from other cyanobacteria in symbiotic systems. In evaluating the role of the symbiotic nitrogen fixation in the ecosystem, the data reported by Guo-fan et al. (1993) are an interesting contribution for *Cycas panzhihuaensis* growing in a dry-hot river valley in the south-tropical region of China. The living coralloid roots of current seedlings amounted to 8 g per plant in natural community, whereas in plants of over 100 years in artificial community coralloids averaged 370 g. The nitrogen fixation activity is usually from 1.8 to 11.1 nmol C₂H₄/gfw h⁻¹ in autumn, and is obviously influenced by light and soil moisture. The amount of fixed nitrogen from a plant of *Cycas* in natural community varied from 4.39 to 12.02 mg h⁻¹ whereas in artificial community it varied from 0.64 to 18.68 mg h⁻¹.

7. Concluding remarks and perspectives

The Cycad-cyanobacteria association shows many peculiar aspects compared to plant-bacteria or plant-mycorrhizal fungi association. For a high number of genera and species of Cycads there is only a very limited number of genera of cyanobionts, namely *Nostoc*, *Anabaena*, *Calothrix* (Grilli Caiola, 1974; 1975 a and b; Grilli Caiola, 1990b). All cyanobionts belong to the Nostocales, having both heterocysts and akinetes (Grilli

Caiola and de Vecchi, 1980; Huang and Grobbelaar, 1989), although the akinetes are very rare in symbiosis. No other microorganism accompanies cyanobionts whose relation to the host is regulated in a manner that suggests a dominance of the Cycad over the symbiont. In fact, the Cycad is able to regulate the growth of *Nostoc* outside the root, by inducing hormogonia, and also inside the coralloid roots by stimulating an enlargement of the vegetative cells and the differentiation of heterocysts at rates ten to twenty times those of free-living cyanobacteria. In addition, the Cycad promotes the conditions for a maximum of nitrogen fixation and subsequently the utilization of nitrogen produced by the cyanobionts. How cyanobionts survive in the degenerated coralloid roots and in the soil is still unclear. However, when considering the influence of the host on the cyanobionts, the association seems to be mainly or totally in favor of the Cycad. Cyanobacteria could induce an enlargement of the cyanobacterial zone and provide themselves with a protected environment; however, this is only a short term protection and cyanobionts appear as prisoners in the host.

More research aiming at exploring the basic mechanisms of the formation of Cycad coralloid roots is still needed to better define this symbiosis. The nature of the signaling molecules involved in the first step of the symbiosis has to be clarified. Also, the processes of infection, penetration and diffusion need yet much research. Difficulties are met in working with Cycads because of their very slow growth. However, great progress has been made with respect to the reconstitution of *Nostoc*-coralloid root symbiosis as well as other symbioses with *Nostoc* (Adams, 2000). Other interesting topics concern the mechanisms adopted in regulating the oxygen concentration in the heterocysts. The surviving forms of the symbiont in the coralloid roots and in the soil is another stimulating topic, and also the possibility that heterocysts may germinate in coralloids (Spratt, 1911; Grilli Caiola, 1990a) opens a new insight on the reversibility of heterocyst differentiation.

Biotechnological tools might help in preparing *Cycas revoluta* plants with improved characteristics to grow in nurseries, such as a higher capacity to produce coralloid roots active in nitrogen fixation. The ever increasing demand for *Cycas revoluta* plants for ornamental purposes should stimulate research toward the development of molecular and biotechnological tools to establish new symbioses of Cycads with active symbionts to grow in different areas. The main goal is to obtain plants which do not require a nitrogen supply, resulting in energy saving and avoidance of polluting fertilizers.

8. References

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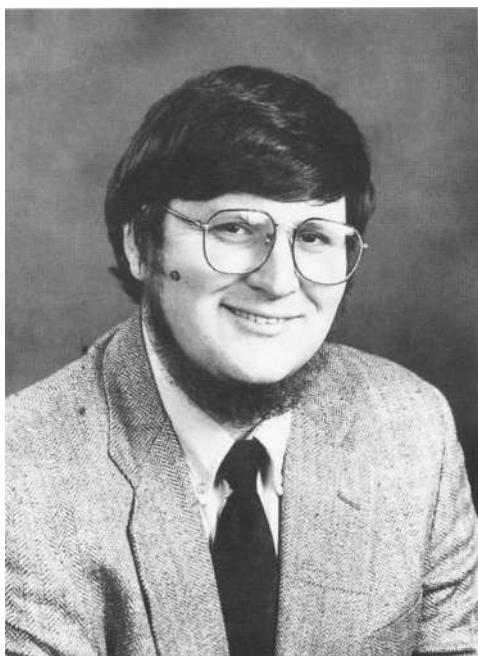
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EVOLUTION OF *EPICHLOË/NEOTYPHOIDIUM* ENDOPHYTES AND OTHER CLAVICIPITALEAN BIOTROPHS

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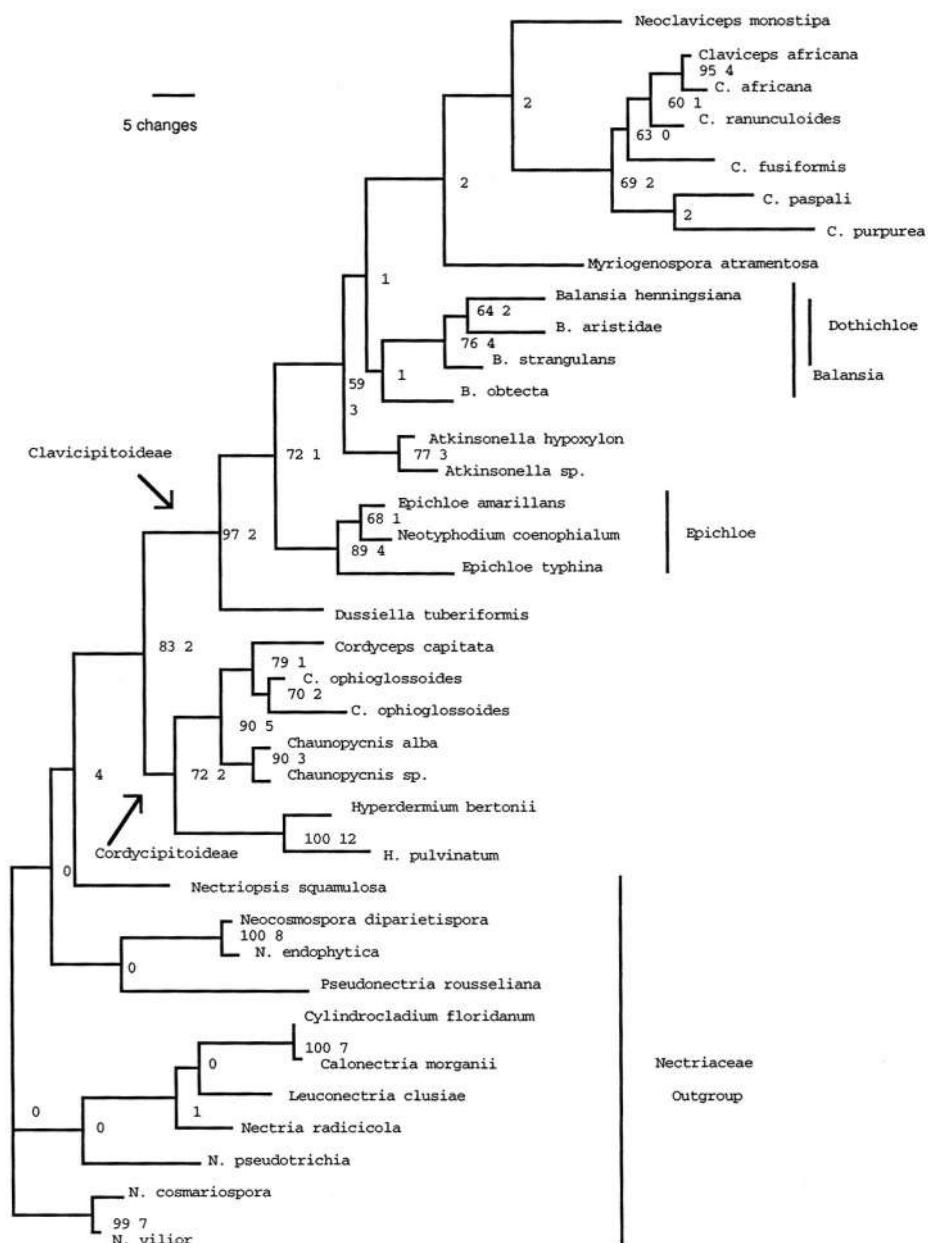
1. Introduction

The fungal family Clavicipitaceae (Hypocreales) contains numerous species that are important economically. *Cordyceps subsessilis* (anamorph *Tolypocladium inflatum*) is a source of the powerful immune suppressant drug cyclosporin, used prevent rejection after organ transplants (Drefuss and Chapela, 1994; Hodge *et al.*, 1996). Ergonovine and other indole alkaloids, employed in the treatment of migraine headaches and to induce uterine contractions, are derived from *Claviceps purpurea*. (Lewis and Lewis, 1977). This fungus was responsible for ergotism, which historically afflicted numerous human populations consuming sclerotia mixed with grain. The same fungus was the original source of lysergic acid diethylamide (**LSD₂₅**), a fungal product that gave rise to the psychedelic era of the 1960's (Schultes and Hoffmann, 1979). Alkaloids produced by *C. purpurea* have been used in western medicine for hundreds of years (Lewis and Lewis, 1977). Derivatives of ergot alkaloids are also used in medicine as treatments for migraine headaches and for induction of contractions to initiate childbirth. Anthropological evidence demonstrates that another member of the Clavicipitaceae, *Balansia cyperi*, is employed among women of the amazonian Achuar Jívaro tribe to facilitate child birth (Lewis and Lewis, 1990; Plowman *et al.*, 1990). In addition to the pharmaceutical uses and potentials, recent investigations in the graminicolous Clavicipitaceae have suggested that the group is ecologically significant to host plants due to beneficial effects, such as increased resistance to herbivory by insects and mammals (Clay, 1988, 1989; West, 1994), increased drought tolerance (West, 1994), and increased fungus disease resistance (Clarke *et al.*, In press). Clay (1988) has characterized the relationship between mycosymbiont and host as a defensive mutualism.

2. Modes of Parasitism

The Clavicipitaceae may be parasites of insects, other fungi, or plants. Generally, insect or fungal parasites pertain to the subfamily Cordycipitoideae; while plant parasites belong in subfamilies Oomycetoideae or Clavicipitoideae (Diehl, 1950) (Figure 1). Plant parasitic species extract nutrients from hosts as epibionts or endophytes (endobionts) of

Figure 1. One of 3 equally parsimonious trees (length 501, CI 497, RI 0.706, HI 0.503) obtained from analysis of large subunit rDNA data using PAUP. Percentages (above 50%) from 500 bootstrap replications and decay indices are given to the right of nodes.



the plants. A few species are parasites of both insects and plants. Species of genus *Hyperdermium* (Cordycipitoideae) infect scale insects, consume them completely, and continue to develop epiphytically surviving on the sugars that leak from the phloem of the plant (usually a shrub in family Asteraceae) through the wound left by the scale insect stylet. Species of *Hyperdermium* are necrotrophs of scale insects but biotrophs of plants since they kill their insect hosts but require a living plant to maintain themselves. *Hyperdermium* effectively makes a transkingdom shift in hosts each turn of its life cycle. This peculiar genus of fungi demonstrates how easily the Clavicipitaceae could have jumped from insect hosts to plant hosts. Epibiotic species such as the leaf infecting *Myriogenospora atramentosa* establish infections with hosts early in host tissue development and modify the host tissues during development, perhaps using auxins or auxin-like compounds to prevent development of the waxy cuticle layer (White and Glenn, 1994). Like *Myriogenospora*, most species of plant-infecting Clavicipitaceae form their fruiting structures on meristematic parts of plants, including inflorescence primordia and nodal or leaf meristems. Species of genus *Balansia* and *Epichloë* are endophytic in grasses. Species of these genera produce mycelia in the intercellular spaces of leaf mesophyll and culm parenchyma. The fruiting structures of endophytic species often develop so that they connect internally to the xylem and phloem tissues of the plant and thus establish a mechanism for direct extraction of nutrients from the host. In *Epichloë/Neotyphodium*, the endophytic mycelia are sometimes incorporated into ovules of the grass. There they infect the embryo and are thus seed transmitted to the next generation of host. Partly because of the seed-transmission capacity of many of these endophytes, they have become abundant and widespread in cool-season grasses. The endophytic species of *Balansia* are not seed transmitted and are not as common as are the *Epichloë/Neotyphodium* endophytes.

3. A Defensive Mutualism

A large body of literature documents the toxicity of clavicipitalean endophytes to herbivores (e.g., Bacon *et al.*, 1975; Mantle, 1978; Porter and Thompson, 1992; Porter *et al.*, 1993). Endophytes are the sources of several types of secondary compounds that are the cause of toxicoses. Loline, peramine, indole acetic acid, ergonovine, ergovaline, lolitrem B, and terpenoids and other secondary compounds have been documented to be produced by endophytes (Lane, Christensen, and Miles, 2000). Alkaloids such as ergovaline and lolitrem B are generally implicated as the causes of animal toxicoses. Lysergic acid amide is suspected to cause the sleepygrass narcosis affecting animals that consume the endophyte-infected grass *Achnatherum inebrians* and *A. robustum* (Lane, Christensen, and Miles, 2000). Animals that consume relatively small quantities of endophyte-infected sleepy grasses may suffer a sleep-like narcosis that lasts up to three days (White, 1987; Petroski, Powell, and Clay, 1992).

Clavicipitalean endophytes have also been shown to enhance the fitness of their grass hosts. Various grasses infected with endophytes are more resistant to insect pests than comparable endophyte-free plants. Prestidge *et al.* (1985) demonstrated that the Argentine stem weevil was deterred from feeding on endophyte-infected ryegrasses. Clay and Cheplick (1989) demonstrated that endophyte-infected grasses were more resistant to Fall armyworms than comparable endophyte-free individuals. Lewis, White,

and Bonnefont (1993) found that various species of grasses infected with endophytes repelled the feeding of migratory locusts; while uninfected host individuals were readily consumed. Peramine and lolines have been implicated as primarily anti-insect compounds (Lane, Christensen , and Miles, 2000).

Some species of endophyte-infected grasses appear to increase abiotic stress tolerance. Tall fescue grass infected by the endophyte *Neotyphodium coenophialum* shows a greater ability to survive drought stress than endophyte free tall fescue (West, 1994). Clavicipitalean endophytes also appear to make some grasses more resistant to phytotoxic metal ions in soils (Belesky and Malinowski, 2000). Endophyte-infected tall fescue and ryegrass are now extensively used as forages and turfgrasses because of the enhanced hardiness of the associations.

Endophytes have been shown to protect their hosts from plant diseases. Shimanuki (1987) found that endophyte-infected timothy (*Phleum pratense*) was more resistant to the leafspot disease caused by *Cladosporium phlei* than endophyte-free plants. Recently it has been found that fine fescues infected by *Epichloë festucae* show an almost absolute resistance to dollar spot disease caused by *Sclerotinia homeocarpa* (White, unpublished data). Moy *et al.* (2000) identified an epiphyllous network of mycelium on surfaces of leaves that was demonstrated to belong to the endophyte *E. festucae*. Epiphyllous nets were hypothesized to function as a defensive barrier to protect leaves from colonization of potentially pathogenic fungi. Thus, niche exclusion was proposed as at least one mechanism of increased fungus disease resistance. Due to the many ways that endophytes have been shown to protect host grasses, Clay (1988) proposed the “defensive mutualism hypothesis” that held that the basis for the symbiotic association is the increased defensive capacity of the symbiotic union due to activities of the endophytes.

4. Life Cycle Patterns in Genus *Epichloë*

Three life cycle patterns of *Epichloë/Neotyphodium* endophytes have been described (White, 1988; Table 1). Type-I endophytes are those species of *Epichloë* that are not seed transmitted, instead a high percentage of the host culms bear stromata. Type-II endophytes have both stroma development on some culms and seed transmission on other culms. The percentage of culms that bear stromata may be a function of environmental conditions such as soil fertility (Funk and White, 1997). Seasonal variation is also evident. In some years grass crops of fine fescues containing *E. festucae* produce many stromata, while in other years, fewer stromata are formed (unpublished observations). Unknown environmental conditions may alter the balance between endophyte and plant and effect the degree of stroma development. Type-III endophytes very rarely or never form stromata. These endophytes appear to rely on seed transmission as the primary means of dispersal. Asexual *Epichloë* spp. are typically classified by their anamorphs in genus *Neotyphodium* and are frequently referred to as *Neotyphodium* endophytes.

TABLE 1. *Epichloë/Neotyphodium* species, hosts, and lifecycle type

Selected species	Hosts	Assoc. Type
<i>E. amarillans</i>	<i>Agrostis hiemalis</i>	II
<i>E. baconii</i>	<i>Agrostis stolonifera</i>	I
<i>E. brachyelytri</i>	<i>Brachyletrum erectum</i>	II
<i>E. bromicola</i>	<i>Bromus ramosus</i>	II
<i>E. clarkii</i>	<i>Holcus lanatus</i>	I
<i>E. elymi</i>	<i>Elymus canadensis</i>	II
<i>E. festucae</i>	<i>Festuca rubra</i>	II
<i>E. glyceriae</i>	<i>Glyceria striata</i>	II
<i>E. sylvatica</i>	<i>Brachypodium sylvaticum</i>	II
<i>E. typhina</i>	<i>Dactylis glomerata</i>	I
	<i>Lolium perenne</i>	
	<i>Poa pratensis</i>	
<i>N. coenophialum</i>	<i>Festuca arundinaceae</i>	III
<i>N. lolii</i>	<i>Lolium perenne</i>	III
<i>N. starrii</i>	<i>Festuca arizonica</i>	III
<i>N. uncinatum</i>	<i>Festuca pratensis</i>	III
<i>N. tembladerae</i>	<i>Poa huecu</i>	III
	<i>Festuca argentina</i>	

5. Evolution of Type-III Endophytes

Recent research suggests that hybridization between sexually-reproducing types-I and -II endophytes may be an important factor in development of the asexual type-III endophytes (Tsai *et al.*, 1994; Schardl and Wilkinson, 2000). Evidence of hybridization is the presence of polymorphisms in several genes within individual endophyte genomes (Tsai *et al.*, 1994; Schardl *et al.*, 1997; Leuchtmann and Schardl, 1998; Cabral *et al.*, 1999). The gene polymorphisms are suggested to be remnants of species origin events. However, it seems equally plausible to hypothesize that gene polymorphisms may be the result of a parasexual process that is a function of the epiphyllous stages of endophytes. Anastomosis of the mycelium of endophytes on leaf surfaces is a frequently observed phenomenon (White *et al.*, 1996). Chung and Schardl (1997) demonstrated that *Epichloë/Neotyphodium* endophytes show interspecies vegetative compatibility. It may be that vegetative fusions on leaf surfaces are part of a parasexual process that endophytes employ to compensate for the loss of stromata and sexual reproduction and may be an important means whereby such asexual endophytes evolve and change genetically.

An alternate hypothesis for origin of type-III endophytes and the loss of sexual reproduction is selection against the stroma stage in the life cycle of endophytes. The stroma is the structure on which the spermatia and perithecia of the endophyte develop. Stromata are composed of a combination of plant and fungal tissues. The plant tissues, including inflorescence primordium and leaf sheath, are trapped in the fungal mycelium and modified so that they do not impede the flow of nutrients and moisture into the mycelium (White, Bacon, and Hinton, 1997). The stromal mycelium passes moisture and nutrients to the developing perithecia of the endophyte on the stroma surface. Because the cuticle never develops on the epidermal surfaces trapped within stromata,

moisture freely flows into the mycelium of the stroma. Evaporation from the moist surface of stromata is continuous and water losses are several times that observed from healthy plant tissues (White and Camp, 1996). It has been proposed that the enhanced evaporation from the surface of the stroma is important because it provides the mechanism whereby the fungus accumulates nutrients from plants (White, Bacon, and Hinton, 1997). High evaporation from the surface of stromata causes development of a flow of water from plant tissues to fungal stroma that carries the nutrients needed by the fungus for reproduction. This mechanism, termed ‘evaporative-flow nutrient acquisition’ may also provide the impetus for environmental selection against stroma development (White, Bacon, and Hinton, 1997). In soils where water is limiting, the development of stromata on plants may be selected against due to the increased losses of water through evaporation from the surface of stromata. In the type-II endophytes, whether the endophyte forms stromata or is seed transmitted is largely a function of the growth rate of the endophytic mycelium on carbohydrates in the expanding inflorescence primordia (Kirby, 1959; White *et al.*, 1991). Strains of endophytes that grow rapidly are capable of outgrowing the inflorescence primordium and trapping it in a stroma. Slower growing strains of endophyte are incapable of trapping the inflorescence primordium in a stromal mycelium. To favor seed transmission over stroma development faster growing strains would be selected against. The majority of the type-III endophytes grow more slowly than the type-I or -II endophytes. It is also notable that plants bearing stromata tend to be located in soils that are frequently flooded; while in very dry soils only plants bearing type-III endophytes may be encountered.

6. Northern Hemisphere Center of Diversity of *Epichloë/Neotyphodium* Endophytes

Preliminary surveys of grasses in North and South America reveal that grasses are infected in comparable frequencies, ranging 20-40% of species in genera *Festuca* and *Poa* (White 1987; Cabral and White unpublished). Analyses of rDNA ITS, 5.8S, and ITS2 sequences and protease genes (Cabral *et al.*, 1999) of *Neotyphodium* spp. from Asia, Europe, North America, and South America reveals that South American collections associate with the *E. festucae* grouping of isolates. Further, of 10 isolates from South America, 8 were grouped in a single *N. tembladerae* subclade; and two grouped in another subclade. Numerous species from Europe and North America are types-I and -II endophytes known to produce stromata and the *Epichloë* teleomorph. Stromata are not formed by the South American endophytes, appearing instead to comprise exclusively type-III endophytes. Stromata have not been encountered in infected host populations or in herbarium collections at the University of Buenos Aires or La Plata, Argentina. The apparent lack of diversity in absence of the sexual cycle and narrow range of variation in rDNA sequences is in contrast to the diversity seen in Eurasian and North American collections (Figure 2). *Epichloë/Neotyphodium* endophytes of Eurasia and North America constitute a diverse group of species. Some 10 different species of *Epichloë* have been documented from Europe and North America. For example, ribosomal DNA ITS sequences of endophytes of the Northern Hemisphere grass genus *Achnatherum* are distributed in multiple clades. The endophyte of *A. sibiricum* #2 (from Inner Mongolia, China) appears in the *E. festucae* clade near

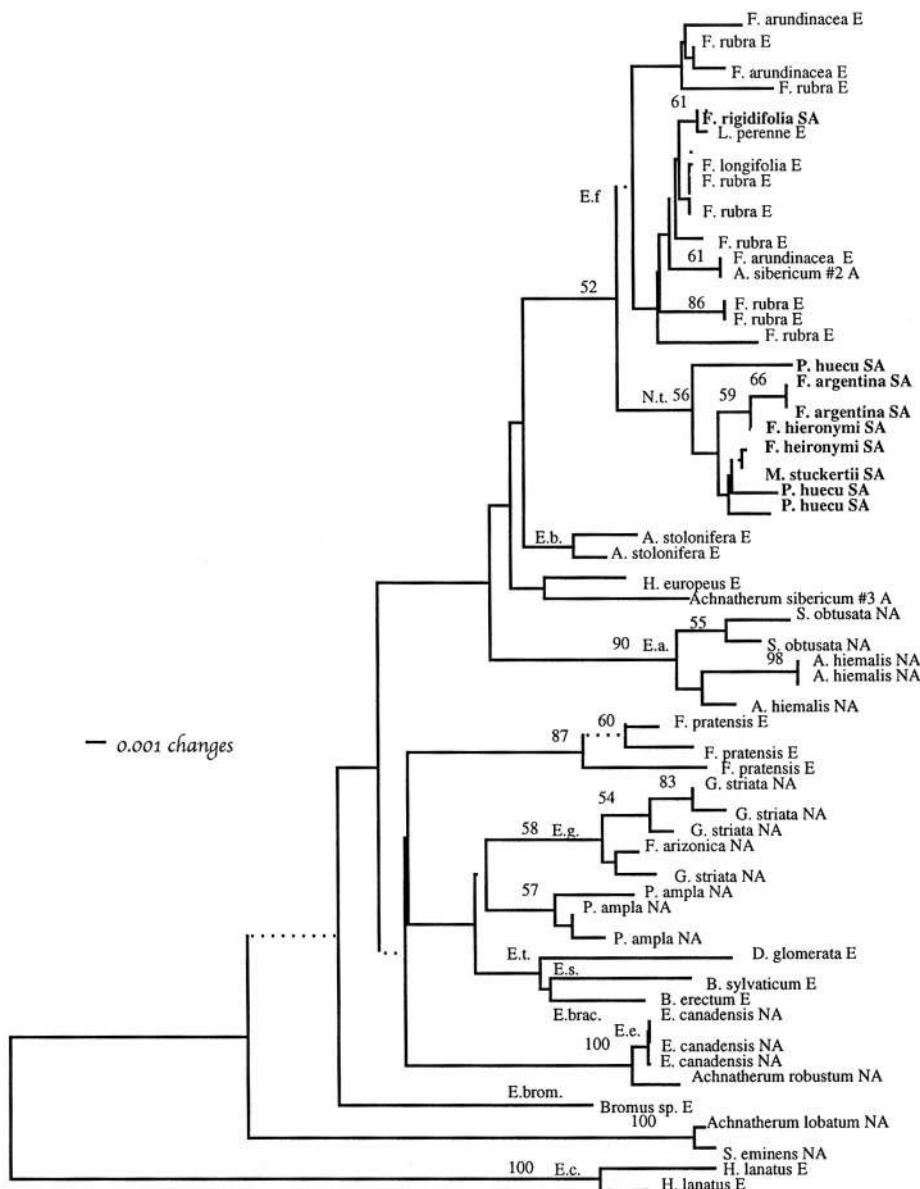
an isolate from *Festuca arundinaceae*. The endophyte of *A. sibericum*#3 (from Inner Mongolia, China) appears near an endophyte from *Hordyelymus europeus* (from Spain). The endophyte of *A. robustum* (from North America) appears in the North American *E. elymi* clade. Hybridization between species of endophytes may, in part, account for the diversity in rDNA ITS sequences among *Achnatherum* endophytes as has been demonstrated to occur among the asexual endophytes (Schardl *et al.* 1997). Regardless of the mechanism of diversity generation, it is clear that there is a paucity of variation among the endophytes of South America so far sampled.

We hypothesize that the low diversity seen in our sample of South American endophytes is due to a paucity of genotypes that colonized South America. *Epichloë* may have evolved in the Northern Hemisphere in Asia, Europe, or North America and later migrated into the Southern Hemisphere, entering South America a limited number of times. The oceans are obvious barriers to colonization of South America by endophytes. Additionally, the tropical zone separating North and South America may prevent the movement of the cool-season grass hosts of *Epichloë/Neotyphodium* into South America. Regardless of the barriers, the colonization of South America appears to have occurred at least twice by individuals of *E. festucae*. It is probable that the emigrant endophytes were incapable of stroma production and probably were well adapted for seed transmission and persistence. It is notable that the exclusively seed-transmitted *N. coenophialum* survives longer in seeds than stroma forming *E. festucae* from which it evolved (Funk and White, 1998). Loss of stroma development may have preadapted the ancestors of the South American endophytes to survive the long distance trip to South America either from Eurasia or North America. The founding populations in South America may have evolved and adapted to various hosts, which would account for the variation encountered in the *N. tembladerae* clade. The close sequence similarity of the *Poa rigidifolia* endophytes to the endophytes of Eurasian grasses such as *Festuca rubra* and *Lolium perenne* may be an indication that it is a more recent colonizer of South America than endophytes of the *N. tembladerae* group.

7. The Fungus-Fly Symbiosis (Insect Mediated Fertilization)

Epichloë relies on a symbiotic association with species of plant-eating flies in genus *Botanophila* (formerly *Phorbia*) to complete its sexual cycle (Bultman *et al.*, 1995). The flies serve as vectors of spermatia of the fungus in a heterothallic mating process. The life cycles of the endophytes and flies are correlated so that as stromata develop on inflorescence primordia of grasses, the flies emerge from over wintering in the soil. Male flies feed on plant material but the female flies feed exclusively on spermatia and mycelium of *Epichloë* on stromata. Females actively seek out stromata, consume mycelium and spermatia, and defecate frequently on them, often walking on stromata dragging the extreme end of the abdomen to spread the feces. Feces of the flies consist of digested mycelium and masses of viable spermatia of *Epichloë*. If the fly has previously visited stromata of the opposite mating type, the masses of spermatia in feces will initiate development of perithecia on the surface of stromata. Once a stroma has

Figure 2. Distance tree (Neighbor-joining, minimum evolution, Kimura 2-parameter model, ME score = 0.38873) made using aligned rDNA (ITS1, 5.8S, and ITS2) sequences of endophytes from numerous grasses from Asia (A), Europe (E), North America (NA), and South America (SA). Endophytes are identified by host species names. Endophyte species abbreviations and bootstrap values (500 reps) are given above branches (E.a. = *Epichloë amarillans*, E.b. = *E. baconii*, E. brac. = *E. brachyletri*, E. brom. = *E. bromicola*, E.c. = *E. clarkii*, E.e. = *E. elymi*, E.f. = *E. festucae*, E.g. = *E. glyceriae*, E.s. = *E. sylvatica*, E.t. = *E. typhina*, N.t. = *Neotyphodium tembladerae*).



been fertilized using spermatia in feces, the fly will deposit an egg on the stroma. The egg hatches as the stromal mycelium thickens due to the developing perithecia. The fly larvae consume a small fraction of the young perithecia for several weeks as they grow. When perithecia are fully formed, the larva drops off of the stroma to the soil and pupates. The fly over winters in the pupa stage, emerging again as an adult the following spring. It is still unknown how many species of flies are symbiotic with *Epichloë*. Whether a particular species of fly associates only with a particular species of *Epichloë* or with several species of *Epichloë* has to be determined. It is also yet to be determined how flies detect stromata of *Epichloë*.

8. Taxonomy

Several species of *Epichloë/Neotyphodium* endophytes have been proposed (Table 1). The majority of these are well supported with phylogenetic data as well as mating compatibility studies (White, 1993; Schardl, 1996). However, there are some problems in recognition of some species. In many of the established species no structural features of the fungi were found that could distinguish them from some other species. This is apparently due to their simple and conserved morphologies. Similar problems are seen among species of bacteria where many different species may possess the same structure. Additionally, some evidence suggests that even endophytes of different species may possess the capacity to hybridize using a parasexual mechanism. Chung and Schardl (1997) demonstrated vegetative compatibility between species of endophytes. Mycelium of *Epichloë/Neotyphodium* endophytes was found to undergo fusions of cell contents in vitro culture. This coupled with the finding that some asexual endosymbionts have also been shown to contain an assemblage of genes from different species of *Epichloë/Neotyphodium* endophytes (Schardl *et al.*, 1997), suggests that parasexual genetic recombination may be occurring naturally. It is thus possible that there is some gene flow between distinct species of these fungi.

9. Other groups of Endophytes and Epibionts in the Clavicipitaceae

Balansia includes both endophytes and epibionts that infect predominantly warm season grasses. Species in this genus are known throughout the world. The majority of the endophytes are classified in the subgenus *Dothichloë*, and the epibionts in subgenus *Eubalansia* (Diehl, 1950). Subgenus *Dothichloë* is characterized by the possession of stromata that develop on leaves or culms of grasses; while subgenus *Eubalansia* is defined to include species with stromata that develop on inflorescences. Future research will need to address whether subgenera in *Balansia* represent natural (i.e., phylogenetic) categories. Several genera have been allied with genus *Balansia* including *Atkinsonella*, *Balansiopsis* and *Myriogenospora*. Diehl (1950) defined genera largely on the basis of anamorph (=sexual spore state). Species were classified in *Balansia* if they possessed only an *Ephelis* anamorph, and in *Atkinsonella* if they possessed an *Ephelis* anamorph and an *Acremonium*-like synanamorph. *Balansiopsis* included species that did not appear to produce any anamorph. *Myriogenospora* produces and ephelialidial conidial

state but was distinguished from these genera on the basis of differences in perithecial, ascus, and ascospore morphology (White and Glenn, 1994).

10. *Neoclaviceps* and the Evolution of *Claviceps*

Most species of *Balansia* infect entire inflorescences, culm nodes, or leaves of grasses. *Neoclaviceps monostipa* is similar to species of genus *Claviceps* (e.g. *C. purpurea*) in that it infects individual florets of the host grass and that the perithecial stroma is stipitate. However, the conidial state in *Claviceps* is sphacelial (*Sphacelia* spp.), conidia are enteroblastic, and they do not resemble the ephelidial state of *N. monostipa*. While *N. monostipa* infects ovaries of its host as do species of *Claviceps*, sclerotia are not formed. In *Claviceps*, a sclerotium develops from the sphacelium and replaces the ovary. In *N. monostipa*, infection of the ovary results in development of a mycelium within the ovary, perhaps consuming only the unfertilized ovule or young embryo. This mycelium (hypothallus) remains confined within the ovary, except for a white collar that emerges from the style base at the time the stipe develops. The mycelium within the ovary is a loose network of hyphae rather than a dense pseudoparenchyma. This hypothallus likely functions in absorption of nutrients from the ovary, rather than as energy storage as in sclerotia. *Neoclaviceps* is an evolutionary intermediate between *Balansia* and *Claviceps* (Figure 1).

11. Evolutionary Trends and Inferences

The graminicolous Clavicipitaceae comprise a monophyletic grade through *Epichloë*, *Balansia*, *Myriogenospora*, *Neoclaviceps*, and terminate in the *Claviceps* clade (Figure 1). Some changes in features of these groups are evident in this grade. *Epichloë* and *Balansia* are known to possess a heterothallic mating system (White and Bultman, 1987; White and Owens, 1992). *Myriogenospora* and *Claviceps* have a homothallic mating system (White and Glenn, 1994; Tudzynski, 1999). We do not know whether *Neoclaviceps* is also homothallic, although we predict that it is. Because the most basal species in the graminicolous Clavicipitaceae clade are heterothallic, it is a reasonable hypothesis, that the ancestral condition in the graminicolous Clavicipitaceae was heterothallism.

In species of *Epichloë* and *Balansia* subgenus *Eubalansia*, stroma development results in sterility of host grasses since stromata envelop grass inflorescence primordia. In *Myriogenospora*, stromata form on leaves, while in *Neoclaviceps* and *Claviceps* they are limited to individual florets. Restriction of infection to ovaries is thus suggested to be the derived condition.

12. Conclusions

There is good support for the hypothesis that the *Epichloë/Neotyphodium* endophyte-grass association is a defensive mutualism. The endophytes may defend hosts from abiotic stresses such as drought and mineral toxicity and biotic stresses such as insects,

mammal herbivores, and fungal diseases. The evolution of endophytes appears to have been influenced by environmental factors but hybridization may also play an important role in creating variation among endophytes. Ribosomal DNA diversity studies suggest that the center of diversity and perhaps the region of origin of *Epichloë/Neotyphodium* endophytes was in the Northern Hemisphere (Eurasia or North America). The graminicolous Clavicipitaceae appear to comprise a monophyletic grade through *Epichloë*, *Balansia*, *Myriogenospora*, *Neoclaviceps*, and *Claviceps*. Endophytism may be an ancestral feature of the grass-infecting members of the Clavicipitaceae, since epibiotic species in *Myriogenospora*, *Neoclaviceps*, and *Claviceps* appear more derived than *Epichloë* and *Balansia*.

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THE EFFECT OF ENDOPHYTIC FUNGI ON HOST PLANT MORPHOGENESIS

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1. Introduction

Morphogenesis (growth and differentiation) is to be considered the basis of functioning of green plants at all levels (cell, tissue, organ and organism) of the plant system. The building up of morphogenesis models as depending on molecular genetic patterns, cell to cell signaling, secondary metabolite production, tissue architecture, organ functional morphology is a subject in the mainstream of nowadays plant biology (Bonfante *et al.*, 2000 and literature therein). Moreover plants, the modular organisms *par excellence*, are easy to be exploited for agricultural and biotechnological purposes, by controlling their morphogenetic patterns *in vitro* and in the field. Therefore a deeper insight in morphogenesis is of importance for many biological applications. For example, plant micropropagation, somatic embryos mass production and the synthesis of specific metabolites in organs can be implemented by controlling morphogenetic patterns at cell, tissue and organ level (Luttmann *et al.*, 1994; Mori and Sakurai, 2000; Maffei and Scannerini, 2000). Usually plant morphogenesis is studied through the mathematical evaluation of organism and organ forms (Leverenz *et al.*, 2000), cyto-histological mapping (Dolan *et al.*, 1993; Barlow, 1996), biochemical-physiological techniques (Maffei *et al.*, 1999), and what is more, by molecular-genetic dissection of mutants (Coen, 1991; Di Laurentio *et al.*, 1996). Unfortunately, the bulk of evidences concerning morphogenesis are usually obtained and discussed by the assumption that a "healthy" plant is an axenic organism depending only on its own genome and abiotic environment (e.g. light, water and mineral resources). Possibly, this is one of the reason why *Arabidopsis thaliana*, a non-mycorrhizal species, has been choose as the *par excellence* reference organism in experimental plant biology.

Summing up, until today the role played by mutualistic symbioses in plant morphogenesis has been underestimated. When a general model is to be built up both in plants and animals, legume-*Rhizobium* is the only system presented as a significant symbiotic interaction in plant morphogenesis.

By contrast, mycorrhizal fungi, a heterogeneous group of soil fungi that colonize the roots of about 240.000 terrestrial plant species, are well known owing to the beneficial effects - better growth and higher ecological fitness - they exert on the host (Bonfante, 2001). Moreover this host-fungus interaction involves a sequence of morphogenetic events and modifications of plant and fungal host leading to a functioning mycorrhiza (Smith and Read, 1997). Mycorrhizae are classified in different groups (basically ecto- and endomycorrhizae) in keeping with a trend from extracellular to intracellular colonization by the fungus and to functional morphology of the host-fungus *interfaces* (Scannerini and Bonfante, 1982; Bonfante, 2001). Biodiversity of mycorrhizal fungi is very high and more and more fungi are revealed as mycorrhizal. For example the number of works dealing with novel species or isolate genetic diversity is increasing among root-associated fungi of the Ericales (Perotto *et al.*, 1996).

In addition, non-mycorrhizal fungi that colonize symptomlessly the living internal tissue of their host plant for all or nearly all their life cycle (endophytic fungi or endophytes, in keeping with Petrini, 1991) are surprisingly common both in grass and woody plants. Biodiversity of endophytic fungi and patterns of host-endophyte interaction show similar or higher degrees of complexity with respect to mycorrhizae. Trends in host-endophyte interactions, despite differences in organs involved (stem, leaves and flowers) parallel more or less those of mycorrhizae as a sequence from extracellular to intracellular. By contrast the effects of endophytes on their host plants are more differentiated. They can act as pathogens, defenders against predators, growth promoters, competitors of microbial pathogens (see references within the text). As a consequence the exact meaning of the term endophyte has been long debated, owing to the difficulty to clearly distinguish between an endophytic colonization and a latent infection (Sinclair and Cerkauskas, 1996; Stone *et al.*, 2000). We should therefore assume as endophytes a wide range of fungi, from fungal plant pathogens and saprophytes that have extended latency periods before disease or external signs of infection appear (Sinclair and Cerkauskas, 1996) to specialized fungi in grasses that are considered obligate mutualists (Glenn *et al.*, 1996). In this chapter we will focus only on mutualistic symbionts and saprophytes with long latency periods, avoiding any references to pathogens.

Until today little attention has been paid to host-endophyte patterns and their correlation to morphogenesis of the host. Nevertheless, endophytes are of importance in plant growth and development in keeping with their ability in secondary metabolite production, their ability to colonize all types of host tissues and organs, their trends in cell to cell interactions (see references within the text).

The aims of this chapter are 1. to stress the importance of plant-fungus symbioses in host morphogenesis at all levels of its architecture (cell, tissue, organ, and organism). 2. to suggest that host-endophyte interaction could be of major importance in plant morphogenesis both *in vitro* and in the field. To implement these suggestions host-endophyte patterns of interactions at cell, tissue and organ level will be discussed in comparison with those of mycorrhizae.

2. Trends in Endophyte–Host Relationships

2.1. GRASS ENDOPHYTES

Trend in colonization of endophytic niches starting from the epibiotic condition, is evident when clavicipitaceous grass symbionts are considered (Glenn *et al.*, 1996). They are systemic endophytes that can exert a wide range of biological effects on growth and reproduction of host grasses, pathogens and herbivores of grasses, and natural enemies of herbivores (West *et al.*, 1988; Carroll, 1988; Clay, 1988a; Clay, 1990a,b; Clay *et al.*, 1993; Breen, 1994). These fungi go from totally epiphytic species, like *Atkinsonella hypoxylon*, to fungi in which the progressive colonization of internal tissues of the host, accompanied by reduction of detrimental effects and the establishment of a mutualistic interaction is achieved, as documented for *N. coenophialum* (White and Morgan-Jones, 1996; Moy *et al.*, 2000). Colonization of grasses by *Atkinsonella hypoxylon* often result in plant sterility due to the formation of stromata on developing inflorescences (Clay, 1984). Host sterility is accompanied by vegetative growth enhancement, interpreted with the diversion of nutritional substances from the developing inflorescence to vegetative organs (White and Morgan-Jones, 1996).

Epichloë and *Neotyphodium* endophytes show a highly decreased tendency to form stromata on the plant host, relying instead to vertical seed transmission, through infection of host ovaries. This avoids fungal egression from host tissues and ensures their widespread distribution in pooid grasses (Clay, 1988b).

2.2. ENDOPHYTES OF WOODY PLANTS

In the case of woody plants, infections by endophytes are usually tissue or organ specific and highly localized within leaves, petioles (Petrini, 1991; 1996), tree branches (Kowalski and Kehr, 1996; Petrini and Fisher, 1990), aquatic roots (Fisher *et al.*, 1991), needles of conifers (Carroll *et al.*, 1977; Sieber-Canavesi *et al.*, 1991), bark or xylem (Stone *et al.*, 2000). Little is known about the existence of systemic infections among these endophytes.

Woody plant endophytes are closely related to pathogenic fungi and probably evolved from them through the extension of the latency period and the reduction of virulence (Petrini *et al.*, 1992). In this regard, a parallel may be drawn with the aforementioned grass endophytes, which are thought to have evolved from the choke grass pathogens of the genus *Epichloë* (Saikkonen *et al.*, 1998), through a progressive loss of their pathogenic behavior.

3. Patterns of Infection of Endophytic Fungi

The cytological pattern of colonization of grasses by clavicipitaceous endophytes, as typified by *Neotyphodium* endophytes of *Lolium* and *Festuca* spp. is characterized by an extensive colonization through a network of intercellular hyphae, which do not

reproduce on living or dead tissues (Hinton and Bacon, 1995). In contrast, the leaf endophyte of *Pseudotsuga menziesii*, *Rhabdocline parkeri* (Stone, 1987), and that of the perennial species of *Juncus* (Cabral *et al.*, 1993), show the intracellular colonization of single epidermal cells in healthy plant tissues.

3.1. HOST COLONIZATION IN GRASSES

Leaf Colonization. Differently from mycorrhizal fungi, *Neotyphodium* fungal endophytes grow entirely in the apoplast of the host tissues, without producing absorbing structure such as haustoria or arbuscules, so they obtain nutrients directly from the intercellular spaces. These species are variably distributed along the host leaf, with decreasing biomass concentration going from the base of leaf sheath to the upper part of the blade (Schmid *et al.*, 2000). By the mean of a GUS reporter gene linked to a promoter of the endophyte allowing visualization of the endophytic mycelium, Schmid and colleagues (2000) have recently observed that if the endophyte colonize a new apex at its early stage of development, it has the chance to enter in the apex when the cell leading to the formation of the blade are still dividing, while in later colonization it can enter only in the developing sheath (Schmid *et al.*, 2000).

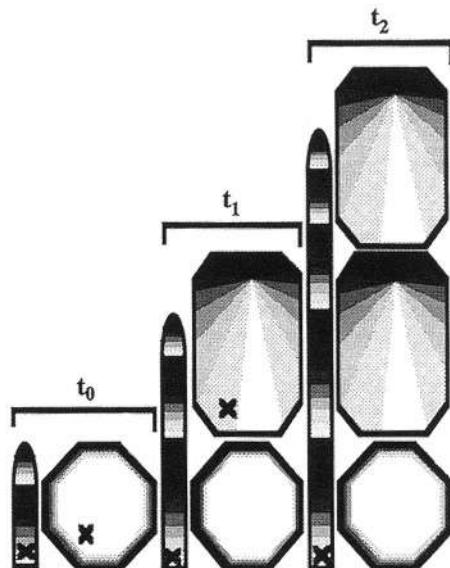


Figure 1. Apical extension of hyphae simultaneous with basal addition and elongation of new plant cells during leaf expansion requires the sliding of hyphae in intercellular spaces. Crosses marks positions in a hypha of a neighboring plant cell which are initially adjacent (t_0), but which slide apart over time (t_1-t_2) (Reprinted from Schmid *et al.*, 2000, in: *Microbial Endophytes*, by courtesy of Marcel Dekker Inc., N.Y.)

Another typical feature of diverse species of *Neotyphodium* and *Epichloë* examined by Schmid and colleagues (2000) is that endophytic unbranched hyphae run parallel within host tissues. The authors have hypothesized that in the host plant, tissue extension and endophyte hyphae extension are to occur simultaneously (Fig. 1), and the entire length of the hypha must elongate through the intercellular space where it is located. Hyphal branching in these conditions would be only an impediment to leaf progressive colonization by the endophytic mycelium (Schmid *et al.*, 2000) and as a consequence, branching is limited (Fig. 1). After leaf expansion is complete, hyphae do not expand any more. An explanation of this phenomenon could be envisaged in the presence of chemical signals whose concentrations could vary in relation to the activation or ceasing of host meristematic activity (Schmid *et al.*, 2000).

The control mediated by chemical signals could be important also in the case of reproductive stromata formation. In this region, a sustained meristematic activity of developing tissues together with the diversion of plant nutrients to sustain floret formation, allow extensive fungal growth (Kirby, 1961). Increased transfer of sugars has been reported in plants infected with *Epichloe* and *Balansia* endophytes, and in some cases this can be ascribed to an increased photosynthetic rate in infected leaves (Bacon and White, 2000) or to changes in translocation patterns (Thrower and Lewis, 1973; White and Camp, 1995).

The ability of fungal endophyte to sense chemical changes occurring while the plant cell differentiates or divides is a fascinating subject and deserves possible extension to other symbiotic systems.

3.2. ULTRASTRUCTURAL CHANGES IN HOST COLONIZATION BY *NEOTYPHOIDIUM* spp.

Hyphal Distribution within Host Tissues. By the means of light and electron microscopy, Hinton and Bacon (1995) have accurately described the distribution and ultrastructure of the endophyte of tall fescue in the meristematic tissues of the shoot apex, leaf sheath, lateral buds and rhizomes at different stages of tiller formation. Within the leaf sheath, hyphae are found in the intercellular spaces of the parenchyma and chlorenchyma of the leaf mesophyll. When in spring the grass produces inflorescences, the fungus invades the young floret before anthesis and could be detected in ovaries at their early development. In mature seed the hyphae were randomly or uniformly distributed between the endosperm and the epithelial cells of the scutellum. Hyphae did not penetrate any part of the embryo (Hinton and Bacon, 1995).

Cell to Cell Contact. At the point of contact between fungal cell and host cell walls, an amorphous electron dense material has been described (Hinton and Bacon, 1995). Hypha and host cell walls results in intimate contact in areas where the fungal cell wall is not surrounded by sheath material. This contact is not continuous since hyphae are coiled within the intercellular spaces of the host. Hinton and Bacon (1995) found also the presence of particular structures consisting in multivesicular bodies (lomasomes) and cell membrane whorls present both in hyphae and in the photosynthetic tissue of the host. These structures may indicate a secretory activity by the fungus.

Ultrastructural relationships of the endophytes and its host appear generally similar to those found in non-haustoriolate lichens and certain mycorrhizae (Hinton and Bacon, 1995).

Philipson (1991) has similarly described the endophytic colonization of ground parenchyma and leaf air lacunae in tall fescue by the *Phialophora-like* endophyte. Mucilage deposition and modifications at the fungal plasma membrane level occurs as in the case of the *Neotyphodium* association described by Hinton and Bacon (1995).

Alterations and Modifications of Host Tissues by Clavicipitaceae. Reproductive structures of *Epichloë*, the perfect form of *Neotyphodium*, consist in spermatia later followed by perithecia, which develop on the surface of stroma. The last are the resultant combination of fungal mycelium and host tissues, referred as “stromal shoots” or “stromal leaves”.

White and colleagues (1997) have studied the manner in which the Clavicipitaceae extract nutrients from hosts. As mycelium develops on the epidermis, walls of the epidermal cell become softer and thinner up to the appearance of fungal perforation within these walls (White *et al.*, 1997). Hyphae seem not to be allowed to come in direct contact with the host cytoplasm, through the persistence of a thin layer of host wall material, between fungal hypha and the plasma membrane and the deposition of a dark staining material penetrating the outer layers of the plant cell wall. This phenomenon has been interpreted as the result of the loosening of the wall by fungal activities, in a manner similar to that observed for many pathogenic strategies (Keen *et al.*, 1987).

Stromata formation in epibiotic endophytes like *Myriogenospora*, determine changes in the epidermal cell size and shape. This has suggested that growth regulatory substances are produced either by *Myriogenospora* and secreted into the host or the growth substance is produced by the host in response to the presence of the fungus (Bacon and White, 2000). Epidermis penetration by the epibiont is part of the fungus strategy to increase the nutritional exchange surface and to facilitate this exchange through the removal of cuticle and softening of tissues (White *et al.*, 1997).

The stroma is a structure that is maintained through the selective modification of host tissue to maximize flow of nutrients and water to fungal fruiting bodies. Nutrients are obtained in a manner similar to ectomycorrhizae without the production of haustoria and with the least damage to the delicate cell structure of the host (White *et al.*, 1997).

3.3. HOST COLONIZATION IN WOODY PLANTS

In contrast to *Epichloë* and the associated anamorphic endophytes of grasses, fungal endophytes of trees are horizontally transmitted via spore and are not known to grow in seeds of their host, even if the adhesion of reproductive propagules to seed coats may favor following colonization of cotyledons and leaves of the plantlet. For some endophytes, presumably those that show a high specificity of hosts, should exist very specific chemical recognition pathways, that identify the host as compatible and start spore adhesion and following colonization (Wilson, 2000).

Woody plant endophytes may overwinter in abscised leaves or stay silent in the bark, reinfecting leaves during the following spring. Besides this, the plant may have some control or have a role at least in regulating infection levels, since as stressed by Wilson

(2000) the host plant seems to have a saturation level of infection, which is not possible to increase via artificial inoculation.

In host trees, leaves still in the bud are typically endophyte-free and contrary to grass endophytes and mycorrhizal fungi, very little is known about the spatial aspects of colonization of plants by fungal endophytes.

Leaf Colonization. Within – leaf colonization patterns of trees are common, but not the same in all tree species. The most common distribution gradient of endophyte frequency in conifer needles as well in broadleaf trees, is along the axis of the midrib, where the petiole end of the leaf is more heavily colonized with respect to the distal end of the lamina.

Wilson and Carrol (1994) have proposed that differential spore deposition on the leaf surface and differential patterns of leaf expansion could explain many of the leaf ways of colonization. Many endophytes are rain dispersed, thus the affinity of the different parts of the leaf lamina to water, the phylloplane characteristics and the presence of leaf hairs (Allen *et al.*, 1991) may control endophyte spore deposition and germination. As noticed before for grass endophytes, infections occurring at different levels of leaf expansion may condition also the position of deposited spores, and changes of this original position occur as leaf expands.

Endophytes tend to occupy highly localized sites and do not grow away from the infection site during at least the latent phase (Wilson, 2000). Following penetration, the fungi may be restricted to a single epidermal cell as shown for *Rhabdocline parkeri* in Douglas fir needles, or be localized in intercellular subcuticular and epidermal areas as seen in *Discula umbrinella* from beech leaves (Wilson, 2000).

4. Mutualism in Endophytes

4.1. EFFECTS OF ENDOPHYTES ON GRASS HOSTS

Mutualism has been the prevailing concept under which the evolution and ecology of endophytes have been interpreted. Grass endophytes deter herbivores as plant mutualists acquired defenses. This defensive role results from the production of a diverse array of fungal alkaloids, that released within the host tissues may deter vertebrate and invertebrate herbivore as well as plant pathogens (Porter, 1994; Siegel and Bush, 1996). In turn, plant host provide endophytes with a specific ecological niche, nutrients and in the case of vertically transmitted fungi, an efficient way of dispersal and transmission to the next generation of hosts. Other works on this topic showed that the endophyte may provide the plant with other fitness enhancing properties. Endophyte-infected tall fescue grows and persists whereas noninfected isogenic material succumbed to stresses associated with the environment e.g., water deficit, insect attacks, nematode diseases, high temperatures, soil acidity (Bouton *et al.*, 1993). The competitive advantage consists in morphological responses such as larger and more numerous tillers (Hill *et al.*, 1991), greater leaf elongation (Belesky and Fedders, 1996), leaf area, leaf thickness, stem length (Belesky and Malinowski, 2000) and altered root architecture (Malinowsky *et al.*, 1999).

Endophyte-infected tall fescue and meadow fescue show greater drought stress tolerance than non infected plants (West, 1994; Hill *et al.*, 1996). Belesky has suggested that endophyte may exert a control on the hormonal regulation of stomatal apparatus in infected plants, affecting the cell level of abscisic acid in leaves of drought stressed tall fescue (Belenski and Malinowski, 2000). The accumulation of osmotic active substances, e. i. loline alkaloids possibly responsible of the regulation of cell turgor in endophyte infected plants could represent another explanation of the drought resistance acquisition (Beleski and Malinowski, 2000).

Under a morphogenetic point of view, it must be remembered that changes in root biomass and alterations of root architecture as those demonstrated in many grass-endophytes systems could also enhance plant access to water in soils, through the increase of total absorbing root area. Greater root mass and root density change also the soil structure and its chemical and physical properties, like field capacity, thus further affecting plant growth and resistance. As in the case of stromata formation by epibiotic species, *Neotyphodium* endophytes can similarly induce leaf area reduction in host plants, through leaf senescence or rolling of the lamina. These two mechanisms could contribute to reduction of the total leaf area and thus of the leaf surface involved in the transpiration water loss (West *et al.*, 1990). Similar variation in growth and morphogenetic response of infected plants were noticed in water logged soil conditions, where non-infected plants were less productive than endophyte-infected ones. As resumed by Belesky and Malinowski (2000), endophytic-infected tall fescue adjusts physiologically and morphologically to drought stress, and to some degree may also modify the soil structure and stability, as a result of modifications in plant root architecture.

It has also been stressed that infected plants use nitrogen more efficiently than non-infected plants, but little is known about possible explanation of this common finding. If herbaceous and woody species often benefit from association with mycorrhizae in acidic or marginal nutrient soil conditions (Smith and Gianninazzi-Pearson, 1988), one can argue if an endophyte confined to the above ground part of the plant host could have a similar influence on nutrient acquisition and plant growth of its host. Effectively, it has been found that endophyte hyphae (*N. coenophialum*) contained inorganic P structures similar to those found in cytoplasm of mycorrhizal fungi and that endophyte hyphae may act as a sink of phosphorous in media grown tall fescue plants. As in the case of nitrogen, phosphorous uptake and herbage production were greater in infected than non infected plants, when soil P availability was low. Infected plants grown at low P availability had greater specific root length and concentrations of P, Mg, and Ca in roots and shoots than did non-infected plants. Two endophyte-related mechanisms for P uptake appear to be operating in tall fescue grown in P-deficient soils: 1) altered root morphology and 2) increased activity of root exudates (Belesky and Malinowski, 2000). Belesky and Malinowsky (2000) found that infected tall fescue produced roots with smaller diameter and longer root hairs than non infected plants regardless of P level (Fig. 2). Using a simple colorimetric reaction they showed that more reducing activity, associated with phenolic-like compounds (occurring in greater amounts in roots and shoots of infected plants) was present in roots of endophyte infected than by non infected plants in P-deficient media. Phenolic-like compounds exuded from roots of

endophyte infected tall fescue might be involved in Al tolerance, due to their chelating ability (Belesky and Malinowski, 2000).

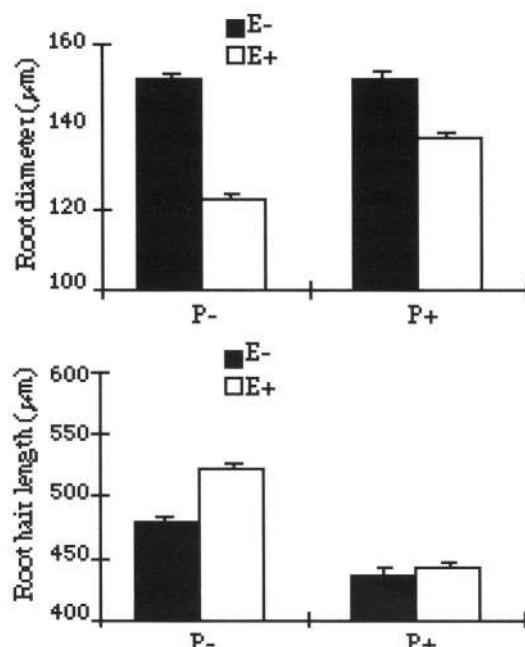


Figure 2. Average root diameter and root hair length of endophyte infected (E+) and non-infected (E-) tall fescue in response to P concentrations in nutrient solutions. Bars indicate standard errors (Reprinted from Belesky and Malinowsky 2000, in. *Microbial Endophytes*, by courtesy of Marcel Dekker Inc., N.Y.)

So we can resume that shoot-localized endophyte, specifically *Neotyphodium* species, can induce changes in root morphology and root function. Benefits to grass host arising from infection with *Neotyphodium* species appear similar to the well-known benefits resulting from some symbioses involving mycorrhizae. The observed changes in rhizosphere chemistry, root morphology and root function in endophyte-host relationships, show that infected plants adapt to biotic and abiotic stresses through different morphological, physiological and biochemical modifications (Belesky and Malinowski, 2000).

4.2. INDUCED EFFECTS OF ENDOPHYTES ON WOODY PLANTS AND PERENNIAL GRASSES

Endophytes may affect host plant morphology, metabolism and physiology, but ultimately what changes is plant ecology. Matta (1971) recognized the potential interactions between temporally and spatially separated fungi colonizers of plants via induced changes in the host plant.

Recently, the ecological role of fungal endophytes of tree leaves has received much interest and discussion, in particular for their role in preventing host colonization by other more aggressive parasites, through different kinds of competitive mechanisms or true natural pruning of branches (Kowalski and Kehr, 1996) and senescence of leaves (Wilson, 2000). Fisher and colleagues (Fisher *et al.*, 1986) were the first to raise the question whether the presence of endophytes within healthy plant tissues may elicit the onset of senescence and thus influence the lifecycle of its host plant. From that, many observations regarding endophyte-induced acceleration of leaf senescence and abscission have been reported (Wilson, 2000).

Fungal endophytes can cause induced changes in plant metabolism and morphogenesis and may therefore alter host subsequent interactions with other endophytes or pathogens (Wilson, 2000). Endophyte infection of leaves around feeding sites of grazing insects resulted in negative or deterrent effects on insect feeding, with consequent host protection (Edwards and Wratten, 1985; Preszler *et al.*, 1996).

It has been postulated that endophytes might act as aerial counterparts of mycorrhizal fungi. Even if endophytic fungi do not have the same morphological characteristics of mycorrhizae, they might have a role in nutrient acquisition from substances on the plant surface or dissolved in rainwater falling on leaves and stems. Relatively easy experiments employing tracking radiolabeled nutrient solutions on both endophyte-free and infected plants have been proposed (Wilson, 2000).

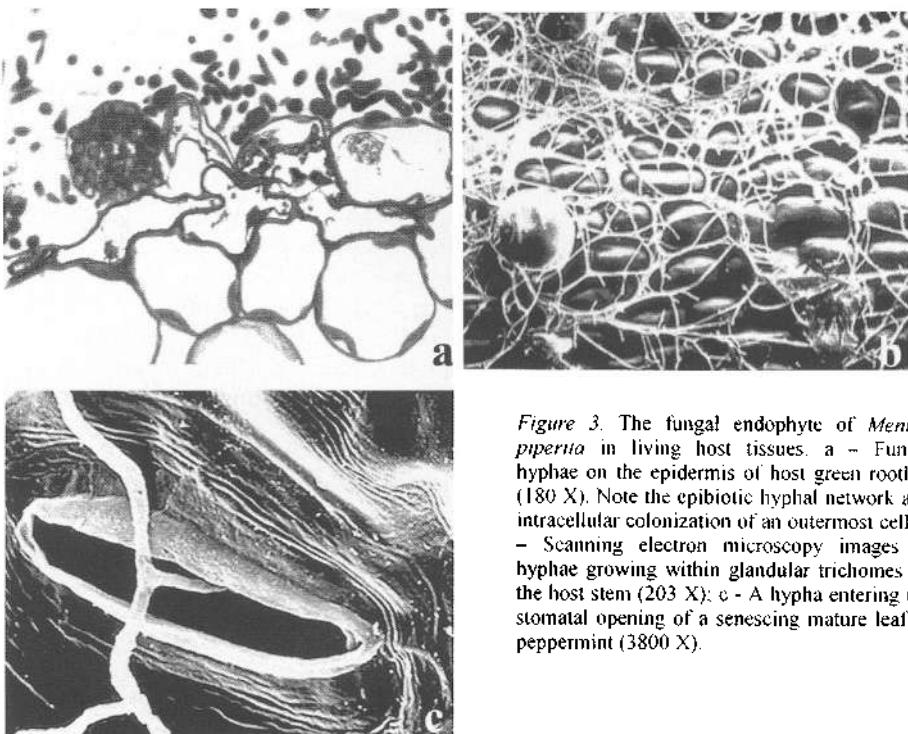


Figure 3. The fungal endophyte of *Mentha piperita* in living host tissues. a - Fungal hyphae on the epidermis of host green rootlets (180 X). Note the epibiotic hyphal network and intracellular colonization of an outermost cell. b - Scanning electron microscopy images of hyphae growing within glandular trichomes on the host stem (203 X); c - A hypha entering the stomatal opening of a senescent mature leaf of peppermint (3800 X).

Endophytes clearly have the potential to affect many different ecological, physiological and biochemical processes in plants, yet there are very few detailed studies that have investigated much more than insect antagonism (Wilson, 2000).

The same can be said for mutualistic endophytes inhabiting dicotyledonous herbaceous hosts. The great bulk of scientific reports concern taxa sampled for Xylariales inhabiting leaving trees (Petrini and Petrini 1985; Bills 1996; Stone *et al.* 2000), while it is growing the number of fungi isolated from herbaceous plants, as well as those from *Lupinus* spp. (O'Dell and Trappe, 1992), *Stylosanthes* spp. (Pereira *et al.*, 1993), *Dryas octopetala* (Fisher *et al.*, 1995), *Baccharis coridifolia* (Bertoni *et al.*, 1997). In some cases the analysis of the endophytic population of a given species led to the identification of new species and genus as in the case of two new ophiostomoid endophytes isolated by infructescences of *Protea* (Marais *et al.*, 1998). These taxa are believed to be evolved first as opportunistic pathogens, having acquired only later in their evolution a total asymptomatic behavior. Due to their almost recent discovery and to the lack of specific studies, relationships between fungal endophytes and perennial grasses are almost totally unknown. The inability to identify mycelia sterilia, which have been reported in various proportions in most of the studies of Dicot endophytes (Quo *et al.*, 2000), could represent a further limitation to the knowledge of their distribution and ecology in natural habitats.

Recently we have discovered an endophytic sterile isolate, inhabiting shoots and leaves of *Mentha piperita* (Mucciarelli *et al.*, 2001 *in press*). The peppermint isolate produces both epiphyllous and endophytic hyphae, localized in the shoot apex and in axillary buds. The individual hyphae of the epiphyllous mycelium were observed to run sparsely within peltate and capitate glandular hairs of leaf surfaces and stems (Fig. 3), with no apparent signs of suffering in both partners. When the symbiosis was re-established *in vitro*, by the means of tissue culture techniques, extension of the fungus to green rootlets (Fig. 3) and a conspicuous plant growth enhancement have been observed. Plant-fungus biochemical interactions, mentioned before for tree inhabiting endophytes, are highly probable in this situation, especially when considering the secondary metabolism of peppermint host. Terpenoids due to their volatilization from the plant, go to enrich the plant headspace, thus reaching the epiphytic mycelium at the interface of leaf surfaces. If we consider the role of terpenoids as plant defense compounds because of their effects on fungal growth and reproduction (Inouye *et al.*, 1998), the isolation of a fungus inhabiting peppermint leaves poses the question whether these secondary metabolites can contribute to the observed symptomless and beneficial plant-fungus interaction. Relationships of leaf endophytes with host volatile terpenoids were studied in *Sequoia sempervirens* (Espinosa-Garcia and Langenheim, 1990). *Sequoia* terpenoids variability and their differential activity on fungal species have been shown to be important in redwood endophytes interactions (Espinosa-Garcia and Langenheim, 1990).

Owing to the colonization of a unique plant niche, the presence of a mutualistic endophyte in the genus *Mentha* represents a novel finding among beneficial plant-fungus symbiosis in non-graminaceous plants.

5. Trends in Mycorrhizae

5.1. SYMBIONTS IN ECTOMYCORRHIZAE

A relatively small number of phanerogams (Pinaceae, Fagaceae, Myrtaceae) which, however, occupy a large part of the terrestrial land surface and a large number of fungal species, mostly belonging to Basidiomycetes and Ascomycetes and a few Zygomycetes (*Endogone* sp. pl.) are responsible for ectomycorrhizae (Smith and Read, 1997). Biodiversity of these plant-fungus associations is noteworthy: different species of fungi with broad host range (Trappe, 1962) are able to colonize roots of the same host. Moreover it is well known for long time that different fungal symbionts are present at different time on the same host root (Marks and Foster, 1967; Fontana *et al.*, 1982). Finally, what is more, compatibility between symbionts as well as mycorrhiza efficiency (growth effect) are also depending on different strains of fungal species (see e.g. Debaud *et al.*, 1995, Guidot *et al.*, 1999).

5.2. SYMBIONTS IN ENDOMYCORRHIZAE

Fungi of Endomycorrhizae (Smith and Read, 1997 and literature therein) are different in keeping with their cell to cell interactions, interfacies and host plants. Namely: an enormous variety of host plants (angiosperms, gymnosperms and pteridophytes) are colonized by aseptate obligatory symbiotic fungi in the order of Glomales (Zygomycotina) to building up vesicular arbuscular mycorrhizae (VAM). Ericales mycorrhizae are produced by some ectomycorrhizal Basidiomycotina (arbutoid and monotropoid) or by a few Ascomycotina and Basidiomycotina (Ericoid). Finally the majority of fungi responsible for the intriguing mycorrhizal or not mycorrhizal associations with Orchids are referred to Rhizoctonia (Marchisio *et al.*, 1985). Summing up - in natural environment - plants are always mycorrhizal, stressing the importance of symbiosis for their life.

6. Pattern of infection of mycorrhizal fungi

The development of the symbiosis involves a sequence of events during which morphogenetic modifications in the plant and the fungus occur (Smith and Read, 1997). Long time ago cell to cell interactions of different mycorrhizal types were considered as a sequence running from extracellular to intracellular: ectomycorrhizae, Ericales, endomycorrhizae, arbuscular mycorrhizae (Bonfante-Fasolo and Scannerini, 1983).

Emphasis on Ericales mycorrhizae has turned to taxonomy and molecular genetics of their fungal isolates (Perotto *et al.*, 2000), and therefore host plant morphogenetic responses, if any are present, have been neglected. Orchid mycorrhizae are different from the other mycorrhizal associations: adult mycorrhizae are a matter of debate in their functioning, orchid protocorms are strictly dependent on fungal colonization but

their developmental patterns are not so clear (Smith and Read, 1997 and literature therein).

A deeper insight of the host side of the table in these symbiotic interactions is needed to allow their use for comparative analysis with green tissue endophyte-plant associations.

For these reasons, in this chapter we will discuss patterns and functioning of VA mycorrhizae and ectomycorrhizae only.

6.1 ECTOMYCORRHIZAE

Ectomycorrhizal fungi generally form a mantle sheath around the root and an intercellular Hartig net. The mantle is pseudoparenchymatous, its formation begins in a susceptible zone basipetal to the meristem and or in the root cap region (Wilcox, 1996) and proceeds with hyphal growth inwards, into the remains of root cap cells which are constantly formed. The elongation of the short root causes a progressive shift backward along the root (Fusconi, 1983). The Hartig net (Fig. 4a) forms between young epidermal cells (Wilcox, 1996); it is an intercellular network of highly branched and packed hyphae confined to the epidermis in most angiosperm species, but penetrating the cortex in most conifers (Smith and Read, 1997). It shows a labyrinthic branching pattern, which increase the apoplastic and symplastic exchanges between the symbionts. However fungal and host cell walls show only little changes in their morphology during the colonisation process, even if changes in wall composition may occur. For example specific proteins, probably involved in cell adhesion have been found in hyphae attached to the root surface or the root hairs of *Pisolithus tinctorius* (Laurent *et al.*, 1999). In the cap zone hyphae of some ectomycorrhizal fungi take a tight contact with the host cells and may growth beneath the outer layer of the cell wall, but, deeper in the root, they cause only little change in thickness and texture of the plant cell walls, without any substantial change in their composition (Bonfante, 2001).

Some ectomycorrhizae can lack the mantle (E-strain fungi), others the Hartig net; the latter were termed “peritrophic” or “superficial” (see Wilcox, 1996). Between these, the ectomycorrhizae formed by *Pisonia grandis*, which are surrounded by a basidiomycetous fungal sheath, are characterized by wall ingrowths on the outer tangential and radial walls. Wall ingrowths of *P. grandis* resemble those of the transfer cells at sites where high rates of nutrient transfer between the apoplast and symplast occur and their development is considered an alternative to Hartig net formation (Ashford and Allaway, 1982; Ashford, 1985). The same basidiomycete produces ectomycorrhizae when inoculated directly on short lateral roots of *Picea sitchensis*, with more intercellular penetration and no wall ingrowths. It is thus possible that the structure of the interface between the mycobiont and higher plant depends on the plant species involved, in parallel with the situation where fungi form typical ectomycorrhizae with conifers and arbutoid mycorrhizal with ericaceous species (Cairney *et al.*, 1994).

6.2. V.A. MYCORRHIZAE

In most associations the fungus penetrate the epidermal cells and spreads in the root cortex via intercellular hyphae. It produces short side branches, which penetrate the

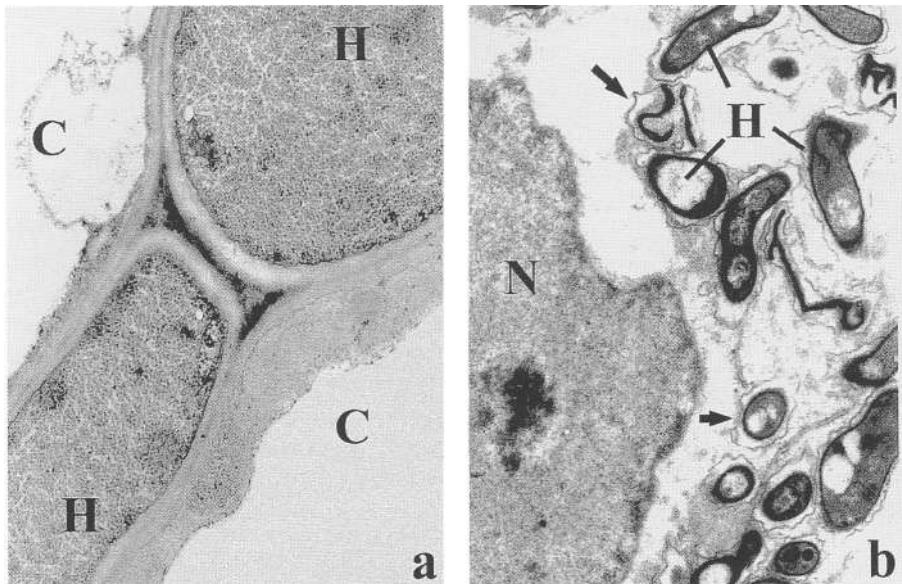


Figure 4. Transmission electron microographies of (a) ecto- and (b) arbuscular mycorrhizas. a- Hartig net hyphae (H) between two cortical cells (C) (21900 X); b- Arbuscular hosting cortical cell with hyphal branches (H). N, host nucleus, arrows, invaginated host membrane (9900 X)

cortical cells, and branch dichotomously giving rise to the arbuscules, the fungal structure mostly involved in plant-fungus exchanges (Fig. 4b). It never penetrates the apical meristems and the central cylinder of the roots. Along with the progress of root colonisation, hyphal walls grow thinner and the fibrils of chitin became shorter; the arbuscule branches display the thinnest and most simplified wall (Bonfante, 2001).

Arbuscule formation causes a profound reorganization of the host cells. By invagination of the host membrane around the fungus, wall components are deposited between fungal wall and host membrane giving rise to a new compartment, the interface. It is a complex mixture of molecules including xyloglucans, arabinogalactans, β -1,4 glucans and proteins rich in hydroxyproline (Balestrini *et al.*, 1997; Bonfante, 2001). The plasma membrane activities are modified in respect to that of uncolonized cells, the central vacuole fragments, the configuration of microfilaments and microtubules change (Genre and Bonfante, 1997) and the nucleus move from the periphery of the cell to a central position, often inside the arbuscule branches (Balestrini *et al.*, 1992). The nucleus shows significant modifications related to the changes in gene expression during the establishment of AM colonisation (Berta and Fusconi, 1998). They consist in

hypertrophy in various AM systems (Berta and Fusconi, 1998) caused by chromatin decondensation, and, in *Lycopersicon esculentum* + *Glomus mosseae*, an increase in the percentage of polyploid nuclei in the arbuscule hosting cells (Berta *et al.*, 2000).

7. Mutualism in Mycorrhizae

Mycorrhizal fungi enhance the acquisition of nutrients, particularly phosphorus and nitrogen, by the host plant increasing its biomass. Moreover they can exclude toxic ions, to control the spread of pathogens and to influence the photosynthetic and water relation of the plant (Read, 1999).

7.1. EFFECTS OF ECTOMYCORRHIZAL FUNGI ON THE ROOT SYSTEM

Most ectomycorrhizal hosts have two root classes: persisting long roots, mainly involved in exploring the soil, and ephemeral, feeder branches, with small diameter and length. Ectomycorrhizal fungi generally colonize both types of roots, however only the latter show profound alterations of their shape following infection, and give rise to a characteristic swollen appearance to the root system. Root epidermal cells show a reorientation of growth following the initiation of the Hartig net and expand more in a transverse than longitudinal plane than uninfected roots. The result is an increase in the root diameter and the formation of a rounded instead of a pointed apex, also due to a reduction of the longitudinal extent of the meristem caused by the fungal sheath surrounding it (Clowes, 1951; 1981). Moreover ectomycorrhizal fungi induce dichotomy of the apical meristem of short branches in the genus *Pinus* (Piché *et al.*, 1982) and the repeated production of lateral roots in many species (Smith and Read, 1997).

7.2. EFFECTS OF VESICULAR ARBUSCULAR MYCORRHIZAS ON THE ROOT SYSTEM

In contrast to ectomycorrhizae, AM associations do not cause evident changes in the root shape, however they influence the pattern of root morphogenesis. AM lower the root:shoot ratio, however the total root length (Schellenbaum *et al.*, 1991; Trotta *et al.*, 1991; Tisserant *et al.*, 1992; Berta *et al.*, 1995; Dixon *et al.*, 1999; Torrisi *et al.* 1999), and sometimes also the mean diameter of the roots (Daniels-Hetrick *et al.*, 1988; Fusconi *et al.*, 1994; Berta *et al.*, 1995) increases in respect to controls in most of mono- and dicotyledonous plants. Root morphology is also modified by colonization: the effect of AM fungi on root length, even if frequent, is extremely variable in different associations whilst a general effect seems to be an increase in lateral root formation which generally gives rise to a more branched root system. However the order of the roots involved varies: in the monocot *Allium porrum* colonized by *Glomus* sp. strain E3, the number of lateral roots per unit length of adventitious root is significantly higher in AM plant (Berta *et al.*, 1990). On the contrary the degree of branching of the axes of the woody dicotyledonous plants *Prunus cerasifera* (Berta *et al.*, 1995) and *Platanus acerifolia* (Tisserant *et al.*, 1992; 1996) is almost unaltered. The greatest morphological effect of AM colonization is the intensity of branching of first order lateral roots in the former and of the development of laterals of higher order in the latter. The dynamics of

the root system development in mycorrhizae is thus characterised by modifications in the hierarchical appearance of different root orders, which cause a different contribution of the different root orders to the whole root apparatus. For example in *P. cerasifera* the first order lateral roots make up a relatively constant proportion of the root system in control plants, while in AM plants the proportion decreases markedly with time whilst the proportion of second lateral roots increases (Berta *et al.*, 1995)

Variations in root system morphology are mainly related to a different behavior of their apices and, even if AM fungi never penetrate the root apical meristems, it has been demonstrated that AM fungi can alter their structure and activity. Data on this matter however are very scarce and the only results about a mycorrhizal association grown in controlled condition were obtained on *A. porrum* plants infected by *G. sp. E3* (Berta *et al.*, 1990, 1991). Adventitious root of *A. porrum* have a determined pattern of growth, in mycorrhizal plants the percentage of active apices decrease more rapidly than in controls, and in highly infected plants most of the root apices were inactive or necrotic (Fusconi *et al.*, 1986; Berta *et al.*, 1990). The apices of adventitious roots of mycorrhizal plants do not change in structure with respect to those of uninfected plants but increase drastically in size, had larger proximal meristems with more cell-files in the cortex and central cylinder, and larger caps. This increase is due partly to a higher number of meristematic cells, and partly to an increase in cell size (Fusconi *et al.*, 1994). In spite of the larger size, AM apices had a lower metabolic activity than controls (Fusconi *et al.*, 1994) and the mitotic cycles of AM apical meristems become longer with increasing infection (Berta *et al.*, 1991). Lastly, AM fungi block meristem activity in mycorrhizal plants and this is the direct cause of the alterations of the root system of *A. porrum* reported above.

The precocious inactivation and loss of the meristem of the AM root apices of *A. porrum* does not lead to the senescence of the differentiated, colonised root tissues. On the contrary, in these tissues senescence is delayed. In fact in *A. porrum* it has been demonstrated that arbuscular mycorrhization delays chromatolytic and pycnotic degeneration of cortical nuclei in respect to controls (Lingua *et al.*, 1999).

Very little information, however, exists about the influence of AM on root longevity in other A.M. associations. In the root system of poplar, where a high proportion of the root system is made up of roots higher than second order (Hooker *et al.*, 1992), a strong decrease in root longevity was observed for colonised roots (Hooker *et al.*, 1995). Fungal effect on root longevity could be related to the root function, as *A. porrum* adventitious roots are primarily engaged in exploration, whilst higher order roots of poplar are primarily engaged in absorption.

Mycorrhizal roots are more efficient in P uptake than those of uncolonized control plants, as it is demonstrated by the higher concentration of P of their tissues (Smith and Read, 1997). Add of P usually stimulates the development of the root system and of branching (Amije *et al.*, 1989; Bruce *et al.*, 1994) and hence the modification of the root growth in mycorrhizal plants could depend, at least in part, from the enhanced growth of the entire plant.

In *A. porrum + G. E3* the differences between non-mycorrhizal and mycorrhizal plants in total and adventitious root length and branching decreased with applied P concentration, and disappeared or were reversed at high P levels (Trotta *et al.*, 1991). These differences are related to the activity of their root apices: P causes a lengthening

of the mitotic cycle comparable to that induced by mycorrhization at low P level. However the slowing down of the mitotic cycle does not cause a block of the mitotic activity, as shown by the low percentage of active apices, and resulted in a slow and steady growth of the adventitious roots (Fusconi *et al.*, 2000). First and second order laterals, however, do not respond to P nutrition: they are shorter in mycorrhizal plants at each P level tested (Berta *et al.*, 1993). These results suggest that the morphological effects of AM fungi on the root system are not completely P mediated and phytohormones are probably involved in the morphogenesis of AM. Altered levels of auxin, cytokinin and ABA have been reported in mycorrhizal plants, even though data about variations of the growth regulators balance in the roots are very fragmentary (Dannenberg *et al.*, 1992; Esch *et al.*, 1994; Beyrle, 1995; Barker *et al.*, 1998; Kaldorf and Ludwig-Müller, 2000; Torelli *et al.*, 2000). Moreover a P independent hyperpolarization of the root cortical cells has been demonstrated in AM of *A. porrum* + *G. E3* (Fieschi *et al.*, 1992).

8. Conclusions and Future Directions

Both Endophytic and Mycorrhizal Fungi Improve Plant Fitness, by Contrast a True Morphogenetic Effect has been Demonstrated only in Mycorrhizae

Mycorrhizal associations are generally true mutualistic symbiosis, resulting from plant and fungal host coevolution. On the contrary, mutualism can't be considered a feature common to all specialized endophytes, and obligate mutualists are restricted to *Neotyphodium* grass endophytes. Systemic colonization of herbaceous perennial plants by endophytic fungi has been little explored. Some important mechanisms of chemical host recognition must be present anyway and presumably be of great general importance.

In some mycorrhizal associations (e. i. in orchids) as for many other leaf and wood endophytes, it is not always easy to distinguish when plant growth enhancement is a true morphogenetic response to endophyte colonization rather than a most generalized improvement of host fitness by acquired tolerance to biotic and environmental stresses. This is especially true when the mutualistic fungus induces the acquisition of chemical defenses towards host pathogens and herbivores, as in the case of endophytes of green tissues.

Mycorrhizal fungi improve mineral nutrition of the host plant and stimulate its growth. Moreover they steadily influence root system morphology by affecting size and mitotic activity of their apices. When considering fungal endophytes, the array of biological effects encountered by analyzing growth and reproduction of the host plant, is so wide to limit the comprehension of cell mechanisms responsible of morphogenesis.

Through the means of *in vitro* cell and tissue culture techniques, molecular biology is probably the best scientific approach to solve many of the problems concerning leaf and wood fungal associations and to highlight the true nature of their symbioses.

The endophyte we have isolated from *M. piperita* is a promising model system for this purpose. It may be placed in the group of mutualists as demonstrated by recent experiments of inoculation on the roots of *in vitro* growing plants, where some modifications of root patterns running from growth improvement to plant necrosis were observed. Specific incompatibility was frequently observed in the peppermint isolate

i.e., when inoculated in sterile conditions on non-host plants, thus stimulating new research in the field of host specificity in leaf endophytes.

Green Tissues Endophyte-Plant Associations are best Candidates for Experimental Studies on Host- Mutualistic and/or Pathogenic Balance

Endophytic fungi represent an interesting experimental model for studies concerning biochemical and genetic mechanisms regulating the equilibrium between pathogenesis and mutualism in plant-fungus interactions. They may be cultivated *in vitro* and applied to plant hosts under controlled experimental conditions in order to analyze their potential on plant morphogenesis and secondary metabolism.

Green Tissues Endophytes Deserve a Deeper Insight in their Effect on Plant Morphogenesis

Despite the growing body of literature describing the various aspects of endophytic colonization, many questions remain to be answered on biodiversity, ecology, cell-to-cell interactions of these important symbionts and, what is more, are to be checked as responsible of evolutionary innovation in plants.

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VII. Association with Protozoa

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ANAEROBIC CILIATES AND THEIR METHANOGENIC ENDOSYMBIONTS

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1. Introduction

Ciliates constitute a very species-rich taxon of unicellular organisms. They are characterized by a conspicuous nuclear dimorphism and a complex infraciliature. The size range of the various ciliates extends over three orders of magnitude: it spans the whole interval between a few micrometers and several millimeters. Ciliates occupy the most divergent ecological niches, from freshwater sediments, over Arctic and Antarctic ice clefts and saline sands to the gastro-intestinal tracts of vertebrates and invertebrates. They can thrive as symbionts, commensals and parasites in pelagic, benthic, sapropelic or intestinal ecosystems. Their morphological complexity, their sensory capacities and their behavioral repertoire are mind-boggling – it is hard to believe that ciliates are “just” unicellular organisms.

2. Symbiotic Associations of Ciliates

Ciliates exhibit a fascinating spectrum of symbiotic associations with prokaryotes but also with unicellular eukaryotes. Certain ciliates seem to be able to collect chloroplasts and to keep them in a functional state as *kleptochloroplasts* over extended periods of time (Stoecker et al. 1987; Schüssler and Schnepf 1992; Gustafson et al. 2000). Symbiotic associations with green, unicellular algae transform *Paramecium bursaria* and a variety of other ciliates into photosynthetic, autotrophic organisms that can thrive in anoxic niches thanks to their endogenous oxygen production (Finlay et al. 1996; Nishihara et al. 1998). The association between various *Paramecium* species and eubacterial “symbionts” belonging to the genera *Holospora* and *Caedibacter* might be

interpreted as a chronic infection by obligate intracellular parasites. The only benefit to their hosts seems to be the acquisition of a killer-phenotype that is provided by the bacterial “symbiont” (Kusch et al. 2000). Certain associations between ciliates and eubacteria resulted in a vital dependence from the endosymbiont; e.g. the β -proteobacterium *Polynucleobacter necessarius* appeared to be essential for the survival of its host, *Euplotes aediculatus* (Springer et al. 1996). Other ciliates, such as *Strombidium purpureum* host endosymbiotic purple-non-sulfur photosynthetic bacteria (Fenchel and Bernard 1993), whereas the surface of the mouthless ciliate *Kentrophoros fasciolata* is covered by a dense layer of sulfide-oxidizing bacteria (Fenchel and Finlay 1989). An extremely baffling symbiotic association evolved in ciliates belonging to the genus *Euplotidium* where *Verrucomicrobia*-like extrusive ectosymbionts defend their host against predators by undergoing suicide (Petroni et al. 2000). Lastly, many anaerobic ciliates live in a symbiotic association with methanogenic Archaea (Vogels et al. 1980; van Bruggen et al. 1983): these consortia produce methane, one of the most important gases involved in global warming.

This short *tour de horizon* regarding the symbiotic associations of ciliates pinpoints all the inherent problems that rise when one attempts a description of symbiotic associations. How can we define *symbiosis*? How do we know whether a given association is really *symbiotic* – or (using another frequently used definition) *mutualistic* (De Bary 1879). When might such an association be regarded as *parasitic* - when as *commensalistic*? How frequently are we just studying an accidental association - a rare infection, for example, that becomes manifest when we try to culture the host? How can we assess benefit or harm in the case of ciliates? Even the acquisition of photosynthetic algae which confers photoautotrophy to the host, is advantageous for the host only as long as the sun shines: in the dark, these symbionts eventually become parasites that thrive heterotrophically on the host’s resources (Douglas 1994). Consequently, even in this unequivocal case of classical symbiosis, the mutual benefits largely depend on ecophysiological conditions under which this particular association operates. Even when applying the most neutral definition for symbiosis – assuming a long-lasting association between two organisms – how can we prove whether such an association is specific? Are we able to assess as to whether a host–symbiont association is the result of long lasting co-evolution? Do we really understand the functional or the metabolic basis of these associations? Notably, it requires substantial efforts just to prove that a certain association is regular, or at least long-lasting. To be honest, we have to admit that we are very ignorant even about well-known associations. Since the associations between anaerobic ciliates and methanogenic Archaea provide unique possibilities for studying many facets of symbiotic associations, we will review the symbiosis between anaerobic ciliates and methanogenic Archaea in more detail.

3. Anaerobic Ciliates and Their Energy-Generating Organelles

In at least 8 of the 22 orders of ciliates as classified by Corliss (1979), anaerobic or microaerophilic species evolved that can live permanently in the (nearly) complete absence of oxygen. Three more orders, i.e. the *Karylectides*, *Hypotrichs* and

Prostomatids, encompass a number of facultative anaerobes. All ciliates – also the strictly anaerobic ones - possess energy-generating organelles - either mitochondria or hydrogenosomes. Hydrogenosomes are membrane-bounded organelles approximately 1 μm in size that produce hydrogen and ATP. They were first discovered in parabasalian *Trichomonads* and subsequently also in a number of only distantly related unicellular anaerobes such as amoeboflagellates and chytridiomycete fungi (Müller 1993; Fenchel and Finlay 1995; Embley et al. 1997; Roger 1999; Rotte et al. 2000). The “anaerobic mitochondria” of ciliates remained rather elusive until now. There is evidence that one or the other anaerobic ciliate might be capable of nitrate-respiration (Finlay et al. 1983; Finlay 1985). However, the information about ciliate mitochondria that can use nitrate as a terminal electron acceptor is circumstantial, and we have to admit that we are completely ignorant as to whether the mitochondria of other anaerobic ciliates are capable of a kind of fumarate respiration like, for example, the anaerobic mitochondria of trypanosomatids, lugworms, bivalves or trematodes (Tielens and van Hellemond 1998).

Hydrogenosomes evolved in seven out of the 22 taxa of ciliates, (Fenchel and Finlay 1995) – however, both the phylogenetic and the functional relationships between these hydrogenosomes and the mitochondria of their aerobic sibs remained largely unclear until now. There is evidence that the hydrogenosomes of the various anaerobes are not the same (Coombs and Hackstein 1995; Embley et al. 1997; Hackstein et al. 1999) and there is evidence that even ciliate hydrogenosomes are different. The few studies that have been performed with anaerobic ciliates revealed obvious differences in metabolism, and most of the anaerobic ciliates have never been studied in more detail (c.f. Yarlett et al. 1985; Lloyd et al. 1989; Goosen et al. 1990a). The vast majority of ciliates with hydrogenosomes were only identified by their association with endosymbiotic methanogens (see below). A small number of species have been studied by electron microscopy: their hydrogenosomes clearly resembled mitochondria (van Bruggen et al. 1983, 1984, 1986; Goosen et al. 1988, 1990b; Zwart et al. 1988; Finlay and Fenchel 1989; Gijzen et al. 1991; Akhmanova et al. 1998). In a few species of ciliates, hydrogen/methane production has been measured using gas chromatography (Fenchel and Finlay 1992; van Hoek et al. 2000b). In *Plagiopyla nasuta* and *Trimyema compressum* hydrogenase activity has been demonstrated inside the hydrogenosomes with the aid of a cytochemical reaction (Zwart et al. 1988). Notably, with respect to anaerobic ciliates, we are dealing with the unique phenomenon that the presence of a cellular organelle, the hydrogenosome, is deduced from the presence of characteristic symbionts, i.e. endosymbiotic methanogens.

4. Methanogenic Archaea

Methane formation requires a set of unique enzymes and cofactors (Ferry 1993,1999). One of the cofactors, F_{420} , exhibits a characteristic autofluorescence. Because it is present in large amounts in most of the methanogens, the cells of methanogenic Archaea exhibit a strong blue autofluorescence when excited with UV-light of a wavelength of 420 nm. Therefore, it is possible to detect even a single methanogen among thousands of

eubacteria using epifluorescence microscopy (Doddema and Vogels 1978). Because a single methanogen can produce approximately 1×10^{15} mol methane/hour (Fenchel and Finlay 1992) methane formation by samples containing more than 10^6 methanogens can easily be studied with the aid of a gas chromatograph. Using photoacoustic laser techniques methane production can be monitored even below ppb level (Bijnen et al. 1996).

These unique characteristics allowed detecting methanogenic Archaea in virtually all anaerobic ecological niches on earth, including the intestinal tracts of vertebrate and invertebrate animals (Ferry 1993; Whitman et al. 1998). For example, a systematic screening of arthropods and mammals for the presence of intestinal methanogens revealed that (i) the taxonomy of their hosts plays an important role, and (ii) that the presence of methanogens seems to be a prerequisite for the presence of anaerobic protozoa (Hackstein and Stumm 1994; Hackstein and van Alen 1996; Hackstein 1997; Brune and Friedrich 2000). The putative specificity of these associations, and the enormous number of potential hosts suggests that the intestinal tracts of the various animals represent a kind of "Galapagos islands" that provide niches for the evolution of an unfathomed biodiversity of intestinal methanogens and anaerobic protozoa.

5. Endosymbiotic Methanogens

The fascinating capability of methanogens to thrive in various anaerobic niches led to the evolution of ecto- and endosymbioses with a variety of anaerobic protozoa (Vogels et al. 1980; van Bruggen et al. 1983, 1984, 1986; Goosen et al. 1988; 1990b; Broers et al. 1990; Gijzen et al. 1991, Fenchel and Finlay 1995). Electron microscopy and cytochemistry confirmed that these protozoa possessed hydrogenosomes (Zwart et al. 1988; Fenchel and Finlay 1995). Hundreds to thousands of methanogens were found in close association with the hydrogen-producing organelles, sometimes even penetrating into complexes of hydrogenosomes (van Bruggen et al. 1986, 1988; Goosen et al. 1988, 1990b; Broers et al. 1990; Gijzen et al. 1991; Fenchel and Finlay 1995). The benefit for the methanogens seems obvious: they are at the closest possible distance to organelles that supply them with their favorite substrates, H₂ and CO₂, and they are protected from predation. The benefit for the ciliate is less obvious. However, the formation of hydrogen is a reversible reaction, and the removal of hydrogen by the formation of methane, will "pull" the reaction to hydrogen formation (Adams 1990). The presence of endosymbiotic methanogens might therefore improve the function of the hydrogenosomes and, consequently, the energy balance of the protist host (Fenchel and Finlay 1991, 1995).

The potentially mutualistic character and the obvious stability of these associations fostered speculations about the specificity of associations between ciliates and methanogens. However, it appeared to be rather difficult to prove the specificity of these associations. Before the advent of molecular techniques that allow an identification of symbionts without culturing, the assessment of their specificity had to rely on morphological traits and the eventual culturing of endosymbionts. The group of Vogels and Stumm succeeded in cultivating a number of putative methanogenic endosymbionts from the anaerobic ciliates *Metopus striatus*, *Metopus contortus*, and *Plagiopyla nasuta*,

from the amoeboflagellate *Psalteriomonas vulgaris* and the giant amoeba *Pelomyxa palustris* (see Fenchel and Finlay 1995 for references and discussion). The conclusion from these studies was that the endosymbionts were similar if not identical to well-known free-living methanogens. Only the putative endosymbiont from *Metopus contortus* seemed to represent a new type of methanogen, i.e. *Methanoplanus endosymbiosis*. However, before a confirmation by DNA sequencing and *in situ* hybridization became feasible, it remained unclear whether isolates were derived from the putative endosymbiont population or merely contaminants (c.f. Fenchel and Finlay 1995).

6. Culture-Independent Approaches for the Identification of Methanogenic Endosymbionts and their Hosts

The polymerase chain reaction (PCR) and the fluorescent *in situ* hybridization (FISH) allowed the identification of organisms in their natural environments without the need for culturing (Amann et al. 1995, Spring et al. 2000). In addition, the growing rDNA database enabled the construction of the first meaningful phylogenetic trees of ciliates (Schlegel 1991). It was not until 1992 that Embley and his co-workers identified the first methanogenic endosymbionts by rDNA sequencing and *in situ* hybridization (Embley et al. 1992a,b; Dyal et al. 1995). The identification of additional endosymbionts and their hosts revealed an astonishing puzzle (Embley and Finlay 1994; Fenchel and Finlay 1995). Firstly, a particular host species hosted a single species of methanogens – notwithstanding the presence of morphologically different endosymbionts. Therefore, the different morphology of endosymbionts in certain ciliates was not due to the presence of different methanogens. Rather, different morphs of one and the same methanogen seemed to be indicative for morphological adaptations to intracellular life (Embley et al. 1992b). Secondly, phylogenetic analysis of the symbionts' rDNA genes revealed that endosymbionts were very similar, but definitively not identical to well-known, free-living methanogens. Surprisingly, closely related ciliates hosted rather different methanogens and the phylogenetic trees of hosts and endosymbionts did not match. Apparently, the endosymbionts must have been acquired independently in the different taxa (Embley and Finlay 1994, Fenchel and Finlay 1995).

This puzzle could be explained by the assumption that hydrogenosomes evolved repeatedly in the various orders of ciliates (Embley and Finlay 1994; Embley et al. 1995; Hirt et al. 1998). Just by chance, different endosymbionts might have been acquired that co-evolved with their particular hosts after the evolution of a hydrogenosome in a given lineage in the course of its adaptation to anaerobic environments. However, the demonstration of co-evolution between ciliates and their endosymbionts was seriously hampered by the fragmentary data. Only a very small number of rDNA sequences from ciliates and methanogens was available for the construction of the phylogenies. Eventually, the addition or the removal of a species caused substantial changes in the topology of the phylogenetic trees of both hosts and endosymbionts. Obviously, this observation raised the question whether a co-evolution between ciliates and their methanogenic endosymbionts could be demonstrated unequivocally. In addition, it became evident that the deep branching of ciliates could not be resolved using 18S

rDNA sequences (van Hoek et al. 2000b; 2001; Fig. 1). Therefore, the occurrence of hydrogenosomes in rather distant lines of ciliates might not necessarily be the result of multiple “inventions” of these organelles. It might also reflect the evolution of a hydrogenosome early in the radiation of ciliates (c.f. Martin and Müller 1998; Rotte et al. 2000) In this scenario, the methanogenic endosymbionts might have been acquired once - together with the evolution of ciliate hydrogenosomes. However, these “original” endosymbionts must have been replaced repeatedly by free-living methanogens in the course of evolution of the ciliates and their adaptation to the various anaerobic niches (Fig.2).

7. Acquisition of Environmental Methanogens by Endosymbiont Replacements

A detailed analysis of endosymbionts in a well-studied, monophyletic group of ciliates was required to solve the puzzles provided by the unexpected lack of co-evolution between ciliates and their endosymbionts. Van Hoek et al. chose for an analysis of the anaerobic heterotrichous ciliates, i.e. the Armophoridae and Clevelandellidae (van Hoek et al. 1998; 2000b). This group consists exclusively of anaerobic ciliates. They occupy very divergent ecological niches such as freshwater- and marine sediments, but also the intestinal tracts of vertebrates and invertebrates. In other words, the anaerobic ciliates from freshwater- and marine sediments, and those from the intestinal tracts of cockroaches, millipedes and frogs share a recent common ancestry (van Hoek et al. 1998, 2000b). Moreover, the hydrogenosomes of these ciliates appeared to be monophyletic, too; there is evidence that all hydrogenosomes in this taxon have retained a mitochondrial-type genome. Phylogenetic analysis of organellar SSU rDNAs revealed their monophyly with ciliate mitochondria and strongly supports a common ancestry of the hydrogenosomes of the Clevelandellids and Metopids (Akhmanova et al. 1998, van Hoek et al. 2000a). This observation implies that the evolution of hydrogenosomes preceded the radiation of their hosts and their adaptation to the various anaerobic environments. In other words, the last common ancestor of all Clevelandellids and Metopids already possessed hydrogenosomes. Since the function of hydrogenosomes is hampered in the absence of endosymbiotic methanogens, it is likely that already the first ciliate with a hydrogenosome attracted methanogenic Archaea. Consequently, endosymbiotic methanogens found in ciliates of this lineage should be monophyletic like their hosts and their hydrogenosomes. Alas, this appeared not to be the case (van Hoek et al. 2000b).

This observation is in conflict with the observation that methanogenic endosymbionts are vertically transmitted. All life-stages, including the cysts, host methanogens, and in transfaunated animals, we could not find any indication for an uptake of new endosymbionts by intestinal ciliates after their establishment in the “wrong” animal host (van Hoek et al. 1999). Also, closely related, free-living ciliate species from the same sampling place in freshwater sediments possess clearly distinct

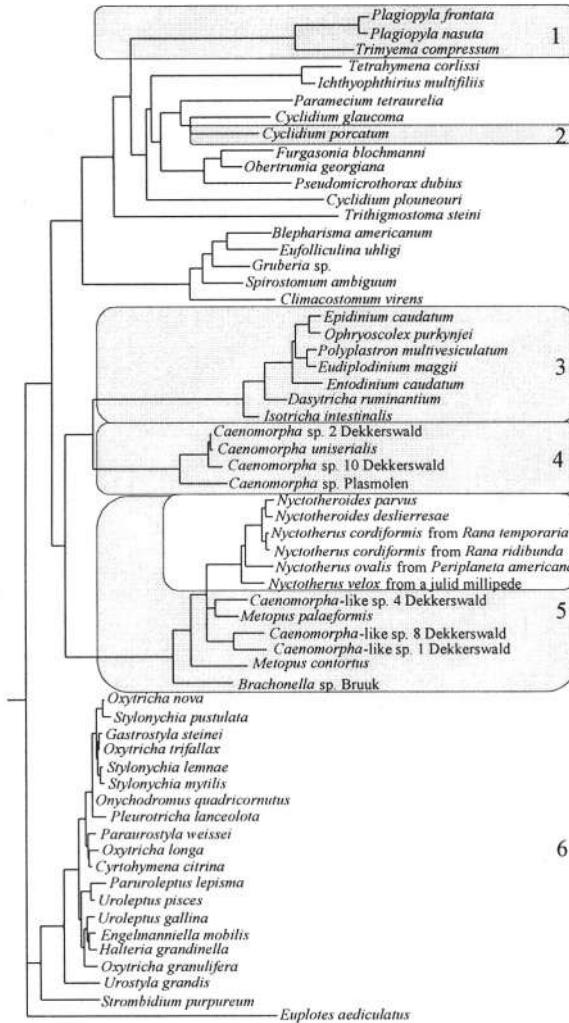


Fig. 1: Phylogenetic tree (Molphy Star Decomposition, Adachi and Hasegawa, 1996) demonstrating the evolution of the relevant ciliate taxa on the basis of their 18S rDNA. 1-5 taxa with hydrogenosomes, all other, non-boxed, taxa possess mitochondria. There is not sufficient statistical support for the deep branching, i.e. it cannot be excluded that all hydrogenosome-bearing ciliates share a common origin. On the basis of the genome organization, the *Stichotrichida* (6) are the closest relatives to the anaerobic heterotrachs (5), also named Armophoridae and Clevelandellids. The light box in 5 indicates intestinal ciliates.

endosymbionts, whereas identical ciliates from different sediments host identical endosymbionts. Moreover, endosymbionts of ciliates living in the intestinal tract of frogs (*R. ridibunda* and *R. temporaria*, van Hoek et al. 2000b) are specific for their particular ciliate (and amphibian) host. Notably, they belong to an intestinal and not a fresh-water-type of endosymbionts – notwithstanding that a wealth of free-living methanogens populates the ponds where frogs and their larvae thrive. Thus, all available evidence argues for a high specificity of the association and a long-lasting genetic isolation of ciliates and their endosymbionts. Nevertheless, it is not possible to match the phylogenies of ciliates and their methanogenic endosymbionts – even if the analysis is restricted to ciliates from one and the same ecosystem (van Hoek et al. 2000b).

The lacking match between host and endosymbiont phylogenies might be due to multiple acquisitions of endosymbionts – as suggested by Embley and Finlay (1994) – but not because of multiple origins of hydrogenosomes. A helicopter view at the phylogenetic position of the methanogenic endosymbiont reveals that various endosymbionts in a monophyletic group of ciliate hosts with monophyletic hydrogenosomes do belong to different orders of methanogens (van Hoek et al. 2000b). Notably, the endosymbionts are clearly related to the free-living methanogens thriving in the same ecological niche, but not identical with any of their free-living relatives. The mere existence of specific endosymbionts in the ciliates that live in the same environment excludes a regular uptake of endosymbionts from the environment. Given the host-specificity and the strictly vertical transmission of the endosymbionts, methanogenic Archaea must have been acquired, albeit very infrequently, from the particular environment where the host thrives. Since the hosts and their hydrogenosomes are monophyletic, there is no other solution to the dilemma but postulating endosymbiont replacements after the radiation of their hosts in the course of their adaptation to particular environments (Fig. 2).

One can only speculate about the frequency of these replacements and its mechanisms. The frequency must be very low – given the host-specificity and the significant divergence of the 18S rDNA sequences of endosymbionts from their free-living relatives. The mechanism remains speculative. Since the endosymbionts represent a small, isolated population of clonal descent, deleterious and slightly deleterious mutations will accumulate (“Muller’s ratchet”). Unless specific mechanisms evolve that enable the endosymbionts to cope with the mutational load – like in *Buchnera* and other intracellular symbionts in insects (Shigenobu et al. 2000; Moran and Baumann 2000), the fitness of the methanogenic endosymbionts will decrease in time. Since grazing ciliates regularly take up bacteria and free-living methanogens, one or the other methanogen has a rare chance to escape digestion and to survive in a food vacuole to replace eventually an “aged” population of endosymbionts suffering from its genetic load (c.f. Doolittle 1998).

This hypothesis can explain both the limited co-evolution between ciliates and their methanogenic endosymbionts on the one hand, and the relationship between endosymbionts and free-living methanogens on the other hand. The mechanism of the endosymbiont replacements requires more detailed studies. There is evidence that

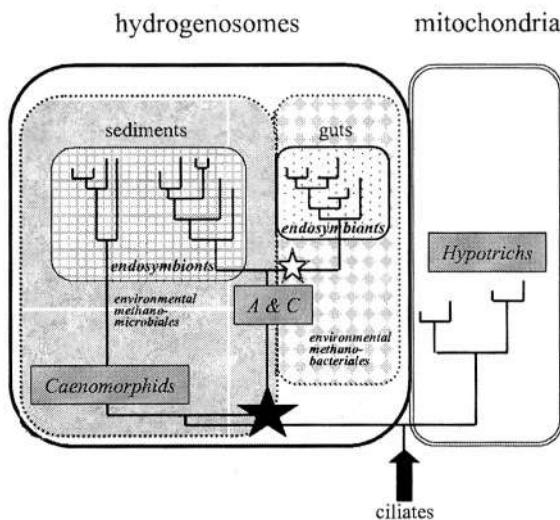


Fig. 2: Cartoon summarizing the evolution of anaerobic heterotrichous ciliates (A&C, Armophoridae and Clevelandellids) and their endosymbiotic methanogens (c.f. van Hoek et al. 2000b). As described in the text, ancestral ciliates (black arrow) diverged into aerobic, mitochondria-bearing ciliates (most likely the hypotrichs) and anaerobic, hydrogenosome-bearing heterotrichs. The black asterisk identifies the first acquisition of methanogenic endosymbionts that precedes the adaptation of the ciliates to the various ecological niches. Subsequently, the ciliates diverge (black lines), and both *Caenomorphids* and part of the A&C radiate in freshwater sediments. Their endosymbionts are closely related to environmental, free-living *Methanomicrobiales*. Those A&C species that adapt to life in the gastro-intestinal tract acquire endosymbionts that are related to intestinal *Methanobacteriales*. This adaptation was accompanied by the replacement of the ancestral endosymbionts by intestinal methanogens.

endosymbiont replacements are not an isolated phenomenon: also in the symbiosis between proteobacteria and certain bivalves belonging to the genus *Solemya* endosymbiont replacements must have occurred (Krueger and Cavanaugh 1997; Distel 1998;). Therefore, it is likely that future studies on the diverse symbiotic associations eventually will reveal that endosymbiont replacements are a common theme for many stable, long-lasting, and specific symbiotic associations.

8. Conclusions

The endosymbiotic association between ciliates and methanogenic Archaea represents only one of the many symbiotic associations of ciliates. However, this association is an especially well-studied symbiosis, that, besides its bearing for an understanding of the evolution of the eukaryotic cell (Martin and Müller 1998), has a global impact because the consortia between the hydrogen-producing ciliates and their methanogenic endosymbionts contribute to the greenhouse effect. It is not the aim of this review to

discuss the environmental consequences of biological methane production. Here we have reviewed the literature dealing predominantly with the molecular, cell-biological and evolutionary aspects of the symbiosis between anaerobic ciliates and methanogenic archaea. We have shown that the symbiosis between ciliates and methanogens has its origin in the evolution of hydrogenosomes – organelles that share a common origin with the mitochondria. Hydrogenosomes provide the nutritional basis for the symbiosis: they generate hydrogen and carbon dioxide, the presumed substrates for the endosymbiotic methanogens. By the removal of both compounds, the endosymbionts improve the function of the hydrogenosomes, and consequently, the performance and “fitness” of the ciliates. Therefore, the symbiotic association between ciliates and their methanogenic endosymbionts seems to provide the “cleanest” example of a mutualistic symbiosis. However, we have to admit that we are far from understanding all the facets of just this one symbiotic association. Are hydrogen and carbon dioxide really the only substances that the endosymbionts take from their hosts? How can we be sure that the ciliates do not digest one or the other of their endosymbionts? How do the ciliates and their endosymbionts communicate? How do they synchronize their cell divisions? Which mechanisms help the endosymbionts to cope with “Muller’s ratchet”? What is the role of the host and endosymbionts, respectively, to survive an accidental exposure to oxygen? There are many questions and no answers yet. Thus, we have to commit that we are still very ignorant about many of the facets of the phenomenon “symbiosis”.

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ENDOSYMBIOSIS OF BETA-PROTEOBACTERIA IN TRYPANOSOMATID PROTOZOA

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1. Introduction

All trypanosomatid protozoa are usually associated with other animals or plants. A few digenetic species, living alternately in two different hosts, have been the subject of intensive research because they are etiological agents of well-known human diseases, such as Chagas', African sleeping sickness and leishmaniasis. Additional species are also known to cause diseases in domestic animals and in plants.

TABLE 1. Symbiotic-containing trypanosomatids isolated from insects

Trypanosomatid	Insect	Reference
<i>Blastocrithidium culicis</i>	<i>Triatoma infestans</i>	Novey et al. 1907
<i>Critchidia deanei</i>	<i>Zelus leucogrammus</i>	Mundim et al. 1974
<i>Critchidia desouzai</i>	<i>Ornithia obesa</i>	Fiorini et al. 1989
<i>Critchidia oncopelti</i>	<i>Oncopeltus fasciatus</i>	Newton and Horne 1957
<i>Herpetomonas roitmani</i>	<i>Ornithia obesa</i>	Fiorini et al. 1989 Faria e Silva et al. 1991

Endosymbionts have been observed and initially described as "bipolar bodies" or "diplosomes" in some trypanosomatids, most of which are monogenetic non-pathogenic species of insect origin (Table 1). The ease of isolating these protozoa in culture with simple media has greatly facilitated the study of their endosymbionts. Their number is usually limited to a single bacterium per protozoan, suggesting that the symbionts divide in synchrony with their host cells. All past attempts to culture these endosymbionts outside of their host have failed, indicating that they are obligate intracellular microorganisms. Occasionally, similar intracellular structures were also described from some digenetic species of *Trypanosoma*, but their identity as true endosymbionts has not been proven.

The review presented here serves to introduce this unique system of endosymbiosis as a potentially useful model to study the evolution of eukaryotic cells. Especially relevant is the evolutionary event of the transition from an endosymbiont to an organelle, in keeping with the endosymbiosis theory for the monophyletic origin of mitochondria.



Fig. 1. The trypanosomatid *Blastocerithidia culicis* contains, during the division process, a pair of endosymbionts as "diplosomes". Kinetoplasts and nuclei are also observed in these protozoa.

2. Endosymbiont Morphology and Ultrastructure

The endosymbionts of the insect trypanosomatids were originally recognized as "extra" intracellular bodies additional to the normal complement of cell organelles common to all eukaryotes. The endosymbionts are also distinct from cell structures unique to all trypanosomatid protozoa, e. g. glycosomes, kinetoplasts (mitochondrial DNA) and paraxial rods. Glycosomes contain several enzymes of the glycolytic pathway and thus are involved in energy metabolism. The kinetoplast is a region of the mitochondrion, which contains its genome, constituting up to ~20% of the total cellular DNA.

Endosymbionts are rod-shaped structures, approximately 0.3 to 1.0 μm wide and 1.3 to 2.3 μm long. They are present in singlet or in doublet and are localized in the cytoplasm, usually near the host nucleus (Novey et al. 1907; Newton and Home 1957; Mundim et al. 1974; Chang 1974; Fiorini et al. 1989) (Fig. 1). Ultrastructural analyses by transmission electron microscopy revealed that these endosymbiotic bacteria display an electron dense cytoplasmic matrix composed of ribosomes interspersed with electron lucid areas with fibrous DNA typical of prokaryotic genomes (Fig. 2). Although endosymbionts are each enclosed by two unit membranes, tightly associated at some points, the typical Gram-negative peptidoglycan layer is not visible (Chang 1974; Motta et al. 1991a; Soares and De Souza 1988). A residual peptidoglycan layer is thought to exist, since the endosymbionts changed their morphology from rods to pleiomorphic forms when the host trypanosomatids were grown in the presence of ampicillin or cephalixin. This interpretation receives support by finding the putative target of these β -lactam antibiotics as membrane-bound penicillin-binding proteins in purified symbionts after labeling with [^3H]-benzylpenicillin (Motta et al. 1997a).

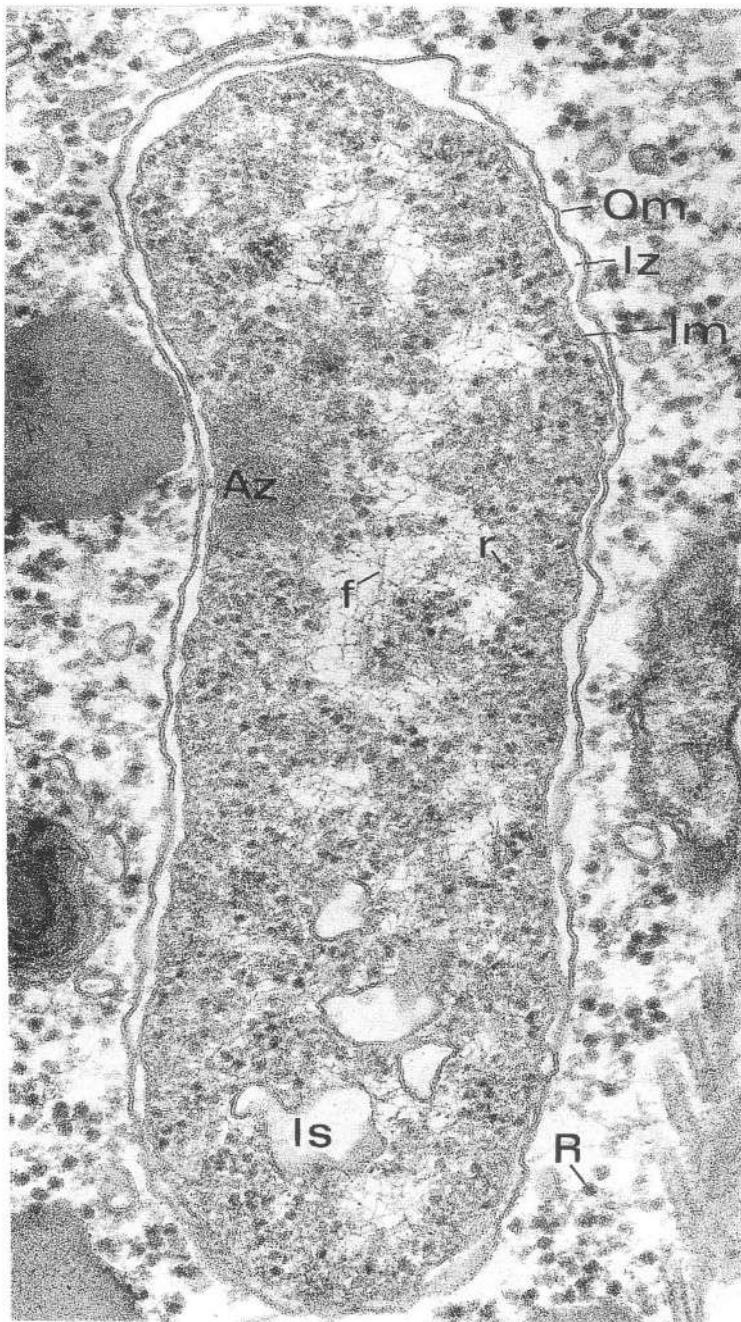


Fig. 2. Ultrathin profile of an endosymbiont in the trypanosomatid protozoa, *B. culicis*. AZ, Amorphous zone; f, DNA fibers; Im, Inner membrane; IS, Internal membranous structures; Iz, Intermembrane zone; OM, Outer membrane; R, Host protozoan ribosome; r, Symbiont ribosome. From Chang 1974.

Freeze-fracture and etching profiles of the endosymbionts revealed filaments between the two symbiont membranes. Whether these filaments may correspond to the remnants of the peptidoglycan is unknown (Motta et al., data not shown).

3. Phylogenetic Status of Symbionts and Symbiont-Host Coevolution

The phylogenetic status of the insect trypanosomatid symbionts was inferred from their ssu-rDNA sequence data. Analyses of these sequences revealed that these symbiotic bacteria from different insect trypanosomatids are very similar and are phylogenetically clustered with *Bordetella bronchiseptica* in the beta division of Proteobacteria (Du et al. 1994a). Two trypanosomatid endosymbionts were subsequently named as *Kinetoplastibacterium blastocrithidiae* and *K. crithidiae* (Du et al. 1994b). Other endosymbionts from the free-living protozoa, *Euplotes aediculum* also belong to the beta division (Springer et al. 1996), while those of *Amoeba proteus* belong to the gamma group (Jeon 1997). Mitochondria have been inferred to originate from the alpha group of Proteobacteria. From the phylogenetic status of the extant intracellular symbionts, it appears that distinct groups of Proteobacteria may have developed into organelles during the evolution of different eukaryotic lineages.

The intracellular bacteria cluster to specific phylogenetic groups of protozoa, suggestive of symbiont-host coevolution. Monophyly of endosymbiont-containing trypanosomatids was first noted on the basis of their clustering into the same zymodeme determined via isoenzyme electrophoretic mobility analysis (Faria e Silva 1991; Motta et al. 1991b). This notion was strengthened by ssu-rDNA sequence analyses of different symbiont-containing trypanosomatid species (Du and Chang 1994; Du et al. 1994b). Taken together, endosymbiosis is predicted to have arisen from the association between an ancestral beta-Proteobacterium and an ancestral flagellate in a single event some 20–120 million years ago. This event was presumably followed by their co-evolution, giving rise to the extant symbiont-containing species (Du et al. 1994b). A similar event has been proposed to account for the origin of mitochondria found in all eukaryotic cells.

4. Symbiont-Host Relationships Involving Known Metabolic Pathways

Symbiosis has presumably evolved by natural selection for a partnership under conditions otherwise detrimental to the individual organisms involved. Although the precise function of the endosymbionts in the natural setting is unclear, they have long been known to benefit their host protozoa nutritionally *in vitro*. The symbiont-containing species grow readily in simple defined media containing few amino acids and vitamins (Newton, 1957; Mundim et al. 1974). The same media do not support the growth of all other naturally symbiont-free species unless fortified with additional nutrients, including a purine base and hemin. The hemin-sparing effect of endosymbionts (Chang and Trager 1974) is due to their ability to complement defects in the trypanosomatid pathway for heme biosynthesis. Clearly missing in these protozoa is the third enzyme in the pathway, i. e. porphobilinogen deaminase (PBGD). This enzyme

was found in isolated symbionts, in symbiont-containing flagellates, but not in those rendered symbiont-free (Chang et al. 1975). Complementation of the defective heme biosynthesis pathway by endosymbionts was further confirmed by the presence of porphyrin precursors (Salzman et al. 1985). More recently, transfection of naturally symbiont-free species with genes encoding heme pathway enzymes was carried out. The results obtained so far point to multiple defects in the heme biosynthesis pathway of trypanosomatid protozoa beyond PBGD (Chang and Sassa, data not shown). It is likely that the endosymbionts may supply their host with either protoporphyrin XI or heme itself.

There is circumstantial evidence to suggest that the endosymbionts may obtain ATP from the host protozoa as their energy source, similar to the obligate intracellular pathogens, like *Rickettsia* and *Chlamydia* (Hatch et al. 1982; Weiss 1973; Weiss and Wilson 1969; Winkler 1976). Cytological models obtained by three-dimensional reconstruction of the symbiont-containing flagellates suggest that endosymbionts are in close association with glycosomes (Faria e Silva et al. 2000; Motta et al. 1997b).

Biochemical and cytochemical analyses of these cells further showed that symbionts contain very low levels of functionally active enzymes in the respiratory chain. In addition, ATPase activity was detected in the isolated endosymbionts, presumably serving as a transporter for the uptake of essential nutrients from the protozoa, such as metal ions and adenosine (Motta et al. 1997b).

5. Genetic Approach needed to Further Elucidate Symbiont-Host Interactions

Some structural or physico-chemical changes of the host flagellates have been attributed to the presence of endosymbionts. Notable in some endosymbiont-bearing trypanosomatids and the cured strains derived from them, but not in those naturally symbiont-free, are the following cytological features (Freymuller and Camargo 1981): (i) The appearance of mitochondrial or kinetoplast DNA as atypical fibrous arrays; (ii) The apparent absence of the flagellar paraxial rods; and (iii) Close apposition of the mitochondrial branch membrane against the cytosolic face of the plasma membrane at places devoid of subpellicular microtubules. When the protozoa were rendered symbiont-free, changes were noted in their externally exposed saccharide residues (Dwyer and Chang 1976; Esteves et al. 1982) and in their surface charge (Oda et al. 1984). It is not known if these surface alterations are related to the observations that endosymbiont-bearing species are more avidly ingested by phagocytes and more resistant to intracellular degradation by these cells (Rozental et al. 1987). The efficiency of *Herpetomonas roitmani* differentiation, from promastigotes into opistomastigotes, is higher in endosymbiont-free trypanosomatids (Faria e Silva et al. 1994).

It is unknown whether these differences observed between symbiont-containing and symbiont-free flagellates may result from symbiont-host mutual adaptation or even gene transfer during their long-term coevolution. One obvious approach to answer this question is to recover the phenotypes in question after reconstitution of endosymbiosis. The inability of endosymbionts to grow *in vitro* makes this approach difficult. Attempts to re-introduce the endosymbiont in the host protozoa were unsuccessful by mixing and incubation of symbiont-free flagellates with mechanically isolated symbionts (Tuan and

Chang 1975). These failed attempts may be accounted for by a loss of viability of the isolated symbionts or the lack of phagocytic activity of the flagellates or both. More recently, attempts to infect symbiont-free flagellates with *B. bronchiseptica* were also unsuccessful (Chang and Moulder, Unpublished), despite its phylogenetic relatedness to the trypanosomatid endosymbionts. The absence of infection in this case clearly results from an inability of the flagellates to take up the bacteria. Ultimately, a genome sequence project for these endosymbionts will have to be undertaken to generate the data needed for elucidating the genetic basis of their functional significance in the host protozoa.

6. Concluding Remarks

The endosymbiont-bearing trypanosomatids represent a useful model to study prokaryote-eukaryote interactions for integration relevant to the origin of cell organelles. Protozoa of the Trypanosomatidae family themselves live mostly in association with other animals or plants. The interrelationships of these associations with the origin of the trypanosomatid endosymbionts are unknown. Many digenetic trypanosomatid species are well-known and have been extensively studied as the disease-causing agents. Interestingly, only monogenetic non-pathogenic species, which have one host in their life cycle, are naturally infected with prokaryotic endosymbionts. Their phylogenetic affiliation with beta-Proteobacteria is unique. With few exceptions, other intracellular symbiotic and pathogenic bacteria mostly belong to the alpha-Proteobacteria. The trypanosomatid endosymbionts are each enclosed by two "unit" membranes, but apparently with a degenerative cell wall. Each protozoan usually contains only one symbiont, resulting apparently from their synchronous division. Integration of the symbionts into the physiology of the hosts is thus indicated. However, the symbiont-containing protozoa can be rendered permanently symbiont-free by antibiotic treatment. Biochemical and nutritional comparisons of the symbiont-containing and aposymbiotic strains are thus made possible. It is clear from such studies that the symbiont supplies its host with essential nutrients, i. e. heme, amino acids and vitamins, while the symbiont probably obtains ATP from the host in return. Interestingly, some symbiont-free and symbiont-containing flagellates differ in their externally exposed saccharide residues, surface charges and other cytological features. Characterization of the symbiont genome is crucial for further elucidation of symbiont-host interrelationships in this system by molecular genetic approach.

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ECTOSYMBIOSIS IN CILIATED PROTOZOA

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1. Introduction

Symbiotic associations are thought to have played an important role in the evolution of a variety of organism lineages (Margulis and Fester, 1991). It is difficult, however, to reconstruct the different stages of the processes through which symbiosis operated. Many authors think that indications may be provided by the study of the symbiotic associations found in Protozoa at the present time. An initial step in eukaryotic evolutionary processes may be represented by ectosymbiotic associations originated as an adaptative tool against environmental difficulties. For these reasons the study of ectosymbiosis in protozoa may be important from both an evolutionary and ecological point of view.

Ectosymbiotic associations between flagellates and bacteria, in which the symbionts clearly confer advantages to their host, have been described. A well known example is the case of the termite flagellate *Mixotricha paradoxa* which is propelled by its associated locomotory *Spirochetae* (Cleveland and Grimestone, 1964; Margulis, 1993). Another peculiar motility system has been adopted by devescovinid flagellates from termites: their rapid, gliding movement in contact with a substrate is powered by the flagella of thousands of rod bacteria which live on their surface (Tamm, 1982).

As concerns ciliates, ectosymbiotic bacteria have been repeatedly observed on different aerobic and anaerobic species. The regularity of many of these observations suggests that these associations are potentially important for both bacteria and their eukaryotic hosts. Although each of these associations may have its own peculiarities, certainly the ectosymbiotic bacteria take advantage by the increased motility and disposal of organic or inorganic material for their metabolism. It is more difficult to understand or, at least, to generalize the significance of the association for ciliates. Indeed different types of benefits due to the presence of ectosymbiotic bacteria on ciliates have been recognized. For example the body of the mobiline peritrich *Trichodinopsis* (endosymbiont of a terrestrial prosobranch mollusc) is covered with attached spirochetes - formerly thought to be cilia - that presumably aid the host in locomotion (Corliss, 1969). Different cases in which the functional role of the ectosymbionts has been demonstrated are reported in the following sections. They have been organized in groups according to the kind of benefit the bacteria confer to the host.

2. Ectosymbionts as food

Most Ciliates possess a "mouth", i. e. a specialized region of the body, delimited by a simple cell membrane, for food uptake by phagocytosis. The structure of the mouth or cytostome is highly variable among species of ciliates; it may be at the cell surface or at the end of a more or less deep depression of the body, equipped with a differentiated oral ciliature, called peristome. The food mainly consists of bacteria, microalgae or protozoa; once ingested the food organisms are enclosed and digested in cytoplasmic vacuoles. The peritrich ciliate *Trichodinopsis*, beside harboring ectosymbiotic spirochetae as mentioned above, has a lateral pocket at the level of the cytopharynx (the terminal part of the peristome) in which many bacteria are always present. These bacteria actively reproduce in the pocket: given that the pocket opens toward the exterior, they can be considered ectosymbionts. They are not simple commensals: electron microscopy (Grassé et Mugard, 1963) revealed that many food vacuoles in the cytoplasm of the host contain these same bacteria at different stages of digestion. Through the apical large peristome *Trichodinopsis* catches and ingests other kinds of organisms. Thus the pocket appears to be a sort of "pantry" where bacteria are stored and grown as reserve food for times of famine. Other comparable cases in which bacteria are "cultured" by ciliates and used as supplement of food are reported in the classic literature (for review see Hovasse, 1984). The case of the sand ciliate genus *Kentrophoros*, which appears to depend completely on ectosymbiotic bacteria as food, is peculiar. These ciliates are flat organisms with a ciliated side referred to as the ventral side as it is usually in contact with the substratum and a non ciliated (except for two marginal kineties) dorsal side whose surface is covered with bacteria oriented perpendicularly to the host cell surface. These bacteria contain sulfur granules and are characterized by having a longitudinal fission. It has been calculated (Fenchel and Finlay, 1989) that they account for about half the biomass of the symbiotic consortium. The bacteria are embedded in a thick mucus layer, produced by the ciliate, to keep the symbionts in place (Foissner, 1995). In *K. fistulosus* the symbionts are further protected from being removed by mechanical forces, by a tube-like involution of the ciliate body (Fig. 1a, b). While *K. fistulosus* and *K. fasciolata* (Fenchel and Finlay, 1989) harbor only one type of bacterium, in *K. latum* ectosymbiotic spirochetae were also found (Raikov, 1974). The functional role of the latter organisms is unknown.

Kentrophoros ciliates have not a mouth or, as in the case of *K. fistulosus* (Foissner, 1995), they have greatly reduced and functionless oral structures; they phagocytose the ectosymbionts (Fig. 1c) through the cell surface of the entire dorsal side. Newly formed vacuoles contain single symbionts; then they fuse to form large digestive vacuoles containing bacteria at different stages of the digestive process (Raikov, 1971, 1974; Fenchel and Finlay, 1989). In the same papers Raikov suggested that the ectosymbiotic bacteria are chemolithoautotrophic sulphide oxidisers. This idea was experimentally confirmed by Fenchel and Finlay (1989) who also described the behavioral properties of the ciliate host. *Kentrophoros* is microaerophile; it shows a chemosensory response to oxygen tensions and tends to accumulate at a pO_2 somewhat less than 5% atm sat. This may account for the characteristic vertical distribution in sediments, just at the chemocline between oxidized and anaerobic sulfide reducing layers, observed in the natural environment (Fenchel, 1969). Through this behavior the ciliate seems to

optimize the activity of the symbionts which, indeed, are able to divide on its surface. In sands, the vertical position of sulfide layers varies seasonally and according to the general conditions of the sea. The motility of the ciliate is certainly advantageous as allows the bacteria to exploit these variations more efficiently. Since *Kentrophoros* depends on the bacteria as food both the behavior and the flattened shape (which means a larger surface area available for the bacteria) may be considered adaptive traits which favor bacterial growth. Thus *Kentrophoros* has been reported as a ciliate with a "symbiotic kitchen garden" (Fenchel and Finlay, 1989).

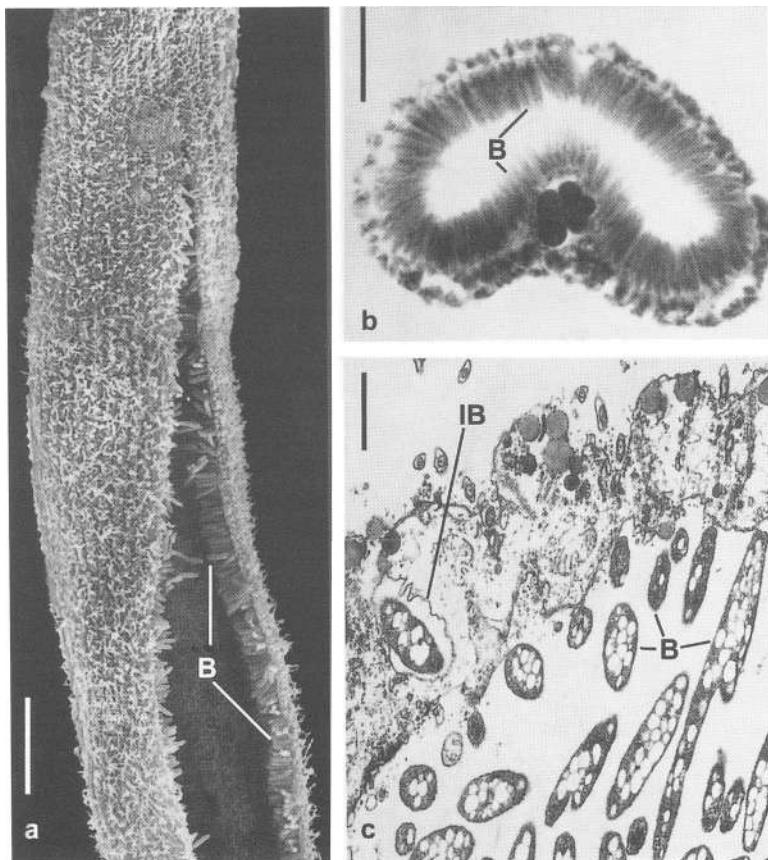


Fig. 1. *Kentrophorus fistulosus* a) The body is involuted tube-like with the symbiotic bacteria lawn inside. From Foissner (1995); scale bar 50 μm . b) Cross section of the body at the optical microscope. Scale bar 20 μm . c) Cross section at the electron microscope. From Raikov (1971); scale bar 1 μm . B = bacteria; IB = internalized bacterium.

3. Ectosymbionts as a response to environmental challenges

The importance of the sulfur cycle is determined by the features of sulfur as a chemical element. Due to the large scale of valencies that sulfur has, its compounds can serve both as electron donors and acceptors. In sediment and water column they are the main energy link between aerobic and anaerobic processes, separated by the redox zone.

Oxidation of reduced sulfur compound in the aerobic zones takes place either chemically or with the participation of sulfur bacteria, while in anoxic layers sulfate and elemental sulfur are reduced to hydrogen sulfide by specialized sulfate reducing bacteria which use them as electron acceptors during anaerobic sulfur respiration. Hydrogen sulfide production by bacteria is widespread especially in marine environments. Thus microaerobic and anaerobic zones are colonized by abundant and diversified prokaryotic communities that might be an important alimentary resource for protozoa or little metazoa; yet these habitats, characterized by the presence of toxic hydrogen sulfide and the paucity or absence of oxygen, are scarcely compatible with eukaryotic life. Cooperation with prokaryotic organisms often proved to be an important aid in solving the problem.

3.1 SYMBIOSIS WITH SULFUR OXIDIZING BACTERIA.

Symbiotic sulfur oxidizing bacteria are known to be distributed across a broad range of invertebrate animals belonging to different taxa, occurring as intracellular endosymbionts or, more rarely, as extracellular ecto or episymbionts (Felbeck and Distel, 1992; Cavanaugh, 1993). The invertebrates bearing sulfur oxidizing bacteria live in environments whose common feature is the coexistence of reduced inorganic sulfur compounds and oxygen as hydrothermal vents, sea grass beds and the superficial layers of anoxic sediments. As concern ciliates no endosymbiotic associations with sulfur oxidizing bacteria have been reported. We previously mentioned the ectosymbiotic association in the Loxodid *Kentrophoros*. More recently a large and fast growing sessile colonial ciliate, the peritrich *Zoothamnium niveum* was discovered in mangrove channels of the Caribbean Sea (Bauer-Nebelsick et al., 1996). The whole surface of the *Z. niveum* colonies is covered with ectosymbiotic bacteria (Fig. 2a) characterized by a conspicuous white color. The white color is assumed to represent inclusions of elemental sulfur, used by the bacteria as storage within a sulfide-oxidizing process. These bacteria, like those of *Kentrophoros* (Fenchel and Finlay, 1989) contain ribulose-1,5 biphosphate carboxylase oxidase. Thus, in all likelihood, they obtain energy by oxidizing reduced inorganic sulfur compounds and fix inorganic carbon via the Calvin-Benson pathway. Culture and incubation experiments showed that *Z. niveum* is in obligatory association with its symbionts; it is unable to survive without them, even for a short period of time. Moreover, to survive and reproduce, it needs the simultaneous presence of oxygen and sulfide. The sulfur oxidizing symbionts may provide the host with a substantial source of reduced organic carbon as they do in invertebrates (Distel et al., 1994). In addition the massive presence of ectosymbiotic sulfur oxidizing bacteria on their surface reduces the toxicity (due to the presence of sulfide) in the immediate surroundings of the *Zoothamnium* colonies.

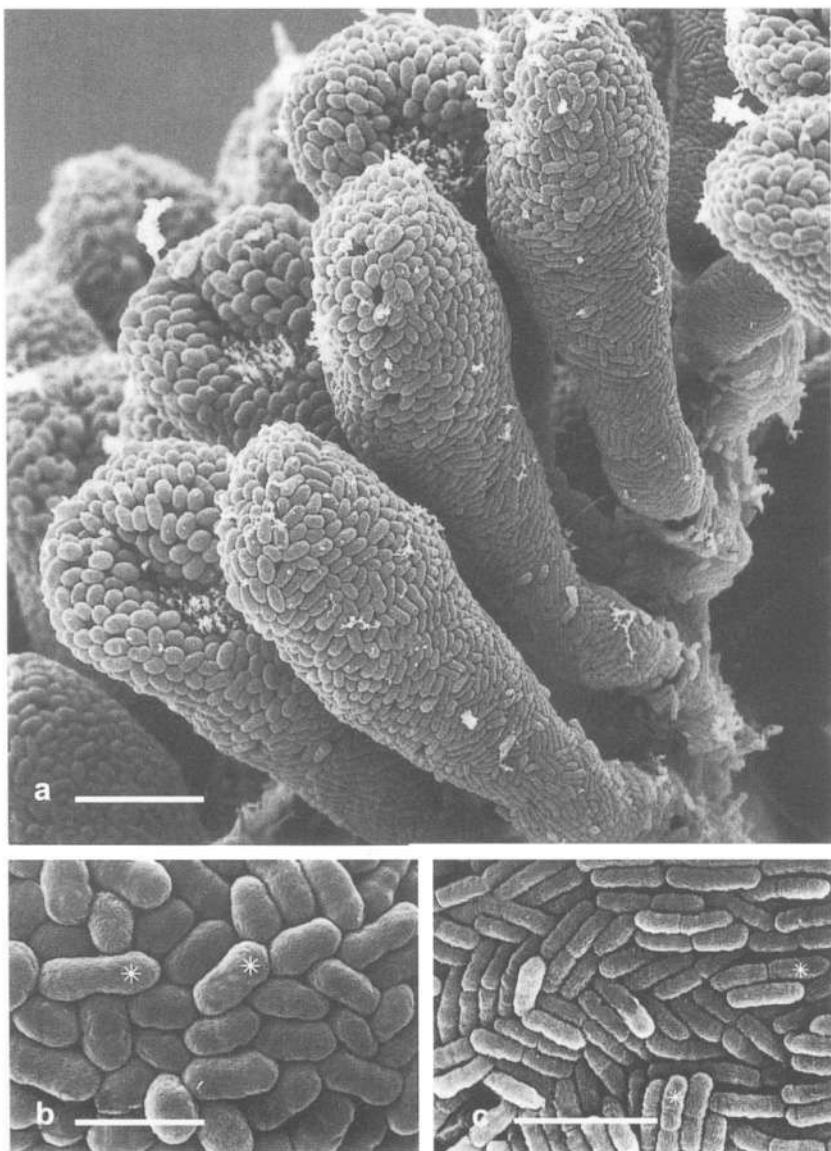


Fig. 2 Scanning electron micrographs of *Zoothamnium niveum*. From Bauer-Nebelsiek et al. (1996). a) Microzooids with bacterial coat. Scale bar 10 μm . b) Detail of morphotype 1 bacteria, asterisks marking dividing cells. Scale bar 4 μm . c) Detail of morphotype 2 bacteria, asterisks marking dividing cells. Scale bar 4 μm .

The colony of *Z. niveum* is a unit composed of individuals at different stages: microzooids (trophic stage), macrozooids (telotroch stage) and terminal zooids (capable of longitudinal fission). The bacteria on the microzooids are coccoid, slightly dumbbell-shaped (Fig. 2b), while the bacteria covering all the rest of the colony are rod-shaped (Fig. 2c). Within the life history of *Zoothamnium* new colonies arise from macrozooides detached from the mother colony. The macrozooids are always covered by rod-shaped bacteria. It is very likely that the cocci and rods are two morphotypes of a single bacterial species and the cocci of the microzooids in the new colony develop from the rods rather than arising from repeated colonizations. Ectosymbiotic bacteria were reported, with no special attention, in other species of *Zoothamnium* (Fauré-Fremiet et al., 1963; Bauer-Nebelsick, 1996). From the data reported in the literature it cannot be judged whether the presence of the ectosymbionts is vital in all cases; in any case, the fact that these associations are apparently widespread within the genus, is indicative of a very old and well established relationship.

3.2. SYMBIOSIS WITH SULFATE REDUCING BACTERIA

The great majority of ciliate species are aerobic but an anaerobic lifestyle has evolved independently in many unrelated groups.

Most anaerobic ciliates, either free-living or commensals of higher organisms, possess hydrogenosomes, organelles whose function is to ferment pyruvate produced by glycolysis into acetate and hydrogen. All evidence indicates that hydrogenosomes are modified mitochondria (Hackstein et al., 1998). Many of these anaerobic ciliates also have other organisms living inside and/or attached to their external surfaces. Most of these associations may be considered true symbiosis as they are permanent and involve specific partners. The symbionts belong to three different prokaryotic groups: purple non sulfur photosynthetic bacteria, methanogens bacteria (both living inside the cell), and sulfate-reducing bacteria living on the surface of the host. All three groups have a common trait: they can consume hydrogen as a substrate. This suggests that the production of H_2 by the ciliates at the hydrogenosomal level is significant in maintaining these symbiotic associations. As the productivity of H_2 evolving fermentation depends on the maintenance of a low H_2 pressure, one could argue that the evolution has favored the retention of these types of functional consortium to increase the efficiency of the anaerobic ciliate metabolism. Purple photosynthetic bacteria have been described in the benthonic oligotrich *Strombidium purpureum* (Fenchel and Bernard, 1993). Methanogens, that are easily recognizable due to their autofluorescence, are widespread in anaerobic ciliates and their multiple acquisition by the different hosts has been recently demonstrated (van Hoek et al., 2000). They occur in marine as well as in limnic free-living anaerobic ciliates. In contrast ectosymbiotic bacteria occur almost exclusively in marine forms some of which also harbor endosymbiotic methanogens.

These ectosymbiotic bacteria have different morphologies even in ciliate species of the same genus and attach to their host in different ways (Fenchel et al., 1977; Fenchel and Finlay, 1991). For example the bacteria on the outer surface of *Metopus vestitus* are oriented perpendicularly to the host surface and held by mucus while in *M. contortus* (Fig. 3a) they are arranged irregularly and are mainly located on the posterior end of the cell. In *Parablepharisma pellitum* (Fig. 3b) the bacteria are long rods with a

brown pigmentation; they are oriented perpendicularly to the cell surface and are inserted in depressions in the cell membrane; in *Caenomorpha* species the bacteria live in sheaths that attach to the host cell membrane.

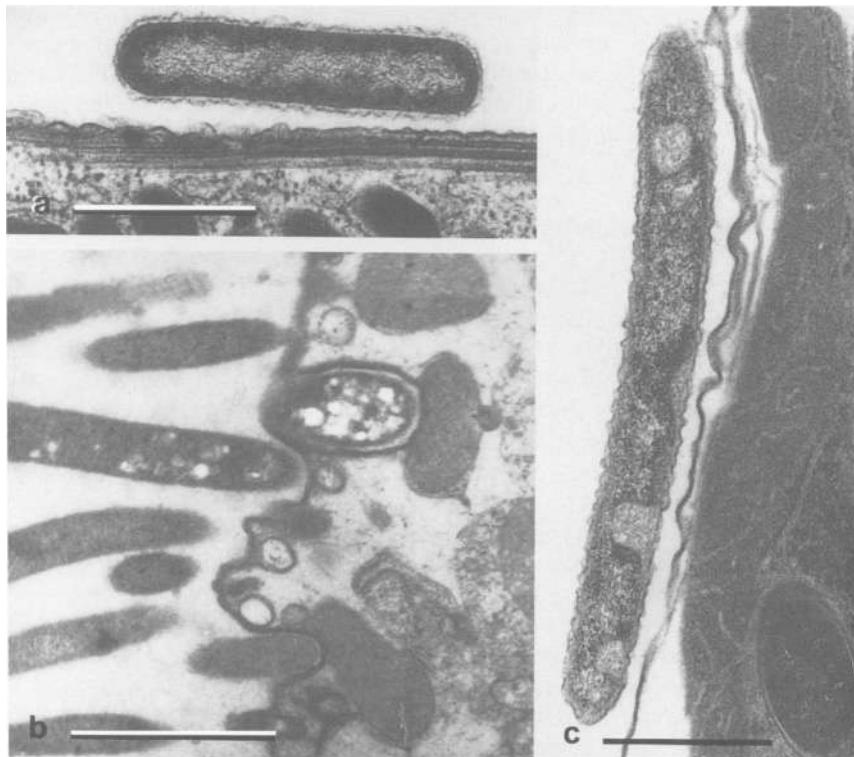


Fig. 3. Symbiotic sulphate reducing ectosymbiotic bacteria in different ciliates. a) *Metopus contortus*. From Fenchel and Finlay (1991); b) *Paralepharisma pellitum*. From Fenchel and Finlay (1991). c) *Isocyclidium globosum*. From Esteban and Finlay (1994). Scale bars 0.5 μm .

The possibility that these ectosymbiotic bacteria are sulfate reducing was firstly suggested by Fenchel and Finlay (1991). At that time ectosymbiotic bacteria were known only in ciliate marine species which live in a sulfate-rich environment. The identity of these bacteria was then studied using fluorescent-dye conjugated oligonucleotides complementary to short sequence elements of 16S ribosomal RNA (Fenchel and Ramsing, 1992). All the bacteria tested hybridized with an eubacterial probe and those of *Metopus contortus* and *Coenomorpha levanderi* hybridized with a general probe for sulfate-reducing bacteria. These results strongly support the idea that the ectosymbionts of marine anaerobic species, are indeed sulfate reducers. In the meantime bacteria attached to the external surface were found in the anaerobic freshwater ciliate species *Caenomorpha medusula* (Finlay et al., 1991) living in the hypolimnion of a sulfide-rich solution lake in Spain. Later Esteban and Finlay (1994)

observed rod shaped bacteria, partially covering the external surface of the scuticociliate *Isocyclidium globosum* only when retrieved from sulphate-rich freshwater (Fig. 3c). These findings are in line with the hypothesis. Moreover the ectosymbionts may compete with intracellular methanogens for common substrates. Interestingly the ectosymbionts are significantly more abundant in species which do not have endosymbiotic methanogens. On *Parablepharisma*, for example, they may account for the 10-15% of the host biovolume, whereas in methanogens bearing species they account for only 1-2% (Fenchel and Finlay, 1995). The adaptive significance of this symbiosis, from the bacterial point of view, is evident: the bacteria receive phosphates from the host. A likely result is that a low H₂ intracellular pressure is maintained in the host.

4. Ectosymbiosis as a defensive tool against predation

Hypotrich ciliates of *Euplotidium* genus harbor peculiar ectosymbionts, referred to as epixenosomes (Verni and Rosati, 1990; Rosati, 1999), typically localized along a cortical band running along the right and left borders of the cell body and forming a sort of "scarf" at the dorsal anterior end (Fig. 4a). Epixenosomes were studied in detail in *E. itoi* (for review Rosati, 1999) and *E. arenarium*. These studies revealed that these ectosymbionts have a complex life history in which two main stages are recognizable. In stage I (Fig. 4b) they are roundish in shape, 1 μm in diameter, have a typical prokaryotic structure and divide by direct binary fission. Stage II cells (Fig. 4c) are larger, egg shaped and show a complex organization, more complex than the majority of prokaryotic organisms. The following structures are always present: 1) an apical dome-shaped zone in which DNA and proteins have been evidenced; 2) the extrusive apparatus, immersed in a proteic matrix different from the remaining cytoplasm, consisting of a coiled ribbon tightly rolled up around a central core; 3) a network of tubules 20-24 nm in diameter, delimited by a wall made up of globular structures, which are sensitive to antitubulin drugs and react positively with antitubulin antibodies (Rosati et al. 1993). It has been demonstrated that these features are gradually acquired following a well defined pattern during the transformation from stage I to stage II. The multiplication and the transformation of epixenosomes are correlated with the host cell cycle. This indicates a well established association. Membrane receptors, located at the top of the organism, detect external signals (of an unknown nature). The consequent activation of the adenylate cyclase-cyclic cAMP system triggers the ejection (Rosati et al., 1997). During the ejection the ribbon of the extrusive apparatus unrolls from the inside and forms a hollow tube about 40 μm long. It terminates with a "head" mainly consisting of the genetic material of the epixenosome (fig. 4d, e).

The nature of epixenosomes has only recently been recognized by means of comparative sequence analysis of amplified small subunit rRNA genes and in situ hybridization with fluorescently labelled rRNA probes: they are bacteria phylogenetically related to *Verrucomicrobia* (Petroni et al., 2000). Epixenosomes are to date the first report of symbionts in this recently discovered bacterial division.

The nature of the symbiotic association was investigated in particular in *E. itoi*. It appears constant in nature. Indeed every specimen examined soon after collection, in the

course of several years, carried the typical band of epixenosomes. In the laboratory *E. itoi* stocks tend to lose epixenosomes when moderate starvation slows their cell cycle.

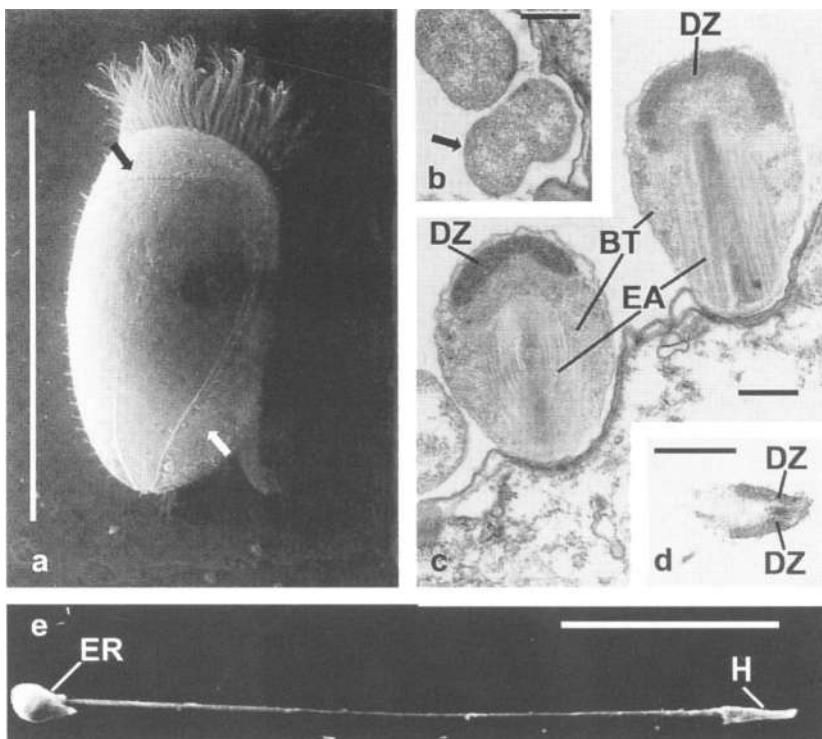


Fig. 4. Epixenosomes and their host. From Petroni et al. (2000). a) Dorsal view of *Euplotidium itoi*. Arrows indicate epixenosomes in the cortical band.). Scale bar 100 μ m. b) Stage I epixenosomes. Arrow indicates a dividing one. c) Stage II epixenosomes sectioned at different levels. DZ, apical (dome-shaped) zone; EA, extrusive apparatus; BT, microtubule-like elements. d) Section through the head of an ejected epixenosome. Scale bars 1 μ m. e) The tube at the end of the ejection. ER, epixenosome remnant; H, head. Scale bar 10 μ m.

The lack of epixenosomes does not modify either the behavior or the fission rate of the ciliate (Giambelluca and Rosati, 1996). So *E. itoi* can live and reproduce even without epixenosomes. This observation led the authors to hypothesize that, in the natural environment, the presence of the ectosymbionts might play a crucial role, such as a defense against predators. This hypothesis was experimentally verified by comparing the behavior of the raptorial feeding ciliate *Litonotus lamella* when preying upon *E. itoi* without epixenosomes and *E. itoi* with epixenosomes. *Litonotus* was chosen as it shares its natural habitat with *E. itoi* and as its feeding behavior is well known; this behavior involves several steps: the detection of the prey, the discharge of toxicysts (extrusomes that contain a toxic substance able to paralyze the prey) upon cell to cell contact, the search of the stricken prey, and the ingestion of the prey (Ricci and Verni 1988; Ricci et al., 1996). The results obtained (Rosati et al., 1999) strongly support the

hypothesis that epixenosomes provide their host with an efficient defensive tool against predation. Indeed, *L. lamella* discharged its toxicysts with the same efficiency against *E. itoi* without epixenosomes and *E. itoi* with epixenosomes. Nevertheless, while it was able to ingest the former (Fig. 5a) it never ingested the latter. Thus it appears that something prevents the predator from finding and eating *E. itoi* bearing epixenosomes, although it is able to attack and paralyze them. Since the main difference between the two prey types used in our experiments is the absence versus the presence of epixenosomes, it may be concluded that epixenosomes themselves defend paralyzed *E. itoi* against engulfment. It has also been demonstrated (Rosati et al., 1999) that in both prey types, about 60% of the individuals attacked by *Litonotus* toxicyst discharge are able to recover their normal behavior once transferred into pure sea water. The probability of survival is certainly higher for *E. itoi* with epixenosomes which are never eaten by the predator than for *E. itoi* without epixenosomes where the prey engulfed by the predator do not have the chance to recover. Thus, in the natural environment, where the toxic substance of the toxicysts can be readily dispersed, the survival probability, following the attack of the predator should be high for symbiotized *E. itoi*.

How can epixenosomes play this defensive role?

In another series of experiments (Rosati et al., 1999) some *E. itoi* with epixenosomes, were eaten by *Litonotus*, following a treatment with alloxan. Based on these results it can be inferred that the ejection itself is involved in the defensive function. Indeed a mild treatment with alloxan, an inhibitor of the enzyme adenylate cyclase, prevents the ejection of the epixenosomal extrusive apparatus (Rosati et al., 1997). Thus it is possible that the toxicyst discharge by the predator functions as the stimulus triggering the ejecting process; the ejected tubes might perturb the "toxic area" that surrounds the paralyzed prey thus hindering the finding and the ingestion by the predator (Fig. 5b).

The undoubtedly advantage conferred to *Euplotidium* by the presence of epixenosomes on its surface is very likely an important factor in stabilizing and maintaining such a specialized symbiotic relationship. This could also explain the apparent lack of non symbiotized specimens in the natural environment and the presence of epixenosomes on different *Euplotidium* species, even if the association has proven to be non vital, at least for the ciliate.

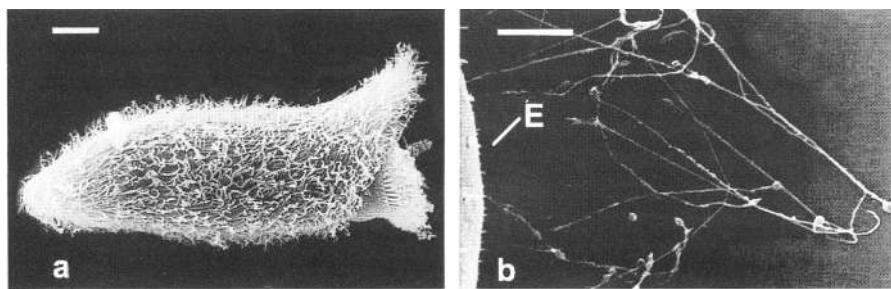


Fig. 5. From Rosati et al. (1999) a) *Litonotus lamella* that has engulfed *Euplotidium itoi* without epixenosomes. Scale bar 20 μm b) *Euplotidium itoi* (E) with ejected epixenosomal tubes nearby. Scale bar 10 μm .

5. Ectosymbiosis of unknown nature and significance

The list of ciliated protozoa that harbor ectosymbionts was extended by Epstein et al. (1998) who described the association between the microaerophilic ciliate *Geleia fossata*, living in marine sediments, and rod-shaped bacteria. From $2\text{-}10 \times 10^3$ bacteria per ciliate were always positioned on the ciliate surface, apparently in spatial association with dikinetids. In many cases there were 2 or 3 bacteria per kinetid; they appeared to form imperfect vertical rows. All the bacteria observed at the transmission electron microscope were morphologically similar. The uppermost bacteria were placed in deep cell membrane invaginations, so that over half of the bacterium was below the ciliate surface. These bacteria were typically oriented at approximately 45° to the cilium axis and the cell surface. This distinct association pattern suggests that there is a close integration between bacteria and their host. Moreover epibiotic bacteria were also observed on the surfaces of other sediment ciliates from *Geleia* and other genera like *Loxophyllum*, *Tracheloraphis*, *Paraspardidium*. The bacteria found in *G. fossata*, the only ones studied at the electron microscopical level, do not possess either internal membranes typical of metanogens and nitrifiers or sulfur granules typical of sulfide-oxidizing bacteria. So it is not clear whether or not the symbionts are chemolithoautotrophs. A number of bacteria were observed in apparently membrane bounded vesicles positioned below the bacteria in contact with the outside of the host cell. However, as serial sections have not been examined, the possibility that these vesicles are in contact with the cell surface (i.e. that they are not completely internal) cannot be excluded. Moreover bacteria in stages of progressive digestion were never observed. So in all likelihood *Geleia* does not use bacteria as food as *Kentrophorus* does. At present the significance of these kind of symbiosis remains obscure. In any case, according to the authors, these findings indicate that epibiotic bacteria/protozoa associations are widespread in the marine benthic environment not only in anaerobic forms but in microaerophilic and aerobic species as well.

6. Conclusions

The picture of the ectosymbiotic associations between ciliates and bacteria that can be drawn from this paper is complex, although, in all likelihood, it is far from complete: other different, as yet undiscovered relationships may exist. From the ecological point of view these kinds of associations certainly contribute to the differentiation of the ecological niches of both partners and favor them in the struggle for survival. On the other hand the different degrees of integration between the eukaryotic host and its prokaryotic ectosymbionts (unspecific, specific, obligatory), demonstrated in the studies reported above, might be really indicative of the steps of the eukaryotic cell evolution preceding the internalization of a symbiont. The idea that the monopolization of the outside may be an important precondition for entry into the host body, has already been proposed (Smith, 1979). It appears now reinforced by some comparative 16S rRNA sequencing analysis. For example sulfur oxidizing, ectosymbiotic bacteria of marine sediment inhabiting nematodes were found at the base of two endosymbiotic bacteria groups (Polz et al., 1994).

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LIVING SANDS: SYMBIOSIS BETWEEN FORAMINIFERA AND ALGAE

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1. Introduction

Foraminifera belong to a unique lineage of marine protists which have granular reticulopods with bi-directional streaming. The majority of forms have durable calcareous or agglutinated tests which are abundant in the fossil record from the beginning of the Paleozoic. Some groups of modern Foraminifera are exceptionally large (10-100X larger than their ancestors) and these are all hosts to endosymbiotic algae. Although they are not as prominent in undersea vistas as are the giant clams and corals, larger foraminifera are quite abundant in the same tropical and semitropical habitats. Once you know about them, they are quite conspicuous, easily visible to the unaided eyes. They are spectacularly large considering the fact that they are protists. "Larger foraminifera" is a collective, rather than a taxonomic, term. These foraminifera share in common two characters: they are large (0.1-6 cm), often 10 times larger than their ancestors, and they form associations with endosymbiotic algae. In addition to their large size and great surface to volume relationships, larger foraminifera have developed complex internal structures, seemingly as adaptations to symbiotic relationships (Lee and Hallock, 1987). Modern larger foraminifera belong to seven families in two different orders (Table 1). Endosymbiotic algae are also found in five modern families of planktonic foraminifera (Lee and Anderson 1991).

It is reasoned by analogy that some groups of large foraminifera (fusulinids) which teemed in the middle and late Paleozoic seas were probably also hosts to algal symbionts. The identities of the algae that fueled putative symbioses in these ancient seas are not known. The largest of these pencil-shaped protistian giants grew to sizes >10 cm. The best known fossils are the coin-shaped and coin-sized nummulites which formed the limestone which was used to build the Egyptian pyramids. The longevity of the phenomenon of symbiotic associations by foraminifera as a group suggests that they are exceptionally good hosts for symbiotic algae. They are hosts to a wider variety of algal types than any other protist or invertebrate group in the sea. In today's seas different families of foraminifera are hosts to endosymbiotic chlorophytes, dinoflagellates, diatoms, and unicellular red algae (Table 1.). Red cyanobacteria have also been found as secondary symbionts. Several families have genera which practice kleptochloroplast retention of diatom plastids (Correia and Lee, 2000).

TABLE 1. Algal groups which form symbioses with contemporary large benthic tropical and semitropical foraminifera from shallow well illuminated seas.

H O S T	Order	Miliolida				Rotalida*		
		Soriticiae						
	Family	Alveolinidae	Soritiaceae	Peneroplidae	Archaiidae	Calcariidae	Amphisteginidae	Numulitidae
S Y M B I O N T	Diatoms	X				X	X	X
	Dinoflagellate		X					
	Cyanophytes		X					
	Rhodophytes			X				
	Chlorophyte				X			

*Five families of planktonic foraminifera host dinoflagellate and chlorophytic endosymbionts

2. What Biological Features Underlie The Predisposition of Foraminifera to Form Endosymbioses with Algae?

Food is captured in the spider web-like pseudopodial network of foraminifera. Algal husbandry microhabitats within cells are well separated from host digestive activities (Müller-Merz and Lee 1976, Lee and Hallock 1987). Cytochemical assays using naphthol AS-BL phosphate for the presence of acid phosphatase (Sigma #387A) on 14 species of foraminifera, some with symbionts, others without, showed that digestion begins in the pseudopodial web (Faber and Lee, 1991). Acid phosphatase activity was found in the web around the periphery of the foraminifer, near the apertures, or in the last few chambers. This digestive enzyme was never found near the location of the endosymbionts. If the basic multicameral (multiple chambers) nature of most foraminifera is regarded as a mechanism to separate cellular activities, (i.e. digestive functions and symbiosomes), then it is reasonable to argue that extracameral initial digestion, coupled with intracameral partitioning, could be a fundamental foraminiferal property. This has predisposed foraminifera toward the establishment and maintenance of those endosymbiotic algae which avoid initial external digestion.

The diversity of symbiotic types and the non-finical, or looseness of fit, relationships shown in some of the associations, are evidence that foraminifera are generally potentially good habitats for the establishment of symbiosis (Lee and McEnery 1983, Leutenegger 1984, Lee and Anderson 1991). The diatom-bearing hosts have been the most extensively studied in this respect. Including the diatoms isolated from Caribbean hosts (Lee et al 1995b), the results of isolations from almost 3,000 hosts have been published (Lee et al 1980a & b, 1989, 1992, 1995b). While two species, *Amphistegina lessoni* (681 individuals) and *A. lobifera* (975 individuals), made up more than half of those sampled (60.4%), significant numbers of 10 other diatom-bearing hosts [*A.*

gibbosa (50), *Heterostegina depressa* (313), *H. antillarum* (18), *Borellis schlumbergi* (65), *Operculina ammonoides* (77), *Neorotalia calcar* (105), *Calcarina spengleri* (37), *C. defrancei* (51), *C. gaudichaudii* (167), and *Baculogypsina sphaerulata* (170)] were also sampled. The relationship between host species and endosymbiotic diatom species is not finical. Any of several dozen pennate diatoms were found in individual host specimens; however, 6 species, *Nitzschia frustulum* var. *symbiotica* (Fig 1A.), *N. laevis*, *N. panduriformis* var. *continua*, *Fragillaria shiloi*, *Amphora roettgeri* (Fig. 1B), and *A. erezi*, accounted for 75% of all of the associations in various hosts. Many individual hosts harbored more than one species of diatom at the same time.

In the Gulf of Eilat, Red Sea, significant numbers of *N. frustulum* var. *symbiotica*, *N. laevis*, *N. panduriformis* var. *continua* were found in hosts harvested at every depth. However, other diatom species were more commonly collected from shallow waters (e.g. *F. shiloi*) or deeper waters (>25m) (e.g. *Achnanthes maceneryae*, *Protokeelia hottingeri*). We have done several searches of the habitats of larger foraminifera looking for endosymbiotic diatom species (Lee et al 1989, 1992). One hypothesis was that the foraminifera were temporary hosts for the most abundant diatoms in their habitat. If that were true then we would expect a close correspondence between the endosymbionts isolated and the abundance of the same species in the habitat. That was not the case. Endosymbiotic diatom species (isolated from hosts harvested from the same place) were rare (<<1%) or absent from the habitat.

Several experiments have been aimed at testing whether the symbiotic diatoms in a host could be replaced by other species of endosymbionts (Lee et al. 1983, 1986). Specimens of *A. lessonii* were rendered nearly aposymbiotic by incubating them in sea water with DCMU (10^{-5} M} in tissue culture flasks in the high light (at 5m depth) in the Gulf of Eilat. After 5 days in the sea with DCMU, and no food, the foraminifera were fed mixtures of 10 different diatoms and chlorophytes. Each mixture of three species contained two species of endosymbiotic diatoms and a free living diatom isolate, or a chlorophyte. The foraminifera and the mixtures of algae were placed in flasks with fine membrane filters and incubated in the sea at normal depths (10m & 20m). After a week most of the foraminifera regained color. The results of the isolations from treated "rebrowned" foraminifera showed that some endosymbiotic diatom species were selected over, or were more competitive, than others. Many introduced symbiont species replaced the endosymbionts previously established within the hosts. Bleached controls, which were not fed, but which were incubated in the light, also "rebrowned" due to the regrowth of their original symbionts. In histological preparations, we observed that as many as 15% of the diatoms were in cell division at the time of preparation. None of the free-living algae incubated with the foraminifera survived within the hosts. At 10m depth *N. laevis* was co-dominant with *N. valdestriata* in the "pecking order"; at 20m *N. valdestriata* was dominant.

Although not as deeply explored, because taxonomic distinctions are more labor intensive, it is clear that there is considerable diversity in the algae in dinoflagellate-(Fig 2A-G) and chlorophyte-bearing hosts (Fig. 3A-C). The ssrRNA sequences we obtained from two different dinoflagellate-bearing hosts, *Amphisorus hemprichii* and *Marginopora kudakijimensis*, were not sister clades, but each was close to the endosymbionts from two very different coelenterate hosts (Lee et al. 1995a). A third sequence from a *Symbiodinium* sp. isolated from *Sorites orbiculus* was a sister clade to

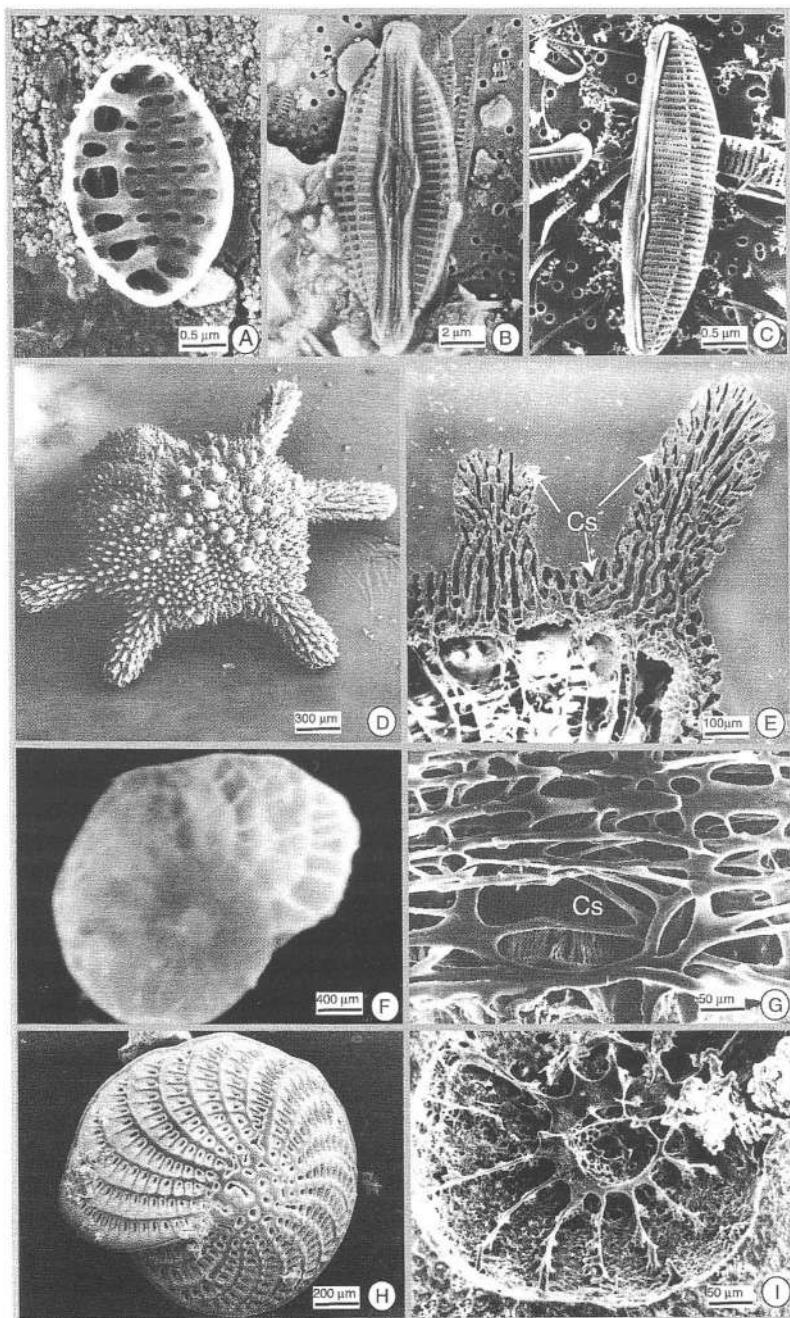


Figure 1 - Symbiotic diatoms and their foraminifera hosts. A - Most common endosymbiont, *Nitzchia frustulum symbiotica*; B - *Amphora roetgerii*. C - *A. tennerima*; D and E - *Calcarina gaudichaudiai* and it's marginal canal system. F and G - *Hesterostegina depressa* and it's canal system. H - *Elphidium crispum*. I - Canal system of *Elphidium* sp.. Cs=Canal system.

the *M. kudakajimensis* isolate (Langer and Lipps, 1994). Lee et al. (1995a) speculated that the later evolving soritids did not co-evolve with their zooxanthellae but acquired them from environmental pools contributed by other hosts with zooxanthellae. If they co-evolved with their zooxanthellae one would expect that the ribosomal sequences would be sister clades, closely related to each other. This does not imply that the soritids did not continually have zooxanthellae, only that some time in the past they acquired new unrelated zooxanthellae. This is easy to imagine because there is tremendous selection pressure for success; foraminiferal zygotes have to acquire fresh zooxanthellae, or perish. Our most recent research has shown that the same host species at the same location and at different locations can harbor more than one genotype of symbiont, but they all fall within the clades particular to foraminiferal zooxanthellae. These are sister groups to the coral zooxanthella clades but interestingly they are not co-mingled. This suggests that there is some genetic distance between the symbionts of both types of hosts (Pawlowski et al. submitted). Belasky (1996) postulated that the same factor, temperature, seems to control the distribution of both the scleractinian corals and the larger foraminifera in the Indo-Pacific. If the present is a key to the past, then it is reasonable to believe that these two unrelated groups and their dinoflagellate endosymbionts have been similarly linked in the past. Molecular comparisons of *Chlamydomonas hedleyii* and *C. provasolii*, two endosymbiotic-symbiotic chlorophytes from the family Archaiidae (our isolates), were also very distantly related (Bucheim, personal communication). This finding entreats further isolations of the symbionts in chlorophyte bearing hosts to see if they too have less finical relationships.

Flexibility in acceptance of different potential endosymbionts by some larger foraminifera helps to explain the long and continued evolutionary success of the group. It is an adaptive mechanism to exploit new habitats, and remain in changing habitats. Our conceptual framework for the evolutionary development of the endosymbiotic phenomenon in the foraminifera is that they did not co-evolve with their endosymbiotic algae because they are better off by not doing having such a tight relationship. They mainly reproduce asexually a processes that insure transmission of endosymbionts for many generations. When they do reproduce sexually they better their chances for adapting to a broader range of environmental parameters such as illumination, temperature, by being able to enter relationships with alternative algae with differences in their environmental responses

Another factor favoring endosymbiosis, in as yet unexplained way, is an in-place signaling system in foraminifera which seems to alter the surface of all the types of endosymbiotic algae involved in the phenomenon. The algae fail to form envelopes typical of their kind when they are in their hosts (Leutenegger 1977, 1984, Müller-Merz and Lee 1976, McEnery and Lee 1981). Fortunately, when the algae are released from their hosts into suitable culture media, they form envelopes. In the case of the diatom endosymbionts, they form frustules, which makes identification a relatively straightforward task.

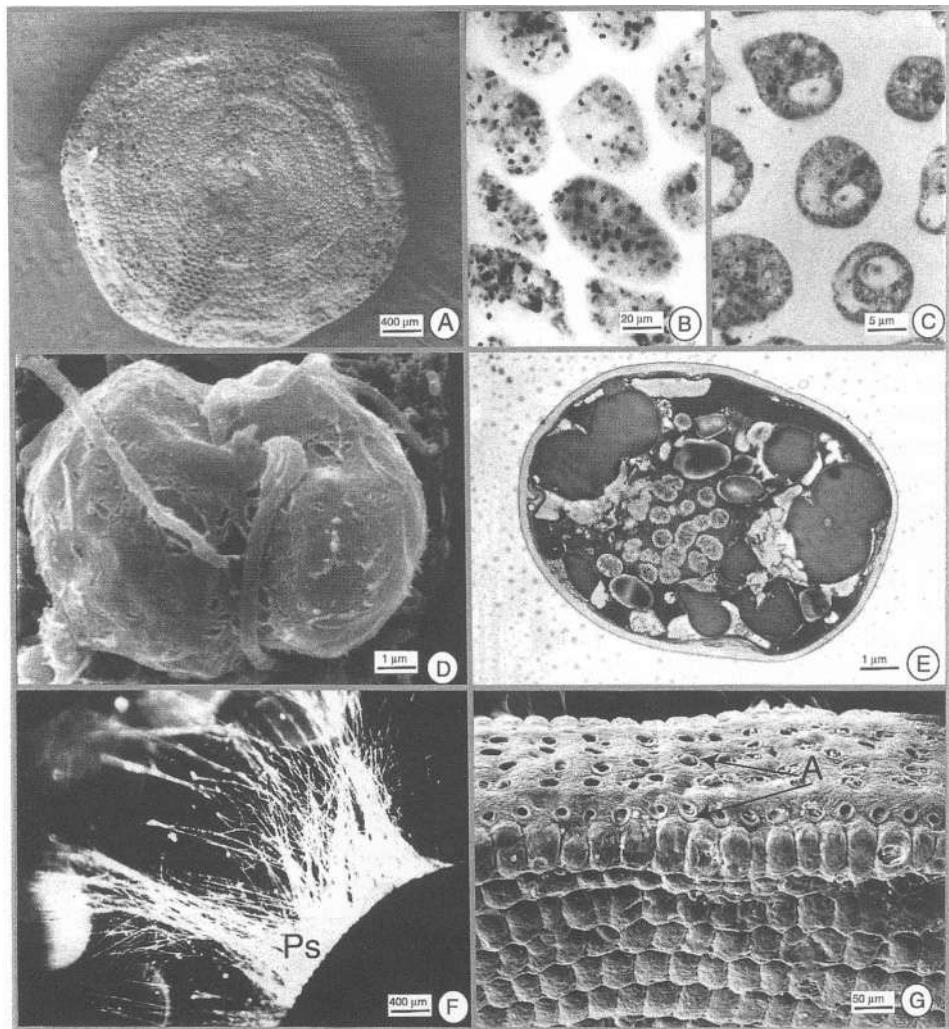


Figure 2 - The foraminifer *Amphisorus hemprichii* and it's dinoflagellate symbiont. A- *Amphisorus hemprichii*. B and C - Dinoflagellate symbiont within *Amphisorus hemprichii*. D and E - Dinoflagellate symbiont in culture isolated from *Amphisorus hemprichii*. F - Pseudopods emerging from apertures in *Amphisorus hemprichii*. G - Apertures in the test of *Amphisorus hemprichii*. Ps=Pseudopods; A=Apertures.

3. New Characters Developed by Larger Foraminifera

It is hard to make generalizations which are appropriate for all larger foraminifera. Some living sands are quite simple externally and internally (e.g. *Peneroplis* Fig.3D). However, the morphological complexities of some taxa are remarkable! Certainly few would argue that these symbiont-bearing foraminifera are among the most morphologically intricate protists known. In many cases, we observe test complexity which we interpret as adaptations to symbioses (reviewed in Hallock et al. 1991). For example, the interior of the pores of *Amphistigina* sp. are expanded to form little cups, each of which serves as a socket to hold a single endosymbiotic diatom. Although there are membranes at the bottom of the pores, in addition to the cell and symbiosome membranes separating the diatoms and the sea, each symbiont has almost a direct pipeline to a fresh supply of sea water with its nutrients and dissolved gases. A marvelous casting technique developed by Hottinger has given us a tool to study the cytoplasmic connections and test structures in three dimensions (Hottinger, 1979, Hottinger and Leutenegger, 1980). The casting technique has shown us that the shells of "star sands" and nummulites are honeycombed with chamberlets and very complex canal systems (Figs 1 D-G.). Although it is reasonable to believe that they serve to increase the circulation of sea water to and from the symbionts, carefully designed experiments to test this idea have not yet been done. The adaptations can also be viewed as mechanisms for compartmentalizing cellular activities. There is some variation even among genera of the same family. *S. marginalis*, for example, is quite regionalized. It is disc-shaped with concentric rings of chamberlets. The outer zone has digestive vacuoles, symbionts are restricted to the middle zone, and the micronuclei (generative nuclei), which give rise to the next generation, are found in the center of the disc (embryonic chambers) (Müller-Merz and Lee, 1976). In contrast, the symbionts of the even more complex *M. vertebralis* in the same family, are distributed throughout their host (Lee et al 1997).

4. Algal Characters

4.1 LIGHT

Field observations and experiments with intact host-symbiont systems showed that there are ranges of light intensity which play an important role in the associations (Lee et al., 1980 b, c, Röttger 1976, Hallock, 1981). The photosynthetic and growth responses of four symbiotic species grown in axenic culture, *N. valdestriata*, *N. laevis*, *N. panduriformis*, and *F. shiloi*, have been studied (Lee et al. 1982). Two of the species, *F. shiloi* and *N. laevis*, isolated from *A. lessonii*, grew best at high light intensity ($312 \mu\text{Wcm}^{-2}$), while the other two isolated from *H. depressa*, a deep water species, or one which is found in shaded places, grew best at lower light levels ($175 \mu\text{Wcm}^{-2}$). Photocompensation rates approximated 2% of the light level measured in spring in the sea at a depth of 1m. If the algae were free, the photocompensation depth would be reached between 40-50m in the sea at Eilat

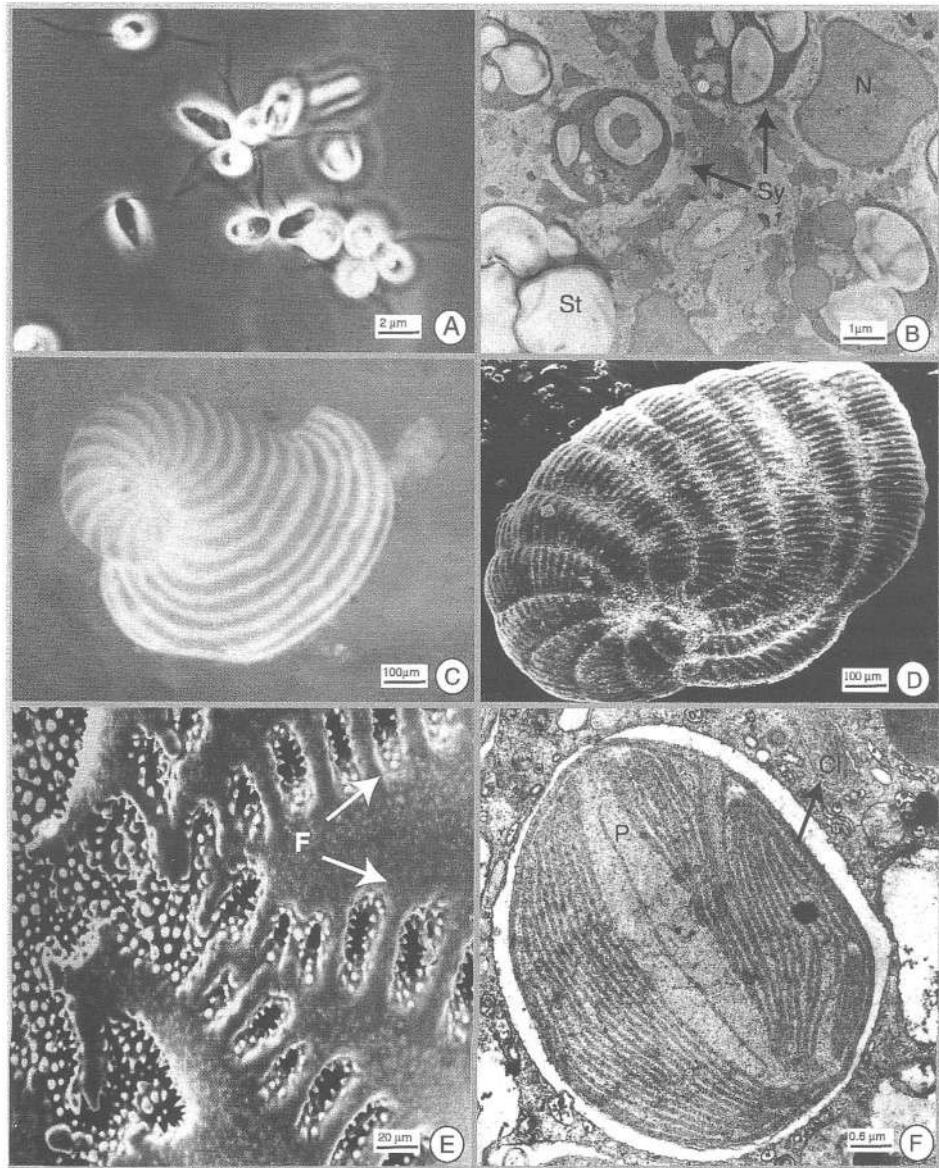


Figure 3 A- *Chlamydomonas hedleyii* in culture. B - *Chlamydomonas hedleyii* inside the host cytoplasm. St=Starch, Sy=Symbiont. C-*Archaias angulatus* host of the above. D - *Peneroplis planatus* host of a red algal symbiont. E - *Elphidium incertum* host of the retained chloroplasts showing fossae (F) with denticles. F - Retained chloroplast within the cytoplasm of *Elphidium*. Cl=Chloroplast, P=Pyrenoid.

4.2 CELL ENVELOPE ANTIGENS

Since initial steps of digestion were shown to begin in the reticulopodal web shortly after contact with potential prey, and since, in the case of the diatom-bearing hosts, many different species could be endosymbiotic in the same host species, it seemed reasonable to look for potential host signaling molecules in endosymbiotic diatoms. Symbiotic diatoms were disrupted by sonification. Their frustules were separated from the rest of the cell fractions by centrifugation, washed several times and then injected as antigens to raise polyvalent antibodies in rabbits (Lee et al., 1988a). The antibodies were tested against a series of diatoms, some of which were symbiotic, others of which, were not (Chai and Lee 1999). A 66 kDa protein was found in the frustules of all the diatoms tested; a 104 kDa protein (CSSA, common symbiont surface antigen) was found only on the surfaces of the endosymbiotic diatom species. Diatoms, labeled by growing them in media with $\text{Na H}^{14}\text{CO}_3$ as a tracer, were incubated with one or the other of the two proteins. The diatoms were washed free of unreacted antibodies and then fed to *A. lobifera*. Unreacted diatoms were used as a control in these blocking experiments. More symbiotic diatoms were digested when the foraminifera were fed organisms which had their surfaces blocked with the 104 kDa antibody, than untreated controls, or those treated with the 66 kDa protein. Receptors which recognize the CSSA have been found on the surface of the reticulopodia (Chai and Lee 1999b) and the CSSA also seems required for the maintenance of the relationship (Chai and Lee 2000).

4.3 NUTRITION

There have only been a few studies on the nutrition of the endosymbiotic algae isolated from foraminifera. In axenic culture seven species of endosymbiotic diatoms (*N. panduriformis*, *N. laevis*, *N. valdestriata*, *N. frustulum*, *A. tenerima*, *Navicula reissi*, and *F. shiloi*) required thiamin (Lee et al., 1980a). Biotin stimulated the growth of six species. Only one clone of *N. frustulum* required vitamin B_{12} . We speculate that the requirements for these vitamins is satisfied by the food organisms eaten by the host. Optimal concentrations of NO_3^- varied among the species tested ($0.2\mu\text{M}$ - 2mM), but all grew best at level of NO_3^- which exceeded by several orders of magnitude the levels found in the Gulf of Eilat ($1\mu\text{g l}^{-1}$), where the hosts were captured.

Nutritional studies of *C. provasolii* from *Cyclorbiculina compressa*, *C. hedleyi* from *Archaias angulatus* and *Symbiodinium* sp. from *S. marginalis* gave very similar results (Lee et al. 1974, 1979). Growth of *C. hedleyi* was tripled in the presence of thiamin, but it did not have an absolute requirement for this vitamin. Biotin also stimulated growth when the medium was also supplemented with several amino acids. Growth was twice the level when urea ($20\mu\text{M}$) was used as nitrogen source than when NO_3^{1+} or NH_4^{1+} ($20\mu\text{M}$) were used as nitrogen sources. Optimum phosphate concentrations (0.1 - $1\mu\text{M}$) varied with the nitrogen source used. The growth of *C. provasolii* was tripled in the presence of vitamin B_{12} and doubled in the presence of biotin. *Symbiodinium* sp. from *S. marginalis* had absolute requirements for vitamin B_{12} and thiamin. Both of these latter symbionts from Key Largo Sound, Florida, required very high optimum levels of nitrogen ($\text{NO}_3^{1+}/\text{NH}_4^{1+}$; 0.2mM) and phosphorus (PO_4^{3-} ; 0.1mM).

4.4 UNIQUE MORPHOLOGICAL CHARACTERS

In addition to the fact that many of the endosymbiotic diatoms are new species (e.g. *F. shiloi*, *N. hanseniana*, *A. roettgerii*), and new genera (*Protokeelia* and *Canopioiphorum*), a number of the diatoms found as endosymbionts have unusual characteristics. Presumably, due to the fact that the very small sizes of these pinnate diatoms fall below the generally accepted limits for sexuality and auxospore formation in the diatoms used as models for this phenomenon (Geitler 1932). *N. muscatini* which grows as a naked frustule-less zooxanthella in its foraminiferal host has an unusual life cycle in culture which includes large multinucleate cells. Infundibuliform frustules were formed in primary isolation cultures of *F. shiloi* isolated from hosts collected at two Pacific habitats (Lee 1996).

5. Host-symbiont interactions

5.1 LIGHT-CALCIFICATION/GROWTH

How does light exert its effects on the host / symbiont system? The most obvious answer is to consider photosynthesis by the symbiotic algae. The potential is large. In some oligotrophic habitats (e. g. Gulf of Eilat, Red Sea) there is often more chlorophyll in a single 1-3 mm diameter larger foraminifer than in the phytoplankton in a cubic meter of sea water above it. ^{14}C tracer techniques have been used to study rates of primary production in two species of *Amphistegina* (Muller 1978, Hallock 1981). Rates of primary production were quite high ($2\text{-}3 \times 10^{-5}$ mg C/H/foraminifer). At Lizard Island, Great Barrier Reef, *M. vertebralis* fixed $0.05 \text{ ng C min}^{-1}$. (Smith and Wiebe, 1977). One species of "living sand", *H. depressa*, survives and grows well in the light in the absence of any obvious concentration of food (Röttger, 1976). The other host/symbiont systems studied, *Peneroplis* spp., *Amphistegina* spp., *A. hemprichii*, require food, as well as light, for growth (Faber and Lee, 1991, Lee and Bock, 1976, Lee et al., 1988b).

Another advantage conferred by the symbiosis to larger foraminifera is enhancement of growth. For example, the calcification rates of three Caribbean soritid species, *A. angulatus*, *C. compressa*, and *S. marginalis*, are two to three times greater when incubated in the light than in the dark. In these species, calcification rates are proportional to light intensity in the range of $0\text{-}200 \mu\text{Em}^{-2} \text{s}^{-1}$ (Dugay 1983). At light saturation the total carbon fixed into the organic fractions of *A. angulatus* was $170 \text{ ng C mg dry weight}^{-1} \text{h}^{-1}$ (Dugay and Taylor 1978). Erez, ter Kuile, and collaborators have studied the details of the uptake of inorganic carbon and calcium in *A. lobifera* and *A. hemprichi* (review, ter Kuile, 1991; and ter Kuile and Erez 1987; ter Kuile et al. 1987, 1989 a, b). They found evidence for differences in the uptake, kinetics, and internal cycling of carbon in these two larger foraminifera. Using pulse-chase tracer experiments they demonstrated the transfer of photosynthetically fixed carbon into the shell of *Amphistegina lobifera*, but not into the shell of *Amphisorus hemprichi*.

The importance of calcification in the overall carbon budget varies with the growth stage (ter Kuile 1991). Young perforate foraminifera use a greater proportion of incoming inorganic C for building their tests than do older specimens. In *A. hemprichii*, which was used to model imperforate larger foraminifera, younger specimens had slightly higher ratios of calcification to photosynthesis. In older specimens the reverse was found (ter Kuile 1991). Carbon budgets have been calculated for *A. lobifera* and *A. hemprichi* (ter Kuile 1991) which serve, respectively, as good models for carbon cycling in perforate or imperforate species (ter Kuile 1991).

5.2 LIGHT-BEHAVIOR

Several simple experimental studies have demonstrated phototaxis in larger foraminifera. *A. lessonii* was phototoxic at photonic fluxes of 10^{11-12} and unresponsive at lower light levels (Zmiri et al., 1974). *A. lobifera* was positively phototactic at an incident illumination of 0.1-1 klx and negatively phototoxic at higher light levels (Lee et al., 1980a). *A. hemprichi* was positively phototoxic at some point between 6 klx and 11 klx but photoinhibited above 22 klx. This latter organism, which sets up feeding territories in cultures, overcame its territorial behavior in its phototoxic responses (Lee et al., 1980a).

5.3 NUTRIENT TRANSFER

There is very little evidence on nutrient transfer from symbionts to hosts. It was found that *C. hedleyi* freshly released from their host, *A. angulatus*, were filled with starch grains. By TEM examinations, similar starch grains were also found in the host cytoplasm. One could speculate that the algae undergo autolysis, or that the host causes the algae to be digested, or lysed, but static pictures have not yet given us an answer. It is also possible that *in situ* *C. hedleyi* releases soluble metabolites. In $H^{14}CO_3$ -labeled media axenic *C. hedleyi* released large quantities of mannitol (Lee et al. 1974). More fixed carbon was found in the medium (57%) than in the cells (43%). Kremer and co-workers (1980) also used tracer labeled ($H^{14}CO_3$) media to study the photosynthetates and their products in six larger foraminiferal associations. They identified floridoside and polyglucan in extracts of the rhodophyte-bearing *P.s arietina* and *P. pertusus*. They found that 74% of the photosynthate in the dinoflagellate-bearing *Amphisorus hemprichi* was in unspecified lipids and 3.5% was in glycerol. In the diatom-bearing *A. lessonii*, *A. lobifera* and *H. depressa*, a large percent of the label also was found in lipids (31, 51, 33% respectively) and as glycerol (5, 6, 11% respectively). More refined techniques would be needed to locate the labels in either the algae and/or their hosts.

In pulse chase experiments, radionuclide (^{14}C) labeled algae provided evidence of transfer of C ingested in food to the symbionts (Lee et al. 1988a). After feeding with tracer-labeled food the foraminifera, *A. lobifera* and *A. hemprichi*, were incubated for 24 h with cold food and then fixed and prepared by standard histological methods. The sections were stained, dipped in radioautographic emulsion, and incubated in the dark. Label was found in food vacuoles, in residua (feces), and cytoplasm and in the symbionts. This type of experiment can be interpreted only in a general way. Carbon (in some form[s]) flows from the food organisms, through the host, to the symbionts. The

label could have been catabolized and respired to CO₂ before it reached the algae and/or it could have been organic molecules released from food vacuoles and taken up from the host's cytoplasm by the algae.

Sterile host homogenates (Lee et al 1984) increased the levels of photosynthate release by diatoms to their medium. *N. valdestriata*, released 76% of its photosynthate to the medium in the presence of host homogenate. The least affected species released about half (36%) as much. The concentration of photosynthates was too low in the medium to be identified by thin layer chromatography. Next steps would be separation and identification of the active factor(s). Without further work, we remain cautious about implying that the release of photosynthates, in the presence of homogenate in the experiment, indicates some kind of an integrating mechanism peculiar to the symbiotic relationship. We can imagine quite a number of physiological conditions and surface active molecules which could induce rapid leakiness by alteration of cell membrane properties. Even more subtle factors might be operating in the host *milieu*.

5.4 ENVELOPE CHANGES

In a series of experiments we exposed axenic log phase cells of endosymbiotic diatoms to filter sterilized homogenates of crushed *A. lobifera* and *A. lessonii* (Lee et al 1984). To various degrees, depending upon species, the host homogenate affected the formation of new frustules of growing and dividing cells. *F. shilo* was the most sensitive species to the homogenate. New cells were spherical with no, or little, vestiges of a frustule. The inference is that host "substances" are probably responsible for the maintenance of the frustule-less state *in vivo* and if ingested potential endosymbionts escape digestion, they could become frustule-less after growth and cell division. This hypothesis, however, needs a rigorous test. Some work in progress in our laboratory suggests that the frustules of ingested symbionts may be resorbed. A fine structural examination of *Peneroplis* with their endosymbiotic *P. purpureum* seemed to suggest that the sheath of the red alga was digested as it was being formed (Lee 1990). These symbionts, however, are quite unusual because they are the only foraminiferan endosymbionts which lie naked in the cytoplasm of their hosts; they are not surrounded by a symbiosome membrane.

6. Chloroplast Husbandry

Three families of foraminifera, Elphididae, Nonionidae, and Rotaliellidae have members that retain large numbers ($\sim 1 \times 10^{2-4}$ cell⁻¹) of functional chloroplasts that they capture from partially digested food (Fig. 3F; Lopez 1979, Lee and Lee 1990.). Tracer labeling studies using ¹⁴C_O₂ to measure primary production by the chloroplasts suggested that *Elphidium williamsoni* was fixing carbon at a rate of 2.3 µg C mg ash-free dry weight⁻¹ h⁻¹ and *Haynesina germanica* fixed at a rate of 0.5. µg C mg ash-free dry weight⁻¹ h⁻¹ (Lopez 1979). Specimens of *E. crispum* from the Red Sea fixed dry 1.5 µg C mg weight⁻¹ 48 h⁻¹ (Lee et al., 1988c). Fine structural studies and pigment analyses suggest that the chloroplasts are derived from diatoms (Lopez, 1979, Knight and Mantoura, 1985, Lee et al 1988c, Correia and Lee, 2000).

Many questions arise about the nature of the chloroplasts which are retained by foraminifera. Are the chloroplasts of all algae equally viable in the foraminifera? If not, why? How long do chloroplasts last after they have been captured? Are there any differences of the phenomenon in different hosts? These questions have barely been explored. The results of a HPLC study of *H. germanica* suggested that there was very little digestion of the chloroplasts because little phaeophytin was found (Knight and Mantoura, 1985). They reasoned that if a large proportion of the chloroplasts were being digested, or autolysing, all the time, they would have detected this by the presence of a high percentage of degraded pigments (phaeophytin). In a study of *E. williamsoni* and *H. germanica* from Limfjörden, Lopez (1979) estimated that under normal light and dark conditions individuals of the former species needed to capture at least 65 chloroplasts h^{-1} while individuals of the latter needed to capture only 20 chloroplasts h^{-1} . Using an approach of feeding and then starving, Lee and Lee (1990) made some calculations on the turnover time of the chloroplasts of *H. germanica* and several species of *Elphidium*. When *H. germanica* was starved and incubated in the dark it survived only 9 weeks, but when it was starved and incubated in the light it survived 13.5 weeks. In the dark there was a steady decline in the number of chloroplasts; the chloroplast half-life was estimated as 2 weeks. In the light the loss of chloroplasts in starved individuals was more gradual than in the dark. There was an initial drop in chloroplast number to half by 6 weeks, after which the loss became more gradual. In the dark starved *E. crispum*, from Drake's Island, rapidly lost chloroplasts ($T_{1/2} \sim 3$ weeks), and all perished after 10 weeks. In the light there was a biphasic curve with an initial rapid loss ($T_{1/2} \sim 3.5$ weeks) followed by a second slower phase ($T_{1/2} \sim 10$ weeks). Feeding experiments with different types of algae showed that only chloroplasts from diatoms are retained (Correia and Lee, 2000).

There seems to be an unresolved paradox about the morphology of the chloroplast husbanding foraminifera. The morphology of the tiny rotaliellids is quite ordinary. On the other hand, the elphidiids and nonionids are quite modified. Their apertures are highly modified as are their sutures (junction of chambers). Large funnel-like fossettes lined with denticles are found in the sutures of *Elphidium* spp. (Figs 1H, 3E). The pseudopodia emerge through these orifices in the test, and it is believed that the denticles act like sieves to hold back diatom frustules while permitting chloroplasts to be drawn into the shell. The umbilical region of *H. germanica*, through which its pseudopodia emerge is also lined with denticles which might have the same function. Casts prepared by Hottinger's method are quite useful in demonstrating the canal system which is connected to the ends of the fossettes (Figs 11). The utility of this complex system needs an explanation, particularly since the rotaliellids are so simple.

7. Symbionts and Organelles

The last several decades have seen the development and general acceptance of ideas on the origin of eukaryotic cells through integration of endosymbiotic prokaryotic cells into new functional organellar units. Presumably the acquisition of new adaptive abilities is a powerful evolutionary leap which has very high selective power. In view of this, the question is why have the intracellular algal symbionts in larger foraminifera not become

completely integrated with their hosts to become organelles? This would be of particular advantage to organisms which, although reproducing asexually, do have a sexual phase in their life cycles. One could argue that the barrier to the next step of integration could be the presence of the symbosome vacuolar membrane around each symbiont. When this membrane is lost, the potential for lysosomal fusion with the vacuole is lost and exchanges between compartments are facilitated. A possibility, yes, but not the whole answer. When we examined, for the first time, the red algal endosymbiont, *P. purpum*, inside the foraminifer, *P. planatus*, we were surprised. The symbiont is not surrounded by a second membrane (symbosome) outside its own cell membrane. The same is true in the other species of *Peneroplis* examined. Compared to its appearance in axenic culture, the envelope (sheath) of the alga in its host is drastically reduced to a thin layer of fibrils (Lee 1990b). Since these algae lie independently in the cytoplasm of their hosts, as do mitochondria or chloroplasts, we had to ask ourselves whether these strains of endosymbiotic *Porphyridium* have achieved organellar status, or not. As is true for all larger foraminifera, the hosts cannot live very long, grow, or reproduce, without their photosynthetically functioning algal symbionts. But, the reverse is not true. When the hosts are broken open, and *Porphyridium* are inoculated into simple media they grow rapidly and show no loss of vigor over many serial transfers. When simple inorganic nutrients are given to them, they do not seem to need their hosts. Others may argue differently, but I would argue that the minimal evolutionary step for a symbiont to become an organelle, is loss of some genes required by the symbiont, but present in the host's genome, or transfer of symbiont genes to the host's genome. Thus I considered the red algae inside the peneroplids to be symbionts and not organelles. This, of course, obfuscates the answer to the question. Perhaps the time scale, >40 million years, is too short for greater genetic integration. Given the changes observed in other host characteristics (shell and behavioral) this is hard to believe. Are there advantages to maintaining cellular independence which outweigh greater cellular integration? The answer to this question may have been given in an earlier section in this review. Less finical relationships between the host and its symbionts may be more adaptive to changing or new habitats. What advantages would there be if there were more integration? It is clear that larger foraminifera represent lines of evolution which led to increasing cellular complexity rather than multicellularity. Does this fact underlie the lack of greater cellular integration? Our failure to satisfactorily answer this basic cellular question, challenges us to probe it in new ways.

8. Association Regulation

This is a topic which has not been studied extensively in foraminiferal associations. Our studies of the mineral nutrition requirements of axenic cultures of endosymbiotic algae isolated from larger foraminifera suggested that the algae required levels of NO_3^- and PO_4^{3-} which are 100 to 1000 times higher than they could get if they were growing free in the Gulf. This explains why they are not abundant there. Although this has not been tested experimentally, it is reasonable to suggest that there is a benefit to the algae in the association. As the host feeds on bacteria and algae in its habitat, the symbiotic system

gains scarce nitrogenous and phosphorous compounds needed by both symbiont species. When we incubated several larger foraminifera (*A. lobifera* and *A. hemprichii*) in mineral nutritional experiments with levels of nitrate and phosphate which were optimal for their algae, the hosts and their symbionts died (Lee et al 1991b). In batch culture, optimum growth of the foraminifera took place in the light, if the hosts were feeding, if the levels of nitrate and phosphate were low, and if the medium was changed weekly. Similar results were obtained in chemostats. These results lead us to speculate that the regulation of the numbers of algal symbionts in their hosts may be dependent on transfer of nitrogenous and phosphorous compounds to them. This speculation may tie in with some field observations made at stations where domestic, and similar wastes, enter seas. While remains in the sediment suggest that living sands were once abundant at these stations, living larger foraminifera (work in progress) are no longer present in seriously affected areas. Our present interpretation is that the excess of nutrients at these locations has upset the balance, or regulation, of the host-algal symbiont relationships in these larger foraminifera.

9. Conclusions

Larger foraminifera are, in some respects, a high point in the evolutionary development of protistan cells. Their cellular functions are compartmentalized by rather complex morphological adaptations whose functions are really not well understood. Because they are filled with endosymbiotic algae, and can not live without them, we could, perhaps, regard them as a type of algal colony. This however, is a very unsatisfactory conceptual framework. Foraminifera have had a long and successful association with algae in well illuminated warm shallow seas. While we already know some aspects of these associations, most aspects of the phenomenon in these giant-sized protists remain unexplored in depth. The challenges are there waiting for us to explore them. The prospects of results of new probes are exciting to contemplate.

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PROTISTAN-PROKARYOTIC SYMBIOSSES IN DEEP-SEA SULFIDIC SEDIMENTS

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1. Introduction

Symbiotic relationships between prokaryotes and eukaryotes in the deep sea have been the object of intense study for the last two decades (e.g., Felbeck 1981, Sibuet and Olu 1998). The discovery of high concentrations of large animals living in close proximity to deep-sea hydrothermal vents was soon followed by the revelation that symbiotic relationships existed between bacteria and these metazoans (Cavanaugh et al. 1981). Chemosynthetic autotrophy supports these associations and involves the oxidation of hydrogen sulfide or methane by endosymbiotic bacteria housed within the animals. Identical or similar symbioses have been documented from other marine environments such as cold seeps (Barry et al. 1996) and the edge of silled basins (e.g., Distel and Felbeck 1988).

The study of symbioses between prokaryotes and protists in the marine environment has a long history (Fenchel and Finlay 1995, Ott 1996). These associations span a broad spectrum of metabolisms and physical associations from endosymbiotic methanogens in ciliates to epibiotic hydrogen-sulfide oxidizers on euglenoids. Although the majority of these associations are described from flagrantly reducing environments (Fenchel and Finlay 1995, Ott 1996), they are also recorded from more benign environments (Epstein et al. 1998). The shallow marine niches are well covered by the aforementioned references, however, the deep sea (>200 m) remains relatively unexplored for symbioses between protists and prokaryotes. The difficulties in sampling deep-sea environments and in separating organisms from the fine grained sediments (i.e., silts and clays) that characterize deep-sea sediments have been two factors conspiring against the broader recognition of the deep sea as an environment rich in symbioses.

Several recent works examine symbiotic relationships between protists and prokaryotes from the deep sea. An oxygen-depleted basin (Santa Barbara Basin, Bernhard et al. 2000) and cold seeps (Monterey Bay, Buck and Barry 1998, Buck et al. 2000, Bernhard et al. in press) from the California margin are the loci for these studies. These two environments share several attributes, namely depth greater than 500 m, high concentrations of mat-forming chemoautotrophic bacteria (e.g., Beggiatoaceae) and high concentrations of hydrogen sulfide. However, there are marked differences as well. The deepest part of Santa Barbara Basin totally lacks the megafauna (e.g. vesicomyid clams

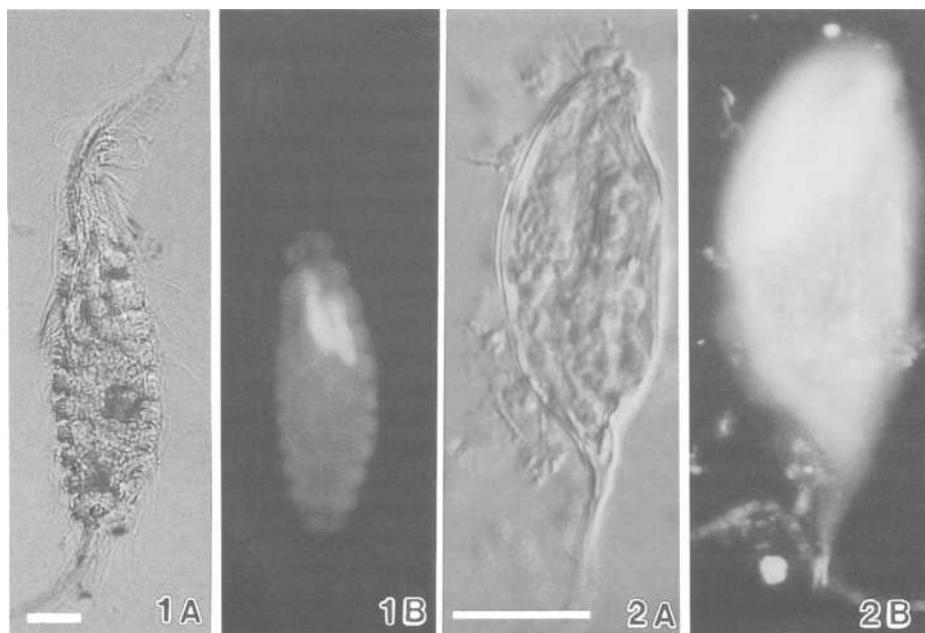
and others) that inhabit cold seeps but is characterized by a higher biomass and diversity of protists (Bernhard et al. 2000, Buck and Barry 1998). The vast majority of the protists from Santa Barbara Basin and most of those recovered from Monterey Bay cold seeps possess either epi- and/or endobiotic bacteria. We discuss our findings from these two environments, elaborating on the issues of diversity of protists with, and types of, symbiotic associations. We will present this information in a phylogenetic context, examining three major groups of deep-sea protists: euglenoids, ciliates and foraminifera.

2. Methods Employed

Establishing the existence of prokaryotic-protistan symbioses can be difficult because many protists are small (i.e., <50 μm) and many of the methods typically employed to definitively show metabolic exchange between host and symbiont can only be done with large amounts of material. To date, such approaches are not feasible because we do not have these organisms in culture. A variety of microscopy methods can, however, be used to establish prokaryotic-protistan associations. In particular, we employ electron microscopy as well as epifluorescence microscopy to elucidate such consortia. The fluorescent DNA-intercalating dye 4,6-diamidino-2-phenylindole (DAPI) is especially useful to illustrate associations between protists and prokaryotes. Because protists typically have one or only a few nuclei, the fluorescent signal imparted from eukaryotic DNA does not mask the prokaryotic contribution to the signal (Figs. 1, 2). Furthermore, DAPI is useful for identifying both epibionts and endobionts and can be employed to survey large numbers of specimens. Both scanning and transmission electron microscopy (SEM and TEM, respectively) are useful tools in identifying protistan-prokaryote associations (Figs. 3-14). While SEM is obviously useful to show relationships between host and epibiont, only TEM indicates sub-cellular morphology of the putative symbionts, whether epi- or endobionts.

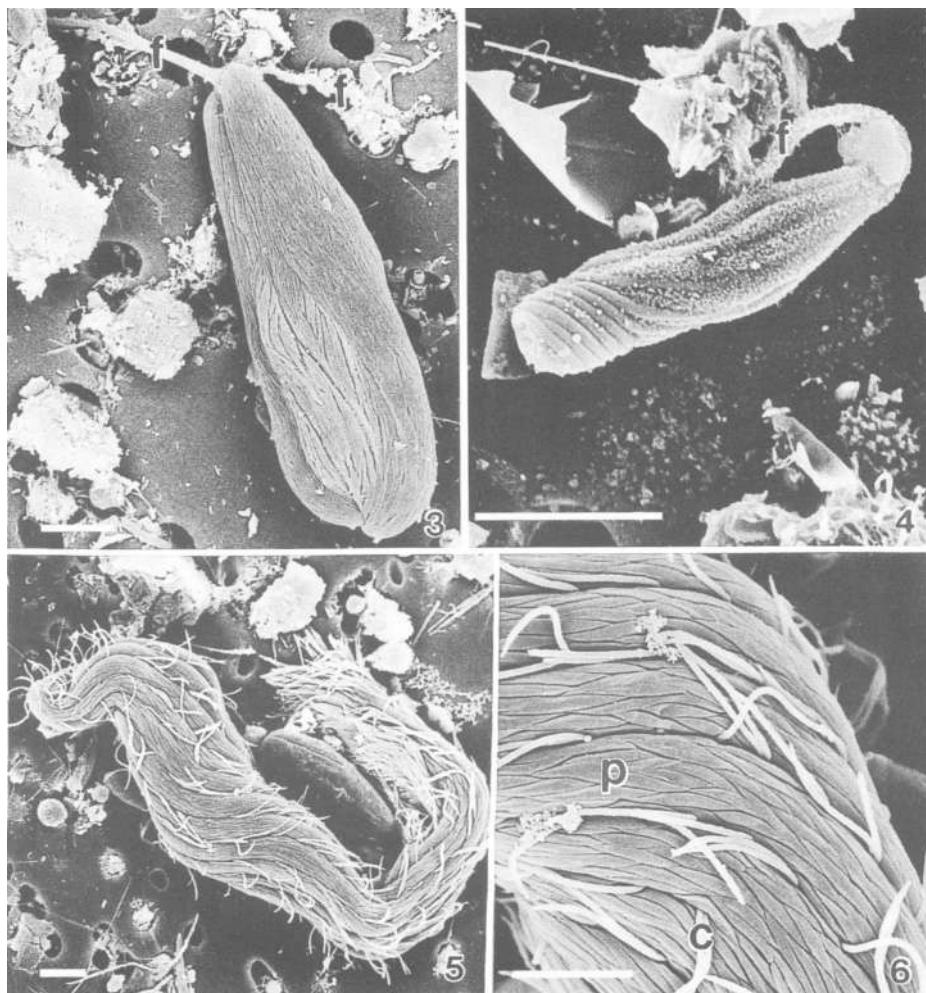
3. Euglenoids

Euglenoids are the most abundant protist found in deep-sea sediment. In Santa Barbara Basin their concentration exceeded 6×10^5 cells ml^{-1} in the top cm. Taxonomic identification relies, in large part, on the observation of living organisms, hence not much is known about the diversity of this group in the deep sea. Based upon morphology preserved in fixed material, however, it appears that a large number of taxa are present. Presently only epibiotic associations between euglenoids and prokaryotes have been documented. The prokaryotes are rod shaped, sometimes quite elongate and are arranged parallel to the cell axis in many cases (Fig. 3), however in other instances they follow the pattern of the pellicle imparting a spiral pattern (Fig. 4). The pellicle may be covered or the coverage may be limited to its posterior (Figs. 1, 3 & 4). Currently the only metabolic pathway attributed to the epibiotic prokaryotes found in association with marine euglenoids is chemoautotrophy (Buck et al. 2000). The presence of membrane-bound elemental sulfur spherules in the rod-shaped prokaryotes vesting euglenoids from the



Figures 1-2. Paired differential interference contrast (DIC) and corresponding epifluorescence micrographs of DAPI stained protists, showing associated epibionts. *Figure 1.* *Metopus verrucosus* from Santa Barbara Basin. Note transverse bands of epibionts as well as ciliate nuclei in B. *Figure 2.* *Calkinsia aureus* from the Soledad Basin, off Baja Mexico. Although the flagellate nucleus is bright, the veil of epibionts can also be seen in B. Scale bars = 10 μ m.

Monterey Bay cold seep is regarded as evidence that these are sulfur (presumably sulfide) oxidizers (Buck et al. 2000, Figs. 5 & 6). Sulfur oxidizing bacteria are commonly found as epibionts on a wide diversity of animals in the marine environment (Polz et al. 2000), however this was the first report on a marine flagellate. Other protists with epibiotic sulfur-oxidizing prokaryotes involve active grazing of the prokaryotic coat by the host (Fenchel and Finlay 1989, Bauer-Nebelsick et al. 1996) although this was not observed in the Monterey Bay case. It was speculated that local detoxification of the immediate environment surrounding the host might benefit the euglenoid. The symbiont would be provided a substrate for attachment and perhaps be exposed to varying concentrations of sulfide and the terminal electron acceptor (O_2 or NO_3^-), similar to what is believed to be the case in other symbioses of this type. Other possible metabolic pathways possessed by euglenoid symbionts might include sulfate reducers (see Ciliate section) and heterotrophic prokaryotes. Initial fluorescent *in situ* hybridization attempts with sulfate-reducing-group specific probes produced positive results on both filamentous free-living bacteria from Monterey Bay and for epibiotic bacteria on euglenoids from Santa Barbara Basin (unpublished data).



Figures 3-6. Scanning electron micrographs of protistan-prokaryote epibiotic symbioses from two deep-sea sulfidic sites in California. **Figure 3.** Euglenoid from Monterey Bay cold seep with rod-shaped prokaryotes completely covering the cell. **Figure 4.** Euglenoid from Santa Barbara Basin with rod-shaped prokaryotes lying in pellicular grooves at the posterior end of the cell. **Figure 5.** Ciliate from Monterey Bay cold seep with rod-shaped prokaryotes completely covering the cell. **Figure 6.** High magnification of specimen shown in Fig. 5 showing the prokaryote morphology and the pairing of cilia. f, flagella; p, prokaryote; C, cilia. Scale bars = 5 μm .

4. Ciliates

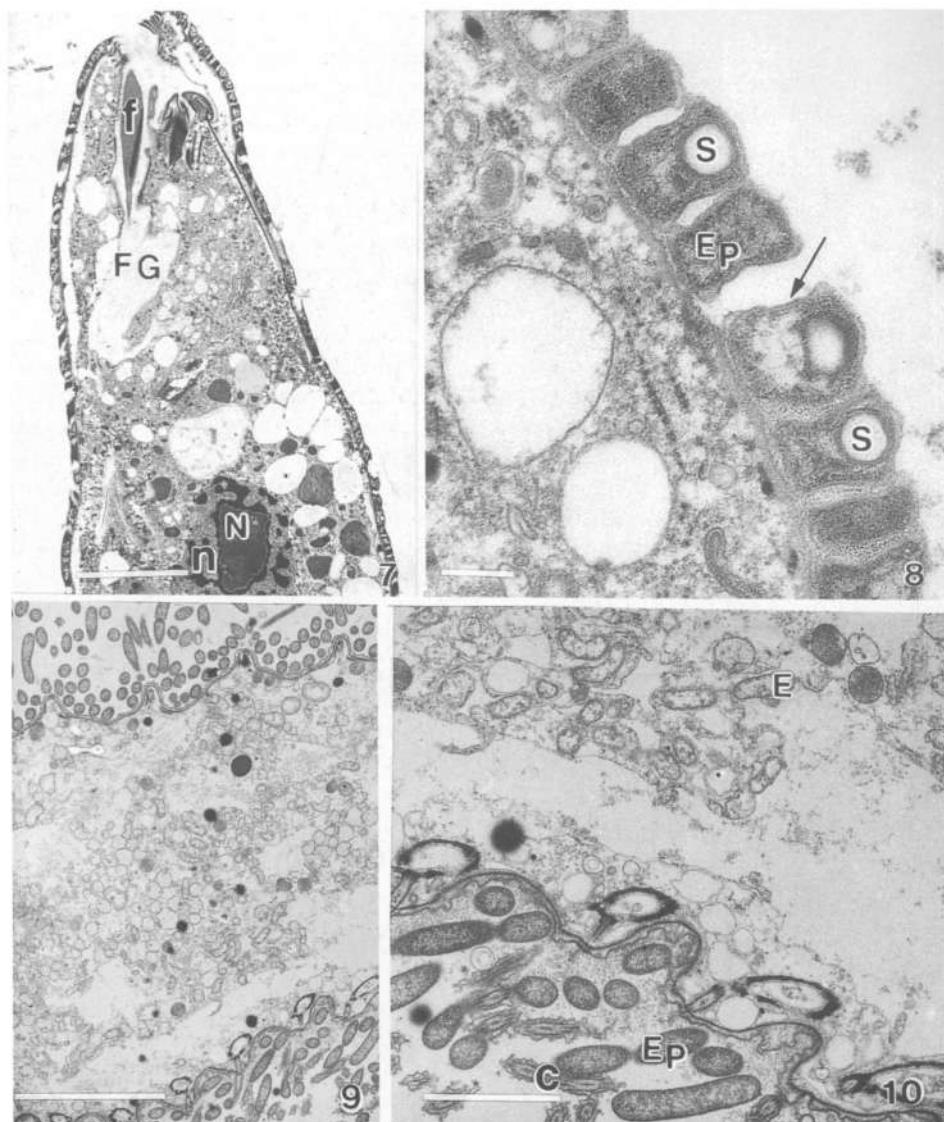
Ciliates are less abundant than euglenoids in the deep-sea sulfidic environments observed to date, however, their larger size makes their biovolume contribution to total meiofaunal biovolume comparable with euglenoids (Buck and Barry 1998, Bernhard et al. 2000). It has been predicted that ciliates would decrease in abundance as water depth increases owing to the concomitant decrease in both sediment grain size and the size of available pore space in deep-sea sediments (Fenchel and Finlay 1995). Although this prediction is validated for oxygen-replete sites that we have sampled, we observe an enhancement of ciliates in sulfidic sediments when compared to euglenoids (Buck and Barry 1998, Bernhard et al. 2000).

Ciliates are probably the best-documented group of marine protists that forms symbiotic associations with prokaryotes (see Fenchel and Finlay 1995). In shallow benthic environments, ciliates can possess epibiotic sulfide oxidizers (Bauer-Nebelsick et al. 1996) and sulfate reducers (Fenchel and Ramsing 1992) as well as methanogenic endosymbionts (Finlay and Fenchel 1993). The diversity of symbiont-bearing taxa/morphotypes is higher in ciliates than in the other two groups of abundant protists from the two deep-sea sites we have examined (Bernhard et al. 2000). The manner in which the epibiotic prokaryotes attach themselves to the protist also exhibits more variability than with euglenoids. The rod-shaped prokaryotes can either be tightly packed on the outer surface of the ciliate (Figs. 1, 5 & 6) as we have seen with euglenoids (Fig. 2) or they can be attached by their end to the cell surface of the ciliate (Figs. 9 & 10). We have not seen sulfur spherules inside any ciliate epibionts (Fig. 10) surveyed with TEM from either site although this has been reported for other environments (Bauer-Nebelsick et al. 1996). Ciliates from other sulfidic environments have associated epibiotic sulfate reducers (Fenchel and Ramsing 1992). A number of ciliates from both sites have been identified with endobionts (Bernhard et al. 2000, Figs. 9 & 10), however the classical methanogen-hydrogenosome complex depicted in many species of *Metopus*, for example, from shallow European sites (Fenchel and Finlay 1995) have not been seen in the TEM preparations we have surveyed.

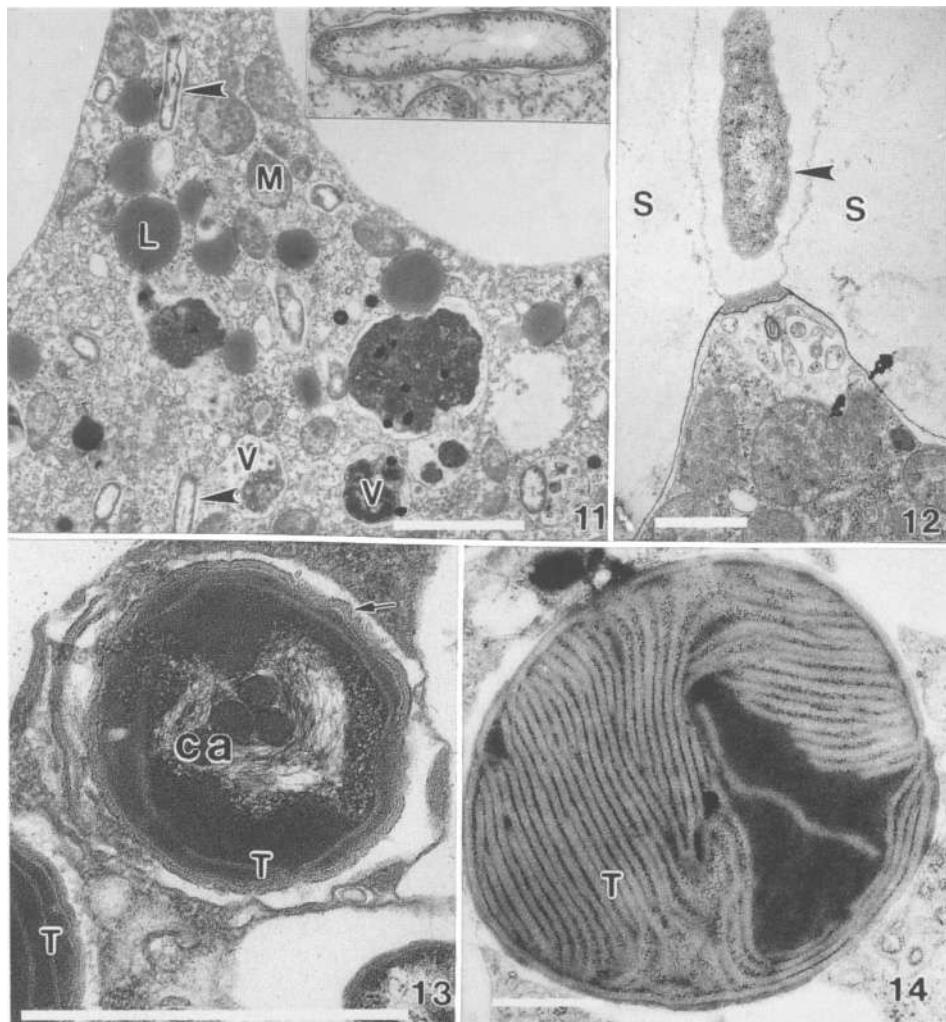
5. Foraminifera

Foraminifera typically comprise a large proportion of the eukaryotic biomass in some deep-sea sulfidic sediments (Bernhard et al. 2000). In fact, in the Santa Barbara Basin, foraminifera can be the dominant biomass contributor (Bernhard et al. 2000). Although typically outnumbered by other protists, foraminifera commonly outnumber metazoan meiofauna in certain sulfidic sediments (Gooday et al. 2000). For a recent review on foraminifera of oxygen-depleted environments, including sulfidic sediments, see Bernhard and Sen Gupta (1999).

To date, the foraminifera inhabiting sulfidic deep-sea sediments show inconsistency regarding symbionts. Monterey Bay cold seep specimens lack symbionts (Bernhard et al. in press), although one instance of epibionts was observed. In this case, rod-shaped bacteria were observed in the holes or pores of the calcareous shell of a *Uvigerina peregrina* (Bernhard et al. in press, Fig. 12). Abundant Santa Barbara Basin foraminiferal



Figures 7-10. Transmission electron micrographs of protistan-prokaryote epibiotic symbioses from two deep-sea sulfidic sites in California coastal waters. **Figure 7.** Anterior end of a euglenoid from Monterey Bay showing the continuous coat of prokaryotes and protistan organelles such as the nucleus, nucleolus and the flagellar groove, as well as a flagellum. **Figure 8.** High magnification of specimen shown in Fig. 7 illustrating the epibionts with their characteristic cell membranes (arrow) and sulfur spherules. **Figure 9.** A portion of a ciliate from Santa Barbara Basin with epibiotic and putative endobiotic prokaryotes. **Figure 10.** High magnification of specimen shown in Fig. 9 illustrating the epibionts, as well as endobionts and cilia. n, nucleus; N, nucleolus; FG, flagellar groove; f, flagellum; Ep, epibiont; S, sulfur spherule; C, cilia; E, endobiont. Scale bars = 5 μm for Figs. 7 & 9, 2 μm for Figs. 8 & 10.



Figures. 11-14. Transmission electron micrographs of foraminiferal symbioses from two deep sea sulfidic sites in California. **Figure 11.** *Buliminella tenuata* from Santa Barbara Basin. Note numerous prokaryotes, which can be distinguished from food vacuoles. Inset. Higher magnification view of intracellular prokaryote. **Figure 12.** *Uvigerina peregrina* from Monterey Bay cold seep showing prokaryote nestled in pore plug. The calcareous shell of the foraminifera would have been in the area denoted S. **Figure 13.** *Furciferia rotundata*, from Santa Barbara Basin with endosymbiotic *Synechococcus* sp. Cell membranes, parietal thylakoids and 4 carboxysomes are all visible. **Figure 14.** *Nonionella stella* from Santa Barbara Basin showing sequestered chloroplast. T, thylakoid; arrow, cell membrane; ca, carboxysomes; arrowheads, prokaryote; V, food vacuole; L, lipid; M, mitochondrion; S, shell. Scale bars = 2 μm for Figs. 11 & 12, 1 μm for Figs. 13 & 14.

species generally have endobionts, although these putative symbionts are of different types. The foraminifer *Buliminella tenuata* has rod-shaped bacteria throughout its cytoplasm (Bernhard 1996, Bernhard et al. 2000, Fig. 11); these prokaryotes are not in digestive or food vacuoles (Fig. 11). The identity of these prokaryotes is not known. It is interesting to note that *B. tenuata* living at Monterey Bay cold seeps do not have these rod-shaped bacteria (Bernhard et al. in press), suggesting that the relationship between host and prokaryote is not obligatory. Other cases of prokaryote-foraminiferal associations have been observed in shallower-water sediments that may have been sulfidic (i.e., *Globocassidulina* cf. *biora*, Bernhard 1993).

The second type of symbiosis observed in certain foraminifera from sulfidic sediments involves the plastidic prokaryote *Synechococcus* sp. (Bernhard et al. 2000). *Synechococcus* forms associations with heterotrophic protists in the upper water column (Buck and Bentham 1998) and we have now identified it with epifluorescence microscopy and TEM in the benthic foraminifer *Fursenkonia rotundata* from Santa Barbara Basin (Fig. 13). The phycoerythrin fluorescence, when excited in the blue or green portion of the spectrum, is very distinctive as are the parietal thylakoids and carboxysomes characteristic of actively metabolizing cells (Fig. 13). We have also observed sequestered chloroplasts in foraminifera from Santa Barbara Basin (reviewed in Bernhard and Bowser 1999). Although eukaryotic chloroplasts are obviously not prokaryotes, the relationship between foraminifer and chloroplasts is considered by some to be a special type of symbiosis (Leutenegger 1984, Lee and Lanners 1988), so we include a short discussion here. A variety of foraminifera sequester or "husband" chloroplasts from other eukaryotes (commonly diatoms, Leutenegger 1984); this association is not particularly noteworthy except that in at least two instances the plastid-sequestering foraminifer inhabits a sulfidic and presumably dark environment (i.e., *Nonionella stella* in Santa Barbara Basin, Bernhard and Bowser 1999, *Stainforthia fusiformis* in Norwegian fjords; Bernhard and Alve 1996). In the Santa Barbara Basin, the foraminifer *N. stella* dominates biomass and sequesters a plethora of diatom chloroplasts (Fig. 14; Bernhard and Bowser 1999). The water depth of this near-shore basin is ~600 m, therefore it is unlikely that enough sunlight filters through the water column to fuel photosynthesis by *Synechococcus* or the sequestered chloroplasts. It is possible that (1) a previously unknown dark reaction occurs in the cyanobacteria or chloroplasts or (2) a source besides the sun provides enough light for deep-sea photosynthesis. Ongoing research seeks to explain this "chloroplast conundrum".

6. Concluding remarks

The most abundant protistan groups (euglenoids, ciliates and foraminifera) from two deep-sea sulfidic sedimentary environments possess a variety of symbiotic associations with prokaryotes. These associations include both epibiotic and endobiotic relationships. To date, endobiotic symbioses in euglenoids are not well documented while numerous examples of endosymbioses exist in ciliates and foraminifera. In all protistan-prokaryote symbioses observed to date from these deep-sea environments the exact benefit to the protist as well as the prokaryote remains only speculative. Quite clearly the biomass

enhancement of these three groups in these deep-sea sulfidic environments, compared to nearby aerated sites is likely related to the rich and diverse symbioses we observe.

In addition to the questions of the nature of the host-symbiont relationships, future research will likely focus on the trophic mechanisms of the host (e.g., raptorial vs. epibiont phagocytosis) and symbiont metabolism (e.g., sulfur oxidizing, sulfate reducing, methanogenic). Only sulfur oxidizing epibionts have positively been identified with euglenoids when many other possibilities have been shown for ciliates.

Exploration of other deep-sea sulfidic environments for their symbiotic communities and their symbiotic consortia and utilization of molecular techniques such as fluorescent *in situ* hybridization are two promising approaches to resolving some of these questions. We occasionally observe symbioses between protists and prokaryotes in more aerated or less reduced sites in both shallow and deep marine settings (Epstein et al. 1998, Bernhard et al 2000). Are these relationships fundamentally different from those we see at sulfidic sites or are the organisms refugees from sulfidic microhabitats not normally observed by classic benthic ecological approaches?

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VIII. Symbiosis in Insects and Higher Animals

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SPONGE/ALGAL SYMBIOSES: A DIVERSITY OF ASSOCIATIONS

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1. Introduction to the Sponges

Fossil records, dating back to the Proterozoic era 1000 million years ago, show sponges to have been among the first multicellular animals. Since this time, members of the phylum Porifera have persisted as major elements of benthic aquatic fauna, without any apparent major advance in their body plan (Reiswig, 1973; Pavans de Ceccatty, 1974; Muller, 1982). All members of the phylum Porifera are sessile aquatic metazoans. The specialised body structure is built around a water canal system through which the surrounding water is circulated to obtain what is necessary for growth and reproduction (Pavans de Ceccatty, 1974). Sponges lack distinct anterior and posterior ends. They have neither true tissues nor organs and the cells display a considerable degree of independence, leading many scientists to question whether sponges are in fact true multicellular animals or merely aggregations of specialised unicellular organisms. In no other multicellular organism can the body be completely dissociated into component cells and then reform into a functioning unit (Humphreys, 1963).

Sponges make up an important part of most benthic marine communities wherever rocks, shells, submerged timbers, coral or even soft sand or mud provide a suitable substratum. They abound in all seas from the tropics to the poles, from shallow waters to depths of over 1000 m, in both well lit and dark habitats.

In tropical regions, the biomass, and ecological tolerances, of sponges frequently exceed those of the hermatypic corals (Rützler, 1978; Wulff & Buss, 1979). In addition, sponges have been shown to be important in the consolidation of rubble and sediments on coral reefs (Glynn *et al.*, 1972; Wulff & Buss, 1979; Wulff, 1984).

2. Sponges and Symbiosis

Symbiotic associations between animals and photosynthetic microorganisms are found in many marine invertebrate phyla and are particularly common in modern coral reefs (Vacelet, 1981). In both tropical and subtropical environments, sponges are frequently found in association with algae. Sponge/algal associations also occur in temperate waters but there are fewer, in terms of both diversity and abundance, than in the tropics. Sponge symbioses with bacteria are also widespread, and it has been suggested that all marine sponges contain non-photosynthetic symbiotic bacteria (Wilkinson, 1984).

Symbiotic bacteria are found in varying abundances in marine sponges; in some sponges they occupy less than 1% of the tissue volume of the sponge, whereas in others, they may occupy up to 40 % of the tissue volume. Consequently, symbiotic bacteria are presumed to have varying levels of importance in the physiology of different sponge species. Bacteria may contribute to sponge structural rigidity, particularly if the bacteria produce sticky, mucoid colonies (Wilkinson *et al.*, 1981). It has also been suggested that large populations of symbiotic bacteria within sponges may take up dissolved organic matter from seawater, thereby filling any shortfall in the nutritional requirements of the sponge if insufficient food is obtained through filter feeding. However, it is not known if sponges are able to utilise any dissolved organic matter taken up by symbiotic bacteria (Reiswig, 1971). Wilkinson and Garrone (1980) showed that when the sponge *Chondrosia reniformis* was incubated with the amino acid proline, the proline was incorporated initially into bacterial cells associated with the sponge. Longer incubation times were necessary to detect the amino acid in the sponge cells and intracellular matrix.

One species of sponge, *Mycale fistulifera*, is also known to form an association with a colonial scyphozoan, in which the association is facultative for the sponge and obligate for the scyphozoan. This mutual symbiosis provides protection, as a number of predators were observed to avoid the symbiotic sponges, and enables the sponge to increase in size more rapidly due to its ability to utilise the scyphozoan as a substitute skeleton (Uriz *et al.*, 1992; Meroz & Ilan, 1995).

In spite of the widespread occurrence of other symbioses in sponges, this chapter will deal only with the wide range of symbioses between sponges and algae.

Symbioses between sponges and photosynthetic organisms are very diverse; in fact, sponges form symbioses with more species of algae, from more Divisions, than any other animal phylum. The class Demospongiae contains about 90% of all sponge species and it is members of this class which contain algal symbionts. Algal symbionts may occur either extracellularly, when sponges occur with large, multicellular algae, or intracellularly. Intracellular symbionts may inhabit almost all cell types within the body of the sponge, with the exception of the cells covering the external surface and those of the exhalant canals, those which secrete spicules, which form part of the skeletal structure of the sponge, and cells which contain other granular material (granulocytes) (Sailer, 1989). Algal symbionts may comprise up to 75% of the cellular tissue of the sponge/algal association (Rützler, 1981; Wilkinson, 1987a; Trautman *et al.*, 2000). Some species may also naturally occur either with or without algal symbionts.

Sponges can be found in association with unicellular cyanobacteria including *Aphanocapsa spp.* and *Synechocystis sp.*; multicellular cyanobacteria, including *Oscillatoria spp.*; unicellular green algae, including *Chlorella spp.* (in fresh water sponges); dinoflagellates of the genus *Symbiodinium*; cryptophytes, including *Zoocryptella spp.*; and diatoms, including *Nitzchia sp.*, and other unidentified diatoms found within Antarctic sponges. Descriptions of many of these algal symbionts have been reviewed by Wilkinson (1992). Documented associations between sponges and large multicellular eukaryotic algae include associations with the green alga, *Struvea deliculata* (Weber Van Bosse, 1890), and the red algae *Codiophyllum flabelliforme*, *Codiophyllum spongiodes*, *Thamnoclonium dichotomum* (Scott & Wetherbee, 1982;

Scott *et al.*, 1984), *Ceratodictyon spongiosum* (Price *et al.*, 1984), and the calcified red algae *Jania adherens* and *J. calliparia* (Rützler, 1990).

In marine sponges, the most commonly found photosynthetic symbionts are cyanobacteria, and it is these associations that have received considerable scientific attention (Wilkinson, 1978a, 1980b, 1981, 1983, 1987b; Rützler, 1988; Wilkinson *et al.*, 1988; Cheshire & Wilkinson, 1991). Detailed studies on symbioses involving other types of algae are virtually non-existent and consist largely of descriptions of gross morphology and records of occurrence. In light of the diversity of sponge/algae associations and the limited data on all aspects of the ecology and physiology of sponges with photosynthetic symbionts, it is apparent that there is vast scope for research.

3. Population Dynamics

Despite the importance of sponges in marine ecosystems, characteristics of sponge population dynamics remain essentially unknown for most species.

Availability of food, sedimentation, physical turbulence and predation are all factors which limit local distributions of some sponge species and cause significant morphological variability in others. In addition, for sponges with photosynthetic symbionts, available photosynthetically active radiation and the availability of inorganic nutrients to support algal growth are important to their population dynamics.

3.1. THE BIOMASS OF SPONGE/ALGAL SYMBIOSSES

There appears to be an inverse relationship between latitude and the numbers of sponges which contain photosynthetic symbionts. Sponges containing algal symbionts are an important component of the biota of coral reefs (Reiswig, 1973; Wilkinson, 1981), but there are relatively few of these types of associations in temperate and polar waters.

In tropical regions, the large biomass of phototrophic sponges, that is sponge/algae symbioses which are net primary producers, may make a major contribution to the benthic primary productivity in the areas in which they are found. Despite this abundance, few quantitative studies of their biomass or productivity have ever been done in reef systems. One recent study has shown that the symbiotic association between the sponge *Haliclona cymaeformis* and the red macroalga *Ceratodictyon spongiosum*, which is common on shallow reef flats where few other macroscopic organisms can survive, could contribute up to 50% of the total reef flat primary productivity on these reef flats (Trautman, 1997).

3.2. FACTORS WHICH MAY AFFECT THE DISTRIBUTION OF PHOTOTROPHIC SPONGES

3.2.1 Light

Major differences exist between the ecological roles and requirement of symbiotic and non-symbiotic sponges. Irradiance has been found to be the major factor limiting the

growth of phototrophic sponges. Below 30 m, there is usually not enough light for the photosynthetic symbionts of sponges to maintain photosynthetic productivity. Reducing the level of irradiance, either through shading or transplanting sponges to deeper waters, is known to inhibit growth in a number of different sponge/algae symbioses.

In shallower waters, ultraviolet (UV) light may inhibit the growth of some coral reef sponges (Jokiel, 1980; Cheshire & Wilkinson, 1991); however, in some areas of the Great Barrier Reef off eastern Australia, phototrophic sponges are particularly prevalent in reef lagoons between depths of 1 and 5 m. The mechanism used by these sponges to escape UV damage is unknown; one possibility is that some species contain UV blocking compounds similar to those found in corals (Dunlap & Chalker, 1987). Another possibility is that symbiotic cyanobacteria may shield sponge tissue from some deleterious effects of both high irradiation and UV light (Wilkinson, 1981). Other possible mechanisms, for example specialised granular cells found within some aposymbiotic sponges, or thick layers of surface mucus, may function as UV shields and may be important in some symbiotic sponges.

3.2.2 Sediment and Turbulence

Studies by Wilkinson and Vacelet (1979) and Wilkinson and Evans (1989) have shown that the species composition of sponge populations varies considerably with the levels of suspended sediment and turbulence. The flattened morphology and horizontal orientation of many phototrophic sponges enhances the capture of light, but may make them unsuitable for environments where there are large amounts of fine sediments suspended in the water column. Sediment particles can clog inhalant canals, causing a reduction in pumping rates (Gerrodette & Flechsig, 1979) and this may result in the sponge ceasing to pump water for extended periods (Reiswig, 1974). Prolonged reduction in the pumping rates of sponges can have adverse effects on such vital functions as feeding and respiration.

Few sponges have skeletons which are rigid or tough enough to withstand the high levels of turbulence often found in exposed or shallow water habitats (Palumbi, 1984). Wilkinson and Evans (1989) reported that on reef slopes, few sponges are found within the first 10 m. However, the symbiotic algae associated with some sponges may enhance their ability to survive in more turbulent environments. For example, the symbiotic red macroalga *Ceratodictyon spongiosum*, which grows only in symbiosis with the tropical sponge *Haliclona cymaeformis*, forms a tightly anastomosed mesh within the sponge, forming branches which are generally at least 1 cm thick. Consequently the symbiotic association is quite tough and survives well on reefs less than 2 m deep (Trautman *et al.*, 2000).

3.3. GROWTH OF SPONGE/ALGAL SYMBIOSSES

Phototrophic sponges have been found, in a number of instances, to grow faster than aposymbiotic sponges (Wilkinson & Cheshire, 1988). This difference in growth rate has been attributed to translocation of products of photosynthesis from the symbiotic alga to the sponge (Wilkinson & Vacelet, 1979; Frost & Williamson, 1980; Rosell & Uriz, 1992). For example, Wilkinson and Cheshire (1988) found that the fastest

growing sponges on a reef which had been devastated by a hurricane were those which contained symbiotic cyanobacteria.

3.4. REPRODUCTION OF SYMBIOTIC SPONGES

Despite the fact that sponges are a significant component of the benthic marine fauna, there have been very few studies of the reproductive biology of this phylum (Fromont, 1994). In particular, there is no information available on the transmission of symbiotic algae to successive generations. The presence of symbiotic bacteria has been reported in the larvae of only five sponge species; it was postulated that these bacteria were transferred from the parent sponge early in embryonic development, via the formation of collagen-like 'umbiculi' from the parent sponge to the embryo (Kaye, 1991). Such connections have never been observed in sponges which contain photosynthetic symbionts. However, some species of sponges can acquire their symbiotic algae directly from the environment, as do many other marine invertebrate species with symbiotic algae. Sailer (1989) showed that when aposymbiotic individuals of the freshwater sponge *Spongilla lacustris* were fed with the unicellular green alga *Chlorella*, the cells were ingested by the sponge, phagocytosed by archeocytes (amoeboid cells) and transferred to mesenchyme. Six hours after exposure to the alga, most of the sponge cells contained *Chlorella*. The marine sponge *Anthosigmella varians* also acquires its symbiont, the dinoflagellate *Symbiodinium sp.*, from the water. Hill and Wilcox (1998) transplanted a number of individuals of this sponge from a depth of 20 m to a depth of 1 m and observed that after 12 days some of the sponges had completely bleached; but then about 40 days after bleaching had occurred, the sponges began to slowly regain pigmentation. It was shown that these recovered sponges harboured a strain of zooxanthellae which was different from the original symbiont. Genetic analysis showed that the new symbionts matched those isolated from a common local anemone. Antarctic sponges have also been shown to take up both planktonic and benthic diatoms actively from the environment. Once the diatoms have been incorporated into the sponge, it may utilise extracellular polysaccharides produced by the diatoms as energy substrates (Bavestrello *et al.*, 2000).

Fromont (1994) reported that in the association between the sponge *Haliclona cymaeformis* and the red macroalga *Ceratodictyon spongiosum*, the partners both reproduce during the summer, although the sponge and algal reproductive products are spatially separated along the branches of the association. It is not known when or how the symbiosis is re-established, but it is assumed that this occurs after release of the algal spores and the sponge larvae, since there is no obvious association between these sponge and algal propagules before release.

Fragmentation may be one of the primary forms of reproduction in some species of phototrophic sponges, and since it does not involve separation of the two symbionts, it ensures the survival of the association. It is known that in some species of sponges virtually all recruitment into established populations is by asexually produced fragments (Wulff, 1986). Fragments can easily be dispersed by water movement before adhering to solid substrata and becoming established as independent individuals (Wulff, 1985, 1986).

Further investigations are obviously required to establish clearly the sequences of events leading to the propagation of sponge/algal symbioses.

4. Alterations in the morphology of sponges and algae as a result of symbiosis

In some symbioses, particularly those between sponges and macroalgae, the morphology of both partners in the association is modified. Although the symbiotic sponge *Haliclona cymaeformis* has never been isolated and grown in culture, it is likely that its growth would be significantly altered in culture, as the alga *Ceratodictyon spongiosum* provides most of the structural support for this association (Bergquist & Tizard, 1967).

The alga *C. spongiosum* certainly does change its growth form when it is isolated from the *Haliclona/Ceratodictyon* association. When it is associated with *H. cymaeformis*, the filaments of the alga fuse frequently to form a dense reticulum, whereas in monoculture, the algal filaments remain quite separate and are relatively lightly pigmented (Price *et al.*, 1984). However, when a homogenate of sponge cells is added to cultured *C. spongiosum*, the growth form of the alga changes so that the branches of the alga become thicker, do not grow as long, and become more heavily pigmented (Grant, Trautman, Hinde and Borowitzka, unpublished data).

One sponge species which alters its morphology as a result of symbiosis is *Petrosia ficiformis*. In well illuminated habitats, this species is found in association with the cyanobacterium *Aphanocapsa feldmanni*, and has a flattened morphology. In shaded conditions, the numbers of cyanobacterial symbionts decrease and the sponge becomes cylindrical in shape. In darkness, the species is white, small and cylindrical (Sarà *et al.*, 1998). Such species represent useful models for the study of sponge adaptation to algal symbioses.

5. Metabolism Of Symbiotic Sponges: Photosynthesis and Respiration

Knowledge of the photosynthetic and respiratory rates of symbiotic marine sponges is confined to a few studies performed by Wilkinson and colleagues (Wilkinson, 1981, 1983, 1987b; Cheshire & Wilkinson, 1991; Cheshire *et al.*, 1995; Cheshire *et al.*, 1997), and Pironet and colleagues (Pironet, 1985; Borowitzka *et al.*, 1989; Hinde *et al.*, 1994). Photosynthesis and respiration have also been studied in the *Haliclona/Ceratodictyon* association (Trautman, 1997), and the freshwater symbiotic sponge, *Spongilla lacustris* (Gilbert & Allen, 1973a, b; Frost and Williamson, 1980; Sand-Jensen & Pedersen, 1994). In most instances, the photosynthetic rates of both the marine and freshwater symbiotic sponges were significantly higher than the rate of respiration, indicating that these organisms are net primary producers and may be highly productive components of the environments in which they are found.

5.1. PHOTOADAPTATION

A long-term reduction in light availability can alter the function of marine communities severely. Coastal reef populations in particular may be at great risk as coastal development and dredging increase the levels of sediment, reducing the levels of photosynthetically active radiation available to photosynthetic organisms (Rogers, 1979). For example, Fricker (1980) found that shaded specimens of the *Haliclona/Ceratodictyon* association and those growing in deeper waters, showed markedly reduced photosynthetic and growth rates, and concluded that this association requires high levels of irradiance to maintain photosynthetic productivity.

Most algae, whether free living or in a symbiotic association, will display some form of photoadaptation in response to low light levels or changes in irradiance. In a number of studies investigating the productivity of sponge/cyanobacterial associations, Wilkinson and colleagues (Wilkinson, 1978b, 1983, 1987b; Cheshire & Wilkinson, 1991; Cheshire *et al.*, 1995, 1997) have discussed the probable occurrence of adaptation to low light in these associations. Based on the compensating light intensity, where photosynthetic oxygen productivity just balances respiratory consumption, Cheshire and Wilkinson (1991) predicted that the depth limit for phototrophic sponges, without photoadaptation, would be 25 m. They have consistently found the depth limit for these sponges to be 30 m, and have attributed the greater depth range to photoadaptation by the cyanobacterial symbionts (Cheshire *et al.*, 1997). Cheshire and Wilkinson (1991) also noted that many phototrophic sponges are consistently thin and distinctly flattened, apparently as a mechanism to enhance light capturing efficiency. These morphological features become accentuated in specimens from deep water.

The symbiotic cyanobacteria in deep water sponges could also adapt by changing the size and the morphology of their spiral thylakoids. In some associations this increases from one or two spirals in cyanobacteria near the sponge surface, to seven or more in cyanobacteria in the highly shaded, internal regions of the sponge (Wilkinson, 1978b). In other symbiotic relationships between sponges and algae, the structure of the sponge may provide favourable conditions for the growth of the alga. For example, in the sponge *Tethya seychellensis*, the symbiotic chlorophyte, *Ostreobium sp.*, grows only in close association with the silica spicules of the sponge. It has been suggested that the arrangement of these spicules, in groups running inwards radially from the surface, may allow the spicules to act like optical fibres, trapping light and guiding it towards the centre of the sponge. Thus *Ostreobium* may grow in an environment which would otherwise be unsuitable for the growth of a photosynthetic organism (Gaino & Sarà, 1994, Cattaneo-Vietti *et al.*, 1996). Similarly, in the sponge *Petrosia ficiformis*, the arrangement of the spicules changes according to the level of light received by the sponge. In well lit habitats the spicules lie tangential to the surface, but in shaded habitats a dense coat of vertical spicules is also present. The vertical spicules are again absent in the specimens living in darkness (Sarà *et al.*, 1998).

5.2. EFFECTS OF OXYGEN CONCENTRATION

In marine environments, such as coral reef lagoons, or temperate reefs dominated by macroalgae, reef organisms may be subjected to high concentrations of dissolved

oxygen. High concentrations of oxygen can cause oxidative damage to cells, including the breakdown of proteins and membranes (Grant *et al.*, 1993), and can inhibit photosynthesis (Downton *et al.*, 1976). Because symbiotic algae are enclosed within their hosts high concentrations of oxygen may be a problem for both host and algae. There have been few studies of the effect of oxygen concentration on the photosynthetic rates of algae in symbiotic associations. However, it is possible that in sponge symbioses, circulation of water through the sponge may remove this excess oxygen more rapidly than would occur in other symbioses, for example, corals. In addition, the respiratory consumption of some of the oxygen by the algae and the sponge may maintain the oxygen concentration around the algal cells at a level where the algae can maintain near optimal productivity (Szuch *et al.*, 1978). For example, in the freshwater sponge *Spongilla lacustris*, oxygen produced by the intracellular symbiotic algae may fulfil part or all of the host's oxygen requirements. During summer, the oxygen produced by algal photosynthesis satisfies most of the oxygen requirements of both the sponge and algal tissues; consequently, rate of oxygen uptake in the light is lower than in the dark for this association (Szuch *et al.*, 1978).

5.3. EFFECTS OF NUTRIENT ENRICHMENT

Because of their capacity to pump large volumes of seawater, large sponges, or dense populations of sponges, may influence nutrient concentrations in seawater (Vincente *et al.*, 1991). The only study of possible changes in the productivity of a sponge/algae symbiosis in response to nutrient enrichment showed that the photosynthetic and respiratory rates of the symbiotic association between *Haliclona cymaeformis* and the red alga *Ceratodictyon spongiosum* were unaffected by enrichment with nitrogen and/or phosphorus at concentrations 10 times higher than those they would normally encounter (Trautman, 1997).

6. Metabolism of Symbiotic Sponges: Heterotrophic Nutrition

Symbiotic sponges obtain the bulk of the nutrients they require for growth and survival by two methods; the capture of particulate organic matter (Reiswig, 1971; Frost, 1980; Willenz *et al.*, 1986; Riisgård *et al.*, 1993), and the uptake of dissolved organic material which has been released by the photosynthetic symbionts (Wilkinson, 1980a, b; Pironet, 1985). The digestion of symbiotic algae or bacteria has been observed in a number of species, but appears to be rare and is possibly related to eliminating diseased or degenerate symbionts, rather than to the nutrition of the sponge (Sarà, 1971; Wilkinson 1978b; Berthold *et al.* 1982; Saller 1989). Sponges may also obtain some of their nutrition via the uptake of dissolved organic matter from the surrounding water, but this process has not been investigated for sponge/algae symbioses.

6.1. UPTAKE OF PARTICULATE ORGANIC MATTER

Coral reef waters are generally very low in particulate matter. Despite this, Reiswig (1971) and Wilkinson *et al.* (1988) demonstrated that the pumping efficiencies of

tropical sponges are significantly greater than those of other suspension feeders, allowing them to maintain large populations in nutrient poor waters.

The relative importance of filter feeding may vary with geographical region. Phototrophic sponges are very abundant on the coral reefs of the Indo-West Pacific, including the Great Barrier Reef, but are virtually absent from Caribbean reefs. It is thought that marked differences in habitat between Caribbean coral reefs and the Great Barrier Reef have resulted in the evolution of dissimilar sponge faunas between the two regions, and the differences in the relative importance of filter feeding and nutrition from photosynthetic symbionts (Wilkinson, 1987a). The numbers of sponge species containing cyanobacteria in the two oceans are roughly similar; however, in the Caribbean, these sponges contain only a thin layer of cyanobacteria which does not significantly contribute to the nutrition of the association (Wilkinson, 1987a). On the Great Barrier Reef, higher densities of cyanobacteria are found in many of the symbiotic sponge species, and these are believed to make a significant contribution to the nutrition of the sponge (Wilkinson, 1980b, 1983; Arillo *et al.*, 1993).

The effects of algal symbionts on the rates of uptake of particulate matter by sponges have been examined in only a few species. In a number of species, the presence of the alga appears to reduce the filter feeding ability of the sponge. For example, the freshwater sponge *Corvomeyenia everetti* (Frost *et al.*, 1997) and the marine sponge *Phyllospongia sp.* (Wilkinson *et al.*, 1988) both have lower filter feeding rates than aposymbiotic species found in the same areas. In addition, the marine sponge *Petrosia ficiformis* shows variable numbers of inhalant pores. In full sunlight inhalant pores are very rare, but large numbers are found in those living in dark environments (Sarà *et al.*, 1998). Conversely, Frost and Williamson (1980) compared symbiotic and aposymbiotic individuals of the freshwater sponge *Spongilla lacustris*, and reported that the presence of algal symbionts had no effect on the clearance rates of this species, either during the day or at night.

6.2. TRANSLOCATION OF PHOTOSYNTHATES FROM THE SYMBIOTIC ALGAE TO THE SPONGE

Many of the sessile animals on coral reefs obtain at least part of their nutrition from photosynthetic symbionts, through the translocation of photosynthetically fixed carbon to the host's tissues during photosynthesis. They retain the mechanisms required for heterotrophic feeding, but the translocation of photosynthetic products may considerably reduce the need to expend energy on water filtration or prey capture (Schlichter, 1982; Szmant-Froelich & Pilson, 1984; Wilkinson *et al.*, 1988; Smith, 1991; Arillo *et al.*, 1993). This may be of particular value in oligotrophic waters.

It is well known that symbiotic corals may obtain a significant proportion of their daily nutrition from their symbiotic dinoflagellates (Hinde, 1988). It has only recently been established that sponges, with their array of different types of algal symbionts, may also obtain some of the products of photosynthesis from their symbiotic algae. Wilkinson and Vacelet (1979) showed that sponges with symbiotic cyanobacteria grew nearly 4 times as fast in well illuminated positions compared to those found in shaded positions, and inferred that the significant increase in sponge growth rate in the light was due to the translocation of photosynthetically fixed organic nutrients by the

symbiotic cyanobacteria. Similarly, Frost and Williamson (1980) showed that the aposymbiotic freshwater sponge, *Spongilla lacustris*, grew at only 20% of the rate of symbiotic *S. lacustris* under the same conditions. Wilkinson and Cheshire (1988) also found that two species of symbiotic sponges from Jamaica grew faster than other, non-symbiotic, species after a hurricane, and speculated that this difference was due to supplementary nutrition from the cyanobacterial symbionts.

Wilkinson (1980b) was the first to demonstrate that cyanobacteria isolated from six species of coral reef sponges did in fact release products of photosynthesis. Rützler (1981) confirmed that translocation does occur in the intact association between species of the sponge *Ulosa* and their symbiotic cyanobacteria. Following incubation with ^{14}C sodium bicarbonate, strong ^{14}C activity was detected in the sponge cells, demonstrating transfer of photosynthate from alga to sponge. Similar results were obtained by Pironet (1985) using the sponge *Dysidea herbacea* which is symbiotic with the cyanobacterium *Oscillatoria spongiae*. In Wilkinson's (1980b) experiments, isolated cyanobacteria released 5 to 12% of the total carbon fixed, mostly in the form of glycerol. This rate is at the lower end of the range found in cnidarian/dinoflagellate symbioses. Since this time, it has been reported that sponges could potentially obtain between 20 and 126% of their respiratory carbon requirements from algal photosynthesis (Wilkinson, 1983, 1987b; Demidov *et al.*, 1993; Cheshire *et al.*, 1997).

6.3. EXTRACELLULAR DIGESTION OF SYMBIANTS

It is generally accepted that the growth rates of the symbionts and sponges are somehow linked, keeping the symbiont population at the optimum level (Wilkinson, 1987c; Rützler, 1988); however, little is known of the population dynamics of symbiotic bacteria or algae, or how the host sponge controls their numbers. Excess symbionts may be expelled from the sponge in a manner similar to that used by the sponge to expel large, non-symbiotic cells or inorganic particles. When these cells enter a sponge they may be phagocytosed by amoeboid cells (archeocytes), which then carry them to an exhalant canal where they are expelled (Cheng *et al.*, 1968a, b). One example of the failure of this balance between host and symbiont has been observed in the sponge *Geodia papyracea*. Under certain conditions, the cyanobacteria found in this association multiply faster than host archeocytes can eliminate the excess. High densities of cyanobacteria often lead to extensive histolysis of the host tissue, possibly caused by toxic excretions (Rützler, 1988).

Another possible method for a host to control the numbers of symbiotic cells is by phagocytosis and digestion of the symbionts, by the sponge. The digestion of symbiotic algae has been reported by several authors, but the extent to which it occurs and its nutritional significance have not yet been determined. Sarà (1971) suggested that the transfer of nutrients from cyanobacteria to host may be achieved through the digestion of the symbionts. By examining sponges under an electron microscope, he showed that at least some species of sponges appeared to digest some of their endosymbionts. Digestion of symbiotic cyanobacteria, in different species of sponges, has also been reported by Wilkinson (1978b), Berthold *et al.* (1982) and Saller (1989), but few phagocytosed cells were observed in each of these studies.

In marine sponges the digestion of symbionts is presumed to be more important in the removal of defective or damaged symbionts from the sponge tissue than as a form of feeding (Rützler, 1988). Due to the limited numbers of phagocytosed symbionts which have been observed in sponges, the transfer of the products of photosynthesis to the animal tissue is likely to occur mainly by translocation from living algae, rather than by digestion of algae (Wilkinson, 1980b).

7. Nitrogen Recycling In Sponge/Algal Symbioses

Nitrogen recycling, where nitrogenous wastes released by one symbiont are taken up by the other, is known to occur in corals (Muscatine & D'Elia, 1978; Burris, 1983; Ferrier-Pages *et al.*, 1998), but has not been studied in detail in symbiotic sponge associations. There is some evidence that symbiotic *Petrosia ficiformis* release less ammonia than aposymbiotic ones. This supports the hypothesis that cyanobacterial symbionts use waste nitrogen from the host (Sarà, 1998). A more detailed study of nitrogen metabolism in two species of symbiotic sponges by Corredor *et al.* (1988) showed that the sponge *Chondrilla nucula*, which contains cyanobacterial and bacterial symbionts, releases large amounts of nitrate, which may then be taken up by other reef organisms. The second species of sponge, *Anthosigmella varians*, which contains symbiotic dinoflagellates and smaller populations of bacteria, releases significantly less nitrate, probably due to the incorporation of some of the released nitrate by the symbiotic zooxanthellae.

8. Other Benefits Of Symbiosis

The main benefits of invertebrate/algal associations have been reported to include protection of the algae inside the animal, the receipt of products of algal photosynthesis by the host animal, nitrogen and phosphorus recycling, provision of oxygen, removal of wastes and protection from ultraviolet light. However in sponges, due to the diversity of symbioses with algae, there appears to be a greater range of benefits to both partners.

Protection from predators, through the production of toxic compounds, may originate either from the sponge or from the symbiotic alga. Where they are produced by the alga, they may protect the sponge, the reverse of the more common situation where the algae are protected from herbivores. Cyanobacteria in particular are known to produce toxic secondary metabolites which can act as defence substances for symbiotic sponges or other organisms which contain these algae (Sarà, 1971; Pardy & Lewin, 1981; Unson *et al.*, 1994). Similarly, a number of species of sponges are also known to produce secondary metabolites which deter predators (Schupp *et al.*, 1999; Wilson *et al.*, 1999; Becerro, *et al.*, 1998). The arrangement of spicules in the sponge may also protect the alga. The rhodophytes *Thamnoclonium dichotomum* and *Codiophyllum flabelliforme* are both covered with thin layers of unidentified species of sponges. These sponges may protect the algae with 'defensive spicula' which are oriented at right angles to the underlying algal surface (Scott *et al.*, 1984).

Antarctic sponges often host large populations of planktonic and benthic diatoms, and these diatoms have been shown to consume carbohydrates which have been produced by the sponge, thereby utilizing the sponges' metabolic products as an energy source. This relationship enables the diatoms to survive at depths of 100 to 120 m where irradiance is very low (Bavestrello *et al.*, 2000).

In freshwater sponges, zoothorellae are often incorporated within gemmules. These gemmules, which are formed during autumn, contain food-filled archeocytes and are surrounded by a layer of spicules and collagen-like material so that a thick shell is formed that is resistant to freezing and drying. The presence of symbiotic algae within a gemmule enables the gemmule to hatch sooner and develop into a sponge more quickly once conditions become suitable for sponge survival (Brøndsted & Løvtrup, 1953; Sarà *et al.*, 1998). In marine species, sponge/zooxanthellae symbioses appear to be more stable than coral/zooxanthellae symbioses, since environmental factors, such as high temperature or increased exposure to UV light, which cause the algae to be expelled from corals (i.e. bleaching), do not appear to affect sponges. It is not known why these sponge associations are more resistant to environmental fluctuations than coral associations (Hill & Wilcox, 1998).

Finally, symbiotic associations with algae, and in particular macroalgae, allow the sponges to increase their growth rates either directly, by the sponge feeding on organic matter translocated from the alga, or indirectly, by utilizing the symbiont as a substitute skeleton, thereby reducing the investment in sponge collagen and spicule production (Uriz *et al.*, 1992).

As a result of these benefits, symbiotic sponges may be larger, survive longer, be less susceptible to predation, and exploit more environmental niches than if they lacked symbionts.

9. Conclusions

Given that we have only limited data, for a few species, on any aspect of the biology of sponges with photosynthetic symbionts, there is clearly a need for much more research on these associations. This is particularly clear when their diversity is taken into account; they include associations with prokaryotic and eukaryotic symbionts, and with unicellular and multicellular forms (from a wide range of Divisions, in the case of the eukaryotes) of each. The proportions of sponge and symbiont in the associations vary greatly; some associations are net primary producers, while in others photosynthesis may contribute very little to the nutrition of the sponge host. Given the diversity of sponge/algal associations, there will undoubtedly turn out to be an equally great diversity in their ecology, physiology and biochemistry. Areas of particular interest for future research include the population dynamics of sponge/algal associations of all kinds, which will help us to understand their roles in reef and other ecosystems; their physiology; their contributions to the carbon and nitrogen budgets of ecosystems; their responses to environmental change, including human-induced changes, such as global warming, eutrophication, other forms of pollution, and increased sediment. In addition, if we are to understand the true nature of each symbiosis, we need data on the translocation of both inorganic and organic nutrients between the symbionts and on

synthesis of secondary metabolites. Interactions between alga and sponge at the cellular level, the apparent morphogenetic effects in some associations and the mechanisms by which the association is carried forward from one generation to the next are intriguing problems which are important to understanding how each association functions.

What is known so far suggests that in coral reefs, sponge/algae associations may be functionally similar to the coral/dinoflagellate associations, in that they are often net primary producers operating at more than one trophic level, and there is preliminary evidence that they may recycle nitrogen. The existence of coral reefs, with their high biomass and diversity, in oligotrophic waters is attributed to these characteristics of the coral/algae symbiosis. Since phototrophic sponges are often very abundant on coral reefs, they must also play an important role in making these communities possible, and better understanding of the functioning and conservation of coral reefs requires better knowledge of sponge/algae associations.

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THE TAXONOMY AND EVOLUTION OF THE ZOOXANTHELLAE-CORAL SYMBIOSIS

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1. Introduction

Coral reefs are the most biologically productive of all marine ecosystems and comprise the largest structures created by living organisms. The symbiotic association between corals and their endosymbiotic unicellular microalgae (the zooxanthellae) has been a contributing factor in the ecological success of reef-building corals since their appearance in the Triassic period (Heckel, 1974). Associations between unicellular algae and invertebrates such as corals, sea anemones, bivalves, sponges, foraminiferans, flatworms and hydra, known as photosymbioses , are found in sea water and freshwater (Muscatine, 1971). The most common, and prominent is the exclusive marine symbiosis of coelenterates with zooxanthellae that are located in vacuoles, usually within the host's endoderm cells (Glider *et al.*, 1980; Trench, 1979; Trench, 1987), Fig. 1)

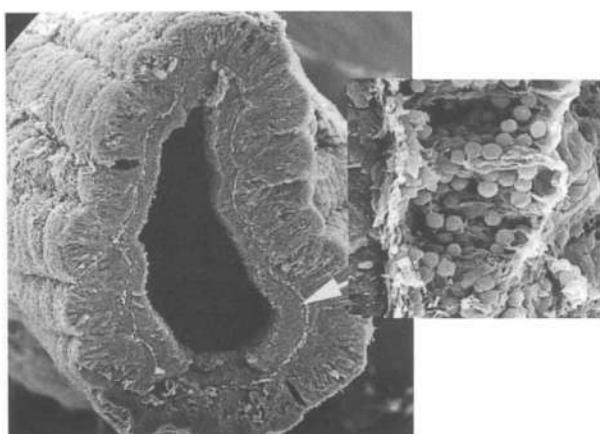


Figure 1. Scanning electron micrograph of cross section through tentacle of *Anemonia sulcata*.

These specialized vacuoles may have evolved from food vacuoles, and are termed symbiosomes (Trautman and Hinde, 2000; Wood, 1999). In the case of the Tridacnid clams the symbionts are found within a branched tubular structure that has no direct connection with the hemolymph (Norton *et al.*, 1992).

In hermatypic, or reef-building corals, the algae are arranged in a single layer in the tissue with a mean density of 1 million cells per cm^2 (Drew, 1972). This value may vary in different locations in the colony (Titlyanov, 1991), as a function of the light distribution within it. Another example of such heterogeneity is the absence of zooxanthellae from the tips of rapidly growing branches in many coral species (Fang *et al.*, 1989). Furthermore, nutrient concentrations are also known to affect the areal densities of these algae (Dubinsky *et al.*, 1990)).

The symbionts mediate the flux of carbon and nutrients between the host and the environment (Muscatine, 1990). This mutualistic relationship allows corals and coral reef communities to succeed in spite of the low concentrations of nitrogen and phosphorus typical of the oligotrophic waters surrounding reefs (Muscatine and Porter, 1977). Actually, the zooxanthellae-coelenterate association evolved specifically in response to the otherwise limited nutrient concentrations typical of the “blue deserts” of the tropical oceans where coral reefs dominate shorelines.

During the last decades reefs are facing multiple anthropogenic stressors, including a progressive rise in sea surface temperature, UV radiation, as well as eutrophication and increased sedimentation (Birkeland, 1997; Dubinsky and Stambler, 1996). Elevated temperatures such as those associated with El-Niño episodes and increased UV radiation are widely believed to operate synergistically in the collapse of the algal symbiosis between corals and other invertebrates, causing the mass expulsion of algae resulting in bleaching (Glynn, 1993), that can lead to widespread coral mortality (Wilkinson, 1998). The study of the origins and nature of the symbiotic algae-host association is of great importance for the understanding of the future of coral reefs under different Global Climate Change scenarios, and for remediation efforts in damaged reef systems.

2. Historical Background

Unicellular algae inhabiting various marine invertebrates have been designated mainly as zooxanthellae or zoothorellae. These algae have been called zooxanthellae if they were yellow-brown and zoothorellae if they were green (Brandt, 1881, 1882). Although these terms are based on their color, like algal taxonomy in general, they indicate valid taxonomic affiliation, which includes many cellular and life cycle features in addition to pigmentation (Chapman, 1968). During many years the relationship between the algae and the invertebrates was investigated. Brandt, in 1881, recognized that these colored cells did not belong to the animals, although they were found in them. Cienkowski (1871) thought that the green cells found in radiolarians were “parasitic algae” capable of surviving the death of the animal host. Haeckel (1879) viewed them as animal gametes, whereas Hamann (1881) considered the zooxanthellae of the sea anemones to

be unicellular animal glands, but thought that those found in radiolarians were parasites. Brandt (1881, 1882, 1883, 1885) did not accept this suggestion and described the latter as symbiotic algae in radiolarians, and placed them in the new genus *Zooxanthella*. Subsequently (Brandt, 1885), following the work of Kelbs (1884), Brandt accepted they belonged to the Dinoflagellata. Chatton (1923) placed the zooxanthellae of radiolarians into the Dinoflagellata. Hovasse (1922, 1923) assigned the symbionts of the chondrophora *Velella velella* to the new genus *Endodinium* as *E. chattonii*, but believed that they were parasites. In 1937 Hovasse showed that although most zooxanthellae have dinoflagellate affinities, the algal symbionts in the turbellarian *Convoluta roscoffensis* were nondinoflagellate. Kawaguti (1944) was the first to observe that coccoid algae isolated from the coral *Acropora corymbosa* produced gymnodinioid motile "swarmers" in culture, and he assigned them to the dinoflagellate genus *Gymnodinium*. McLaughlin and Zahl (1959) showed that under axenic conditions the vegetative cells of zooxanthellae from a variety of marine coelenterates produce a motile which is unequivocally dinoflagellate in character. In 1962, Freudenthal described the life history of the algae from *Cassiopeia* and *Condylactis* in culture, and referred them to the new genus *Symbiodinium* as *S. microadriaticum*, placing them in the parasitic dinoflagellate family, Blastodiniaceae. In 1967, Parke and Manton succeeded in culturing the symbiont of *Convoluta roscoffensis* described as *Plastymonas convolutae* and later in 1969, Apelt identified it as a diatom. Subsequently, other diatoms were identified as symbionts in Foraminifera (Lee *et al.*, 1982), and sponges (100).

Benthic foraminifera, which are ecologically important members of many tropical reef communities, harbour zooxanthellae, which unlike those of corals, belong to the diatoms (Lee *et al.*, 1965; Lee, 1998; Lee and Anderson, 1991).

Zoochlorellae are associated with many of the fresh-water *Hydra* species (Muscantine, 1971) and in the sea anemone *Anthopleura xanthogrammica* (Bates, 2000; O'Brien and Wyttensbach, 1980).

The coral symbionts are all zooxanthellae, which in the course of recent work were recognized as belonging to different dinoflagellate taxa (see taxonomy part).

At present the photosynthetic symbionts in various animal hosts are grouped as follows: If they are blue-green algae (Cyanobacteria, or Cyanophyta), Zoocyanellae, the Chlorophytes (=green algae) Zoochlorellae, and the Chromophyta, including diatoms (=Bacillariophyta) and Dinoflagellata retain the name zooxanthellae. So far no generic name has been assigned to the few Rhodophytes described as symbionts in Foraminifera and mollusca (Hallock, 1988; Trench, 1979).

3. Taxonomy of Symbiotic Zooxanthellae

While zooxanthellae include a few diatoms, members of the Bacillariophyceae, and some Cryptophyceae, the overwhelming majority, belong to the Dinoflagellates, a group of the Pyrrophyta. Current taxonomy based on the various criteria and methods listed below, place zooxanthellae in at least seven genera and four orders of dinoflagellates

(Banaszak *et al.*, 1993). Of these genera, only *Symbiodinium* Freudenthal (1962) lacks known free-living species in nature (Rowan, 1998).

The dinoflagellate symbionts of coelenterates mainly belong to various species of the genus *Symbiodinium* but in some cases such as in *Millepora* they may belong to the genus *Amphidinium* (Banaszak *et al.*, 1993; Trench, 1979, 1997). Early studies based on the apparent morphological similarities between *Symbiodinium microadriaticum* (Fig. 2) and symbionts isolated from a variety of other hosts, considered these organisms to be members of that single pandemic species (Taylor, 1974). Implicit to the concept of a single widespread "zooxanthella" would be the establishment of promiscuous associations between several hundred invertebrate species, of various genera and several families, with a genetically uniform symbiont taxon, therefore, precluding the existence of specificity (Iglesias-Prieto and Trench, 1997).

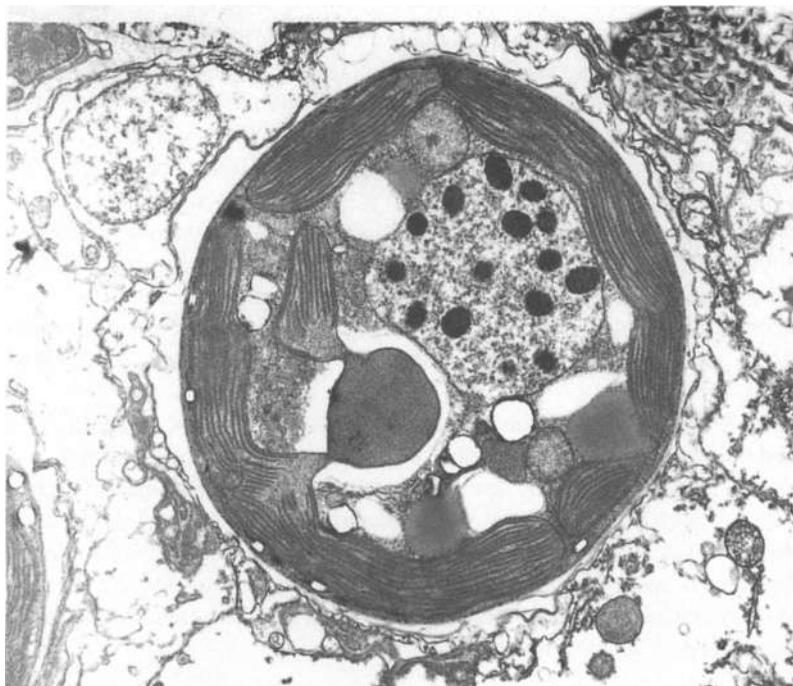


Figure 2. Transmission electron micrograph of *Symbiodinium microadriaticum* from *Stylophora pistillata*.

Over the last two decades a variety of independent evidence has shown that zooxanthellae are an extraordinarily diverse assemblage rather than one single species (Blank and Huss, 1989; Blank and Trench, 1985; Carlos *et al.*, 2000; Fitt *et al.*, 1981; Rowan *et al.*, 1997; Rowan and Powers, 1991a, 1991b, 1992; Schoenberg and Trench, 1980; Trench, 1993). Different zooxanthellae species, strains and isolates have been described taxonomically after isolation and cultivation *in vitro*, using isoenzyme patterns (Colley, 1983; Schoenberg and Trench, 1980), biochemistry (Chang and Trench, 1982; Govind *et al.*, 1990; Iglesias-Prieto *et al.*, 1991), morphology (Schoenberg and Trench, 1980; Trench and Blank, 1987), physiology (Chang *et al.*, 1983; Fitt *et al.*, 1981; Fitt and Trench, 1983a, 1983b), karyotyping (Blank and Trench, 1985), ultra-structure (Glider *et al.*, 1980), photoacclimation (Baillie *et al.*, 2000; Chang *et al.*, 1983; Iglesias-Prieto and Trench, 1994) and molecular approaches (Blank and Huss, 1989; Carlos *et al.*, 2000; McNally *et al.*, 1994; Rowan and Powers, 1991a, 1991b).

Sequence data from small-subunit ribosomal RNA genes (ssrRNA) have been employed by various investigators to distinguish between the different strains of *Symbiodinium* (Carlos *et al.*, 2000; Langer and Lipps, 1995; Lee *et al.*, 1995; McNally *et al.*, 1994). Polymerase chain reaction (PCR) was used to amplify symbiont ssrRNA with "universal" primers or, where the algae were significantly contaminated with host tissue, with "zooxanthella-specific" primers targeted to less conserved sequences (Rowan and Powers, 1991a, 1991b).

Baker *et al.*, (1997) pointed out that host DNA contamination can also be avoided by amplifying part of the large ribosomal subunit RNA genes (IrDNA) with "universal" primers, in which case host and symbiont PCR products differ in size and thus can be separated prior to analysis. Sequence-based phylogenetic reconstructions are the result of analyses of single genes, and the trees produced are "gene trees". The current phylogeny of *Symbiodinium*, is inferred from partial small subunit rRNA sequences.

Similarly, ssrDNA sequence differences between *Symbiodinium* groups A, B and C leave little doubt that these molecules in different groups represent distinct species of zooxanthellae (Rowan and Knowlton, 1995; Rowan and Powers, 1991a, 1992), and sequence diversities within groups B and C imply that each group contains multiple species (Rowan and Powers, 1991a). RFLPs in large subunit ribosomal rRNA genes and molecular sequencing of large subunit rDNA distinguished 17-19 symbiont genotypes in four clades of *Symbiodinium* (A, B, C, D) from about 110 species of coral reef from around the world (Baker, 2000a, 2000b; Carlos *et al.*, 1999; Rowan and Powers, 1991a).

Under natural conditions the combinations between hosts and symbionts are far from random (Trench, 1993). McNally *et al.*, (1994) even suggested that there is no correlation between host taxon and symbiont taxon; meaning that closely related hosts may harbor unrelated symbionts while taxonomically distant and geographically separated hosts may harbor very closely related (but not identical) ones.

Rowan and Knowlton (1995), based on small-subunit ribosomal DNA (ssrDNA) comparisons, showed that several host species harbored multiple, yet uncharacterized taxa of zooxanthellae. More than 35% of the scleractinian coral species surveyed contained multiple symbiont genotypes even in a single coral colony (Baker, 2000a,

2000b). These numerous different “strains”, were isolated from a variety of host species and geographic locations, even though there is a lack of evidence of sexual recombination. However, existing evidence does not suffice to establish that these isolates or clades represent distinct biological species.

There is evidence that various clades of zooxanthellae have competitive advantage under different environmental conditions. Coral colonies often show light-related patterns of zonation of zooxanthellae clades , both among colonies at different depths and colonies on sunlit and shaded surfaces (Baker, 2000a, 2000b).

Mixed algal populations of *Acropora digitifera* and *Tridacna* showed general taxonomic consistency over the different seasons (Belda-Baillie *et al.*, 2000). That tendency differed from the seasonal variation that was found in the composition of the symbiont community within single colonies of *Acropora palifera* from Taiwan (Yang *et al.*, 2000). *Symbiodinium* from *Plesiastrea versipora* from sub tropical and tropical water hosts belongs to clade C, and at high latitude to clade B (Rodriguez-Lanetty *et al.*, 2000). Zooxanthellae from locations with different clades clearly differed physiologically. *Symbiodinium bermudense* of *Aiptasia pallida* from Bermuda belong to clade B while in Florida they belong to clade A. These clades respond differently to temperature and irradiance (Goulet and Cook, 2000).

The acquisition of new symbionts is explained by the adaptive bleaching hypothesis: loss of zooxanthellae by invertebrate hosts under stressful conditions provides the opportunity for acquisition of genetically different types of zooxanthellae potentially leading to a more fit symbiosis. The bleaching may allow coral reef symbioses to respond more rapidly to environmental change (Baker, 2000a). Thus, a lower number of genotypes in bleaching susceptible coral species (*Acropora digitifera*, *Stylophora pistillata* and *Seriatopora hystrix*) may contribute to host bleaching sensitivity (Loh *et al.*, 2000).

Although the taxonomy of symbiotic dinoflagellates is far from complete, it is clear that diversity and specificity are two important properties of the endosymbioses involving dinoflagellates and marine invertebrates. The diversity of symbiotic dinoflagellates, as currently understood, is summarized in Table 1.

4. Transmission

The transmission of symbionts from generation to generation is one of the most puzzling aspects of the alga-coelenterate association. The establishment of the association ranges from certain, based on the presence of symbionts already in the unfertilized egg, all the way to the seemingly unlikely event of its formation *de novo* in every generation. The regular occurrence of the latter mode is of special interest, since it requires the meeting between the as yet, azooxanthellate, newly released planula, and a free-living symbiont of the “right” species. One is tempted to speculate that the more ancient associations are the ones with the most ascertained mode of symbiont transmission, whereas the more recent ones require acquisition in every reproductive cycle.

TABLE 1: The diversity of symbiotic dinoflagellates.

CLADES	MICROALGA	SITE AND HOST	CITATION
A	<i>S. microadriaticum</i>	Caribbean; jellyfish.	Rowan and Powers, 1992; Wilcox, 1997.
A	<i>S. pilosum</i>	Caribbean; zoanthid.	Rowan and Powers, 1992; Wilcox, 1997; Sadler <i>et al.</i> , 1992.
A	<i>S. corculorum</i>	Indo-Pacific; bivalve.	McNally <i>et al.</i> , 1994.
A	<i>S. meandrinae</i>	Caribbean; coral.	McNally <i>et al.</i> , 1994.
A	<i>Gymnodinium-linucheae</i>	Atlantic; jellyfish.	Wilcox, 1997.
A	Symbiont	Red Sea; benthic foraminiferan.	Lee <i>et al.</i> , 1995.
A	Symbionts	Caribbean; corals, sea anemones.	Rowan and Powers, 1991b; Rowan and Knowlton, 1995.
A	Symbionts	Indo-Pacific; giant clam, hydrocoral	Wilcox, 1997; Rowan 1998
A	<i>S. bermudense</i>	Bermuda, <i>Aiptasia palida</i>	Goulet and Cook, 2000
A	<i>Symbiodinium</i>	Hawaii, Free living (benthic).	Carlos <i>et al.</i> 1999
A or B	<i>Symbiodinium</i>	Octocorallia (newly settled polyps)	Coffroth, 2000
A-B	Symbiont	Mid-Pacific; coral.	Rowan and Powers, 1991b.
A-B	<i>Gymnodinium varians</i>	South Pacific; free-living.	Rowan and Powers, 1992; Wilcox, 1997.
A or C	Symbionts	Philippines, giant clam,	Silvestre <i>et al</i> 2000.
B	<i>S. pulchroru</i>	Mid-Pacific; sea anemone.	Rowan and Powers, 1992; Wilcox, 1997; McNally <i>et al.</i> , 1994.
B	<i>S. bermudense</i>	Atlantic; sea anemone.	Rowan and Powers, 1992; Wilcox, 1997; McNally <i>et al.</i> , 1994.
B	Symbionts	Atlantic; Caribbean; corals, gorgonian, sponge.	Hill And Wilcox, 1998; Rowan and Powers, 1991b; Rowan and Knowlton, 1995
B	<i>S. bermudense</i>	Florida , <i>Aiptasia palida</i>	Goulet and Cook, 2000
B	Symbiont	East Pacific; sea anemone.	Rowan and Powers, 1991b
B	<i>Symbiodinium</i>	High latitude <i>Plesiastrea versipora</i>	Rodriguez-Lanetty <i>et al.</i> 2000
B	<i>Symbiodinium</i>	Octocorals (adults)	Coffroth,2000
B	Symbionts	Curacao Netherlands Antilles, 2-5m <i>Madracis</i>	Dickmann, 2000

C	Symbionts	Caribbean; corals, sea anemone.	Rowan and Powers, 1991b; Rowan and Knowlton, 1995.
C	Symbionts	Mid-and-Indo Pacific; benthic foraminifera, corals, zoanthids.	Rowan and Powers, 1991b; Langer and Lipps, 1994; Lee et al., 1995.
C	Symbionts	Atlantic; sponge.	Hill and Wilcox, 1998.
C	Symbionts	<i>Gymnodinium beii</i> , Atlantic; pelagic foraminifera	Gast and Caron, 1996.
C	Symbionts	GBR, <i>Tridacna</i> , <i>Acropora</i> , <i>Heliofungia</i> , <i>Stylophora</i> , <i>Capnella</i> .	Aisvah et al. 2000
C	<i>Symbiodinium</i>	Sub tropical and tropical waters, <i>Plesiastrea versipora</i>	Rodriguez-Lanetty et al. 2000.
?	<i>Gloeodinium viscum</i>	Red Sea; hydrocoral	McNally et al., 1994.
?	<i>Amphidinium belauense</i>	Indo-Pacific; flatworm	McNally et al., 1994.
?	Symbionts	<i>Scrippsiella nutricula</i> , Atlantic; radiolaria, chondrophore.	Gast and Caron, 1996.
A, B, C	<i>Symbiodinium</i>	Caribbean Scleractinian corals	Baker and Rowan 1997.
C	<i>Symbiodinium</i>	Eastern Pacific Scleractinian corals	Baker and Rowan 1997.
D	<i>Symbiodinium</i>	Palau, Sponge <i>Haliclona koremella</i>	Carlos et al. 1999
D or E	Symbionts	GBR, <i>Zoanthus robustus</i>	Aisvah et al. 2000
C and D	Symbionts	Taiwan, <i>Acropora palifera</i>	Yang et al. 2000.
A-D	<i>Symbiodinium</i>	Scleractinian corals	Baker, 2000

During clonal reproduction in foraminiferans, radiolarians and coelenterates, algal symbionts are transmitted directly to offspring. This occurs in the course of cell division of protists, when the symbionts are distributed between daughter cells. The same is the case of colonial organisms such as corals and hydrocorallians, whenever the colony “reproduces” by fragmentation. However, during sexual reproduction of hosts, two possibilities exist: (1) the algae are transmitted directly via the egg (maternal inheritance) as in the hydroid *Myriophyllum*, or the algae and the egg are released simultaneously, the eggs acquiring the adhering algae after fertilization, as in the coronate medusa *Linuche* (Montgomery and Kremer, 1995). In scleractinians, the spawning corals *Pocillopora verrucosa*, *P. eydouxi*, *Montipora effusa* and *M. Verrucosa*, and the brooding species *Porites porites* contain zooxanthellae in the oocytes, ((Hirose et al., 2000; Mate et al., 1998; Tomascik and Sander, 1987; Yeemin, 1988). When the symbionts are transferred directly from host to offspring as in the above examples, the process is known as **vertical transmission**, or **closed system**, which is the norm in cases of asexual reproduction. Parental algal cells may also be placed in the egg cytoplasm immediately prior to fertilization and subsequent release.

This occurs in many 'broodinhg', but few 'broadcasting', corals (Trench, 1979, 1987) (2) the eggs are released without algae, and larval or juvenile stages acquire the algae from the environment. Symbionts are transmitted to the next host generation by a number of means, which are ultimately linked to the reproductive traits of the host. In other hosts, such as broadcasting corals, scyphozoans and tridacnid bivalves, symbionts must be acquired anew by each generation from the open environment after metamorphosis to the adult form. This process is known as **horizontal transmission** or **open system**. Some host organisms (e.g. *Tridacna*) are restricted to this method, as vertical transmission is made impossible due to structural barriers in the host which bars access of the symbiont to the host gametes. Hosts capable of both sexual and asexual reproduction are able to employ both vertical and horizontal transmission of symbionts.

The rather precarious mode of symbiont "transmission" by re-establishment of symbioses at each generation may have some advantages. Such acquisition offers the potential for "reshuffling" host symbiont associations and the formation of novel, more competitive associations, or such with better survival chances under changing environmental scenarios, like those emerging due to current global climate changes. Dinoflagellates, when outside the host are motile and flagellated, but since corals will not ingest free algae, it has been suggested that they acquire symbionts while feeding on herbivorous zooplankton containing viable algal cells in their guts (Douglas, 1994). Alternatively, motile algal symbionts randomly contact host tissue, or may be chemically attracted to them (100).

Also, this mode of host colonization by symbionts implies that these are widely available for acquisition from the environment, which so far has not been conclusively demonstrated. Yacobovitch *et al.* (2000) showed that isolated algae from the soft coral *Heteroxenia fuscences*, added to laboratory grown primary polyps, were seen swimming toward the polyp mouth opening, and that after 4-12 hours, the symbionts were already present in the primary polyps. Subsequently the location of the symbionts changes with time. Their findings indicate that primary polyps are capable of acquiring symbiotic algae over a 2-3 month period. The concentration of *S. microadriaticum* in the sea under normal conditions is low: this organism is not thought to lead a permanent free living existence, and may only be available for re-infection when newly released from former hosts. Indeed, there is evidence that in many cases the doubling rate of the zooxanthellae exceeds the requirement for their stable areal density, and the excess algae are released to the environment (Stimson and Kinzie, 1991). Trench (1993) hypothesized that hosts accept symbionts that they do not recognize as being foreign (the symbionts are recognized as self). According to that view, the algae produce macromolecules that are compatible with the self-recognition system of a specific host. Microalgae are known to possess surface macromolecules and to release macromolecules to the environment. If two different algal taxa possess surface macromolecules with similar characteristics, then both would be recognized as self, and accepted by a given host. However, the algae may possess distinct adaptive characteristics, which allow one to function better in that environment than the other. As a result, one may replace the other. For example Fitt and Trench (unpublished) found a displacement of the intrinsically slower-growing *S. goreauii* by *S. microadriaticum* in

the scyphostoma of *Cassiopea xamachana*. Warner *et al.*, (1996) showed that algae in certain corals were more temperature sensitive than in other corals. Zahl and McLaughlin (1959) suggested that once settled in the host's tissue, the gymnodinioids develop into the vegetative zooxanthella of classical references, which proliferate subsequently by mitosis. These authors proposed that susceptibility on the part of so many marine vertebrates to gymnodinioid infection might be related to the oligotrophy of tropical waters and the resulting lack of growth factors. This situation presumably induces free-swimming gymnodinioids to actively seek animal tissues where catabolic products containing potential precursors of such factors abound. Upon inclusion in a host vacuole, the motile gymnodinioids lose their flagella and metamorphose to the sessile vegetative form, the zooxanthella. Benayahu *et al.*, (1992) showed that the zooxanthellae from the gastrovascular cavity of *Litophyton arboreum* are endocytosed by the follicular layer and then translocated into future sexual offspring. They showed that the entry of the zooxanthellae into the oocytes of *Litophyton arboreum* involved an elaborate ultrastructural pathway. At all stages of the process, symbionts are translocated while residing within vacuoles, which most probably inhibits lysosomal fusion (Fitt and Trench, 1983a, 1983b). Oocytes of both species of *Pocillopora*, *P. verrucosa* and *P. eydouxi* picked up zooxanthellae 3 to 4 days before spawning. At first, zooxanthellae were evenly distributed in oocytes, and later moved to the hemisphere that contained the germinal vesicle. Their results suggest that only blastomeres that had been determined to develop into gastrodermal cells receive zooxanthellae during cleavage (Hirose *et al.*, 2000).

The follicular cells of young oocyte phagocytose extruded symbiotic algae found within vacuoles and surrounded by a cytoplasmic sheath. Subsequently, gaps open in the underlying mesoglea, and zooxanthellae within vacuoles, are translocated along with follicular cytoplasm into the perioocytic zone, where the symbionts accumulate, suspended within cellular debris. Prior to the breeding season, the symbiont bulges through the oolema into the periphery of the mature oocyte (Wood, 1999).

Adults of the octocorals *Briareum polyanthes* and *Plexaura kuna* harbor zooxanthellae belonging to *Symbiodinium* clade B over a range of habitats and depths. Their planulae initially lack zooxanthellae and acquire algae upon metamorphosis. Newly settled polyps acquired zooxanthellae of *Symbiodinium* clade A or Clade B depending on the location of settling. After 3-6 months the majority of polyps harbored zooxanthellae of the same clade as those found in adult hosts regardless of site. In early ontogeny the host symbiont interaction appears to be more plastic than in the adults, which harbor *Symbiodinium* clade B throughout the species range. This flexibility may be driven by either local algal abundance or selection for the zooxanthellae taxon best adapted for that environment (Coffroth, 2000). There also is evidence for replacement of symbiotic algae in juvenile tridacnids (Belda-Baillie *et al.*, 1999).

Transplanting corals from deep (20-23 m) to shallow (2-5 m), will cause bleaching and lead to symbiont community change, however, transplanting from shallow to deep did not lead to such change (Baker, 2000a).

Corals, which receive zooxanthellae from their mother colonies, may also acquire symbionts from the environment, since symbiotic identity appears to depend on the

locality of the host corals rather than the specific identity of the coral host or its mode of symbiont acquisition. (Hidaka and Hirose, 2000).

5. Photosynthesis and Nutrient Fluxes

The mutualistic symbiotic association between zooxanthellae and coral is the basis for the extraordinary success of corals and coral-reef ecosystems, which virtually form “oases” teeming with life within the otherwise barren “blue desert” of the tropical oceans. Translocation or leakage of photosynthate from the algae to the host provides a major, variable, part of the host’s metabolic needs (Davies, 1977; Muscatine *et al.*, 1984).

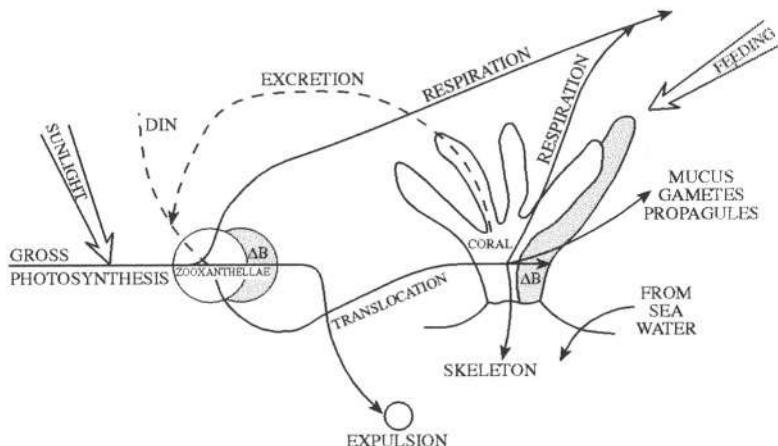


Figure 3 Scheme of energy transfer between zooxanthellae and coral host.

Based on that fraction, corals may be ranked on a niche continuum ranging from heterotrophy to autotrophy, depending on the relative share of symbiont-photosynthesis and prey in their energy budget. In return, the zooxanthellae benefit from “leftovers” of prey digestion by the host and excreted products of the turnover of the coral’s tissues (Fig. 3, (Dubinsky and Jokiel, 1994). The flux of carbon into zooxanthellate corals is controlled by the underwater light intensities (Dubinsky *et al.*, 1984; Falkowski *et al.*, 1984; Falkowski and Dubinsky, 1981; Muscatine *et al.*, 1983, 1984; Porter *et al.*, 1984). Zooxanthellae, like all phytoplankton, photoacclimate within a week to new ambient light levels. The photoacclimation process involves among other accommodations, changes in the pigmentation of the zooxanthellae, and adjustment of all the parameters of photosynthesis as to optimize the harvesting and utilization of the underwater light-field. Thus, shallow water colonies are pale, whereas their conspecifics from shaded

crevices or deeper reef-locations are much darker (Fig. 4). That difference stems from low light induced increases in pigmentation, while the areal density of the zooxanthellae remains largely unchanged (Dubinsky *et al.*, 1984; Falkowski *et al.*, 1984; Falkowski and Dubinsky, 1981; Porter *et al.*, 1984).

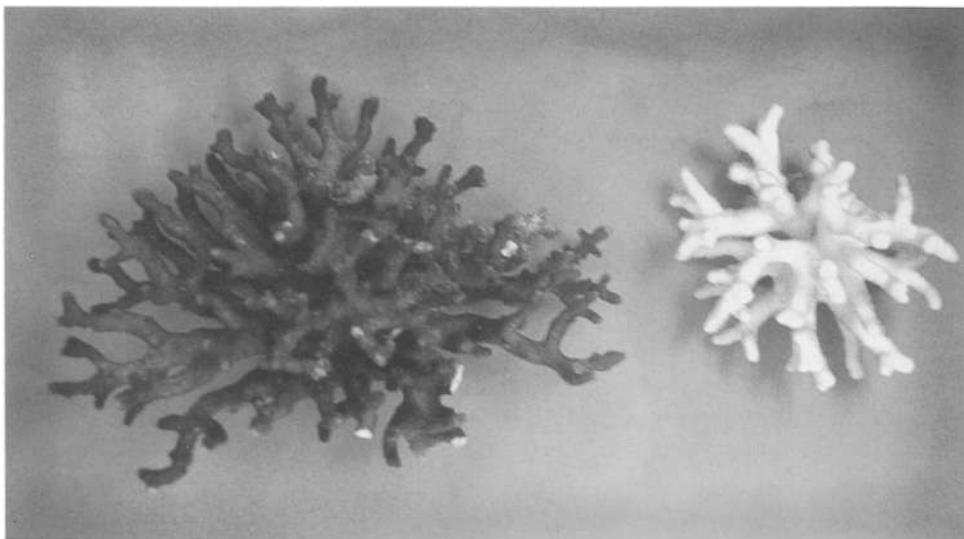


Figure 4 Low- and high- light acclimated colonies of *Stylophora pistillata*

Changes in the population density of the zooxanthellae, which also affect colony color, are due to physiological stress responses to environmental disturbance (Gates, 1990). When exposed to elevated nutrient levels or eutrophication, algal division uncouples from host growth. Such runaway symbiont proliferation is detrimental to the coral, since now the algae retain carbon compounds normally supporting the host (Dubinsky *et al.*, 1990; Muscatine *et al.*, 1989; Stambler *et al.*, 1991). Significant differences in all parameters of photosynthesis were found among 24S genotypes of zooxanthellae belonging to clades A, B, C that were isolated from Bermudan corals (Savage and Douglas, 2000). These parameters included P_{max} , (light-saturated photosynthesis), α the initial slope of the photosynthesis versus irradiance relationship and I_k (saturating light intensity). Indeed these differences are additional parameters underscoring the heterogeneity of the zooxanthellae, which include, besides ultrastructural and molecular

traits differences in their physiological response to various environmental factors (temperature, light, salinity) as found for *Symbiodinium* clades A and C from giant clams (Sison *et al.*, 2000).

6. Origins and Evolution

The search for the origins of photosymbioses has been approached on the basis of the fossil record of morphological and isotopic data from potential hosts, and potential symbionts. In general, photosynthesis preferentially sequesters the lighter ^{12}C isotope leaving a pool enriched in ^{13}C , whereas, respired CO_2 is assumed to be depleted in both ^{13}C and ^{18}O , relative to the CO_2 source. This means that carbonate skeletons precipitated under the influence of photosynthesis are predicted to show enrichment in $\delta^{13}\text{C}$ composition compared to a skeleton of non symbiotic organisms from the same environment. Data from different clades of extant taxa and isotopic studies of extinct species demonstrate that photosymbiosis has been present in planktonic foraminifera for at least 75 million years (d'Hondt and Zachos, 1995). Zooxanthellae isotopic signatures have been sought in fossil scleractinian corals. Material from the Upper Triassic (Norian: Turkey and Italy) sites, the Jurassic (Tithonian: Poland) site and the Eocene (England) site examined geochemically (Mackenzie *et al.*, 1997; Stanley and Swart, 1995). The resultant data show a strong positive correlation between ^{13}C and ^{18}O isotopes in the Jurassic material. The data from the Upper Triassic sites showed isotopic signals similar to that of living zooxanthellae, although the carbon isotopes are 3-5‰ heavier than the Modern. A specimen of the Eocene also showed an isotopic signal similar to those living zooxanthellae corals (Wood, 1999). The origin data of photosymbioses is summarized in Table 2.

Prior to the Triassic, based on their morphology, the symbionts were chlorophytes, rhodophytes or cyanophytes. Unlike the contemporary zooxanthellae associations such symbioses might not have been limited to low nutrient environments (Wood, 1993). The dinoflagellate symbiont, *Symbiodinium* that is so successful at overcoming the defense systems of the host, did not evolve until the Triassic (Moldowan *et al.*, 1996), when it replaced all other symbiont taxa. The symbiosis between the hermatypic corals and the algae allowed in the course of the co-evolution of both partners the development of coral reefs in increasingly oligotrophic waters. During the evolution steps most hermatypic coral species acquired algal symbionts, and became zooxanthellate whereas few others never did. A noteworthy exception is the temperate coral *Astrangia danae* which may growth with or without zooxanthellae at different depths (Szmant-Froelich and Pilson, 1977; Veron, 2000).

The dynamic nature of symbiotic combinations in corals may have allowed them to persist through hundreds of millions of years of rapid, and sometimes extreme, environmental change (Buddemeier, 1997). One is tempted to wonder whether these remarkable, ancient associations will evolve also to survive the perils of the current global warming, anthropogenic eutrophication and increased UV flux. Unfortunately, currently it is estimated that as many as 70% of the worlds reefs have declined to the point of no return (Wilkinson, 1998).

Table 2: The origin data of photosymbioses (adapted from Wood, 1999)

HOST	SYMBIONT	FIRST APPEARANCE OF SYMBIONT	FIRST APPEARANCE OF HOST	INFERRRED TIMING OF ACQUISITION BY HOST
Scleratinian corals	Dinoflagellate <i>Symbiodinium</i>	?Proterozoic abundant by Upper Triassic (Norian) ~206Ma*	Middle Triassic (Anisian) ~228Ma	At least by Upper Triassic (Norian) ~206Ma
Rotaliine foraminiferans	Diatom	Late Cretaceous ~65Ma	Late Cretaceous ~65Ma	Late Cretaceous ~65Ma
Milioline foraminiferans	Dinoflagellate	?Proterozoic abundant by Upper Triassic (Norian) ~206Ma	Upper Triassic ~206Ma	Upper Triassic ~206Ma
Tridacnid bivalves	Dinoflagellate	?Proterozoic abundant by Upper Triassic (Norian) ~206Ma	Eocene ~33.5Ma	Eocene ~33.5Ma

*Ma, million years

7. References

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ALGAL SYMBIOSIS IN FLATWORMS

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1. Introduction

Flatworm-algal symbiosis may be the most understudied group of associations considering the numerical abundance, diversity of taxa, range of habitats, and geographic distribution of these symbioses. They occur in both fresh water and marine habitats, and have been reported in temperate and tropical oceans where they are a common, though often overlooked, component of both littoral and pelagic environments. The simplicity of the body plan and small size hide a complex balance between specificity and plasticity in these interactions. Although the symbionts, and sometimes the hosts, of many of the associations have not been described, associations that have been studied show host specificity to particular algal genera; however, different algal strains can be dominant under different environmental conditions. Within the Platyhelminthes as a whole, separate host species can form stable associations with distantly related algae including dinoflagellates, diatoms, and chlorophyceans.

Even some of the leading researchers in the field have downplayed their importance. In a review of algal symbioses in acoels, Douglas (1992) writes “The algal symbioses in acoel turbellaria are not ecologically or economically important and... the principal rationale for their study is to test the generality of hypotheses developed from investigations of other alga-invertebrate symbioses”. While it is clear that the small size, short generation time, aposymbiotic larvae, and diversity of symbionts do provide excellent experimental systems, the ecological importance of natural populations remains unclear. The complete absence of solid ecological studies from the entire last century shows only that we do not understand the ecological interactions, it does not quantify them. The paucity of information on these associations results from the small size of the flatworm hosts, absence of hard parts which facilitate preservation, difficulty of morphological identification, and lack of attention.

Much of the recent literature is focused on the “discovery” of new organisms, new habitats, and new modes of reproduction suggesting we have yet to discover much of the diversity of flatworm-algal symbiosis. The literature suggests symbiotic flatworms

have been introduced into new habitats multiple times, but it is only recently that Rivest *et al.* (1999) described the introduction of *Convoluta convoluta* to the Gulf of Maine as an invasion, and thus somewhat comparable to the many cases of introduced species known to cause ecological damage. Throughout the range of the invasive population, *C. convoluta* occurs at high densities of 19 individual cm⁻², and like many symbiotic flatworms continue to feed as adults. Other symbiotic flatworms occur at high densities as well. A presumably native population of *Amphiscolops* sp. in Belau occurs at densities of 1.28x10⁶ m⁻³ (Trench and Winsor, 1987). It is difficult to imagine any metazoan population at average densities of greater than a million per cubic meter as ecologically insignificant.

Biologists from several disciplines have gained a renewed interest in the biology of the Platyhelminthes as a result of their newly recognized importance for understanding metazoan evolution and the resolution of deep branching phylogenies. The application of molecular systematics has brought greater attention to these questions, although many of the relationships have yet to be resolved. As additional sequence data accumulates, it not only facilitates further systematic work, but also investigations directly relevant to the study of symbiosis, including population dynamics of both host and symbionts, biogeography, and the evolution of symbiotic interactions.

The growing recognition of the importance and prevalence of flatworm-algal symbioses worldwide, and active research into the systematics of the hosts is heartening. Together with the inherent advantages of these associations for laboratory studies, these signs suggest the near future may hold great revelations within the field of flatworm-algal interactions, and thus to symbiosis as a whole. It is for this purpose that we undertake this review of the field. It is not meant to be a recognition of what has been achieved as much as to provide a foundation for future studies.

2. General considerations

There is an extensive literature on symbiotic interactions with members of the Platyhelminthes forming associations with larger organisms (see reviews by Jennings, 1974, Jennings, 1997, and also González and Salazarvallejo, 1996, Kato, 1951, Villalba *et al.*, 1997). In these associations the flatworms are not the host organisms as they are in flatworm-algal systems. Instead they are typically the smaller partner in associations that are often parasitic or commensal. Flatworms are also known to serve as a host for symbioses with organisms other than algae, such as chemosynthetic bacteria (Lundin and Hendelberg, 1995, Ott *et al.*, 1982), Rickettsiae (Williams, 1991), protozoa (Watson and Jondelius, 1997), and viruses (Justine *et al.*, 1991); however, these interactions will not be considered here.

Some knowledge of the distribution of the host organisms is essential for understanding the basic life histories. A detailed description of the geographic range of the various host organisms is beyond the scope of this work. Thus the geographic range will only be included in reference to aspects of the biology such as invasions.

It is remarkable that the diversity of flatworm algal symbioses extends to nearly every aspect of the interactions including systematics of the associations, location of

habitats, modes of reproduction, and degree of metabolic and anatomical integration. Given that little is known about many of these associations, it is often necessary to focus on the few well studies examples primarily and use other examples to illustrate the range of currently known possibilities. This necessity is not meant to imply that these examples represent a typical condition. Instead the reader should remain aware of the need for good comparative work in this field.

3. Host diversity

The taxonomic makeup of the Platyhelminthes is far from clear. Recent work suggests the Platyhelminthes may be paraphyletic (Carranza *et al.*, 1997). Placement of the Acoela is particularly difficult given the rather simple architecture of the body plan and high rate of molecular evolution of the 18S rDNA molecule among this group (Carranza *et al.*, 1997). As most of the symbiotic flatworms that have been characterized are acoels, the placement of the Acoela is particularly relevant to the study of these symbioses. Although most molecular analyses place the Acoela within Platyhelminthes (see Berney *et al.*, 2000, Campos *et al.*, 1998, Litvaitis and Rohde, 1999), or find placement of the acoels uncertain (Littlewood *et al.*, 1999, Telford *et al.*, 2000), one study has suggested the Acoela represent a distinct lineage (Ruiz-Trillo *et al.*, 1999). Recently, further efforts to clarify the phylogenetic position of the group included analysis of sequences for a different gene, elongation factor 1-alpha, without the long branch attraction problem of acoel 18S sequences. Results for *Symsagittifera* (=*Convoluta*) *roscoffensis* and five other Platyhelminthes show the Acoela branching within Platyhelminthes suggesting they remain within that group (Berney *et al.*, 2000). Molecular analyses have been helpful with many taxa where morphological characters were absent or confusing (Distel *et al.*, 1988, Rowan, 1991), but have yet to provide a clear picture for the Platyhelminthes.

It is likely that the relationships within the Acoela will continue to change as well. According to the current taxonomy, symbiosis is restricted to only two of the 17 families of acoels (Tyler and Bush, 1998), namely Convolutidae and Sagittiferidae. There are no molecular based studies designed specifically to elucidate the relationships among the symbiotic acoels; however, the analyses of Littlewood *et al.* (1999), in both molecular and combined molecular and morphological data sets, show relationships inconsistent with the current taxonomy. In these analyses, *C. convoluta* and *Praesagittifera* (=*Convoluta*) *naikaiensis* are sister taxa more closely related to *Amphiscolops* spp. than to *Convoluta pulchra* (Littlewood *et al.*, 1999). It is possible this reflects an artifactual arrangement due to low sampling density among the Acoela with *P. naikaiensis* the only representative of the Sagittiferidae, but clearly more work needs to be done. Taxa used throughout this chapter and their taxonomic affiliations are summarized in Table 1. Of note to those familiar with flatworm algal symbiosis is the use here of *S. roscoffensis* in place of the familiar "*Convoluta roscoffensis*".

The freshwater flatworms have undergone less revision. All are Rhabdocoels with *Dalyellia viridis* and *Dalyellia penicilla* of the family Dalyelliidae, and *Phaenocora typhlops* and *Typhloplana viridata* of the Typhloplanidae.

4. Diversity of algae

The taxonomic relationships among many of the symbiotic algae remain to be resolved for the simple reason that most of the symbionts have yet to be fully described. It is clear that several separate lineage's can form symbioses with flatworms including members of the Bacillariophyta (Diatoms), Dinophyceae (Dinoflagellates), and Chlorophyta (Trench and Winsor, 1987, Taylor, 1971, Parke and Manton, 1967, Ax and Apelt, 1965). The symbionts characterized to date represent specific lineage's within those diverse groups. The dinoflagellates all appear to belong either to the genus *Amphidinium* or *Symbiodinium*. Only one diatom association is known, and apparently is restricted to members of the genus *Licmophora*. Chlorophyte symbionts of the marine flatworms are all characterized as Prasinophytes of the genus *Tetraselmis*. Two morphologically distinct subgenera are recognized, *Tetraselmis* and *Prasinocladia*. Only one freshwater symbiont has been adequately characterized and is identified as *Chlorella vulgaris* var. *vulgaris*, which is a Trebouxiophyte.

Although initially believed to be parasites by Graff (1882), Keeble and Gamble (1907) first recognized the symbionts as algae in *S. roscoffensis*. Since that time their placement has been changed several times, but the symbionts are currently recognized as members of the genus *Tetraselmis*, with the most common symbiont being *T. convolute* (Douglas, 1992, Norris *et al.*, 1980, Parke and Manton, 1967, Taylor, 1971). Laboratory experiments show all *Tetraselmis* species tested are capable of forming symbioses with *S. roscoffensis*, but *S. roscoffensis* containing *Tetraselmis* cells of subgenus *Tetraselmis* grow faster and produce more offspring than those containing subgenus *Prasinocladia* (Douglas, 1985, Douglas, 1988, Douglas, 1992). Field collected *S. roscoffensis* typically has *T. convolute* as the dominant symbiont (see review by Douglas, 1992). Other *Tetraselmis* spp. dominate as symbionts only in *S. roscoffensis* from Aberthaw Beach, UK, where *T. convolutae* is apparently absent (McFarlane, 1982a, Douglas, 1985).

Tetraselmis appears to be the most common genus of symbionts among the marine Acoels (Table 1). Some caution is warranted as many of the reports of *Tetraselmis* symbionts for hosts other than *S. roscoffensis* rely on cursory examination of the symbiotic state which lacks many of the morphological traits of the free living forms (see Bush, 1984, Devaney and Eldredge, 1987, Hanson, 1961, Stoecker *et al.*, 1989 and also Taylor, 1974). One notable exception is the recent work by Balzer (1999) where she has isolated and cultured the symbiont of *Convolvularia longifissura* and verified it is indeed a previously unknown species of *Tetraselmis*.

Symbionts of other marine flatworms are distinguished by their brown coloration in contrast to the green associated with the *Tetraselmis* spp. symbionts. Taylor (1971) identified the symbiont of *Amphiscolops langerhansi* as the dinoflagellate *Amphidinium klebsii*. Trench and Winsor (1987) identified the symbionts of two other flatworms. *Amphiscolops* sp. and *Haplodiscus* sp. as dinoflagellates as well. The symbionts were identified as *Amphidinium* sp.* for the *Amphiscolops* sp., and two morphologically distinct dinoflagellates were described for *Haplodiscus* sp.. *Haplodiscus* sp. appears to routinely maintain both *Amphidinium* sp. and *Symbiodinium* sp. types simultaneously (Trench and Winsor, 1987).

Another marine acel with brownish coloration, *C. convoluta*, harbors diatom symbionts. Ax and Apelt (1965), and Apelt (1969), describe the symbionts as members of the genus *Licmophora* including the related species *Licmophora hyalina* and *Licmophora communalis*. Taylor (1971) questions whether *Licmophora* is the true symbiont of *C. convoluta* as aposymbiotic juveniles can be infected with environmentally collected material containing *Licmophora* sp., but cultured *Licmophora* sp. were unable to infect (Apelt, 1969). The symbionts have never been successfully cultured from an infected host, but analyses using molecular sequence data are underway. Comparison of 18S rDNA sequences confirms the symbionts are diatoms, and is consistent with their identification as *Licmophora* sp., but the paucity of 18S sequences for *Licmophora* spp. make it impossible to verify its position within the genus at this time (McCoy, unpublished data).

Among the freshwater flatworms, *Chlorella vulgaris* var. *vulgaris*, the symbiont of *P. typhlops*, is the only symbiont which has been reliably identified (Eaton and Young, 1975a). Haffner (1925) reports the symbiont of *D. viridis* to be "*Chlorohydra viridissima*", but provides inadequate detail for reliable confirmation by current taxonomic methods. The others can only be described as zoochlorella (Buchner, 1965, Genevois, 1924, Limberger, 1918), a term that is fundamentally descriptive, not taxonomic in nature.

It is clear that much work remains to be done on the identification and characterization of the symbionts of flatworms. Perhaps the realization that relatives of *Tetraselmis* also form symbioses with other marine organisms such as foraminifera will rekindle interest in the identification of flatworm symbionts (Gast *et al.*, 2000). Many of the *Tetraselmis* spp. are culturable which allows the reliable identification and description of new species. As sequence data becomes available it will likely prove as useful to the identification of flatworm symbionts as it has in symbionts of other marine invertebrates (Distel *et al.*, 1988, Lee, 1998, Rowan, 1991, Rowan, 1998).

* Trench and Winsor (1987) report culturing *Amphidinium* sp. from *Amphiscolops* sp. but not from *Haplodiscus* sp.. A later report, McNally *et al.*, (1994) describes "*Amphidinium belauense* Trench isolated from the flatworm *Haplodiscus* sp. (Trench and Winsor 1987)". We have chosen to ignore the specific epithet until we can verify the origin of the culture material.

Table 1. Taxonomic affiliation of flatworm taxa described in the text and their associated algal symbionts.

Host organisms	Algal symbionts
Rhabdocoela	
Family Dalyelliidae	Chlorellales (Chlorophyta)
<i>Dalyellia viridis</i>	Family Chlorellaceae
<i>Dalyellia penicilla</i>	<i>Chlorella (vulgaris?)¹</i>
	<i>Unidentified²</i>
Family Typhloplanidae	
<i>Phaenocora typhlops</i>	<i>Chlorella vulgaris var. vulgaris³</i>
<i>Typhloplana viridata</i>	<i>Chlorella (vulgaris?)⁴</i>
Acoela	
Family Sagittiferidae	Chlorodendrales (Chlorophyta)
<i>Symsagittifera roscoffensis</i>	Family Chlorodendraceae
<i>Symsagittifera psammophila</i>	<i>Tetraselmis convolutae</i> (and other spp.) ⁴
<i>Convolutriloba longifissura</i>	<i>Tetraselmis</i> sp. ⁵
<i>Convolutriloba retrogemma</i>	<i>Tetraselmis</i> sp. ⁶
<i>Praesagittifera naikaiensis</i>	Unidentified, probably <i>Tetraselmis</i> sp. ⁷
	<i>Tetraselmis</i> sp. ⁸
Family Convolutidae	Gymnodiniales (Dinophyceae)
<i>Amphiscolops langerhansi</i>	Family Gymnodiniaceae
<i>Amphiscolops</i> sp. (Brazil)	<i>Amphidinium klebsii⁹</i>
<i>Amphiscolops</i> sp. (Belau)	<i>Amphidinium</i> sp. ¹⁰
<i>Haplodiscus</i> sp.	<i>Amphidinium</i> sp. ¹¹
<i>Convoluta pulchra</i>	<i>Amphidinium</i> sp. and <i>Symbiodinium</i> sp. ¹¹
	None (nonsymbiotic host species) ¹²
<i>Convoluta</i> sp. (Hawaii)	Chlorodendrales (Chlorophyta)
<i>Convoluta</i> sp. (N. Atlantic)	Family Chlorodendraceae
<i>Convoluta</i> sp. (E. Atlantic)	<i>Tetraselmis</i> sp. ¹³
	<i>Tetraselmis</i> sp. ¹⁴
<i>Convoluta convoluta</i>	Unidentified Prasinophyte ¹⁴
	Bacillariales (Bacillariophyta)
	Family Tabellariaceae
	<i>Licmophora</i> spp. ¹⁵

1. Douglas (1987); 2. Heitkamp (1979); 3. Eaton and Young (1975a); 4. Douglas (1992); 5. Sarfatti and Bedini (1965); 6. Balzer (1999); 7. Åkesson and Hendelberg (1989); 8. Yamasu (1982); 9. Taylor (1971); 10. Lopes (1994); 11. Trench and Winsor, (1987); 12. Smith and Bush (1991); 13. Devaney (1987); 14. Stoecker et al. (1989); 15. Apelt (1969).

5. Ultrastructure

Considering the diversity of algal types, which form associations with flatworms, it is not entirely surprising that there is variation in ultrastructural arrangements. Algae have been reported to be located intracellularly or extracellularly. Douglas (1992) suggests all acoel symbionts may be located intracellularly and points out that the early reports of extracellular locations for the *T. convolutae* symbionts of *S. roscoffensis* (Oschman and Gray, 1965), and *A. klebsii* symbionts of *A. langerhansii* (Taylor, 1971), were based on micrographs of sections. Subsequent studies on *T. convolutae* in *S. roscoffensis* and *Amphidinium* sp. in *Amphiscolops* sp. (from Belau) have indicated an intracellular location for these two systems (McFarlane, 1982b; Trench and Winsor, 1987); however, neither the host *Amphiscolops* sp. nor the symbiont *Amphidium* sp. studied by Trench

and Winsor (1987) are the same as those studied by Taylor (1971). In fact, Trench and Winsor (1987) note structural differences between their samples and those of Taylor (1971) including reduction of theca, loss of flagella, and lack of the ordered arrangement of the symbionts seen in *A. langerhansii*.

Licmophora symbionts of *C. convoluta* are another example where the symbionts are reported to be extracellular based solely on microscopic evidence (Apelt, 1969). Although definitive studies have yet to be performed, neither visible membranes nor host nuclei are apparent adjacent to symbiont cells in squashes, or wet mounts of whole or partial *C. convoluta* which is consistent with an extracellular location (McCoy, pers. obs.). Thus, until definitive evidence becomes available, it seems most prudent to rely on the existing experimental literature for each species. An extracellular location of the symbionts is reported for *C. convoluta* (Apelt, 1969), *C. longifissura* (Bartolomaeus and Balzer, 1997), and *A. langerhansii* (Taylor, 1971), as well as for the symbionts of the freshwater flatworms *D. viridis* (Haffner, 1925), and *P. typhlops* (Buchner, 1965). In contrast, *S. roscoffensis*, and *Amphiscolops* sp. (from Belau) are reported to house their symbionts intracellularly (McFarlane, 1982b, Trench and Winsor, 1987).

Loss or reduction of the algal cell wall, or theca, is also variable among many of the flatworm symbionts. The diatom symbionts of *C. convoluta* lose their frustules in the symbiotic state but continue to divide within the host (Apelt, 1969). It is possible the loss of frustules could be one difficulty in culturing these symbionts, but the diatom symbionts of foraminifera also lack frustules while within the host yet readily acquire them upon dividing after isolation (Lee, 1998). As noted above, *Amphidinium* sp. in *Amphiscolops* sp. (from Belau) show a reduced theca (Trench and Winsor, 1987), while *A. klebsii* in *A. langerhansii* show no reduction in theca (or flagella) (Taylor, 1971). Additionally, *A. klebsii* assume a characteristic uniform orientation inside the host tissue that is interpreted as an adaptation for optimal light uptake (Taylor, 1971). *Tetraselmis* symbionts also show variation in cell wall retention. *Tetraselmis* sp. symbiotic with *C. longifissura* retain the cell wall inside the host (Balzer, 1999), while all species of *Tetraselmis* spp. tested thus far lose the pectinaceous cell wall during infection of *S. roscoffensis* (Douglas, 1992, Provasoli *et al.*, 1968).

All of the host flatworms studied to date produce aposymbiotic eggs contained within a mucilaginous egg case. Potential symbionts, and other algae, have been found on the egg cases of several host species (Apelt, 1969, Dorey, 1965, Douglas and Gooday, 1982, Eaton and Young, 1975a, Keeble and Gamble, 1907). Douglas (1982) has demonstrated that the occurrence of *T. convolutae* on egg cases of *S. roscoffensis* represents a physical property of the egg cases, and not the result of chemoattraction and this is likely true for other species as well. There is no evidence for direct transfer of symbionts from parent to sexually derived offspring in any flatworm algal symbiosis.

6. Habitats and distribution

Flatworm-algal symbioses are found in a surprising diversity of habitats. Less is published on the freshwater associations than their marine counterparts, but *P. typhlops* and *D. viridis* are likely typical in that they live on and burrow in the mud (Eaton and Young, 1975a, Eaton and Young, 1975b, Haffner, 1925). Marine acoels are common in

littoral environments, and the best studied symbiotic acoel, *S. roscoffensis*, lives interstitially at high tide, and rises to form mats on the sandy surface at low tide (Doonan and Gooday, 1982, Douglas, 1984, Douglas, 1992). Other symbiotic acoels such as *C. convoluta* can also be found littorally, but are more common in the sublittoral environment. It is typically found at highest densities as epifauna on subtidal algae, but can also occur on urchin barrens, soft sediment, or bare rock (Apelt, 1969, McCoy, unpublished data, Rivest *et al.*, 1999).

There is a growing body of evidence that algal symbiosis may be more common among pelagic flatworms (see Bush, 1984, Dörjes, 1970, Lopes and Silveira, 1994, Stoecker *et al.*, 1989). Pelagic flatworms with algal symbionts were first described as early as 1911 (Loehner and Micoletzky, 1911); however, Stoecker *et al.* (1989) were the first to document the widespread occurrence of these symbiotic acoels. Lopes and Silveira (1994) point out the pelagic acoel, *Amphiscolops* sp., which frequently occur in blooms in estuarine and coastal waters of Brazil, are not reported by the taxonomic studies on acoels in that area. Stoecker *et al.* (1989) suggest the acoels have been overlooked in most studies of oceanic plankton because they become unrecognizable with standard fixation protocols. This problem can often be resolved by anesthetizing in MgCl₂ prior to fixation (Smith and Tyler, 1984).

The discovery of new acoels is not limited to the pelagic realm. Smith and Bush (1991) note acoels are common in marine interstitial habitats in North America, but most species found to date are undescribed. Yamasu (1982) reports a similar situation in Japanese waters in his description of five new species of the genus *Convoluta* (now *Praesagittifera* and *Symsagittifera*). The description of two new species of *Convoluta* from the Philippine Islands notes the inability to find a previously described species from this area (Bush, 1984).

It is difficult to identify biological invasions when the endemic fauna are not characterized; however some evidence for invasions of flatworms does exist. Rivest *et al.* (1999) document the invasion of *C. convoluta* into the Gulf of Maine in the Western North Atlantic. This does not appear to be the first habitat expansion of this species. Although known from the North Sea, *C. convoluta* was first found in the Baltic Sea in 1973 (Karling, 1974). It is now known from the Western and Eastern North Atlantic, European Arctic, Baltic Sea, Canary Islands, Mediterranean Sea, and Black Sea (Bush, 1981, Dörjes, 1968, Karling, 1974, Rivest *et al.*, 1999). Human activity may not be the only means of transport, however, as *C. convoluta* has been found associated with marine snow (Bochdansky and Herndl, 1992). Other species are likely invaders as well, but solid evidence is lacking.

There is a seasonal component to the densities of symbiotic flatworms in some temperate habitats. The freshwater flatworm *P. typhlops* in Britain shows the most dramatic seasonal variation as it overwinters in a cocoon stage with a single egg per cocoon. These eggs appear to have an obligatory diapause and in the field most hatched within a period of three to four weeks (Eaton and Young, 1975a). In the Gulf of Maine, *C. convoluta* also show a seasonal variation in abundance possibly as a result of redistribution of the worms by storm activity (B. R. Rivest, pers. comm.). Additionally, egg development in *C. convoluta* is rapid at summer temperatures, but can exceed three months at winter temperatures effectively shutting down reproduction in the winter (McCoy, unpublished data). In the British Channel Islands, the size of the *S.*

roscoffensis population is low in early summer and high in autumn and winter. It is interesting that the variation was observed in colony size, but not density of animals within a colony (Doonan and Gooday, 1982).

7. Specificity

The *S. roscoffensis-Tetraselmis* spp. system is best known as a system for the study of specificity in algal-invertebrate associations. Juvenile *S. roscoffensis* fed a range of algae ingest only free living *Tetraselmis* species and *Chlamydomonas coccooides*, believed to have similar cell walls (Douglas, 1983a). *Tetraselmis* sp. freshly isolated from *S. roscoffensis*, and other algae, are not ingested suggesting a component of the theca triggers phagocytosis (Douglas, 1983a). Pretreatment of the algal cells with lectins or proteases, and inhibition of algal photosynthesis with DCMU failed to inhibit initial uptake, but killed algae were only ingested at a low rate. After ingestion, another aspect of specificity exists as *C. coccooides* failed to persist for more than 12-24 hours (Douglas, 1983a). In the light, *Tetraselmis* cells ingested by *S. roscoffensis* lose their theca and flagella and are transported to the periphery of the parenchyma (Douglas, 1983a, Provasoli *et al.*, 1968). If the animals are maintained in darkness through the initial phases, they do not retain the algae (Douglas, 1992). After transport to the periphery of the parenchyma, *S. roscoffensis* can still selectively eliminate algae via the mucus glands (Provasoli *et al.*, 1968). Presumably the selection is based on an as yet unidentified nutritional interaction as the retained algae always promotes faster growth of the host (Douglas, 1983a, Douglas, 1992, Provasoli *et al.*, 1968).

Field observations provide interesting hints at specificity in *S. roscoffensis* as well. Free living *T. convolutae* are typically present in the environment where *S. roscoffensis* are found, and in those locations it is the dominant symbiont (Douglas, 1992, Provasoli *et al.*, 1968); however, at one location, Aberthaw, South Wales, *T. convolutae* are absent. *Convoluta roscoffensis* at this site are found with *Tetraselmis* species from two morphologically distinct subgenera, *Tetraselmis* and *Prasinocladia*, but never both together (McFarlane, 1982a). Douglas (1988) interprets this duality to the rarity of free living *Tetraselmis* at this site due to the desiccating conditions. *Prasinocladia* can tolerate the desiccating conditions more readily (McFarlane, 1982b), but in competition experiments *Prasinocladia* algae are always expelled in favor of the *Tetraselmis* types (Douglas, 1985, Douglas, 1988). As *S. roscoffensis* feed only as juveniles, it appears that those juveniles which encounter algae of subgenus *Tetraselmis* form symbioses with these types and those which only encounter the more abundant *Prasinocladia* cells allow those cells to persist (Douglas, 1992). As the symbiosis appears obligate to the host in all flatworm-algal systems investigated to date, but are acquired from the environment with each host generation, the geographical range of the host may be limited by the distribution of suitable symbiont populations (Douglas, 1992).

Considerably less is known about specificity in other flatworm-algal associations, but clearly some degree of specificity exists. Feeding trials with *A. langerhansi*, which normally hosts a dinoflagellate symbiont, and *C. convoluta*, which hosts diatoms, failed to infect either host with any of several *Tetraselmis* species (Keeble, 1908, Taylor, 1971). Likewise, juvenile *S. roscoffensis* do not feed on the dinoflagellate *Amphidinium*

sp. (Douglas, 1983a). Other than the notable case of *Haplodiscus* sp., all the algae within an individual host are morphologically similar. *Haplodiscus* sp. is notable in that it harbors two distinct dinoflagellates, an *Amphidinium* sp. and a *Symbiodinium* sp., simultaneously. In fact, both types can occur within a single host cell, and the ratio of *Symbiodinium* cells to *Amphidinium* cells appears nearly constant among individuals (Trench and Winsor, 1987).

Trench and Winsor (1987) found an *Amphiscolops* sp., which naturally harbored an undescribed *Amphidinium* sp., could also be infected with *A. klebsii*, but not any *Symbiodinium* spp.. This is consistent with the assertion of Douglas (1992) that these associations are specific to the level of algal genera. Admittedly even a single genus such as *Symbiodinium* can harbor a great deal of variation with potentially profound consequences for the symbiotic interaction (McNally *et al.*, 1994, Rowan *et al.*, 1997, Rowan, 1998). Symbionts of *C. convoluta* are reported to be specific to diatoms of the genus *Licmophora* with two species, *L. hyalina* and *L. communis*, shown to be capable of forming a symbiosis in the lab (Apelt, 1969). Small subunit (18S) rDNA sequences for the symbionts of *C. convoluta* from several sites along the Atlantic coast of North America, which represent a recently invasive population (Rivest *et al.*, 1999), are identical to sequences from samples collected in Sweden (McCoy, unpublished data). This is consistent with a single symbiont species being dominant despite the geographic separation. The more variable ribosomal internal transcribed spacer (ITS) region does show some variation among samples allowing comparisons within and between hosts, sites, and lifestyles (symbiotic or free-living). It will be interesting to see what insights into specificity and biogeography of the association unfold as these studies progress.

8. Nutritional Interactions

The photosynthetic ability of acoel symbionts was demonstrated before the symbionts were recognized as algae. Geddes (1879) noted the accumulation of starch and production of oxygen from what he believed to be chloroplasts in *S. roscoffensis*. Welsh (1936) provided the first rigorous study of O₂ production by an algal-flatworm symbiosis utilizing *A. langerhansi*. In the dark, symbiotic *A. langerhansi* consume oxygen, but in the light symbionts provide enough O₂ for animal respiration (Welsh, 1936). The freshwater flatworms *D. viridis* and *D. penicilla* both respire O₂ produced by their symbionts (Heitkamp, 1979). Likewise, symbiotic *P. typhlops* survive low oxygen conditions longer than their aposymbiotic counterparts which may be of advantage in the freshwater pools it inhabits (Eaton and Young, 1975b). Although providing O₂ for animal respiration may be necessary only under certain conditions, using rates of O₂ production as an estimator of photosynthesis provides an excellent tool for studying nutritional interactions more directly. Taylor (1971) provides a quantitative study of the rates of oxygen production and consumption in symbiotic *A. langerhansi*. Unfortunately, this predates the use of O₂ production rates for calculations of translocation from symbiont to host (Muscatine *et al.*, 1984). Readers unfamiliar with these techniques should consult the reviews of Muscatine (1980, 1990).

Traditionally, carbon-14 tracer studies have been used to evaluate the partitioning of photosynthetically fixed carbon between host and symbiont in a number of systems.

Eggs and mucus of host origin become labeled when symbiotic *S. roscoffensis* are incubated with $\text{NaH}^{14}\text{CO}_3$ in the light (Muscatine *et al.*, 1974). Difficulties in separating host and symbionts without damage to the algae complicated estimates of the portion of fixed carbon translocated to the host. Boyle and Smith (1975) resolved this problem by the development of a novel method of symbiont isolation. They discovered that *S. roscoffensis* will eject most of their algae if CO_2 is briefly bubbled through the media. These algae appear healthy and fix carbon at rates approaching those of the intact association. Rates were further enhanced if the released algae were incubated at pH 5.5, which is believed to be the internal pH of the worm (Boyle and Smith, 1975).

Algae ejected from the host only release about 2% of their fixed carbon to the media with the major release products being amino acids (Boyle and Smith, 1975). The main soluble fixation product, however, is mannitol (Muscatine *et al.*, 1974, Boyle and Smith, 1975). If symbionts are ejected after photosynthetic labeling with ^{14}C , they contain about half of the total fixed carbon suggesting there is substantial translocation to the animal. Boyle and Smith (1975) suggest the translocated products may still be primarily amino acids, but that is based largely on the findings of Muscatine *et al.* (1974), who showed the animal is incapable of metabolizing externally supplied mannitol. In another variant of isotopic tracer studies, Meyer *et al.* (1979) demonstrated that *S. roscoffensis* lacks the ability to synthesize de novo long-chain fatty acids and sterols. Instead *S. roscoffensis* relies on utilizing or modifying products obtained from the symbionts which are their only source of nutrition (Meyer *et al.*, 1979).

Other associations do not rely entirely on symbiont derived nutrition, but only limited data exists on rates. Mixotrophic planktonic flatworms fixed from $0.6\text{-}27 \text{ ng C worm}^{-1} \text{ h}^{-1}$ (Stoecker *et al.*, 1989). In the freshwater associations with *Typhloplana viridata* and *D. viridis* and their *Chlorella* like symbionts, Douglas (1987) showed the symbionts translocate 30-40% of the photosynthetically fixed ^{14}C to the animal tissues. The primary release product from freshly isolated symbionts was maltose with the maximal release at pH 4 (Douglas, 1987).

The major nutritional interaction in these associations may not be strictly carbon based but may instead be related to nitrogen recycling (or nitrogen conservation, see Douglas, 1987). Cultures of *T. convolutae*, the primary symbiont of *S. roscoffensis*, can utilize a wide range of organic nitrogen compounds as the sole nitrogen source including uric acid (Gooday, 1970, Taylor, 1974). In culture, *T. convolutae* has an active uricase and the specific activity of the uricase increases three fold when uric acid is the sole nitrogen source (Gooday, 1970). Symbionts in the intact association appear to have a specific activity comparable to that for cultured cells grown in the presence of uric acid if host proteins are accounted for (Boyle and Smith, 1975, Douglas, 1983c). Juvenile *S. roscoffensis* contain solid crystals of uric acid when hatched. Uric acid content decreases to undetectable levels 15-20 days after larvae are infected with symbionts, but aposymbiotic juveniles accumulate additional uric acid (Douglas, 1983c). Uric acid increased in symbiotic animals incubated in ammonium, but not nitrate, suggesting use of uric acid is subject to ammonium repression. Incubation in DCMU also caused increases in uric acid content demonstrating utilization is promoted by photosynthetic activity (Douglas, 1983c).

Whatever compounds form the basis for the nutritional interaction, it is clear that the degree of host dependence on symbiont derived compounds is variable. For example, *S.*

roscoffensis adults are totally dependent upon their symbionts as the adults do not feed (Keeble and Gamble, 1907, Douglas, 1992). In nature, *Symsagittifera* (= *Convoluta*) *psammophila* feeds as an adult, but laboratory cultures can be maintained in a defined mineral medium in the light without feeding (Sarfatti and Bedini, 1965, Taylor, 1971). Several other hosts regularly feed as adults including *C. convoluta*, all known pelagic flatworms, and *A. langerhansi* (Apelt, 1969, Lopes and Silveira, 1994, Stoecker *et al.*, 1989, Taylor, 1971). In fact, aposymbiotic *A. langerhansi* can grow to adult size, though not sexual maturity, if fed sufficiently (Taylor, 1971). It is likely that the nutritional interactions in most associations rely primarily on translocation and not digestion of symbionts, but digestion of at least some symbionts has been noted in a few hosts (Åkesson and Hendelberg, 1989, Balzer, 1999, Eaton and Young, 1975a).

It is clear that reproductive output is tied to the nutritional interactions of these symbioses. Hanson (1960) first described the ability of *A. langerhansi* to undergo both sexual and asexual (architomy) modes of reproduction the result of which can be distinguished by the lack of a statocyst in asexually derived progeny. Although no nutritional link was suggested in this work, it was noted that asexual progeny appear after a dark period, and illumination could halt the fission like asexual division (Hanson, 1960). Taylor (1971) extended this work by demonstrating that aposymbiotic *A. langerhansi* grew more slowly than their symbiotic counterparts and were more likely to undergo architomy. Symbiotic *A. langerhansi* also underwent architomy if unfed, but reproduce sexually when fed suggesting architomy in this species is a response to nutritional deficiency (Taylor, 1971).

Asexual reproduction appears to be the normal, and perhaps only, mode of reproduction in several other species including an undescribed species of *Convoluta* (Marcus and Macnae, 1954), *C. retrogemma* (Hendelberg and Åkesson, 1988), and *C. longifissura* (Bartolomaeus and Balzer, 1997). In at least *C. retrogemma*, reproductive potential is still tied to feeding as fed animals reproduced at higher rates than those relying only on symbiotic algae (Åkesson and Hendelberg, 1989). Likewise, reproductive output is correlated to nutrition in some strictly sexually reproducing associations. In the now familiar example of *S. roscoffensis*, egg production ceases when animals are incubated in DCMU suggesting that algal photosynthesis contributes to egg production (Douglas, 1983b). Additionally, *S. roscoffensis* containing symbionts of the subgenus *Tetraselmis* produced egg capsules containing more embryos than *S. roscoffensis* containing symbionts of subgenus *Prasinocladia* which is believed to be a result of the higher nutritional output of *Tetraselmis* cells (Douglas, 1985).

9. References

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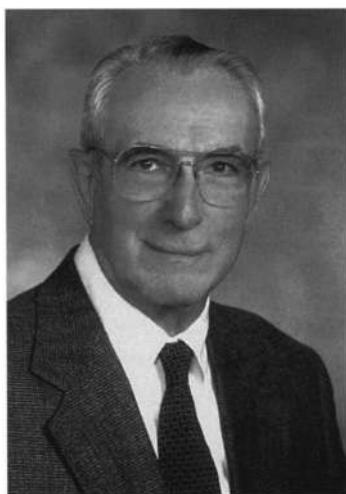
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Biodata of **Robert W. Lichtwardt**, author of “*Trichomycetes: Fungi in relationship with insects and other arthropods.*”

Dr. Robert W. Lichtwardt is Professor Emeritus of the Department of Ecology and Evolutionary Biology at the University of Kansas (USA) where he served twice as the chairman of the Botany Department. He obtained his Ph.D. in Botany (Mycology) at the University of Illinois in 1954. Dr. Lichtwardt's main interest is in mycology of fungi that parasitize or are symbiotically associated with insects, with emphasis on the systematics, physiology, nutrition, ecology, morphogenesis, ultrastructure, biogeography and evolution of the Trichomycetes (a class of fungi that live obligately within the guts of arthropods). Among his numerous publications, he is the author of *The Trichomycetes: fungal associates of arthropods* published by Springer-Verlag, New York (1986). Also, he co-authored (with Misra, J.K.) the *Illustrated genera of Trichomycetes: fungal symbionts of insects and other arthropods* [Science Publishers, Inc. Enfield, New Hampshire (2000)]. Dr. Lichtwardt has gained several awards. He served as the president of the Mycological Society of America (MSA), obtained awards for Distinguished Mycologist and for Teaching Excellence in Mycology, was elected as Honorary Life Member of MSA, and was the Editor-in-Chief of Mycologia. The Japanese Mycological Society made him an Honorary Member, and he is a Centennial Fellow of the British Mycological Society.

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TRICHOMYCETES: FUNGI IN RELATIONSHIP WITH INSECTS AND OTHER ARTHROPODS

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1. The symbiotic relationship

Environments that are unusual or extreme invariably require special adaptations if organisms are to succeed. Trichomycetes live as obligate fungal symbionts in the guts of insects, millipedes, and various kinds of crustaceans (crabs, anomurids, isopods, and amphipods). Their association with such hosts is unique among fungi. The evolutionary events in adapting to the gut environment required not only the ability to withstand the digestive processes of the gut, but also a range of morphological and physiological changes that distinguish them from their closest relatives in the Zygomycota. These include special spores to ensure transmission from host to host, the ability to attach firmly to the gut cuticle, and a sensitivity to host development. The host seeks out food and an optimal environment, which in turn benefits the fungus. Though gut habitation offers advantages, existence of these gut fungi is nonetheless precarious. This is because of their attachment to the linings of the gut—the cuticle of the foregut or hindgut, or the peritrophic membrane of the midgut—that are shed along with the fungi during each molting cycle. This has required a reliable means of continually reestablishing the fungi in another suitable gut.

Trichomycetes do not appear to harm the host in most instances, and are therefore commensalistic. But, as described below, there is evidence that at least under special circumstances the fungi can contribute to the welfare of the host by supplying organic metabolites that may be deficient in the external environment. At least one species of gut fungus is lethal to mosquito larvae, and there are other species capable of invading the ovaries of insects, leading to their sterilization and consequent reduction of fitness to some degree within the host population.

2. Evolutionary success and possible origins

Success of Trichomycetes can be judged by their presence in hosts that live in a wide variety of habitats, by the range of arthropod host types they infest, and their distribution on all continents, except Antarctica, as well as many islands throughout the world (Lichtwardt, 1986). Insect larvae in freshwater streams at highest altitudes and marine

crustaceans at abyssal depths contain gut fungi (Van Dover and Lichtwardt, 1986), as do a wide variety of terrestrial arthropods.

Currently, 55 genera and 225 species are described (Misra and Lichtwardt, 2000), with new taxa being recorded at a rapid rate. It can be assumed that only a fraction of extant species of these cryptic fungi have been discovered, possibly many in new kinds of arthropods not yet studied. Published trichomycete taxa, hosts, and habitats are given in Table 1. It should be noted that current molecular studies are expected to lead to taxonomic revisions of major taxa.

TABLE 1. Taxa of Trichomycetes^a (Zygomycota), arthropod hosts, and habitats

Order	No. of genera/species	Host types and habitats
Harpellales	33/141	Aquatic insect larvae: Diptera (Nematocera), Ephemeroptera, Plecoptera, Trichoptera, Coleoptera, Isopoda
Asellariales	3/11	Isopoda from freshwater, marine and terrestrial habitats; Insecta (Collembola)
Eccrinales	17/61	Crustacea from freshwater, marine, and terrestrial habitats; terrestrial Coleoptera and Diplopoda
Amoebidiales ^a	2/12	Aquatic insect larvae and Crustacea

^a Amoebidiales, though traditionally classified in Trichomycetes, are protozoans not phylogenetically related to the other three orders (Benny and O'Donnell, 2000); they are often encountered in or on some of the same hosts with Trichomycetes.

The known distribution of genera varies widely, from being cosmopolitan to regionally endemic. Examples of worldwide genera include *Harpella*, *Smittium*, and *Stachylina* in aquatic dipteran larvae, and *Enterobryus* in millipedes. Other genera—to the extent current data indicate—may be geographically restricted, such as *Allantomyces* in Western Australian mayfly nymphs (Caenidae), *Caudomyces* in Japanese crane fly larvae, and *Graminelloides* in Costa Rican blackfly larvae. Most genera are found on more than two continents. Evidence suggests that the distribution of gut fungi is entirely dependent on the hosts' vagility and dispersal patterns, and that propagules of Trichomycetes serve only to reinfect their hosts within a population and over short distances.

In the absence of a fossil record, knowledge about the origins of Trichomycetes is circumstantial. Three observations suggest that these specialized fungi have an ancient origin. First, there is some evidence from morphology, serology, and sequencing of DNA that Harpellales may share a common ancestor with some of the Kickxellales (Zygomycetes) (Young, 1969; Sangar *et al.*, 1972; Moss and Young, 1978; Peterson and Lichtwardt, 1987; O'Donnell *et al.*, 1998; Gottlieb and Lichtwardt, 2001). Second, Harpellales are found almost exclusively in the most primitive aquatic insects, namely Plecoptera, Ephemeroptera, and the lower dipteran suborder Nematocera, whose origins go back some 190–250 million years B.P. Third, one can cite the wide host range of the

class Trichomycetes and the degree to which the fungi have integrated their development with different host types (see below), which suggest a long evolutionary history. The hypothesis is that Harpellales may have begun their evolution shortly after the first aquatic insects began evolving, that major tectonic processes played a significant role in the early distribution and vicariant speciation of arthropods already infested with gut fungi, leading to the Trichomycetes' present successful worldwide establishment as symbionts.

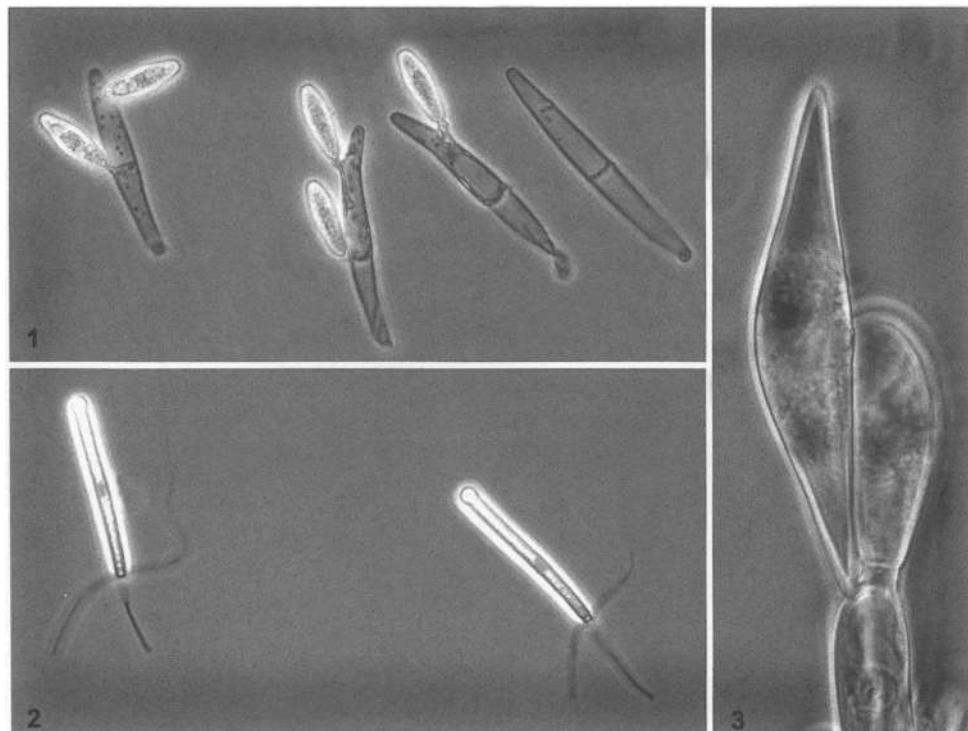
3. Adaptive mechanisms of transmission and dispersal

All of the structures and processes summarized below are unique to Trichomycetes, and are adaptations from a presumed ancestor shared with the free-living Kickxellales.

3.1. TRICHOSPORES AND ZYGOSPORES

The basic reproductive structures of Zygomycota are asexually produced sporangiospores, and zygospores that result from karyogamy following hyphal conjugation. The name *trichospore* was coined for the modified external sporangia of Harpellales, which contain inside a single sporangiospore (Manier and Lichtwardt, 1968). The trichospore normally breaks away at maturity from the cell that produced it (the generative cell), and in almost all genera the trichospore bears at its base one to many appendages, depending upon the genus (Figures 1, 2). Two adaptive features differentiate the trichospore from other fungal sporangia. The trichospore is the disseminating unit, and the sporangiospore within it extrudes and develops into a new thallus only after the trichospore has been ingested by a suitable host (see Section 5 for host stimuli that lead to extrusion). The basal appendages of trichospores, as they form within the generative cell but outside the plasmalemma, have a unique ontogeny and structure (Reichle and Lichtwardt, 1972; Moss and Lichtwardt, 1976). Most species of Harpellales live within insect larvae that require flowing waters—sometimes turbulent—for survival, and appendages apparently aid in successful transmission of the fungus to another host in such an environment by becoming attached to the substrate after passing from the gut. This retains many of the trichospores within the larval population and increases the probability of being ingested by the same or another larva. The efficiency of trichospore transmission is described in Section 3.5 below.

Zygospores of Zygomycetes are typically diploid, spherical, thick-walled structures designed to withstand unfavorable environmental conditions, and they undergo meiosis just prior to germination. In Harpellales it is not known to what extent zygospores are resistant, because they have never been produced in culture and therefore are unavailable for experimentation. But their shape and ploidy appear to be adaptive. Unlike other Zygomycota, all zygospores of gut fungi are biconical or are pointed at one end (Figure 3), rather than spherical (Lichtwardt, 1986). Limited ultrastructural evidence indicates that meiosis occurs prior to zygospore formation, such that the zygospore contains a single, haploid nucleus (Moss and Lichtwardt, 1977). Little is known about zygospore germination in Harpellales, but occasionally these have been observed to extrude from



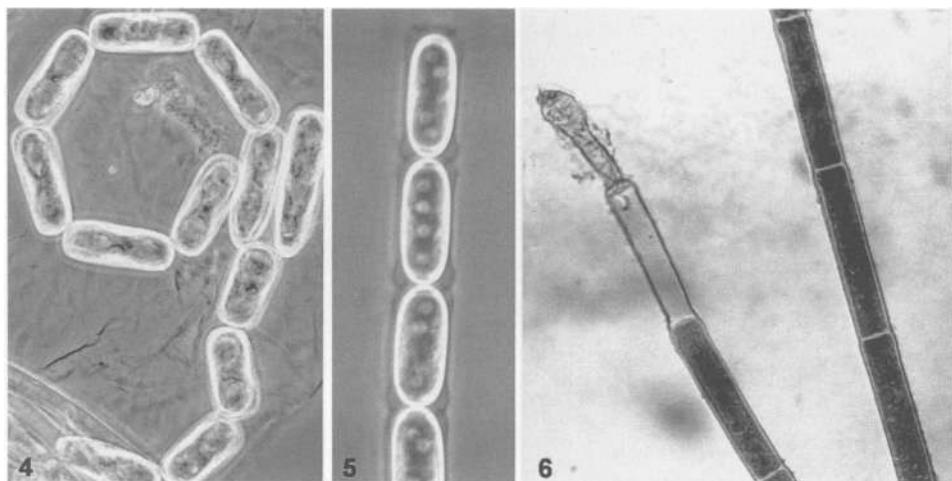
Figures 1–3 Reproduction in the Harpellales. 1. Thalli of *Stachyliina nana* producing trichospores, as seen through the peritrophic membrane of a midge larva (Chironomidae) from Costa Rica. 2. Released trichospores of *Glotzia tasmaniensis*, each with 3 appendages, from the hindgut of a Tasmanian mayfly nymph (Ephemeroptera). 3. Biconical zygospore of *Pennella simulii* from the hindgut of a Newfoundland blackfly larva (Simuliidae).

one of the pointed ends, a process that results in the inner protoplast almost explosively leaving the outer wall, similar to trichospore extrusion (Whisler, 1963; Moss, 1970; Lichtwardt, 1972). The haploid nucleus means that an ingested zygospore, when it reaches the appropriate location in the gut, can immediately germinate, attach to the cuticle, and produce a new haploid thallus (the normal somatic condition in Harpellales) before it becomes expelled from the gut. Otherwise it might require many hours or days to undergo meiosis and germination that is typical in the Zygomycetes.

3.2 ARTHROSPORES

Arthrospheres are produced in Asellariales (Figure 4), but have been studied only in *Asellaria ligiae* from the hindgut of marine isopods (Manier, 1963; Lichtwardt, 1973, 1986). Those of the other asellarid genus, *Orchesellaria*, appear to be quite different. *Asellaria ligiae* is a branched fungus whose cells at maturity disarticulate (Figure 4). These have been interpreted as modified sporangia. When they germinate they closely resemble the modified trichospores of *Carouxella* (Harpellales), which do not detach from the generative cell, but rather disseminate as a trichospore/generative cell unit (Lichtwardt,

1973). Manier *et al.* (1961) suggested that such dissemination units might also germinate and produce thalli in the originating gut, much as secondary sporangiospores of Eccrinales do (see next Section).



Figures 4–6. 4. Arthrospores disarticulating from branches of *Asellaria ligiae* (Asellariales) in the hindgut of an isopod (*Ligia* sp.) from Hawaii. 5. Thick-walled primary infestation sporangiospores of *Taeniella carciin* (Eccrinales) in an anomurid, *Callianassa fitholti*, from South Island, New Zealand. 6. Secondary infestation sporangiospores in *Enterobryus elegans* (Eccrinales) from the millipede *Narceus americanus* in the USA; sporangiospores have escaped from the two terminal sporangia, and the sporangial wall of one is being decomposed by bacteria.

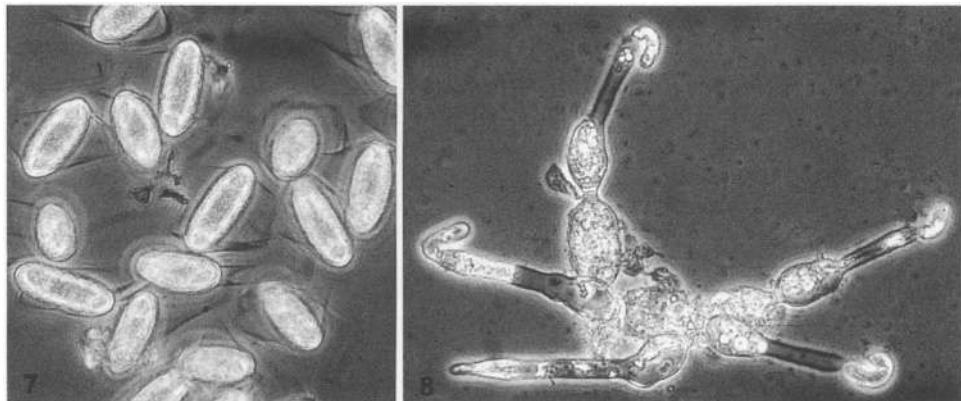
3.3. SPORANGIOSPORES

Unlike sporangia of Zygomycetes, those of the trichomycete order Eccrinales are produced in basipetal succession at the ends of the unbranched ecrinid thalli, each normally containing a single spore. Two basic types exist. One has been called the primary infestation sporangiospore (Lichtwardt, 1986), which serves to transmit the fungus from one gut to another. Primary spores of most ecrinid genera are thick walled (Figure 5), and presumably resistant to unfavorable external conditions. More commonly produced are secondary infestation sporangiospores (Figure 6). Such spores are released from the sporangium and germinate within the gut that produced them, in this way providing new thalli endogenously. This can be viewed as an adaptive mechanism to increase reproduction following initial gut infestation by an ecrinid, because each individual thallus alone produces but a limited number of spores during the intermolt period.

3.4. OVARIAN CYSTS

Harpellales were believed to live exclusively in the larval stages of aquatic insects. Consequently, it was not understood how these common gut fungi could so successfully

disperse from pond to pond, from one drainage area to another, or upstream. Yeboah *et al.* (1984) in Newfoundland, Canada, found several types of cysts in the ovaries of blackflies (Simuliidae), but thought they might be a stage of a zoospore-producing water mold. Moss and Descals (1986) proved that the cysts they found "oviposited" on simuliid egg masses in Great Britain were, in fact, *Harpella melusinae* (Figure 7), perhaps the most common and widespread simuliid gut fungus. It appears that this growth of the fungus from the larval gut into the developing ovaries occurs at particular times of the year, but what induces the parasitic phase in the life cycle remains to be elucidated.



Figures 7, 8. Fungal cysts of *Harpella melusinae* (Harpellales) removed from the ovaries of an adult blackfly (*Simulium* sp.) from New York State, USA. 8. A cluster of cysts that have germinated in water and are beginning to produce the coiled trichospores of *H. melusinae*.

This interesting means of dispersing the fungi by the flying adult converts the benign, commensalistic fungus in the larval gut into a pathogen that sterilizes the female. In the laboratory, some of the cysts have been germinated in water to produce trichospores (Figure 8). Those trichospores presumably can infect larvae in nature, perhaps larvae that have hatched from egg masses on which fungal cysts have been oviposited (Moss, 1998). Several species of harpellid cysts have now been found in the ovaries of simuliids and chironomids. At this time, *Harpella*, *Genistellospora*, and *Smittium* have been identified through trichospores that develop from the cysts (Labeyrie *et al.*, 1996; Moss, 1998), and other cyst types are currently being identified by DNA sequencing in the author's laboratory (M.M. White, unpubl.).

Trichomycete-infested mosquito and biting midge (Ceratopogonidae) larvae have been found in disjunct habitats such as rock holes, tree holes, discarded beer cans, cut bamboo stems, and cemetery flower vases (Manier *et al.*, 1961; Lichtwardt, 1986; Lichtwardt and Williams, 1990; Lichtwardt *et al.*, 1999). As yet no ovarian cysts have been found in these dipterans, but it is likely that they exist. In fact, one could hypothesize that all Harpellales have evolved a similar mechanism of dispersal by means of ovarian cysts.

3.5 EFFICIENCY OF TRANSMISSION FROM HOST TO HOST

Most fungi produce abundant spores to ensure dissemination and survival. Individual thalli

of Trichomycetes, on the other hand, produce relatively few propagules for transmission from host to host, yet they succeed admirably well. One may conclude that the efficiency of this process is high. An extreme example of low trichospore production, yet with the potential for most larvae in a population to be infested, can be found in species of *Harpella* and *Stachylina*. On the average many species of these genera produce about four to eight trichospores per unbranched thallus. *Stachylina nana* (Figure 1), for example, currently known in lotic chironomid larvae from France, Costa Rica, and Japan usually produces two to eight trichospores (Lichtwardt, 1984, 1997; Lichtwardt *et al.*, 1987). *Stachylina minima* from New Zealand and Argentina produces one to four trichospores per thallus (Williams and Lichtwardt, 1990; Lichtwardt *et al.*, 1999). To survive as a species, this means that on the average one of those trichospores would have to leave the host gut, be ingested by another suitable host, extrude in the midgut, attach to the peritrophic membrane, and sporulate. Fitness in such cases should be measured more in terms of successful transmission from host to host rather than in numbers of propagules produced per thallus.

3.6 FUNGAL RESPONSES TO THE MOLTING PROCESS

The molting process in arthropods, which expels their fungi, is especially critical to the mycobionts. In some genera of Eccrinales (e.g., *Taeniella*, *Eccrinidus*) thick-walled primary infestation sporangiospores (Fig. 5), presumably resistant to adverse external conditions, begin to form prior to molting of their crustacean or millipede hosts. The hindgut exuvia may contain numerous resistant spores.

It is assumed in Harpellales that zygosporae are resistant, but this has not been demonstrated experimentally because to date zygosporae have not been produced in cultured species. Some harpellid genera (e.g., *Legeriomycetes*, *Simuliomyces*, *Plecopterozymyces*) form masses of zygosporae at the end of the last larval stage, usually with a cessation of trichospore production and even death of the thalli following zygosporae formation.

Such morphogenetic shifts indicate that these fungi are sensitive to the initiation of ecdysis. Unfortunately, none of the fungal species that exhibit this response is culturable, and consequently the factors that trigger resistant spore formation remain unknown. There exist genera of all three trichomycete orders that apparently do not produce resistant spores, including species of the very common and widespread genus *Enterobryus* (Figure 6). Successful as they are, other mechanisms must function that ensure continued reinfestation by the voided fungi, such as the act of consuming part of their molt containing viable spores.

4. Culturability

The more than 225 axenic isolates of Harpellales maintained in the author's laboratory belie the fact that they represent only 9 genera and 30 species (plus a number of unnamed species). No Eccrinales or Asellariales has been cultured. Among Harpellales, 80% of the cultured species belong to the large genus *Smittium* (Figure 9). Other genera—and even many *Smittium* spp.—have proved to be intractable to culturing *in vitro*, even where

species that are culturable coexist in the same gut. In such cases, one would expect their gut environment to be identical.

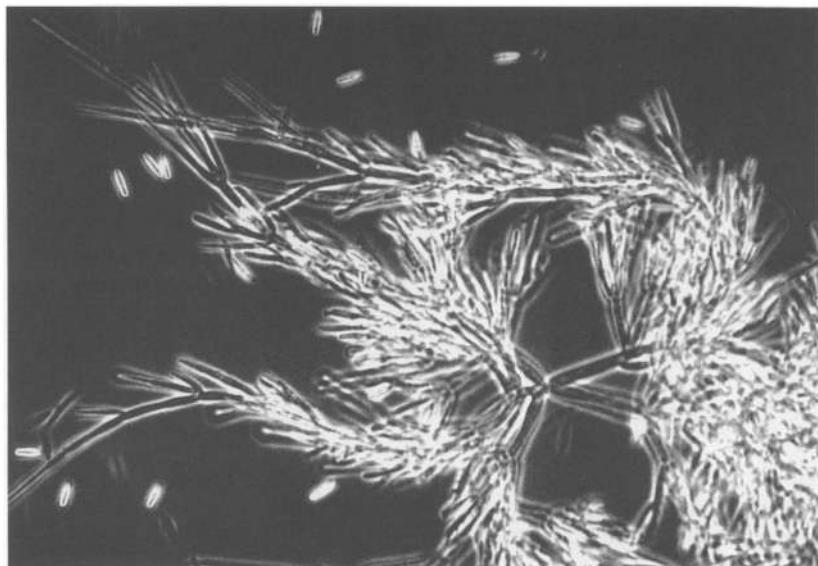


Figure 9. Axenic culture of *Smittium culisetae* (Harpellales) obtained from a mosquito larva, *Culiseta impatiens*, from Colorado, USA., with some released trichospores.

Most axenic isolates have been obtained and maintained on one of two media. Most commonly used has been a diluted commercial brain-heart infusion (Lichtwardt, 1986; Misra and Lichtwardt, 2000). The other is a tryptone-glucose-salts medium. Antibiotics are used to control bacteria that are universally present in arthropod guts. Harpellales are aquatic fungi, and their growth on agar media usually requires an overlayer of water.

5. Host specificity

There are many records of Trichomycetes infesting a single host species. In some cases this may be the actual case, but in other instances it may simply reflect a lack of sufficient examination of related hosts. More common is a generic or a familial host range. In *Enteromyces callianassae* (Eccriniales), the range can span both decapods and anomurids.

Studies on host specificity are difficult to conduct unless culturable fungi are used, preferably with arthropods that can be raised in the laboratory. Horn's experiments (1989a, b, 1990) addressed the phenomenon that trichospores *in vitro*, with very few exceptions, remain quiescent until ingested by a suitable host, at which time development occurs rapidly as the trichospores pass through the gut. Development in these unisporous sporangia begins with a rapid extrusion of the sporangiospore from the sporangium (the trichospore outer wall). Horn used cultures of *Smittium culisetae* and *S. culicis* from mosquito larvae, and discovered that the *in vitro* stimulus for sporangiospore extrusion involved two

phases. The first required exposing trichospores to a pH value of 10 in the presence of potassium ions. When the pH was lowered to 7 in phase two, within minutes extrusion occurred. These are stimuli that trichospores encounter as they pass from the foregut into the hindgut of mosquito larvae where growth of thalli occurs. Cultured harpellid species from other host types did not respond to the same stimuli for extrusion. This suggests that the trichospore is sensitive to its particular host. If an unsuitable host ingests a trichospore, the spore presumably passes through the gut intact, perhaps providing it an opportunity to be ingested by another larva within which it can develop.

6. Pathogenicity

Harpellales reveal two types of pathogenicity. The first, in *Smittium morbosum*, has been reported to kill various species of mosquito larvae. This fungus has been recorded in Australia, Italy, USSR, Japan, and Argentina (Sweeney, 1981; Coluzzi, 1966; Dubitskii, 1978; Sato *et al.*, 1989; López Lastra, 1990; García *et al.*, 1994). It apparently does so when the thalli attached to the hindgut cuticle grow forward and slightly penetrate the midgut epithelium. At that region in the gut, a small and externally visible melanization occurs (Sweeney, 1981). Apparently the infected larvae are incapable of molting and die, but the fungus does not consume the larvae.

The other type of pathogenicity in Harpellales affects fertility of adult insects when gut fungi invade the ovaries and produce fungal cysts that replace egg development, as described above in Section 3.4. Some such form of transmission of the gut fungi from site to site is conceivably present in all Harpellales which otherwise grow only in the larval gut.

7. Mutualism

Using a culture of *Smittium culisetae* and *Aedes aegypti* raised axenically in order to eliminate effects of bacteria and other gut organisms, Horn (1980) (Horn and Lichtwardt, 1981) demonstrated that larvae raised in nutrient-deficient media survived better to pupation, and developed at a faster rate, when larvae were infested with the gut fungus. He tested the larvae in separate media each of which was deficient in either sterol or one of several B-vitamins. Extrapolations from these experiments indicate that some Trichomycetes, while living as commensal in their hosts, essentially become mutualists in situations where one or more essential nutrients that the fungus can provide are suboptimal or absent in the environment.

8. Geographic distribution and co-evolution

These brief comments on distribution of Trichomycetes acknowledge the fact that our understanding of where extant species of gut fungi occur is deficient, despite considerable field work in many parts of the world in recent decades. Excluded here are widespread distributions of some arthropods with fungal symbionts that may be attributable to human

activities. These include springtails (Collembola), some species of millipedes and mosquitoes, and bloodworms (Chironomidae), among others (Lichtwardt, 1986).

The majority of marine crustaceans with ecrinid gut fungi are known from or near the intertidal zone. *Arundinula galathea* was found at depths of 15-25 m (Manier and Ormières, 1962), and *A. abyssicola* was reported by Van Dover and Lichtwardt (1986) from a galatheid squat lobster living around hydrothermal vents at depths around 2600 m. Some marine Trichomycetes have a distribution in several oceans [e.g., *Taeniella carci* (Figure 5) and *Enteromyces callianassae* in crabs and anomurids, *Palavascia sphaeramae* and *Asellaria ligiae* (Figure 4) in isopods]. Thus, one species of fungus may infest many species of hosts. Apparently as the hosts became widely distributed, they carried their respective gut fungi with them which remained evolutionarily stable during the radiation of their hosts.

A similar situation is found in some terrestrial arthropods. For example, *Leidyomyces attenuatus* has been found in the anterior hindgut of many genera and species of passalid beetles ranging from eastern North America to southern Brazil (Lichtwardt *et al.*, 1999). Another fungus, *Passalomyces compressus*, has also been found in many different Passalidae, but may be restricted to tropical beetles. Such a wide host range and geographical distribution indicates that these fungal species must have been symbiotically associated with their hosts during their early radiations, as also appears to be the case of the harpellid fungus, *Genistelloides hibernus* in many *Allocapnia* spp. (Plecoptera, Capniidae) in eastern North America (Lichtwardt *et al.*, 1993).

Widely disjunct distributions of some Trichomycetes may be attributable not only to insufficient surveys but also to past extinctions of host populations. *Furculomyces*, with two species in Chironomidae in Australia until recently was thought to be limited to Australia, but a new species was discovered in the Rocky Mountains of Colorado, USA (Misra *et al.*, 1999). *Carouxella* spp. have been found in France, Australia, and Argentina (Lichtwardt, 1986; Lichtwardt *et al.*, 1999), all in different species of *Dasyhelea* (Diptera, Ceratopogonidae). *Harpellomyces* spp. occur in *Thaumalea* spp. (Diptera, Thaumaleidae) in Europe, eastern North America, and Japan (Lichtwardt and Moss, 1984; Lichtwardt *et al.*, 1987; and unpubl.). In the last two examples, where different species of one genus of insect harbors different species of one genus of fungus, co-evolution of the symbionts is probable. An analysis of Trichomycetes infesting Chironomidae at the subfamily and generic levels suggests a high degree of co-evolution among these fungi and their hosts (Slaymaker *et al.*, 1999).

9. Concluding remarks

Trichomycetes are unique among symbiotic fungi. Their adaptation to living in arthropod guts has resulted in many structural and functional features that make them distinct from other Zygomycota. A worldwide distribution of Trichomycetes and the wide range of arthropod types and habitats to which they have adapted are evidence of their evolutionary success.

Parasitism that results in death of the host currently is known only in one species, *Smittium morbosum*, in mosquito larvae. However, several Harpellales in blackfly larvae have been found at particular times of the year to invade the ovaries, which become filled

with fungal cysts that are “oviposited” by the sterile female. It is postulated that this mechanism of fungal dispersal by adults may have evolved in all species of Harpellales, given that they normally inhabit only larval stages.

Trichomycetes share arthropod guts with a range of prokaryotes, nematodes, and protozoans. In those symbiotic relationships where effects on the host are subtle, it is necessary to distinguish between the consequences of fungal activity and that of other microorganisms in the gut if the role of the mycobiont is to be experimentally ascertained. A study by Horn (1980) using axenically raised larvae of *Aedes aegypti* and a culture of *Smittium culisetae* showed that the gut fungus could provide a benefit by supplying some of the required sterol and B-vitamins when the mosquito larvae were deprived of those essential nutrients. Thus, in Trichomycetes that normally appear to be commensalistic, their symbiotic roles may not be obvious, or may change, depending upon the species and circumstances.

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EVOLUTION OF ASCOMYCOTA-ARTHROPODA SYMBIOSES

Ascomycete-Arthropod Symbioses

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1. Introduction

The Ascomycota (=ascomycetes) is the largest phylum of the Kingdom Fungi, with greater than 32,000 described species (Hawksworth et al 1995). It is estimated, however, that only 5-10% of all species of fungi have been described and that the majority of species in the Kingdom Fungi remain undiscovered (Hawksworth 1991, 1997, Hawksworth & Rossman, 1997, but see May 1991). The Ascomycota includes species of diverse growth forms (e.g., unicellular yeasts, filamentous, etc.) and diverse ecologies and nutritional modes (e.g., mycorrhizae, plant pathogens, lichens, etc.). They can be found in virtually all habitats on earth from arctic tundras, to deserts, to temperate and tropical forests, to aquatic and marine ecosystems. Throughout these environments, ascomycetes associate with most major groups of arthropods in a multitude of symbioses ranging from beneficial to antagonistic host interactions. This chapter will present a review of the evolution of some of the major arthropod symbioses found throughout the Ascomycota with an emphasis on phylogenetics. I will not cover all known ascomycete-arthropod symbioses, but rather I will limit my discussion to the major symbioses for which we currently possess the most complete phylogenetic and ecological knowledge. Several texts exist that provide a more complete coverage of the general biology of fungal-arthropod symbioses (Batra 1979, Wheeler & Blackwell 1984, Wilding et al 1989), but there exists a definite need for a more current treatment. Also, I will discuss a range of symbioses (e.g., beneficial and antagonistic) and interactions (e.g., dispersal, endosymbionts, etc.), as these have long been considered within the realm of fungal symbioses (de Bary 1887, Blackwell 1994).

2. Fungal Phylogenetics

Prior to discussing ascomycete-arthropod symbioses, a general discussion of the current state of knowledge of fungal phylogenetics is warranted. In the last 10 to 15 years, mycology has experienced unprecedented advancements in the field of fungal phylogenetics and in our understanding of the relationships of the major groups of fungi (reviewed in Alexopoulos et al 1996). The two major reasons for these advancements

are the widespread use of explicit phylogenetic methodology in data analyses (e.g., parsimony, maximum likelihood, etc.) and the ability to collect large amounts of nucleotide sequence data (Bruns et al 1991, Hibbett 1992, Alexopoulos et al 1996). The polyphyly of fungi is well established (Barr 1992, Baldauf et al 2000) and there exists no fewer than three separate clades of eukaryotes that have at one time or another been considered fungi. These include the Kingdom Fungi, the heterokont fungi of the Kingdom Heterokonta (= Kingdom Chromista), and the slime molds of the Kingdom Mycetozoa (Alexopoulos et al 1996, Cavalier-Smith et al 1994, Baldauf et al 2000). Several studies have also demonstrated that the Kingdom Fungi is more closely related to the animals than it is to either plants or numerous groups of algae (Wainwright et al 1992, Baldauf et al 2000). The clade containing the kingdoms Fungi and Animalia may also include diverse groups of "protozoa" such as the choanoflagellates (Barr 1992, Cavalier-Smith et al 1994) and microsporidia (Baldauf et al 2000, Keeling et al 2000).

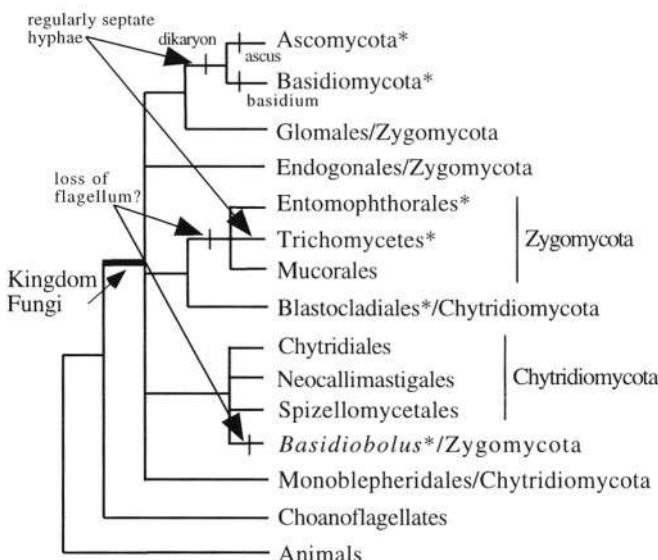


Figure 1. Phylogenetic tree of the Kingdom Fungi adapted from Bruns et al (1991, 1992), Berbee & Taylor (1993), Baldauf & Palmer (1993), Nagahama et al (1995), James et al (2000), Tehler et al (2000), O'Donnell et al (2001). Asterisks indicate those lineages with known fungal symbionts of Arthropoda.

The Kingdom Fungi is currently classified into four phyla, Chytridiomycota, Zygomycota, Basidiomycota, and Ascomycota (Fig. 1). The Chytridiomycota is characterized by motile cells that possess a smooth posterior flagellum and, when present, a thallus of coenocytic filaments or hyphae. Of the four phyla, only the Chytridiomycota possesses a flagellated state at some point in their life cycle. The Zygomycota is a morphologically and ecologically diverse group of fungi that is primarily characterized by the production of a unique meiospores, zygosporangia, and a thallus that is typically coenocytic. Neither the Chytridiomycota nor the Zygomycota are supported as monophyletic phyla (Nagahama et al 1995, James et al 2000) and the points at which they integrate most probably reflect the multiple losses of the flagellum.

during the evolution of the Kingdom Fungi (Fig. 1). Only the Basidiomycota and the Ascomycota are monophyletic and they are supported as members of the most derived clade of the Kingdom Fungi (Bruns et al 1992, Berbee & Taylor 1993, Tehler et al 2000): They are united by the synapomorphies of a body plan composed of a regularly septate mycelium and a dikaryotic phase to their life cycles. The Basidiomycota is characterized by meiospores (basidiospores) that are produced upon club-shaped cells or basidia (sing. basidium). It is the second largest phylum of Kingdom Fungi with approximately 23,000 species (Hawksworth et al 1995), including many of the common macroscopic forest fungi (e.g., mushrooms, shelf fungi, etc.). The Ascomycota is characterized by the production of meiospores (ascospores) within sac-shaped cells or asci (sing. ascus). It includes many commonly encountered fungi that have forever changed human civilization through food (e.g., *Saccharomyces cerevisiae*), medicine (e.g., *Penicillium chrysogenum*), and disease (e.g., *Pneumocystis carinii*).

3. Ascomycota-Arthropoda Symbioses

The Ascomycota comprises a phylogenetically, taxonomically, and ecologically diverse group of organisms. It includes three major classes, the ‘Archiascomycetes’, Saccharomycetes, and Euascomycetes (Nishida & Sugiyama 1994, Alexopoulos et al 1996, but see Eriksson & Wynka 1998 for an alternative nomenclature). The ‘Archiascomycetes’ comprise numerous basal lineages of relatively simple or reduced fungi that display yeast, filamentous, or both (dimorphic) growth forms. It is not well supported in molecular rDNA phylogenies and the extent to which it represents a natural monophyletic group is debatable. The two remaining classes of the Ascomycota are the Saccharomycetes and the Euascomycetes, both of which are monophyletic and represent natural taxa. The Saccharomycetes include most of the fungi that are typically referred to as “yeasts” and the Euascomycetes include most of the ascomycetes that produce filamentous thalli and form organized ascomata. Fungi from these latter two clades will be the focus of this review as the majority of – if not all – ascomycetes that participate in known fungal-arthropod symbioses are members of either the Saccharomycetes or Euascomycetes. Although this chapter is arranged in a systematic or phylogenetic framework, five main ecologies are emphasized including ambrosia fungi, gut endosymbionts, dispersal, ectoparasites, and entomopathogens.

The ambrosia of bark beetle tunnels was first described by Schmidberger (1836) and confirmed as fungal in nature by Hartig (1844). We now recognize this symbiosis to include numerous distantly related species of mostly obligate fungal symbionts of bark beetles in the coleopteran families Platypodiae and Scolytidae. Considerable ecological and behavioral variation exists across the fungus-bark beetle associations, but in general the fungi are intimately associated with the beetle throughout their life cycles. In the majority of species, the adult female beetles possess a specialized structure, a mycangium, in which they maintain an inoculum of the ambrosia fungus. Among species of ambrosia beetles, the mycangium varies greatly in morphology and complexity ranging from membranous pouches at the base of each mandible, to sclerotized pouches in the base of elytra, to intersegmental pouches between the pro-

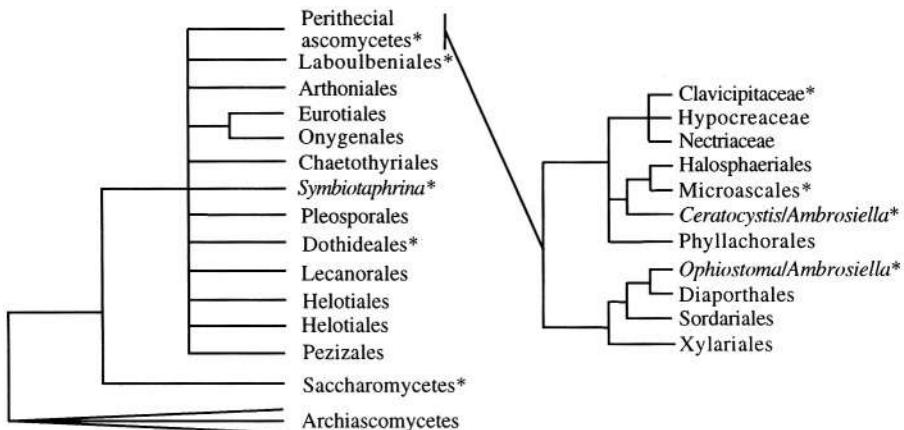


Figure 2. Cladogram of the Euascomycetes adapted from Bruns et al (1992), Berbee & Taylor (1993), Nishida & Sugiyama (1994), Spatafora & Blackwell (1993, 1994), Cassar & Blackwell (1996), Jones & Blackwell (1996), Jones et al (1999), Berbee (1998), Spatafora et al (1998), Tehler et al (2000).

and mesonotum (Norris 1979). The fungus is transferred to the tunnels or galleries by the burrowing action of the insect and the walls of the gallery eventually become lined with the fungus. This fungal lining, or ambrosia, serves as the sole source of food for the developing mycophagous larvae; adults typically consume a wider range of food and are only obligately mycophagous in a few species of bark beetles. Ambrosia fungi are often categorized as primary or secondary symbionts (Batra 1966, 1987). Primary ambrosia fungi are consistently found associated with bark beetle tunnels and serve as the food source for the mycophagous larvae. Secondary ambrosia fungi are infrequently associated with bark beetle tunnels, but do represent a potential food source for those beetle species that also include mycophagous adults. Importantly, several of the ambrosia fungi are closely related to the causal agents of devastating tree diseases such as Dutch elm disease (Cassar & Blackwell 1996, Harrington et al 2001).

Numerous yeast-like species of fungi function as gut endosymbionts of insects. The possession of intracellular mutualistic microorganisms, endocytobiosis, occurs throughout many groups of arthropods and functions in the detoxification and breakdown of numerous plant compounds (Koch 1967, Douglas 1989, Dowd & Shen 1990, Dowd 1991, Jones et al 1999). While the majority of these symbionts are prokaryotes, several species of Coleoptera and Homoptera are known to possess yeast-like fungi instead of bacteria. Of those insects with fungal endocytobionts, the majority of research has been conducted on members of the Anobiidae and Cerambycidae (Coleoptera) (Jurzitz 1979, Nardon & Grenier 1989, Jones et al 1999). The fungi in these systems are incorporated into specific host cells, the mycetocytes, which form an organ, the myctome, located at the junction of the foregut and hindgut. Studies involving aposymbiotic individuals suggest that the fungal endosymbionts provide necessary nutritional and growth factors for developing larvae (Pant et al 1960, Jurzitz 1979). The adult female beetles also possess fungus-bearing pouches near the reproductive organs that insure eggs are inoculated with the fungi during oviposition.

Developing larvae consume the yeast inoculum and thus become infected with the symbiont.

Countless species of ascomycetes rely upon arthropods for transport from one suitable substrate to the next (Malloch & Blackwell 1992, Blackwell 1994). The ascoma, ascus and ascospore of many species of arthropod-dispersed ascomycetes possess a number of unique traits that are often considered to be the result of selection for arthropod dispersal (Cain & Weresub 1957, Cain 1972). In many species the ascii typically do not forcibly eject their spores, as is the case in most ascomycetes, but rather the cell wall of the ascus deliquesces or disintegrates. The ascospores of arthropod-associated ascomycetes are often either ornamented, possess an adhesive region, or are produced in a mucilage. The ascomata possess long perithecial necks or beaks that function to present the spores in an elevated manner. The combination of these unique ascoma, ascus and ascospore traits results in spores that are often presented in sticky droplets at the top of reproductive structures and are dispersed to new substrates or food sources by arthropods.

Parasites and pathogens of arthropods are well represented within the Ascomycota. They range from seemingly benign ectoparasites that impart no apparent harm upon their host, i.e., Laboulbeniales (Thaxter 1924, 1926, 1931, Tavares 1985, Blackwell 1994), to entomopathogenic fungi that ultimately result in the death of their host, i.e., *Cordyceps* (Mains 1958, Kobayasi 1941, 1982, Samson et al 1988). Entomopathogenic fungi in particular hold great promise with respect to biological control of insect pests and are a rich source of secondary compounds that are of pharmaceutical importance. Arthropod associated ascomycetes provide a wealth of biological systems for the study of universal themes in organismal evolution and symbiology (e.g., evolution of host affiliation, symbiont replacement, etc.) and represent an enormous reservoir of eukaryotic biodiversity that is in dire need of expanded research.

3.1. SACCHAROMYCETES-ARTHROPOD SYMBIOSSES

Saccharomycetes are well supported as a monophyletic clade (Kurtsman & Robnett 1994, Berbee & Taylor 1993, Jones & Blackwell 1999), which consists of those fungi that are most frequently referred to as the “yeasts” or “true yeasts”. The basis for this designation is that most species are characterized by life cycles dominated by a unicellular growth form – yeast – and that the best known yeast fungi, *Saccharomyces cerevisiae* and *Candida albicans*, are members of this group. It is important to note, however, the term yeast is best used as a descriptor of growth form and has little phylogenetic utility (Alexopoulos et al 1996). Many species of the Saccharomycetes possess a filamentous growth form (a mycelium) and many species of the mainly filamentous Euascomycetes possess a yeast state in their life cycle. Yeast fungi are also known among the Basidiomycota and Zygomycota. The Saccharomycetes contains one order, the Saccharomycetales. Saccharomycetalean fungi are found in a multitude of environments and niches and those in which they most commonly associate with arthropods are slime fluxes, beetle galleries, and gut endosymbionts.

3.1.1. *Cephaloascaceae*: Cephaloascus

Cephaloascus includes two species *C. fragrans* Hanawa and *C. albidus* Kurtsman, which rely upon arthropods for dispersal of their hat-shaped ascospores. *C. fragrans*, which was originally isolated from the guts of adult *Orthotomicus caelatus* and *Gnathotrichus materiarius*, is the better known of the two species and is generally considered to be a secondary ambrosia fungus (Batra 1963a). It is also known from slime fluxes where it can be associated with numerous beetle species (von Arx & van der Walt 1987). It is unique among the Saccharomycetales in that it produces erect ascophores from which numerous ascogenous hyphae arise. Ascogenous hyphae are generally thought to be lacking from the Saccharomycetes, which typically produce ascii from a fusion cell of two haploid yeast cells. For this reason, some classifications excluded *Cephaloascus* from the Saccharomycetales and included it in the Ophiostomatales of the Euascomycetes (von Arx & van der Walt 1987). The Ophiostomatales include species that may possess a yeast state, produce hat-shaped ascospores, and are known as ambrosia fungi. Phylogenetic studies of ambrosia fungi, however, have clearly shown that *Cephaloascus* is a member of the Saccharomycetes and that it is not closely related to the filamentous ambrosia fungi of the Euascomycetes (Hausner et al 1992, Spatafora & Blackwell 1994, Cassar & Blackwell 1996).

3.1.2. *Dipodascaceae*: Ascoidea and Dipodascus

The genus *Ascoidea* includes numerous species of filamentous Saccharomycetes that function as mutualistic ambrosia fungi (Batra 1963b). *A. hylecoeti*, which associates with the European beetle *Hylecoetus dermestoides*, is one of the better studied systems (Koch 1962, Batra 1987). Hat-shaped ascospores are stored by the female in mycangia; these spores function as an inoculum, which is deposited with eggs during oviposition in bark and crevices. Upon hatching, the larvae burrow into the sapwood and passively transmit the fungus into the developing tunnels. The tunnels then become lined with the fungus, which serves as the sole source of food and necessary growth factors for the developing larvae (Koch 1962, Batra 1987). Upon maturation of the young adults, the fungus forms ascospores, which become lodged in the mycangia and are subsequently dispersed to new substrates by adult females. The fungus is associated with its host in all states of its life cycle and relies upon it for dispersal to new and suitable substrates. Other species of *Ascoidea* and the closely related genus *Dipodascus* also function as ambrosia fungi (Batra 1987).

Members of the Dipodascaceae are also dominant fungi isolated in association with arthropods from slime fluxes of woody angiosperms (e.g., elms, oaks, and maples; reviewed in Batra 1987). These fungi are saprobic in their nutritional mode and rely on insects, mainly Coleoptera and Diptera, for dispersal, but apparently do not exhibit strong tendencies toward specificity of association. Although insects reared on slime fluxes have been demonstrated to grow larger, no direct benefit to the insect from the fungus has been attributed to the association. Batra (1987) classified slime fluxes into two groups, perennial and ephemeral, and found that they were characterized by different fungus-arthropod associations. Perennial slime fluxes, which can persist up to 20 years, were more common on *Ulmus* (elms) and *Acer* (maples) and were dominated by *Ascoidea rubescens* in association with Nitidulidae (Coleoptera) and Drosophilidae (Diptera) (Batra 1963b). Ephemeral slime fluxes, which persist less than a few years.

are more common on *Quercus* (oaks) and are initially dominated by bacteria and strongly fermenting yeast *Saccharomyces* spp., after which they quickly become colonized by species of *Dipodascus* in association with Diptera and Coleoptera (Batra 1987). There is also some evidence that some these yeast species may over winter in association with domesticated and wild bees (Batra et al 1973, Gilliam 1978).

3.1.3. *Saccharomycetaceae*: Candida, Debarromyces, and Pichia

In addition to brewer's and baker's yeast, *Saccharomyces cereviseae*, the *Saccharomycetaceae* includes many arthropod associated species. The genus *Pichia* includes several species that have been isolated from numerous insect associations and insect-dominated microenvironments including slime fluxes and beetle galleries. Some species have even been isolated from specific cactus habitats in association with *Drosophila*, where they may have a beneficial impact on mating and larval development (Starmer et al 1988, 1990). In these associations, a high degree of specificity has been documented with certain species of *Pichia* associated with particular species of *Drosophila* on a given host. *Debarromyces*, a closely related genus to *Pichia*, is also known to be closely associated with insects and has been isolated from the digestive tracts of bees (Batra et al 1973). Most of the insect-associated species in these two genera possess the unique hat-shaped ascospores that are thought to facilitate spore dispersal by insects.

Numerous saccharomycetaceous species also function as gut endosymbionts of insects with the majority of these symbiotic with members of the Anobiidae and Cerambycidae (Coleoptera). All of the endocytobiotic fungi of the Cerambycidae are classified in the asexual genus *Candida* Berkhout (Jurzitzza et al 1960), while the endocytobionts of the Anobiidae have been classified in *Candida*, *Torulopsis* Berl. (=Candida Yarrow & Meyer), and *Symbiotaphrina* W. Gams & Arx. In recent molecular phylogenetic studies, Jones et al (1999) confirmed that the *Candida* isolates from the Anobiidae and Cerambycidae are members of the Saccharomycetales, but that they do not form a monophyletic group. Rather, individual beetle species from both the Anobiidae and the Cerambycidae have independently acquired endocytobionts numerous times from separate lineages of saccharomycetalean fungi. Some of the *Candida* symbionts showed a close phylogenetic affinity with yeasts associated with beetles as ectosymbionts, but others displayed a close phylogenetic affinity with *Candida* species that are known as human pathogens (e.g., *C. albicans*). Furthermore, *Symbiotaphrina* was not supported as being a member of the Saccharomycetales, but is most likely a reduced yeast of the Euascomycetes (Jones & Blackwell 1996, discussed below). The results support the hypothesis that fungal endocytobiosis of arthropods is a dynamic evolutionary process that has involved independent acquisitions of symbionts from phylogenetically distant lineages of fungi. Anobiid and cerambyciid beetles come into contact with a diverse yeast flora through normal herbivory (Lachance et al 1982, Gonzalez et al 1989) with the apparent result being the replacement of the prokaryotic symbiont of the mycetosome followed by repeated acquisitions of novel fungal gut endocytobionts (Jones & Blackwell 1996, Jones et al 1999).

3.2. EUASCOMYCETES-ARTHROPOD SYMBIOSSES

The Euascomycetes is the largest class within the Ascomycota and includes the filamentous, sporocarp forming ascomycetes (e.g., cup-fungi, truffles, etc.). Species of Euascomycetes include many of the quintessential examples of fungal symbioses with algae and vascular plants (e.g., lichens, mycorrhizae), but fungal arthropod symbioses are also well represented. Species of Euascomycetes that participate in arthropod symbioses are found throughout distantly related orders, families, and genera of fungi, associate with species from all major orders of Arthropoda, and have independently evolved numerous times. The nature of these symbioses range from classical examples of mutualism (e.g., ambrosia fungi with bark beetles), to commensalism (Laboulbeniales with Coleoptera), to antagonistic parasitism (e.g., *Cordyceps* with Lepidoptera), and have impacts on arthropod populations from negligible to epidemic (Evans & Samson 1982, 1984). Subclass and ordinal level nomenclature for the Euascomycetes is currently in a state of flux. This instability stems from a lack of resolution in recent phylogenetic studies, the majority of which have been inferred from nuclear ribosomal DNA sequences. While the node supporting the Euascomycetes is reasonably supported by the data, supraordinal relationships within the class are characterized by well-supported terminal clades, but poorly supported interconnecting basal nodes (Berbee & Taylor 1993, Berbee 1998, Gargas et al 1995, Spatafora 1995, Tehler et al 2000). Figure 2 provides a schematic summary of the current resolution of supraordinal relationships within the Euascomycetes; groups that include arthropod symbionts are highlighted.

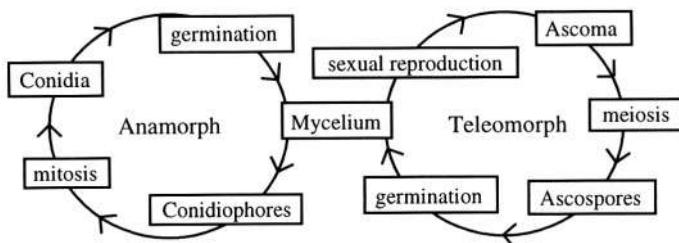


Figure 3. Schematic of a pleomorphic fungus lifecycle that includes both a teleomorph state, which produces meiospores or ascospores, and an anamorph state, which produces mitospores or conidia. Both the teleomorph and the anamorph reproductive structures are produced from a mycelium. They may occur on the same or different host individuals, on different host species, or observed in laboratory conditions. For example, *Cordyceps militaris* is a teleomorph that parasitizes lepidopteran pupae, but when its ascospores are germinated in culture, they produce a *Verticillium* anamorph.

Euascomycete life cycles can be extremely complex and pleomorphic, a phenomenon whereby a single species possesses more than one independent form or spore-producing state in the life cycle (Kendrick 1979, Sugiyama 1987, Reynolds & Taylor 1993). The complete life cycle of a species is referred to as the holomorph and can be categorized into three groups based upon combinations of meiotic (teleomorph) and mitotic (anamorph) reproductive states. A holomorph may include only a

teleomorph, a teleomorph and one to many anamorphs, or only an anamorphic state or states. A major challenge in ascomycete systematics, especially among arthropod-associated species, is elucidating how teleomorphic and anamorphic fungi are phylogenetically related and whether a particular anamorph represents a true asexual mitotic species. Because teleomorphs and anamorphs share few morphological traits, traditional classifications based upon morphological comparisons are limited in their utility and have resulted in artificial classifications that do not reflect phylogenetic relationships. Historic classification included all anamorphic fungi in the artificial class the Deuteromycetes (reviewed in Alexopoulos et al 1996), but this practice has been discontinued along with the general philosophy of recognizing artificial or nonmonophyletic taxa (Taylor 1995, Alexopoulos et al 1996, Hibbett & Donoghue 1998). Molecular systematics has proven to be a powerful source of data in testing hypotheses of teleomorph-anamorph relationships, as the genes used as primary sources of data are ubiquitously distributed across species regardless of reproductive mode. Several of the ascomycetes that form symbioses with arthropods are anamorphic and their relationships to teleomorphs are only recently being elucidated (Cassar & Blackwell 1996, Sung et al 2001).

3.2.1. Symbiotaphrina

The genus *Symbiotaphrina* contains two species, *S. kochii* and *S. buchneri*, that are obligate gut endosymbionts of the anobiid beetles, *Lasioderma serricorne* (cigarette beetle) and *Sitodrepa paniceae* (drugstore beetle). These beetles occur as pests in processed tobacco and other similar plant material and their ability to inhabit these chemically hostile microenvironments has been partially attributed to detoxifying properties of their gut symbionts (Dowd 1989, Dowd & Shen, 1990). Other species of anobiid beetles are known to possess obligate gut endosymbionts from the genus *Candida* (Saccharomycetales), but the taxonomic and phylogenetic affinity of the symbionts of *L. serricorne* and *Si. paniceae* has been disputed for some time. The gut endosymbiont of *Si. paniceae* was originally classified in the genus *Saccharomyces* (Escherich 1900), but was transferred to the genus *Torulopsis* (=*Candida*) due to its inability to ferment glucose (Gräebner 1954). Later it was classified in the genus *Symbiotaphrina* of the Taphrinales, along with the second species *S. kochii*, based on morphological and biochemical characteristics (van der Walt 1961, Jurzitz 1964).

Taphrinales (Archiascomycetes) is a monotypic order that includes the genus *Taphrina*. It comprises numerous species of plant pathogens, but no species of *Taphrina*, or of the ‘Archiascomycetes’, are known to form symbioses with insects. Also, members of the Taphrinales are dimorphic, forming a haploid, saprobic yeast phase and a dikaryotic, filamentous pathogenic phase; a filamentous phase has never been observed for either species of *Symbiotaphrina*. Recent molecular phylogenetic studies have rejected a close phylogenetic relationship of *Symbiotaphrina* with either the Taphrinales or the Saccharomycetales (Jones & Blackwell 1996). Rather, it is supported as a lineage of reduced yeasts among the Euascomycetes. The two species formed a well-supported clade, but their relationship to other major groups of the Euascomycetes remains unclear. This finding is important in that not only have anobiid beetles acquired endosymbionts multiple times within the Saccharomycetales (Jones et

al 1999), but they have also independently acquired them from among the Euascomycetes (Jones & Blackwell 1996).

3.2.2. *Laboulbeniales* and *Pyxidiophora*

Laboulbeniales is a large order of approximately 1800 species (Thaxter 1924, 1926, 1931, Tavares 1985, Blackwell 1994). It is enigmatic among the filamentous ascomycetes in that species of the order do not form an extensive filamentous or mycelial state. Rather, they exhibit determinate growth and form thalli of a defined number of cells. These minute thalli exhibit spectacular morphologies and Thaxter's monographs (1924, 1926, 1931) not only represent an important contribution to the biodiversity and taxonomy of these fungi, but it is also an impressive work of botanical illustrations. The ascii of *Laboulbeniales* are produced and deliquesce within somewhat flask-shaped ascomata. The ascospores are usually two-celled with one end modified as an attachment point to arthropod exoskeletons. The *Laboulbeniales* are found on the exoskeletons of many groups of arthropods, in both terrestrial and marine ecosystems, and may be both host and site specific. That is, not only may a particular species of *Laboulbeniales* associated with a single host, it will also only be found on a particular body part (e.g., antennae, genitalia, etc.) of the host (Thaxter 1924, 1926, 1931, Tavares 1985). For this reason, the transfer of many species of *Laboulbeniales* between hosts is considered to occur through well-defined behavioral practices (e.g. touching of antennae) and in some cases may represent good examples or models of sexually transmitted organisms. The fungus does penetrate the exoskeleton via a special infection cell, a haustorium, apparently deriving nutrients from its host. However, the interaction between *Laboulbeniales* and their hosts is generally considered to be relatively benign with some potential for decreased longevity of individuals (Benjamin 1971, Tavares 1985). See also chapter by A. Weir in this volume.

Pyxidiophora is a poorly known genus of ascomycetes that until recently was of disputed phylogenetic affinity. Phylogenetic analyses of the nuclear SSU rDNA revealed a close relationship between the genus and the *Laboulbeniales* and supported the homology of certain similarities in ascospores between them (Blackwell 1994). For example, like the *Laboulbeniales*, *Pyxidiophora* produces two-celled ascospores, with a darkly pigmented adhesive end, that are dispersed by arthropods. Species of the genus typically occur in ephemeral habitats, such as dung or rotting seaweed, and rely on arthropods for dispersal from one substrate (e.g., dung pile) to a new and suitable habitat (Blackwell & Malloch 1989a, 1989b). Like the *Laboulbeniales*, species of *Pyxidiophora* are not known to impart any harm upon their hosts, but unlike the *Laboulbeniales*, *Pyxidiophora* does show extensive filamentous development in and on substrates and is hypothesized to possibly function as a mycoparasite (Blackwell 1994). These fungi rely on arthropods for dispersal from one substrate to the next, and thus gain access to new food sources (Blackwell et al 1986). It seems that the arthropods are neither benefited nor harmed by the interaction, but very little experimental data exist for the system. Although *Pyxidiophora* and the *Laboulbeniales* form a monophyletic clade, their relationship to other taxa within the Euascomycetes is not resolved by current data and analyses, and as a group they remain a phylogenetic enigma.

3.2.3. Myriangiaceae

The Myriangiaceae is a small group of approximately 20 species of entomopathogenic fungi that occur on scale insects (Homoptera) (Petch 1924, Miller 1938). They are part of large group of fungi that have been classified as the Loculoascomycetes (Barr 1987). In some classifications they are classified as a family within the Dothideales and in others they are classified as the order Myriangiales (Hawksworth et al 1995). They are characterized by crustose to pulvinate stromata and ascii produced in gelatinous locules. Ascii are bitunicate in that they possess two functional wall layers that forcibly eject ascospores in a “jack-in-the-box” manner. The number of known species is almost certainly an underestimate, as few studies have been conducted on the group.

3.2.4. Ceratocystis, Ophiostoma, and Ambrosiella

Ceratocystis (~20 species) and *Ophiostoma* (~100 species) are two genera of morphologically and ecologically similar arthropod-associated ascomycetes that are referred to as ophiostomatoid fungi (Upadhyay & Kendrick 1975, Upadhyay 1981, 1993, Harrington 1987, von Arx & van der Walt 1987, Wingfield et al 1993). They include some of the more notorious plant pathogens (e.g. Dutch elm disease caused by *O. ulmi*) and in many regions of the world they impart considerable environmental damage and significant agricultural and economic losses (Brasier 1990, Harrington 1993, Wingfield et al 1993). Species from both genera produce evanescent ascii in long-necked or beaked perithecial ascomata. Ascospores are often hat-shaped and presented in a droplet at the tip of the ascomatal neck from which they are dispersed by arthropods, mainly beetles of the Platypodiae and Scolytidae. The extent of morphological and ecological similarities among species of the two genera has resulted in species being synonymized in either *Ceratocystis* or *Ophiostoma* (Weijman & de Hoog 1975, Upadhyay 1981, Upadhyay & Kendrick 1975, de Hoog & Scheffer 1984). However, species of the two genera differ across numerous biochemical and developmental traits. Species of *Ophiostoma* are insensitive to the antibiotic cyclohexamide whereas *Ceratocystis* is sensitive (Harrington 1981). *Ophiostoma* is unique in that it possesses cellulose as a cell wall carbohydrate, which is absent in *Ceratocystis* and most true fungi (Smith et al 1967, de Hoog & Scheffer 1984). Also, studies of ascomal development and ascosporogenesis revealed several differences between selected species of *Ceratocystis* and *Ophiostoma* (van Wyk et al 1991, van Wyk & Wingfield 1994).

Several molecular phylogenetic studies of ophiostomatoid fungi revealed that *Ceratocystis* and *Ophiostoma* do not form a monophyletic clade, but rather comprise at least two separate lineages of perithecial ascomycetes (Hausner et al 1993, Spatafora & Blackwell 1994, Cassar & Blackwell 1996). *Ceratocystis* and *Sphaeronaemella*, another ophiostomatoid genus, are members of – or at least closely related to – the Microascales, an order of fungi that also produces insect-dispersed ascospores, and the Halosphaeriales, an order of marine and aquatic fungi (Spatafora et al 1998). *Ophiostoma* is not supported as being closely related to *Ceratocystis* and the Microascales, but is placed in a separate part on the Euascomycete clade. Species of *Ophiostoma* form a monophyletic clade that is most closely related to the Diaporthales, an order of mostly saprobes and plant pathogens, but this relationship is not strongly supported by current data and requires further investigation (Spatafora & Blackwell

1994, Cassar & Blackwell 1996). The discovery of at least two independent origins of ophiostomatoid fungi is consistent with the hypothesis that the ophiostomatoid morphology is the product of convergent evolution. As mentioned previously, the production of long-necked or beaked perithecial ascomata, evanescent ascii, and ornamented ascospores, which are passively released, is thought to be the result of convergent evolution under selection pressure for arthropod dispersal of propagules (Cain 1972, Malloch & Blackwell 1992, Blackwell 1994, Spatafora & Blackwell 1994). This hypothesis, although difficult to test experimentally, is consistent with the polyphyletic origins of ophiostomatoid fungi.

Ambrosiella is a genus of mitotic or anamorphic species that is the dominant and most frequently encountered group of primary ambrosia fungi (Batra 1967, Cassar & Blackwell 1996). All mycophagous species of Platypodidae and many Scolytdiae form obligate associations with species of *Ambrosiella*. The beetles possess mycangia in which they maintain an inoculum of the fungus, which is transferred to the interior of the tree and throughout the galleries by the burrowing action of the beetles. The fungus is dimorphic, exhibiting a yeast-like phase in the mycangium and a yeast-like and filamentous phase in the bark beetle tunnel. *Ambrosiella* spp. serve as the sole source of nutrition for developing larvae and also serve as an important food source in species where the adults are mycophagous. Studies of the Xyleborini have shown that ambrosia fungi provide a necessary sterol, ergosterol, for proper larval maturation to adult and thus colony viability (Kok 1979, Norris 1979). The fungi benefit from the relationship by receiving targeted dispersal to carbon sources and by receiving a rich source of nitrogen from beetle waste products (Batra 1966, 1967).

Phylogenetic studies of *Ambrosiella* have revealed that selection pressures for arthropod association is apparently not restricted to the meiotic or teleomorph states of life cycles, but also acts upon anamorphic states. In a phylogenetic analysis of ribosomal DNA sequences, Cassar and Blackweil (1996) found that species of *Ambrosiella* formed two separate clades; one that was most closely related to *Ceratocystis* and one that most closely related to *Ophiostoma*. This pattern of relationship was also supported by sensitivity to cyclohexamide; those *Ambrosiella* isolates that grouped with *Ophiostoma* were insensitive cyclohexamide whereas those that grouped with *Ceratocystis* were sensitive to the antibiotic. The *Ambrosiella* species that grouped with *Ceratocystis* formed a monophyletic clade, suggesting that they may represent a lineage restricted to mitotic reproduction. However, few species of *Ceratocystis* were sampled and a greater taxon sampling of *Ceratocystis* is needed to determine the extent, if any, that teleomorphs may group within the *Ambrosiella* lineage of *Ceratocystis*. The *Ambrosiella* species that grouped with *Ophiostoma* did not form a monophyletic lineage (Cassar & Blackwell, 1996). Some isolates formed a clade with teleomorphs of *Ophiostoma*, suggesting that either mitotic lineages have been derived more than once within *Ophiostoma* or that some *Ambrosiella* species may be anamorphs of *Ophiostoma* teleomorphs. Again, future analyses of nucleotide data from multiple genes are needed with a greater sampling of anamorphic and teleomorphic species to fully understand the extent to which they are phylogenetically related and in some cases may be conspecific.

3.2.5. Clavicipitaceae

The Clavicipitaceae (Hypocreales) is a diverse family of perithecial ascomycetes that is unusual in being composed entirely of obligate symbionts of other organisms: grasses, arthropods, or other fungi (Rogerson 1970). It includes some of the most common pathogens of insects (Kobayasi 1941, 1982), epiphytes and endophytes of plants (Clay 1988) that may cause serious toxicoses of animals grazing on them (Bacon 1977), and mycoparasites of false-truffles (Mains 1957). Phylogenetic analyses of nucleotide sequence data suggest that the family is more recently derived than its hosts (Spatafora & Blackwell 1993, Glenn et al. 1996), so the observed pattern of host-association cannot have arisen through cospeciation. Instead, the evolutionary history of the Clavicipitaceae reflects an unusually active record of inter- and intrakingdom host-shifts that have resulted in the current range of host affiliation and specificity (Nikoh & Fukatsu 2000, Sung et al 2001).

Cordyceps is the largest and most diverse genus of the Clavicipitaceae, both in terms of number of species and in its morphology and remarkably wide host range (Rogerson 1970). Morphology of meiotic reproductive structures varies according to species from stipitate stromata that emerge from an insect corpse to minute structures that are produced superficially on the exoskeleton of the dead host. An estimated fifteen to twenty species parasitize species of the false-truffle genus *Elaphomyces* (Ascomycota). The remaining (~300) species of *Cordyceps* and the 60 species of its putative sister genus *Torrubiella* are fatal pathogens of insects and other arthropods. Numerous different orders of arthropods serve as hosts for *Cordyceps*, including Arachnida, Coleoptera, Diptera, Hemiptera, Homoptera, Lepidoptera, Orthoptera. Any one *Cordyceps* species, however, is typically restricted to a single host species or genus (Mains 1958, Kobayasi 1982). *Torrubiella*, which produces superficial reproductive structures, is restricted in its host range to Arachnida and Homoptera (Kobayasi 1982, Hywel-Jones 1995, Hywel-Jones et al. 1997). In those systems that have been studied, hosts are infected by spores, which penetrate through the exoskeleton via proteases, lipases, and chitinases (St. Leger 1986a, 1986b, 1991). Once inside the host body cavity, the fungus grows in a yeast phase, circulating toxins that ultimately kill the host. Upon death of the host, the fungus reverts to filamentous growth, consuming the contents of the body cavity and ultimately producing a spores-producing stroma that ruptures through the exoskeleton and releases spores into the environment. In most cases the exoskeleton of the host is not significantly damaged or utilized by the fungus.

The systematics of *Cordyceps* and its allies is currently in a state of flux and in need of taxonomic revision, an undertaking made difficult by a lack of robust phylogenetic hypotheses. Past and current classifications of the Clavicipitaceae, *Cordyceps*, and its relatives rely heavily upon host affiliation (Diehl 1950, Rogerson 1970, Mains 1958, Kobayasi 1941, 1982). Initial phylogenetic analyses do not support such subfamilial classifications and suggest that several of the major taxa within the Clavicipitaceae, including *Cordyceps*, are not monophyletic (Sung et al 2001). The ancestral symbiosis of the family was most likely a pathogen of arthropods and through a series of inter and intrakingdom host shifts the family has diversified onto hosts from three kingdoms of eukaryotes (Nikoh & Fukatsu 2000, Sung et al 2001). Possible inter- and intrakingdom host shifts during the evolution of the Clavicipitaceae include jumps from subterranean arthropod hosts (e.g., cicadas) to ascomycetous truffles (Nikoh & Fukatsu 2000), jumps

from arthropods onto the grass family Poaceae (Sung et al 2001, J. W. Spatafora unpublished), and multiple bidirectional jumps among the major orders of arthropods (e.g., Coleoptera, Homoptera, Lepidoptera) (Sung et al 2001).

Unraveling the evolutionary history of *Cordyceps* and the Clavicipitaceae is further complicated by the large number of anamorphic entomopathogenic fungi that are known, or hypothesized, to be closely related to *Cordyceps* and *Torrubiella* (Kobayasi 1941, 1982, Mains 1958, Gams 1971, Samson et al 1988). Several species of *Cordyceps* are known to produce anamorphic forms in culture and numerous anamorphic entomopathogenic fungi are collected in nature. However, the nature of phylogenetic relationships and potential conspecificity is known for only a few species (Gams 1971, Gams & van Zaayen 1982, Hywel-Jones et al. 1997, Hodge et al. 1996, 1998). Recent molecular phylogenetic studies demonstrated that numerous species of *Beauveria*, *Engyodontium*, *Hirsutella*, *Metarhizium*, *Paecilomyces*, *Tolypocladium*, and *Verticillium* are members of the Clavicipitaceae and are closely related to numerous teleomorphs of *Cordyceps* (Zare et al 2000, Sung et al 2001). In addition, the species of these anamorphic genera exhibit host ranges that are not entirely congruent with that of teleomorphs of *Cordyceps*. For example, species of *Verticillium* are pathogens of nematodes, rotifers, and mushrooms, and thus greatly expand the host range for the Clavicipitaceae and potentially *Cordyceps*. These results raise the question whether species with dual host life cycles – a teleomorph state on one host and an anamorph state on another – exist within the Clavicipitaceae and represent the means for sampling new and potential hosts on an evolutionary scale.

In addition to pathogens of arthropods, the Clavicipitaceae include species that display beneficial interactions with arthropods that could be interpreted as mutualistic symbioses. The grass symbionts of *Claviceps* and *Epichloë* are pathogens of the grass family with some species of *Neotyphodium*, an anamorph of *Epichloë*, considered mutualistic with their grass hosts (Clay 1988, Glenn et al 1996, Schardl et al 1991, 1997). Teleomorphs from these genera also form interactions with arthropods that appear to be quite specific in nature. Flies of the genus *Phorbia* lay their eggs in the reduced stromata of *E. typhina* and upon hatching the mycophagous larvae consume a substantial portion of it as a food source. Although this may appear to be strictly antagonistic for the fungus, the adult flies function as vectoring agents of *E. typhina* spermatia (Bultman et al 1988, 1995). *E. typhina* is an obligate outcrosser with opposite mating types required for sexual reproduction. Adult flies transmit spermatia of one mating type to a compatible stroma of the opposite mating type. Without this insect mediated “pollination”, sexual reproduction in *E. typhina* can not occur. Similarly, *Cl. purpurea* produces a honeydew state that is known to attract flies from the genus *Minettia* (Lemon, 1992). Individuals of *M. lupulina* which feed upon the honeydew were shown to carry spores both on their bodies and in their guts, and spores from both external and internal sources were viable (Lemon, 1992).

Recently, it has been discovered that fungal endosymbionts of some homopterans are of clavicipitalean origin (Fukatsu 1998, T. Fukatsu pers. comm., Suh et al 2001). These fungi are yeast-like symbionts that function in sterol synthesis and nitrogen recycling for the host (Noda et al 1979, Sasaki et al 1996, Hongoh & Ishikawa 1997). They occur in the host fat body and are transmitted to the offspring through the ovaries (Noda 1977). Phylogenetic analyses of nuclear ribosomal DNA rejected a close

phylogenetic relationship of these yeast-like symbionts with other known lineages involved in arthropod endosymbioses, i.e., *Candida* and *Symbiotaphrina*, (Noda H. et al. 1995, Fukatsu & Ishikawa 1996). Phylogenetic studies that included a more thorough sampling of perithecial ascomycetes confirmed a clavicipitalean origin of these symbionts within *Cordyceps* (Suh et al 2001, T. Fukatsu pers. comm.). Suh et al (2001) sampled symbionts from three rice planthoppers and one aphid and the results suggest a single origin of the mutualistic symbionts of Homoptera within the otherwise pathogenic *Cordyceps*. Additional yeast-like clavicipitalean symbionts of additional homopterans have been discovered and appear to be part of the same clade, although additional analyses are needed (T. Fukatsu pers. comm.). Mutualistic arthropod symbioses of the Clavicipitaceae have either been unknown (e.g., yeast-like symbionts of Homoptera) or have been largely ignored (e.g., insect “pollination” in *Epichloë*) when considering the ecology and evolution of the family. These symbioses undoubtedly hold clues to understanding the dynamics of host affiliation and host-jumping and the character state polarity of fungal-arthropod symbioses, i.e., antagonistic to mutualistic. Phylogenetic analyses that involve more inclusive taxon sampling of both a greater breadth of host range and nature of symbioses is needed to more accurately understand the evolution of arthropod symbioses with the Clavicipitaceae.

4. Summary

Symbioses involving eukaryotes of the Ascomycota and Arthropoda are numerous and exhibit the complete gamut of interactions from mutualistic (e.g. ambrosia fungi) to antagonistic and fatal parasites (e.g. *Cordyceps*). Ascomycete-arthropod symbioses provide valuable experimental systems for numerous global questions in evolutionary biology including convergent evolution of morphological and biochemical traits necessary for establishing symbioses, independent origins and repeated replacement of endosymbionts, and host-jumping as a means of speciation in pathogenic taxa. One overarching theme to ascomycete-arthropod symbioses is that most – if not all – major symbioses have been derived multiple times. Whether it is for spore dispersal of the fungus or detoxification of recalcitrant and noxious plant compounds in arthropod diets, the evolutionary opportunities and symbionts’ abilities to establish fungal-arthropod symbioses are ever present and represent an on-going and dynamic process.

As stated earlier, it is estimated that only approximately 5-10% of the earth’s fungal species have been described (Hawksworth 1991, 1997, Hawksworth & Rossman 1997). This number was largely calculated through comparison of ratios of numbers of fungi associated with plant species in well-documented temperate regions and extrapolated world wide for the estimated number of plant species. Although this number as been criticized as being an overestimate, mainly due to lack of presumed host specificity of fungal-plant interactions in tropical ecosystems (May 1991), this estimate of diversity did not involve any fungi known to be associated with Arthropoda, the largest single phylum on earth. Fungal-arthropod symbioses involve two of the more successful groups of eukaryotes in the history of life on earth, and represent a vast, under-documented repository of fungal biodiversity.

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6. References

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THE LABOULBENIALES – AN ENIGMATIC GROUP OF ARTHROPOD-ASSOCIATED FUNGI

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1. Introduction

In discussions among mycologists at scientific meetings members of the fungal order Laboulbeniales do not figure prominently. Indeed few mycologists would admit to ever having seen representatives from this large and diverse assemblage. Only a small handful would be brave enough to claim more than a superficial knowledge. This apparent “neglect” may be due, in large part, to a perceived economic or scientific insignificance, but is also likely linked to our current inability to culture and manipulate these fungi in the laboratory, and to collect potential arthropod hosts in the field.

Yet the Laboulbeniales have, since their discovery, featured prominently in the literature on phylogenetic speculations of the higher fungi (Weir and Blackwell, *in press*), and have more recently been promoted as a “model” group in the exploration and elucidation of fungal and parasite biodiversity patterns (Weir and Hammond, 1997). Their continued study will undoubtedly shed light on other topics of interest to the broader biological and scientific communities, such as questions related to the processes and mechanisms involved in speciation, coevolution, and host-parasite interactions. A major impediment to our understanding of the biology of Laboulbeniales fungi has been our inability or reluctance to move beyond the basic description and classification of species. My intention here is to provide an overview of the current state of knowledge regarding these enigmatic fungi. Inevitably, this is largely based on morphology, development, and observed patterns of host utilization. However, I will also use this opportunity to discuss new approaches to the study of Laboulbeniales and the potential future advances that might accrue. It is my hope that a broader audience will come to appreciate that the Laboulbeniales have much to offer the inquiring mind, and will be attracted into this exciting field of research.

2. Occurrence , Distribution and Diversity

The order Laboulbeniales as currently recognized is a well-defined, yet extremely diverse, group of minute parasitic fungi. In the traditional sense, the Laboulbeniales included taxa with a reduced hyphal system and determinate thallus development. All of these were known to be obligate parasites of living arthropods. More recently, a second

distinct lineage, the *Pyxidiophoraceae*, a group of mycelial ascomycetes which utilize arthropods for dispersal, have been included within the order (Hawksworth *et al.* 1995).

Virtually all of the 2000 described species occur on insects although both millipedes (Diplopoda) and mites (Arachnida, Acari) are also known to carry infection. Amongst the Insecta, representatives from ten orders serve as hosts viz: Blattodea (cockroaches and allies), Coleoptera (beetles), Dermaptera (earwigs), Diptera (true flies), Heteroptera (true bugs), Hymenoptera - Formicidae (ants), Isoptera (termites), Mallophaga (bird lice), Orthoptera (crickets and allies), and Thysanoptera (thrips). However, the vast majority of known parasite species both globally (79%), and in well-studied temperate mycobiotas (ca80%) have been recorded from beetles, with the contribution made by most other insect orders being very low (Weir and Hammond, 1997).

Although studied by few mycologists, Laboulbeniales are known from a wide range of ecosystems including tropical, temperate, and polar habitats. As this distributional data is, for the most part, based on *ad hoc* collecting at various geographical locations, well-supported hypotheses regarding the effect of latitudinal gradient on species-richness are not yet available. However, quantitative data presented by Weir and Hammond (1997) provided preliminary evidence that Laboulbeniales assemblages in the moist tropics (in this case Indonesia) were much richer than their counterparts in temperate regions (UK). The greater species-richness of such tropical assemblages may be roughly proportional to the higher local species-richness of their principal host groups, although there are some indications that parasite species-richness is, in fact, proportionally somewhat higher. As yet very few Laboulbeniales have been described from more arid tropical environments and there is no evidence to support rich Laboulbeniales assemblages in these areas. Research is currently focused on south-temperate mycotas (Weir and Hughes, unpublished data) but it seems likely from the foregoing, that the greater part of laboulbenialean species richness is associated with (1) beetles as hosts; and (2) the moist tropics.

In keeping with estimates for the global fungal resource (Hawksworth, 1991) current attempts to quantify the likely diversity of arthropod-associated fungi (in particular Laboulbeniales) encompass a wide spectrum. Based on quantitative samples of arthropods from Indonesia and other available data on host exploitation patterns at various spatial scales, Weir and Hammond (1997) arrived at a global figure for Laboulbeniales exploiting Coleoptera only to be in the range of 10,000-50,000 species. Obviously, this figure takes no account of Laboulbeniales diversity on other host groups and given that new host families are regularly reported, overall Laboulbeniales diversity is likely to be significantly higher.

3. Specificity

As a direct consequence of their obligate relationship with their hosts Laboulbeniales fungi exhibit an often high degree of host specificity. This was recognized early in the study of these organisms and in varying degrees is characteristic of most obligate parasites. Examination of published host-parasite lists (Frank, 1982; Hulden, 1983; Weir, 1996) mainly from temperate regions, clearly shows that the host range for any given parasite appears, with very few exceptions, to be restricted taxonomically-generally encompassing only species that belong to the same genus or group of closely

related genera. These somewhat anecdotal observations have been, to a large extent confirmed, in lab experiments (Cépède and Picard, 1908; Richards and Smith, 1954). More recently, lab-based studies working with *Laboulbenia slackensis* questioned these earlier experimental results and concluded that this fungus could be successfully transferred to 19 atypical host species (DeKesel, 1996). This study showed that establishment of *L. slackensis* on a new host was determined by several factors that include characteristics of the host integument, availability of nutrients and the microclimate on the surface of the host. DeKesel (1996) concluded that the observed specificity of *L. slackensis* on its host *Pogonus chalceus* (Carabidae) in nature resulted from its reinforced ecological isolation caused by obligate ectoparasitism, the lack of free-living stages, host specificity (nutrients), and dependence on a specific environment that is rigorously selected by its host and few other potential host species.

An even more intriguing aspect of Laboulbeniales biology are the numerous reports of so-called "position specificity"- the often precise occurrence of thalli on very restricted areas of the body of the host. This phenomenon was first observed by Peyritsch (1875) who noted that *Stigmatomyces baeri* usually grew on the upper surface of the female house fly (*Musca domestica*) and on the lower surface of the male. Whilst these observed patterns may be explained by assuming that ascospore transmission is optimized during copulation, others, such as Thaxter's (1926) description of 16 species of *Chitonomyces*, each growing in a precise location on the integument of a single species of African whirligig beetle (Coleoptera, Gyrinidae) can not. The phenomenon of position specificity has both intrigued and frustrated all investigators of these fungi. Until recently a rigorous method for determining the identity and morphological stability of these taxa has been lacking. Recent development of a DNA extraction protocol and PCR-based amplification procedure (Weir and Blackwell, 2001) may facilitate a long-overdue analysis of these observations. Preliminary analysis of approximately 1kB of SSU rDNA sequence data from two morphologically different species of *Stigmatomyces* found on the same dipteran host proved to be almost identical (1 bp difference), casting doubt on the existence of position specificity in this instance (Weir and Blackwell, 1998). However, data acquisition for more variable DNA regions of more individuals is needed before position specificity in these taxa can be equivocally rejected.

A third type of specificity phenomenon called 'sex-of-host specificity' was described by Benjamin & Shanor (1952) in their work on the parasites of *Bembidion picipes*. Here 6 parasite species were distinguished on a single host, 2 of these were found only on male beetles, a third species was apparently restricted to females. The restricted nature of this specificity phenomenon has also more recently been questioned (Scheloske, 1976; Rossi, 1998). Further detailed experimental and genetic studies using modern molecular techniques are undoubtedly required in order to further clarify some of the issues regarding species concepts and specificity raised here.

4. Structure, Development and Thallus Organization

Although most of the published work on the Laboulbeniales has been concerned with taxonomy and has involved the description of morphological features, detailed morphological and developmental observations have only rarely been presented

(Benjamin, 1985, 1986; De Kesel, 1989; Tavares, 1965, 1966; Weir and Beakes, 1996). In less detailed studies the structure and organization of the thallus have been frequently misinterpreted (Tavares, 1985). Here I will attempt to describe the structure, development and variability of the principal components of the thallus primarily following the terminology used by Tavares (1985).

Ascospores: The ascospores of all of the Laboulbeniales (including Pyxidiophoraceae) are hyaline, spindle-shaped or aciculate, and consist of two unequal cells surrounded by an enveloping gelatinous sheath. The shorter cell of the ascospore appears always to be oriented downward in the perithecium prior to spore discharge. The longer, distally-oriented cell has an expanded sheath pad around its tip which appears to function in attachment to the host following release from the perithecium. In *Hesperomyces virescens* the ascospores elongated on contact with the integument of the host and the tip region of the longer segment differentiated to form the foot (Weir and Beakes, 1996). The vast majority of known taxa of Laboulbeniales form this organ. The foot subsequently becomes heavily melanized and swollen. Weir and Beakes (1996) using a combination of light- and scanning electron microscopy (SEM) recently showed that removal of foot cells from the surface of the host revealed a small (ca 1 μm) circular penetration hole and evidence of remnants of an adhesive gasket-like "O-ring". A relatively extensive, branched, nonseptate rhizoidal haustorial apparatus was also observed. It is assumed that similar structures are produced by all members of the Laboulbeniales and that the foot region serves dual functions of attachment and nutrition.

Receptacle: The receptacle arises directly from repeated divisions of the longer of the two ascospore cells and gives way to the development of the perithecium. In most of the known genera the receptacle is 3-celled (typically labelled I [basal], II [suprabasal] and III [uppermost]). Exceptions include genera where there is no division into separate II and III cells (eg *Amorphomyces* Thaxt. and *Rhizopodomycetes* Thaxt.). Some genera of the Laboulbeniales develop secondary axes and these lie outside the primary axis of the original ascospore. These structures are known as secondary receptacles (Tavares, 1985). The nature and extent of the primary receptacle and the presence or absence of a secondary receptacle have been important in the delimitation of families and sub-families. In addition, the precise morphologies and position of the receptacular cells have provided important taxonomic characters for distinguishing genera. More recently, molecular data derived from 18S rDNA sequences (Weir, unpublished data) are challenging the validity of these traditional characters. In particular, the large tribe Stigmatomycetinae which presently encompasses some 42 genera, appears to be polyphyletic as currently circumscribed (Weir, unpublished data).

Appendages: The primary appendage is formed by the division of the upper cell of the ascospore and is usually a direct continuation of the primary receptacle axis. Frequently antheridia are formed from cells of the primary appendage. Any sterile or antheridial branches arising from the lower ascospore segment are designated as secondary appendages. The structure and size of the primary appendage, the presence or absence of secondary appendages and the position of appendages in relation to the receptacular axis and perithecium have been used in the recognition of genera.

Antheridia: Historically, the mode of formation of spermatia has been used as the main criterion for the separation of families within the order (Thaxter, 1896).

Antheridial characters remain important in current classificatory concepts with three distinct mechanisms for the production of spermatia distinguished:

Exogenous – with spermatia borne on intercalary cells or terminally from cells of the appendage

Simple endogenous – in which spermatia are formed within a flask-shaped cell which usually has an attenuated neck

Compound endogenous – in which antheridial cells are variably united into a compound structure so that their spermatia are discharged into a common chamber prior to exit through a single opening.

Exogenous spermatial production is unusual and found mainly in those species associated with aquatic hosts. The simple endogenous antheridium is the one most commonly encountered in the Laboulbeniales. Compound endogenous antheridia are known only in representatives of the subfamilies *Peyritschielloideae* and *Monoicomycetoideae*.

Molecular sequence data from a limited number of taxa appear to support the recognition of relationships based on these antheridial characters (Weir, unpublished data).

Peritheциum: The laboulbenialean ascocarp is essentially a peritheciium almost always formed as an outgrowth from the suprabasal cell (II) of the receptacle. The typical peritheciium is composed of stalk cells (VI, VII), and basal cells (*m*, *n*, *n'*) the latter surmounted by one outer and one inner layer of wall cells, each layer consisting of 4 vertical rows of cells. Perithecia develop either by upgrowth of wall cells around the carpogonium, as in the Laboulbeniinae, or by intrusion of the carpogonium upward between the rows of wall cells as in the monogeneric Herpomycetinae. Tavares (1985) emphasized perithecial characters in her recent classification of the order. In particular, in addition to the fundamental difference in perithecial development outlined above, she used the position of the stalk cells of the peritheciium in relation to the primary axis of the thallus to delineate the families Ceratomycetaceae, Euceratomycetaceae and Laboulbeniaceae. The number, shape, and relative heights of perithecial wall cells in each vertical row provided further distinguishing characters at the sub-family, tribal and generic levels. Analysis of preliminary sequence data (18S and 28S rDNA) for a limited number of taxa (Weir, unpublished data) produces phylogenetic reconstructions that appear to be largely congruent with those resulting from analysis of morphological perithecial characters.

5. Phylogeny

Although the precise phylogenetic placement of many fungal taxa has been controversial when viewed in an historical context, there can be few groups who share the confused taxonomic history of the Laboulbeniales. The existence of strange “hairlike structures” on the exoskeleton of beetles was independently reported at two scientific meetings in 1849 (Tavares, 1985). These same structures would later be identified and formally described as abnormal cuticular outgrowths (Mayr, 1853), acanthocephalan worms (Kolenati, 1857), or basidiomycetes and zygomycetes (Karsten, 1869). This early confusion is, perhaps understandable, given the exceptional morphological variations observed, and the apparently intimate association with the

integument of arthropods. Eventually, detailed cytological (Faull, 1906a, b, 1911, 1912), ultrastructural (Hill, 1977), life-history (Blackwell and Malloch, 1989) and molecular (Blackwell, 1994; Weir and Blackwell, *in press*) studies have provided strong evidence for placement of Laboulbeniales within the Ascomycota.

Recognition and growing acceptance of their ascomycetous affinities, in combination with their unique characters also led investigators to consider them in phylogenetic speculations regarding evolutionary origins of the higher fungi. For example, both Karsten (1869) and Sachs (1874) proposed a derivation of Laboulbeniales and, by inference, all ascomycetes, from floridean red algal ancestors. Evidence for this relationship was based primarily on the gross morphological similarity of sexual organs in the two groups. There have been more recent revivals of the "floridean hypothesis" (Dennison and Carroll, 1966; Kohlmeyer, 1973, 1975; Demoulin, 1975, 1985) although strong molecular evidence refuting this has since accumulated (Kwok *et al*, 1986; Bhattacharya *et al*, 1990; Hendriks *et al*, 1991).

More recently, Laboulbeniales have been linked with *Pyxidiophora*, a genus of mycoparasitic filamentous ascomycetes with an arthropod-dispersal stage in the life cycle (Blackwell and Malloch, 1989). Alternative hypotheses include the recent proposal by Cavalier-Smith (1998) to remove the Laboulbeniales from the Ascomycota and place these with other invertebrate-associated fungi including Trichomycetes, Entomophthorales and Zoopagales in a newly-erected SubKingdom Eomycota, Phylum Archemycota, Class Zoomycetes. This latter controversial hypothesis has been refuted on the basis of phylogenetic analysis of partial sequences of 18S rDNA, including parsimony analysis and maximum likelihood ratio tests applied to trees constructed using different topological constraints (Weir and Blackwell, *in press*).

Although their placement within the filamentous ascomycete clade is now well-supported, a robust phylogeny of Laboulbeniales taxa must await further molecular work and integration with a morphologically-based cladistic analysis. The ancestral form in the Laboulbeniales and subsequent evolutionary trends are still matters of some debate. Thaxter (1908) "...found himself unable to arrive at any satisfactory conclusions" and the more recent treatise by Tavares (1985) also concludes with uncertainty regarding these important questions. Once again preliminary molecular data, representing an as yet incomplete data set (Weir, unpublished data) of taxa, provide possible clues to the questions raised above. In particular, members of the Ceratomycetaceae including representatives from the genera *Rhynchophoromyces*, *Autoicomyces*, and *Ceratomyces* appear basal in phylogenetic reconstructions. These taxa share unspecialized exogenous spermatia and are found exclusively on aquatic hosts. *Coreomyces*, (an unusual genus found only on aquatic water-boatmen [Heteroptera, Corixidae] and characterized by unique perithecial development and production of antheridial cells on the primary axis below the perithecium) appears to represent an intermediate stage between these basal taxa and the more advanced members of Laboulbeniaceae. The latter are typified by the presence of more specialized endogenous antheridia and occurrence largely on terrestrial insects. No firm conclusions can yet be drawn from these data sets however, since many critical genera remain to be sequenced. Nevertheless, the answers to many intriguing questions regarding the evolution of these enigmatic fungi finally appear to be within grasp. It should now be possible to formulate and test higher-level phylogenetic hypotheses regarding Laboulbeniales; to investigate morphological plasticity and relate this to host,

position, and sex-of-host specificities; to test morphology-based species concepts within the order- linking these to observed patterns of geographical distribution; and to examine the patterns of coevolution occurring between Laboulbeniales and their arthropod hosts. It is my hope that resolution of these and other enigmas displayed by Laboulbeniales will allow them to be more firmly incorporated into the mainstream of mycological thought.

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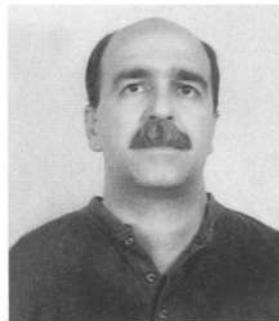
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WOLBACHIA-INDUCED CYTOPLASMIC INCOMPATIBILITY

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1. Introduction

1.1. TAXONOMY, DISTRIBUTION AND NOMENCLATURE

Wolbachia belong to the α subdivision of proteobacteria (O'Neill *et al.* 1992). As all other members of their family (Rickettsiaceae), they are obligatory endocellular symbionts (Weiss and Moulder, 1984). First observed in 1924 by Hertig and Wolbach in the mosquito *Culex pipiens*, and described in details by Hertig in 1936, they were since detected in various Arthropod groups (Insecta, Collembola, Crustacea, Arachnida) (Werren *et al.* 1995a; O'Neill *et al.* 1997a; Vandekerckhove *et al.* 1999) as well as filarial Nematodes (Sironi *et al.* 1995; Bandi *et al.* 1998). Systematic surveys of insect communities revealed that at least 15% of the species are infected, making *Wolbachia* one of the most abundant endocellular bacteria (Werren *et al.* 1995a). Recent studies based on highly sensitive detection methods suggest to extend this estimation to 76% (Jeyaprakash and Hoy, 2000).

As inferred from molecular data, *Wolbachia* form a monophyletic group (Roux and Raoult, 1995), among which five clades (A, B, C, D and E) can be distinguished. A and B diverged ~60 MY ago and form a monophyletic group including most Arthropod-infecting *Wolbachia* (Werren *et al.* 1995b). The E group is now represented by a unique *Wolbachia* strain, infecting a single arthropod species (Hexapoda, Collembola) (Vandekerckhove *et al.* 1999). A, B and E form together a monophyletic group (Vandekerckhove *et al.* 1999), out of which fall C and D, represented by Nematode-infecting *Wolbachia* (Bandi *et al.* 1998). Several genes have been used for phylogenetic purpose, such as 16S rDNA (O'Neill *et al.* 1992; Rousset *et al.* 1992; Stouthamer *et al.* 1993) and different protein coding genes (Werren *et al.* 1995b; Zhou *et al.* 1998; Van Meer *et al.* 1999), which all confirmed the main groupings. Highly variable ones also allowed understanding phylogenetic relationships on a finer scale (Zhou *et al.* 1998; Van Meer *et al.* 1999).

In spite of such a diversity, *Wolbachia* are denominated under a unique species name (*Wolbachia pipiensis*). During an international meeting held in June 2000 (reported by Cook and Rokas, 2000; Charlat and Merçot, 2000), a nomenclature system was proposed. It was suggested to maintain this unique species name, but to name separately strains that had been shown to differ by any trait, either DNA sequences or phenotypic characters. Names should then be written using *w* (for *Wolbachia*) followed by two or three characters and a subscript, indicating the strain origin and host species. As an example *wNo_{D.sim}* refers to a *Wolbachia* strain naturally infecting *Drosophila simulans* in populations from Noumea (New Caledonia) while *wCer1_{R.cer}* refers to one of the two *Wolbachia* strains infecting the cherry fruit fly *Rhagoletis cerasi* (M. Riegler, pers. com.). We will follow such a rule in this review.

1.2. WOLBACHIA TRANSMISSION MODE: A PARADOX?

Owing to its high predictive value, transmission mode is an essential trait of endosymbiont biology (Ewald, 1987). It allows the classification of endosymbionts along a continuum, ranging from complete vertical transmission (dependent on host reproduction) to complete horizontal transmission (independent from host reproduction). These two extreme strategies impose very different constraints on the evolution of symbiont/host interactions. This being so, horizontally transmitted symbionts can be deleterious to their host, while vertically transmitted ones more often provide benefits. Although horizontal transfers can occur, *Wolbachia* are mainly vertically transmitted, through egg cytoplasm. Thus, mutualistic relationships are to be expected. Such a situation is indeed observed between *Wolbachia* and Nematodes, in pathogenic filaria (Hoerauf *et al.* 1999), a major cause of morbidity throughout the tropics. In this respect, researches on *Wolbachia* are offering serious opportunities for medical applications. Conversely, in most cases, *Wolbachia* in Arthropods do not strictly speaking benefit their host. A solution to this apparent paradox is given by considering the amazing effects of *Wolbachia* on their host reproduction: feminization, male killing, thelytokous parthenogenesis and Cytoplasmic Incompatibility (CI). The three first phenotypes have in common to increase the proportion of females in infected females' broods, and thus directly advantage *Wolbachia* infected cytoplasms (for a review, see Pintureau *et al.* in this volume). CI-inducing *Wolbachia*, the subject of this chapter, have slightly different and probably more perverse consequences.

We will first describe the CI phenomenon, its distribution as well as the current knowledge about the mechanisms involved. Next, we will investigate the evolutionary dynamics of the associations of CI-*Wolbachia* with their hosts, which undoubtedly condition the long-term fate of this symbiosis. We will then discuss the evolutionary consequences of CI. Finally, the potential of CI-*Wolbachia* as biological control agents, as well as last advances in *Wolbachia* genomics, will be considered.

2. *Wolbachia*-induced CI

2.1. GENERAL DESCRIPTION

In 1952, Ghelelovitch reported the occurrence of reproductive isolation between different mosquito populations. He showed that owing to a maternally inherited factor, males from a given strain failed to produce progeny when mated with females from other strains, while the reverse cross was compatible. Laven (1967) further showed that in some cases, incompatibility occurred in both directions of cross. *Wolbachia* was identified as the causative agent, by Yen and Barr in 1971, which allowed to describe CI as an embryonic mortality of various intensity, occurring when *Wolbachia*-infected males mate with uninfected females (unidirectional CI) or females infected by a different *Wolbachia* strain (bidirectional CI) (figure 1).

Figure 1.

	W1 +	W2 +	Ø +
W1	W1	+	+
W2	+	W2	+
Ø	W1	W2	Ø

Unidirectional incompatibility is illustrated in crosses between infected (W1 or W2) and uninfected (Ø) individuals. Bidirectional incompatibility is illustrated in crosses between individuals infected by two different *Wolbachia* strains. Infection status of the descent is indicated in circles.

As a consequence of unidirectional CI, *Wolbachia* can, as a first approximation, spread through uninfected populations. Indeed, while mating with infected males is detrimental to uninfected females reproduction, infected females are compatible with both infected and uninfected males. Infected cytoplasms are thus indirectly selected for, in a positive frequency dependent manner: as infection frequency increases, uninfected cytoplasms are more and more disadvantaged. Thus, CI induction allows *Wolbachia* to spread and then remain within natural populations. Other factors than CI, that may affect invasion dynamics, will be considered in detail later on in this chapter.

Since the time of its initial discovery in mosquitoes, CI has been described in Arachnida (Breeuwer, 1997), some Crustacea (Legrand *et al.* 1985; Moret *et al.* 2001) as well as in numerous insect orders, making it the most frequent and widely distributed of *Wolbachia* induced phenotypes (O'Neill *et al.* 1997a). Phylogenetic analysis suggested that CI-*Wolbachia* do not form a monophyletic group with respect to the *Wolbachia* strains that cause other phenotypes (Werren *et al.* 1995b; Zhou *et al.* 1998). In fact, the distribution of CI within *Wolbachia* general phylogeny makes parsimonious to assume that it was an ancestral *Wolbachia* property.

2.2. MECHANISMS

The so-called mod/resc model provides a general framework for the investigation of CI (Werren, 1997a). It assumes the existence of two bacterial functions: (i) mod (modification), the poison, is expressed in the male germline before *Wolbachia* are shed from maturing sperm and (ii) resc (rescue), the antidote, is expressed in the egg. If sperm has been affected by mod, zygote development will fail unless the appropriate resc is expressed in the egg. Although their molecular nature is currently unknown, the mod and resc functions are now characterized through a number of properties, which we report below. These properties will have to be accounted for by any hypothesis regarding the molecular nature of mod and resc.

mod intensity is variable. The percentage of unhatched eggs observed in crosses between infected males and uninfected females, which we will refer to as CI level, shows quantitative variations, ranging from 0 to 100 % (Poinsot *et al.* 1998). Thus, the molecule(s) involved in the mod function must potentially show variation, in quantity and/or activity. In some cases, variations in CI levels are caused by *Wolbachia* inherent properties (Giordano *et al.* 1995, Rousset and de Stordeur, 1994; Poinsot and Merçot, 1999). Interestingly, variations due to host effects were also shown to exist. Indeed, injection experiments, allowing transferring *Wolbachia* strains between species, demonstrated that CI level is affected by host nuclear background. As an example, a “strong” strain naturally infecting *Drosophila simulans* expresses a low CI level when injected into *D. melanogaster* (Boyle *et al.* 1993). Conversely, a “weak” strain naturally infecting *D. melanogaster* expresses a high CI level when injected into *D. simulans* (Poinsot *et al.* 1998).

mod and resc interact in a specific manner, as shown by the occurrence of bidirectional incompatibility. Simply speaking, any *Wolbachia* strain is only compatible with itself, suggesting that the molecules involved can exist under various forms, allowing specific recognition. Proteins are, of course, the best candidates. It is notable that several mod/resc interactions can take place within a single embryo as suggested by crossing experiments involving doubly infected individuals (infected simultaneously by two *Wolbachia* strains). Patterns of compatibility are exactly the ones expected if each resc interacts only with its mod counterpart: doubly infected males are compatible with doubly infected females only (Rousset and Solignac, 1995; Merçot *et al.* 1995; Perrot-Minnot *et al.* 1996; Sinkins *et al.* 1995). Interestingly, mod/resc recognition can, in some cases, be partial. Indeed, partial compatibility between different *Wolbachia* was reported in *Drosophila* by Poinsot *et al.* (1998). Surprisingly, the bacterial strains involved were phylogenetically distant. Two alternative explanations can be proposed to account for this result: (i) either bidirectional incompatibility did not evolve yet, or (ii) compatibility was lost and subsequently restored by evolutionary convergence. More data on the evolutionary rate of compatibility types is required for choosing between these two alternatives.

mod and resc are probably separate functions. Depending on the presence or absence of the mod and resc functions, four different CI-*Wolbachia* types can theoretically exist: mod+/resc+, mod-/resc-, mod+/resc- and mod-/resc+. The mod+/resc+ type corresponds to most strains described so far: they induce CI and rescue their own CI phenotype. The mod-/resc- type was shown to exist in *D. simulans* (Hoffmann *et al.* 1996). It

corresponds to strains that are both unable to induce CI and rescue the CI phenotype of other mod+ strains. The mod+/resc- type is suicidal and has never been observed: it cannot theoretically be maintained in natural population as it counter-selects its own presence. On the contrary, the mod-/resc+ type, unable of inducing CI but capable of rescuing CI induced by other strains, was actually shown to exist (Bourtzis *et al.* 1998; Merçot and Poinsot, 1998a; Poinsot and Merçot, 1999). The existence of such a *Wolbachia* strain strongly suggests that mod and resc are separate functions: if not separate genes, at least different gene domains. Other interpretations can however be proposed. mod and resc could represent a single molecule, with mod requiring higher concentrations. Alternatively, the mod-/resc+ strain could have a sex specific gene expression pattern, with a unique function being expressed in the female, not in the male. Let us emphasize here that the resc- status of a *Wolbachia* strain cannot be definitively fixed by crossing experiments. Indeed, such bacteria may be able to rescue the mod function of other strains, still undiscovered.

mod intensity is linked to bacterial density. The possibility of a relationship between *Wolbachia* density and CI level was investigated. Let us first consider studies focusing on bacterial density in male testes. It was shown in *D. simulans* that as CI level decreases with male aging (Hoffmann *et al.* 1986), so does *Wolbachia* density in male testes (Binnington and Hoffmann, 1989), as well as the number of infected spermocysts (Bressac and Rousset, 1993). CI level was also shown to correlate positively with the number of infected spermocysts in *D. melanogaster* (Solignac *et al.* 1994), and *D. simulans* (Merçot *et al.* 1995). Thus it seems that when comparisons involve variations associated to a given *Wolbachia* strain within a given species, the relationship between CI level and density in testes is clear. Interestingly, experimental interspecific transfers showed that this relationship was also observed in comparisons involving different hosts species: when *wMelp.mel* is transferred from *D. melanogaster* to *D. simulans*, a shift from low to high CI level is accompanied by a shift from low to high number of infected spermocysts (Poinsot *et al.* 1998). Do CI level and density in male testes still correlate when different *Wolbachia* strains are compared? In *D. simulans*, *wMeld.mel* and *wRid.sim* infect the same frequency of spermocysts and induce similar CI levels (Poinsot *et al.* 1998). However, discrepancies appear if other *Wolbachia* strains are considered (in a single host or different hosts): some strains harbor low CI levels and high densities, while others harbor strong CI levels and low densities (Rousset and de Stordeur, 1994; Bourtzis *et al.* 1998; see also Bourtzis *et al.* 1996). Thus, it appears that the relationship between CI level and density in male testes, although well demonstrated when comparisons involve a single *Wolbachia* strain (in a single host or different hosts), breaks down when different *Wolbachia* strains are compared. Are similar conclusions drawn from density measurement in eggs? Here again, a positive correlation is observed when comparisons involve a single *Wolbachia* strain within a single host (Boyle *et al.* 1993), but it breaks down as soon as different *Wolbachia* strains and/or different hosts are compared (Giordano *et al.* 1995; Hoffmann *et al.* 1996; Clancy and Hoffmann 1997). Poinsot *et al.* (1998) included both types of measurements: while a strong correlation is observed between CI level and density in testes, density in eggs and CI level appeared to be independent. Such a result is not surprising: one would indeed expect mod intensity to be more intimately linked to density in testes than in eggs, as mod is expressed during spermatogenesis.

mod prevents condensation of paternal chromosomes after fertilization. Cytological observations revealed that in crosses between males and females of different infection status, fertilization takes place normally (Ryan and Saul, 1968; Yen, 1975; Kose and Karr, 1995). In *Drosophila*, pronucleus fusion also occurs but paternal chromosomes show abnormal behaviors, remaining undercondensed while maternal chromosomes undergo mitosis (Callaini *et al.* 1996, 1997; Lassy and Karr, 1996). In the hymenopteran *Nasonia vitripennis*, paternal chromosomes are entirely lost, inducing complete haploidy (Breeuwer and Werren, 1990), while in *Drosophila*, they segregate more or less randomly, giving rise to haploid or aneuploid cells. The precise consequences on zygote development vary between species. Especially, diploid and haplodiploid organisms must be distinguished. In diploids, death occurs more or less shortly after fertilization (Callaini *et al.* 1996, 1997; Lassy and Karr, 1996). A more diverse range of outcomes occurs in haplodiploid species, owing to the fact that in such organisms, arrhenotokous parthenogenesis (male development from unfertilized haploid eggs) is commonly observed in the absence of infection. In *Nasonia*, *Wolbachia* induced haploidy leads fertilized eggs from incompatible crosses to develop into males (Ryan and Saul, 1968; Breeuwer and Werren, 1990). Conversely, CI induces the death of all fertilized embryos in *Leptopilina heterotoma*, another hymenoptera (Vavre *et al.* 2000) suggesting that in this case, embryos are aneuploid. In the haplodiploid acarian *Tetranychus*, a proportion of fertilized eggs develop into females while the others die (Breeuwer, 1997), suggesting that part of the embryos are diploid (not affected by CI) while the others are aneuploid. Such patterns are of high interest with regard to the investigations of CI mechanisms. Indeed, they provide opportunities to observe variations in the property of the mod function and, potentially to understand the origins of these variations.

3. Dynamics of CI-*Wolbachia*/host associations

Here we discuss the evolutionary dynamics of the associations between CI-*Wolbachia* and their hosts. Starting from a description of the different events involved in the invasion of a new species, we then consider the evolution of CI, and other relevant factors, once infection is established. This evolution undoubtedly conditions the long-term fate of CI-*Wolbachia*/host associations, which we finally discuss.

3.1. HOW CI-WOLBACHIA INFECT SPECIES

3.1.1. Co-speciation or horizontal transfer?

Two underlying processes can be envisaged when considering the present distribution of *Wolbachia* among arthropods: co-speciation and Horizontal Transfers (HTs). As numerous sequence data were obtained for *Wolbachia* and their hosts, it became possible to investigate this issue through a phylogenetic approach (O'Neill *et al.* 1992; Werren *et al.* 1995b; Zhou *et al.* 1998). Host and symbiont phylogenies appeared to be often incongruent, suggesting that if co-speciation may occur, it must be limited and cannot explain, on its own, *Wolbachia* distribution. HTs between species must thus be invoked, although it remains difficult to estimate their frequency.

By which means can *Wolbachia* jump between species? Several recent studies have focused on this issue. Based on the idea that such transfers require between-species intimate relationships, possibilities of *Wolbachia* HTs between parasitoids and their host were considered. Phylogenetic data suggested that *Wolbachia* can skip from host species to parasitoids (Vavre *et al.* 1999). Conversely, the reverse transfer (from parasitoids to hosts) seems to be less straightforward, and in any case less frequent (which may be due to the fact that when hosts are not killed by parasitoids, these latter are encapsulated, preventing from any exchange). Let us emphasize that such results, based on a phylogenetic approach, are weakened by the recent discovery that *Wolbachia* strains can exchange DNA: Werren and Bartos (2001) showed the 5' and 3' ends of a *Wolbachia* gene to have clearly distinct evolutionary origins, while F. Jiggins (pers. com.) demonstrated a strong lack of congruency between *Wolbachia* phylogenies based on two different loci. The existence of *Wolbachia* recombination, although of high interest, weakens any phylogenetic trees inferred from DNA sequences. If reliable phylogenetic data is to be obtained, the use of several different genes, allowing to identify robust nodes, is thus highly recommended.

HTs from hosts to parasitoids were also experimentally demonstrated. Indeed, Heath *et al.* (1999) reported that *Wolbachia* was transferred from *D. simulans* to its parasitoid *Leptopilina boulardi*, at a frequency near 1%, when uninfected wasps oviposit into infected host larvae. Furthermore, the newly acquired *Wolbachia* infections were maintained over generations, demonstrating that the germline had been efficiently colonized. Intraspecific HTs were also demonstrated. In isopod crustaceans, transfers have been shown to occur by a simple hemolymph contact, a route likely to be used in natural populations (Rigaud and Juchault, 1995). Furthermore, Huigens *et al.* (2000) recently reported that in *Trichogramma* wasps, when infected and uninfected individuals infest the same egg, *Wolbachia* transfers from infected to uninfected individuals occur at frequencies higher than 30%. These results demonstrate that in some conditions *Wolbachia* have the ability to be horizontally transferred, either within, or between species. It remains to be determined if the conditions required are very limited and if *Wolbachia* infection can efficiently develop and colonize germ cells in any new host.

Injection experiments between different host species provide possibilities for investigating the latter question. When performed between closely related species, such transfers are usually successful (Boyle *et al.* 1993; Giordano *et al.* 1995; Clancy and Hoffmann, 1997; Poinsot *et al.* 1998). The outcomes of injections between distantly related species are less straightforward. Injections from mosquitoes into *Drosophila* (Braig *et al.* 1994), and from Hymenoptera into *Drosophila* were successful, but in the latter case, the infection was lost after several generations (Van Meer and Stouthamer, 1999). Furthermore, two of us (S.C. and H.M., together with M. Riegler) recently undertook injections between two closely related Diptera families. Current results suggest that the infection is highly unstable, due to a very low maternal transmission efficiency. It appears that the potential of *Wolbachia* to colonize efficiently the germline of new hosts is variable and might, in some cases, be a limiting factor of HTs.

3.1.2. Spreading of CI-inducing *Wolbachia*

Since the discovery of CI in mosquitoes, experimental and theoretical studies focused

on its invasion dynamics in natural populations. Early models (Caspari and Watson, 1959) demonstrated the unusually high invasion abilities of CI-*Wolbachia*. As a better knowledge of this symbiont biology has been gained, new parameters have been included, providing more realistic views (Fine, 1978; Hoffmann *et al.* 1990). Here, we present an overview of CI-*Wolbachia* invasion dynamics, without getting into equations. For a more detailed review on this issue, see Hoffmann and Turelli (1997).

What happens after a new CI-*Wolbachia* strain has been efficiently transmitted, either by HT or migration, into an uninfected population? Let us consider here the simplest situation: a *Wolbachia* strain perfectly transmitted (that is, infected females produce 100% infected progeny) and not affecting the fitness of its bearer (apart from its CI effect). As mentioned above, uninfected females suffer a fertility disadvantage when mating with infected males (the higher the CI level, the higher this disadvantage). Infected females thus reproduce more efficiently than uninfected ones: *Wolbachia* induce a selection against uninfected cytoplasmic lines, which indirectly advantages infected ones. Eventually, *Wolbachia* infection is expected to be fixed, even if CI is not 100%. Nevertheless, in this latter case, invasion will be slower.

Should other factors than CI be taken into account? Theory suggests that two parameters could have determining effects on the evolution of CI-*Wolbachia* frequencies: fitness effects on females and maternal transmission efficiency. Caspari and Watson (1959) considered the effect of a fitness reduction suffered by infected females (noted here f , varying from 0 to 1) together with that of CI (mod intensity, noted here m , varying from 0 to 100%). The main conclusions were the following: (i) 0 and 1 (i.e. extinction and fixation) are the only stable equilibrium frequencies and (ii) $p = f/m$ is an unstable equilibrium frequency, representing a threshold point below which frequency goes to 0 and above which it goes to 1. Thus, if $f \geq m$, *Wolbachia* is always lost (if it is not already fixed), and if $m > f \geq 0$, *Wolbachia* gets fixed if it reaches p (which is possible through random events). An important feature of this model is that it does not predict the stable coexistence of infected and uninfected individuals, which can yet be observed in natural populations (Turelli and Hoffmann, 1995). Fine (1978) and Hoffmann *et al.* (1990) showed that considering the effect of imperfect maternal transmission (noted μ , the fraction of uninfected eggs produced by infected females, varying from 0 to 1) could explain such a polymorphism. The main conclusions are the following: (i) 0 and 1 are stable equilibrium frequencies, (ii) p_s is a stable equilibrium frequency determined by f , m and μ and (iii) p_u is an unstable equilibrium frequency determined by f , m and μ , representing a threshold point below which frequency goes to 0, and above which it goes to p_s . Thus, the introduction of imperfect maternal transmission affects the value of the threshold frequency predicted by Caspari and Watson, but more importantly, it allows the stable coexistence of infected individuals (at frequency p_s) and uninfected ones (at frequency $1-p_s$).

Few case studies are complete enough to allow the testing of such theoretical models. Cage population experiments confirmed *Wolbachia* invasion abilities (Nigro and Prout, 1990). What about invasion dynamics in the wild? The most complete analysis to date concerns *D. simulans* and the spread of the *wRiD.sim* infection in California (Turelli and Hoffmann, 1995). The authors provided field estimates of the three key parameters mentioned above: fitness cost, transmission efficiency, and CI level. Although females seemed to suffer a slight fecundity reduction in laboratory conditions, this effect was not

detected in the field ($f=0$). Conversely, transmission efficiency was shown to be perfect in laboratory conditions, but it was estimated that infected wild females produce in average 4% of uninfected progeny. Finally, CI was estimated to be close to 100% using males from laboratory stocks, but only 55% if wild males were assessed (since CI level strongly decreases with male age (Hoffmann *et al.* 1986), it was proposed that such a reduction of CI in the field was mainly due to the fact that, in average, wild males are older than males commonly used in CI experiments). Remarkably, field parameter estimation allowed authors to predict the infection spread at a rate comparable to the one observed in the wild through a monitoring of several independent populations over 5 years. Furthermore, predicted equilibrium frequencies from most natural populations were accurately concordant with observed ones. From this study, it appears that CI-*Wolbachia* invasion dynamics and equilibrium frequencies are well predicted by current theory. Let us note however that a few locations from the above study showed anomalous frequencies with regard to predictions of the model, possibly representing a tension zone between highly infected and uninfected populations. Another discrepancy between theory and reality comes from *D. melanogaster* infection frequency data. In this species, parameter estimates from the wild lead to predicted equilibrium frequencies clearly different from those observed in natural populations (Solignac *et al.* 1994; Hoffmann *et al.* 1994; Hoffmann *et al.* 1998). Although low and undetectable fitness advantages were invoked, such results suggest that other important parameters (possibly new *Wolbachia* induced phenotypes) remain to be discovered. The existence of non-CI-inducing strains in *D. simulans* (Hoffmann *et al.* 1996; Merçot and Poinsot, 1998a), considered in more details further in this chapter, suggests similar remarks.

Let us mention here that estimation of the three key parameters often suggest that *Wolbachia* negative effects on host fitness are rare and limited (reviewed in Hoffmann and Turelli, 1997; Poinsot, 1997). Thus, CI level and transmission efficiency appear to be the main parameters.

3.2. WHAT HAPPENS AFTER SPREADING?

How do CI, transmission efficiency and fitness cost evolve once infection has reached its equilibrium frequency? The answer to this question conditions the fate of CI-*Wolbachia* host associations in the long term: it determines to what extent *Wolbachia* infections are stable over time and hence, what type of relationships between CI-*Wolbachia* and their hosts are expected to evolve. We first discuss the evolution of these three key parameters from the bacterial point of view, then from the host side. Finally, we explore the outcomes of the evolution of bacterial and host determinants in combination.

3.2.1. Evolution of bacterial determinants

CI levels. Since CI-*Wolbachia* invade host populations owing to CI induction, one is intuitively tempted to consider that high CI levels are selected for. However, Prout (1994) and Turelli (1994) showed that this is not to be the case when the competing variants are compatible with each other. In order to illustrate this conclusion, let us consider a *Wolbachia* strain **mod_a/resc_a** infecting a panmictic host population. Consider

now a mutant $\text{mod}_{\alpha^*}/\text{resc}_\alpha$ harboring a stronger mod intensity ($\text{mod}_{\alpha^*} > \text{mod}_\alpha$) but compatible with the original strain (resc_α can rescue mod_{α^*}). Will $\text{mod}_{\alpha^*}/\text{resc}_\alpha$ variants invade the population? The answer is no: males infected by $\text{mod}_{\alpha^*}/\text{resc}_\alpha$ induce a higher embryonic mortality than $\text{mod}_\alpha/\text{resc}_\alpha$ males (when mating with uninfected females), but $\text{mod}_{\alpha^*}/\text{resc}_\alpha$ and $\text{mod}_\alpha/\text{resc}_\alpha$ females are equally compatible with all types of males. Since *Wolbachia* are maternally transmitted, $\text{mod}_{\alpha^*}/\text{resc}_\alpha$ and $\text{mod}_\alpha/\text{resc}_\alpha$ variants have the same fitness. In fact, the occurrence of mod_{α^*} will induce an overall increase of the infection frequency, but this benefit goes both to $\text{mod}_{\alpha^*}/\text{resc}_\alpha$ and $\text{mod}_\alpha/\text{resc}_\alpha$ variants. Thus, it appears that from the bacterial point of view, variations in mod intensity between compatible strains are selectively neutral and thus evolve under genetic drift only.

Transmission efficiency and fitness cost. Turelli (1994), generalizing Prout's model (1994), has shown that selection among bacterial variants acts to maximize the number of infected progeny produced by infected females, which is directly determined by transmission efficiency and fitness costs. Thus, from the bacterial side, long-term evolution is expected to lead to high transmission efficiency and low fitness costs.

3.2.2. Evolution of host's determinants

Here we describe the selective forces acting on host genome with regard to the evolution of the three key factors. Let us notice that mitochondrial genome is not considered here: "host genes" refer to nuclear genes only.

CI levels. What selective pressures act on host genes that affect CI levels? Turelli (1994) showed that a reduction of CI levels is selected for. To illustrate this conclusion, let us distinguish the four types of individuals present in a host population: infected females (IF), infected males (IM), uninfected females (UF) and uninfected males (UM). IF and UM do not suffer from CI but are not advantaged either by strong CI levels. IM and UF do suffer from CI, and are selected for reducing CI levels. In other terms, host genes decreasing the incompatibility between IM and UF are selected for. Two types of such genes can be envisaged: those reducing mod intensity, selected for in IM and host rescue genes, selected for in UF. Thus, low CI levels are expected evolve. Let us note that this conclusion is drawn only if infection is not fixed. More generally, the dynamics of host genes reducing CI will depend on the infection equilibrium frequency (which depends itself on CI levels).

Transmission efficiency and fitness cost. Turelli (1994) showed that regarding these two parameters, similar selective pressures act on hosts and symbionts. Low fitness cost are selected for, while maternal transmission tends to be maximized (because uninfected offspring are not protected from CI). Thus from the host side, long-term evolution is expected to lead to low fitness cost and high transmission efficiency. An interesting exception to this rule was recently shown to occur in hymenopteran species where CI induces male development instead of death. Vavre (2000) showed that in such species, host are selected for a reduction of transmission efficiency, owing to the fact that nuclear genes are efficiently transmitted in incompatible crosses.

3.2.3. Combined evolution of bacterial and host determinants

What if selection on hosts and symbionts are considered together? Mod intensity seems to be neutral for *Wolbachia*, while a reduction of CI levels is expected from selection on

hosts. High transmission efficiency and low fitness costs are selected for from both sides (if the special case of haplodiploids is not considered). Thus, if these three key factors do not interfere, host/symbiont co-evolution is expected to lead to low CI levels, low fitness costs and high transmission efficiency.

3.2.4. Interference between the three key factors

Turelli (1994) suggested that CI levels, transmission efficiency and fitness cost might be linked through a unique feature: bacterial density. This would in theory lead to trade-off densities, since selection pressures act in different directions: selection on CI level and transmission efficiency favor an increase of density, while selection on fitness cost tends to minimize density. Such interference would explain the above-mentioned discrepancies between field and laboratory parameter estimates in *D. simulans* (lower CI level, lower transmission and lower cost in the field, possibly due to a lower density).

What arguments actually support such relationships between the three key factors and density? As previously stated, CI level and density were shown to be positively correlated, but this relationship may have limited applications. Furthermore, there is no *a priori* reason to assume that density in embryos determines mod intensity: the way is long from the embryo to its spermatozoa... Is there any evidence for a correlation between density in embryos and transmission efficiency? Such a link is suggested by logic. However, few studies focused on this issue. Indirect evidence come from Poinsot *et al.* (2000). What about the relationship between infection cost and density? Here again, a link would not be surprising, but no empirical data support it.

The three key parameters, if linked to density, are probably not linked to the same aspect of this latter: CI level must be linked to density in males reproductive tissue, transmission efficiency may be facilitated by high densities in ovaries, while the infection cost is probably affected by overall density (not tissue specific). Thus, any assumption of simple relationship between the three key factors should be considered cautiously.

3.2.5. Empirical data

Theory predicts that *Wolbachia*/host coevolution will lead to low CI levels, low infection cost and high transmission efficiency. Are such tendencies observed in reality? Injection experiments provide insights into this question. By comparing the parameter estimates in both natural and naive host (that is naturally uninfected), it is possible to determine the outcomes of co-evolution. Thus, when $wRi_{D.sim}$, naturally infecting *D. simulans* is transferred into *D. serrata* (naturally uninfected), CI level is increased while transmission efficiency is decreased (Clancy and Hoffmann, 1997). However, while $wRi_{D.sim}$ induces a fecundity deficit in its natural host, no such cost was detected in the novel one. Thus, injection into a naive host partially support the predictions.

Interestingly, when $wRi_{D.sim}$ is transferred into *D. melanogaster*, a species naturally infected by another *Wolbachia* strain, a shift from high to low CI level is observed (Boyle *et al.* 1993). Conversely, when $wMel_{D.mel}$, naturally infecting *D. melanogaster*, is transferred into *D. simulans*, a shift from low to high CI levels is observed (Poinsot *et al.* 1998). These results put together suggest that *D. melanogaster* reduces CI in a non-specific manner. Solignac *et al.* (1994) suggested that *D. melanogaster* had experienced

Wolbachia infection for a longer time than *D. simulans*, which would explain such controls of CI levels. However, recent investigations show that some infections in *D. simulans* might be ancient (Charlat *et al.* unpublished results), suggesting to consider this explanation cautiously.

3.3. WHAT MAINTAINS CI?

In the above section, we concluded that CI levels were neutral from the bacterial point of view, while a reduction was selected from the host side, possibly leading to the loss of the mod function. However, CI-inducing *Wolbachia* are widespread and CI levels are often very high (Hoffmann and Turelli, 1997). Accordingly, it is very likely that CI is selected for. Three kinds of explanation can be proposed, which will be detailed here. First, the ability of *Wolbachia* to invade new species and to be maintained depends on its ability to induce CI. Second, population structure may affect the evolution of mod intensity. Finally, bidirectional incompatibility, and the evolutionary process leading to it, might favor an increase of CI levels.

3.3.1. Invasion and maintenance abilities

We proposed elsewhere to compare arthropods to a metapopulation within which the current distribution of *Wolbachia* strains was the consequence of the dynamics of extinction (loss) relative to colonization (gains) (Charlat and Merçot, 2000). Such a conception provides arguments for the evolutionary maintenance of CI.

First, CI inducing strains are more efficient than non-CI ones in colonizing new species. Indeed, the higher the intensity of mod, the lower the threshold frequency (that must be reached through random events for *Wolbachia* to invade deterministically), and the faster the invasion process. Thus, *Wolbachia* potential to be horizontally transferred induces a selection for high CI levels.

The second argument invokes selection at the population level. Although, within populations, variants inducing high CI levels are not selected for, bacterial populations that induce CI are less likely to go extinct than populations that do not. Thus, through a process of group selection (Hurst and McVean, 1996) CI may be evolutionary maintained.

3.3.2. Host population structure

Considering the competition between compatible strains harboring different CI levels, Franck (1997) showed that if infection is not fixed, and if host population is structured, high CI levels might be selected for. Indeed, population structure means that bacterial clones are not evenly distributed. A clone that induce a higher CI level will increase the infection frequency, but this increase will be more important locally. Thus, through a process of kin selection, strains inducing high CI levels advantage themselves by aiding relative symbionts in neighboring females. Franck (1997) suggested that weak population structuration is sufficient to explain the maintenance of high CI levels. Let us notice that such an argument also stands for selection on host: if population is structured, hosts genes that increase CI levels benefit themselves by aiding relative genes in infected females.

3.3.3. The importance of bidirectional incompatibility

Different CI-*Wolbachia* variants can infect separate populations within a given species. If populations come into contact, a competition occurs between *Wolbachia* variants. If these are incompatible, two outcomes are possible. The populations may become definitively isolated (a possibility which will be considered in more detail in the section below on CI and speciation). If complete isolation does not occur, one of the two variants will go extinct (Rousset *et al.* 1991). In such a case, selection will favor the strongest variant (in terms of mod intensity, transmission efficiency, and fitness effects). As transmission efficiency and fitness effects are supposed to be optimized by “within population selection”, strains will most probably differ by mod intensity. The highest mod intensity will hence be selected for. Thus, bidirectional incompatibility may be an important factor explaining the maintenance of high CI levels.

Furthermore, our current theoretical work suggest that evolutionary processes leading to new incompatibility types might also favor strong mod intensity (Charlat *et al.* unpublished results). By contrast to previous studies on this issue, our work includes recent results suggesting that mod and resc are independent functions (Merçot and Poinsot, 1998a; Poinsot and Merçot, 1999). Such an assumption allows the evolution of new compatibility types under a wider range of conditions than previously thought (Turelli, 1994; Werren, 1997b). It also suggests that new incompatibility types will invade more easily if mod intensity is at the same time stronger. Such a process may be a further explanation of why CI still exists.

3.4. INFECTION LOSS OR EVOLUTIONARY STABILITY?

We described some evolutionary forces that act to decrease CI level, and others that act to maximize it. The outcome of such conflicts is not straightforward to predict, since it is likely to depend on host biological traits such as population structure. When selection maximizes CI, infection is probably maintained in the long term.

Empirical data demonstrate that, at least in some cases, CI might be lost. Indeed, in *D. simulans*, two *Wolbachia* strains have been described that do not induce any detectable phenotype and probably derive from CI-inducing strains since they are very closely related and infect the same species as the latter. Apparently, their frequency is low and stable in the wild (Hoffmann, *et al.* 1996; James and Ballard, 2000; Charlat *et al.* unpublished results). Models do not predict that such mod- strains can be maintained, unless they confer a fitness advantage, which was yet not detected (Hoffmann *et al.* 1996). These may be too small to be experimentally spotted. Alternatively, undetected mod+ strains, compatible with these mod- variants, may occur at low frequencies in natural populations. Whatever the causal factor, it remains that mod- strains are maintained, albeit at a low frequency, suggesting that CI loss does not necessarily mean evolutionary instability. Most notably, since non-CI inducing strains were almost undetectable before PCR became available, it is possible that their discovery will become a relatively common event in the coming years.

4. Evolutionary consequences of *Wolbachia*-induced CI

4.1. CONSEQUENCES ON HOST POPULATION BIOLOGY

4.1.1. CI lowers population mean fitness

During the invasion process, the population average fitness is reduced. Considering the simplest case (no cost, perfect transmission and 100% CI), the average fitness drops to a minimum of 0.5 when bacteria infect 50% of the population (i.e. on average, half of the eggs do not hatch because of CI). Let us note that the population mean fitness is also lowered if infection equilibrium frequencies is not 1, that is, as soon as transmission is not perfect. Furthermore, our current work on the evolution of bidirectional incompatibility suggests that polymorphic situations may be maintained by selection (Charlat *et al.* unpublished results). Such a polymorphism can actually induce a mean fitness reduction of 50%. Thus, it seems that in diverse situations, the population mean fitness, and thus its capacity to grow, is strongly affected by CI-*Wolbachia*. In this respect, these symbionts represent an important feature of host demography and may strongly affect the structure of species communities.

4.1.2. Mitochondria hitchhike along with CI-*Wolbachia*

It has been well demonstrated that as *Wolbachia* invade, so do associated mitochondria (Nigro and Prout, 1990; Ballard *et al.* 1996). As a consequence, *Wolbachia* spread induces a strong reduction of mitochondrial diversity. Interestingly, it was demonstrated that even in cases where *Wolbachia* equilibrium frequency is not 1, the mitochondrial haplotype associated to the infected cytoplasm gets fixed. This is due to the fact that when *Wolbachia* is at equilibrium frequency, uninfected individuals derive from infected ancestors (Turelli *et al.* 1992). Such *Wolbachia* effect should be considered very seriously when inferring population histories from mitochondrial data. As an example, the patterns of mitochondrial diversity induced by CI-*Wolbachia* may mistakenly be interpreted as founder events.

Let us note that occasional paternal transmission of *Wolbachia* or HTs within populations may break the association between *Wolbachia* and mitochondria. In *D. simulans*, possibilities of rare paternal transmission seem to exist in laboratory conditions (Hoffmann *et al.* 1990). Furthermore, intraspecific HTs were shown to occur at very high frequencies in some parasitoid species (Huigens *et al.* 2000). In any case, population genetic studies in *D. simulans* demonstrate a linkage between *Wolbachia* and mitochondrial haplotypes (Ballard *et al.* 1996).

4.1.3. CI may promote speciation

Because of its ability to induce partial or complete isolation between host populations, CI has been investigated as a potential promoter of speciation (Werren, 1997b). The most complete study to date with regard to this issue concern three parasitoid wasp species of the genus *Nasonia*: *N. giraulti*, *N. longicornis* and *N. vitripennis* (Werren, 1997b). The first two diverged ~250,000 years ago, while their common ancestor diverged from *N. vitripennis* ~800,000 YA (Campbell *et al.* 1993). All three species are doubly infected by *Wolbachia*. Infection was shown to induce complete reproductive isolation between one of the older species pairs: *N. giraulti* and *N. vitripennis*.

(Breeuwer and Werren, 1990; Bordenstein *et al.* 1998). Following antibiotic curing, fertile F1 hybrids are produced but there is severe F2 hybrid breakdown (Breeuwer and Werren 1995). These data demonstrate that CI-*Wolbachia* is involved in reproductive isolation, but since other reproductive (pre- and post-mating) barriers exist between these species, it is not known if *Wolbachia* were the original cause of speciation. Bordenstein *et al.* (2001) analyzed reproductive barriers in other species pairs and have shown that *Wolbachia* is involved in reproductive isolation in all cases. Furthermore, in the younger pair (*N. giraulti* and *N. longicornis*), CI seems to be the only isolating barrier to gene flow (except for weak sexual isolation), suggesting that if these species came into contact, *Wolbachia* could play a causal role in speciation.

Other systems potentially provide relevant information. Bidirectional incompatibility was shown to occur in *Culex pipiens* (Guillemaud *et al.* 1997). However, the involvement of *Wolbachia* was not clearly demonstrated. Furthermore, there is no evidence that geographical races in this species are due to *Wolbachia* (Werren, 1997b). In *D. simulans*, different incompatible strains also occur, but no genetic structuration, at the nuclear level, was observed (Ballard, 2000), suggesting that if this is a case of incipient speciation, we are still at the very first steps. Let us notice that in this species, CI is not complete (Merçot and Poinsot, 1998b), and that the infection is not fixed in natural populations (James and Ballard, 2000), thus allowing for significant gene flow. The potential involvement of unidirectional incompatibility in speciation should also be considered. Although it limits gene flow in only one direction of cross, Shoemaker *et al.* (1999) recently provided evidence that unidirectional incompatibility can be a component of reproductive barriers. Indeed, the isolation between *Drosophila recens* and *D. subquinaria* was shown to be mediated by behavioral components in one direction of cross, while unidirectional incompatibility was an important factor in the reverse cross.

Although the involvement of CI-*Wolbachia* in speciation events is strongly suggested, no complete and direct evidence is available, as it is often the case in speciation study. However, the widespread occurrence of CI-*Wolbachia* and the potential of uni- and bidirectional incompatibility to cause reproductive isolation strongly motivates further investigations.

4.2. MUTUALISTIC RELATIONSHIPS

CI-*Wolbachia* are selected for a reduction of fitness costs. Going further, benefits to host are also to be expected, if infection is stable enough for these to evolve. Mutualistic relationships were actually observed in Hymenoptera (Girin and Bouletreau, 1995; Dedeine *et al.*, 2001), and invoked (but not detected) to explain the maintenance of non-CI strains in *Drosophila*. A striking example of the consequences that mutualistic endosymbiosis can have on evolution is that of mitochondria. Their endosymbiotic origin is well documented, especially from phylogenetic data. Strikingly, mitochondria fall within the **α -proteobacteria** subdivision of Eubacteria (Yang *et al.* 1985; Gray *et al.* 1989), as does the Rickettsiaceae family, to which *Wolbachia* belongs. Such a relatedness makes it tempting to speculate on the long-term evolutionary fate of *Wolbachia*. Complete genome sequencing projects, presented as a conclusion of this chapter, will undoubtedly tell us more on this issue, as remarkably illustrated for other endocellular symbionts (Andersson *et al.* 1998; Shigenobu *et al.* 2000).

5. Applied biology of CI-inducing *Wolbachia*

Wolbachia has been suggested as a potential tool for the development of novel, environmentally friendly, biotechnological strategies for the control of arthropod species that are major agricultural pests or disease vectors to humans, plants, and livestock or for the improvement of beneficial species (Beard *et al.* 1993a; Bourtzis and O'Neill, 1998; Bourtzis and Braig, 1999). Below are the potential applications for CI-inducing strains of *Wolbachia*.

First, *Wolbachia*-induced CI might be used to suppress natural populations of arthropod pests in a way analogous to Sterile Insect Technique (S.I.T.). S.I.T. technology involves the mass production and release of irradiated sterile male insects and is the current strategy used for the control of certain insect agricultural pests. One of the limitations of the S.I.T. programs is the competitiveness of released males. Radiation doses commonly used to sterilize males introduce secondary deleterious effects that reduce the fitness of these males. CI provides an alternative method to produce non-irradiated "sterile" males and as such reduces the cost of a given S.I.T. program by increasing the competitiveness of released males and thereby reducing the numbers need to be released for effective control. CI has been used in the past to introduce sterility into wild populations of mosquitoes. Indeed, several trials, sponsored by the World Health Organization, were undertaken in the mid 1960s in Burma and India to eradicate the filariasis vector species *Culex pipiens* and *C. quinquefasciatus*. By mass rearing and then releasing males that were incompatible with the target population, it was possible to effectively sterilize wild females and in one field trial completely eradicate mosquitoes from a Burmese village (Laven, 1967). Also, in the 1970's an international collaborative project took place in Central Europe and used CI strategies to control the European cherry fruit fly, *Rhagoletis cerasi*. Several successful field trials were performed but for financial reasons this project was never completed (Blümel and Russ, 1989; Boller, 1989). In addition to these field experiments, a number of laboratory and warehouse experiments in the United States of America have successfully applied *Wolbachia*-induced CI as a means of genetic control of the stored product pest, the almond moth, *Cadra (Ephestia) cautella* (Brower, 1978; 1979; 1980; Kellen *et al.* 1981). However, in order to use CI as an effective method to produce "sterile" males, it has to be combined with an effective sexing system, since released females that also carry *Wolbachia* would be capable of successfully mating with released males (Laven, 1967). In the absence of such technology, CI could be used in conjunction with lower doses of radiation than are currently used and still achieve higher competitiveness of males and also sterilize the few females that escape conventional sexing systems. This strategy has been experimentally tested in the mosquito *Culex pipiens* (Curtis, 1976) and it has been shown that application of low radiation doses can generate sterile females and cytoplasmically incompatible males are equally competitive to non-irradiated males (Sharma *et al.* 1979; Arunachalam and Curtis, 1985; Shahid and Curtis, 1987).

Second, *Wolbachia*-induced CI might be used as a mechanism to spread desirable genotypes into wild arthropod populations. For example, current research projects aim to develop genetically modified arthropods that will not be capable to transmit pathogens to humans, plants and livestock (Curtis, 1994; Pettigrew and O'Neill, 1997;

Ashburner *et al.* 1998; O'Brochta and Atkinson, 1998). However, an important practical concern exists over the efficacy of a given transgene to spread where population replacement is required (Ashburner *et al.* 1998). *Wolbachia* infections can be used as a spreading means in order to genetically engineer arthropods to replace natural target populations. In an elegant study by Turelli and Hoffmann (1995), it was shown that the *Wolbachia*-infected *D. simulans* Riverside strain was spreading at a rate of approximately 100 km a year, replacing the uninfected population in the Central Valley of California. Similar spreading of *Wolbachia* infections have been reported in other species such as the small brown plant hopper *Laodelphax striatellus* and the moth *Cadra cautella* (Ahmed *et al.* 1984; Hoshizaki and Shimada, 1995; Hoshizaki, 1997). Bidirectional incompatibility and multiple infections provide further tools for repeated sweeps into target natural populations. Indeed, bidirectional incompatibility phenomena and double infections have been described in natural populations of several arthropod species (O'Neill and Karr, 1990; Rousset and Solignac, 1995; Werren *et al.* 1995b; Perrot-Minnot *et al.* 1996; Bordenstein and Werren, 1998; Merçot and Poinsot, 1998b; Wenseleers *et al.* 1998; Zhou *et al.* 1998; Jeyaprakash and Hoy, 2000). In addition, double and triple infected strains have been artificially generated in the laboratory. These strains are stable, express high levels of CI and replace double, single and uninfected strains in experimental cage populations (Sinkins *et al.* 1995; Rousset *et al.* 1999). Moreover, the identification of the *Wolbachia* genes responsible for CI will allow the introduction of these genes into the host nuclear genome and the induction of CI without the presence of *Wolbachia*. Theoretical models suggest that nuclear-coded CI genes will spread their host replacing target naïve populations along with any other chromosomally linked gene(s) (Sinkins *et al.* 1997; Curtis and Sinkins, 1998).

Third, *Wolbachia* might be also used as an expression vector in para-transformation strategies. Para-transformation is the method that uses symbiotic bacteria as vehicles for the introduction and expression of genes of interest into a target arthropod species and has been suggested as an alternative approach for the genetic manipulation of arthropods (Beard *et al.* 1993a; Ashburner *et al.* 1998). The symbiotic bacteria of the assassin bug *Rhodnius prolixus* (actinomycetes *Rhodococcus rhodnii*) and of tse-tse flies (S-endosymbionts) have already been used as expression vehicles (Beard *et al.* 1992, 1993b; Durvasula *et al.* 1997; Cheng and Aksoy, 1999). Moreover, a para-transformation approach is currently being evaluated for field releases of *Rhodnius prolixus* aiming to reduce the prevalence of the causative agent of Chagas' disease, *Trypanosoma cruzi* (Durvasula *et al.* 1997). It has to be noted that both *Rhodococcus rhodnii* and S-endosymbionts can be cultured in a cell-free medium and their genetic transformation was easily achieved by using shuttle plasmid vectors (Beard *et al.* 1992, 1993b). As regards *Wolbachia*, which is an obligatory intracellular bacterium, both a cell-free culture and a genetic transformation system are still missing. The fact that these bacteria can now be maintained in different insect cell lines (O'Neill *et al.* 1997b; K.B. unpublished data) and the recent isolation and characterization of endogenous phages and insertion sequences (Masui *et al.* 1999) will certainly facilitate current efforts for the genetic engineering of *Wolbachia*. In addition, homologous recombination approaches were successfully used for the genetic manipulation of other intracellular bacteria such as *Rickettsia*, *Chlamydia* and *Coxiella* (Tarn *et al.* 1994; Suhan *et al.* 1996; Rachek *et al.* 1998, 2000) and are currently being applied to

Wolbachia as well (K.B. unpublished data). In each case, the genetically manipulated *Wolbachia* need to be reintroduced into the target hosts and express the desired gene in a spatially and temporally correct manner and finally to replace their native counterparts. These goals can be easily achieved since *Wolbachia* have been detected in all major tissues and transferred by a variety of methods into different hosts where they induced CI (Boyle *et al.* 1993; Braig *et al.* 1994; Chang and Wade, 1994; Rousset and de Stordeur, 1994; Giordano *et al.* 1995; Rigaud and Juchault, 1995; Clancy and Hoffmann, 1997; Bouchon *et al.* 1998; Grenier *et al.* 1998; Poinsot *et al.* 1998; Dobson *et al.* 1999).

Wolbachia-based applications may be of broad use since these bacteria are present in a wide range of arthropod species and can also be transferred into naïve hosts. Perhaps, the ability of these bacteria to establish new infections and persist into their hosts for long time may be related with their potential to “escape” the host’s innate immune system (Bourtzis *et al.* 2000). However, several important factors need to be considered since they may influence the strength of CI expression. These include male host age, repeated copulation (Bressac and Rousset, 1993; Karr *et al.* 1998), larval density and diapause (Sinkins *et al.* 1995; Perrot-Minnot *et al.* 1996; Clancy and Hoffmann 1998) and environmental factors such as temperature (Hoffmann *et al.* 1986, 1990; Snook *et al.* 2000), food quality and natural occurring antibiotics (Stevens and Wicklow, 1992).

6. *Wolbachia* genomics, proteomics and post-genomics studies

Molecular, biochemical, genetic and classical microbiological studies have been hampered in *Wolbachia* because of their fastidious unculturable nature. However, recent advances in genomics have allowed deciphering the biology of obligatory intracellular bacteria such as *Rickettsia* and *Buchnera* (Andersson *et al.* 1998; Shigenobu *et al.* 2000). A European *Wolbachia* Consortium has recently been established, consisting of eight laboratories from six countries, and co-ordinated by one of us (K.B.). The aim of this Consortium, funded by European Commission, is to identify *Wolbachia* and host genes involved in *Wolbachia*-arthropod symbiotic associations, including the *Wolbachia* genes responsible for the induction of CI, parthenogenesis and feminization, by using an integrated genomics, proteomics and post-genomics (microarrays and bioinformatics) approach. The Consortium also aims to develop a genetic transformation system for *Wolbachia* that will facilitate further functional studies and genetic manipulation of the bacterium for applied purposes. The genomics component of the project consists of the complete and annotated genome sequence of three *Wolbachia* strains, respectively responsible for the induction of CI (*wNo_{D.sim}* strain from *D. simulans*), parthenogenesis (*wUni_{M.uni}* strain from *Muscidifurax uniraptor*) and feminization (*wVul_{A.vul}* strain from *Armadillidium vulgare*). Currently, the genome of the *wNo_{D.sim}* strain is being sequenced. Genome analysis will be complemented by proteomics and microarrays of the host and *Wolbachia* comparing RNA and protein extracts from: a) infected versus uninfected host strains and b) inducing a reproductive phenotype versus non-inducing *Wolbachia* strains. The identification of the genes involved in host-*Wolbachia* interactions will be a major breakthrough in deciphering the biology of this unculturable bacterium, understanding *Wolbachia*-host symbiotic

associations and uncovering the evolution of intracellular symbiosis. In parallel with the European *Wolbachia* project, another initiative, funded by the National Institute of Health of USA and New England Biolabs in collaboration with the Yale University (Dr. Scott O'Neill's laboratory), is in progress aiming to sequence two *Wolbachia* strains at the "Institute for Genomic Research" (Rockville, USA). The first strain induces CI in *D. melanogaster* (*wMelD.mel* strain) while the second is present in the filarial nematode *Brugia malayi*. Interestingly, phylogenetic analyses have suggested a mutualistic relationship between the bacteria and their nematode hosts that is also documented by antibiotic treatments (Bandi *et al.* 1998). Indeed, tetracycline treatments inhibit development in early stages and reduce worm fertility (Genchi *et al.* 1998; Langworthy *et al.* 2000). Recent studies also showed that an endotoxin or lipopolysaccharide (LPS) from *Wolbachia* is a major cause of inflammatory responses induced directly by the filarial nematode (Taylor and Hoerauf 1999; Taylor *et al.* 2000). Comparative genomics of *Wolbachia* is expected to identify potential drug targets for filariasis control. Also, comparing the genome of *Wolbachia* with that of *Rickettsia prowazekii* (Andersson *et al.* 1998) may result in the identification of factors that determine host specificity and virulence of these intracellular pathogens. It is also expected that comparing the genomes of several intracellular organisms such as *Wolbachia*, *Rickettsia*, *Buchnera* including mitochondria will help to unravel the molecular pathways for the establishment of intracellular symbiosis.

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8. References

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HOW DO WOLBACHIA SYMBIANTS INCREASE THE PROPORTION OF FEMALES IN THEIR HOSTS?

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1. Introduction

Until the last three decades, it was assumed that sex determination in arthropods was mainly governed by chromosomal factors and some environmental factors, also called epigenetic factors. The nutritive quality of the plant or host, larval competition, the quality of the egg laying substrate, and photoperiod and temperature have all been identified as external factors able to influence sex determination (Bull, 1983; Laugé, 1985). Some of these factors do not involve sex determination per se, but induce biased sex ratios via the female choice (whether the egg is fertilized or not) or via differential mortality between sexes. On the contrary, some other factors, such as photoperiod and temperature, have an actual sex reversal effect (Bull, 1983; Naylor *et al.*, 1988).

A new era in sex determination appeared with the discovery of a cytoplasmic factor implicated in the transformation of males into functional females in some crustaceans and in the induction of parthenogenesis in hymenopteran insects. The observation of a biased sex ratio, along with a sensitivity to high temperature and antibiotics, were at the origin of these discoveries. These findings led researchers to suspect a microorganism as being the responsible agent. Studies by transmission electron microscopy allowed for the identification of this agent as an intracellular bacterium of the Rickettsiaceae family: it is located in intracytoplasmic vacuoles and is discernible with 3 enveloping membranes (Martin *et al.*, 1973; Louis & Nigro, 1989). Molecular tools have led to the identification of these bacteria as alpha Proteobacteria, belonging to the clade of *Wolbachia pipiensis* (Rousset *et al.*, 1992). Recently, they were specifically visualised within their host by Fluorescence In Situ Hybridization (FISH), an improvement on standard histo-cytological methods (Heddi *et al.*, 1999; Pintureau *et al.*, 2000b). *Wolbachia* were thought to be mainly present in eggs, ovaries and testes. Nevertheless, it is now acknowledged that *Wolbachia* infections are more widely distributed in host

tissues than was previously appreciated. Somatic, as well as germ line tissues, of various arthropod species may be infected (Rigaud *et al.*, 1991; Dobson *et al.*, 1999).

It is now accepted that *Wolbachia* represent probably the most abundant endosymbiotic bacteria (Werren *et al.*, 1995a). Recently, thanks to a new technique of long PCR amplification of DNA from *Wolbachia*, Jeyaprakash & Hoy (2000) showed that the *wsp* gene sequence of this symbiont was present in 76% of sixty-three arthropod species belonging to twelve orders of insects and one of arachnid (mite). The main insect orders represented were: Thysanura, Odonata, Isoptera, Orthoptera, Dermaptera, Homoptera, Thysanoptera, Coleoptera, Siphonaptera, Diptera, Lepidoptera, and Hymenoptera. *Wolbachia* were also detected by standard PCR from isopods (Bouchon *et al.*, 1998), nematodes (Sironi *et al.*, 1995) and spiders (Oh *et al.*, 2000).

The effects of *Wolbachia* on their hosts are variable. In one example, in *Drosophila*, a deleterious action of this symbiont has been described (Min & Benzer, 1997). *Wolbachia* may also influence host fecundity. Nevertheless many results have not been definitely assessed, probably because fecundity depends on many other biotic and abiotic factors. Both a negative and a positive effect on this parameter have been observed in *Drosophila simulans* (Hoffmann *et al.*, 1990; Poinsot & Merçot, 1997). A negative effect was observed in *Oniscus asellus* (Rigaud *et al.*, 1999b), whereas a positive effect was reported in *Trichogramma bourarachae* (Girin & Boulétreau, 1995; Vavre *et al.*, 1999b). In *Nasonia vitripennis*, the positive effect on fecundity described by Stolk & Stouthamer (1995) was no longer found by Bordenstein & Werren (2000) after strict control of the genetic background of the hosts.

In most species, however, *Wolbachia* are responsible for reproductive alterations in their hosts. Cytoplasmic Incompatibility (CI) is probably the most frequent trait associated with these symbionts (see Chapter by Charlat *et al.* in this book). *Wolbachia*-induced CI has been described in most insect orders (Diptera, Coleoptera, Hymenoptera, Orthoptera, and Lepidoptera) (Stouthamer *et al.*, 1999), in mites (Breeuwer, 1997), and in the terrestrial isopod *Porcellio dilatatus* (Rousset *et al.*, 1992).

In numerous other cases, *Wolbachia* are responsible for increasing the proportion of females in their hosts. This augmentation can occur through three mechanisms: by feminization of males into functional females, by thelytokous parthenogenesis induction in females, or by a male-killing phenomenon. The feminization mainly occurs in crustaceans (terrestrial isopods), and was recently discovered in some lepidopteran insects (Kagayama *et al.*, 1998). The thelytokous parthenogenesis induction is especially observed in Hymenoptera, mainly the parasitic species. The male-killing due to *Wolbachia* (i.e. the death of young male embryos) is described in some Coleoptera (Fialho & Stevens, 2000; Majerus *et al.*, 2000) and Lepidoptera (Jiggins *et al.*, 2000). In this chapter we focus only on sex ratio distortions, by reviewing the means by which these phenomena are induced. We also suggest some potential targets, not yet established, through which *Wolbachia* might bias their host sex ratio.

2. Symbiont transmission and advantages obtained from the augmentation of the proportion of females in the host population

2.1. SYMBIONT TRANSMISSION

2.1.1. Vertical natural transmission

As with most endocytobionts, *Wolbachia* are typically maternally transmitted by the host from infected females to their progeny. Although *Wolbachia* can be found in both ovaries and testes, the host male seems to have no role in the symbiont transmission between generations, probably because of the low cytoplasmic content of sperm (Werren, 1997; O'Neill *et al.*, 1997). An exceptionally low level of paternal transmission was described in *Drosophila simulans* (Hoffmann & Turelli, 1988), but this has not been confirmed, or only very rarely, in the field (Hoffmann *et al.*, 1998).

Vertical transmission could be of variable efficiency, as has been demonstrated in different species of *Trichogramma*. The efficiency of the vertical transmission may be related to the degree of infestation of the species or population considered. In *Trichogramma*, many populations are polymorphic for the *Wolbachia* infection. The perfect transmission of *Wolbachia* observed in *T. cordubensis* may explain, at least partly, the complete thelytoky characterizing this species, and an imperfect transmission may explain the partial thelytoky of *T. evanescens* and *T. pretiosum* (Pintureau *et al.*, 1998). In the woodlouse *Armadillidium vulgare*, *Wolbachia* inducing feminization of genetic males are transmitted to most of the offspring (about 90% on average). The rates of transmission are different in female biased progeny and male biased progeny (Rigaud & Juchault, 1992; Rigaud *et al.*, 1999b).

2.1.2. Horizontal natural transmission

The comparison of both symbiont and host phylogenies reveals the existence of natural horizontal transmission. Several cases where the host phylogeny is not congruent with the bacterial phylogeny suggest that horizontal transfers have occurred (O'Neill *et al.*, 1992; Schilthuizen & Stouthamer, 1997).

Intertaxon horizontal transmission of *Wolbachia* was clearly demonstrated by studying *ftsZ* gene phylogeny in A, as well as in B, supergroups of *Wolbachia* (see Zhou *et al.*, 1998, for the supergroup definition). The transmission between parasitoids and their hosts is a possible exchange mechanism, as shown between *Nasonia* and *Protocalliphora*: both host and parasitoid exhibit very similar *Wolbachia* (Werren *et al.*, 1995b). Natural horizontal transmission was demonstrated in another host-parasitoid couple: from infected *Drosophila simulans* to its parasitoid wasp *Leptopilina boulardi* (Heath *et al.*, 1999). Vavre *et al.* (1999a) gave five examples of very high similarities between *Wolbachia* from *Drosophila* hosts and their parasitoids.

In addition to these indirect observations, direct evidence of natural horizontal transfer was found recently, through multiple parasitism implicating strains which do or do not harbour *Wolbachia*. Horizontal transmission of parthenogenesis-inducing *Wolbachia* from infected to uninfected lines, with expression of parthenogenesis, was obtained in *Trichogramma kaykai* sharing a common host egg (*Trichoplusia ni*) (Huigens *et al.*, 2000). This transfer could be explained by the good adaptation that exists between parasitoid species and *Wolbachia*. Transfer attempts by food between

different species of *Trichogramma* have been unsuccessful so far, and thus the existence of wounds (for example after a larval fight) could explain this horizontal transmission. Indeed, in woodlice, hemolymph contact as a result of wounds is sufficient to obtain horizontal transfer (Rigaud & Juchault, 1995). This suggests that transfers by this means are possible in the wild, and this strengthens the possibility of transmissions in host-parasitoid complexes following contact between parasitoids and infected host fluids.

The detection of *Wolbachia* of the A supergroup (mostly encountered in insects) in the golden orb-weaving spider *Nephila clavata* indicates that the bacteria was probably horizontally transferred from insects to the spider via predator-prey relationships (Oh *et al.*, 2000). If this assertion is confirmed, the transfer would also probably necessitate wounds and contacts because transfer through the intact digestive tract is unlikely.

However, in another group of species of Trichogrammatidae parasitoids, no evidence has been found for transmission between host and parasitoids (Schilthuizen & Stouthamer, 1997). In the same way, the parasitoids *Torymus bedeguaris* and *Habrocytus bedeguaris*, and their common host *Diplolepis rosae*, carry three different *Wolbachia* indicating that the parasitoids have not acquired their symbionts from their host (Schilthuizen & Stouthamer, 1998).

The prevalence of *Wolbachia* in arthropod populations is often high, and *Wolbachia* host range and frequency are probably more widespread than previously suspected (Jeyaprakash & Hoy, 2000). This suggests that horizontal transmissions between individuals in the same population/species and between species are an essential component in the maintenance and distribution of *Wolbachia* in arthropod populations (Rigaud & Rousset, 1996; Werren & Windsor, 2000).

2.1.3. Horizontal artificial transfer

Experimental transfers of *Wolbachia* between species or taxons, quite phylogenetically distant, were initially attempted to give a better understanding of the mode of action of the symbionts on their hosts, and secondly to try to separate the role of the symbiont itself on the phenotype induction from its interaction with the host genome. All the experiments carried out so far have demonstrated that the transfers could be obtained, in many cases, implicating isopods, Diptera and Hymenoptera. Successes or failures of these transfers depends on several factors (phylogenetic closeness between the native and the new hosts, expression of a phenotype in the new host, etc.) (e.g. Boyle *et al.*, 1993; Braig *et al.*, 1994; Rigaud & Juchault, 1995; Meer & Stouthamer, 1999).

An example can be used to illustrate these findings. *Wolbachia* were successfully transferred from *Trichogramma pretiosum* to *T. dendrolimi* (a population deprived of *Wolbachia*) by microinjection of symbiont extract into pupae grown on an artificial diet (Grenier *et al.*, 1998). Following these transfers between *Trichogramma* species, the study of the dynamics of the *Wolbachia* population revealed differences between the transfected lines and the naturally infected species. In transfected lines, the eggs are polymorphic for the infection and the *Wolbachia* symbionts are scattered in the ooplasm, contrary to naturally infected species. Even if the *Wolbachia* infection could persist over 40 generations in *T. dendrolimi*, the genetic background of the recipient population naturally deprived of *Wolbachia* symbionts could explain the difficulty for the introduced *Wolbachia* to become established as a permanent symbiont effective in parthenogenesis induction. Therefore, the necessity of a co-adaptation between

Wolbachia and their natural hosts may reduce the possibilities of natural horizontal transfers (Pintureau *et al.*, 2000b).

2.2. EVOLUTIONARY ASPECTS: ADVANTAGES OF INCREASING THE PROPORTION OF FEMALES IN HOSTS

The pre-eminence of vertical/horizontal transmission of the symbionts could explain different evolutive strategies in host exploitation. A significant horizontal transmission would eventually lead to an increase in the virulence of the symbiont (Lipsitch *et al.*, 1996), i.e. a strategy favouring the pathogenicity of *Wolbachia* to the host. Strict vertical transmission would lead to mutualism with a good co-adaptation of the two partners (Fine, 1975) or to reproductive parasitism (Werren & O'Neill, 1997). Coexistence of both vertical and horizontal transmissions might characterize a mixed strategy, with the possibility of promoting one or the other according to the environment (Agnew & Koella, 1997), but no example has been extensively documented so far. *Wolbachia* possesses a day-to-day vertical transmission, but can be horizontally-transmitted on an evolutionary scale. So this provides the symbiont with the possibility of using mixed strategies to exploit its host. Indeed, exploitation strategies are variable, but the more original amongst them probably concern sex ratio distortion (see Hurst, 1993, for a review).

Vertically-inherited symbionts are often transmitted by the female hosts only (Buchner, 1965; O'Neill *et al.*, 1997). Thus, these symbionts will gain in fitness in favouring the females they infect relative to those they do not infect, but they have no evolutionary interest in favouring the males where they live because they will not directly improve their fitness. Ideally, such a symbiont is typically a selfish cytoplasmic element (Werren *et al.*, 1988) that should be beneficial to the female hosts they infect but not to the males.

In the case of feminization and parthenogenesis-induction, the advantage for the symbiont is obvious: by forcing an increase in the proportion of infected females, the symbiont increases its probability of transmission and survival in the host population. In fact, while the infected females produce more daughters on average than uninfected females, the prevalence of the infection will increase in the population (Bull, 1983; Werren, 1987; Taylor, 1990; Hatcher & Dunn, 1995; Stouthamer, 1997). This is a demographic advantage for the symbiont.

In the case of male-killing symbionts, the advantage is less obvious because the symbiont induces an increase in the proportion of females in infected female progenies, but not an increase in the total number of daughters relative to uninfected females. Male-killing symbionts occur mainly in species where intra-sibling cannibalism exists, this behaviour providing a fitness gain for offspring that eat their siblings (Hurst *et al.*, 1997). By killing male embryos, symbionts, in a way, provide a first meal to the offspring that will emerge, i.e. the females. As the rate of cannibalism is stronger in broods where male-killing is expressed, compared with broods without male-killing (the dead embryos can be easily exploited as a food resource), the fitness of females from infected mothers will be higher relative to those of uninfected mothers (Hurst *et al.*, 1997). Other advantages invoked for male-killing symbionts are the avoidance of

inbreeding depression and the avoidance of competition for food or antagonistic interactions (Skinner, 1985). All deal with the fact that male-killing provides an indirect advantage to infected vs. uninfected females.

These arguments, and the conventional population genetics theory, therefore predict the spread of sex ratio distorting symbionts in populations (e.g. Taylor, 1990; Hurst, 1991; Stouthamer, 1997). But there is more evidence of populations where these symbionts are not fixed, and where they persist at relatively low frequencies (see Hatcher, 2000, for a review). Several processes might explain such a prevalence, including structuring of the host populations and reaction of the host genome.

3. Targets of *Wolbachia* to increase the proportion of females in their host population

3.1. SEX DETERMINATION AND DIFFERENTIATION IN ARTHROPODS

3.1.1. Chromosome determination

In insects. Insects are typically gonochoristic with bisexual reproduction. However parthenogenesis is a frequent phenomenon (cf. § 3.2.1). Apart from autosomes, most insects show a karyotype with heterochromosomes or sex chromosomes, different from one sex to another. We will consider only gonochoristic insects, excluding hermaphroditism and parthenogenesis. The mechanisms involve simple or multiple sex chromosomes. Basically, the XX-XY (or ZW-ZZ) system is involved: one heterochromosome is present in both sexes while the second is present only in one sex. Either the male or the female could be the heterogametic (or digametic) sex. The males are heterogametic in Diptera (XY), the females being homogametic (XX). The Y chromosome is sometimes lacking in several orders, such as Odonata, Orthoptera, various Heteroptera and Coleoptera, the males being XO. In Lepidoptera, the females are heterogametic (ZW or ZO) and the males homogametic (ZZ). Several more complicated situations are observed with more than two sexual chromosomes, such as XXY, XYY, in the same or in other insect orders. In some Heteroptera and Homoptera, we can even find $2AX_1X_2X_3X_4X_5X_6$ in the male, and $2AX_1X_1X_2X_2 \dots X_6X_6$ in the female (Laugé, 1985).

In acari (mites and ticks). Similar mechanisms to those described in insects are generally involved, i.e. simple and multiple sex chromosomes. XX females and XY or XO males are systems present in many species. A few species possess multiple sex chromosomes, such as $X_1X_1X_2X_2$ in females and X_1X_2Y in males. Parthenogenesis may be of various types: arrhenotokous, thelytokous, and deuterotokous (Oliver, 1977).

In Crustacea. Although many studies have shown that the classical heterogametic system for sex determination exists in crustaceans (both XX/XY and WZ/ZZ systems are found, see Legrand *et al.*, 1987, and Juchault & Rigaud, 1995, for reviews), the morphological evidence for differentiated sexual chromosomes is very rare in this arthropod group. This is in part due to the fact that chromosomes are very numerous and very tiny in most crustaceans (Lécher *et al.*, 1995) and are therefore difficult to study.

More importantly, sex chromosomes are often at an incipient stage of differentiation in crustaceans (see Rocchi *et al.*, 1984, and Juchault & Rigaud, 1995, for discussions). This is supported by the fact that YY or WW individuals obtained by experimental manipulations are viable and fertile in numerous species (Legrand *et al.*, 1987). Most of the heteromorphism of sex chromosomes found in crustaceans results more from chromosomal rearrangements rather than from chromosomal differentiation (e.g. Staiger & Bocquet, 1954). Systems involving complex sex chromosome composition also exist (Legrand *et al.*, 1987).

In other arthropods. In araneids (spiders), the female sex is homogametic with the sex chromosomes $X_1X_1X_2X_2$, the male being X_1OX_2O . Few data are available on the sex chromosomes of other groups, such as scorpions or millipedes.

3.1.2. Determinism by the ploidy

In haplodiploid insects (Hymenoptera, Thysanoptera, rare Homoptera and Coleoptera) and mites (some Acari: Dermanyssina), fertilized eggs develop as diploid females and unfertilized eggs develop as haploid males. In pseudohaploid or parahaploid insects (some Homoptera) or mites (some Acari: Dermanyssina), males and females come from fertilized eggs but the loss of half of the chromosome set occurs in males (Hoy, 1979; Jong *et al.*, 1981; Laugé, 1985).

The multiple locus model (Snell, 1935), the first model proposed to explain sex determination, hypothesized that sex was determined by several loci, each with several alleles. If an individual is heterozygous at one of these loci, it develops as female, if it is homozygous at each locus or hemizygous, it develops as male. The multiple allele model (Whiting, 1940) or single-locus model assumes only one sex locus with several alleles. A female is a heterozygous individual and a male a homozygous or hemizygous individual, as in the Hymenoptera species of the genera *Habrobracon*, *Apis* and *Diadromus* (Cook, 1993).

The genetic balance theory (Cuhna & Kerr, 1957) hypothesized that a series of maleness genes (M) and a series of femaleness genes (F) determine sex, and that $2F > M > F$ in such a way that diploid individuals develop as females and haploid individuals develop as males. The nucleo-cytoplasmic balance model (Crozier, 1971) proposes that sex is determined by the ratio affected by the ploidy level “amount of products of particular nuclear genes / amount of products of particular cytoplasmic genes”. A new theory, the imprinting hypothesis, was described in 1992 (summarized by Cook & Crozier, 1995). It assumes that females imprint the sex loci, inactivating a product in unfertilized eggs which develop as males, and that males do not imprint so the sex loci are active in fertilized eggs which develop as females.

Nevertheless, diploid males detected in numerous species (Stouthamer *et al.*, 1992) reinforce the single-locus model. Inbreeding does not lead to such diploid males in all species, and the multiple locus model is also probably often involved. Whatever the number of sex loci, it is possible to hypothesize one or some duplications of these loci with a fixed mutation on one duplicated locus, and a variability of the gene product without heterozygosity. In such a case, a diploid individual, homozygous or heterozygous, would develop as female and a haploid individual would develop as male.

3.1.3. Sexual differentiation

Instead of directly manipulating the chromosomes, endosymbionts can manipulate the sex differentiation of their hosts at any stage between the genetic information and its translation in effective sex differentiation. However, it is hard to imagine that the symbiont genome possesses all the information necessary to actively induce the whole pathway leading to the differentiation of its host sex. In all cases where sex determination and differentiation have been studied in detail, the ultimate molecules that allow an individual to differentiate into male or female are issued from a long and complex control cascade between activator and repressor genes or gene products (Marin & Baker, 1998). The symbionts might therefore, in theory, disrupt one of these steps in the sex-determining pathway to reverse an individual genetically programmed to differentiate into one sex towards the other sex. However, all sex-determining patterns are not susceptible to this manipulation (Rigaud, 1997; Rigaud *et al.*, 1997). For example, cell-to-cell sex determination requires the symbionts to be present in all cells of its host to completely reverse its sex, a condition that is rarely fulfilled (otherwise a mosaic of male and female tissues would be created, probably non-functional). Sex differentiation via circulating hormones, as found in vertebrates and in crustaceans, is much easier to disturb since the symbiont can fulfil one "simple" task, i.e. preventing the information from reaching its targets. Whatever the process, this manipulation needs to occur within the early steps of sex determination or differentiation to avoid incomplete gonadogenesis that would lead to a sterile intersex individual.

3.2. TARGET REACHED BY WOLBACHIA

3.2.1. Effect on chromosomes

In many haplodiploid species, especially Hymenoptera species (Table 1), *Wolbachia* of the B supergroup induce thelytokous reproduction, i.e. the production of daughters without fertilization, and thus the restoration of diploidy. Induction of thelytoky by *Wolbachia* is also suspected in some species not listed in table 1: Hymenoptera species, including *Trichogramma agrotidis*, *T. daumalae*, *T. dendrolimi* (Pintureau, 1994) and *T. pintoi* (Zhang S.-Y., Trichogramma News Nr. 5.), and Thysanoptera species (Pintureau *et al.*, 1999).

The symbiont acts on the behaviour of the chromosomes. During anaphase of the first mitotic division, the two sets of chromosomes do not separate and the number of chromosomes is doubled (Stouthamer & Kazmer, 1994). The resulting diploid cell contains two copies of the same set of chromosomes and is entirely homozygous. The subsequent mitotic divisions are normal.

Complete homozygosity in females produced by *Wolbachia*-associated thelytoky led to re-examination of the theories of sex determination. The multiple locus model and the single-locus model became unsuitable (Stouthamer & Kazmer, 1994; Pintureau, 1997). The genetic balance theory, the nucleo-cytoplasmic balance model or the imprinting hypothesis could be reinforced, although the multiple or single-locus models, the best established theories in bisexual Hymenoptera, could be adjusted. A *Wolbachia* DNA fragment could be incorporated in the host cytoplasm where it could mime a sex gene and induce heterozygosity. According to this hypothesis, *Wolbachia* would act not only

TABLE 1. Insect species infected by *Wolbachia* associated with thelytokous parthenogenesis

Order	Super family	Family	Species	References
Coleoptera	Curculionoidea	Curculionidae	<i>Naupactus tesselatus</i>	Werren <i>et al.</i> , 1995b
Hymenoptera	Chalcidoidea	Aphelinidae	<i>Aphytis diaspidis</i>	Zchori-Fein <i>et al.</i> , 1995
			<i>A. lingnanensis</i>	Zchori-Fein <i>et al.</i> , 1994
			<i>A. yanananensis</i>	Werren <i>et al.</i> , 1995b
			<i>Encarsia formosa</i>	Zchori-Fein <i>et al.</i> , 1992
		Encyrtidae	<i>Apoanagyrus diversicornis</i>	Pijls <i>et al.</i> , 1995
		Pteromalidae	<i>Muscidifurax uniraptor</i>	Stouthamer <i>et al.</i> , 1993
		Trichogrammatidae	<i>Spalangia fuscipes</i>	Werren <i>et al.</i> , 1995b
			<i>Trichogramma brevicapillum</i>	Stouthamer & Werren, 1993
			<i>T. chilonis</i>	Stouthamer <i>et al.</i> , 1990
			<i>T. cordubensis</i>	Rousset <i>et al.</i> , 1992
			<i>T. deion</i>	Stouthamer <i>et al.</i> , 1990
			<i>T. dianae</i>	Wang & Smith, 1996
			<i>T. embryophagum</i>	Pintureau <i>et al.</i> , 2000a
			<i>T. evanescens</i>	Stouthamer & Werren, 1993
			<i>T. kaykai</i>	Schilthuizen <i>et al.</i> , 1998
			<i>T. nubilale</i>	Schilthuizen & Stouthamer, 1997
			<i>T. oleae</i>	Rousset <i>et al.</i> , 1992
			<i>T. platneri</i>	Stouthamer <i>et al.</i> , 1990
			<i>T. pretiosum</i>	"
			<i>T. semblidis</i>	Pintureau <i>et al.</i> , 2000a
			<i>T. sibericum</i>	Schilthuizen & Stouthamer, 1997
Cynipoidea	Cynipidae		<i>Diplolepis rosae</i>	Meer <i>et al.</i> , 1995
	Eucoilidae		<i>Leptopilina australis</i>	Werren <i>et al.</i> , 1995b
			<i>L. clavipes</i>	"
Proctotrupoidea	Platygasteridae		<i>Amitus fuscipennis</i>	Meer <i>et al.</i> , 1999
	Scelionidae		<i>Telenomus nawai</i>	Arakaki <i>et al.</i> , 2000

on diploidization but also as a sex allele in diploid individuals (Pintureau, 1997). Moreover, if the above-mentioned theory assuming a duplication is verified, it is unnecessary to hypothesize an action of *Wolbachia* as a sex allele.

3.2.2. Effect on embryo viability

In *Adalia bipunctata* (Col.: Coccidellidae), *Tribolium madens* (Col.: Tenebrionidae), *Acraea encedon* and *A. encedana* (Lep.: Nymphalidae), *Wolbachia* induce early male-killing (Majerus & Hurst, 1997; Hurst *et al.*, 1999; Fialho & Stevens, 2000; Jiggins *et al.*, 2000; Majerus *et al.*, 2000). The symbiont kills male but not female hosts during embryogenesis and increases the probabilities of viability of female embryos and larvae. Here, the target of *Wolbachia* is probably also the sex chromosomes. To discriminate between male and female embryos, the symbiont might use dosage compensation and selectively disrupt mitosis during the early development. However, the fact that the system of chromosomal sex determination is different in Coleoptera (heterogametic males) and Lepidoptera (homogametic males) does not favour the hypothesis of a unique mode of action in male-killing.

3.2.3. Effects on sex differentiation

Sex differentiation targets have been reached by *Wolbachia* endosymbionts in numerous isopod crustaceans (Martin *et al.*, 1973; Juchault *et al.*, 1993; Bouchon *et al.*, 1998), and probably in some insect species. Table 2 gives some examples of feminization. In Insects, a feminizing effect associated with microorganisms has only been detected in the Lepidoptera Pyralidae *Ostrinia furnacalis* (Kagayama *et al.*, 1998) and *O. scapulalis* (Sasaki *et al.*, comm. at the XXI Int. Cong. Entomol.). While the mechanism of this feminization is unclear in insects, the main target reached by *Wolbachia* in crustaceans appears to be the androgenic gland (AG) and its product. This gland is critical in sex differentiation in crustaceans (Charniaux-Cotton & Payen, 1985). During its differentiation under the influence of male sex-determining trigger genes it produces a circulating androgenic hormone (AH), which induces the male differentiation of gonads and other sexual characters. In crustaceans, this hormone is a glycosylated protein (Martin *et al.*, 1999). In the absence of the hormone, i.e. when the gland does not differentiate, the female phenotype and the ensuing physiology are "autodifferentiated" (Charniaux-Cotton & Payen, 1985). In fact, it appears that both males and females possess the gene set necessary for the differentiation of either sex (Legrand *et al.*, 1987). This offers, to inherited symbionts, the opportunity to simply disrupt the sex differentiation cascade: the avoidance of AG differentiation, or AH activity, will initiate female development. *Wolbachia* endosymbionts can act at both levels to feminize genetic males in isopods. First, they inhibit the development of the AG: infected males of the host species *Armadillidium vulgare* do not show any trace of AG differentiation, and are similar morphologically and physiologically to "normal" genetic females. It therefore seems that *Wolbachia* are able to disrupt, by an unknown molecular mechanism, the cascade leading to AG differentiation.

A second level of action, found in some woodlice species, was expressed when the inhibition of AG differentiation did not occur. If, for several reasons discussed by Rigaud & Juchault (1998), the first level of symbiont action is avoided, an initiation of male differentiation can occur. This male phase is nevertheless short in these

TABLE 2. Prevalence of *Wolbachia* endosymbionts with a feminizing effect (F), or an unknown (?) but putative feminizing effect, in host populations. Only the populations known to be infected are listed here, but populations with no infection exist in most infected species (e.g. in Bouchon *et al.*, 1998)

Host species	Prevalence of <i>Wolbachia</i> infection (<i>N</i> populations with <i>Wolbachia</i> infection; <i>N</i> individuals tested)	Effect of <i>Wolba-</i>	References
INSECT			
<i>Ostrinia furnacalis</i>	0.23 (1; 13)	F	Kageyama <i>et al.</i> , 1998
CRUSTACEANS			
<i>Armadillidium album</i>	0.75 (1; 8)	F	Bouchon <i>et al.</i> , 1998
<i>A. nasatum</i>	0.17 – 0.21 (2; 25)	F	"
<i>A. vulgare</i>	0.06 – 0.67 (11; 467)	F	Juchault <i>et al.</i> , 1993; Rigaud <i>et al.</i> , 1999a
<i>Chaetophiloscia elongata</i>	0.33 - 1.0 (2; 19)	F	Juchault <i>et al.</i> , 1994; Bouchon <i>et al.</i> , 1998
<i>Haploftalmus danicus</i>	0.2 (1; 5)	?	Bouchon <i>et al.</i> , 1998
<i>Helleria brevicornis</i>	0.14 – 0.25 (2; 11)	?	"
<i>Ligia oceanica</i>	0.5 (3; 15)	F	"
<i>Oniscus asellus</i>	0.25 - 0.46 (3; 88)	F	Rigaud <i>et al.</i> , 1999b; Bouchon <i>et al.</i> , 1998
<i>O. lusitanus</i>	0.07 (1; 15)	?	Bouchon <i>et al.</i> , 1998
<i>Philoscia muscorum</i>	0.18 – 1.0 (3; 46)	F	Bouchon <i>et al.</i> , 1998; Moreau & Rigaud, 2000
<i>Porcellio dispar</i>	0.29 (1; 7)	?	Bouchon <i>et al.</i> , 1998
<i>P. scaber</i>	0.25 – 0.50 (6; 31)	?	"
<i>P. spinicornis</i>	0.33 (1; 3)	?	"
<i>P. variabilis</i>	0.66 (1; 3)	?	"
<i>Porcellionides pruinosus</i>	0.4 – 1.0 (7; 66)	F	Marcadé <i>et al.</i> , 1999

Wolbachia-infected individuals, and a female physiology rapidly develops. This often results in a sterile individual possessing characteristics of both sexes, with gonads showing intermediate stages between testes and ovaries (Legrand *et al.*, 1987). These individuals nevertheless produce AH, but the hormone seems to be unable to reach its targets. It was therefore proposed that *Wolbachia* inhibit the accessibility of the AH to its receptors (Juchault & Legrand, 1985). In some species, this "physiologic" effect does not exist, showing that the two *Wolbachia* effects evolved independently and suggesting that the second effect could have been selected after the direct effect, to ensure the microbe of a more complete feminizing effect (Rigaud *et al.*, 1999b).

3.3. POTENTIAL TARGETS FOR WOLBACHIA

3.3.1. Effect on chromosomes, sperm competition or embryos

Numerous other potential targets could be considered for the symbiont in order to increase the proportion of females in their hosts (Hurst *et al.*, 1997). In species with sex chromosomes, *Wolbachia* could increase the female proportion by suppressing meiosis and inducing thelytokous parthenogenesis. In this case, an infected female XX or WZ would produce oocytes XX or WZ able to develop. In haplodiploid species, diploidization could also be achieved by suppressing meiosis. These chromosome manipulations are not unrealistic to invoke, since *Wolbachia* are known to manipulate chromosomes during the early events of mitosis (see 3.2.1, and Chapter by Charlat *et al.* in this book).

It is also possible that *Wolbachia* eliminate one chromosome during meiosis. A sex chromosome suppression could lead to inactivation of Y sperm and production of only X sperm. The inactivation of Z oocytes has less chance of occurring since it would induce a high decrease in fecundity. A similar phenomenon of offspring loss, however, occurs in the case of male-killing. On the other hand, a production limited to W oocytes is possible by meiotic drive (Cazemajor *et al.*, 2000), i.e. the Z drive to the polar body. Gamete elimination, drive of sex chromosomes and further hypotheses could not induce thelytoky and mating, thus the presence of males is necessary. A state of equilibrium between infected and non-infected individuals has to be preserved.

Wolbachia could also influence the sperm competition or the maternal choice among sperm, or give an advantage to the X sperm leading to a differential fertilization. The symbiont cannot alter the WZ system in this way.

Other phenomena of early male-killing or alteration of male fitness should finally be considered: the mortality of haploid embryos in haplodiploid species, the weakening of viability in more or less young males (i.e. late male-killing), etc. These latter phenomena have been found to be induced by intracellular parasites in various species, but never associated with *Wolbachia* endosymbionts (e.g. Gherna *et al.*, 1991; Hurst, 1991; Hurst *et al.*, 1997).

3.3.2. Effects on reproductive physiology or behaviour

Another possibility for a symbiont to increase the female proportion in haplodiploid (arrhenotokous) hosts, in addition to chromosome manipulations, is to increase the fertilisation frequency in infected females. This has been described in *Nasonia*

vitripennis, where an as yet unknown "cytoplasmic factor" induces the production of more than 90% of fertilised eggs (Skinner, 1982). This phenotype, however, has not been found related either to *Wolbachia* or to any other bacterial symbiont or parasite, and the pathway explaining this phenomenon is also unknown. Among other possibilities, this could occur through a symbiont-induced physiological or behavioural manipulation of the female, or a modification of her means of perceiving her environment. The symbiont may affect the nervous system of the females, and make them unable to perceive parameters necessary for selecting the optimal proportion of males vs. females. Given the rate of *Wolbachia* infection in haplodiploid arthropods, and the wide adaptive potential of these endosymbionts (Rigaud, 1999), it is likely that they could induce effects on reproductive physiology not yet discovered.

4. Conclusion

Several "cytoplasmic agents" or microorganisms have a similar effect as *Wolbachia* on the host. In crustacean amphipods, microsporidia and paramixidia endoparasites are responsible for feminization, in a way similar to that found in isopods (Ginsburger-Vogel & Desportes, 1979; Dunn *et al.*, 1993; Terry *et al.*, 1999). In insects, bacteria of the genus *Arsenophonus* (in *Nasonia vitripennis*, Gherna *et al.*, 1991), *Spiroplasma* (in *Drosophila willistoni*, Williamson & Poulsen, 1979), *Rickettsia*, or *Flavobacteria* (in coccinellids, Hurst *et al.*, 1996, 1997) induce male-killing phenomenon. However, as far as we know, only *Wolbachia* seems able to induce thelytokous reproduction.

As we suggested, there are many targets that could be reached by *Wolbachia*, and to which the symbiont could become adapted. We suspect that our knowledge of these potential adaptations is, at present, limited by the number of biological systems that have been extensively and carefully investigated so far.

The control of presence vs. absence of *Wolbachia*, associated with investigations on their effects, in populations of beneficial arthropods used in biological control strategies is of prime importance, especially in predicting the possible negative effects of CI on the population dynamics. For example, the occurrence of *Wolbachia* causing CI in some populations of *Cotesia sesamiae*, a braconid parasitoid of lepidopterous stem borers, in a restricted region of east Africa may have a negative impact on the biological control of cereal stem borers in this region (Ngi-Song & Overholt, comm. at the XXI Int. Cong. Entomol.). *Wolbachia* also have the potential to be beneficial agents by themselves, directly or indirectly. *Wolbachia* could be proposed as a potential drive agent for inserting "selected" genes into populations (Turelli & Hoffmann, 1999). *Wolbachia* inducing parthenogenesis is a potential agent for increasing the efficiency of insects used in biological control, e.g. of *Trichogramma* species, when transferred to bisexual species. A higher rate of females would reduce the effective cost of production and improve the efficacy in the field.

The presence of *Wolbachia* has also been noted in some predator insects, such as *Orius* bug (Miura *et al.*, comm. at the XXI Int. Cong. Entomol.) and coccinellids (Majerus *et al.*, 2000) used in biological control strategies. Neither negative nor beneficial effects were hypothesized concerning the role of the symbionts in the predation efficiency.

Finally, another potentiality of *Wolbachia* biology remains to be investigated. *Wolbachia* occur in blood-sucking insects. They have been detected in three tse-tse flies of the genus *Glossina*, associated with two other symbiotic microorganisms (Cheng *et al.*, 2000), and in the sand fly *Phlebotomus papatasi* (Cui *et al.*, 1999). It would be interesting to test whether the symbionts could be transmitted to the mammal hosts. *Wolbachia* belong to the Rickettsiaceae and are closely related to the genus *Rickettsia* (O'Neill *et al.*, 1992), in which many species are agents of diseases sometimes transmitted to people. Day after day the range of hosts for *Wolbachia* increases, so why not a vertebrate or even a mammal as host?

5. References

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GENOME OF *BUCHNERA* SP. APS, AN INTRACELLULAR SYMBIOTIC BACTERIUM OF THE PEA APHID *ACYRTHOSIPHON PISUM*

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1. Introduction

Most, if not all, aphid species (Homoptera, Insecta) contain intracellular bacterial symbionts, called *Buchnera*, in the bacteriocyte (Buchner, 1965). These aphids contain several tens of bacteriocytes in the fat body, whose cytoplasm are packed with the *Buchnera* symbionts. *Buchnera* are Gram-negative bacteria 1-2 :m in diameter and belong to the 3 subdivision of the Proteobacteria, closely related to *Escherichia coli* (Munson *et al.*, 1991). They are maternally transmitted to eggs and parthenogenetic embryos of aphids through host's generations (Baumann *et al.*, 1995). Phylogenetic analysis, based on 16S rDNA sequence of *Buchnera* from numerous aphid species, has revealed that the symbiotic relationship was established 200-250 million years ago and led to cospeciation of the hosts and their symbionts (Moran *et al.*, 1993). In the course of cospeciation, the two developed intimate genetic and physiological interactions, and the association became so obligate that neither partner could reproduce independently (Houk and Griffiths, 1980; Ishikawa, 1989).

Aphids feed exclusively on phloem sap of plants, which is rich in carbohydrates but poor in nitrogenous and other compounds. This explains why aphids cannot reproduce independently of *Buchnera*. One important role of *Buchnera* is the supplementation of the aphids' diet. There is now strong evidence that *Buchnera* provide essential amino acids (Sasaki and Ishikawa, 1995; Douglas, 1998) and riboflavin (Nakabachi and Ishikawa, 1999) to host insects. Conversely, the fact that *Buchnera* cannot reproduce independently is most likely associated with changes of their genome because of their long symbiotic relationship with aphids. In an effort to look into the evolutionary changes from free-living bacteria to intracellular symbionts, we recently examined the *Buchnera* genome in terms of its size, copy number, and gene repertoire.

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2. Genome Size of *Buchnera*

The endosymbiosis hypothesis by Margulis (1970) lays stress on a dramatic reduction of the genome size of bacteria that have eventually become eukaryotic cell organelles, such as mitochondria and chloroplasts. It is needless to say that many lines of evidence now support this hypothesis. *Buchnera*, in a sense, resemble these organelles in that a eukaryotic cell harbors them, and that they are essential to the host. This tempted us to estimate the genome size of *Buchnera* at an early stage of the study on this symbiosis. In 1987, we estimated the genome size of *Buchnera* sp. APS to be about five times as large as that of *E. coli* (Ishikawa, 1987). At that time, the estimation of the genome size was performed based on re-association kinetics of denatured DNA fragments, which is an indirect method notoriously liable to give erroneous results due to contamination by foreign nucleic acids and proteins. Thus, to re-examine this previous result, we estimated the genome size using pulse-field gel electrophoresis (PFGE), a direct method (Charles and Ishikawa, 1999).

In this experiment, we took advantage of the extremely low G+C content of the genomic DNA of *Buchnera* (Ishikawa, 1987), which is probably due to a strong AT pressure (Sueoka, 1988) imposed on most intracellular bacteria (Moran, 1996). When digested with restriction endonucleases with recognition sequences rich in G and C, the *Buchnera* DNA gave rise to fragments whose sizes were suitable for analyses on PFGE. The restriction endonuclease I-CeuI that recognizes the sequence conserved in the *rrl* genes linealized the genomic DNA without fragmentation, suggesting that the genome is a closed circular molecule, and that it contains a single copy of *rrl*, as indicated previously (Baumann *et al.*, 1995). The average genome size, presumed on the sum of the sizes of these restriction fragments, was between 652 and 657 kb. The sequence analysis of the *Buchnera* genome, to be described below, later demonstrated the exact size to be 641 kb. The slight overestimation of the size was probably due to the high A+T content of the *Buchnera* DNA, which interferes with the mobility of large DNA fragments on PFGE (Pyle *et al.*, 1988).

Comparison of sequences of many genes clearly indicates that *Buchnera* are monophyletic with *E. coli* (Clark *et al.*, 1998). The gene arrangement in the *Buchnera* genome was also more similar to that of *E. coli* than to *Haemophilus influenzae* (Charles and Ishikawa, 1999). Consequently, the result that the genome size of *Buchnera* is only a seventh of that of *E. coli* (4.67 Mb) suggests that *Buchnera* have lost many of those genes that they shared with *E. coli* since divergence from the common progenitor of the two, probably in the course of their intracellular life for 200 million years (Moran and Baumann, 1994). Thus, any difference in gene repertoire between *Buchnera* and *E. coli* can be a direct consequence of intracellular symbiosis over 200 million years. By the time we estimated the genome size, more than 100 genes had been identified in the *Buchnera* genome by Southern hybridization and PCR using their *E. coli* homologues as probes and primers (Clark *et al.*, 1998). Although this approach is effective to pick up the genes that have remained in the *Buchnera* genome, it is beyond its scope to reveal those that have been lost from the genome during evolution. Moreover, in view of the genome size of *Buchnera*, the latter must be several times

more in number than the former. With this in mind, we set about the sequence analysis of the *Buchnera* genome, the result of which will be described in a later section.

3. Copy Number of the *Buchnera* Genome

Notwithstanding a dramatic reduction in the genome size of *Buchnera* in comparison with that of *E. coli* (Charles and Ishikawa, 1999), *Buchnera* cells are much larger in volume than *E. coli* cells, and divide only very slowly, compared with free-living bacteria. These observations led us to suspect that, unlike the great majority of bacteria, *Buchnera* may be bacteria with many copies of the genome in a single cell.

First, we estimated copy numbers of two particular genes in the single cell of *Buchnera* by dot-blot analysis (Komaki and Ishikawa, 1999). It is known that, as *E. coli*, *Buchnera* contain a single copy of *groE*, an operon that codes for two molecular chaperones, per genome (Charles and Ishikawa, 1999). We amplified by PCR a 580 bp *groE* fragment of *E. coli* and blotted it quantitatively onto a membrane. We extracted genomic DNA from the same number of *E. coli* cells as the copy number of the *groE* fragment blotted above, and blotted it onto the same membrane. The *groE* fragment labeled with ^{32}P were hybridized with the two membrane-bound DNA samples mentioned above. As a result, approximately the same amount of radioactivity was detected from the two samples, reflecting the occurrence of a single *groE* operon per *E. coli* cell. We performed a similar experiment using a PCR-amplified 580 bp fragment of the *Buchnera groE* and the *Buchnera* genomic DNA. As a result, the radioactivity detected from the genomic DNA was about 100 times greater than that from the *groE* fragment. Since the genomic DNA was extracted from the same number of *Buchnera* cells as that of the *groE* fragment used, this result indicates that each *Buchnera* cell contains about 100 copies of the *groE* operon. A similar dot-blot analysis was performed using 16 rDNA of *E. coli* and *Buchnera*. Whereas the *E. coli* genome contains 7 copies of this sequence, that of *Buchnera* only one. However, the dot-blot analysis indicated that the *Buchnera* cell contains several tens times more copies of 16 rDNA than the *E. coli* cell.

At this stage of experiments, one may well suspect that both *groE* and 16S rDNA are locally amplified as many extrachromosomal elements. To eliminate this possibility, we compared the DNA content of the single cell among *Buchnera*, *E. coli* and *Saccharomyces cerevisiae* by fluorimetry using a video-intensified microscope photon-counting system (VIMPCS) (Kuroiwa *et al.*, 1986). The result provided convincing evidence that in the *Buchnera* cell not particular genes, but the entire genome has been amplified (Figure 1). It was estimated that each cell of *Buchnera* contains an average of 120 genomic copies (Komaki and Ishikawa, 1999). This result was completely confirmed by real-time quantitative PCR, too (Komaki and Ishikawa, 2000).

A dramatic reduction in the genome size (Charles and Ishikawa, 1999) accompanied by an extreme increase in the genomic copy number in *Buchnera* is reminiscent of eukaryotic cell organelles such as mitochondria and chloroplasts (Gray *et al.*, 1999). Loss of the ability to divide outside the eukaryotic cell is also a common attribute of *Buchnera* and these organelles. It is likely that these changes are an inevitable consequence for the genome of prokaryotes that have been confined in the eukaryotic

cytoplasm for an evolutionary length of time. Special restriction may cause prokaryotes to abandon their common attribute of proliferating rapidly by frequent cell division. However, they still retain the ability to replicate their genome once a while, which may

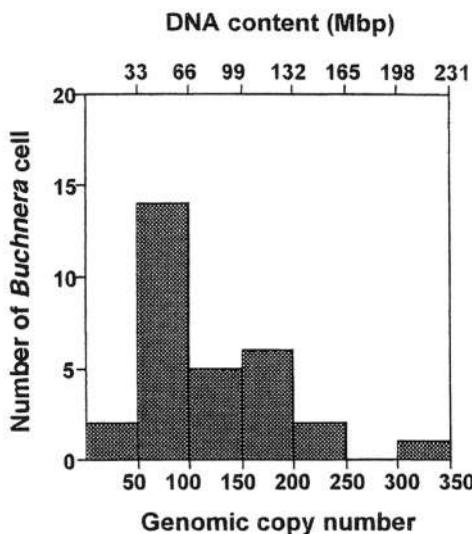


Figure 1. Variation of DNA content and genomic copy number among 30 *Buchnera* cells (Komaki and Ishikawa, 1999). The fluorescence emitted by each *Buchnera* cell was compared with that emitted by a haploid or diploid yeast cell in the same visual field using VIMPCS (Kuroiwa *et al.*, 1986).

account for the high copy number of the genome of intracellular symbionts and cell organelles. Indeed, *Buchnera* cells isolated from the bacteriocyte synthesize DNA vigorously but never divide (Ishikawa, 1982). Both the reduction of the genome size and A+T richness of the genomic DNA (Ishikawa, 1987; Ohtaka and Ishikawa, 1993) will tend to favor repeated replication of DNA.

4. Gene Repertoire of the *Buchnera* Genome

According to the genome size, *Buchnera* have discarded about six sevenths of the genes that they used to share with *E. coli*. In view of this fact, it is a plausible explanation that *Buchnera* cannot reproduce independently of the host because they lost most of the genes essential for an independent life. To test this possibility, we recently determined the genome sequence of *Buchnera* from the pea aphid (Shigenobu *et al.*, 2000). This represents the first genome analysis of an obligatory, intracellular symbiont. Although the small size of the *Buchnera* genome was favorable for such the analysis, a serious disadvantage was that this bacterium cannot be cultured outside the bacteriocyte of aphids. Thus, the only way to obtain pure genomic DNA from *Buchnera* was to dissect

many individuals of aphids manually to free their bacteriocytes, out of which the bacterial cells were purified.

For the genome analysis of *Buchnera*, we dissected about 2,000 pea aphids. The bacteriocytes were crushed by pipetting and subjected to filtration through a 5 m filter to obtain *Buchnera* cells. Genomic DNA was prepared from *Buchnera* cells by a standard phenol/chloroform protocol. Sequencing of the *Buchnera* genome was performed by the whole genome random shotgun sequencing method (Fleischmann *et al.*, 1995). We used two strategies for identifying open reading frames (ORFs). An initial set of ORFs was identified by the GeneHacker program, a system for gene structure prediction using a hidden Markov model (Yada and Hiroswa, 1996). Predicted ORFs were compared against a non-redundant protein database using the BLAST programs. Combining these results, we identified and annotated the ORFs (Shigenobu *et al.*, 2000).

4.1. GENERAL FEATURES OF THE *BUCHNERA* GENOME

The result demonstrated that the genome of *Buchnera* sp. APS from *A. pisum* consists of one circular chromosome and two circular plasmids, pLeu and pTrp, which were identified in a different *Buchnera* species in an earlier work (Lai *et al.*, 1994; Bracho *et al.*, 1995). The chromosome turned out to be 640,681 bp, the smallest of the completely sequenced genomes, except for that of *Mycoplasma genitalium* (Fraser *et al.*, 1995). As predicted, the overall G+C content of the *Buchnera* genome is very low (26.3%). We identified 583 ORFs, whose average size is 988 bp occupying about 88% of the entire genomic sequence. This gene density in the genome is much the same as in that of *E. coli*. There are a single copy of gene for each of the three classes of ribosomal RNA and 32 transfer RNA genes in the *Buchnera* genome. Of interest is that the predicted isoelectric points (pIs) of the products of the ORFs are, on average, much more basic than those of polypeptides of other bacteria. The average pI of *Buchnera* polypeptides is 9.6, whereas that of *E. coli* polypeptides is 7.2. This difference has been already noted for several particular proteins (Sato and Ishikawa, 1997; Matsumoto *et al.*, 1999), and is mostly due to the increased usage of lysine in the *Buchnera* polypeptides, which probably results from the A/T pressure imposed upon the *Buchnera* genome. It is intriguing to know whether the increased pI reflects a consequence of *Buchnera*'s adaptation to the intracellular milieu (Table 1).

Table 1. ORFs in the *Buchnera* genome

Number of ORF	583
Average length	988 BP
Average pI	9.6
Functional category	
Known	500
Unknown but conserved	80
Unique to <i>Buchnera</i>	3

Similarity search permitted the functional assignment of 500 out of the 583 ORFs, and other 79 ORFs were similar to hypothetical proteins deposited for other bacteria. Whereas 4 ORFs seemed to be unique to *Buchnera* at first, recently it turned out that one of them is shared with the plant pathogen *Xylella fastidiosa* (Simpson *et al.*, 2000) and the opportunistic pathogen *Pseudomonas aeruginosa* (Stover *et al.*, 2000). Of the 583 ORFs in the *Buchnera* genome, as many as 569 have orthologs in the *E. coli* genome. Moreover, in general, the most similar counterparts of *Buchnera* proteins are those of *E. coli*, and the gene order in *E. coli* operons is well conserved in *Buchnera*. These findings strongly suggest that the *Buchnera* genome is a subset of the *E. coli* genome, reflecting the evolutionary relationship between the two bacteria.

Buchnera is similar to endocellular and epicellular parasites such as *Rickettsia prowazekii* (Andersson *et al.*, 1998) and *M. genitalium* (Fraser *et al.*, 1995) in that its genome size has been extremely reduced. However, the genes left behind in the *Buchnera* genome are remarkably different from those in the parasites. The parasitic bacteria depend on their hosts for most nutrients, and the reduction of their genome size is, at least partly, due to the loss of genes for biosynthesis of those nutrients. By contrast, in the symbiosis between *Buchnera* and aphids, *Buchnera* is more of a provider than a recipient of biosynthetic products including essential amino acids and vitamins (Sasaki and Ishikawa, 1995; Douglas, 1998; Nakabachi and Ishikawa, 1999).

4.2. MUTUAL DEPENDENCE BETWEEN *BUCHNERA* AND THE HOST AS SEEN IN THE GENE REPERTOIRE

This difference between the symbiont and parasite is typically reflected on the gene repertoire. While parasitic bacteria contain only a few, if any, genes involved in biosynthesis of amino acids, *Buchnera* sp. APS contains 54 genes in this category, which amount to almost 10 % of its total genes. *E. coli* contains about twice as many as genes in this category, and is capable of biosynthesis of all the necessary amino acids. Of these genes, *Buchnera* have selectively lost those for biosynthesis of the non-essential amino acids that animals can synthesize, suggesting that *Buchnera* depend on the host for these nutrients. Instead, unlike parasitic bacteria, *Buchnera* retain virtually all the genes for biosynthesis of the essential amino acids that animals themselves cannot synthesize. Evidence suggests that these genes are expressed (Nakabachi and Ishikawa, 1997). This complementarily and mutual dependence seen in the gene repertoire shows how successfully the symbiosis is operating, in that *Buchnera* provides the host with what the host cannot synthesize, and vice versa.

A similar mutual dependence is predicted for biosynthesis of coenzyme A (CoA). According to the gene repertoire, *Buchnera* can synthesize pantothenate from pyruvate, whereas there are no genes for the pathway from pantothenate to CoA. By contrast, it is generally known that animals are able to produce CoA from pantothenate, but not pantothenate from pyruvate. Despite a dramatic reduction of the gene number, *Buchnera* possess complete gene sets for the sulfur reduction pathway and biosynthesis of cysteine. The host must benefit much from these abilities of *Buchnera*, because insects, in general, cannot reduce sulfate to sulfide. Unlike parasitic bacteria whose genomes have been sequenced to date, *Buchnera* retain almost complete gene sets for

biosyntheses of nucleotides. It is not known whether the pathways are only for *Buchnera*'s own use or not (Table 2).

4.3. ESSENTIAL GENES MISSING FROM *BUCHNERA*

In contrast to relative richness in those genes involved in biosynthesis of nutrients, many genes, including those that seem essential to maintain the identity as a cell, are missing from *Buchnera*.

Buchnera lacks many transporter genes that are shared in common by all bacterial species sequenced to date. This is an unexpected finding because such an intimate association as that between *Buchnera* and the host should necessarily be based on

Table 2. Comparison of gene repertoires among several bacteria

Function	Bu	Ec	Hi	Rp	Mg
Amino acid biosynthesis	55	131	71	6	0
Nucleotide metabolism	34	58	50	14	19
Energy metabolism	51	243	143	67	33
Lipid metabolism	6	48	34	25	8
Transport and binding	18	427	165	38	33
Regulation	7	178	64	14	5
Total genes	583	4289	1709	834	480

Bu, *Buchnera*; Ec, *Escherichia coli*; Hi, *Haemophilus influenzae*.

Rp, *Rickettsia prowazekii*; Mg, *Microplasma genitalium*

frequent exchange of various substances between the two associants. For example, *Buchnera* has only a few genes for the ABC transport system, which is a major class of cellular translocation machinery with many paralogous genes. As for phosphoenolpyruvate-carbohydrate phospho-transferase systems (PTSs), the gene repertoire suggests that *Buchnera* can import glucose and mannitol, which has been confirmed by biochemical studies (Matsumoto *et al.*, 1999). Apart from these transporters, no other substrate-specific transporter genes are not present in the *Buchnera* genome.

Genes for various regulatory systems are almost completely missing from the *Buchnera* genome. Among these are those for two-component regulatory systems that generally control gene expression in response to environmental changes. This can be a reflection of the stable habitat where *Buchnera* live. It is particularly noteworthy that no transcriptional regulator of amino acid biosynthesis is present despite the conservation of many genes for amino acid biosynthesis, as mentioned above. It is probable that, so far as substrates are available, *Buchnera* produce essential amino acids as much as the

host demands them. Genes of *Buchnera* do not have leader sequences, and that *Buchnera* are not provided with a transcriptional attenuation system. It is possible that gene expression of *Buchnera* is not controlled by itself, but by the symbiotic system as a whole.

The gene repertoire of *Buchnera* clearly indicates that this bacterium is of an almost defenseless cell. Although RecA is the most crucial component for the homologous recombination reaction, the recA gene is missing from *Buchnera*. Similarly, in the uvr excision repair system, *Buchnera* lacks *uvrABC*. Taken together with the absence of genes responsible for the SOS system, and those for DNA methylation and restriction, this suggests that the *Buchnera* genome is very vulnerable to DNA damage. Although *Buchnera* are protected in the stable environment by the host cell from DNA damage, they will inevitably accumulate deleterious changes of DNA over years if their defense system is useless in this way. It is possible that the high copy number of the *Buchnera* genome (Komaki and Ishikawa, 1999; see in Section 3) plays a role in eliminating such the damaged DNA.

4.4. HOW DOES *BUCHNERA* MAKE UP FOR MISSING GENES ?

In addition to those mentioned above, numerous genes are missing from the *Buchnera* genome, though their direct or indirect products are obviously present in the *Buchnera* cell. One typical example is the genes for phospholipid biosynthesis. Although phospholipid is an indispensable component in the formation of the membrane lipid bilayer, genes necessary for its biosynthesis are completely missing from *Buchnera*. Possibly, *Buchnera* either imports phospho-lipid from the host or synthesizes it, using relevant enzymes transferred from the host cell, like mitochondria and plastids do.

A more mysterious example is represented by enzymes responsible for respiration. *Buchnera* inhabits the bacteriocyte, which receives an ample supply of oxygen through the trachea and contains numerous mitochondria in the cytoplasm, suggesting that this bacterium respires aerobically. *Buchnera* has complete gene sets for glycolysis and the pentose phosphate pathway, whereas it lacks genes for fermentation and anaerobic respiration. In addition, in the *Buchnera* genome, the NADH dehydrogenase operon, the cytochrome o operon, and an F_0F_1 type ATP synthase operon are conserved. All these findings indicate that *Buchnera* is able to produce ATP using the proton electrochemical gradient generated by the electron transfer system (ETS). Surprisingly, however, the genome of this bacterium does not contain a gene set for operation of the citric acid cycle except genes for the 2-oxoglutarate dehydrogenase complex. Thus, *Buchnera* is the first aerobic organism revealed that does not have genes for the citric acid cycle.

This immediately raises an intriguing question: How does *Buchnera* secure electron donors for ETS, such as NADH? Three possibilities may readily occur to us. First, glycolysis alone may generate much NADH enough to operate ETS in *Buchnera* since the host insect ingests phloem sap that contains an ample amount of carbohydrate. As *Buchnera* is without the ability of fermentation, there will be no other way of regenerating NAD⁺ than oxidation of NADH through ETS. In this case, pyruvate produced by glycolysis may be used for production of amino acids and pantothenate (see in Section 4.2). Second, there may be unknown mechanisms by which *Buchnera* imports electron donors indirectly from mitochondria in the vicinity. Whereas these two

mechanisms assume the lack of the citric acid cycle in *Buchnera*, the third possibility to be considered is based on its presence, and assumes importation of enzymes responsible for the cycle from the host. Implicit in this mechanism is the presumption that the relevant genes have been transferred from *Buchnera* to the host, like in evolution of cell organelles.

In addition to these three possibilities, our previous findings hint that some of other proteins make up for missing enzymes for the citric acid cycle. It is known that symbionin, a GroEL homolog of *Buchnera*, not only is molecular chaperone, but also can function as phosphotransferase (Morioka *et al.*, 1993). Evidence suggests that amino acid substitution at a particular site evolutionarily conferred the GroEL homolog on this new function (Komaki *et al.*, 1996). As a result of this modification, symbionin is able to relay the phosphoryl group in an energy-coupling manner, like sensor molecules in the two-component regulatory system (Morioka *et al.*, 1994). As already mentioned, genes responsible for two-component systems are completely missing from the *Buchnera* genome. It is an interesting possibility that in *Buchnera* symbionin substitutes for the sensor molecule that has been lost during evolution. If such a change of protein is not limited to the GroEL homolog in the course of evolution of *Buchnera*, it is possible that multifunctional proteins, thus generated, may make up for missing gene products of *Buchnera*. Indeed, comparison of nucleotide sequences of various genes among bacteria suggests that the evolution of *Buchnera* genes is distinct from that of free-living bacteria (Shigenobu *et al.*, in preparation).

5. Summary

Aphids contain intracellular bacterial symbionts, *Buchnera*, in their bacteriocytes. *Buchnera* are exceptional bacteria in that they possess many, usually more than 100, genomic copies per cell. Whereas *Buchnera* are phylogenetically close to *Escherichia coli*, their genome size has been reduced to 1/7 of that of *E. coli*. The *Buchnera* genome retains genes necessary for biosynthesis of essential amino acids that the host insect cannot synthesize. Instead, *Buchnera* have lost most of the genes for biosynthesis of non-essential amino acids, and presumably depend on the host for these nutrients. Such mutual dependence is unique to the symbiosis between *Buchnera* and the host, and has never been observed in the association between parasitic bacteria such as *Mycoplasma* and *Rickettsia* and their hosts. Many essential genes are missing from the *Buchnera* genome, most of whose products may be imported from the host. It is also possible that multifunctional proteins unique to *Buchnera* substitute for part of these missing gene products.

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THE FUNCTIONS OF SYMBIOTIC MICRO-ORGANISMS IN INSECTS

A Perspective from Studies on Aposymbiotic Aphids

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1. Introduction

Many species in various insect groups, including cockroaches, aphids, lice and beetles, are absolutely dependent on their association with symbiotic micro-organisms (Buchner 1966; Douglas 1989). The micro-organisms are borne in specialised insect cells, called mycetocytes or bacteriocytes, and are transmitted vertically from the mother insect to her offspring, usually via the cytoplasm of the egg. Although these symbioses have been recognised for about a century, they have received relatively little study in comparison, for example, to nitrogen-fixing symbioses in plants and algal symbioses in corals and allied marine animals. This is because the insect-microbial symbioses are widely perceived as being intractable, linked to the unculturability of most of the microbial partners.

The incidence of microbial symbioses in insects has been related to the metabolic capabilities of animals. The lineage giving rise to most animals was metabolically impoverished, lacking the capacity to synthesize 9 of the 20 amino acids in proteins (known as essential amino acids) and many of the organic coenzymes needed for functioning of enzymes central to metabolism (these are many of the vitamins). Insects additionally cannot synthesize sterols, such as cholesterol which is fundamental to the architecture of membranes. The requirement of insects and other animals for a ‘balanced diet’ is a direct consequence of these biosynthetic limitations. Various animals, however, have bypassed the limitations by forming symbioses with micro-organisms possessing these biosynthetic capabilities (Douglas 1994). The distribution of microbial symbioses in insects is consistent with this scenario. The symbioses are largely restricted to insects feeding on three types of food: vertebrate blood, deficient in B vitamins; plant sap, deficient in essential amino acids; and wood, deficient in amino acids, vitamins and probably sterols (Buchner 1966; Douglas 1989). However, the proposed nutritional function of symbiotic micro-organisms in insects has been explored experimentally in very few systems.

By far the best-studied insect-microbial symbiosis is the association between phloem-feeding aphids and **γ -Proteobacteria** of the genus *Buchnera* (Munson et al. 1991; Baumann et al. 1995; Douglas 1998). Aphids require their complement of *Buchnera* for sustained growth and reproduction (Sasaki et al. 1991; Douglas 1992; Douglas et al.

2001). Current understanding of the function of *Buchnera* derives in large part from research on aposymbiotic insects, i.e. aphids whose symbiotic bacteria have been eliminated experimentally, usually by treatment with antibiotics (Wilkinson 1998). In the usual experimental design, the capabilities of aposymbiotic aphids are compared with untreated aphids bearing *Buchnera*, commonly known as ‘symbiotic aphids’, and the function of the bacteria deduced from the difference between symbiotic and aposymbiotic aphids. However, supplementary experiments are commonly required to identify whether specific differences between aposymbiotic and symbiotic aphids reflect the loss of *Buchnera* or compensatory responses of the insect to the loss of symbiotic bacteria. Otherwise, the results of experiments involving aposymbiotic insects can be misinterpreted.

Approaches to interpret data obtained with aposymbiotic aphids can be illustrated by recent research on amino acid and sugar metabolism in the aphid-*Buchnera* symbiosis, reviewed in this article. These topics were selected for study because amino acids and sugars are the principal nitrogen and carbon compounds, respectively, in the phloem sap of plants.

2. Amino acid metabolism in the aphid-*Buchnera* symbiosis

The amino acids in plant phloem sap are of unbalanced composition for animals (Douglas 1993; Sandström & Pettersson 1994; Sandström & Moran 1999), as can be illustrated by comparison of the amino acids in the black-bean aphid *Aphis fabae* and the phloem sap of one of its host plants, the broad bean *Vicia faba* (Fig. 1). Most of the amino acids in aphids are in the protein fraction, of which the 9 essential amino acids (which animals cannot synthesize) make up 47%; but the phloem sap of *V. faba* is dominated by one non-essential amino acid, asparagine, and has an essential amino acid content of just 16%. For this dataset, every essential amino acid except histidine is at a higher percent concentration in the aphids than in their diet of phloem sap.

One important factor contributing to the capacity of aphids to utilize phloem sap is the provision of supplementary essential amino acids by their symbiotic bacteria. The direct evidence comes principally from metabolic analyses of amino acid biosynthesis by symbiotic and aposymbiotic aphids. Aphids metabolize the nonessential amino acid ¹⁴C-glutamic acid to a variety of amino acids (Fig. 2); both symbiotic and aposymbiotic aphids incorporate radioactivity from ¹⁴C-glutamic acid into nonessential amino acids, including aspartic acid and alanine, but radioactivity is also recovered from the essential amino acids isoleucine, lysine and threonine only in symbiotic aphids (Febvay et al. 1995; Douglas et al. 2001).

The gene content of *Buchnera* provides the strongest support for these physiological studies. The genome of *Buchnera* is much-reduced in size compared to related free-living species (Charles and Ishikawa 1999; Wernegreen et al. 2000). For example, the DNA content of the chromosome of *Buchnera* in the pea aphid [s] *Acyrthosiphon pisum* is 641 kb, less than 20% of that of *Escherichia coli*, and the gene complement of the *Buchnera* has been described as “a subset of the *E. coli* genome” (Shigenobu et al.

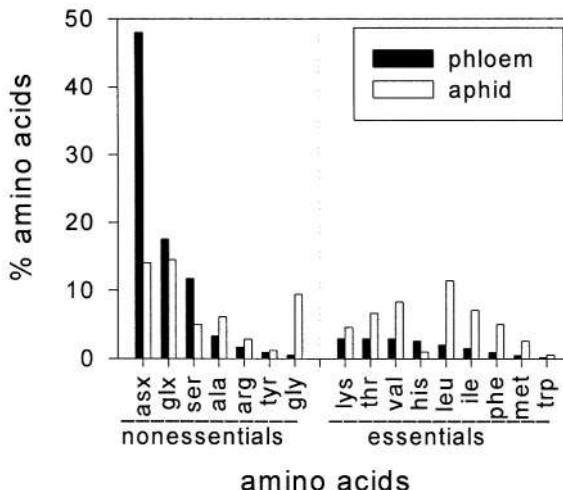


Fig. 1 Amino acid composition of the phloem sap of *Vicia faba* and the black bean aphid *Aphis fabae*. Abbreviations: ala, alanine; arg, arginine; asx, asparagine + aspartic acid; gtx, glutamine + glutamic acid; gly, glycine; his, histidine; ile, isoleucine; leu, leucine; lys, lysine; met, methionine; phe, phenylalanine; ser, serine; thr, threonine; trp, tryptophan; tyr, tyrosine; val, valine. Proline and cysteine (both nonessential amino acids) were not quantified in this analysis. [Data from Douglas et al. (2001) and unpub. results of C.R. Tosh, K.F.A. Walters & A.E. Douglas]

2000). Although *Buchnera* has only 35% of the genes present in *E. coli*, it has retained 45% of the genes coding for amino acid biosynthesis (Table 1). Furthermore, genes coding for enzymes in synthesis of tryptophan (*trpEG*) and leucine (*leuABCD*) are amplified on plasmids (Lai et al. 1994; Bracho et al. 1995; van Ham et al. 1997; Birkle & Douglas 1999), so increasing the genetic capacity of the bacteria to synthesize these two amino acids.

The loss of *Buchnera*-derived essential amino acids has major metabolic consequences for aposymbiotic aphids. The rate of protein synthesis and protein content per unit weight are depressed, and the free amino acid content is elevated in aposymbiotic aphids (Prosser & Douglas 1991; Liadouze et al. 1995; Wilkinson & Douglas 1995; Douglas 1996; Douglas et al. 2001). Typically, all but one-to-several essential amino acids in the free amino acid fraction is raised. For example, in *A. fabae* studied by Douglas et al. (2001), the free amino acid content of aposymbiotic aphids was nearly double that of symbiotic aphids of the same age, and the only amino acids with lower concentration in the aposymbiotic aphids was the essential leucine (Fig. 3).

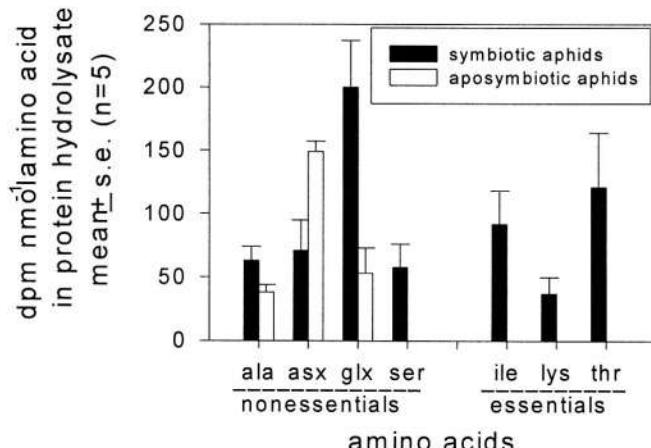


Fig. 2. Incorporation of radioactivity from ^{14}C -glutamic acid into protein amino acids of *Aphis fabae*. See legend to Fig. 1 for explanation of abbreviations [Unpublished data of L.M. Minto & A.E. Douglas]

These data have been interpreted as evidence that leucine limits protein synthesis in these aposymbiotic aphids, resulting in the accumulation of nonlimiting amino acids.

The free amino acid fraction is predominantly in the insect haemolymph ('blood') and the elevated concentration of free amino acids in aposymbiotic aphids results in an increase in the osmotic pressure of the haemolymph. For example, the osmotic pressure of haemolymph in the pea aphid *Acyrthosiphon pisum* is significantly higher in aposymbiotic aphids, at 1.07 MPa, than in symbiotic aphids, at 0.92 MPa (Wilkinson et al. 1997).

3. Sugar metabolism in the aphid-*Buchnera* symbiosis

Plant phloem sap poses a major osmotic hazard for aphids and other phloem-feeding insects. The osmotic pressure of phloem sap is 2-4 times higher than that of the body fluids of insects, largely because of the high concentration of phloem sugars, often dominated by sucrose (Ziegler 1975). Phloem-feeding insects avoid catastrophic osmotic collapse arising from the passage of water from body fluids into the gut lumen by gut-mediated down-regulation of the osmotic pressure of ingested phloem sap (Kennedy and Stroyan 1959). In aphids, which have been studied in particular detail, this is mediated primarily by the transformation of ingested sucrose into long-chain

TABLE 1. Distribution of cellular functions of gene products of *Buchnera* and *E. coli* [Data from Shigenobu et al. (2000) and Riley (1993)]

Functional category	<i>E. coli</i>	Number of genes <i>Buchnera</i> sp.
Biosynthesis of small molecules		
Amino acid synthesis ¹	119	53
Other	237	68
Intermediate metabolism ²	383	74
Macromolecule metabolism	419	209
Other	607	213
TOTAL	1765	617

1. Includes *trpEG* and *leuA-D*, which are plasmid-borne in *Buchnera*, and *trpG*, excluded from the genes listed for *E. coli* in Riley (1993)

2. Genes functional in sulphur metabolism (*cysC*, *cysD*, *cysE*, *cysH*, *cysI*, *cysN*, *cysQ* in both *E. coli* and *Buchnera* sp., and *cysP* in *E. coli*) are included in the functional category 'intermediate metabolism', and not 'amino acid synthesis'.

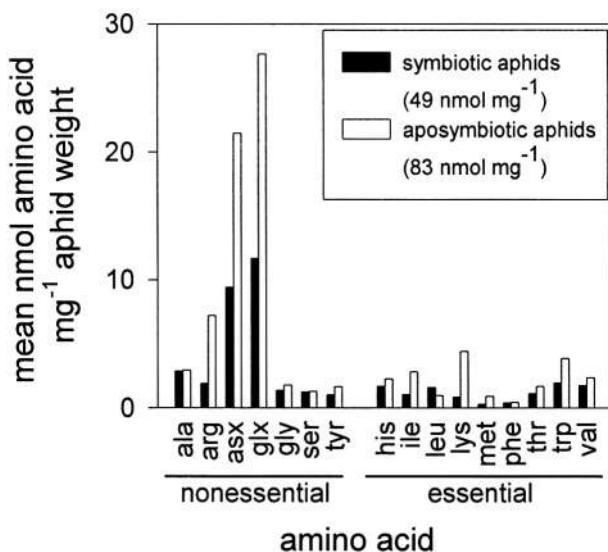


Fig. 3. The concentration of amino acids in the free amino acid fraction of *Aphis fabae*. Abbreviations as in legend to Fig. 1. [Data from Douglas et al. (2001)]

oligosaccharides (Fisher et al. 1984; Rhodes et al. 1996). The oligosaccharides are synthesized in the gut lumen and are composed of glucose (Walters & Mullin 1988; Ashford et al. 2000).

Studies on honeydew production by the aposymbiotic pea aphid *Acyrtosiphon pisum* raised the possibility of a symbiotic dimension to the osmoregulatory synthesis of oligosaccharides. When reared on *V. faba*, symbiotic aphids produce a watery honeydew containing mono- and di-saccharides, but aposymbiotic aphids produce a viscous honeydew with a high content of oligosaccharides (Wilkinson et al. 1997). However, further research did not support the simplest interpretation of these data, namely that *Buchnera* participates in the osmoregulatory capability of the insect by restricting the synthesis of oligosaccharides. The key evidence came from two experiments conducted by Wilkinson et al. (1997) using aphids on chemically-defined diets with sucrose concentration systematically varied between 0.15 and 1.0 M. First, the osmoregulatory capability of the aphids is unaffected by aposymbiosis. On diets with osmotic pressure up to four times greater than that of the aphid haemolymph, the aphids maintained a stable haemolymph osmotic pressure (Fig. 4A); the osmotic pressure is elevated in aposymbiotic aphids, which have high concentrations of free amino acids (see section 2). Second, both symbiotic and aposymbiotic aphids are capable of oligosaccharide production, but the oligosaccharides are synthesized at lower dietary sucrose concentrations in aposymbiotic aphids than in symbiotic aphids (Fig. 4B). The honeydew of symbiotic and aposymbiotic aphids feeding from *V. faba* differs in composition because the concentration of sucrose in *V. faba* phloem sap is sufficient to trigger oligosaccharide synthesis in aposymbiotic, but not symbiotic, aphids.

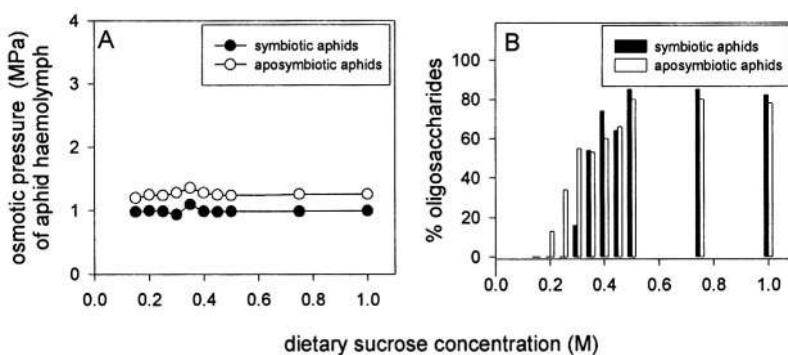


Fig. 4 Osmotic relations of the aphid-*Buchnera* symbiosis in the pea aphid *Acyrtosiphon pisum* feeding from chemically-defined diets containing 0.15-1.0 M sucrose (equivalent to osmotic pressure of 1-4 MPa). (A) osmotic pressure of aphid haemolymph; (B) contribution of oligosaccharides to the total sugar content of aphid honeydew [Redrawn from Wilkinson et al. (1997).]

Why are oligosaccharides synthesised at lower dietary sucrose concentration in

aposymbiotic than in symbiotic aphids? Oligosaccharide synthesis occurs in the distal part of the midgut and the rate of reaction is determined by the concentration of the products of sucrose hydrolysis after assimilation of sugars by the aphid. The proportion of ingested sugars assimilated is much higher in symbiotic aphids, at 72%, than aposymbiotic aphids, at 47% (Wilkinson & Ishikawa 1999); and consequently, for a given concentration of ingested sucrose, the concentration of sugars in the gut lumen is higher for aposymbiotic aphids than for symbiotic aphids.

4. Concluding Comments: The Distinction between Functions of Symbiotic Micro-organisms and Consequences of Aposymbiosis

Recent research reviewed in this article has revealed differences between symbiotic and aposymbiotic aphids in the metabolism of both amino acids and sugars. The causal relationships among the various effects of aposymbiosis can be construed as follows. The loss of bacterial-derived essential amino acids limits protein synthesis in the aphid, and this results in: first, the accumulation of nonlimiting amino acids in the haemolymph (Fig. 3) and linked increase in the osmotic pressure of the haemolymph (Fig. 4A); and, second, depressed aphid growth and resultant reduction in the assimilation of ingested sucrose and increase in oligosaccharide synthesis at low dietary sucrose concentrations (Fig. 4B). The key issue is that all these various effects arise from a single function of *Buchnera*: essential amino acid synthesis.

To generalise, the characteristics of aposymbiotic aphids can be attributed to two distinct processes:

- *functions of the symbiotic micro-organisms*, i.e. properties of the micro-organism that are of selective value to the insect host, and
- *consequences of elimination of the symbiotic micro-organisms*, including various compensatory responses of the insect to loss of bacterial function.

Detailed analysis of the processes underlying any difference between symbiotic and aposymbiotic insects is commonly needed to discriminate between the function of the symbiotic micro-organisms and secondary consequences of their elimination. This is essential if studies of aposymbiotic insects are to continue to contribute fully to our understanding of the microbial symbioses in insects.

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ANTS, AGRICULTURE, AND ANTIBIOTICS

A Quadripartite Symbiosis

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1. Introduction

The ability to cultivate food is unique to humans and a few groups of insects. Although ‘agricultural’ symbioses are rare, organisms that are able to grow their own food can be extremely successful. This is illustrated by the important role that food production has had in establishing the dominant position of humans in the world (Diamond 1997). Like humans, attine ants cultivate their own food. These ants, commonly referred to as fungus-growers, culture fungus, providing it with substrate on which to grow, protection from competitors, and dispersal to new colonies. In exchange, the fungus serves as the ants’ main food source.

The evolution of agriculture in fungus-growing ants has led to their dominant role within neotropical ecosystems. They have a vast geographic distribution, occurring as far north as New Jersey (lat. 40° N) and as far south as central Argentina (lat. 44° S). They are both abundant and diverse throughout most of this range, especially in wet tropical regions. Their success is best illustrated by the leaf-cutters, so named because of their conspicuous behavior of cutting fresh vegetation that the ants carry back to their colonies and use to culture their fungus gardens. Leaf-cutter ants belonging to the genus *Atta* can form colonies with millions of workers and dozens of fungal chambers and can live for more than 10 years (Astuori 1941, 1950; Weber 1966, 1972). Leaf-cutter ants have one of the most complex social systems of all insects, with workers of different caste sizes specialized to perform different behaviors (Wilson 1980a, 1980b). They also play an important role in the functioning of neotropical ecosystems, facilitating nutrient cycling and stimulating new plant and root growth (Haines 1978; Hölldobler and Wilson 1990). In addition, they are considered the dominant herbivore in the New World (Hölldobler and Wilson 1990), cutting perhaps as much as 17% of the vegetation produced in tropical forests (Cherrett 1986).

The mutualism between fungus-growing ants and their fungal cultivars has received substantial attention from biologists for more than a century. The majority of this research has focused on the biology of the leaf-cutters. This trend has been driven

by the agricultural pest status of these ants (Hölldobler and Wilson 1990). Nevertheless, recent studies of fungus-growing ants and their fungi have begun to illustrate the potential for this mutualism to shed new light on the dynamics of symbiosis (e.g., Chapela et al. 1994; Hinkle et al. 1994; North et al. 1997; Mueller et al. 1998; Herre et al. 1999; Currie et al. 1999a, 1999b). In this review, I will present the latest contributions to our understanding of this ancient association, illustrate some evolutionary trends within this mutualism, and argue that this is a model system for the study of symbiosis.

2. The symbionts

2.1. FUNGUS-GROWING ANTS

The ants that cultivate fungus gardens are members of the myrmicine tribe Attini (Formicidae) and include 210 species in 12 genera (Schultz and Meier 1995). Attine ants can be divided into the seven ‘primitive’ (phylogenetically basal) genera, commonly referred to as ‘lower attines’, and the five ‘advanced’ (phylogenetically derived) genera, called the ‘higher attines’. Although all species are fungus-growing ants, only the two most derived genera, *Acromyrmex* and *Atta*, use fresh plant material for manuring their gardens, and are therefore called leaf-cutters.

Fungus-growing ants are apparently derived from a single ancestor (Schultz and Meier 1995), indicating a single evolutionary origin for the ants’ ability to culture fungi. The origin of this ant tribe is not resolved; however, it appears that the closest relatives of attine ants are two sister genera, *Blepharidatta* and *Wasmannia* (Shultz and Meier 1995; Mueller et al. 2001). Mueller et al. (2001) point out that a promising approach to determining the origin of fungus-growing in attine ants is to examine the biology of both the sister group of the Attini and the most phylogenetically basal species (see section 3.1).

The difference between extant species of fungus-growing ants has fascinated biologists and led authors to suggest that there is an evolutionary trend towards improved fungus culturing in these ants (Wheeler 1910; Wilson 1971; Weber 1972; Hölldobler and Wilson 1990). In fact, based on colony size, worker polymorphism, and substrate upon which the fungus garden is cultured, Wilson (1971) suggests that the genera may be divided into three groups representing primitive, transitional, and advanced genera (Table 1). The lower (phylogenetically basal) attine ants represent the likely ancestral form of fungiculture: they have small colonies (tens to hundreds of workers), there is no polymorphism in workers, and they mostly use insect feces and dead vegetable matter to manure their gardens. The transitional form may be represented by the genera *Sericomyrmex* and *Trachymyrmex* of the higher attines, which have medium sized colonies, the beginnings of slight worker polymorphism, and use mostly dead vegetable matter as substrate. Representing the advanced form, leaf-cutters in the genera *Acromyrmex* and *Atta*, as noted above, can have colonies with hundreds of thousands to millions of workers, can live for more than 10 years, have strong worker

TABLE 1. Characteristics of fungus-growing ant genera, including their worker and colony size, worker polymorphism, and substrate used to manure the gardens (based on Weber 1966, 1972; Wilson 1971; Hölldobler and Wilson 1990). Genera listed represent the most phylogenetically basal to the most phylogenetically derived (Schultz and Meier 1995; Wetterer et al. 1998) and are placed in three groups representing proposed primitive, transitional, and advanced genera (Wilson 1971).

Attine group and genera	Worker and colony size, Worker polymorphism	Garden substrate
'Primitive', lower attines: <i>Myrmicocrypta</i> , <i>Mycocepurus</i> , <i>Apterostigma</i> , <i>Mycetarotes</i> , <i>Mycetosoritis</i> , <i>Cyphomyrmex</i> , <i>Mycetophylax</i>	Small to medium workers and colony size Workers monomorphic	Insect feces, Woody matter, Insect corpses
'Transitional' higher attines: <i>Sericomyrmex</i> , <i>Trachymyrmex</i>	Medium worker and colony size Workers monomorphic to slightly polymorphic	Dead vegetative matter
'Advanced' higher attines: <i>Acromyrmex</i> , <i>Atta</i>	Large worker size, large to very large colony size Workers polymorphic to strongly polymorphic	Fresh leaves and flowers

polymorphism, and use only fresh vegetation as substrate (Autuori 1941, 1950; Weber 1966, 1972; Wetterer 1999).

The process of fungiculture begins with workers bringing foraged material back to the colony. The material is licked, masticated, and added to the garden matrix. Pieces of fungal inoculum, typically from lower in the garden, are brought to the top, inoculated onto the substrate, and carefully tended. The maintenance of stable fungus gardens by attine ants is a complex process that is still not completely understood (see Currie 2001a).

The life cycle of fungus-growing ants is centered on the growth and reproduction of the colony. To reproduce, the colony needs to reach sufficient size to support the production of reproductive individuals. Colonies grow through the accumulation of garden biomass and the production of a larger population of workers. As in all ants, the workers are female, and males are produced only to participate in mating flights, shortly after which they die. Mated queens establish new nests (called incipient colonies) by starting a new fungus garden. The fungal inocula that is used to start new gardens is collected by virgin queens from their parent colonies prior to the mating flight. Foundress queens care for the fungus in isolation until their first brood of workers is produced.

2.2. FUNGAL CULTIVARS

Thomas Belt (1874) was the first to discover that the leaf-cutter ants have a fungal partner. He realized that the conspicuous trails of ants transporting leaf fragments deep underground were not using this vegetative material as food, but instead as manure to grow fungus on which they feed. Möller (1893) followed up Belt's work with an extensive mycological study of this mutualism, including the first observations of the

ants consuming the fungus. Shortly after Möller's work, it was established that queens carry fungal inoculum from their parent colony with them on mating flights, further illustrating that the symbionts are closely connected (Ihering 1898; Huber 1905).

The fungi that are cultivated by attine ants are all members of the large order Agaricales (Basidiomycota). Members of the Agaricales, also referred to as agarics, are fungi that produce fruiting bodies commonly called mushrooms. However, the agarics cultivated by fungus-growing ants are grown as mycelium and do not appear to fruit in nature (Möller 1893; Hervey et al. 1977; Muchovej et al. 1991). The absence of the taxonomically informative fruiting bodies has resulted in a poor taxonomic and phylogenetic understanding of these fungi; however, with the development of molecular phylogenetic techniques, an improved understanding has recently been achieved (Chapela et al. 1994; Hinkle et al. 1994; Mueller et al. 1998).

The majority of the lower attines cultivate fungi in the genera *Leucocoprinus* and *Leucoagaricus* (Agaricales: Lepiotaceae: Leucocoprinae) (Chapela et al. 1994; Mueller et al. 1998). These fungi are mostly tropical litter decomposers (Dennis 1952; Singer 1986). Most members of the lower attine genus *Apterostigma* cultivate fungi from the Tricholomataceae (Agaricales), likely in the genus *Gerronema* (Moncalvo et al. 2000). This is believed to be a secondary switch by the ants from growing leucocoprineous cultivars (Chapela et al. 1994). One group of lower attine ants, in the "rimosus" group (genus *Cyphomyrmex*), cultivates its fungus in a single-celled yeast form. Interestingly, these yeast cultivars were not secondarily acquired but instead evolved from the leucocoprineous fungi cultivated by other lower attines (Mueller et al. 1998). The cultivars of the higher attines appear to be a diverse and highly derived assemblage of fungi also evolved from leucocoprineous fungi (Chapela et al. 1994).

2.3. SPECIALIZED PARASITES OF FUNGUS GARDENS

The gardens of fungus-growing ants are also host to a virulent and specialized pathogen in the genus *Escovopsis* (Currie et al. 1999a; Currie 2001a, 2001b). *Escovopsis* is a genus of microfungi (Ascomycota) that is apparently allied with the order Hypocreales. There are currently two species described (Kreisel 1972; Muchovej and Della Lucia 1990; Seifert 1995), however, this is likely a vast underestimate of the diversity of these fungi (Currie 2000). *Escovopsis* has been isolated from the gardens of both lower and higher attines, indicating that it occurs throughout the phylogenetic diversity of this mutualism (Currie et al. 1999a). It is apparently specialized to the gardens of fungus-growing ants, having been isolated only in association with attine ants (Seifert 1995; Currie et al. 1999a; Currie 2001a; Bot et al. 2001). In addition, it is very common in the gardens of fungus-growing ants, having been isolated from a low of 33% to a high of 77% of colonies sampled (Currie et al. 1999a; Currie 2001b). Within infected gardens, *Escovopsis* is often prevalent, typically occurring throughout most of the garden biomass (Currie et al. 1999a).

Escovopsis has the ability to completely devastate an attine ant colony by rapidly overgrowing the whole garden (Currie et al. 1999a; Currie 2001a). The impact of *Escovopsis* on fungus-growing ants was first noted by Möller (1893), but he mistakenly assumed it to be the asexual stage of the ants' cultivar growing out of control. Many

other biologists have also observed colonies being overgrown by a fungus that was most likely *Escovopsis* (Stahel and Geijskes 1941; Weber 1966, 1972). However, they either wrongly followed Möller's conclusions or concluded that these fungi were just airborne molds invading and overwhelming gardens only in the artificial conditions of the laboratory. *Escovopsis* does not typically overgrow ant fungus gardens, but instead forms persistent infections within the matrix of the garden (Currie et al. 1999a). The fungus apparently lives off of the garden, draining away energy that would otherwise be used to the benefit of the mutualists. It appears that *Escovopsis* consumes the fungal cultivar; however, this is not well established, and it is still possible that *Escovopsis* is a highly evolved 'weed' competing with the ants' fungus for the garden substrate (Currie 2001a). Regardless of the mechanisms of pathogenicity, the persistent infection of gardens by *Escovopsis* has been shown to result in a substantial decrease in the growth rate of the garden and ant biomass in the leaf-cutter *Atta colombica* (Currie 2001a). It is not clear under what conditions the pathogen *Escovopsis* overwhelms the fungus garden or forms a persistent infection.

2.4. ANTIBIOTIC-PRODUCING BACTERIA

Recent work has also identified the presence of a fourth symbiont within this ancient mutualism: filamentous bacteria (actinomycetes). Fungus-growing ants have a whitish 'bloom' on their exoskeletons that originally was thought to be a wax produced by the ants (Weber 1972), but is in fact a filamentous bacterium (Currie et al. 1999b; Currie 2001a, see Figure 1a). The bacterium has been found to be associated with all attine ant species and all colonies studied thus far (Currie et al. 1999b; Currie unpublished data). The bacterium can be very abundant on the cuticles of ants, often covering the entire surface (see Figure 2 in Currie 2001a). The location of the bacterium is genus specific, occurring under the forelegs in the phylogenetically basal genera (e.g., *Apterostigma*) and on the laterocervical plates of the propleura in the more phylogenetically derived genera (e.g., *Acromyrmex*, Figure 1b). The laterocervical plate appears to be modified in some genera, perhaps to optimize the growing conditions for the bacterium (Currie unpublished data). Although the bacterium is apparently associated with the most derived genus, *Atta*, its location has not been determined (Currie et al. 1999b). New queens carry the bacterium on their cuticle during the nuptial flights, indicating it is transmitted vertically from parent to offspring colony.

Actinomycetes, such as those that are present on fungus-growing ants, are well known for their ability to produce potent antibiotics. In fact, most antibiotics developed for human pharmaceutical use are derived from actinomycetes (Wakesman and Lechevalier 1962; Goodfellow and Cross 1984). Like humans, it appears that fungus-growing ants use actinomycetes for the production of antibiotics (Currie et al. 1999b; Currie et al. 2001). Bioassays revealed that the bacterium produces antibiotics that suppress the growth of the specialized parasite *Escovopsis*, but have no effect on a diverse collection of general fungi (Currie et al. 1999b). The production of secondary metabolites is energetically costly and requires complex biosynthetic pathways, so the maintenance of metabolites specific towards *Escovopsis* presumes a benefit to the actinomycete and thus is strong evidence that this association is mutualistic.

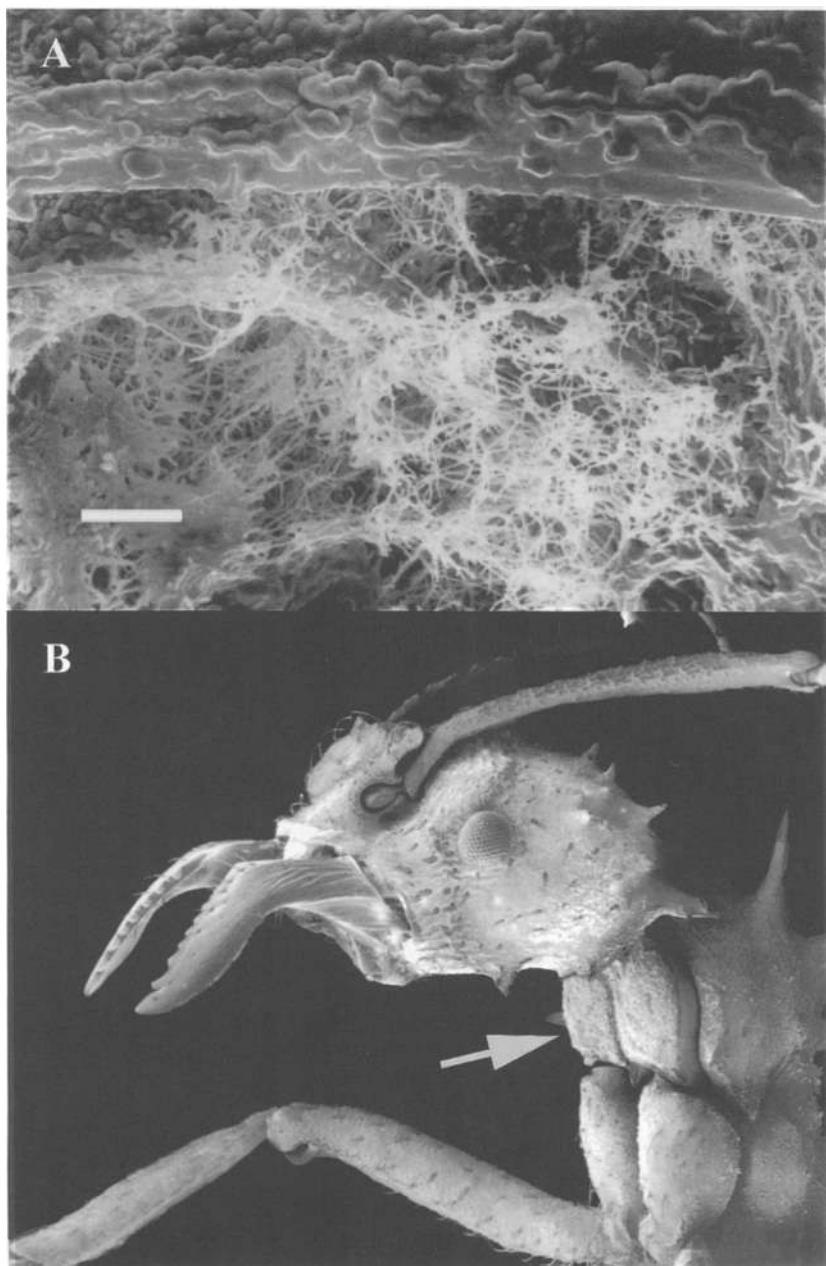


Figure 1. Scanning electron micrographs of actinomycete growing on the fungus-growing ant *Acromyrmex octospinosus*. A. View of the filamentous growth form of the actinomycete, illustrating the mat of bacterium that is present on the cuticle. Scale bar represents 20 μm . B. View of the head and thoracic region of the ant, illustrating the specialized location of the bacterium on the laterocervical plates of the propleura (indicated by the arrow).

Experimental work using sub-colonies of the leaf-cutting ant *Acromyrmex octospinosus* confirm that the ants are better able to deal with infection by *Escovopsis* when the actinomycete is present as compared to when it is removed (Currie et al. 2001). Currie et al. (2001), using sub-colonies of *Acromyrmex octospinosus*, conducted a two-by-two factorial design experiment, crossing the presence/absence of the actinomycete with the presence/absence of *Escovopsis*. Using this design, it was established that sub-colonies with the bacterium present were able to significantly reduce the prevalence and impact of *Escovopsis* infections as compared to those with the bacterium removed. In addition, as would be predicted if this bacterium is important for the maintenance of the health of the garden, garden-tending workers were found to have a higher abundance of the bacterium than foraging individuals.

The bacteria obtain several benefits from being symbionts of fungus-growing ants. Fungus-growing ants represent a unique, abundant, and diverse niche previously not occupied by actinomycetes. In fact, it appears that the ants provide nourishment for the growth of the bacteria (Currie et al. 2001; Currie unpublished data). The ants also disperse the bacterium from parent to offspring colony.

3. Evolution of a quadripartite symbiosis

3.1 THE ORIGIN OF THIS SYMBIOSIS

Fungus cultivation by attine ants is ancient, likely originating 45–65 million years ago (Wilson 1971; Mueller et al. 2001). It is not clear, however, how this mutualism originated (see Mueller et al. 2001). At least seven hypotheses have been generated regarding the transition from hunter-gatherer life to the cultivation of fungi. Each of these differs with respect to the substrate the fungus is thought to have grown on before being cultured by the ants. Using new phylogenetic information on the fungal cultivars and information on the biology of extant attine ants and their close relatives, Mueller et al. (2001) narrow the choices down to the two most plausible hypotheses. One possibility is that the ants evolved to culture the fungi they encountered on walls of nests built in leaf litter (Emery 1899). A second, and perhaps more likely alternative, is a system of fungal myrmecochory (Mueller et al. 2001). In this scenario, the ancestor of the attine ant cultivars was specialized to be dispersed by ants and then evolved to be cultured by the ancestor of attine ants (Bailey 1920; Mueller et al. 2001).

The discovery of a specialized parasite and the bacterial mutualist raises two additional questions regarding the origin of this mutualism. First, when did these two new symbionts become involved in this symbiosis? Second, how did they evolve to occur within this mutualism?

It appears that both *Escovopsis* and the filamentous bacteria have had a long evolutionary history within this mutualism. Both of these symbionts have been found to be associated with fungus-growing ants across much of their geographic distribution: from southern Brazil to Texas (Möller 1893; Currie 2001a; Currie unpublished data). In addition, they are associated with fungus-growing ants representing the phylogenetic diversity of the ants, from the lower to higher attines (Currie et al. 1999a, 1999b; Currie

2001a). In fact, the species of *Escovopsis* occurring in the gardens of lower attines is morphologically distinct from species occurring in the higher attines (Currie unpublished). This suggests a long coevolutionary history between the fungus-growing ants and *Escovopsis*. Also, as noted above, the bacteria occur in genus specific locations on attine ants, suggesting they have coevolved with the ants. Although this evidence suggests that all four symbionts are ancient symbionts, it remains unclear how long the actinomycetes and *Escovopsis* have been associated with fungus-growing ants.

Likewise, it is unknown how these two symbionts evolved a symbiotic association with fungus-growing ants. In fact, there are currently no hypotheses for how these interspecific associations originated. There are some scenarios for the origin of each symbiont within this mutualism that seem more plausible than others. One promising scenario for the origin of the parasite *Escovopsis* is that it evolved from being a pathogen of the ants themselves to infecting their gardens. Another possibility is that the ancestor of *Escovopsis* was a parasite of the free-living form of the ants' cultivar brought into the mutualism with the domestication of leucocoprineous fungi.

The origin of an association between actinomycetes and fungus-growing ants is more difficult to speculate upon. Because actinomycetes are common in the soil and various substrates that come in contact with the ants, there are numerous possibilities for the origin of the actinomycete mutualist. It is possible that the ants first came in contact with the bacterium through the substrate used to manure the garden or in the soil they nest. It is also possible that early fungus-growing ants foraged on material high in actinomycete content to treat the garden for diseases, subsequently evolving a more intricate association.

Further insights into the origins of *Escovopsis* and the ant-associated actinomycete should be possible with the reconstruction of the evolutionary history of these two symbionts. Such attempts should also include the goal of determining the sister species for both of these symbionts so that the natural history of these microbes may be examined.

3.2 EVOLUTION OF THE CULTIVARS

It traditionally has been assumed that the evolution of the mutualism between attine ants and their cultivars has been shaped by the practice of foundress queens carrying inoculum from their natal nests to establish their new gardens. This vertical mode of transmission suggests that the cultivars are ancient clones that are strictly coevolved with their hosts. However, recent work has established that ant–cultivar evolution is much more complicated than previously assumed, involving a diversity of fungi and distinct patterns of evolution occurring within different groups of fungus-growing ants (Chapela et al. 1994; Hinkle et al. 1994; Mueller et al. 1998). Here, I will highlight the main differences between the lower and higher attines based on our current understanding.

As previously mentioned, the majority of species of the lower attines cultivate leucocoprineous fungi (Chapela et al. 1994; Mueller et al. 1998). Mueller et al. (1998) surveyed a large collection of free-living and ant-cultivated leucocoprineous fungi and used population genetics and phylogenetic patterns to determine the fascinating

evolutionary history of these two mutualists. They established three important patterns of cultivar exchange. First, lower attines apparently acquire new cultivars from free-living species, although it is currently not clear how frequently this might occur. Second, single ant species can cultivate a diversity of fungi. Third, distantly related ant species can cultivate the same cultivars. Thus, ant–fungus evolution in the lower attines is shaped by clonal propagation (queens carrying it on mating flights), by occasional lateral transfers of fungal cultivars between ant colonies and species, and by domestication of novel cultivars.

A detailed understanding of ant–cultivar evolution has not been completely analyzed for the higher attines. However, based on work by Chapela et al. (1994) and Hinkle et al. (1994) it appears that the evolutionary history of the higher attines and their fungi is less complex than that of the lower attines. First, it appears that the fungi cultivated by higher attines are ancient asexual clones. Second, there appears to be much greater phylogenetic congruence between the ants and their fungi, with distantly related ants cultivating distantly related cultivars. Third, higher attines do not appear to domesticate new cultivars from free-living species.

3.3 THE EVOLUTION OF GARDEN PATHOGENS

Because the ants and their fungi are mutually dependent, the maintenance of stable fungal cultures is critical to the survival of the mutualists. It is clear that the health of fungus gardens is threatened by a diverse assemblage of microbes. In fact, these microbes can be grouped into two distinct sets. First, gardens are continuously exposed to the microbes present on both the substrate added to the garden and the ubiquitous microbes found in the soil and air. Second, as noted above, gardens are host to at least one specialized pathogen that has coevolved with the ants and their fungal cultivars (Currie 1999a; Currie 2001a, 2001b). The methods employed to defend fungus gardens against ‘general’ alien microbes and ‘specialized’ pathogens are likely different, because overcoming the general defenses employed by the mutualists is a requisite condition for a microbe to become a specialized pathogen. To subsequently defend against this specialized pathogen, the mutualists must evolve new defenses. This suggests that garden defense likely involves one set of methods for dealing with general contaminants and perhaps a different set for defending against specialized pathogens.

Recent work has suggested that the higher attines have evolved improved defenses against generalist contaminants. Currie et al. (1999a) conducted extensive isolations for non-mutualistic filamentous fungi from the gardens of more than 200 colonies of fungus-growing ants in Panama. A total of 2,480 garden pieces were sampled from a diverse assemblage of attines, including both lower (6 species) and higher attines (7 species). They found that the frequency of fungal contamination steadily decreased from the lower to the higher attines, with the highest level of contamination being almost 55% of pieces in a phylogenetically basal lower attine, *Apterostigma*, and the lowest being less than 27% of pieces in the most phylogenetically derived genus, *Atta*. In fact, if the specialized parasite *Escovopsis* is removed from these totals, general contamination levels range from a high of 38.5% in *Apterostigma* to a low of 8.1% in *Atta*. This pattern suggests that the higher attines have evolved better defenses against

generalist fungi over the evolutionary history of this tripartite mutualism. Additional support for this conclusion comes from the observation that colonies of lower attines are occasionally overgrown in the laboratory by common substrate and airborne contaminants such as *Penicillium*, *Aspergillus*, and *Trichoderma* (Currie unpublished data). In contrast, these general contaminants do not appear capable of overgrowing colonies of leaf-cutters, even when sprayed in large quantities onto fungus gardens (Bass and Cherrett 1994; Currie 1999a; Currie unpublished data). Attempts to confirm these observations experimentally should be undertaken. If the higher attines are indeed better at defending their gardens from alien microbes than are lower attines, determining the mechanisms of this defense would provide new insights into this mutualism.

In addition to observing a decrease in the frequency of general contaminants from the lower to higher attines, Currie et al. (1999a) observed an increase in the prevalence of *Escovopsis* in the higher attines. In colonies of the lower attines, *Escovopsis* represented fewer than 30% of the contaminants isolated, while in the higher attines it was typically greater than 70% of the contaminants isolated. These finding are supported by the observation that *Escovopsis* appears to be the only fungus that is capable of overgrowing fungus gardens of higher attines, but is only one of several fungi that can devastate the gardens of lower attines (Currie 1999a; Currie 2001a; Currie unpublished data). Currie et al. (1999a) speculate that this apparent evolution of increased *Escovopsis* virulence in the higher attines is a result of these ants cultivating ancient asexual clones.

3.4 OVERALL EVOLUTIONARY PATTERNS?

As I have outlined in this review, the quadripartite association is marked by some large differences between the lower and higher attines, which may reflect some important evolutionary trends or transitions (e.g., colony size, use of fresh vegetation: see Table 1). The lower attines are characterized by small colony size, no worker polymorphism, use of mostly insect feces and dead vegetation as garden substrate, acquisition of novel cultivars, higher susceptibility to general contaminants, and lower susceptibility to *Escovopsis*. In contrast, the higher attines are characterized by the potential to reach large colony size, substantial worker polymorphism, use of dead or fresh vegetation as garden substrate, clonally propagated cultivars, lower susceptibility to general contaminants, and a greater susceptibility to *Escovopsis*. An additional evolutionary transition recently has been identified, with queens of lower attines mating only with a single male, while leaf-cutter queens mate with multiple males (Boomsma et al. 1999; Villesen et al. 1999). For the most part, the correlative versus causative nature of these evolutionary patterns is unknown, and thus there are many significant questions to address within this symbiosis using a comparative approach. For example, has the transition to greater worker polymorphism resulted in the ability to cut and use fresh vegetation to manure the garden, as suggested by Wilson (1986), or has the switch to fresh vegetation allowed the evolution of greater worker polymorphism? Has the transition towards strict clonal cultivar propagation in the higher attines resulted in greater colony size, or has the evolution of larger colonies resulted in the ability to

cultivate clonal cultivars? Does the cultivation of clonal cultivars result in greater susceptibility to *Escovopsis* and the improved ability to deal with general contaminants? Additional layers of complexity will likely be added as greater understanding of this system is achieved. For example, there likely have been changes in the ant-associated actinomycete over the history of this association, but there is currently insufficient understanding to discern any patterns. Determining what is driving these evolutionary trends will provide unique insights into this mutualism as well as symbiosis in general.

4. A model system of ectosymbiosis

The ancient, highly evolved, and complex mutualism among fungus-growing ants, their fungal cultivars, antibiotic-producing bacteria, and the specialized garden pathogen is becoming a model system for studying symbiosis. Recent work on this symbiosis has provided significant insights into the dynamics of symbiosis in regards to: coevolution versus lateral switching of symbionts (Chapela et al. 1994; Hinkle et al. 1994; Mueller et al. 1998; Herre et al. 1999), the interaction of symbionts (North et al. 1997), parasites of mutualisms (Currie et al. 1999a; Adams et al. 2000; Currie 2001b), and the complexity of symbioses (Currie et al. 1999b; Currie 2001a).

As a model system for the study of symbiosis, this ancient mutualism has a few advantages over others. It is composed of a diverse assemblage of extant species, with a complex set of interactions (see section 2). In addition, the three microbial symbionts (i.e., the ants' cultivars, *Escovopsis*, and the ant-associated actinomycete) can all be cultured. In contrast, the microbes in many other symbioses involving bacterial or fungal symbionts will not grow in culture (McFall-Ngai 1998). The ability to culture the microbes makes it easier to utilize population genetics and molecular phylogenetic techniques and it provides an opportunity for greater experimental work, such as culture bioassays and the switching of symbionts between hosts (e.g., Currie et al. 1999b). Finally, fungus-growing ants are easily maintained in the laboratory, providing the opportunity to conduct laboratory experiments within this symbiosis.

Developing this quadripartite symbiosis as a model system will not only provide important insights into the dynamics of symbiosis, but also has potential implications for human survival (Currie et al. 1999b; Schultz 1999; Wilkinson 1999). The most obvious example of this is in relation to the evolution of resistance to antibiotics by human pathogens. Microbial pathogens of humans have developed resistance to our antibiotics rapidly over the 60-year history of antibiotic use by humans. In contrast, fungus-growing ants apparently have been using actinomycetes successfully for the production of antibiotics for more than 45–65 millions years. Studies examining how these ants have successfully used antibiotics for such an extended time period could provide important insights into our own use of these incredibly important pharmaceuticals. In addition, the host-pathogen dynamics between *Escovopsis* and the tripartite mutualist could provide general insights into the evolution of virulence in pathogens. Such insights would result in a better understanding of the pathogens of humans, their agricultural crops, and domesticated animals.

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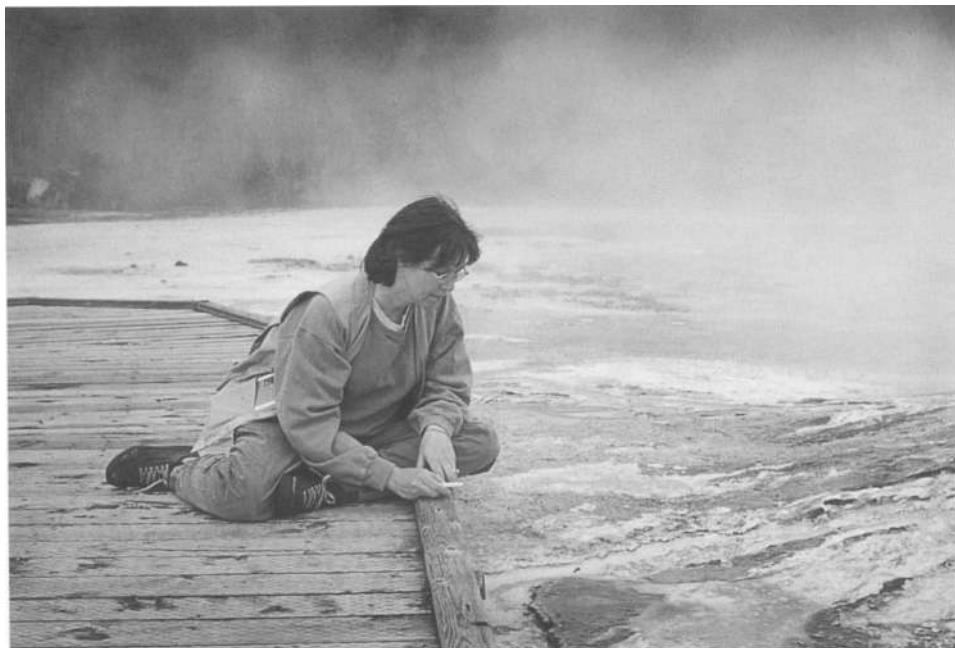
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TERMITE HINDGUT SYMBIANTS

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1. Introduction: The Challenges of Cellulose Digestion

The most abundant supply of fixed carbon in the biosphere is in the form of the biomass of photosynthesizers (plants, algae, and some bacteria). The exceptions of course, are those rare but fascinating communities based entirely on the autotrophy of chemosynthetic bacteria. For example, see the chapter in this volume on deep sea vent communities. Cellulose is a major component of biomass, representing much of the base of a simplified trophic pyramid. Many herbivores and saprophytes at the second level of the pyramid derive their energy and substance from that cellulose-rich biomass and their feeding activities are an essential link in the cycling of carbon. However cellulose is by no means an easy substrate upon which to feed. The massiveness and longevity of trees is testament to the resilience of cellulose (and also of hemicellulose and lignin) that comprise their structures.

Relatively few plant eaters have evolved the enzymes to allow them to feast upon pure wood or even an exclusively leafy diet. A seed-rich diet is another matter. Seeds as reproductive structures contain a balance of more digestible nutrients. Cellulose digesters are found in some bacterial groups and throughout the fungal kingdom; the cellulolytic capabilities of some protists are less clear. Only two protists from termites have been grown in axenic culture with cellulose as the sole source of carbohydrate (Yamin, 1978 and 1981) There may be no animals that can completely digest cellulose, in spite of the diversity and numbers of animals that consume tough plant parts as their exclusive diet. Watanabe *et al* (1998) have isolated a cellulase from a termite salivary gland that seems to be of termite origin and not a result of recent horizontal transfer from bacteria. However they acknowledge that this cellulase cannot complete an "exhaustive" digestion of native cellulose. Other researchers are tracking down cellulases of other animals with enough success that a total absence of animal cellulases should not be assumed in herbivores. The role of cellulolytic symbionts may be to greatly enhance the digestibility of wood rather than to be solely responsible for it.

How do herbivores manage? Without exception, animals existing on cellulose-rich diets rely upon symbionts either bacterial or fungal (internal or external) for their most essential nutritive functions. An examination of the digestive system of almost any "cellulose-digesting" animal reveals that one section or another has been enlarged, forming a sort of culture chamber for a myriad of cellulolytic microorganisms. The ruminant animals (e.g. cattle, sheep, giraffe) tend to be shaped like fermentation vats to

accommodate a huge stomach chamber. Other herbivorous animals such as some rodents, horses, iguanas, and very likely even the extinct sauropod dinosaurs have (or had) enlargements of the hindgut for their symbionts. Yet other animal herbivores demonstrate their reliance on external symbionts by their elaborate activities. Most noticeable are the fungal "gardeners" (some groups of ants, termites, and beetles) which maintain cellulolytic fungi on plant material.

2. The Success of Termites and their Symbionts in using Cellulose

Termites (Isoptera) are an enormously successful order of insects, having evolved almost exclusively a wood-eating habit, "xylophagy" with the help of their symbionts. For some diverse tropical forests it has been estimated that 30% of the dry mass of all of the animals is comprised of termites and ants, with all of the former and many of the latter busy with the digestion of plant material. (Wilson, 1996). The termite families are divided into "lower" and "higher" taxa. The lower termites, which are all of those with protistan symbionts within their enlarged hindguts are the subject of this chapter. These are the families Termopsidae, Hodotermitidae, Mastotermitidae, Kalotermitidae, Rhinotermitidae, and Serritermitidae. The higher termites, the Termitidae have evolved a fungal-gardening habit in which fungi digest cellulose and in turn are food for the termites. Of the 2776 termite species described as of the mid 1990's, 803 are lower termites (as reviewed and updated by T.G. Myles <http://www.utoronto.ca/forest/termite/termite.htm>) Yamin (1979) catalogued all of the protists described in lower termites along with close relatives of the termites, the wood roach, *Cryptocercus*. There were a total of 434 trichomonads, oxymonads, and hypermastigotes described from 205 species of termites and two of *Cryptocercus*. Most other lower termites, nearly 600 species, await examination for symbionts.

Termite phylogeny (as well as symbiont phylogeny) may be viewed as the outline of a great experiment in the evolution of symbioses. It all started when, some ancestral roach (*Cryptocercus*-like) living on decaying wood, presumably acquired and retained symbionts from microorganisms in the wood, and from that arose all of the groups of termites. Cellulolytic bacteria along with nitrogen fixing bacteria appear to be the essential symbionts for digestion and nutrition. However termite hindguts are rich communities, many of which include a diversity of large, morphologically complex protists as well as other bacteria not of direct importance in the symbiosis. The symbionts (both bacterial and protistan) have evolved along with their hosts, diversifying into 100's of species. Many of the protists are unique to their particular lineage of termite host and, while not all are essential as symbionts many have interesting features in their own right, worthy of study. Yamin's monograph (1979) is, to date, the most comprehensive treatment of the co-phylogenies of termites and their protists. His work collects all of the previous taxonomic information on the protists in the literature, much of it published by Harold Kirby (e.g. Kirby 1941, 1942 a,b, 1945, 1949).

3. Identification and Characterization of the Bacterial Symbionts

Until recently the bacterial symbionts of termites have been difficult to study and identify because many are not easily cultured and few are morphologically distinct. However now with the ability to isolate DNA by PCR (polymerase chain reaction) and to probe the hindgut community with that DNA, more precise identifications are possible. Studies (mostly from the lab of Kudo and Ohkuma) have focused on phylogenies of both termites and their bacterial symbionts based on DNA sequences and thus considerable progress has been made in determining which bacterial symbionts are present as well as confirming some of the morphology-based phylogenies of the protists. Berchtold et al (1994) working on the symbionts of *Mastotermes darwiniensis* have identified spirochetes as well as various alpha proteobacteria, delta proteobacteria (sulfate reducers), Gram positives and *Cytophaga-Flavobacterium* types. Ohkuma and Kudo (1996) have confirmed the presence of proteobacteria (enterics and sulfate reducers), spirochetes, *Bacteroides*, and Gram positives. In particular the spirochetes are unique and diverse relatives of *Spirochaeta* and *Treponema* (Berchtold et al, 1994; Ohkuma et al 1999a). Nitrogen fixing bacteria (Ohkuma et al 1999b) and methanogens Ohkuma et al 1999c) have also been identified. Bauer et al (2000) have determined the presence of lactic acid fermenters.

Other bacterial studies have been successful in deciphering the relationships in the hindgut community. A notable example is the on-going research of Breznak (reviewed in Breznak, 1982) concerning the cycling of nitrogen. Xylophagous diets are exceptionally poor in nitrogen with perhaps only 0.03-0.1% nitrogen available in wood. In many different experiments nitrogen fixing bacteria have been sought and found and presumed to provide a significant part of termite nutrition. Furthermore termites are known to conserve nitrogen by recycling of their wastes, tissues, shed exoskeletons, dead termites, and dead microbes. Bacteria that metabolize uric acid also are present and are assumed to contribute to the conservation of nitrogen.

4. Identifications of the Protistan Symbionts

Three major phylogenetic groups of protists are found in termite hind guts: Hypermastigida and Trichomonadida (both of which are within the Parabasalia) and Oxymonadida (as reviewed in Ohkuma et al 2000) Trichomonadida is an excellent example of the adaptive radiation that has occurred in termite protists. The group includes trichomonads, monoceromonads, devescovinids, and calonymphids. The former two are found in many habitats while the latter two (along with closely related hypermastigotes) are exclusive to termites and are astounding in the diversity of their sizes and shapes (as reviewed by Ohkuma et al 2000 and as catalogued by Yamin 1979 much of that based on Kirby e.g. 1941, 1942 a,b, 1945,1949)

The phylogeny of the protistan symbionts continues to be sorted out with the aid of the polymerase chain reaction and DNA sequencing and probing. Again the work from the lab of Kudo and Ohkuma has been voluminous and a welcome supplement to the morphological studies of the past. Through sequencing studies the oxymonads have been determined to be on a deep branch of the eukaryotes (Moriya et al 1998). Furthermore, Kudo et al (1998) and Dacks and Redfield (1998) found some hypermastigotes and trichomonads (the parabasids) to be monophyletic on a deep

branch of the eukaryotes. However because the parabasalids are both numerous and diverse they have been more difficult to completely decipher (Keeling et al 1998). For example, Ohkuma et al (2000) did considerable work to establish a family tree for parabasalids but concluded that there are still ambiguities and gaps in the data.

5. Practical Laboratory Techniques with Termites

Termites and their hindgut occupants are among the easiest anaerobic systems to maintain and study in the lab. Termites are highly accessible model systems for a diversity of studies in microbial ecology, symbiosis, and evolution. Even beginning level students can quickly learn the techniques and perform meaningful experiments. (Dyer, 1997)

A typical termite hindgut community can be an astonishing sight to a first-time viewer and continues to be so even for experienced researchers. The community swarms with a diversity of protists and bacteria, some of remarkably large size and with vigorous movements, whirling, spiraling, and gliding past each other. It is well worth the trouble of acquiring termites and preparing them for the light microscope. Termites may often be purchased through biological supply houses and maintained on dampened woods or paper. Alternatively, local termites may be sought by lifting up rotting boards and logs or even by consulting with an exterminator.

The best choices for study are worker termites, the most abundant members of the colony, relatively undifferentiated and often with darkened abdomens full of wood pulp. Have ready a saline solution of 0.6% NaCl (a modified Trager's solution) and a slide and coverslip. Grip a worker termite with forceps by the head and hold it in a drop of saline on a slide. Cut off the head with a needle probe and then grip the anterior end of the decapitated body with the forceps. With a second pair of forceps or with a needle probe, grip the posterior segments and gently draw the two instruments apart, pulling out the hindgut. (Failing that, simply mince up the entire abdomen to make a messier but still useful preparation.) The drawn out hindgut should look quite enlarged and brown compared to the rest of the digestive system; it may even pulse a bit as a result of the turmoil of microbial activity within. Immediately mince the hindgut and place a coverslip on it. Many of the microorganisms are not tolerant of oxygen. Sealing the coverslip edges with petroleum jelly is an option for a longer term preparation. On the other hand, protists dying of oxygen will temporarily display structures not obvious when they are in a more active state, (e.g. Dyer, 1997)

6. Relationships in the Termite Hindgut: Who is doing what?

Analyzing the various niches and relationships of bacteria and protists of termites is like taking apart a complex puzzle and trying to understand the roles of the various pieces. One research approach has been the selective use of antibiotics and other inhibitors to remove one or a few types of microbes at a time and to observe the effects on the remaining community and on the wellbeing of the termite. Such studies are facilitated by the willingness of many termites to be cultured on filter paper moistened with antibiotic solutions thus administering their own doses. (e.g. Grosovsky, 1983) Among other observations, it has been noted that many members of the microbial community

are likely to be commensal (not strictly symbiotic), taking advantage of the species richness and the constant input of digested wood. Other microorganisms are clearly essential as for example the many bacteria involved with nitrogen fixing and cycling (Breknak, 1982) as well as for the crucial digestion of cellulose.

Termites may also consume redox dyes and pH dyes on filter paper, thus presenting colorful indications of the states of their digestive tracts. (e.g. Dyer, 1997 for how this method as well as antibiotic studies can be used in the classroom). With such studies, it has been noted that the hindgut is quite anoxic at least on a macroscopic scale. However, it has long been noted by researchers who spend hours looking at slide preparations of hindgut symbionts that not all members of the community are readily poisoned by oxygen. There seems to be a range of strict anaerobes to facultative anaerobes to even aerobes or at least microaerobes. Indeed, more focused studies have revealed that there is a steep gradient of oxygen in the hindgut, with the microbe-covered walls being relatively oxic and the center of the lumen being anoxic. Thus the community interactions are quite complicated spatially with methanogens coexisting in a confined area with consumers or scavengers of oxygen. (e.g Berchtold *et al*, 1999; Brune and Friedrich, 2000) Fenchel and Finlay (1995) have calculated that the enlarged hindguts of termites may be at a sort of lower limit of surface to volume ratio by which any sort of anoxic conditions could be preserved in an oxygen-rich atmosphere.

Analyses of the relationships of the symbionts are of course facilitated by electron microscopy, either TEMS of serial gut sections or SEMS of selected areas. Many such studies have confirmed the importance of location which is not at all obvious with crude preparations for the light microscope in which the organisms are dislodged from their positions. For example, Berchtold *et al* (1999) working on *Mastotermes darwiniensis* reported that many of the bacteria are found in close association with protists and that a dense biofilm of organisms is attached to the gut walls. Fenchel and Finlay (1995) discussed the importance of consortia in low oxygen environments especially for the transfer of hydrogen. Such relationships are often facilitated by intimate spatial relationships of the partners, some of which are described in the next section.

7. Symbionts of Symbionts: Bacterial-Protistan Relationships

Protists of termite hindguts are themselves, excellent candidates to be hosts of symbionts. And indeed many protists can be found with symbiotic bacteria on and in their cells. Two protist-bacteria symbioses (with methanogens and with spirochetes) are particularly easy to detect. Many protists have methanogens as internal symbionts. By using a fluorescent microscope set at a wavelength of about 420 nm, the F420 complex of methanogens can be made to fluoresce a blue-green color. Methanogens of termites whether free-living in the hindgut or as symbionts of protists are major sources of biogenic methane in the atmosphere (e.g. Fenchel and Finlay, 1995)

Spirochetes are found in abundance in most termite hindguts. Most seem to be free living or casually associated with protists. Leadbetter *et al* (1999) hypothesised that some acetogenic spirochetes may be attracted to protists because of the possibility of hydrogen transfer, typical in many anaerobic environments. Some spirochetes attach to protists and depending upon their numbers and the regularity of their attachments, the spirochetes may act as the mechanism for motility of their host. In the best studied of these symbioses, *Mixotricha paradoxa* of the Australian termite *Mastotermes*

darwiniensis, the spirochetes form distinctive holdfasts apparently in cooperation with their host. The spirochetes beat in metasynchrony conferring a stately forward motion to their host. (Cleveland and Grimstone, 1964) Coordination of spirochete motility is not just a characteristic of symbioses nor is such coordination likely to be due to some control mechanism. In general, when free living spirochetes are present in dense numbers they synchronize their movements perhaps because harmonious beating takes less energy than movements with frequent collisions. That is, there may be no particular mechanism for the synchrony than that it is easier than a synchronous.

Termite hindguts are really the only places in which motility symbioses have been reported reliably. The possible reasons for this have been the subject of speculation as follows (e.g. Dyer and Obar, 1994 and Dyer, 1996 abstract). Fermentation is the predominant form of metabolism for the protists of the community. Compared to respiration, fermentation yields relatively few ATPs. Perhaps conservation of ATP has been a selection pressure for some fermenters that have acquired motility symbionts. As for the spirochetes, symbioses provide an opportunity to permanently coordinate movements, thus perhaps conserving energy for the spirochetes themselves. Metasynchronous motility is more efficient than that of single structures. Regularly attached spirochetes may even gain some of the benefits of conserved energy known to occur in schooling fish, migrating birds in V formation and human racers of all kinds drafting off each other. That is spirochetes positioned at optimal angles and distances in respect to each other may expend less energy than if swimming freely. (Dyer and Obar, 1994)

Another possible reason for motility symbioses to have been reported almost exclusively in termites may be a practical one. Termite hindguts are one of the most accessible anaerobic communities. By culturing termites, one can maintain the microbes under fairly natural conditions and have ample opportunity to study the system.

Not all motility symbioses involve spirochetes. Tamm (1982) described a devescovinid, made motile by its flagellated rod-shaped bacteria. One other type of symbiosis may be observed readily in the termite *Zootermopsis* which harbors the protist *Streblomastix strix* which is itself covered with rod shaped bacteria. This seems to be a sensory symbiosis in that *Streblomastix*, devoid of symbionts fails to move efficiently toward a source of acetate (Dyer and Khalsa, 1986).

8. Termite Hindguts as Model Systems for the Study of Evolution

8.1 THE POSSIBLE SIGNIFICANCE OF MOTILITY SYMBIOSSES

Symbioses between spirochetes and protists are fascinating in their own right, as described in the previous section. However motility symbioses, may have a greater significance to the understanding of cell evolution. Some researchers have hypothesized that the origin of eukaryotic motility organelles (e.g. cilia, sperm tails) was as spirochetes that began their relationship as motility symbionts of anaerobic hosts. This chapter is not the place to elaborate on that hypothesis. (For further reading on that topic please see Margulis, 1993 and Dyer and Obar, 1994)

8.2 HYPERSTROPHY OR GIANTISM ASSOCIATED WITH SYMBIOSSES

Kirby's nearly exhaustive cataloguing of protists of termites reveals an interesting trend in the evolution of protist-spirochete symbioses (as analyzed by Dyer, 1996 abstract based on Kirby, 1941.1942 ab, 1945, 1949). Whenever a protistan lineage has acquired bacterial (especially spirochete) symbionts, the host-protists have hypertrophied or increased in size. The devescovinids have many good examples in *Foania*, *Pseudodevescovina*, *Devescovina*, and *Caduceia*. *Foania* is a genus in which the larger species, 30-60 micrometers in length, are mostly those with spirochetes. Relatively few *Foania* of 10-20 micrometers have spirochetes and those that do have sparse numbers of them. However, *Pseudodevescovina* seems to be on the same lineage as *Foania* and is characterized by giantism (70-110 micrometers long) and mostly dense coverings of spirochetes. A similar trend is shown in the lineage of *Devescovina* and *Caduceia* in which *Devescovina* (20-80 micrometers) is sparsely covered in spirochetes while *Caduceia* are often giants of over 90 micrometers and are well covered in spirochetes. One of the more famous examples of giantism accompanied by a dense population of symbiotic spirochetes is *Mixotricha paradoxa* of the canonical motility symbiosis. This behemoth of about 500 micrometers, visible to the naked eye, is essentially an overgrown trichomonad, group more typically characterized by tiny cells.

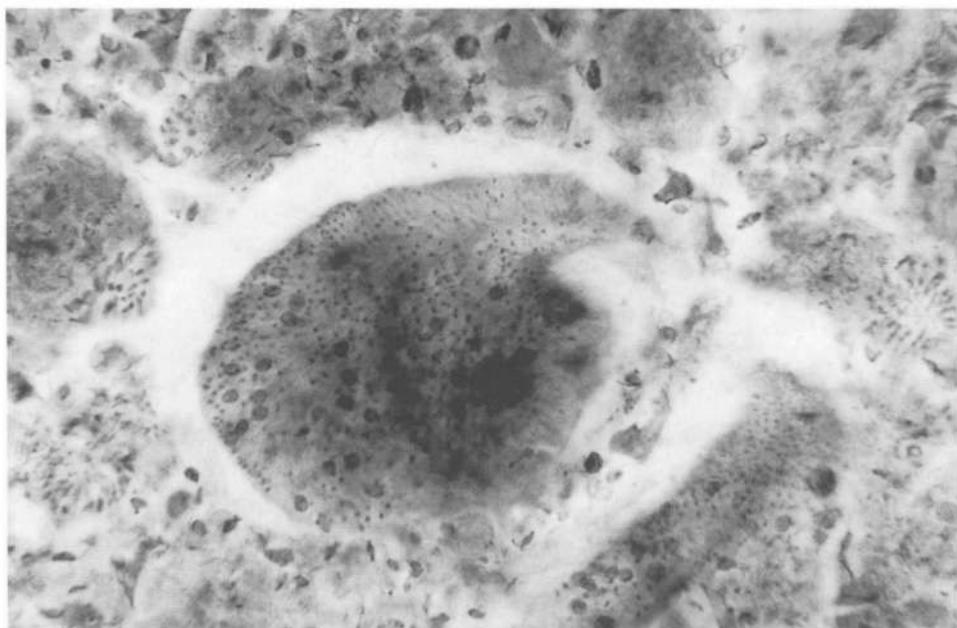


Figure 1 *Caduceia kofoidi* (photographed from Kirby collection TP58010, American Museum of Natural History). This is essentially a very large trichomonad (48-81 micrometers x 24-42 micrometers). It is covered with spirochetes and rod-shaped bacteria, typical of large devescovinids. Very few have been studied live, but a reasonable hypothesis is that many large devescovinids may be partners in motility symbiosis.

8.3 AMITOCHONDRIATE PROTISTS

Termite hindguts are the best systems for the study of some of the deep branches of eukaryotic evolution. In fact there are no other systems as convenient and ready to use in the lab by which a large and diverse assemblage of amitochondriate protists might be studied. Thus several researchers interested in the origins of eukaryotes have focused on termite symbionts. (for example, Margulis, 1993 and Dyer and Obar, 1994)

8.4 THE DIVERSITY OF EUKARYOTIC SEXUALITY

L.R. Cleveland left a considerable body of work on observations of reproduction and sex in termite protists (such as Cleveland, 1947). Unfortunately, little of it has been continued in the lab by other researchers and with just a few exceptions is seldom cited. Nevertheless the origins of eukaryotic sexuality are likely to be found among the deep-branched, amitochondriate termite symbionts some of which display an extraordinary array of variations on meiosis. For reviews and analyses of Cleveland's work as it pertains to the origins of sex see Margulis (1993) and Dyer and Obar (1994).

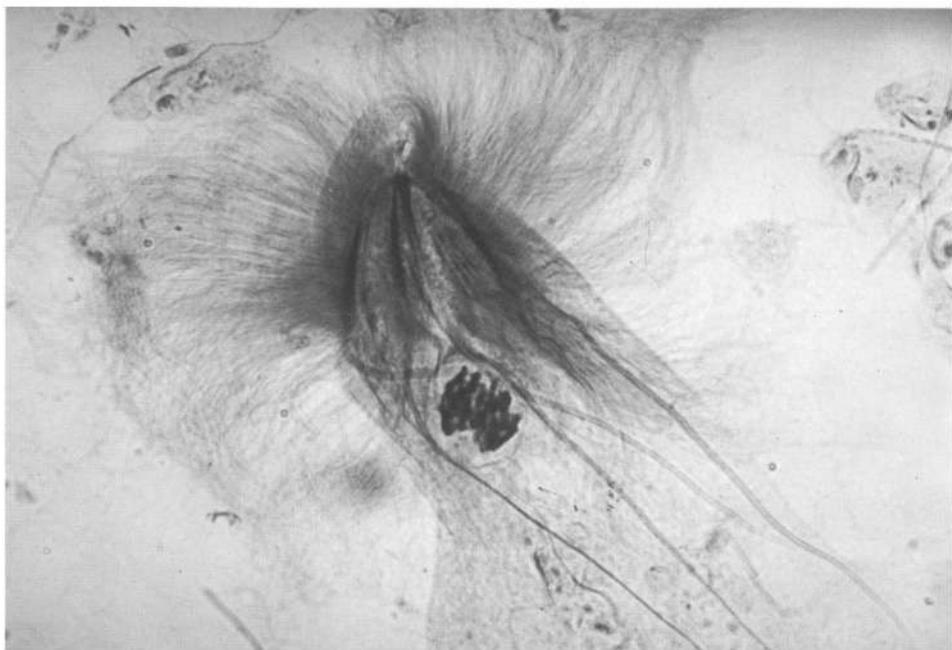


Figure 2 *Staurojoenina assimilis* (photographed from Kirby slide TP163 in the possession of the author) This is one of many hypermastigotes that display large condensed chromosomes throughout the cell cycle, thus enabling many of the observations of Cleveland on cell division

8.5 THE MULTIPLICITY OF CELL UNITS

Harold Kirby produced a wealth of information on termite symbionts in the form of monographs, mostly published by University of California and a large slide collection now archived (and accessible to researchers) at the American Museum of Natural History in NYC. In addition to being an inspiration, Kirby's work continues to be a valuable source of data and methodical, detailed observations for interpretation. A case in point is work both published and in progress about an evolutionary trend by which single nucleated cell units such as are typical of trichomonads are multiplied in related protists such as in the calonymphids. Some of the evolutionary significance has been analyzed by Margulis in a posthumous publication of Kirby (Kirby, 1994) and by Dolan using Kirby's collection at AMNH (Michael Dolan, personal communication and Dolan, 2000).

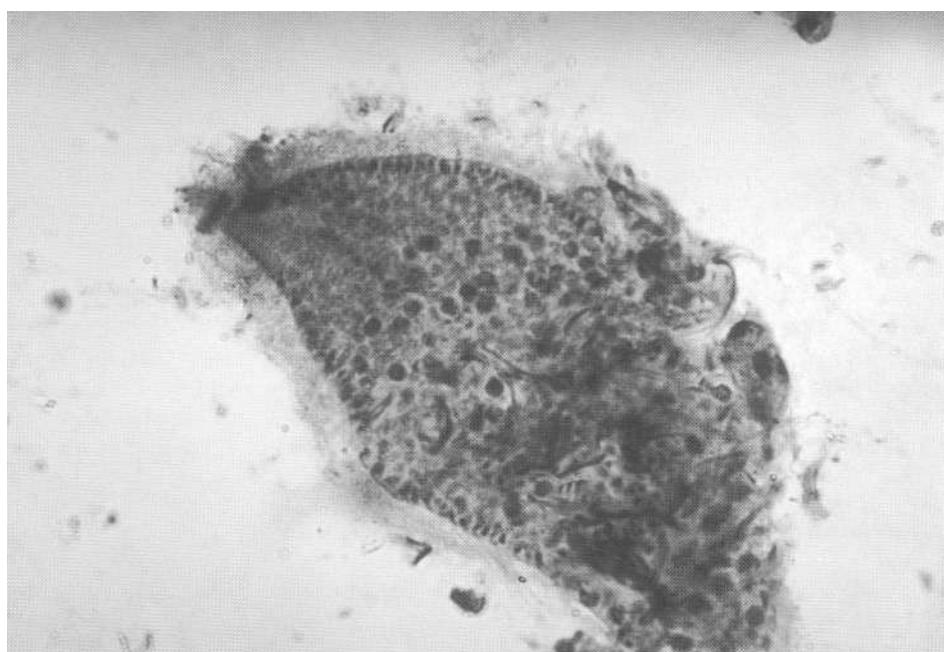


Figure 3 *Snyderella tobogae* (photographed from Kirby slide TP137 in the possession of the author). This is a multinucleate assemblage of hundreds of trichomonad units. This cell is also covered with spirochetes.

8.6 THE FLUIDITY OF MEMBRANES

A fascinating study by Tamm revealed the extreme fluidity of the membrane of a devescovid. The anterior of the cell was observed to rotate steadily and continuously in a clockwise manner along a "shear zone" and without any rewinding. (Tamm, 1979;

Tamm and Tamm, 1983) This observation has remained a sort of "orphan" in that its significance for membranes in general and possibly for the origins of membrane fluidity in early eukaryotes, has seldom been considered except by those in the community of termite researchers. The assemblage of deep-branching protists of termites is in general an underutilized (in spite of being exceptionally convenient) resource for the study of eukaryotic evolution.

8.7 THE PHENOTYPES OF THE TERMITES THEMSELVES

It is easy for researchers concentrating on the hindgut symbionts of termites to almost completely lose consciousness of the termites themselves except as extremely convenient and easy to maintain containers for symbionts. However termites, in their own right, are indeed fascinating subjects for study. One example, within the focus of this chapter, is the way in which termites have co-evolved with their occupants such that distinctive termite characteristics may be viewed as a sort of cooperative phenotype (Dyer, 1989). The sociality of the termites may well be an adaptation for the efficient transfer of symbionts, a process accomplished in various ways including proctodeal feeding of sterile immatures by symbiont-rich workers. The caste system of termites, by which their status as social is elevated to *eusocial*, may be in part a consequence of limited nitrogen. Nitrogen is essential for reproduction but even with nitrogen fixing symbionts and nitrogen recycling this nutrient is limited in termite colonies. The solution may be a centralization to a reproductive pair of whatever excess nitrogen compounds are available and transferable as symbiont-rich gut fluids. (as reviewed in Dyer, 1998) The very shape of termites, with their enlarged abdomens, rotund with symbiont-packed hindguts is certainly a consequence of symbiosis. The ability to consume and digest wood efficiently as a primary diet is extraordinary and in the case of the lower termites a consequence of their internal symbionts. Thus the major phenotypic characteristics of termites in general: colonial eusociality, body shape, unusual feeding practices and a diet of wood are in large part manifestations of symbiosis.

9. Acknowledgements

I thank Phyllis Bradbury, a former student of Harold Kirby who gave me her collection of slides when she retired. I am also grateful to the American Museum of Natural History which houses most of the Kirby collection. I appreciate the work of researchers like Michael Yamin, author of the definitive catalogue of termite symbionts who are organizing the collection.

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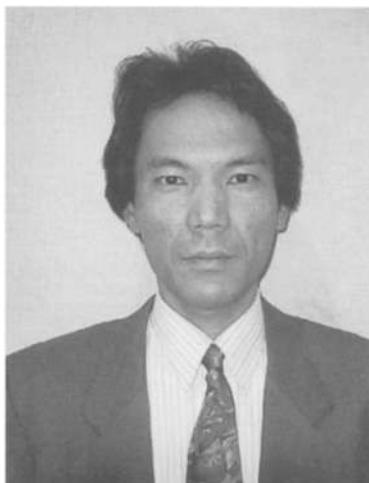
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SYMBIOSIS IN THE TERMITE GUT

Culture-Independent Molecular Approaches

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1. Introduction

Termites harbor a dense and diverse population of microorganisms in their gut. The relationship between termites and the gut microorganisms is a well-known example of symbiosis. Early studies indicated that the presence of cellulolytic protists in the gut is critical to termite survival on a diet of wood or cellulose, and that the protists produce acetate as an energy and carbon source for the termites. The metabolism of lignocellulose, carbon and electron flow in the gut, and the roles of the symbionts in host nutrition have been extensively studied. A large number of studies involving pure cultures of symbionts have contributed to the remarkable progress. Consequently, in addition to lignocellulose digestion, several beneficial roles of the gut symbionts have been revealed so far, such as methanogenesis and acetogenesis from H₂ plus CO₂, nitrogen fixation, recycling of uric acid nitrogen, and so on. A number of excellent reviews are available for more background information (Breznak 1982, 1994; Breznak and Brune 1994; Brune 1998; Brune and Friedrich 2000; O'Brien and Slaytor 1982; Radek 1999; Slaytor and Chappell 1994).

The application of molecular methodology to ecological studies in the past decade has enhanced our ability to assess naturally occurring biodiversity. It is well accepted that in culture-based evaluation only a limited number of inhabitants are sampled, thereby underestimating the real diversity of the microbial assemblages. Culture-independent approaches to investigate a mixed microbial population as a whole are considered advantageous. In these studies, small-subunit rRNA genes (SSL) rDNA in nucleic acid extracted from natural microbial communities are frequently analyzed. Such culture-independent approaches have been applied to analysis of the symbiotic community in the termite gut and have shed new light upon the symbiosis within the termite gut. In this review, we outline the recent advances in this field, especially focusing on the culture-independent studies of the molecular phylogeny of the symbionts.

2. Symbiotic protists as early branching eukaryotes

So-called lower termites harbor both flagellated protists (eukaryotes) and a diverse array of prokaryotes in their gut. The flagellated protists belong to the orders Hypermastigida, Trichomonadida, and Oxymonadida. The former two are classified into the phylum Parabasalia. While trichomonads are found in a variety of habitats and many of them have been cultured, hypermastigotes and oxymonads are unique in nature in that their occurrence has been documented only in termites and wood-eating cockroaches. Before 1995, studies based on molecular sequences began to establish the phylogenetic positions of trichomonads including the gut-dwellers of termites (Berchtold and König 1995; Gunderson et al. 1995). Until recently, however, because of a lack of molecular sequence data for hypermastigotes and oxymonads, there was uncertainty about their phylogenetic positions, particularly in relation to early eukaryotic evolution.

The first molecular sequence data on SSU rDNA from hypermastigotes were identified as sequences originating from members of the family Trichonymphidae (Ohkuma et al. 1998; Dacks and Redfield 1998; Keeling et al. 1998; Fröhlich and König 1999), and subsequently those of the families Eucomonymphidae, Spirotrichonymphidae, and Holomastigotidae (Ohkuma et al. 2000). These sequences were obtained from a mixed population of gut protists, and the origins of the sequences were identified by whole-cell *in situ* hybridization using sequence-specific probes. Also, a number of parabasalian SSU rDNA sequences have been detected in DNA from the gut protist community, but their origins have not been assigned yet. In agreement with morphology-based classification, the hypermastigotes show a sister group relationship with trichomonads. Although Trichomonadida seems to be a monophyletic group, the hypermastigotes form paraphyletic lineages. According to the results of tree-root analysis (Ohkuma et al. 2000), trichomonads have emerged within some lineages of the hypermastigotes. Hypermastigotes are distinguished from trichomonads by having a large number of flagella, and they show a more complex cellular morphology than trichomonads. Based on the evolutionary relationship inferred from the results of analysis of molecular sequence data, loss of cytoskeletal structures and reduction of the number of flagella may have occurred during parabasalian evolution. The results of SSU rDNA sequence analysis have revealed that parabasalids are one of the earliest branching groups of eukaryotes.

The genes for elongation factor-1 α (EF-1 α) have been identified in two oxymonads, *Dinenymphia exilis* (Moriya et al. 1998) and *Pyrsonympha grandis*, and a hypermastigote *Trichonympha agilis* (Moriya et al. 2000). Figure 1 shows a phylogenetic tree based on EF-1 α . A sister group relationship between trichomonads and the hypermastigote is also evident in this case. Parabasalids together with diplomonads, and probably oxymonads, represent earlier extant lineages of eukaryotes, although oxymonads are grouped with recently emerging groups of eukaryotes. All of them lack mitochondria, while parabasalids contain instead an anaerobic metabolic organelle called a hydrogenosome. Oxymonads and diplomonads seem to have neither mitochondria nor hydrogenosomes, and thus a close evolutionary relationship between

them has been proposed. However, a specific sister relationship between oxymonads and diplomonads has been ruled out. Oxymonads and parabasalids are distantly related, although in addition to their amitochondriate nature they share a similar cytoskeletal structure of huge bundled microtubules, called an axostyle. A distant evolutionary relationship of their microtubule systems is also evident as shown by phylogenetic analysis based on protein sequences of α -tubulin (Moriya et al. 2000).

Up to now, only a limited number of symbiotic protists have been cultivated *in vitro*, thus, there have been few biochemical or molecular biological studies examining these protists. In the case of lower termites, the gut protists are important for decomposition of the ingested cellulose as well as hemicellulose (Inoue et al. 1997), but the so-called higher termites typically lack protists. Cellulases (endo- β -1,4-glucanases) of termite origin, which are excreted from the salivary glands or the mid-gut, have been identified and characterized in both lower and higher termites (Watanabe et al. 1998; Tokuda et al. 1999; and references therein). Probably, a substantial amount of the ingested cellulose can be degraded by the cellulases of termite origin, and the cellulose not hydrolyzed in the anterior portion of the gut then travels to the hindgut, where it can be endocytosed

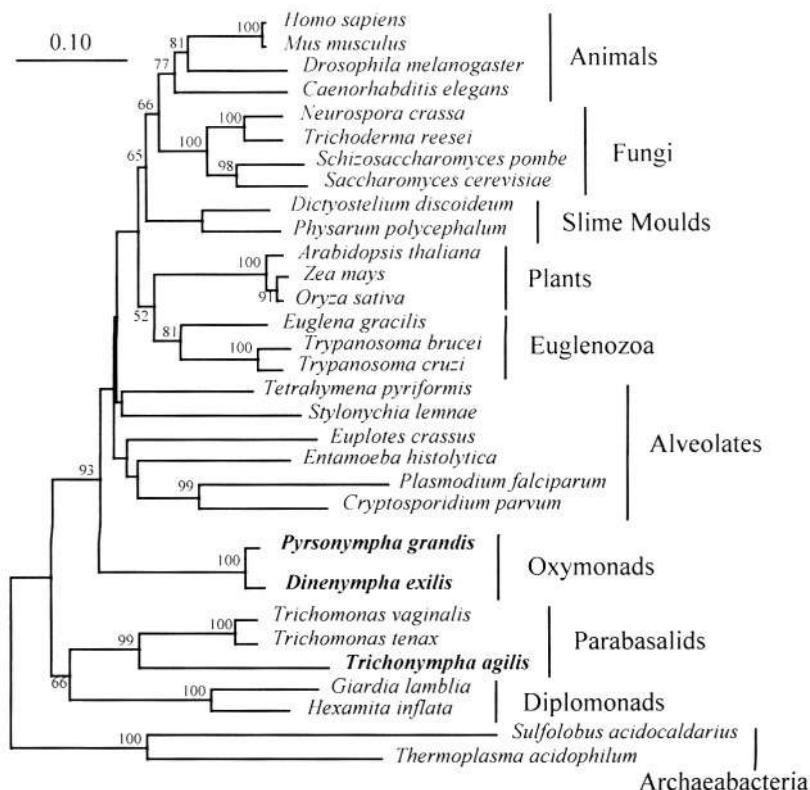


Figure 1. A large-scale eukaryotic phylogeny inferred from EF-1 α amino acid sequences. The tree was inferred by the neighbor-joining method. Numbers at nodes indicate bootstrap proportions (only values above 50 are shown). The scale bar represents 0.10 substitutions per amino acid position.

and fermented by the symbiotic protists in lower termites. The existence of this dual system in lower termites explains their capacity to assimilate wood-glucan to an extent greater than 90%. However, the degradation system of the symbiotic protists remains to be characterized, and studies involving culture-independent identification of cellulase genes of protist origin are now in progress (Ohtoko et al. 2000).

3. Methanogenic archaea in the termite gut

Methanogenic archaea inhabit the gut of termites, and methane emission from termites has often been claimed to be a significant source of global atmospheric methane. The molecular phylogeny of methanogens in the gut of various termite species has been investigated without cultivation, using the whole-gut contents as the starting material (Ohkuma et al. 1995, 1999b; Ohkuma and Kudo 1998; Shinzato et al. 1999). Most of the sequences from lower termites were related to the genus *Methanobrevibacter* whereas the sequences from higher termites were related to the orders Methanosarcinales or Methanomicrobiales in addition to the genus *Methanobrevibacter*. In general, higher termites emit more methane than lower termites (Brauman et al. 1992; Sugimoto et. al. 1998).

From the gut of the lower termite, *Reticulitermes flavipes*, distinct types of methanogen species have been isolated and identified as three novel species of *Methanobrevibacter*, *M. curvatus*, *M. cuticularis*, and *M. filiformis* (Leadbetter and Breznak 1996; Leadbetter et al. 1998). These species are morphologically similar to the cells residing on or near the gut epithelium. In addition to the association with the gut wall, methanogens are also known to occur on and within the cells of the symbiotic protists. Methanogens free in the gut fluid are rarely observed at least in lower termites. The endosymbiotic methanogens of the oxymonad *Dinenymphida* (Figure 2 A and B) and those of the hypermastigote *Microjoenia* phylogenetically identified in the gut of *Reticulitermes speratus* and *Hodotermopsis sjostedti* (Tokura et al. 2000). The cells of these protists were carefully collected by means of a micromanipulator-aided microcapillary and the archaeal SSU rDNA was amplified and analyzed. Also, after careful fractionation of the gut epithelium, the resident methanogens were phylogenetically investigated in both termites. The sequences identified are classified into several distinct groups within the genus *Methanobrevibacter*, including groups of yet-uncharacterized novel phylotypes.

All known members of the genus *Methanobrevibacter*, including three isolates from the termite *R. flavipes*, utilize H₂ plus CO₂, but other substrates are poorly utilized. It is likely that endosymbiotic methanogens remove hydrogen produced by host-protists, resulting in stimulation of fermentation in the host. In lower termites, however, methanogenesis contributes only a small fraction of the carbon and electron flow in the gut fermentation, as CO₂-reducing acetogenesis is predominant (Brauman et al. 1992; Breznak 1994). This feature of the termite system deserves special attention since in most anoxic habitats methanogenesis is almost always predominant rather than acetogenesis. At least in the case of *R. flavipes*, in which protist-associated

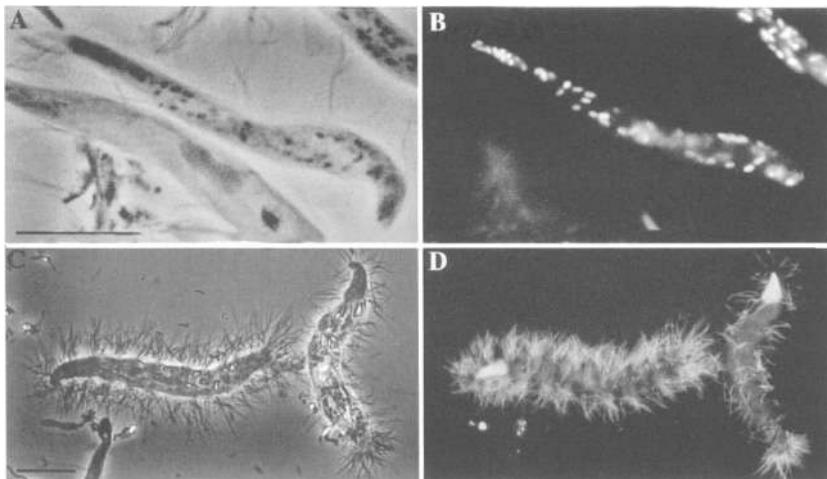


Figure 2. Phase contrast (A) and epifluorescence (B) images of cells of the oxymonad protist, *Dinenymphya parva*. Endosymbiotic methanogens are visible due to autofluorescence of F₁₅₀. The bar represents 10 μ m. Phase contrast (C) and epifluorescence (D) images of cells of the oxymonad protist, *Dinenymphya porteri*. The cells of ectosymbiotic spirochetes were visualized by DAPI staining. The scale bar represents 10 μ m.

methanogens are rarely observed, it is explained by a difference in the distribution of the respective microbial populations and by the presence of a gradient of hydrogen partial pressures in the gut (Brune 1998).

4. Remarkable diversity of symbiotic spirochetes

Spirochetes are one of the most abundant, consistently present, and morphologically distinct groups of bacteria present in the termite gut. Spirochetes rarely occur in nature in a density and morphological diversity as great as that in the gut of termites. Recently, pure cultures of the gut spirochetes have been obtained and these organisms have been shown to catalyze the synthesis of acetate from H₂ plus CO₂ (Leadbetter et al. 1999). This finding helps to explain the predominance of acetogenesis rather than methanogenesis in the termite gut.

The molecular phylogeny of the gut spirochetes in various termite species has been investigated without cultivation of these organisms (Berchtold and König 1996; Lilburn et al. 1999; Ohkuma and Kudo 1996, 1998; Ohkuma et al. 1999a). All of them including the isolates from the termite are affiliated into the genus *Treponema*, but none of them is closely related to any known species. They are divided into two discrete clusters (cluster I and II, see Figure 4). Within the gut of *R. flavipes* alone, no less than 21 different spirochete phylotypes have been recognized as novel species (Lilburn et al. 1999). Although some sequences from evolutionary related termites show close similarity, identical or closely similar sequences rarely occur among the termite genera examined so far, and spirochetes from a single termite species occur in several distinct phylogenetic positions (Lilburn et al. 1999; Ohkuma et al. 1999a). This observation is

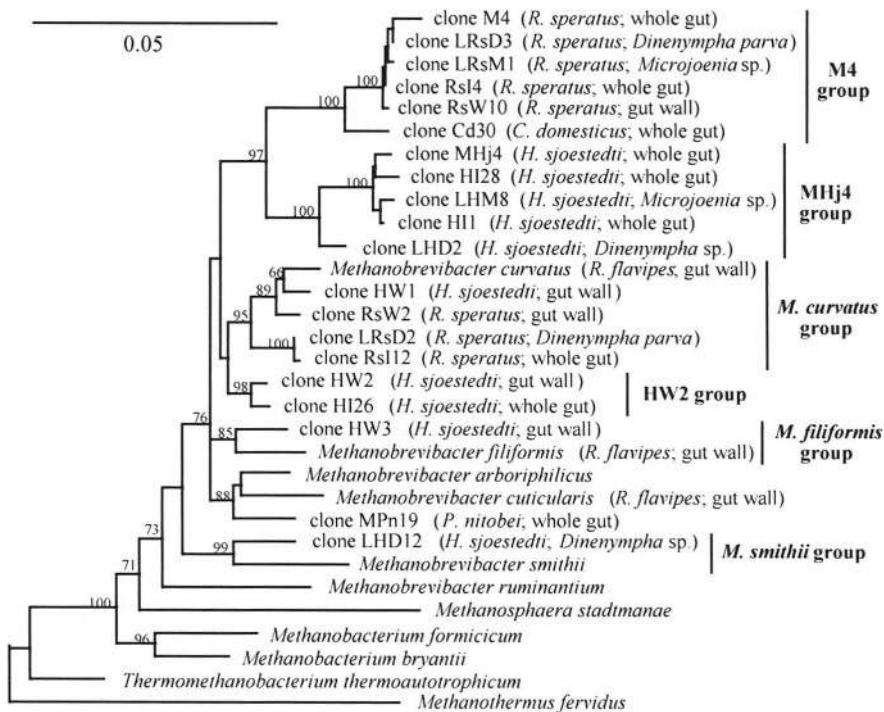


Figure 3. Phylogenetic relationship of methanogens in the gut of termites, *Reticulitermes speratus* and *Hodotermopsis sjoestedti*, among the members of Methanobacteriaceae. The tree was constructed by the neighbor-joining method based on comparison of SSU rRNA sequences. Numbers at the nodes indicate the bootstrap values and the scale bar represents 0.05 substitutions per nucleotide position. The host termites and the fractions from which the sequences were derived are indicated in parentheses. The probable species-level groupings are also indicated on the right side of the tree.

inconsistent with a simple evolutionary scenario that one spirochete species was acquired by an ancestor of termites. Rather, it may suggest that phylogenetically distinct species of spirochetes have been acquired during termite evolution, probably independently in the course of time, by diverse termite species, and then they have evolved within the gut to the present diversity.

It is well known that spirochete-like bacteria are attached to the surface of some protists in the termite gut. At least one case of ectosymbiosis results in a spectacular motility symbiosis. The ectosymbiotic spirochetes have been phylogenetically identified in the oxymonads *Dinenympha* (Figure 2 C and D) and *Pyrsonympha* in the gut of *R. speratus* and *H. sjoestedti* (Iida et al. 2000). The cells of the protists have been collected with the aid of a micromanipulator, and spirochetal SSU rDNA sequences were identified in both clusters I and II (Figure 4). Cluster II of termite spirochetes is clearly divided into two groups (clusters IIA and IIB). A group-specific probe for cluster II can detect a large population of ectobionts of those oxymonads by whole-cell *in situ* hybridization, and interestingly, the proportions of ectobionts

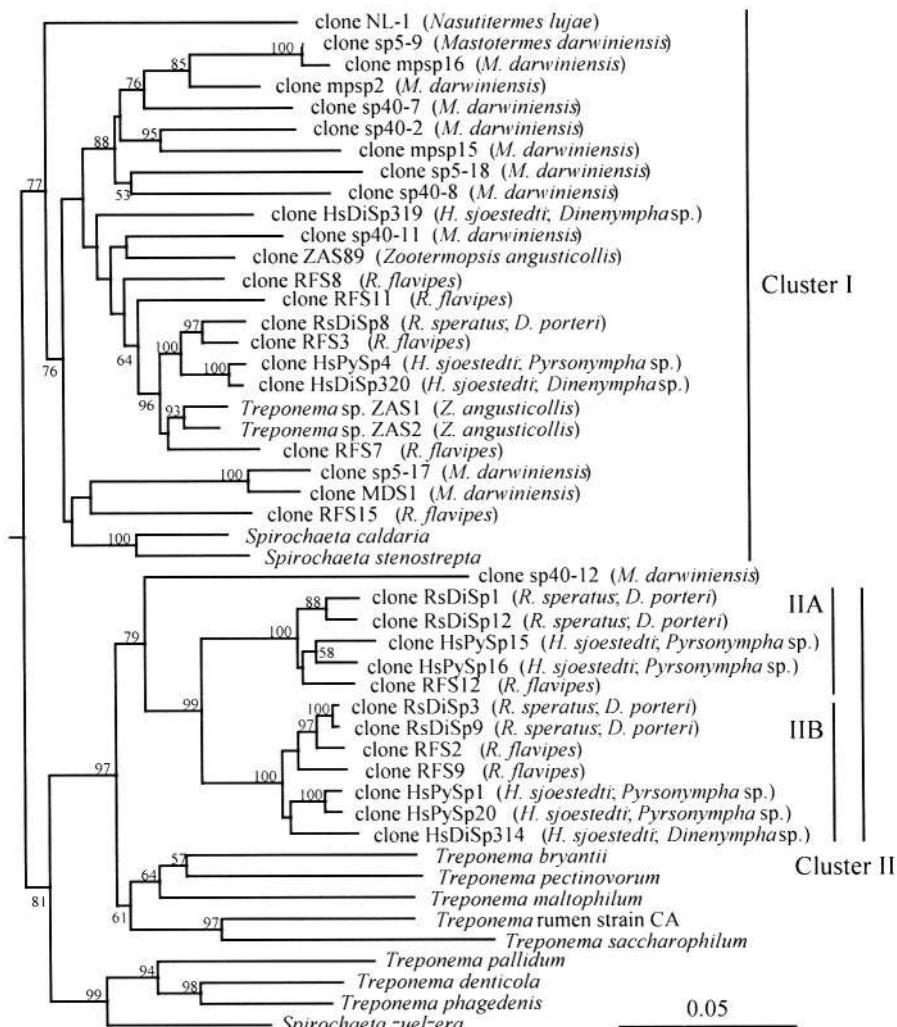


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belonging to cluster II differ among protist species. No spirochete-like cells free in the gut fluid can be detected using this probe. A sequence-specific probe for the members of cluster IIB also detects some of the ectobiotic spirochetes of *D. porteri*, indicating that the population of ectobionts on a single *D. porteri* cell consists of a mixture of more than three species of spirochetes belonging to clusters I, IIA, and IIB. As some spirochetes are CO₂-reducing acetogens, the symbiosis on the surface of the protists

may involve interspecies hydrogen transfer from the protists to the ectobiotic spirochetes as occurs in the case of protist-associated methanogens, but this remains to be proved.

5. The termite gut is rich in novel microorganisms

Not only the methanogens and spirochetes described above, but also a large number of new species of bacteria have been isolated and identified from termite guts. Furthermore, culture-independent molecular studies have revealed the presence of phylogenetically diverse microorganisms within the termite gut, which represent yet-uncharacterized species, unknown to microbiologists. A typical case is the analysis of the gut microbial community in *R. speratus* (Ohkuma and Kudo 1996). In addition to the protists, methanogens, and spirochetes, the SSU rDNA sequences collected are affiliated with *Proteobacteria*, low-G+C-content Gram-positive bacteria (related to clostridia), and relatives of the genus *Bacteroides*. Also, a novel bacterial group which cannot be assigned into any of the known eubacterial divisions (Hugenholtz et al. 1998) has been found. Even though the number of the sequenced clones is small, it is evident that there is significant microbial diversity within the gut of a single termite species. Groups of sulfate-reducing bacteria and lactic acid bacteria have been isolated from diverse termite species, and they are considered to be the major populations in termite guts (Kuhnigk et al. 1996; Bauer et al. 2000). Given the existence of more than 2,000 described termite species on Earth, the gut of termites may be a rich reservoir of novel and diverse microorganisms.

6. The gut community is highly structured

As described above, a large number of prokaryotes are associated with the symbiotic protists as well as the gut epithelium. In this respect, the symbiotic community in the termite gut is a complex system involving many kinds of physical interactions among members of the symbiotic community. Moreover, the termite gut develops segmented structures and/or compartments, which probably house distinct microbial populations. It has been revealed also that the termite gut is, though very small, a highly structured microenvironment with physicochemically distinct microhabitats, rather than serving as a simple anoxic fermentor (see Brune 1998; Brune and Friedrich 2000, and references therein). Studies using microelectrodes have shown the presence of steep gradients of oxygen and hydrogen within the gut. The oxygen gradient drives a continuous influx of oxygen into the gut periphery, rendering a large proportion of the gut microoxic, and hydrogen accumulates in the anoxic lumen. The presence of pH gradients in the axial direction of the gut has also been demonstrated. These gradients would have a significant impact on the microbial activities within the gut.

Recently, cryosections of the gut of *Mastotermes darwiniensis* have been analyzed by *in situ* hybridization using group-specific probes for SSU rRNA, resulting in the

detection of the *Cytophaga-Flavobacterium* group of bacteria and a group of high-G+C-content Gram-positive bacteria in the wall fraction of the posterior region of hindgut (Berchtold et al. 1999). Another study involving sequence-specific *in situ* hybridization targeting SSU rRNA in sections of the so-called mixed segment of the gut of *Nasutitermes takasagoensis* has shown that *Clostridium*-related bacteria are associated with the gut epithelium (Tokuda et al. 2000). As structural integrity is retained upon sectioning of the gut, this approach may be advantageous for *in situ* localization of the symbionts. Fractionation, especially with the aid of a micromanipulator, may also be useful to characterize the resident microorganisms (Fröhlich and König 1999; Iida et al. 2000; Tokura et al. 2000). Such approaches will provide us with more information about the spatial distribution of the symbionts within the gut.

7. Diversity of nitrogen fixation genes

Analysis of rRNA sequences has opened a window to investigate the diversity and composition of natural microbial communities, avoiding the largely unrepresentative nature of microbial cultivation. In some instances, metabolic functions of microorganisms within specific rRNA phylogenetic groups can be inferred, as in the case of methanogens, thanks to the fact that methanogens form a distinct group in the rRNA-based phylogeny. However, the physiological proportions of individual microbial population in a community cannot generally be predicted on the basis of the rRNA sequences only. Under such circumstances, genes encoding metabolically important enzymes can be useful as molecular markers. The marker, if it contains phylogenetic information, may even allow us to deduce the identity of the responsible organisms. The gene *nifH*, which encodes a key enzyme for nitrogen fixation, dinitrogenase reductase, the sequence of which is substantially conserved among diverse nitrogen fixing organisms, has been successfully applied to study the termite gut symbiosis (Ohkuma et al. 1996, 1999c; Noda et al. 1999).

Nitrogen fixation by microorganisms in the gut of termites is one of the crucial aspects of the symbiosis, since termites usually thrive on a nitrogen-poor diet. There is a case in which more than half of the fixed nitrogen in the termite is derived from atmospheric N₂ (Tayasu et al. 1994). However, there are large variations in the rates of nitrogen fixation among termite species, and even within a single termite species (Breznak 1982; Curtis and Waller 1998; O'Brien and Slaytor 1982; Slaytor and Chappell 1994; and references therein). A description of the responsible microorganisms and their contribution is necessary to understand the nature of nitrogen fixation in the termite. The *nifH* genes within the gut community have been analyzed in nine diverse termite species (Ohkuma et al. 1996, 1999c; see also Table 1). Most of the *nifH* sequences from termite guts are distinct from those previously recognized in studies using classical microbiological techniques (Figure 5). Also, there are several sequence clusters consisting of termite derived sequences only, suggesting that some diazotrophic habitats are unique in termite guts. A majority of the *nifH* sequences

from lower termites, which show significant levels of nitrogen fixation activity, can be assigned to either the anaerobic *nif* group consisting of clostridia and sulfate reducers or the alternative nitrogenase (*anf*) group. In the case of higher termites, which show only low levels of nitrogen fixation activity, a large number of the sequences are assigned to the most divergent *nif* group (pseudo-*nif*), probably functioning in some process other than nitrogen fixation. Interestingly, the *nifH* groups detected are similar within each termite family but different among the termite families, suggesting an evolutionary trend reflecting the diazotrophic habitats in the symbiotic community.

TABLE 1. Nitrogen fixation activity of termites and assignment of *nifH* clones from the gut community to phylogenetic groups.

Termite family and species	Nitrogen fixation activity ^a	Numbers of clones			
		Proteo-Cyano	Anf	Anaerobe	Pseudo- <i>nif</i>
Termopsidae					
<i>Hodotermopsis sjostedti</i>	34	0	22	1	0
Kalotermitidae					
<i>Neotermes koshunensis</i>	210	3	10	5	4
<i>Cryptotermes domesticus</i>	33	0	10	8	4
<i>Glyptotermes fuscus</i>	31	0	2	12	6
Rhinotermitidae					
<i>Reticulitermes speratus</i>	16	1	0	18	3
<i>Coptotermes formosanus</i>	79	0	0	23	1
Termitidae					
<i>Nasutitermes takasagoensis</i>	0.7	0	0	11	12
<i>Odontotermes formosanus</i>	ND	0	1	0	23
<i>Pericapritermes nitobei</i>	2.5	0	0	2	20

^aNitrogen fixation activity was measured by the acetylene reduction assay and the activity is expressed as nmol of C₂H₄ formed per hour per g wet weight of termite. ND, not detected.

8. Monitoring gene expression

Although a molecular marker gene is known to be helpful to evaluate a potential function, the mere presence of a gene does not always mean that the biological activity is being expressed. Particularly in the case of the nitrogenase gene, expression is generally strictly regulated. In fact, in spite of the existence of diverse *nifH* sequences in the gut community, some termite species exhibit only slight levels of nitrogen fixation activity. An elegant method monitoring biological activity *in situ* would be detection of the mRNA, and for this purpose, reverse transcriptase PCR (RT-PCR) is advantageous because of its high sensitivity and specificity. Through analysis of *nifH* expression in the gut of *Neotermes koshunensis* it was indeed possible to demonstrate that only a few genes encoding an alternative nitrogenase (*anf* genes) are preferentially transcribed, whereas most of the other genes are not (Noda et al. 1999; see also Figure 5). This points to an important concept in molecular microbial ecology: that not only

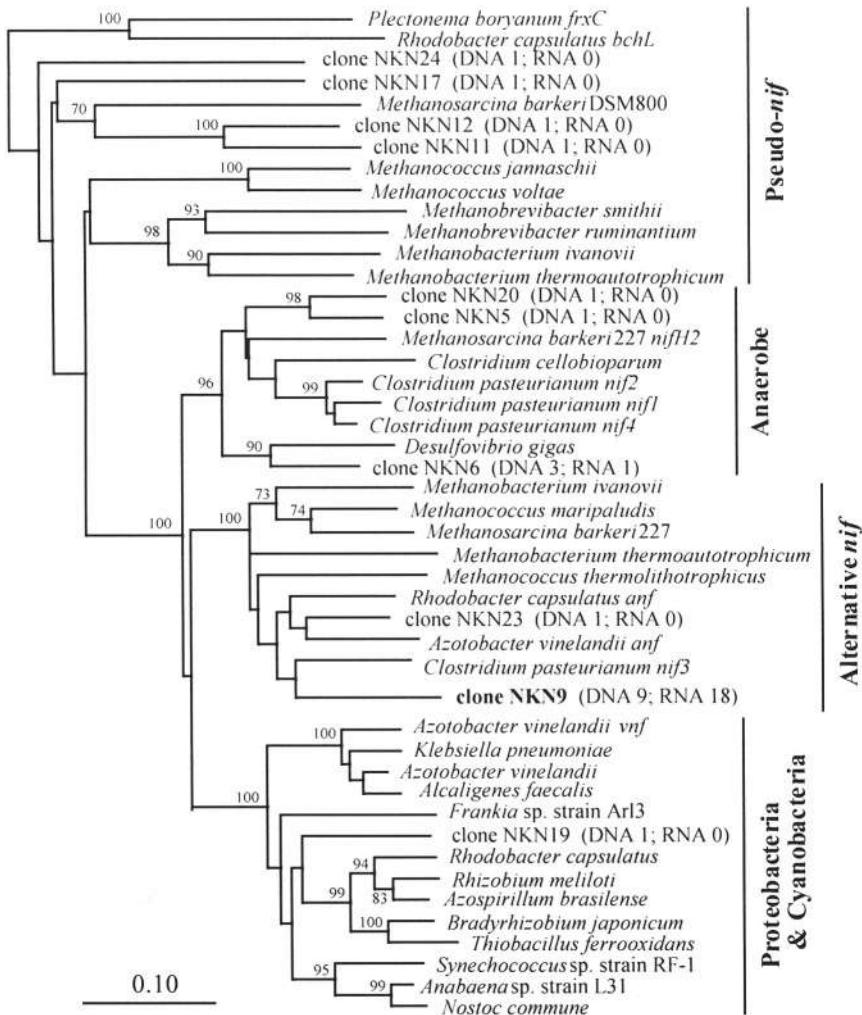


Figure 5. Phylogenetic position of the *nifH* phylotypes identified in analysis of nucleic acids from the microbial community in the gut of *Neoterpes koshunensis*. The tree was inferred by the neighbor-joining method through comparison of *nifH* amino acid sequences. Numbers at the nodes indicate the bootstrap values and the scale bar represents 0.10 substitutions per amino acid position. Within the given phylotypes, the numbers of clones isolated from the DNA and the RNA of the gut community as starting materials for PCR and RT-PCR, respectively, are shown in parentheses. The large *nifH* phylogenetic groups are indicated on the right side of the tree.

the presence of a gene but also its expression should be characterized to evaluate real microbial activity in an environmental sample.

Most of the molecular approaches depend on the PCR technique, and conventional PCR serves to amplify target DNA exponentially, making it difficult to use the technique in a quantitative manner. However, some quantitative PCR methods have

been developed, and one of them is competitive PCR, in which an internal DNA standard is added as a control to correct for the variation among reactions, allowing reliable PCR quantification. Competitive PCR has been applied to evaluate the levels of expression of mRNA from the preferentially transcribed *anf* gene in *N. koshunensis* (Noda et al. 1999). When termites are shifted from a diet of wood to filter paper, nitrogen fixation activity is stimulated. However, when shifted to a diet of filter paper containing nitrogen sources, the nitrogen fixation activity diminishes. The levels of the *anf* mRNA relative to the whole RNA content of the gut are largely congruent with the nitrogen fixation activity displayed by the termite, and the *anf* gene is always the most preferentially transcribed of the *nifH* genes. The amount of the genomic *anf* gene in the gut population shows no significant change, indicating that the level of expression of the *anf* gene is critical for nitrogen fixation activity displayed by the termite.

The gene organization of the region containing the preferentially transcribed *anf* gene in *N. koshunensis* has been determined to be *anfH*-ORF105-ORF122-*anfD*-*anfG*-*anfK* (Noda et al. 1999). This structural feature and the results of phylogenetic analysis of each gene indicate that the *anf* gene encodes a typical alternative nitrogenase of eubacterial origin, except for the presence of ORF105 and ORF122. These two ORFs are related to a *glnB*-like PII protein which probably functions in modulating the nitrogenase activity, and the presence of these ORFs in the region containing the nitrogen fixation genes is a common feature in archaea. These features indicate that a novel group of nitrogen fixing organisms is responsible for nitrogen fixation in termites.

The typical nitrogenase contains molybdenum (Mo) as a cofactor, whereas, alternative nitrogenases are Mo-independent. When a sufficient amount of Mo is available, expression of the *anf* gene is generally repressed, and only an Mo-containing nitrogenase is expressed. In the gut of *N. koshunensis*, expression of the preferentially transcribed *anf* gene is not affected by the presence of Mo in the diet of the termite (Noda et al. 1999). Nitrogen fixation activity is slightly but significantly increased in the presence of Mo, and, interestingly, expression of the mRNA of some *nifH* genes other than the *anf* gene is induced. Probably, the Mo supply is not sufficient to induce expression of the non-*anf* genes under natural condition in the termite. It seems that metal availability is probably a key factor affecting symbiotic nitrogen fixation in termites, determining the presence of nitrogen fixation genes of the *anf* group. The evolutionary trend of the *nifH* genes within the termite symbiotic communities (see Table 1) can be explained in terms of the metal availability through their feeding. The Termopsidae and Kalotermitidae termite species harbor organisms possessing the *anf* group of *nifH* within the gut communities, and they feed on wood in which they live. In contrast, the termites of Rhinotermitidae, which harbor no organisms possessing *anf* genes, show foraging behavior and live in contact with soil. The most evolved group of termites (Termitidae), displaying only low levels of nitrogen fixation activity, shows both extensive foraging behavior and variation of their diet, and then probably they acquire fixed nitrogen from their food.

9. Conclusion and remarks

The recent application of culture-independent molecular approaches provides a new way to characterize the microbial populations in the symbiotic community in the termite gut. Now that the symbiotic community within the termite gut has been shown to be highly structured, many aspects of the interactions between the host and the symbionts and among the symbionts should be studied further. Beyond the mere description of phylogenetic diversity it is necessary to characterize the *in situ* localization of individual populations, and to directly link the identity of individual cells to their functions. New probes for functional marker genes and characterization of their expression *in situ* will lead to remarkable advances. In order to discuss evolution of symbiosis within the termite gut, the structure and functions of the gut community should be extensively characterized in some model termites. A more diverse range of termite species should be analyzed. Finally, it is noted that the symbiotic relationships between the gut protists and their endobionts or ectobionts are attractive research subjects in terms of the symbiosis-accelerated evolution of eukaryotic cells, since these protists represent early emerging groups of eukaryotes.

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CULTIVATION OF SYMBIOTIC FUNGI BY TERMITES OF THE SUBFAMILY MACROTERMITINAE

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1. Introduction

We review the highly specific, but poorly resolved, obligate symbioses between basidiomycete fungi of the genus *Termitomyces* and higher termites of the subfamily Macrotermitinae, comparing this relationship with the animal/microbial mutualisms known in other kinds of termite. The fungus is grown on a structurally complex substrate, derived from primary forage and furnished by the termites, who manipulate the culture to exclude competitive fungi and then consume, at different stages of their development, both ripe conidia and senescent mycelium. *Termitomyces* composts its growth substrate, but it is unclear whether the benefit to the termite is an enrichment of nitrogen, or an enhancement of lignocellulose digestion, or both. Other benefits are possible. Biochemical and molecular data are presented which suggest that *Termitomyces* and its termite host have co-evolved and co-radiated, such that different species of the fungus make differing contributions in their particular symbiosis (mostly relating to the digestion of plant structural polysaccharides), each reflecting the ecological niche occupied by the termite host.

2. Termites as Important Insects

The notion of termites as voracious destroyers of buildings, outdoor timber constructions of all kinds and plantation trees is inaccurate (Bignell and Eggleton, 2000). The vast majority of species are innocuous decomposers of the woody and leaf litters that plants produce in abundance in all natural terrestrial ecosystems (though termites themselves are largely confined to the tropics and subtropics, and in other biomes their decomposer roles are taken by other insects and invertebrates more tolerant of cooler climates). However, what all termites share, and what to a large extent distinguishes them from other animal saprotrophs, is the ability (usually in different species: see Noirot, 1992; Breznak and Brune, 1994; Bignell, 2000; Donovan *et al.*, 2001) to degrade and digest efficiently the more refractory of the residues of dead plant tissues: lignocellulose (the main structural component of wood and secondary cell wall thickening), hemicellulose (a major component of plant cell walls) and humus (a

derivative formed when lignocellulose, hemicellulose and other materials are degraded naturally by decomposer communities including fungi and bacteria; also known as soil organic matter).

Except perhaps under very arid conditions, natural termite assemblages contain a range of species which feed, individually, on materials at different stages in the decomposition process (Bignell and Eggleton, 1995). This integration of termite feeding biology and evolutionary niche separation with the natural decomposition process, together with the generally large abundance and biomass they can achieve through social organisation (Abe, 1987; Higashi *et al.*, 2000), leads to their having an impact on the global terrestrial C cycle exceeding that of any other arthropod group, and probably any other animal, excepting mammalian herbivores (Bignell *et al.*, 1996). The case for termites being considered as soil ecosystem engineers, through their constructions and soil-moving activities, is argued in Lavelle *et al.* (1997).

3. Associations of Termites with Microorganisms

The adaptations of termites to the digestion of refractory plant, or plant-derived materials include appropriately designed cutting and triturating mouthparts, a grinding gizzard and a robust, elongated alimentary canal (Krishna, 1970; Noirot, 1995; Bignell, 1994). However, above all there is also an association with an array of microbial mutualists; most of these inhabit the intestine where the symbiont community always includes prokaryotes and may also be supplemented with unicellular protists (Breznak, 2000; Inoue *et al.*, 2000). The presence of dense microbial populations in termite guts has been well known since the early part of the 20th century, but in more than 75 years of debate and experimentation and despite the widespread use of the termite example in textbooks to illustrate the general phenomenon of symbiosis, their role is yet to be specified exactly. Indeed, not only new members of the intestinal microbial consortium, but also new biochemical functions, are still being discovered (e.g. Ohkuma and Kudo, 1996; Ohkuma *et al.*, 1996; Brauman *et al.*, 1998; Lilburn *et al.*, 2001). In broad terms, the following activities can be ascribed to the intestinal community, including protists where present (for review see Butler and Buckerfield, 1979; Brune, 1998; Bignell, 2000; Brauman, 2000; Breznak, 2000; Brauman *et al.*, 2000; Ji *et al.*, 2000):

- a. dissimilatory carbohydrate metabolism (including cross-feeding reactions), in some cases from cellulose and hemicellulose, or their depolymerization products, and in others from the products of glycolysis, yielding short chain fatty acids which serve as energy sources for other intestinal microbes and/or the termite host.
- b. oxygen consumption (as an electron acceptor), rendering a portion of the gut lumen (generally that nearest the centre) microaerobic or anaerobic.
- c. dissimilatory and assimilatory N metabolism, providing for the conservation of excretory N (produced as uric acid) by the termite host as new microbial biomass, assimilation of the primary products of N₂ fixation in organic form and (probably) transamination balancing the amino acid spectrum available to the host.

- d. electron (or hydrogen) consumption by acetogenesis or methanogenesis, assisting energy conservation by the system as a whole and preserving redox balances.
- e. nitrogen fixation, under N-limiting growth conditions, sufficient in some cases to meet a significant proportion of the N requirements of the termite colony.
- f. demethylation, deacetylation and decarboxylation of aromatic polymers, possibly accompanied by limited aromatic ring cleavage.
- g. humification, or further humification, of organic material passing through the alimentary canal.

All wood- and litter-feeding termites which have been investigated seem able to produce a limited range of cellulolytic and xylanolytic digestive enzymes themselves (the product are simple sugars; reviewed by Slaytor, 2000) and, at least in theory, in large enough amounts to meet their C requirements for growth and energy production. Such an ability is rare in animals (for example there are no vertebrate cellulase producers) and since termite cellulase most resembles that of some bacteria, has possibly been acquired indirectly, for instance after transformation by microbes in a stem group ancestral to both modern-day termites and cockroaches (*cf.* Watanabe *et al.*, 1998; Lo *et al.*, 2000). An ability to digest plant structural polysaccharides without mutualists seems counter-intuitive, but is unquestionably real. It does not help us to understand why the association with microorganisms remains necessary, although this is also unquestionably real, as in experiments no termites have ever been successfully reared without their intestinal microbiotas (*cf.* cockroaches, see Bignell 1981).

The taxonomic affiliations of termite gut microbes are various and include archaea (such as methanogens), eubacteria (including spirochetes, actinomycetes) and eukaryotes (such as flagellates, amebae and yeasts), but filamentous fungi are not reported. In some senses this is unsurprising, given their generally aerobic metabolism (*cf.* the anaerobic chytrid fungi of the bovine rumen described by Bauchop, 1979), the relatively large size of fungal cells and what would be the relatively fragile nature of fungal mycelium inside a highly contractile gut. However, symbioses with fungi should have a potentially high value, given that fungi have a primary role in plant litter decomposition in nature and the group as a whole have been shown to produce an extremely wide range of dissimilatory enzymes, including the most potent known enzymes degrading lignin and lignocellulose (Zeikus, 1983). Such symbioses, focussed on the digestion or softening of wood and other refractory plant materials, have readily evolved in other insects (for reviews see Batra and Batra, 1979; Martin, 1987, 1991), but in no case where mutualism can be demonstrated is living mycelium a part of the permanent intestinal microbial community. The only precedent for such an association appears to be the trichomycete symbionts of aquatic and terrestrial arthropods, including some insect larvae (but the fungi apparently are not mutualists: see Moss, 1979). In termites, a large number of interactions with fungi have been reported where the fungus affects the discovery and consumption of food or its nutrient value, but again these fall short of mutualisms (Becker, 1976; Roulard-Lefèvre, 2000). Only in the nesting

systems of the termite subfamily Macrotermitinae, where fungus-combs of the basidiomycete genus *Termitomyces* are cultivated (outside the alimentary canal, the gut itself containing a microbiota much as in other termites, see Anklin-Mühlemann *et al.*, 1995) and harvested by the termite host, is there a mutual reliance, as neither partner can exist without the presence of the other. This “insect gardening” will be reviewed here, although it is essentially the “composting” component of gardening which forms the main analogy. The association is far from unknown: it has generated a large literature over more than 200 years (for examples and review, see Sands, 1970; Grassé, 1982, 1986; Martin, 1987; Wood and Thomas, 1989; Darlington, 1994; Rouland-Lefèvre, 2000), but the role of the fungus in the life of the termite remains controversial. In this chapter we present a summary of the biology of Macrotermitinae, focussing on the manipulations of the fungal partner by the termite host and the most recent lines of research undertaken to explain the relationship. To reconcile much conflicting evidence, we propose that symbiosis in the Macrotermitinae shows evolutionary diversification, with the fungus contributing to the digestion of refractory substrates in different ways and to varying extents, depending on the nature of those substrates and the degree of feeding specialisation exhibited by the termite host.

4. Basic Biology of Macrotermitinae and the Empirical Facts of the Relationship with Termites

4.1. PHYLOGENETIC POSITION OF MACROTERMITINAE

The Macrotermitinae are a subfamily of the higher termites (Family Termitidae), with a distribution in the tropics from Africa (where diversity is highest) through the Middle East, South and South East Asia, but absent from Central and South America, and from Australasia (Eggleton, 2000). Like other higher termites, they lack intestinal flagellates and show a relatively rigid caste differentiation, with little flexibility in development after the early larval stages (Roisin, 2000). However in other respects, they show a mixture of primitive and advanced features, which makes the group difficult to place phylogenetically. For example, intestinal structure (thought to be a good indicator of phylogeny, see Donovan *et al.*, 2000) is relatively simple and resembles that of lower termites (Bignell, 1994). Further, unlike the other subfamilies of higher termites, they have failed to evolve soil-feeding, but by contrast mound/nest architectures are generally complex, the behavioral repertoire is large and their physiology efficient to the point where they can dominate the termite assemblages of arid and semi-arid environments (Deshmukh, 1989; Noirot and Darlington, 2000; Tranillo and Leuthold, 2000). Emerson (1955), the founder of termite biogeography, placed the origin of the group in Africa in the Oligocene period, postulating a later spread to S. Asia and SE. Asia, where they become progressively depauperate through the Malay-Indonesian archipelago to the Philippines and Sulawesi. Although Emerson worked without the benefit of a widely accepted theory of continental drift, his thesis is attractive in accounting for the absence of Macrotermitinae from S. America and Australasia. However it is inconsistent with the observation that the Apicotermithinae, another subfamily of higher termites which are depauperate in SE. Asia and absent from

Australasia, are present in S. America with high diversity (Eggleton, 2000). Donovan *et al.* (2000) present a modern phylogeny of termites based on a combination of external and internal (largely intestinal) morphological characters. This shows the Macrotermitinae in a basal position within the higher termites, suggesting that they must currently show some suites of characters which were common with, or at least closely resembled, those of the common ancestor of all four subfamilies of higher termites (and where the other three are wholly or partly soil-feeding). Current thinking (Donovan *et al.* 2001; Nalepa *et al.*, 2001) is that the use of soil for nest-building and other constructions (and therefore its inevitable entry into the alimentary canal) may have been a critical event in the derivation of higher termites, as it can account both for the elimination of flagellate symbionts (by abrasion) and the early (in the phylogenetic sense) use of soil as a feeding substrate. Further, the elaborate constructions typical of Macrotermitinae can thus be reconciled with their basal position in the phylogeny of higher termites, although it must be assumed that the modern form of the association with basidiomycete fungi is derived. Soil is a good source of fungal inoculum, and it may be significant that both fungal conidia and mineral soil can be found in the guts of modern worker caste Macrotermitinae (Badertscher *et al.*, 1983).

4.2. THE FUNGUS-COMB

In Macrotermitinae, the symbiotic fungus *Termitomyces* sp. is grown on a special substrate prepared and maintained by the termites, the two together (*i.e.* fungal mycelium and substrate) being known as the “fungus-comb”. This is in turn housed in purpose-built chambers which are either clustered inside a mound nest (which may be buried or epigeal) or dispersed through the soil. The fungus has no free-living stage (other than conidia which are wind-dispersed in some species), and although it can be grown in the laboratory using fairly simple media, it does not sporulate in any manner resembling that *in situ* (Thomas, 1985). Correspondingly, incipient colonies of the termites that fail to establish a viable, growing fungus-comb do not survive (Sands, 1970), and hence the relationship qualifies as obligate symbiosis (this can be taken to mean mutualism, although strictly speaking the nature of the exchanges between the partners has not been established unequivocally). Whereas a gut flora is largely self-organising (Bignell, 2000), Macrotermitinae have evolved complex interactive behaviors to maintain and propagate the external symbiont fungus. Part of these activities are directed to suppressing other fungi which might compete with *Termitomyces*, such that the fungus has lost the ability to compete independently, and when fresh fungus-combs are experimentally separated from their termites, they are rapidly overgrown by other, non-symbiotic species (Wood and Thomas, 1989). Macrotermitinae forage mainly on plant tissues such as leaf litter, dead grass and lying freshly dead wood, that are not much decomposed. The material is passed through the termite gut before being incorporated into fungus-comb, but the passage is rapid and although the termites concerned in comb construction have fully competent cellulases, degradation during this preliminary gut transit appears to be relatively superficial (Martin, 1987). That forage is thus so rapidly exposed to fungal attack, and that the termites then go to such lengths to ensure that the subsequent fungal phase is so carefully regulated, strongly suggests that fungal composting of otherwise quite

refractory materials is a major part of the symbiosis. This may seem obvious to modern eyes, but is in fact a relatively recent interpretation of the role of the “termite fungus-combs”. There is not enough information about the changes that accompany the growth of *Termitomyces* mycelium on processed forage, but Abo-Khatwa (1976), Rohrman (1978) and Rohrman and Rossman (1980) have indicated a relative reduction in lignin, presumably leaving cellulose and other polysaccharides more accessible to the termite. Eventually, almost everything in the original forage appears to be degraded. This is inferred from the fact that Macrotermitinae, unlike many other termites, do not produce large quantities of organic-rich final feces, but rather a dark, slimy substance containing mineral grains, uric acid and, possibly, some humified residue of fungal pigments, which is deposited in the soil away from the main colony. There is also now evidence that *Termitomyces*, like many other basidiomycetes, can degrade lignin (Garnier-Sillam *et al.*, 1988). Consequently, the metabolism of the colony as a whole (including the fungus-combs) is very vigorous, and per unit weight of forage consumed may exceed that of non fungus-growers by 5-6 times (Wood and Sands, 1978). The system therefore produces few residues and the overall assimilation efficiency (into termite and fungal biomass combined, plus CO_2 released to the atmosphere) must be close to 100%. Attempts to budget the production of fungal and termite biomass in Macrotermitinae are made by Wood and Sands (1978) and Darlington (1994).

4.3. NATURE OF THE FUNGUS-COMB

The fungus-comb is largely organic, with a distinctive architecture varying in different taxa. In all cases the structure provides a large surface area, allows the circulation of air and access by worker termites and nymphs, the latter occupying the comb in large numbers without feeding on it directly (Darlington, 1994). New comb is produced from an amalgamation of termite feces (see below), and ripens after a period of several days by the production of clusters of conidia (also known as fruiting bodies, nodules or mycotètes) on the surface of the mycelium. These are eaten by termite workers and at a later stage, when the mycelium is senescent, this too is consumed. The process of comb construction, ripening and consumption is more or less continuous (with some exceptions, see below), but at longer intervals combs are replaced, either by removing them completely or by building new combs in their place. Forage is stored inside the nest in some species before being consumed and not in others. Structural building is always done with sub-soil retrieved from lower horizons and held in the buccal cavity, where it is moistened with salivary secretions which provide an eventual and robust organic binding substance (Wood, 1996).

4.4. ORIGIN OF THE FUNGUS-COMB

Virtually absent from other parts of the nest (Thomas, 1985), *Termitomyces* dominates the comb, which is clearly a selective medium for its growth. Until 1979, there was disagreement between biologists on the derivation of the comb. According to Bathellier (1927) and Grassé (1937; 1959), the comb consisted of fragments of wood, leaves and grass stems macerated by the mouthparts of workers and wetted with saliva. In contrast, other authors maintained the comb was made from worker fecal pellets (e.g. Petch,

1913; Sands, 1960). It was Josens (1971), using plant material colored with lampblack, who showed that the colouring appeared first in the intestine and then subsequently on the superficial regions of the comb, implying that the origin of material in the comb was fecal. Grassé (1978) subsequently distinguished between the fecal pellets added to the comb ("mylospheres", or primary feces) and "final feces", which were the remains of a digestive process. Mylospheres are, *ipso facto*, the growth substrate for *Termitomyces*, but this begs the question of why a preliminary intestinal passage is required before the fungus grows. The answer is not known for certain, although Grassé (1982) suggested that (in all Macrotermitinae) it serves to impregnate the plant material with a growth stimulant for the mycelium of *Termitomyces*. An utilitarian explanation might be that it ensures a thorough mixing of conidia with the growth substrate, something that might ensure the dominance of *Termitomyces* in the early growth stages and therefore the exclusion of competitors. Additionally, it may be that termite secretions are active against competing fungi: surely a possibility that should be investigated, as it might hold the prospect of commercial exploitation.

4.5. CONSTRUCTION OF THE FUNGUS-COMB

As previously mentioned, the comb is constructed from material of fecal origin. The comb is built up progressively to a design which is characteristic of each termite species and almost certainly determined genetically (Grassé, 1982). The variation in size and shape between genera is such that one can identify the termite and the fungus that this associated with it, from the comb morphology. On the basis of comb structure and mode of construction, three main types of comb have been recognized (Josens, 1972; Grassé, 1982; Rouland *et al.*, 1991; Rouland-Lefèvre, 2000).

- Type I, characteristic of the genera *Macrotermes*, *Odontotermes* and *Protermes*. These are alveolar-like combs, light-coloured at the base and dark at the top. There is a more or less continuous addition of material at the top, making the lowest part of the comb the oldest, characterised by the degradative loss (by fungal metabolism) of the brown pigmentation derived from protein-phenol complexes. This modified part is eaten by the termites.
- Type II, characteristic of the genera *Microtermes* and *Ancistrotermes*. These are sub-spherical or globular combs consisting of small spheres, 2–4 cm in diameter, more or less solid and uncoloured (except for a blackened basal zone). The comb is renewed in a fashion similar to that of Type I, except that the turnover of material is in equilibrium, i.e. the addition of new plant material and the consumption by termites occur at more or less constant rates.
- Type III, characteristic of the genera *Acanthotermes* and *Pseudacanthotermes*, but is also found in the (Asian) *Macrotermes carbonarius*. These are combs formed of vertical strips woven together in a complex manner. When first formed, the comb is brown but once the construction is completed, material is no longer added. The strips expand and thicken as they age, losing their colour as fungal metabolism proceeds. They are then completely consumed. For detailed descriptions of fungus-comb

morphology see Grassé (1982; 1986); useful SEM images may be found in Martin (1987) and the frontispiece of Abe *et al.* (2000).

4.6. ROLE OF THE FUNGUS-COMB

Since the discovery and description of comb nodules (first in India by Koenig, 1779; subsequently in tropical Africa by Smeathman, 1781), and the recognition of the termite/fungus relationship by Gibbon (1874), numerous ideas on the role of the fungus have been put forward. Koenig, then Doeblein (1906), suggested the fungus was the food of the royal pair and the larvae, but this first notion of the biological contribution of the fungus was overturned by the studies of Grassé (1937; 1944; 1945) and Noirot (1952). They showed that the queen and king were fed by trophallaxis, noting that the mandibles of larvae were too weak to bite the comb nodules. Smeathman (1781), then Grassé (1937) and Heim (1942) took another view of the termite/fungus relationship, regarding the comb as a nursery, where the velvety mycelial covering of the comb protected the soft cuticle of the larvae from abrasion and damage. However, the observation in *Bellicositermes natalensis* (= *Macrotermes bellicosus*; Ruelle, 1970), that eggs and larvae were present in chambers of the nest without comb, led Grassé to reject the idea. Ghidini (1938) in *Bellicositermes bellicosus* (= *Macrotermes subhyalinus*; Ruelle, 1970), then Lüscher (1951) in *M. bellicosus*, suggested that the comb regulated the microclimate of the nest by stabilising humidity and temperature. However, Grassé and Noirot (1957) showed that in *M. bellicosus* the internal temperatures of individual colonies differed, and they thought this variation was too much to make the thermoregulation hypothesis tenable. The argument was revisited by Korb and Linsenmair (2000), who with the aid of thermistor probes showed that the metabolic heat generated by the living inhabitants of *Macrotermes bellicosus* mounds (*i.e.* termites and fungus-comb) contributed to the maintenance of an even and optimal core temperature of 30°C, but only in large colonies. A fairly large body of evidence has demonstrated that active thermoregulation occurs in mounds of *Macrotermes*, but mediated by termite behavior through control of evaporation (Noirot and Darlington, 2000).

Petch (1913) first proposed a nutritional role for the comb. This is supported by several reports of a high carbohydrate content (Becker and Seifert, 1962; Agarwall, 1978) and a high protein content (Mishra and Sen Sarma, 1985) in the material. The nutritive value of the comb has been clearly shown by experiments on the ability of several species of fungus-growing termites to feed on different substrates (*e.g.* Noirot, 1952; Sands, 1956; Ausat *et al.*, 1960; Rouland *et al.*, 1991). Amongst termites, the Macrotermitinae are by a long way the most effective in degrading plant polymers, especially polysaccharides (Rouland *et al.*, 1988a,b,c) and polyphenols (Mora and Lattaud, 1999). Extensive assays of enzyme activity have pinpointed this strong hydrolytic activity to specific regions of the termite intestine and to the fungus-comb nodules, with negligible activity elsewhere. Oligosaccharide and heteroside degradation is generally higher in the midgut of the termite than in the fungal nodules, except in the termite genus *Pseudacanthotermes*, where activities against all substrates are higher in the fungus (Fig.1). The fungal associates of *Macrotermes subhyalinus*, *M. natalensis*, *P. militaris* and *Odontotermes pauperans* all show higher polysaccharidase activity than

their termite hosts (Fig.2). By way of contrast, the *Termitomyces* symbionts of *Acanthothorax cavithorax*, *M. toumodiensis* and *M. bayerni* show weak activity, and always less than that of the corresponding termite intestine. Thus Rouland *et al.* (1991) proposed the existence of an evolutionary diversification of mutualistic mechanisms within the fungus-growing termites, in which two groupings were distinguishable.

- Group I, where the fungal associate produces little or no enzyme compared with the termite host (*A. cavithorax*, *M. toumodiensis*, *M. bayerni*). The role of the fungus in this group has yet to be determined.

- Group II, where the fungal associate produces enzymes with higher activity than the termite host (*M. subhyalinus*, *M. muelleri*, *M. bellicosus*, *M. michaelseni*, *O. pauperans*, *P. spiniger* and *P. militaris*). In this group the principle of the association is the acquisition (by the termite from the fungus) of a large quantity of enzyme, rich in polysaccharidase. The main evidence for this is the strong enzyme activity detected in the termite midgut, and a demonstrated *in vitro* synergism between enzymes of termite and fungal origin (Martin and Martin, 1978; Rouland *et al.*, 1988a,b,c). In *Pseudacanthotermes*, where oligosaccharidases and polysaccharidases both show higher activity in the fungus, the latter apparently retains the ability to degrade plant material completely.

The presence of acquired enzymes in the digestive tract of termites was first suggested by Martin and Martin (1978; 1979) in *M. natalensis*, then confirmed by Abo-Khatwa (1978) in *M. subhyalinus*. Purification of cellulase- and xylanase-enzyme components in *M. muelleri* and its *Termitomyces* associate together with a comparison of their respective properties, led Rouland *et al.* (1988a,b,c) to the conclusion that the enzymes produced by the fungus were the same as those found in the termite midgut. However, in *M. michaelseni*, Veivers *et al.* (1991, see also Bignell *et al.*, 1994) found the elution profiles of the termite-derived enzymes and those of the fungal associate did not quite correspond and, moreover, the low availability of enzyme of fungal origin (based on estimates of nodule consumption) meant that all the significant cellulolytic enzyme activity in the intestine was of termite origin. Matoub and Rouland (1995) showed the presence of an active fungal xylanase in the intestinal tract of *M. bellicosus*. This supports the acquired enzyme hypothesis in that xylanases are not typical animal products. The conflicting nature of these evidences may reside in the incomplete nature of some enzyme acquisitions in some species of *Macrotermes* (e.g. *M. michaelseni* and *M. bayerni*), whereas in others the process is efficient (e.g. *M. subhyalinus*, *M. natalensis*, *M. muelleri* and *M. bellicosus*). Sequencing data and the establishment of phylogenies for hosts and fungal associates should help to clarify the reasons for this apparent difference in strategies within the same host genus for a process as fundamental as digestion.

The main focus of much recent research on the Macrotermitinae concerns the consumption by worker-caste termites of the fungal conidia produced on ripe comb. Quantitatively, the process is small, *i.e.* the weight of the consumed conidia is tiny in relation to the other materials ingested (primary forage or senescent comb, see Veivers

et al., 1991; Bignell *et al.*, 1994), but the nodules are rich in N (as protein), K, P and sugars relative to the comb itself, and show high specific activities (though low absolute

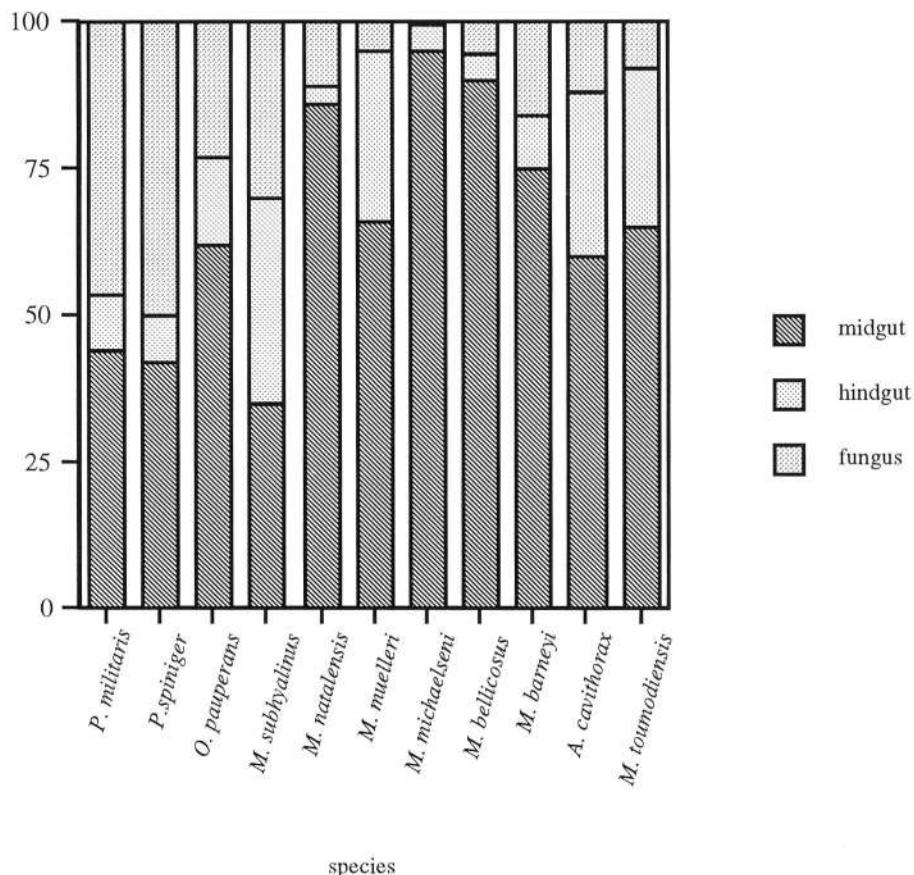


Figure 1. Comparison (by percentages from a standard substrate) of β -glucosidase activities in termite worker midgut and hindgut, and in fungal nodules, in 11 Macrotermitinae species. P, *Pseudacanthotermes*; O, *Odontotermes*; M, *Macrotermes*; A, *Acanthotermes*. *M. tounodensis* is the genus *Microtermes*.

amounts) of certain polysaccharide-degrading enzymes (Abo-Khatwa, 1978; Rouland *et al.*, 1988 a,b,c; Veivers *et al.*, 1991). These observations have led to detailed, but

controversial hypotheses that the nodules are a source of components of a working cellulase complex not produced by the termite itself (Martin and Martin, 1978) or that fungal and termite cellulases (and xylanases) with differing competences towards their

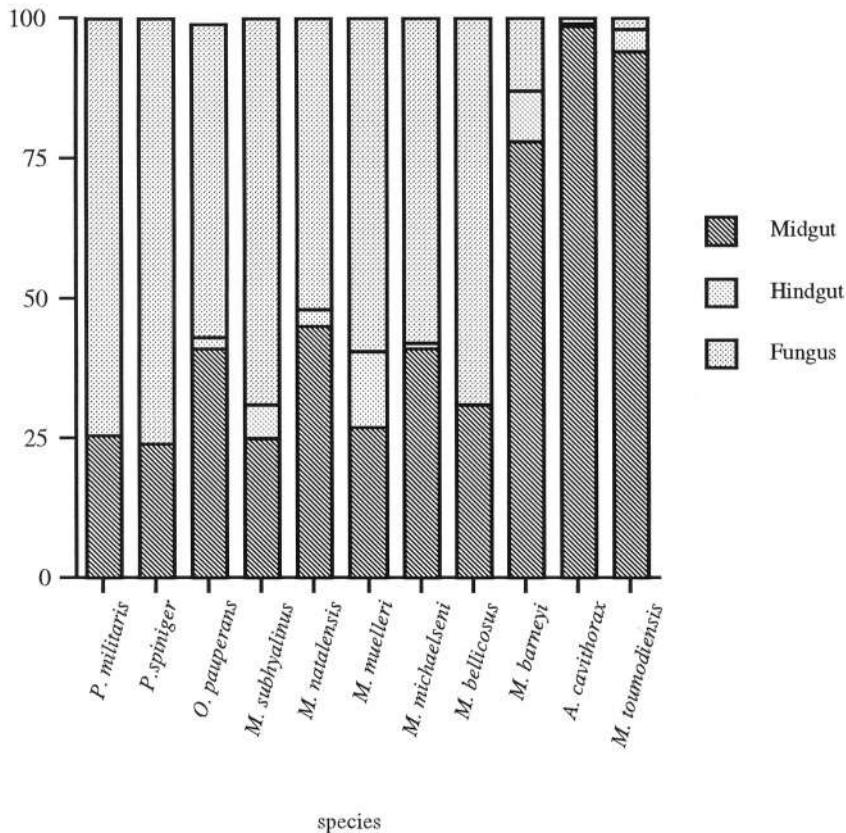


Figure 2. Comparison (by percentages from a standard substrate) of cellulose hydrolysed in the midgut, hindgut and fungal nodules of 11 Macrotermitinae species. P, *Pseudacanthotermes*; O, *Odontotermes*; M, *Macrotermes*; A, *Acanthotermes*. *M. toumodiensis* is the genus *Microtermes*.

substrates synergise in the termite intestine to create a more effective dissimilation of polysaccharide than the termite could achieve by itself (Rouland *et al.*, 1991). The arguments, which make use of detailed concepts of enzyme biochemistry and carbohydrate metabolism, are too complex to repeat here in detail, but reviews by Darlington (1994) and Rouland-Lefèvre (2000) are helpful, and emphasise the

possibility, elaborated below, of differing interactions between fungus and termite host in differing taxa of termites. A critical complication of the issue arises from the work of Badertscher *et al.* (1983), whose careful observations of laboratory-reared colonies of *Macrotermes subhyalinus* revealed that workers were functionally heterogeneous. Workers consuming fungal nodules were predominantly less than 30 days old (as imagoes), and these were also the same termites that consumed primary forage and constructed the fungus comb; older workers increasingly switched to a diet of senescent comb, in which polysaccharide-degrading enzymes have low activity. Thus the interactions between fungus and host may differ between the types of termite present in a colony, as well as between different taxa of termites. Full details of the complete processing of forage by the whole termite colony are known only for one or two species of *Macrotermes*. Extension of behavioural studies to other species and other genera is urgently needed to assess whether the complexity of interactions is universal throughout the sub-family. This might also indicate whether the symbiosis is fundamentally the same in all Macrotermitinae: at present an open question.

5. Management of the Symbiont

Since *Termitomyces* is only encountered within the termite nest, it follows that the optimum conditions for growth are found there, provided by the termites.

5.1. MICROENVIRONMENTAL CONDITIONS

Termitomyces is a poor competitor with other microorganisms (Thomas, 1985), but grows profusely in the fungus-comb of Macrotermitinae. This could be explained by several mechanisms:

- the creation of a microclimate with a temperature regime stabilised at about 30°C, which is optimal for the fungus (Thomas, 1985), high humidity and elevated CO₂ (Matsumoto, 1977). These conditions assist the exclusion of potential competitors such as *Penicillium* and *Xylaria*, which tend to develop spontaneously on fungus comb which is dug out of termite nests and exposed to ambient conditions (Wood and Thomas, 1989).
- a low pH of 4.1 – 4.6, which inhibits bacterial growth (Thomas, 1987b).
- antibiotic secretions produced by the termites which inhibit competitors (Batra and Batra, 1966; Thomas, 1987b).
- growth stimulants produced from the salivary glands or the rectum, which are specific for *Termitomyces* (Grassé, 1982).

The task of maintaining optimum conditions for the growth of *Termitomyces* rests with the worker termites (Wood and Thomas, 1989). Although other fungi, notably *Xylaria*,

are present in a suppressed state on the fungus-comb, their role, if any, in substrate degradation is unknown.

5.2. TRANSMISSION OF THE FUNGAL SYMBIONT.

Several hypotheses have been formulated to account for the transmission of *Termitomyces* from one colony to another (Table 1). In species which do not form fruiting bodies, inter-colony transmission seems to depend on fungal sphaerocysts which resist intestinal transit (Johnson, 1981). Female reproductives ingest conidia before leaving the parental nest (*ibidem*). In other species, such as *Macrotermes bellicosus*, it is the winged male reproductives which transport the conidia at the time of swarming. These asexual fungal structures remain viable within the digestive tract until the new fungus-comb is constructed.

In species which fruit, the fruiting usually takes place about 3 months after swarming, at the time when the first workers from new incipient colonies are foraging on the surface. These workers bring back the spores produced by the carpophores of *Termitomyces* (Sieber, 1983; Leuthold and Badertscher, 1988) and these spores allow the termite to produce the first comb. However, it is difficult to account for the universal presence of *Termitomyces* in the comb by such uncertain transmission process (Grassé, 1982).

TABLE 1. Presence of spores in the guts of swarming reproductives in selected Macrotermitinae and their viability in the fungus-comb of new colonies (after Johnson *et al.*, 1981).

Species	Presence of spores in the gut	Viability on new fungus-comb
<i>Ancistrotermes cavithorax</i>	-	-
<i>Ancistrotermes guineensis</i>	-	-
<i>Macrotermes bellicosus</i>	+	+
<i>Macrotermes subhyalinus</i>	-	-
<i>Microtermes</i> sp. nr. <i>usambaricus</i>	+	+
<i>Microtermes</i> sp. B	+	+
<i>Microtermes</i> sp. C	+	+
<i>Odontotermes pauperans</i>	-	-
<i>Odontotermes smethmani</i>	-	-

6. Diversity within *Termitomyces*

The large body of information accumulated over two centuries on the biology of the termite hosts, summarised above, is not matched by a corresponding knowledge of the fungal symbionts. This ignorance may lie at the root of our inability to specify the relationship between the partners unequivocally. We set out below what can be said about diversity within the genus *Termitomyces*. In doing so, we will demonstrate *ipso facto* the inadequacy of current knowledge of almost every aspect of the life of the fungus.

6.1. TAXONOMIC POSITION

The basidiomycete genus *Termitomyces* is affiliated to the order Agaricales and the family Tricholomataceae, in which 52 other genera are described. Since the first descriptions of termite-associated fungi, their systematic position and phylogenetic relationships have been largely ignored. There was early taxonomic confusion, as different authors had allocated the same termite-associated fungi to different genera, for example *Trichia* (Savorge, 1989, cited by Heim 1977), *Lentinus* (Berkeley, 1896), *Lepiota* and *Armillaria* (Berkeley *et al.*, cited by Grassé, 1982), *Pluteus* (Holtermann, 1899, cited by Heim, 1977), *Ambrosia* and *Volvaria* (Petch, 1906; 1913), before it was established that all termite-associated basidiomycete fungi formed a distinct genus of their own (Heim, 1941).

On the basis of the fruiting process and carpophore structure, Heim (1977) divided the genus *Termitomyces* into two sub-genera:

- the sub-genus *Praetermitomyces* incorporates species which fruit in immediate proximity to the termite colony centre from primordia, the emergence of which the termites themselves engineer from the nest.
- the sub-genus *Eutermomyces* incorporates species which fruit on the termite nest itself.

However, it should be noted that there some *Termitomyces* exist which do not fruit and are therefore difficult to place taxonomically, especially in the absence of physiological and genetic information. In spite of this, the value of the exclusive genus *Termitomyces*, in which all representatives are associated with termites and where the mycelium is managed and cultivated by termites as internal fungus-comb within the nest, is beyond doubt. Indeed, these properties are not known in any other known group of fungi (Thomas, 1987a,b).

6.2. MORPHOLOGY

Termitomyces has three principal components:

- the mycelium, of which the most substantial part is integrated with a large quantity of harvested plant material, forming the fungus-comb, but also constituting a surface web covering each parcel of the comb within the nest.
- the nodules or “mycotêtes”, characteristic for each species, which are aggregations of conidiophores raised from the surface of the mycelium, having the appearance of small white globules, sometimes somewhat conical and either sessile or carried on short stalks. The diameter of the nodules varies between 0.5 and 2 mm.
- the carpophore, the fruiting stage which supersedes the nodules and defines the taxonomic standing of the fungus as a basidiomycete. It is composed of a cord (or pseudorhizoid) connected basally to the fungus comb, and an aerial part which

differentiates into a cap which emerges at the surface of the soil, at the apex of which is found a nipple or perforatorium. The latter is toughened and, with the corkscrew growth of the pseudorhizoid, forces its emergence as an erect structure. The morphology of the perforatorium varies with species and with the hardness of the substratum, and the pseudorhizoid cap therefore permits the various termite-associated fungi in *Termitomyces* to be distinguished, but bearing in mind variations associated with habitat, the host termite species, the forage utilised and the particular soil properties (Heim, 1977). Neither the vegetative and reproductive parts of the mycelium exist other than in contact with the fungus-comb, nor is any free-living species of *Termitomyces* known (Grassé, 1982).

6.3. PHYSIOLOGY

In vivo enzymatic studies of the nodules of different *Termitomyces* have shown differential activities towards the components of the substrates provided by the termite host (Rouland *et al.*, 1991; Mora, 1992; Ikhouane, 1995). To determine the control of these enzyme expressions (whether induced from the environment, or genetic), production has been studied in several species of *Termitomyces* grown in pure culture under the same conditions. It was shown that although enzymes were produced in all species, there were consistent qualitative and quantitative differences between species, such that two physiological sub-groups could be distinguished:

- a group of species in which a suite of enzymes is induced by the substrate and regulated by environmental conditions (Chang *et al.*, 1983; Wood and Thomas, 1989). These may be characterized as “generalists” and each fungus may be associated with several species of Macrotermitinae.
- a group of species in which a restricted range of constitutive enzymes is produced, addressed to specific substrates (Ghosh and Sengupta, 1987; Wood and Thomas, 1989; Sengupta and Sengupta, 1990; Roy *et al.*, 1994; Ikhouane, 1995; Ghosh *et al.*, 1995; Sinha and Sengupta, 1995; Mukherjee *et al.*, 1995). These species each tend to be associated with a limited range of termite hosts, and often just one species.

7. Specificity and Phylogeny of the Fungus

Termitomyces seems to have a variable role in termite nutrition. Not all species are able to form an efficient symbiotic association with all species of Macrotermitinae, so there is some degree of host specificity (Chang *et al.*, 1983; Wood and Thomas, 1989), although not the strict one host species/one fungal species claimed by Heim (1977). As a consequence, it remains unclear whether the cultivation of a particular species of *Termitomyces* by a colony of termites is the result of vertical transmission (*i.e.* co-evolution, with intergenerational transfer via the reproductive caste) or horizontal transmission (availability in a particular geographic zone, with intergenerational transfer by acquisition of compatible spores from the environment). Resolution of this issue would require both knowledge of what brings about contact between the partners and an

analysis of the degrees of affiliation between different species of *Termitomyces*. The latter is inherently difficult, as the existing systematics of basidiomycete fungi are largely based on the structure of the carpophore, while many *Termitomyces* never fruit, in addition to never being found as a free-living mycelium. However, new molecular methods can establish relative affinities and therefore make it possible to sketch a phylogeny, even in such a badly defined group.

7.1. MOLECULAR METHODS

Recent application of molecular sequencing has focussed on the ITS region of *Termitomyces* (Table 2, Rouland *et al.*, 2001). The genomic regions analysed, ITS 1-5. 8S-ITS2, are moderately large (650 bp) in African species of the fungus and larger still in Asian species, for example 1050 bp in *Macrotermes gilvus*, exceeding the norm of 600-800 bp reported for fungi generally (Gardes and Bruns, 1996) and therefore making a good target for taxonomic studies (Fig. 3). Some intraspecific polymorphism in the size of the ITS region has been noted in ectomycorrhizal fungi (Sanon, 1999; Lanfranco *et al.*, 1999), and in the genus *Tuber*, the ITS region can be almost twice the size in some species as in others (Gandeboeuf *et al.*, 1997).

TABLE 2. Designation and termite host of the different *Termitomyces* isolates (Rouland *et al.*, 2001).

<i>Termitomyces</i>	Cultivating termite species	Origin
11 MAT. <i>schimperi</i>	<i>M. subhyalinus</i> 1	Thiès (Sénégal)
12 MAT.18	<i>M. bellicosus</i>	Kolda (Sénégal)
13 MAT. 9	<i>M. mülleri</i>	Franceville (Gabon)
14 MAT.11	<i>M. subhyalinus</i> 2	Garoua (Cameroun)
15 MAT. 12	<i>M. jeanneli</i>	Tanzania
16 MAT. 10	<i>M. sp. Zanzibar</i>	Zanzibar (Tanzania)
17 MAT. 8	<i>M. renouxi</i>	Dimonika (Congo)
28 MAT.16	<i>M. amandalei</i>	Sakaerat (Thailand)
29 MAT. 17	<i>M. gilvus</i>	Sakaerat (Thailand)
210 MAT.15	<i>M. carbonarius</i>	Sakaerat (Thailand)
11 ODT. 4	<i>Odontotermes</i> sp. 1	Thiès (Sénégal)
12 ODT. <i>clypeatus</i>	<i>O. smeahmani</i>	Dakar (Sénégal)
11 PST. <i>eurhizus</i>	<i>P. spiniger</i>	Nkayi (Congo)
12 PST. <i>striatus</i>	<i>P. militaris</i>	Nkayi (Congo)
11 ANT. <i>medius</i>	<i>Ancistrotermes guineensis</i>	Kolda (Sénégal)
11 MIT.2	<i>Microtermes</i> sp.	Thiès (Sénégal)

An analysis of polymorphism in restriction fragments in *Macrotermes* (Fig. 4) confirmed the distinction between African and Asian fungal symbionts, not just in terms of fragment size (as the overall region amplified is greater in the latter group), but also in terms of restriction profiles. Moreover, in the same Asian fungi, a large variability is demonstrated for 210 MAT.5 and 28 MAT.16, with a maximum similarity in comparisons between (termite) species of only 40%. In the African *Termitomyces*, the equivalent similarities ranged from 73% to 100%, except in species 13 MAT.9, which

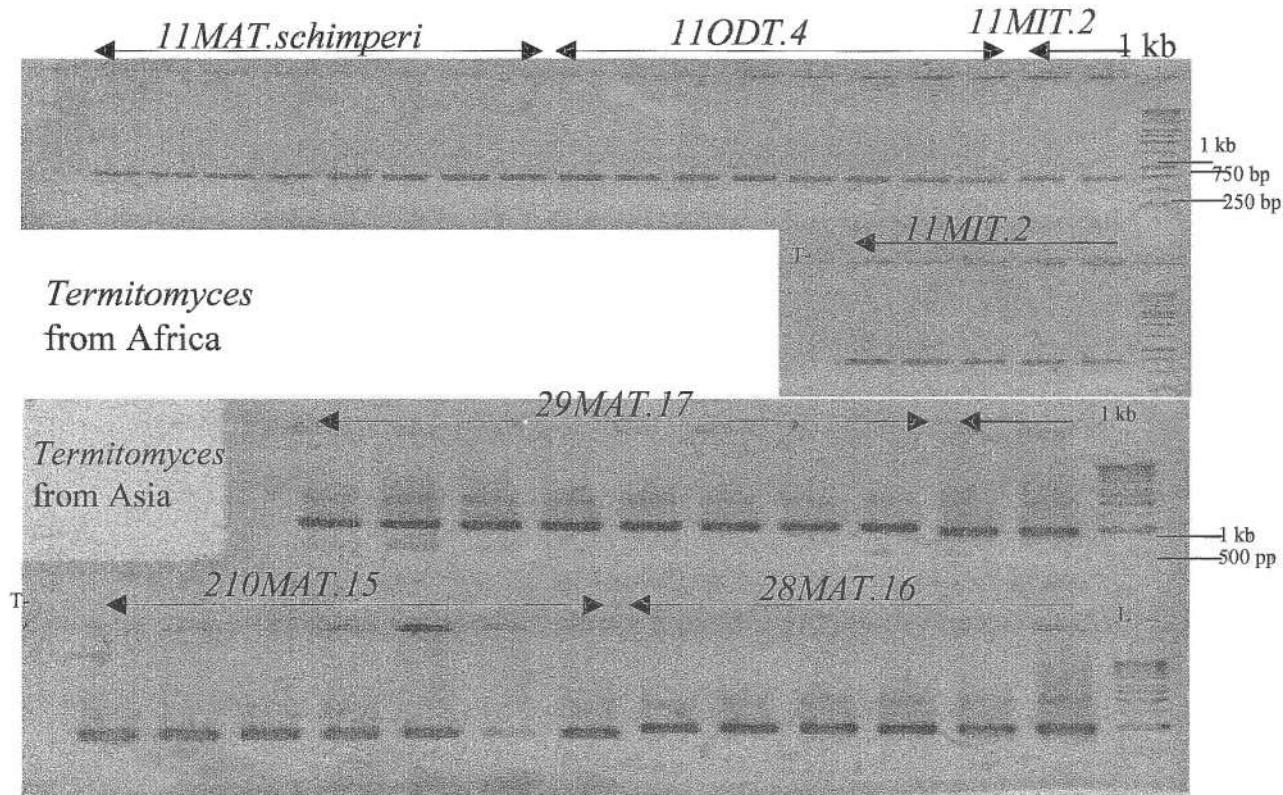


Figure 3. Electrophoregram of ITS from Asian and African *Termitomyces* (Rouland et al., 2001).

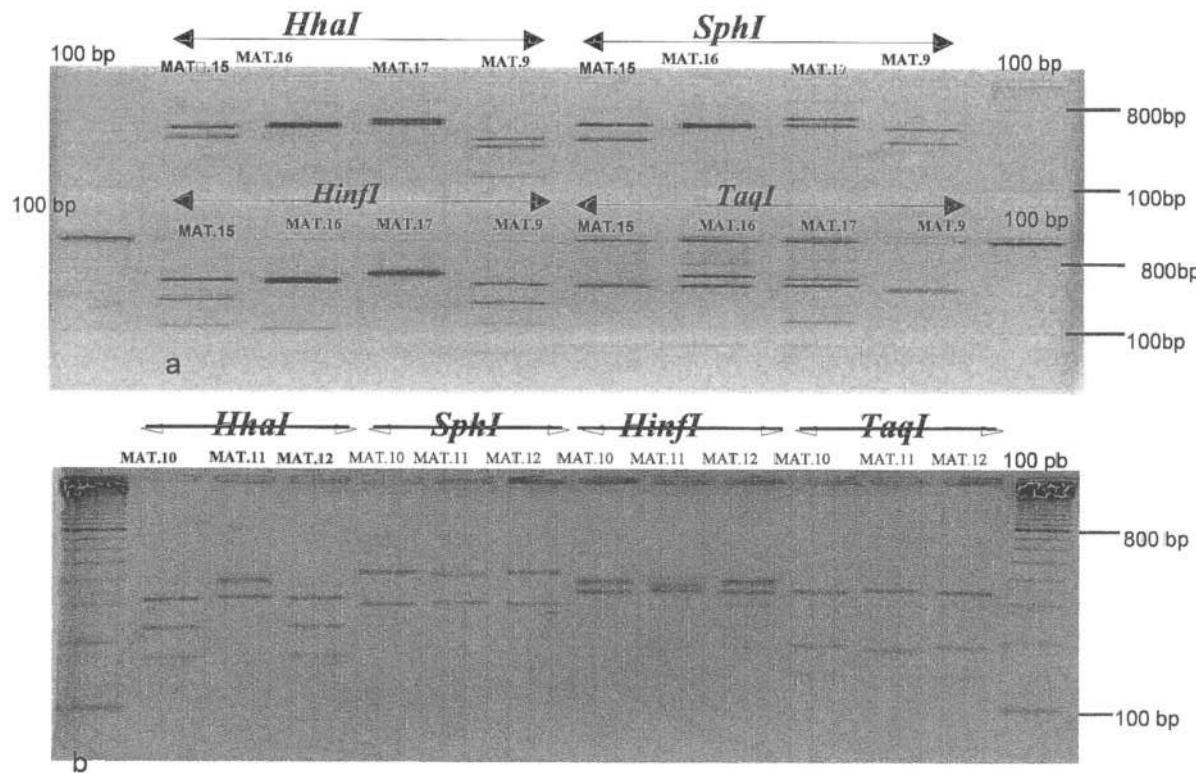


Figure 4. PCR-RFLP of ITS from Asian and African *Termitomyces* (Rouland *et al.*, 2001).

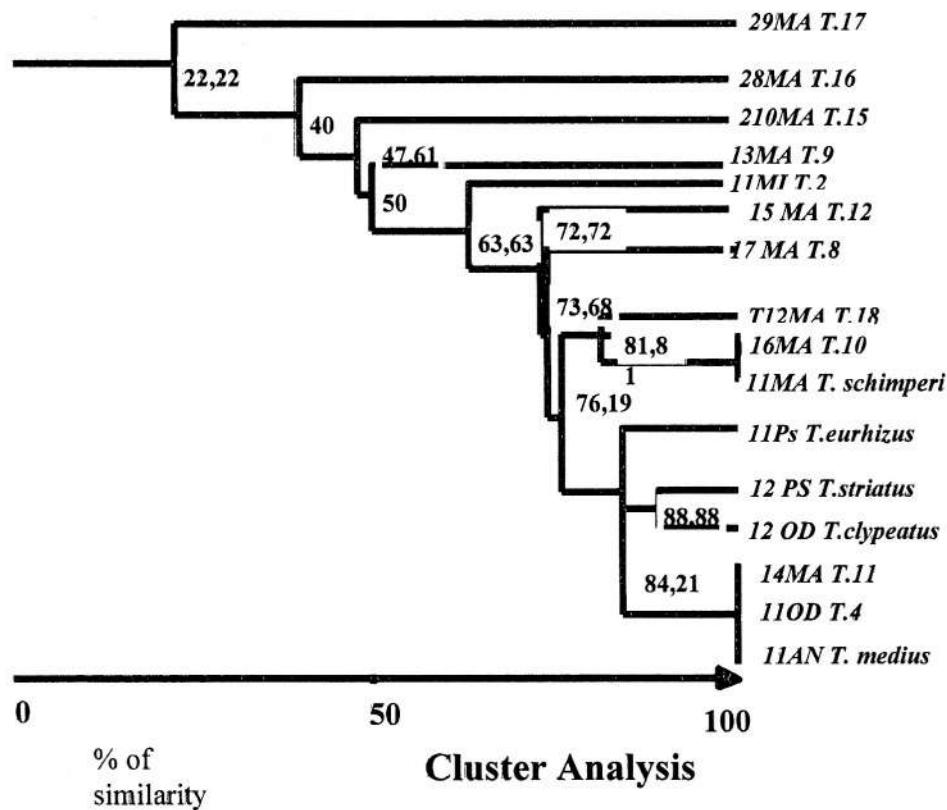


Figure 5. Cluster analysis of the PCR-RFLP of ITS from 16 species of *Termitomyces*.

has only 50% similarity with the others (Fig. 5). The *Termitomyces* symbionts of four other genera of Macrotermitinae examined were well separated on the dendrogram, but against this an apparently anomalous 100% similarity was found between the fungi associated with termite species in three different genera: 14 MAT. 11 (*Macrotermes*), 11 ODT.4 (*Odontotermes*) and 11 ANT. *medius* (*Ancistrotermes*). Though far from perfect, the usefulness of these molecular analyses can be judged against the observation that biochemical enzyme assays have not permitted any divergence to be established within the African *Termitomyces*. Notwithstanding the large overall variation in the ITS genomic region of fungi (O'Donnel, 1992; Kiss, 1997), the usefulness of ITS1-5 and 8S-ITS2 for restriction analysis has been noted more than once (Kiss, 1997; Lanfranco *et al.*, 1998).

The molecular phylogeny of *Termitomyces* does not support the subgroup classification made by Heim (1977) on the basis of carpophore morphology and fruiting behaviour. Heim recognized a basal ancestral form, *Praetermitomyces* (the symbiont of the termite genus *Microtermes*), and an evolved form, *Eutermomyces*, comprising the symbionts of *Macrotermes*, *Pseudacanthotermes* and *Odontotermes*. However, the % of molecular similarity can be lower within *Eutermomyces*, for example between 12 ODT.*clypeus* and 11 MAT.*schimperi*, than between the latter and the *Praetermitomyces* – affiliated 11 MIT.2. Further, the molecular phylogeny suggests that fruiting is not a useful phylogenetic character: of the fungi examined, only 12 MAT.18 and 13 MAT.9, both from the termite genus *Macrotermes*, do not fructify, but they show less similarity to each other than to other fungal symbionts of the same genus. However, there is some evidence to support a thesis of termite/fungus co-evolution (as originally proposed by Heim), namely the regrouping of the *Termitomyces* symbionts of the termite genera *Macrotermes* (12 MAT.9, 12 MAT.18, 11 MAT.*schimperi* and 15 MAT.12) and *Pseudacanthotermes*, although the notion that each species of termite is associated with a separate species of fungal symbiont seems unlikely (for example 100% similarity was obtained from ITS for the fungal symbionts of three species of termites in different genera (*Macrotermes*, sp., Cameroon, *Ancistrotermes guineensis* and *Odontotermes* sp.).

8. Conclusions: Modalities of the Fungus

Rouland *et al.*, (1986) proposed a classification of *Termitomyces* according to the available evidence of the nature of the symbiosis with the termite hosts.

- I. Non-producers of digestive enzymes.
- II. Producers of both oligo- and polysaccharidases.
- III. Producers of polysaccharidases only.

The significance of this functional distinction between species of *Termitomyces* in the wider scheme of termite/fungus co-evolution and phylogeny is currently unknown, but there is no apparent clustering of functional group with the clades identified by molecular markers. Views on the role of the fungal partner are not unanimous; Bignell (2000) summarized the main possibilities as 1. the provision of cellulases and xylanases, especially cellobiohydrolases, to work synergistically with endogenous enzymes

(Martin and Martin, 1979; Rouland *et al.* 1988a,c); 2. the provision of conidia as a concentrated source of organically-combined nitrogen, presumably as protein (Rohrmann, 1978); 3. A general enrichment of nitrogen in foraged foodstuffs, resulting from an intensive metabolism of C-rich carbohydrates and lignin by the fungus (Collins, 1983); 4. the degradation of lignin (adding to fungal biomass and subsequently to termite biomass when fungus comb is consumed, or making subsequent digestion of consumed fungus comb by termites more efficient by improving access to cellulose), combined with detoxification of plant allelochemicals to improve palatability (Grassé and Noirot, 1958; Rohrmann and Rossman, 1980) and 5. a source of heat and metabolic water (Lüscher, 1951; Rohrmann, 1977). The composting of forage prior to consumption is known in other types of termite (Sands, 1960) and the innovation of delegating this function to a single type of fungal symbiont can be seen as a logical development of the external rumen mechanism, thought to have been a critical event in the evolution of termite mutualisms with microorganisms (Nalepa *et al.*, 2001). A final possibility, at least formally, is that *Termitomyces* has no beneficial role for the termites and is maintained at their expense in a parasitic capacity. Such a relationship seems very unlikely, but cannot be entirely excluded on present evidence.

Finally, we suggest that opportunities for future research are available in all areas of the biology of the Macrotermitinae and *Termitomyces*. However, the priority should be to establish whether the diversity of the fungi matches that of the termite hosts and, consequently, whether the benefits they provide are fixed or flexible.

9. References

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Biodata of **Michele K. Nishiguchi** author of "*Cospeciation between Hosts and Symbionts: The Sepiolid Squid-Vibrio Mutualism.*"

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COSPECIATION BETWEEN HOSTS AND SYMBIANTS: *The Sepiolid Squid-Vibrio Mutualism*

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1. Introduction

Mutualistic associations between eukaryotic hosts and their microbial partners have long been an interest of ecologists and evolutionary biologists due to the importance of understanding phylogenetic congruence, coevolution, host specificity, infectivity, and maintaining the precarious balance between pathogenicity and innocuousness. Each partner has an important function in the life history of the symbiosis; the “super organism” functions as a result of combining both lifestyles of host and symbiont, enabling the entire association to take advantage of a broader ecological niche than either one could accomplish independently. These types of functional interactions between host and symbiont can only be established when an avenue for the exchange of information has evolved specifically within the symbiosis. Once this occurs, the discourse between the partners (physiological, genetic) allows the association to flourish and become a competitive and dynamic system in an environment where it may not normally exist. The presence of such interactions between highly divergent taxa provides an excellent opportunity to study the interactive roles of coevolving species in processes such as horizontal gene transfer, interspecies gene regulation, allopatric and sympatric routes of speciation, and the development of population structure and dynamics.

A number of examples exist that indicate that the formation of a symbiotic relationship allows the host or its symbiont to radiate into newly formed niches: chemoautotrophic bacterial residing within tissues of hydrothermal vent or sewage outfall invertebrates (Cavanaugh, 1994); nitrogen fixing bacteria in the nodules of leguminous plants (Wilkinson and Parker, 1996); endosymbiotic bacteria within the body cavities of aphids, termites, and weevils (Moran and Telang, 1998; Nardon and Grenier, 1991; Smith and Douglass, 1987); and luminous bacteria within the light organs of monocentrid and anomolopid fish and sepiolid and loliginid squids (Haygood and Distel, 1993; McFall-Ngai and Ruby, 1991). Although these mutualisms have evolved as part of a new “evolutionary innovation” (Margulis, 1989), there resides the question of how the association is directly affected by either specific host-symbiont interactions, or whether environmental or ecological factors play an important role in the formation of the partnership. Various hypotheses have alluded to the fact that transmission of the symbiosis, whether vertical or horizontal, would also have a major influence in determining whether host-symbiont pairs display parallel modes of speciation or promiscuous patterns of affiliation. Does specificity drive the cospeciation of host-symbiont pairings, or does the fidelity of vertical transmission have a more dramatic effect on determining the coevolution of the mutualism? Does the evolution of specific recognition factors help determine whether a symbiont will maintain fidelity or be promiscuous? Do environmental or ecological factors influence the onset of the symbiosis as well as continually having an additional effect on evolving specificity and recognition?

2. Association, cospeciation, congruency and fidelity of host-symbionts

2.1. UNDERSTANDING THE BASIS OF COOPERATIVE ASSOCIATIONS

Until recently, there has been little evidence that addresses questions of evolution and cospeciation of both partners in a symbiosis. Many symbiotic relationships are obligate; in other words, the host or symbiont depends entirely upon the other for some type of capability or function which they do not possess (Baumann *et al.*, 1997; Cavanaugh, 1994). This co-dependence has made most physiological and biochemical studies of the separated organisms nearly impossible to complete *in vitro*. Microscopic studies of symbionts residing in host tissues have provided only a few clues as to the nature of these relationships and how they were initially established (Buchner, 1965). The inability to culture many of the microscopic partners outside of the symbiotic niche still frustrates biologists, since it does not allow the separate study of either organism without the presence or influence of the other (Distel, 1998). Studies involving the use of antibiotics or other biochemical methods to clear animals of symbionts were inconclusive, since most hosts suffered drastic physiological changes due to the loss of the symbionts (Baumann *et al.*, 1997; Ohtaka and Ishikawa, 1991). Recently, the advent of molecular techniques has allowed investigators to define and identify the nature of the symbioses, characterize the type of symbiont associated with the partnership, and determine whether the symbionts are transmitted vertically or horizontally (Bourtzis and O'Neill, 1998; Cary, 1994; Cary and Giovannoni, 1993; Distel and Cavanaugh, 1994; Kreuger and Cavanaugh, 1997; Munson *et al.*, 1991). These techniques have led to some progress toward an understanding of how symbiotic associations have evolved and whether the presence of a particular symbiont has an influence on host speciation and symbiont recognition.

2.1.1. *The nature of cospeciation and congruency*

Although a number of symbioses have been widely studied, few have proven to be effective models for answering questions concerning the coevolution and establishment of symbiotic associations. Whether the ancestral lineage between host and symbiont holds any clues to the origin of the symbiotic character (i.e., commensalistic, mutualistic or pathogenic), only detailed analyses of genes that are responsible for specific mechanisms within the symbioses can be examined which may uncover any details of the interactions among the individuals involved. Since many of these "symbiotic" genes are yet unknown or may vastly differ between symbiotic organisms, other markers have been chosen to delineate symbiotic lineages that may be evolving in parallel. Phylogenetic analyses of phenotypic characters as well as molecular sequence data obtained from both symbiont and host are helpful in establishing any congruence that may be occurring along these parallel lineages. Previous investigations with various symbiotic associations using molecules and morphology have revealed congruent phylogenetic patterns between the partners (Baumann *et al.*, 1997; Hinkle *et al.*, 1994; Nishiguchi, 2001). These patterns have resolved questions of whether the symbionts were evolving in parallel (cospeciation/coevolution) or have expressed patterns of promiscuity or host switching over their evolutionary lineages. Understanding the evolution of host-symbiont specificity, as well as the effects of the association on life history patterns and interactions between the individual partners has only just begun to be explored among those well studied systems.

2.1.2. *Promiscuity or fidelity?*

The assumption that patterns of coevolution between symbiotic partners were strictly parallel was primarily based upon which type of symbiotic transfer occurred between host and symbiont. Associations where symbionts were transferred directly (vertically) through parental inheritance, assumed that strict patterns of cospeciation would occur

due to the intimate transfer between parent (usually maternal, through the ova) and offspring. It was believed that this direct infection of host offspring with the symbiotic population would eventually form a highly specific association, and that this relationship would produce strict patterns of cospeciation among host species and their particular symbionts. The presence of these vertically transmitted symbioses would also allow the establishment of a hierarchy of geographically distinct populations, where host/symbiont pairs have diverged from a particular ancestral species, but have retained their specificity due to their mode of transfer. An example of this type of tightly congruent association is the evolution of aphids and their bacterial symbionts (Baumann *et al.*, 1997; Moran and Telang, 1998; Munson *et al.*, 1991). Molecular phylogenies based on 16S rDNA sequences of the bacterial symbionts show that the primary endosymbionts have descended from a single common ancestor (Munson *et al.*, 1991). This result corresponds with a number of insect-bacterial studies (Aksoy, 1995; Bandi *et al.*, 1995; Clark *et al.*, 1992; Munson *et al.*, 1993; Schröder *et al.*, 1996), in which the symbionts form a monophyletic group, indicating that each clade was founded by an endosymbiont. Although insect endosymbionts have arisen a number of times from their free-living relatives, there are strict patterns of cospeciation observed among those that represent the symbiont clades (Aksoy, 1995; Bandi *et al.*, 1995; Moran *et al.*, 1993; Munson *et al.*, 1991; Schröder *et al.*, 1996). The congruency observed is strong evidence for parallel cospeciation that has evolved from the prolonged and highly specific vertical transmission of symbionts among hosts. Along with molecular studies, microscopic studies and observations of maternal transmission (Buchner, 1965) have never shown that horizontal transmission is present among these groups of insects. Thus, parallel cladogenesis reflects not only the origin of the ancestor of insect-bacterial symbioses, but also implies that those ancestors were living at the same time.

In contrast, environmentally transferred symbioses allow new symbionts from the free-living population to infect each new generation of hosts (i.e., promiscuous). Therefore, the hosts are obtaining their partners from a "symbiont" pool that may or may not be directly related to the previous generation of symbiotic associates. With this type of infection behavior, one would predict that environmental transfer would provide a variety of closely related strains or species that are promiscuous between the hosts, with the ability to infect each new generation of hosts equally as well. With this assumption, the host would not be able to differentiate between any of the symbionts, and the chances of obtaining any one of the symbionts is equal between all symbionts in the free-living population (given that they are present in equal concentrations). But in reality, most environmentally transmitted symbioses are specific to a particular type of bacterium and a particular host species (Cavanaugh, 1994; Nishiguchi *et al.*, 1998). In these systems where parallel cladogenesis is evident, the underlying hypotheses did not predict the strict phylogenetic nature of the symbioses, thereby creating a paradigm in the "traditional" symbiotic theory. Although correlating traits between interacting organisms are often evidence for corroborating strict cospeciation, the presence of species specificity in associations where the symbiont is transmitted environmentally invalidates this theory (Nishiguchi *et al.*, 1998). Whether the biogeography, ecology, or the life history of the host is a determining factor in the evolution of a specific symbiotic relationship, little is understood about how this specificity arises among symbionts with environmental modes of transfer.

2.1.3. Ancestry and cospeciation

The specificity of each symbiotic association and how the individual partners have accommodated and adapted to each other's presence can be an indicator of how cooperative associations have evolved from ancient lineages. Along with an obligate life history pattern, the mechanism of symbiont transfer between generations may also affect the patterns of parallel cladogenesis. Although transfer mechanisms are not

completely understood in many groups of animals, there are examples where both hypotheses of transmission strategies have been proposed. For example, the presence of symbionts in the reproductive tissues of two families of chemosynthetic bivalves (Vesicomyidae and Solemyidae) suggests that the bacteria may be transferred vertically through the gametes (Cary, 1994; Cary and Giovannoni 1993; Krueger *et al.*, 1996). However, experiments with another family of bivalves (Lucinidae) suggest that the symbionts are obtained anew with every generation, that is, transferred environmentally. Comparisons of host and symbiont phylogenies using 16S rRNA analysis for the symbionts and morphological data from the hosts have shown species specific associations within the chemoautotrophic containing bivalves indicating congruence (Distel *et al.*, 1988, 1994; Distel and Cavanaugh 1994; Krueger and Cavanaugh, 1997). This finding supports the mechanism of vertical transfer among host-symbiont pairs. However, when the phylogenies of these symbionts are compared to other symbionts of chemoautotrophic invertebrates, the associations are much more complex, with some of the bivalve symbionts being more closely related to vestimentiferan worm symbionts (Distel 1998; Distel *et al.*, 1994; Feldman *et al.*, 1997; Krueger 1996). Because all these bacterial symbionts have been classified within the same subdivision of the γ Proteobacteria (Distel, 1998), the evolution of chemoautotrophic symbioses in a number of animal phyla suggests that the history of the associations is complex, and the specialization of these bacteria was established prior to the diversification of host organisms.

2.1.4. A contradiction of theories

Whether host-symbiont specificity has occurred between intimately associated partners cannot be predicted based on transmission strategies alone. If vertical transmission did occur between host and symbiont, and a high degree of fidelity between the partners was observed, then cospeciation patterns would be evident and their respective phylogenies would mirror each other. Additional evidence from the fossil record of host and symbiont may also corroborate the duplicity of the phylogenies (Distel, 1998; Moran and Telang, 1998). Rarely do we find stringent congruencies; usually phylogenetic signals are confounded by rare transfer between hosts species (Goff *et al.*, 1996, 1997) or the establishment of new associations (Wilkinson *et al.*, 1996). Many problems arise in the interpretation of evolutionary relationships amongst pairs of organisms, versus organisms within a single lineage. The symbiotic association has additional aspects to consider when interpreting phylogenetic congruence; not only do the individual lineages of each organism be taken into consideration, but the history of the association must be regarded as well. Even when the evolutionary paths have been teased apart, there can always be room for promiscuity, that is, the loss or gain of new symbionts, the symbionts switching to an entirely new host, or the re-infection of a new type of symbiont. All these factors can also confound the relationships between a host and its symbiont if the phylogenetic composition of the association is not strictly parallel.

3. A case of congruency and specificity: The Sepiolidae-*Vibrio* symbiosis

One association that is shedding more light on the evolutionary history of symbiotic associations is the partnership between a group of shallow-water benthic squids (Family Sepiolidae) and their luminous bacterial symbionts (Genus *Vibrio*, Table 1). This system offers several advantages as an experimentally tractable system in which to study the coordinated influence of bacteria on the parallel evolution and specificity of closely related symbiotic species (Nishiguchi *et al.*, 1998). Unique features that render this symbiosis attractive for such studies are: (i) host animals are easily obtained in quantity, for example, in the Mediterranean Sea where several species of sepiolid squids

Table 1. *Euprymna* and *Sepiola* species

<u>Species</u>	<u>habitat</u>	<u>Location</u>	<u>Symbiont</u>
<i>Euprymna morsei</i> (Verrill, 1881)	continental shelf	Seto Sea, Japan	<i>V. fischeri</i>
<i>Euprymna scolopes</i> Berry, 1913	continental shelf	Kane'ohe Bay, HI	<i>V. fischeri</i>
<i>Euprymna stenodactyla</i> (Grant, 1833)	continental shelf	Solomon Islands	?
<i>Euprymna tasmanica</i> (Pfeffer, 1884)	continental shelf	Crib Point, Australia	<i>V. fischeri</i>
<i>Sepiola affinis</i> Naef, 1912	continental shelf	Mediterranean	<i>V. fischeri</i> <i>V. logei</i>
<i>Sepiola atlantica</i> d'Orbigny, 1839	shelf-edge of slope	NE Atlantic	?
<i>Sepiola aurantiaca</i> Jatta, 1896	outer shelf-upper bathyal	S. Norway-Mediterranean	?
<i>Sepiola intermedia</i> Naef, 1912	continental shelf	Mediterranean	<i>V. fischeri</i> <i>V. logei</i>
<i>Sepiola knudseni</i> Adam, 1984	inner shelf	NW and W Africa	?
<i>Sepiola ligulata</i> Naef, 1912	shelf-edge of slope	Mediterranean and Adriatic	<i>V. fischeri</i> <i>V. logei</i>
<i>Sepiola pfefferi</i> Grimpe, 1921	continental shelf	W. Africa and Mediterranean	?
<i>Sepiola robusta</i> Naef, 1912	outer shelf	Mediterranean	<i>V. fischeri</i> <i>V. logei</i>
<i>Sepiola rondeleti</i> Leach, 1834	shelf and upper bathyal	Mediterranean	?
<i>Sepiola steenstrupiana</i> Levy, 1912	upper sublittoral	Mediterranean	?

co-exist (Mangold and Boletzky, 1988); (ii) both the hosts and the bacterial symbionts are easily cultured and maintained independently under laboratory conditions (McFall-Ngai, 1999; McFall-Ngai and Ruby, 1998; Ruby, 1999a); (iii) the initiation of the association can be maintained under experimental control (Wei and Young, 1989); (iv) molecular genetics can be applied to the bacterial partner (Graf et. al., 1994; Visick and Ruby, 1996); and, (v) the association represents the only known animal symbiosis where cross-colonization experiments can be used to determine the extent of specificity (or symbiotic competence) existing among bacterial symbionts from different host species (McFall-Ngai and Ruby, 1991; Nishiguchi *et al.*, 1998). Along with these characteristics, the symbiosis is environmentally transmitted, which in turn allows the study of whether transmission has a role in the origin of symbiotic lineages and parallel speciation.

Symbioses occur between species of luminous bacteria and squids in two families of cephalopods, the Loliginidae and Sepiolidae, the latter of which consists of four light organ genera: *Euprymna*, *Sepiola*, *Rondeletiola*, and *Semirossia* (Herring *et al.*, 1981; Mangold and Boletzky, 1988; Fig. 1A). In all species within these genera, the bacteria are maintained in a morphologically complex bilobed light organ that lies within the center of the animal's mantle cavity, where it is continually bathed with seawater as a result of normal ventilatory activity (Ruby and McFall-Ngai, 1992; Visick and McFall-Ngai, 2000; Fig. 1B). Observations of the behavior of the host organisms suggest that the bacterially produced light is used in antipredatory behavior (McFall-Ngai, 1990). Emission of the bacterial luminescence is controlled in two ways: (i) by a host-modulated, diel restriction on the luminescent output per bacterial cell (Boettcher *et al.*, 1996); and, (ii) by a series of accessory tissues (Montgomery and McFall-Ngai, 1992) which are functionally analogous to the tissues that control light quality in the eye (Fig. 1C). Although some minor differences exist in light organ morphology, the functionality remains the same among sepiolid species (Foster and McFall-Ngai, 1998; Mangold and Boletzky, 1988).

Bacterial cell numbers in the crypts is controlled in part by diel venting of approximately 90% of the bacterial culture through the lateral pores of the light organ which lead into the mantle cavity and are flushed out into the environment (Boettcher *et al.*, 1996). This venting behavior provides a sufficiently high density of symbiotic-competent vibrios in the water column to promote colonization of the next generation of juvenile squids (Lee and Ruby, 1994a). The remaining 10% of bacteria are capable of establishing a new complement of symbionts during the daylight hours when the squids are buried in the sand and quiescent (Boettcher *et al.*, 1996). It is during this time that the squids do not use any bacterially produced luminescence and may have a decreased metabolic demand from the few symbionts remaining in the light organ (Nyholm and McFall-Ngai, 1998). Bacteria that remain in the light organ are spatially organized near or adhered to the microvilli layer of the crypt epithelium (Lamarcq and McFall-Ngai, 1998; Fig. 1D), enabling them to maintain contact within the tissues of the juvenile squid light organ. However, when two different strains of *Vibrio* are placed in direct competition with each other, the host squid will displace a non-native strain by preferentially venting the non-native *Vibrio* (Nishiguchi *et al.*, 1996). Once inside the light organ, the native strains are closer to the microvilli and crypt epithelium of the light organ compared to their non-native competitors (Nishiguchi *et al.*, 1996).

Within the first few hours after hatching, juvenile squids are colonized by competent *Vibrio* cells, but little is known about the recognition and specificity involved in determining light organ composition. During the initial part of the infection process, symbiosis-competent *Vibrio* strains from the ambient seawater must enter the juvenile light organ through the lateral pores and travel down narrow, ciliated ducts that lead into the epithelium-lined crypts (Montgomery and McFall-Ngai, 1993). These *Vibrio* strains are active participants during the infection process, since the bacteria are not simply swept along by epithelial cell ciliary movement of the host (Montgomery and McFall-

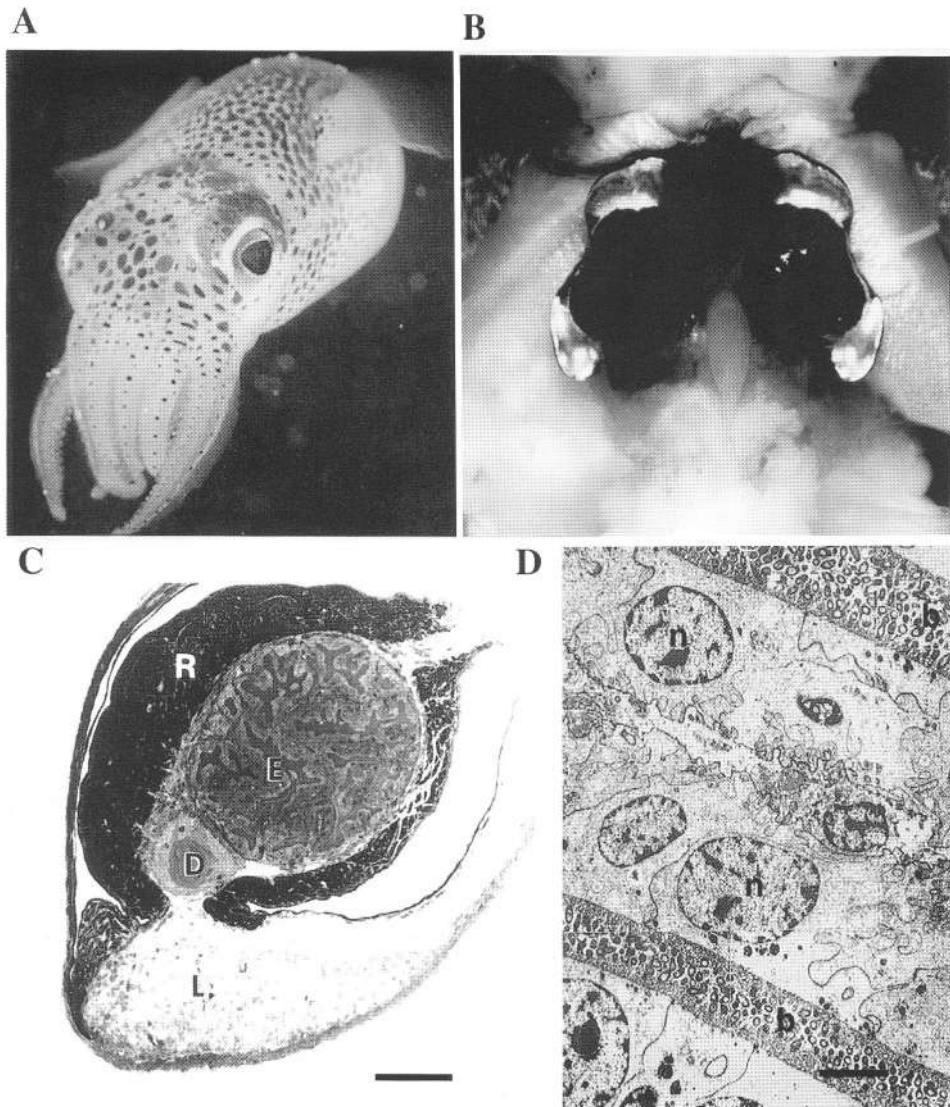


Figure 1. Images of the sepiolid squid-luminous bacterium symbiosis. (A) An adult specimen of *E. scolopes* (5 cm in length). (B) A ventral dissection of the bilobed light organ located within the mantel cavity. (C) A histological section of one lobe (bar = 1 mm), revealing several tissues: (e) epithelial central core tissue housing bacteria, (r) reflector, (l) lens, (d) diverticula. (D) A transmission electron micrograph of an area of the epithelium-line crypts containing symbiotic bacteria: (n) = nucleus of squid, (b) = bacteria in crypts (bar = 5 μ m). Photos: (A) W. Ormerod; (B) S.V. Nyholm; (C) M.J. McFall-Ngai; (D) M.K. Montgomery.

Ngai, 1993), but actually propel themselves into the crypts of the nascent light organ (Graf *et al.*, 1994). Studies identifying the symbiotic bacteria from a variety of Mediterranean sepiolids indicate that each species of sepiolid harbors two species of *Vibrio* (*V. fischeri* and *V. logei*), and that no other species of bacteria are found inside the adult light organ (Fidopiastis *et al.*, 1998; Nishiguchi, 2000). Since the light organ pores are continually open to the surrounding seawater providing access for any type of bacterium, the presence of only *V. fischeri* or *V. logei* in the light organ illustrates very strong species-specific interactions between these bacteria and their host (Nishiguchi *et al.*, 1998).

3.1. EVIDENCE FOR THE PARALLEL EVOLUTION OF ENVIRONMENTALLY TRANSMITTED HOST-SYMBIONT ASSOCIATIONS

Free living, symbiotically competent vibrios have been found in the surrounding environment, as well as other luminescent vibrios that are not able to infect the light organ of the sepiolid squids and monocentrid fishes (Lee and Ruby, 1994a; 1994b). Along with the biogeographical separation among the Indo-west Pacific sepiolid squids, the distinction between related host genera and their specific *Vibrio* symbionts could be hypothesized as evolving from the influences of the surrounding environment of the association (ecology) and not the specific host partner. This hypothesis was first proposed by a number of researchers who believed that the specific *Vibrio* symbionts had originated from the surrounding seawater, and then diverged along with their host partner (Nealson and Hastings, 1991). If this was the case, then we would expect to see similarly related bacterial symbionts from different host organisms found living in the same coastal waters. Previous studies investigating monocentrid fishes found in the same location as sepiolid squids have shown that although the symbionts of both monocentrids and sepiolids are *Vibrio fischeri*, these strains are genetically distinct and cannot cross infect other hosts (Lee and Ruby 1994b; Nishiguchi *et al.*, 1998; Nishiguchi, unpub. data). What is interesting about this preference is the degree of specificity; among the *Vibrio fischeri* strains tested in competition, all *V. fischeri* strains isolated from sepiolid squids can infect juvenile *Euprymna scolopes*, whereas *V. fischeri* isolated from the monocentrid fish *Cleidopus gloriamaris* cannot infect the light organs of *E. scolopes* to the same concentration, even when presented alone. Since the symbioses of both sepiolids and monocentrids are transmitted environmentally, how has the evolutionary radiation among strains of vibrios become so species specific, providing the foundation for patterns of parallel cladogenesis among the phylogenetically analyzed partners?

Phylogenetic congruence has been previously used in other symbiotic systems to test the fidelity of host-symbiont relationships (Baumann *et al.*, 1997; Haygood and Distel, 1993; Hinkle *et al.*, 1994). Because sepiolids in general have been very difficult to identify morphologically (Bello, 1995), molecular methods have recently been used to establish phylogenetic information about the relationships between the existing species complexes (Nishiguchi *et al.*, 2001; 1998). Genetic markers such as the cytochrome *c* oxidase subunit I (COI) and the internal transcribed spacer region of the ribosomal repeat (ITS), have been employed to delineate population level relationships, as well as genus and species level associations (Nishiguchi *et al.*, 1998; Nishiguchi, unpub. data). These data combined with glyceraldehyde phosphate dehydrogenase (*gapA*) sequence data from the symbiotic bacterial partners provide molecular evidence for parallel cladogenesis (Fig. 2). Competition experiments between various symbiotic strains of *Vibrio* and juvenile sepiolids corroborate the phylogenetic data, where a genetic hierarchy exists among closely related symbiotic strains that matches the phylogenetic evidence (Nishiguchi *et al.*, 1998, Table 2). Using a standard squid colonization assay (Ruby, 1999b; Ruby and Asato, 1993), two different strains of *Vibrio* symbionts are mixed in equal concentrations and are presented to the newly hatched aposymbiotic

squids. The infection and growth of the bacteria are monitored over time, and at the end of the assay period (48 hours), the number and strain type of bacteria are quantified by counting the two different competitor strains plated from each juvenile light organ (Nishiguchi 2001; Nishiguchi *et al.*, 1997). Since each strain or species used in the competition experiments can colonize a juvenile light organ equally well when presented by themselves, the existence of possible recognition factors responsible for differentiating native from non-native symbionts supports the hypothesis that specificity has evolved between each host-symbiont pairing.

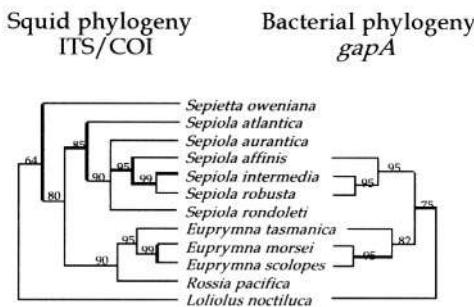


Figure 2. Phylogenetic tree of squid host species and their symbiotic bacteria isolates. Each tree was inferred by comparison of the unambiguously aligned positions of either the ITS or the *gapA* sequence. Identical branching patterns were obtained for each locus when analyzed by either the parsimony or maximum-likelihood methods. The values given at each node are the percentages of 100 bootstrap re-samplings and subsequent heuristic searches that support the relationships between the clades.

TABLE 2. Infection of juvenile *E. scolopes* with mixed inocula of squid light organ symbionts.

Strains used in competition ¹	No. of animals tested ²	Mean proportion found in light organs at 48 h ³
ES114 and EM17	37	98:2
ES114 and ET101	35	95:5
EM17 and ET101	17	75:25
ET101 and SA1	30	90:1
ES114 and LN101	21	100:0

¹Bacterial isolates from squid species are as follows: ES114 - *E. scolopes*, EM17 - *E. morsei*, ET101 - *E. tasmaniaca*, SA1 - *S. affinis*, LN101 - *Photoligot. noctiluca*.

²Total number of animals tested in three or four experiments for each pair of strains. All of the animals became colonized by at least one strain in these experiments.

³Mean averages of individual ratios of bacterial strains were arc sine transformed (Sokal and Rohlf, 1981) to determine the significance of each treatment. All mean values were significantly different to within P ≤ 0.05.

3.1.1. Ecology vs. specificity

As mentioned previously, the underlying hypothesis regarding luminous bacterial symbioses has suggested that the environment determined the type of symbiont each host species acquired (Nealson and Hastings, 1991). Initially, this assumption must be correct, since the partnership must have evolved from two separate organisms living in close proximity prior to the initiation and radiation of the symbioses. If so, are there present day examples where the ecology is driving the speciation of symbiotic

associations? If such associations do exist, then to what level has the partnership evolved such that the symbiont is specific to one host and is also found in many closely related species?

The Mediterranean Sea hosts a large group of luminous sepiolid squid species that offer unique examples of how ecologically driven patterns of evolution might be a precursor to host specificity and parallel cladogenesis. Previously, all luminous bacterial-animal mutualisms were thought to be monotypic, i.e., that only one species of bacterium was associated with a particular host taxon (Nealson and Hastings, 1991; Ruby, 1996). However, Fidopiastis *et al.* (1998) have recently discovered a different species of *Vibrio* (*V. logei*) that resides in the light organs of several *Sepiola* squid species of the Mediterranean, giving rise to a two species consortium (Nishiguchi, 2000). This unique and interesting finding is the first observation of two *Vibrio* species of bacteria residing in the light organs of sepiolid squids (Fidopiastis *et al.*, 1998). What is peculiar about the dual-symbiont relationship is that the two species of *Vibrios* differ not only in their 16S rDNA genotype, but in some of their physiological characteristics as well (Fidopiastis *et al.*, 1998; Lickliter and Nishiguchi, unpub. data). Most notably, growth rates of *V. logei* isolates are higher than *V. fischeri* isolates at lower temperatures (18°C), whereas at higher temperatures (26°C), *V. fischeri* isolates grow at a faster rate (Nishiguchi, 2000; Table 3). Another major difference between the two symbiotic species is their luminescence at different temperatures. Again, *V. logei* isolates are more sensitive to higher temperatures, with decreased luminescence at temperatures above 18°C. The addition of decyl aldehyde to *V. logei* strains grown above 18°C enhanced their luminescence, indicating a temperature sensitivity for the capacity to synthesize natural aldehyde substrate for the luminescence reaction (Fidopiastis *et al.*, 1998). Therefore, *V. logei* symbionts are the more psychrophilic species of the two observed symbionts in *Sepiola* species. Because there are several species of Mediterranean *Sepiola* living in the same coastal habitats as each other, this provides an opportunity to test how environmental factors effect the nature of the symbioses. Does the variety of *Sepiola* species have an effect on the distribution and abundance of *Vibrio* symbionts, or do ecological factors (i.e., temperature) have a greater affect on the distribution and cospeciation of symbiotic associations?

At present, there are ten recorded species of endemic *Sepiola* living in the Mediterranean Sea (Mangold and Boletzky, 1988; Table 1). Of these ten species, five can be found within the same coastal waters off the southernmost part of France (Banyuls-sur-Mer), providing an ideal habitat in which to examine whether specificity or ecology is influencing symbiont distribution. To test whether an ecological (temperature) parameter has a direct effect on the colonization and specificity of symbiotic recognition, several symbiotic strains were analyzed for their competitive ability in infecting light organs of juvenile *Sepiola* squids (Nishiguchi, 2000). Initially, individual strains of *V. fischeri* or *V. logei* were isolated from adult specimens of *S. robusta*, *S. affinis*, *S. ligulata*, and *S. intermedia*. These isolates were used to test whether the species of host determined the symbiotic composition, or if temperature was a significant factor in establishing symbiotic competence. Strains of *V. fischeri* or *V. logei* were added in a 1 : 1 ratio (approximately $10^3/\text{ml}$ total bacteria) to either juvenile *S. affinis* or *S. ligulata* at 18°C and at 26°C. After 48 hours, juvenile squids were homogenized, and plated on seawater tryptone plates to visualize the number and types of vibrios present (Lee and Ruby 1994a). Identification between strains of *V. fischeri* and *V. logei* were accomplished by direct colony lifts (Lee and Ruby, 1992), and probed for the variable region (V1) of the 16S rRNA gene (Fidopiastis *et al.*, 1998). All eight isolates of *V. fischeri* or *V. logei* tested could infect either squid species light organ when the infection was initiated by a single strain (Nishiguchi, 2000). For all competitions held at 26°C, all *S. affinis* and *S. ligulata* juveniles were infected primarily with *V. fischeri* isolates. All light organ competitions held at 18°C resulted in colonization by mostly *V. logei* symbionts in either *S. affinis* or *S. ligulata* juveniles

(Nishiguchi, 2000). Thus, the ecology (in this case, temperature) determines the symbiont composition of the sepiolid light organ, and not the host species. These results are in contrast to observations within the Indo-west Pacific species of *Euprymna*, where symbiont composition was determined by host species, and not ecology (Nishiguchi *et al.*, 1998).

Table 3. Growth constants of *V. fischeri* and *V. logei* isolated from European *Sepiola* species (from Nishiguchi, 2000)

Bacterial strain and species	Host squid	Growth rate constant at 18°C (per hr)	Growth rate constant at 26°C (per hr)
SA1 (<i>V. fischeri</i>)	<i>S. affinis</i>	0.6	0.8
SA8 (<i>V. fischeri</i>)	<i>S. affinis</i>	0.6	1.0
SA6 (<i>V. logei</i>)	<i>S. affinis</i>	0.9	0.7
SA12 (<i>V. logei</i>)	<i>S. affinis</i>	0.8	0.7
SL2 (<i>V. fischeri</i>)	<i>S. ligulata</i>	0.4	1.3
SL8 (<i>V. fischeri</i>)	<i>S. ligulata</i>	0.5	1.3
SL4 (<i>V. logei</i>)	<i>S. ligulata</i>	1.2	0.9
SL12 (<i>V. logei</i>)	<i>S. ligulata</i>	1.2	0.9
SI2 (<i>V. fischeri</i>)	<i>S. intermedia</i>	0.6	1.1
SI4 (<i>V. fischeri</i>)	<i>S. intermedia</i>	0.6	1.0
SI5 (<i>V. logei</i>)	<i>S. intermedia</i>	0.9	0.7
SI7 (<i>V. logei</i>)	<i>S. intermedia</i>	0.9	0.7
SR5 (<i>V. fischeri</i>)	<i>S. robusta</i>	0.4	0.9
SR10 (<i>V. fischeri</i>)	<i>S. robusta</i>	0.5	0.9
SR1 (<i>V. logei</i>)	<i>S. robusta</i>	1.4	1.0
SR18 (<i>V. logei</i>)	<i>S. robusta</i>	1.3	0.9

3.1.2. Assessment of wild-caught sepiolids

Along with laboratory experiments, we have sampled a number of sepiolid squid populations throughout the year, to determine if there is a correlation between temperature and light organ composition in the natural habitat. Adult light organ contents from four species of *Sepiola* revealed that both species of *Vibrio* were present, as mixed assemblages, with one species dominating in concentration over the other (Nishiguchi, 2000). What is intriguing about the *Vibrio* species composition in each squid light organ is the manner in which it varied depending on the season and depth at which the individual was caught. During the summer months (June-September), the Mediterranean near Banyuls-sur-Mer experiences a formation of a thermocline at approximately 20 meters, in which an abrupt change in temperature occurs. Above the thermocline, temperatures average at approximately 23°C, whereas below the thermocline, temperatures drop to approximately 13°C. During winter months, the thermocline disappears, and temperatures on average range between 8-16°C (unpublished data, Laboratoire Arago database). Along with this distinct temperature boundary, one species of sepiolid, *S. affinis*, is mostly found at depths ranging from 5-15 meters. All other species of *Sepiola* sampled thus far (*S. robusta*, *S. intermedia*, and *S. ligulata*) are found at depths ranging from 30-80 meters. Thus, three of the four species of sepiolid squids collected reside at temperatures below 16°C.

Specimens that were primarily populated by *V. fischeri* or were dominated by *V. fischeri* versus *V. logei* were obtained between the first part of July until early September, and were collected at depths above the thermocline (< 20 meters). These specimens were all *S. affinis* individuals whose habitat is found above this thermocline

boundary. Sepiolid squids that were populated primarily by *V. logei* or were dominated by *V. logei* versus *V. fischeri* were collected in late September until late spring (May), during which the thermocline disappears due to winter conditions which consist of wind-driven mixing of surface waters with those below. All specimens that were collected at all depths had a larger proportion of *V. logei* symbionts than *V. fischeri* symbionts. Although the Bay of Banyuls is mainly coastal shelf (approximately 0-150 meters), the change in temperature due to seasonality may be a strong factor influencing symbiont distribution in the squid species that are affected by changes in temperature (*S. affinis*). Since *S. affinis* is found above the thermocline boundary year around, this squid was found to be more susceptible to changes in symbiont composition due to environmental changes rather than host specificity. Sepiolids which reside at depths below the thermocline (*S. intermedia*, *S. ligulata*, *S. robusta*) are rarely found in habitats above the thermocline; therefore these species may be more prone to form species alliances with psychrophilic *V. logei* strains. Although previous *in vitro* experiments with juvenile squids did not show any differences in the infection capability between *S. affinis* or *S. ligulata* juveniles (Nishiguchi, 2000), there have not been sufficient data collected to test host specificity with the other species of squids or their symbiotic vibrios. Future work is planned to include all species of sepiolids found in the Mediterranean, Adriatic, and Atlantic Seas, and to test how concentrations of environmentally transmitted bacteria are related to the presence and distribution of various host squids.

4. Deciphering the mechanisms which drive specificity and parallel cladogenesis

In the preceding paragraphs, I have given examples of how various symbiotic associations either follow expected patterns of cospeciation between the partners or are influenced by abiotic factors such as temperature. Because a particular mode of transmission cannot be used to predict whether two organisms have evolved in parallel with each other, other components such as environmental fluctuations or host-symbiont specificity must be considered. In the examples where there is promiscuity between symbionts and their hosts, there is much speculation about how specific host-symbiont assemblages evolved, and why there is so much diversity expressed in associations where there is a high degree of dependence for some physiological function or capability. It has been previously hypothesized that the benefit of obtaining a symbiont would allow the adaptation to a new environment to exploit a different ecological niche (Saffo, 1992). Obviously, these types of adaptations would increase the survival and fecundity of both organisms. Evolving with a new symbiotic partner can also affect other biochemical interactions not necessarily linked to nutrient exchange (Huger *et al.*, 1985), and may alter sex ratios of hosts with either beneficial or detrimental effects (Nardon and Grenier, 1991). Developmental changes can also be induced with the onset of symbioses (Doino and McFall-Ngai, 1995; Lemus and McFall-Ngai, 2000; Schwemmler, 1989; Visick and McFall-Ngai, 2000), increasing the ability of the symbionts to become an integral part of the host life cycle and therefore having an effect on the overall success of the entire symbiosis. Thus, many of the interactions that occur between host and symbiont have caused one or both partners to undergo complicated and extensive life history changes to accommodate the other's existence.

4.1. ADAPTATION IN PARALLEL?

If several ancestral symbionts adapted to similar host species, thereby allowing independent lineages of symbiotic associations to evolve, then why do we see congruency in host-symbiont pairs where environmental transmission would allow more diversity? Is specificity a less deleterious mode of antagonistic behavior that has been

modified between the host and the symbiont to allow coexistence between the partners? Why have we observed examples where expected congruency is promiscuous (as in vertical transmission) and parallel cladogenesis is evident in environmentally transmitted symbiosis? Do environmental factors have direct effects upon the degree of specificity found in symbiotic associations, or do they magnify the initiation and continuation of a specific host-symbiont relationship?

In the sepiolid-squid mutualism, there are two populations of genera that exhibit both specificity and promiscuity. Law (1985) makes the prediction that mutualistic associations should express less specialization between symbionts that are found in similar host species. Therefore, one would expect that allopatric populations are more prone to divergence than sympatric sister taxa, and that their symbionts would diverge in parallel. Support for this prediction comes from the competitive hierarchy observed between strains of symbionts with closely related host taxa (Nishiguchi *et al.*, 1998). For example, the Indo-west Pacific genus *Euprymna* has several biogeographically isolated species, and their symbiotic partners display a high degree of specificity, even though all symbionts equally infect juveniles of a different host squid when presented alone (Nishiguchi *et al.*, 1998). Although the hosts obtain their symbionts anew with every generation, other mechanisms must influence the precise identification and retention of a particular symbiotic strain. These mechanisms could include: specific receptors on the host epithelia which recognize specific bacterial strains (Hensey and McFall-Ngai, 1992; McFall-Ngai *et al.*, 1998); various adhesin proteins on the bacteria that allow it to settle inside the squid light organ (Jacob-Dubuisson *et al.*, 1993); adaptation to the biochemical milieu of the light organ (Small and McFall-Ngai, 1999; Visick and Ruby, 1998; Weis *et al.*, 1996); the ability of the bacteria to colonize the squid tissues effectively and without competition from other types of bacteria (Graf and Ruby, 1998) and the communication between symbionts and host to enable the symbiosis to effectively work in unison (Doino and McFall-Ngai, 2000; Foster and McFall-Ngai, 1998). Deciphering these mechanisms will shed more light on how symbiotic bacteria and their particular hosts are capable of evolving intricate patterns of specificity and coevolution.

Despite the specific associations shown in *Euprymna*, the genus *Sepiola* has different patterns of physiological specificity. Specificity is observed within the symbiont genus *Vibrio*, and it appears that the most influential factor determining symbiont composition between *Vibrio* species is the ecology of the association. Previous studies investigating the influence of temperature acclimation in *E. coli* (Mongold *et al.*, 1996; Bennett and Lenski, 1993) have shown that the selection criterion of a particular strain has no discernable effect on the future adaptation to a new or different environment. But, in contrast, the evolutionary history of a bacterial strain will influence the future fitness of a strain, particularly when it is acclimated to a temperature which that species has been previously adapted to (Bennett and Lenski, 1998). Assuming that this holds true for bacteria in symbiotic associations, one may conclude that symbiotically competent vibrios capable of infecting squids at certain temperatures are not under any less selection pressure than other species of *Vibrio*, but may have the ability to infect different host species at a better rate or increase their persistence more than strains not acclimated to the infection temperature. This may provide some insight as to how environmental factors can influence the distribution of infective bacteria in a particular host taxon and therefore have direct effects on the biogeography and population genetics of host-symbiont pairs.

Finally, the type of transfer among numerous symbiotic taxa has traditionally been thought of as a good predictor of cospeciation. Recently, however, a number of examples have demonstrated that the systematics underlying these symbioses are not as clear as we have traditionally thought. The conflict between patterns of symbiont transmission arises when there is competition between closely related symbionts; migration leads to this competition from other closely related symbionts, and may have

potential side effects on the hosts (Frank, 1996a). Possible explanations for the variation in patterns of cospeciation have been complicated; the fundamental difference between transfer modes may be linked to the selection pressure on the host's fitness and how the symbiont may or may not affect future generations of host-symbiont pairs (Sniegowski *et al.*, 1997; Frank, 1996a). As well as host fitness, population size, and the availability of hosts that can be infected will affect the dynamics of host-symbiont pairs and the specificity that these pairs will eventually express (Bull *et al.*, 1991, Turelli, 1994; Turner *et al.*, 1998;). The presence of two different species of symbiont that can equally infect different host species may be an initial step in establishing host-symbiont congruency prior to host-symbiont specificity. But how the natural balance between host fitness and symbiont virulence is established, remains to be determined (Frank, 1996b). Because there are several sympatric host species of *Sepiola* living in the area sampled, the possibility of host switching between squids and the establishment of host-symbiont specificity can be studied. Future studies will need to address what degree ecological and/or genetic factors control patterns of cospeciation and congruency, and how we can better predict which patterns arise from these factors. Whether the symbionts determine new avenues for host evolution and radiation into different ecological habitats in this family of squids is just one of the many questions that need to be addressed in future studies.

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