Eugene Rosenberg
Ilana Zilber-Rosenberg

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Eugene Rosenberg
Department of Molecular Microbiology
and Biotechnology
Faculty of Life Sciences
Tel Aviv University
Tel Aviv
Israel

Ilana Zilber-Rosenberg
Givat Shmuel
Israel

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To our offspring holobionts

Preface

As soon as the right method was found, discoveries came as easily as ripe apples from a tree.

—Robert Koch

We are in the midst of a paradigm change in biology. Animals and plants can no longer be considered individuals, but rather, all are holobionts consisting of the host and diverse symbiotic microorganisms. During the last two decades, numerous studies have demonstrated that these symbionts play a critical role in the physiology of all holobionts including metabolism, behavior, development, adaptation, and evolution. In 2007 and 2008, we presented the hologenome concept as a framework for describing and understanding the complex properties of holobionts (Rosenberg et al. 2007; Zilber-Rosenberg and Rosenberg 2008).

We would like to mention briefly how we came upon the hologenome concept. In 1996, we discovered bacterial bleaching of corals (Kushmaro et al. 1996, 1997). After 6 years of studying the mechanisms of infection (reviewed in Rosenberg and Falkovitz 2004), we observed that the coral had become resistant to infection and bleaching by the specific pathogen, *Vibrio shiloi*. Because corals possess a restricted adaptive immune system and do not produce antibodies, we presented the coral probiotic hypothesis (Reshef et al. 2006) to explain the coral development of resistance to infection by *V. shiloi*. The hypothesis posits that the corals acquired stochastically from the marine environment "beneficial" bacteria that prevent infection by the pathogen. If it is possible to have epidemics of pathogens, why is it not possible (even more likely) to have epidemics of beneficial bacteria? They simply generally go unnoticed. Recently, we have published data that support the coral probiotic hypothesis (Mills et al. 2013). A dynamic relationship exists between symbiotic microorganisms and corals under different environmental conditions that select for the most advantageous coral holobiont in the context of the prevailing conditions.

Although the hologenome concept was inspired by our coral probiotic hypothesis, the concept has been developed by consideration of the vast amount of data published by others on the microbiota of plants and animals, including humans. We especially acknowledge Lynn Margulis (1938–2011), who saw before anyone else the importance of bacteria in the evolution of higher organisms (Margulis 1970, 1992).

Let us mention but a few of the pioneers who used culturing and noncultured molecular techniques to examine microbial communities: in animals, David Relman (Kroes et al. 1999), Jeffrey Gordon (Hooper et al. 2001), Forest Rohwer (Rohwer et al. 2001), Margret McFall-Ngai (McFall-Ngai 2002), Harry Flint (Hold et al. 2003), Martin Blaser (Pei et al. 2004), Rob Knight (Lozupone and Knight 2005), and Ruth Ley (Ley et al. 2005); and in plants, Linda Thomashow (Weller et al. 2002), Steven Lindow (Lindow and Brand 2003), Erik Triplett (Tyler and Triplett 2008), Kiwamu Minamisawa (Ikeda et al. 2010), and Davidi Bulgarelli (Bulgarelli et al. 2012), with apologies to the many other important early contributors to this field.

In this book, we will present and discuss a large number of theoretical and experimental studies that have contributed to the paradigm change in biology, placing them within the broad concept of the hologenome. The first step in many ecological studies is determining the numbers of the different species in the study site. Using DNA sequencing techniques, it is now a routine procedure to determine the relative number of bacterial species in any ecological niche. During the last 15 years, these techniques have constantly been improved and applied to analyze microbial and viral communities associated with various plants and animals, including humans. The data demonstrate that all animals and plants contain abundant and diverse microbiota (summarized in Chap. 3 for bacteria and for viruses in Chap. 7). Furthermore, it also has been shown that microbiotas are transferred from parent to offspring by a variety of mechanisms ensuring the continuation of each unique hologenome (summarized in Chap. 4). The biological and biomedical literature contains many reports on correlations between microbial populations and the health and disease of the host. In several cases, the specific contribution of the microbiota to the fitness of the host has been elucidated (summarized in Chap. 5). We will show that putting together these vast data clearly demonstrates that each holobiont (host + microbiota), with its hologenome (host genes + microbiome), is a unique biological entity, with the sum of the dynamic interactions within the holobiont giving rise to the genotype and phenotype of the organism, as we know it. The hologenome concept posits that the holobiont (host + all associated microorganisms, including viruses), being a unique biological entity, acts also as a level of selection in evolution. Accordingly, changes in either the host genome or the microbiome can lead to genetic variation—the raw material for selection and evolution. Chapter 6 introduces several previously underappreciated modes of variation that became apparent when we considered the holobiont as a level of selection. One of the important sources of variation in holobionts is viruses, which are discussed in Chap. 7. How these variations and others can lead to evolution of animals and plants is discussed in Chap. 8.

Microbial pathogens are a special class of symbionts, which depending upon the particular strain and circumstance, can be harmful or beneficial to the holobiont (Chap. 9). Practical applications of the hologenome concept, in the form of probiotics, prebiotics, synbiotics, and phage therapy, show promise in promoting health (discussed in Chap. 10). We argue that understanding the complex

interactions of microbiotas with their hosts to generate holobionts, as unique biological entities, will lead to a greater understanding of many aspects of biology (Chap. 11).

One of the problems we faced in completing this book was each time we completed a draft, important new publications appeared, which caused us to rewrite many of the chapters. This was not surprising because of the fast-moving nature of this subject. Our literature search was completed in November 2013. We thank Ed Kosower, Gil Sharon, Carolyn Elya, and David Gutnick for providing useful references and interesting discussions. It was a pleasure to work with Ursula Gramm and the other editors of Springer-Verlag in bringing the manuscript to publication.

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Contents

Chapter 1
Introduction: Symbioses and the Hologenome Concept

One cannot explain words without making incursions into the sciences themselves, as is evident from dictionaries; and, conversely, one cannot present a science without at the same time defining its terms

—Gottfried Wilhelm Leibniz

The Hologenome Concept

The hologenome concept posits that the holobiont (host + symbionts) with its hologenome (host genome + microbiome), acting in consortium, function as a unique biological entity and therefore also as a level of selection in evolution (Rosenberg et al. 2007; Zilber-Rosenberg and Rosenberg 2008). As discussed in chapters 6 and 8, consideration of the holobiont with its hologenome as a level of selection in evolution brings forth several previously underappreciated modes of variation and evolution. A large body of empirical data, which will be presented in subsequent chapters, provides the foundation for the hologenome concept. The key points are:

(1) **All animals and plants harbor abundant and diverse microbiota. In many cases the number of symbiotic microorganisms and their combined genetic information far exceed that of their host.**
(2) **The microbiota with its microbiome together with the host genome can be transmitted from one generation to the next with fidelity and thus propagate the unique properties of the holobiont and the species.**
(3) **The microbial symbionts and the host interact in ways that affects the physiology, health and fitness of the holobiont within its environment. The sum of these interactions characterizes the holobiont as a unique biological entity.**
(4) **Genetic variation in the hologenome can be brought about by changes in either the host genome or the microbial population genomes (microbiome). Under environmental change and stress, the microbiome can adjust more rapidly and by more processes than the host organism alone and thus can enhance the evolution of the holobiont.**

Major conclusions derived from the hologenome concept and the data that support it include (1) evolution of animals and plants was driven primarily by natural selection for cooperation between and with microorganisms, and (2) microorganisms have played a key role in the origin of new animal and plant species.

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History

Microbiology, like most scientific disciplines, has advanced by observations, asking interesting questions, and developing techniques to understand these observations and answer these questions. Microbiology began with the discovery by Antony van Leeuwenhoek in 1674 of protozoa in freshwater and in 1683 of a wide variety of bacteria in the human mouth. Between 1653 and 1673, Leeuwenhoek developed the curious hobby of constructing microscopes. Although he was not the first to build a microscope, his microscopes were the finest of that time. Leeuwenhoek patiently improved his microscopes and techniques of observation for 20 years before he reported his results in the form of letters, written in "Nether-Dutch," to the secretary of the Royal Society of England. After translation, the letters were published in the Philosophical Transactions of the Royal Society. For details on the life and discoveries of van Leeuwenhoek, we recommend his inspiring biography written by Clifford Dobell (1932).

Following van Leeuwenhoek's initial observations of microbes, it took 200 years for microbiology to develop into an experimental science. The liquid suspensions that Leeuwenhoek and other pioneers in bacteriology examined under the microscope contained a wide assortment of microbes of varying sizes and shapes. Was this because a particular organism could exist in various forms, or was it the result of a mixture of different organisms, each having a fixed form? To answer this question and to test the subsequent germ theory of disease, it became necessary to develop techniques for obtaining pure cultures. The first pure culture was obtained by the English surgeon, Lord Lister, in 1878, using a dilution technique. In 1881, the German bacteriologist Robert Koch developed the simple and efficient "streak-on-agar medium" technique for obtaining pure cultures that is still used today. The technique proved so valuable that by 1900, 21 microbes that caused diseases had been identified.

At about the same time that the Koch/Pasteur school of microbiology was employing pure cultures to isolate and characterize pathogenic bacteria, a second school of microbiology, led by the Russian microbiologist and ecologist Sergei Winogradsky and the Dutch microbiologist and botanist Martinus Beijerinck, argued that the prime focus of microbiology should be the role of microbes in the turnover of matter on the planet (Dworkin 2012). Toward that goal Winogradsky developed what is now called the "Winogradsky Column" to study how a large variety of phototrophic, chemotrophic, autotrophic, and heterotrophic bacteria interact in nature to metabolize organic and sulfur compounds, while Beijerinck invented the enrichment culture, a fundamental method of studying microbes from a mixture collected directly from the environment.

The two schools, Koch/Pasteur as opposed to Winogradsky/Beijerinck, stood for different viewpoints. The former concentrated on diseases and a single causative agent while the latter focused on the environment and the interactions within mixtures of microorganisms. The conflict between the two schools of microbiology was won at the time by the Koch/Pasteur school mainly because of its power in combatting infectious diseases. The students of the Koch/Pasteur school received the academic appointments, became the teachers of microbiology, got most of the research funds, and became the editors of the journals. As we will discuss below, it was not until the end of the twentieth century that the environmental school began to find its rightful place in microbiology.

During most of the twentieth century microbiological research continued to focus on isolating microbes that were causative agents of disease and studying their transmission and mechanism of pathogenesis. Subsequently and not less important, these pure culture techniques were applied as model microbial systems to discover the underlining mechanisms of biochemistry and genetics. To achieve that goal, essentially all research in microbiology was performed with a limited number of pure bacterial cultures under defined laboratory conditions. At that time insufficient tools were available to study the ecology, natural phylogeny, and evolution of microbes.

The advent of techniques for sequencing DNA, coupled with bioinformatics for analyzing the data and the cumulative knowledge that had been gained on pure bacterial cultures opened the door for studying and understanding the vast microbial richness in our world. This has led to a merger of microbiology with botany, zoology, human physiology and medicine, with the microbial realm at the base of global bio-ecosystems. Moreover, it becomes clear today that comprehending the vast role of microorganisms in nature will change the way we understand biology including the principles of ecology, physiology, embryology, immunity, and evolutionary biology.

Whereas a few microorganisms were used in the twentieth century as simple model systems to uncover the principles of biochemistry and genetics, their role in holobionts is more basic: they are not model systems, but an important part of the system in all animals and plants. This will lead to a fundamental change in how we view microbes in the biological world.

Concepts and Definitions

Since we will be using certain specialized terms and concepts throughout this book, we would like to define these terms and briefly discuss the main concepts in the present section before diving into deep waters. The term *holobiont* was initially introduced independently by Mindell (1992) and Margulis (1993) to describe a host and its primary symbiont; the meaning was subsequently expanded to include the host plus all of its symbiotic microorganisms, including viruses (Rohwer et al. 2002). The term *hologenome* is defined as the sum of the genetic information of

the host and its symbiotic microorganisms (Zilber-Rosenberg and Rosenberg 2008). The aggregate of all microorganisms of a holobiont is known as the *microbiota*, while the *microbiome*, a term coined by Lederberg and McCray (2001), describes the sum of the genetic information of the microbiota.

The term *metagenome* is often used in the literature to describe the sum of the genetic information of an environmental sample, including an animal or plant host and its symbiotic microorganisms. However, we consider hologenome to be a more appropriate term for describing the total genomes of an animal or plant for three reasons: First, metagenome is a general term for all the genetic material retrieved from any environmental sample, whereas hologenome is specific to the genetic material of a holobiont; Second, the prefix meta- (from Greek: μετά = "after", "beyond", "adjacent", "self"), is used in English to indicate a concept which is an abstraction from another concept, used to complete or add to the latter, such as in metaphysics, whereas the prefix holo- (from Greek: holos = whole) is more appropriate because it is used in English to denote whole, entire, and entirety. Third, the term hologenome parallels the accepted term holobiont.

What is the relationship between the hologenome concept and the classical phenomenon of *symbiosis*? The term symbiosis was first coined by Anton de Bary (1879) in the nineteenth century as "the living together of different species." His broad definition is still generally accepted. The symbiotic system is usually constructed from a large partner termed the host and smaller partners called symbionts. This arbitrary division by dimension between host and symbiont may not fit all systems because size can be measured by cell number or by genome size, and in the case of many symbioses the microorganisms both outnumber their host and contain more genetic information. In spite of these limitations, the definition of host and symbiont based on size continues to be widely used, although it would be more appropriate to consider both the host and symbionts as constituents of the holobiont. Among the symbionts one finds endosymbionts and exosymbionts, referring to microrganisms living inside or outside host cells, respectively. Symbioses can also take many forms; most of them are defined as different levels of mutualism, when both the host and the symbiont benefit from the interaction. When the symbiont benefits and the host suffers damage, symbiosis is termed parasitism (or pathogenesis). Commensalism is accepted to be a close association of two or more dissimilar organisms where the association is advantageous to one and does not affect the other(s). These types of symbioses may change under different circumstances, for example, parasitic microorganisms can become beneficial under different circumstances (Taylor et al. 2005).

The hologenome concept presents a novel way of looking at symbiosis. Until recently most of the important research on symbiosis has been carried out with a small number of model systems involving a host and a single primary symbiont, e.g., aphids and their endosymbiotic bacterium *Buchnera* sp (Wilson et al. 2010), the squid light organ and *Vibrio fischeri* (Nyholm and McFall-Ngai 2004), hard corals and their photosynthetic microalga of the genus *Symbiodinium* (Buddemeier et al. 2004), and legumes (Fabaceae) and nitrogen-fixing rhizobia (Ott et al. 2009).

Studies on these model systems and others have led to a greater understanding of how symbioses are established, maintained, and how the two partners co-evolved. However, the realization of the general significance and complexity of symbiosis in all animals and plants only became possible when molecular techniques were developed for analyzing complex bacterial communities and when sufficient knowledge of the properties of microbes had been gained by studies with simple model systems. The hologenome concept places importance not only on single established symbionts, but mainly on the enormously diverse associated microorganisms, many of which have only been uncovered in recent years with molecular techniques.

One way to emphasize the importance of microbial diversity to the holobiont is to compare the number of unique genes coded for by the microbiome to that of the host genome. For example, the human genome contains ca. 23,000 genes (Wei and Brent 2006), whereas the human microbiome was reported to contain over 9,000,000 unique genes (Yang et al. 2009), corresponding to a ratio of bacterial to human genes of 390:1. The high ratio of microbiome/host genes is not unique to humans or even vertebrates. Calculations based on the microbial diversity of corals (Gates and Ainsworth 2011) and sponges (Webster et al. 2010) compared to their corresponding host genomes (Srivastava et al. 2010; Shinzato et al. 2011), yields ratios of bacterial/host genes of 50–200.

In our initial publication of the hologenome concept (Zilber-Rosenberg and Rosenberg 2008), we noted that the hologenome concept fits within the framework of the *superorganism* first proposed by Wheeler (1928) and revived by Wilson and Sober (1989). Since that time there has been numerous scientific and popular publications referring to holobionts as superorganisms. It seems that it would now be useful to distinguish between the terms "superorganism" and "holobiont." A superorganism has been defined (Holldobler and Wilson 2008) as a "collection of single creatures that together possess the functional organization implicit in the formal definition of organism." As discussed by Gordon et al. (2013), "the super in superorganism denotes a higher level of organization, an association composed of multiple organisms of the same species." A holobiont, on the other hand, is an animal or plant host plus all of its symbiotic microorganisms. Thus, a holobiont consists not only of multiple species but includes representatives of two or three of the domains of life plus viruses. As a result, the holobiont contains an enormous diversity of genetic material, the hologenome. Another distinguishing difference between superorganisms and holobionts is that, according to Wilson and Sober (1989), "only some groups and communities qualify as superorganisms." In contrast, one of the fundamental principles of the hologenome concept is that all animals and plants are holobionts. We suggest that the term superorganism be reserved for eusocial animals, such as colony-forming ants. Accordingly, an ant colony is a superorganism composed of ant holobionts.

O'Hara and Shanahan (2006) referred to the human gut microbiota as "a forgotten organ." Although this analogy had a significant impact on focusing attention on the importance of the beneficial microbiota, the authors did not point out the fundamental differences between the microbiota and an organ: first, the

microbiota contains thousands of different genomes, whereas all cells in an organ have the same genome; second, the microbiota is in a dynamic state, with the relative numbers of some species increasing and others decreasing as a function of diet and other factors, while the cell number in a mature organ remains relatively constant. As a consequence of these fundamental differences, the microbiota acts not only as an integral and active part of an organism (an organ) but the microbiota is also able to play a significant role in adaptation to new conditions and in the evolution of the holobiont.

We will argue throughout this book on the validity and usefulness of considering the holobiont as a unique biological entity and therefore a primary level of selection in the evolution of animals and plants. The hologenome concept is consistent with the body of ideas known loosely as "multi-level selection theory" (Wilson 1997; Okasha 2006). Multilevel selection theory takes as its starting point the notion that natural selection can operate simultaneously at different levels of the biological hierarchy.

In order to appreciate the role of microbiota in the metabolism, fitness, variation, and evolution of higher organisms, we shall document each of the above four principles in subsequent chapters. We will then discuss how the hologenome concept adds a new dimension to biology in general and evolutionary biology in particular. While doing so, we will also discuss another interesting aspect of the hologenome concept, namely, the emphasis it places on cooperation, rather than competition, in the evolution of complexity.

Key Points

- During most of the twentieth century, following the lead of Pasteur and Koch, microbiologists focused on agents of disease and using pure cultures of a few microbes as model systems to study the underlying principles of biochemistry and genetics. Only at the end of the century, when DNA-based tools became available, was it possible to return to the Winogradsky/Beijerinck environmental viewpoint of focusing on the study of natural microbial communities, such as those associated with animals and plants.
- The hologenome concept considers the holobiont with its hologenome, acting in consortium, as a unique biological entity and therefore also an important level of selection in evolution. The holobiont has been defined as the host organism and all of its symbiotic microbiota, including viruses. The hologenome is the sum of the genetic information of the host and its microbiota.
- The hologenome concept posits that (1) all animals and plants are holobionts, harboring abundant and diverse microorganisms. (2) the microbiota with its microbiome together with the host genome can be transmitted from one generation to the next with fidelity and thus propagate the unique

properties of the holobiont and the species, (3) the microbial symbionts and the host interact in ways that affects the physiology and the fitness of the holobiont within its environment, and (4) genetic variation in the hologenome can be brought about by changes in either the host genome or the microbiome. The microbiome can change more rapidly and by more processes than the host organism alone and thus influences the adaptation and evolution of the holobiont.

- The hologenome concept places importance not only on the primary symbiont but also on the enormously diverse associated microorganisms, many of which have only been uncovered in recent years using molecular techniques.
- Major conclusions derived from the hologenome concept and the data that support it include (1) evolution of animals and plants was driven primarily by natural selection for cooperation between and with microorganisms and (2) microorganisms have played a key role in the origin of new animal and plant species.

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Chapter 2
Origin of Prokaryotes and Eukaryotes

The past the finite greatness of the past/For what is the present after all but a growth out of the past.

—Walt Whitman

Speculation on the Origin of Protocells

The fundamental desire to understand the origin of life is demonstrated by the fact that essentially all human cultures contain a story of how life began. From the tribes of ancient times to the mythologies of more modern cultures, there are countless stories of the origin of life. Some are based in pagan beliefs, while others are based on creation resulting from a holy deity. This collection of myths, legends, and tribal knowledge handed down over generations is the collective expression of how man has attempted to explain his world and his place in it. What is common to all these cultures is that their specific story of how life began is accepted without question. The role of the adults is simply to teach the story to the children.

The distinguishing feature of the scientific approach to understanding the origin of life is that it begins by acknowledging the fact that we do not know how life began. Only by admitting that we do not have the answer, is it possible to put forth and test multiple hypotheses.

The origin of life is one of the most challenging problems in biology. The best evidence is that prokaryotic life (microorganisms lacking a membrane-bound nucleus) first appeared on Earth about 3.8 billion years ago (Zimmer 2009). The abiogenesis theory of the origin of life begins with the experimentally supported concept that simple organic compounds, such as amino acids, organic acids, purines, pyrimidines, and simple sugars, could have been produced spontaneously in the earth's primitive reducing atmosphere (Oró and Kamat 1961), containing CH_4, H_2, H_2O, N_2, and NH_3 (Schaefer and Fegley 2010). Further, these molecules could have been concentrated into a "prebiotic organic soup" in evaporation ponds. Whatever the earliest events on the road to the first living cell were, it is clear that at some point the large biological molecules found in modern cells must have emerged. Considerable debate in origin-of-life studies has revolved also around which of the fundamental macromolecules came first—proteins, DNA, or RNA. To gain insight into this question it is useful to consider the functions performed by each of these polymers in existing organisms.

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The proteins make up about 50 % of the mass of most cells and are the main structural and functional agents in the cell. Catalytic proteins, or enzymes, carry out the thousands of chemical reactions that take place in any given cell, among them the synthesis of all other biological constituents, including DNA and RNA. However, proteins cannot replicate themselves. They require the information contained within the nucleic acids, DNA, and RNA. In all modern cellular organisms, DNA serves as the storage site of genetic information. The DNA contains the instructions for the manufacture of proteins. In the modern cell, protein, DNA, and RNA are each dependent on the others for their manufacture and function. DNA, for example, is merely a blueprint, and cannot perform a single catalytic function, nor can it replicate on its own. Proteins, on the other hand, perform most of the catalytic functions, but cannot be manufactured without the specifications encoded in DNA and transcribed to RNA. This classic "chicken-and-egg" problem made it immensely difficult to conceive of any plausible prebiotic chemical pathway to the molecular biological system.

One possible scenario for life's origins is that two kinds of molecules evolved together, one informational and one catalytic. But scientists studying the origin of life consider this scenario extremely complicated and highly unlikely. Another possibility, currently favored by many theorists, is that one of these molecules, namely RNA, could itself perform the multiple functions of self-replication and catalysis. Catalytic RNAs, also referred to as ribozymes, were discovered in the 1980s (Cech et al. 1981; Guerrier-Takada et al. 1983). Today, RNA molecules are the only molecules known both to store genetic information (as in the RNA viruses and viroids or during transport in the form of mRNA) and to exert biological catalysis, like a protein enzyme. RNA may therefore have supported precellular life and been a major step in the evolution of cellular life. This hypothesis, referred to as the "RNA world" (Gilbert 1986), has gained support in recent years from experiments demonstrating that RNA can perform self-cleavage and autocatalyze its own synthesis (Johnston et al. 2001). The RNA world hypothesis assumes a phase of life, whereby catalytic biopolymers consisted exclusively of ribozymes, hence the name "RNA world." With the appearance of the prototypical ribosome began the translation of RNA sequences into protein sequences. This gave rise to an RNA–protein world with a step-by-step replacement of ribozymes by enzymes (enzymatic take-over). Much later, according to this theory, RNA as the replicating informational molecule was replaced by the chemically more stable double-stranded DNA (Lazcano et al. 1988). Although criticized by several scientists (Bernhardt 2012), the RNA world hypothesis is considered the most promising we currently have to help understand the backstory to contemporary biology.

Another important issue to consider in the origin of life is boundaries, how molecules might have interacted to assemble into the first cell-like structures, or "protocells." The origin of cell membranes is a major unresolved issue of evolution (Peretóa et al. 2004). Contemporary cells are enclosed by membranes that are essentially made out of phospholipid bilayers (or monolayers in many thermophilic prokaryotes) into which different proteins are embedded. Phospholipids are amphipathic (contain both polar and hydrophobic groups) molecules composed

of a glycerol phosphate moiety and two lateral hydrocarbon chains (usually C14–C20). The membranes of eukaryotes also contain cholesterol. Membranes keep the cell components physically together and form a barrier to the uncontrolled passage of large molecules. Sophisticated proteins embedded in the membrane act as gatekeepers and pump molecules in and out of the cell, while other proteins assist in the construction and repair of the membrane. How on earth could a rudimentary protocell, lacking protein machinery, carry out these tasks? Primitive membranes were probably made of simpler molecules, such as fatty acids (which are one component of the more complex phospholipids). Research has shown that membranes could indeed assemble spontaneously from elementary fatty acids, and molecules as large as nucleotides can in fact easily slip across these membranes as long as both nucleotides and membranes are simpler, more "primitive" versions of their modern counterparts. In fact, it has been shown that fatty acid-based membrane vesicles containing a short piece of a single-stranded nucleic acid could incorporate active versions of nucleotides (Richardo and Szostak 2009). The nucleotides crossed the membrane spontaneously and, once inside the model protocell lined up on the nucleic acid which reacted with one another to generate a complementary strand. Some experimental evidence supports the idea that the first protocells contained RNA (or something similar to it) and little else and replicated their genetic material without enzymes.

We would like to emphasize that attempts to explain the origin of life are rich in speculation and poor on evidence. When a particular concept is tested in a laboratory and yields a result consistent with the hypothesis, then the hypothesis moves from pure speculation to a possible hypothesis. For example, the formation of amino acids from gases in the Earth's prebiotic atmosphere is a possible step in the origin of life because it has been experimentally demonstrated. The RNA world hypothesis is still primarily speculation since the abiotic formation of activated ribonucleotides has not yet been shown. It should be pointed out that there are other published speculations on the origin of life, such as the "protein interaction world" (Andras and Andras 2005) and the iron–sulfur world (Wächtershäuser 2010) hypotheses.

An alternative to the concept that life originated on Earth by a series of prebiotic chemical reactions is the Panspermia Theory. The word "Panspermia" comes from the Greek language and means "seeds everywhere." The seeds in this case would not only be the building blocks of life, such as amino acids and monosaccharides, but also small extremophile organisms. The theory states that these "seeds" were dispersed "everywhere" from outer space and most likely came from meteor impacts. Once the earth had cooled sufficiently, these invading microbes from other celestial bodies found the conditions favorable for growth and gave rise to life on this planet.

Recent research has provided some support for the Panspermia Theory. First, we now know that the time from when the earth cooled to permit life and the first evidence of life was relatively short, less than 300 million years; further, the first microfossils were photosynthetic cyanobacteria. Most models of the origin of life have the earliest organisms obtaining energy from reduced chemicals, with the

more complex mechanisms of photosynthesis evolving later. Second, it has been argued that some microbes are resistant to extreme conditions and may be able to survive for very long periods of time, probably even in deep space and, hypothetically, could travel in a dormant state between hospitable environments (Nicholson et al. 2000). Third, researchers have reported the presence of complex organic molecules (Callahan et al. 2011) and possibly extraterrestrial bacteria (D'Argenio et al. 2001) inside meteorites.

The Panspermia Theory has been criticized because it dodges the question. If the earth was infected by microbes, we still have to explain how these microbes came into existence on their native planet. Nevertheless, the Panspermia Theory has been supported by several leading scientists, including Stephen Hawking, Nobel Prize winner Francis Crick, and Leslie Orgel (1973).

Origin of Prokaryotes and Eukaryotes

The oldest direct evidence for life on Earth is well-preserved microfossils of prokaryotes found in 3.9-billion-year-old rocks in Western Australia (Wacey et al. 2011). Prokaryotes (Greek for "before karyon" or "before nucleus") are simple, single cell organisms that lack a membrane-bound nucleus. Eukaryotes (Greek for "true nucleus") divide by mitosis and possess a membrane-bound nucleus, an intricate cytoskeleton, mitochondria, and in the case of algae and plants cells, also chloroplasts. Based on the fossil record, single cell eukaryotes first appeared ca. 1.8 billion years ago (Parfreya et al. 2011). According to these data, prokaryotes were the only cellular form of life for 2.1 billion years. During this time, prokaryotes evolved most of the biochemistry present in all forms of life, including DNA replication, the genetic code, protein synthesis via transcription and translation, photosynthesis, and anaerobic and aerobic metabolism. Furthermore, based on differences in their ribosomal RNA gene sequences, molecular microbiologists have concluded that during this time, prokaryotes split into two groups, or domains, titled Bacteria (or eubacteria) and Archaea (or archaebacteria) (Woese and Fox 1977; Pace et al. 2012).

Since eukaryotes perform the same basic biochemical reactions as prokaryotes, i.e., the unity of biochemistry, it is highly likely eukaryotes descended from prokaryotes. For example, of the approximately 22,000 human genes that code for proteins (Stein 2004), 60 % of them are homologs of prokaryotic genes (Domazet-Loso and Tautz 2008), primarily those involved in intermediate metabolism. Many scientists assumed that eukaryotes evolved from prokaryotes through the familiar process of mutation and natural selection. However, Lynn Margulis argued that a number of parts of the eukaryote cell were acquired in a radically different way, namely by the fusion of separate bacterial species (Sagan 1967). Many studies have bolstered this once-controversial endosymbiont hypothesis. Let us first consider the mitochondrion, a fundamental organelle of all eukaryotic cells. Mitochondria resemble bacteria in many ways. Both are surrounded by a double layer membrane,

and the structural arrangement of the inner and outer membrane of mitochondria is similar to Gram-negative bacteria. Mitochondria and bacteria can use oxygen to generate chemical energy, in the form of adenosine triphosphate (ATP) molecules. The ribosome coded for by mitochondrial DNA is similar to those from bacteria in size and structure. Mitochondria have their own bacterial-like DNA, which they duplicate when they divide, similarly to bacteria, into new mitochondria. Based on DNA analyses, mitochondria are closely related to the intracellular parasite *Rickettsia prowazekii* (Andersson et al. 1998), the causative agent of epidemic typhus. Using similar arguments, it is now generally accepted that chloroplasts of photosynthetic algae and plants arose through incorporation into cells of symbiotic cyanobacteria (Martin et al. 2002). In fact, the data indicate that chloroplasts were acquired through multiple primary endosymbiotic events (Fagan and Hastings 2002) that occurred long after the acquisition of mitochondria (Perasso 1989). If one accepts the bacterial origin of mitochondria and chloroplasts, then the eukaryote cell, itself, is a holobiont.

One of the fundamental differences between eukaryotes and prokaryotes is the presence of a membrane-bound nucleus in eukaryotes. Because the nucleus lacks an obvious homologue or precursor among prokaryotes, ideas about its evolutionary origin are diverse and highly speculative. One hypothesis is that a prokaryotic cell membrane formed an invagination that enclosed the DNA in a primitive prokaryote (probably an Archaea) and this membrane-bound DNA evolved into the nucleus (Jékely 2003). Another hypothesis is endosymbiosis of an archaebacterium within an eubacterium, with the archaebacterium becoming a nucleus (Martin 2005). The reason for assuming that the archaebacterium became the nucleus is that the molecular machinery involved in information storage and retrieval in eukaryotes is more similar to archaebacterial counterparts than to eubacterial counterparts (Reeve 2003). On the other hand, eukaryotic genes involved in metabolic and biosynthetic pathways reflect a eubacterial ancestry (Simonson et al. 2005).

Recently, it has been discovered that certain bacterial species within the phylum Planctomycetes possess features typical of eukaryotic cells, such as intracellular compartmentalization, a lack of peptidoglycan in their cell walls, and a membrane-bound nucleoid analogous to the eukaryotic nucleus (Fuerst and Sagulenko 2011). However, Planctomycetes are definitely bacteria on the basis of gene sequence trees such as those from ribosomal RNA (Fuerst 2010). Planctomycetes have several other dramatically distinctive features separating them from other prokaryotes (Fuerst 1995). They occur in many marine and freshwater aquatic habitats and in soils, as well as in extreme habitats such as the ultra-dry Mars-like soil of the Atacama Desert (Schlesner 1994) and living stromatolite microbial mats in hypersaline marine shallows (Papineau et al. 2005). They are so different from other bacteria that, when first detected by microscopy of lake water, they were confused with fungi since some have stalks easily mistaken for cellular filaments like those of fungi. The compartmentalization of Planctomycetes challenges our hypotheses regarding the origins of eukaryotic organelles. Furthermore, the recent discovery of both an endocytosis-like ability in addition to proteins homologous to

eukaryotic clathrin in a planctomycete marks this phylum as one to watch for future research on the origin and evolution of the eukaryotic cell (Fuerst and Sagulenko 2011).

A third provocative option for the origin of the nucleus revolves around viruses. It has been proposed that a complex membrane-bound DNA or RNA virus became established in a prokaryote and evolved into the eukaryotic nucleus by acquiring genes from the host (Bell 2001). The eukaryotic nucleus shares several properties with certain viruses, such as mRNA capping, linear chromosomes, and separation of transcription and translation. According to this hypothesis, a large virus would take control of a bacterial or archaeal cell. Instead of replicating and destroying the host cell, it would remain within the cell. With the virus in control of the host cell's molecular machinery it would effectively become a "nucleus" of sorts (Forterre 2006). Through the processes of mitosis and cytokinesis, the virus would thus hijack the entire cell—an extremely favorable way to ensure its survival. The hypothesis that a virus was the origin of the eukaryotic nucleus brings forth the question of the origin of viruses.

The recent discovery of giant DNA viruses has provided some additional support for the viral origin of the nucleus hypothesis (Philippe et al. 2013). These virus particles are large enough to be visible under light microscope and contain DNA genomes larger in size and gene content than some bacteria and simple eukaryotes (Xiao et al. 2009). Amongst the more than 1,200 genes present in the giant viruses are several genes thought to be the hallmark of cellular organisms, including a number of genes coding for eukaryotic-like proteins involved in transcription and translation.

It should be pointed out that the three hypotheses are not mutually exclusive and a combination of them may have been involved in forming the nuclei of eukaryotic cells.

Origin of Viruses

Since their discovery in the late nineteenth century (Bordenave 2003; Iwanowski 1892), viruses have challenged our concept of what "living" means. Initially seen as toxic agents that cause disease (Beijerinck 1898), then as life-forms that multiply only in cells, and then as biological chemicals that could be crystallized (Bernal and Fankuchen 1941), viruses are currently thought of as being in a gray area between living and nonliving (Villarreal 2004). The Nobel laureate André Lwoff wrote (Lwoff 1967), "Whether or not viruses should be regarded as organisms is a matter of taste. A virus is a virus." Regardless of whether or not one considers viruses living organisms, it is now clear that they play a major role in controlling geochemical cycles (Rohwer and Youle 2011), structuring cellular populations and, as we shall see, also in the fitness and evolution of holobionts (discussed in Chap. 7).

Three main hypotheses have been put forth to explain the origin of viruses (Wessner 2010):

(1) Viruses arose from intracellular genetic elements that gained the ability to move between cells. According to this hypothesis, viruses originated through a progressive process. Mobile genetic elements, pieces of genetic material capable of moving within a genome, gained the ability to exit one cell and enter another.
(2) Viruses are remnants of cellular organisms. In contrast to the progressive process of hypothesis 1, viruses may have originated via a regressive, or reductive, process. Microbiologists generally agree that certain bacteria that are obligate intracellular parasites, like *Chlamydia* and *Rickettsia* species, evolved from free-living ancestors. It follows, then, that existing viruses may have evolved from more complex, possibly free-living organisms that lost genetic information over time, as they adopted a parasitic approach to replication.
(3) Viruses predate or coevolved with their current cellular hosts.

It should be pointed out that these hypotheses are not mutually exclusive. To distinguish between these hypotheses it would be useful to date the origin of viruses. Unfortunately, it has not yet been possible to detect ancient viruses in fossils (Emerman and Malik 2010). However, there is strong circumstantial evidence that viruses emerged very early in the evolution of life, before the separation of prokaryotes into two domains: Eubacteria and Archaebacteria. The evidence is the striking structural similarities between viruses that infect organisms belonging to the different domains of life. For example, archaeal and bacterial tailed phages show remarkable morphological similarity (Zillig et al. 1996), and the *Sulfolobus islandicus* rod-shaped archaeal virus SIRV shows structural and mechanistic similarities to eukaryotic poxviruses (Filée et al. 2003). If the viruses arose after the separation into the three domains of life, one would expect that each domain would have its own characteristic viruses.

Another argument in favor of the early origin of viruses is that some eukaryotic and prokaryotic viruses exhibit a high level of similarity with regard to the organization of their genomes and replication process (Peng et al. 2001). If viruses arose from intracellular genetic elements (hypothesis 1), or are remnants of cellular organisms (hypothesis 2), then one would expect strong homology between virus genes and cellular genes. In fact, most genes in viromes have no homologues in cellular genomes (Wommack et al. 2009). Even more convincing is the fact that so-called hallmark genes that are central to viral replication and structure are shared by many RNA and DNA viruses but are missing from cellular genomes (Koonin et al. 2006). It has been suggested that the first viruses were RNA viruses that originated during the RNA world and played a critical role in major evolutionary transitions, such as the invention of DNA and DNA replication mechanisms (Forterre 2013).

Although the origin of viruses remains an unsolved mystery, it is highly likely that virus-like entities predated the appearance of modern cells. The common dismissal of an early viral origin on the grounds that all extant viruses are intracellular parasites is over simplistic.

Origin of Multicellular Organisms

Multicellularity developed in prokaryotes prior to eukaryote evolution (Bonner 1998). The fossil record indicates the presence of multicellular cyanobacteria about 2.7 billion years ago, whereas multicellular eukaryotes are first seen only 1.2 billion years ago (Fedonkin 2003). Let us consider some theoretical advantages of multicellularity. To begin with, the smaller the cell the higher the ratio of cell surface to volume. This allows for rapid uptake of nutrients and removal of waste products and is one of the traits that enable bacteria to grow rapidly. However, having a high surface-to-volume ratio also makes the cell more exposed and more vulnerable to the environment. To overcome this predicament, a cell can either grow larger or enter into aggregations with other cells, i.e., become multicellular.

Many types of bacteria form biofilms, which are structured cell aggregates. Bacteria inside the biofilm are considerably more resistant to antibiotics and other toxic materials than free-living cells. Still it is crucial that the multicellular structure be constructed such that water, nutrients, and waste products can flow through it. In addition to protection, multicellularity allows for cell density-dependent reactions (Rosenberg et al. 1977). For example, metabolic utilization of nutrients that contain polymers, such as proteins and polysaccharides, requires extracellular enzymes because polymers generally cannot be transported through cellular membranes. An isolated individual cell does not produce a high enough concentration of enzyme in its surroundings to effectively breakdown the polymer to smaller units that can be taken up by the cell. However, an aggregate of cells, each contributing enzymes, can efficiently breakdown the polymer into smaller units which can be taken up by all the cells in the multicellular structure. The same cell density-dependent argument holds for cell signaling. Cell-to-cell adhesion and signaling are two mechanisms that are widespread in the bacterial world. *Myxobacteria xanthus,* for example, when starved of nutrients, produce signals and aggregate by gliding chemotaxis in order to construct species-specific fruiting bodies consisting of thousands of cells (Dworkin 1996). Interestingly, the signals used by *M. xanthus* include molecules, such as kinases and G proteins, in common with eukaryotes. It should be pointed out that most natural biofilms are composed of a cooperating mixture of different species of bacteria; accordingly, they fit the definition of symbiosis.

The origin of the first multicellular eukaryotic organism has been a topic of intense debate in biology, and many hypotheses have been put forth to explain this evolutionary milestone (Grosberg and Strathmann 2007). It is reasonable to

hypothesize that early eukaryotic cells, formed by the fusion of two or more prokaryotes, had the genetic information that would allow for cell-to-cell interactions and the formation of multicellular structures. Support for this hypothesis comes from the discovery that morphogenesis of a choanoflagellate (one of the closest living relatives of animals) is induced by bacteria in the Bacteroidetes phylum (Alegado 2012). Further, it was shown that the inducing factor is a bacterially produced sulfonolipid. This study provides another example of how bacteria may have contributed to the evolution of animals. The relative ease at which unicellular organisms can evolve into multicellularity is supported by the fact that multicellularity has evolved independently dozens of times in the history of Earth, for example at least once for plants, once for animals, once for brown algae, and several times for fungi, slime molds, and red algae (Bonner 1998).

The earliest animal that still exists is the sponge. What can the sponge tell us about the early evolution of animals? Costerton et al. (1995) has compared modern sponges to biofilms because both lack tissues and organs, but are composed of a three-dimensional matrix that allows for the flow of water, nutrients, metabolites, and oxygen. Modern sponges are well known for containing large complex microbial symbiotic communities. More than half of the biomass of some sponges is bacteria (Taylor et al. 2007). The fossil record of sponges demonstrates their ancient association with bacteria, further indicating that prokaryotic symbionts were essential components of animals from their very beginning. Interestingly, some present sponge symbionts produce proteins which have domains that have cell-attachment activity (Siegl et al. 2010). One could speculate that similar bacterial proteins were involved in providing the "glue" for the construction of the first multicellular eukaryotes. Some evidence exists for specific genes involved in early multicellularity (Rokas 2008).

It should be pointed out that not all manifestations of multicellularity are the same. For example, multicellularity in volvocine green algae likely evolved as a consequence of incomplete separation after cell division, whereas in cellular slime molds multicellularity evolved as a consequence of aggregation (Waggoner 2001). Many, but not all, of the molecular components of the genetic toolkit for multicellularity are also present in the DNA records of unicellular relatives, which suggest that these components were likely present in their last common (unicellular) ancestor (Rokas 2008). A large fraction of the additional genes associated with cell–cell signaling and transcriptional regulation observed in these multicellular–unicellular comparisons can be accounted for by gene duplication (Goldman et al. 2006). Genomic analysis of *M. xanthus* identified more than 1,500 duplications that occurred during the transition to multicellularity, and determined that cell–cell signaling and regulatory genes underwent 3–4 times as many duplications as would be expected by chance.

In light of the available information, we propose that animal and plant cells arose from prokaryotic organisms by fusion, aggregated into multicellular complexes, initially using prokaryotic genetic information, and differentiated into animals and plants, always in close association with microorganisms. During evolution, which is discussed in Chap. 8, animals and plants acquired additional

structures and functions either by changing their DNA or by acquiring new symbionts. Good examples of the latter are ruminants (Dehority 2003) and termites (Brune 2011), which evolved the ability to utilize cellulose as a nutrient by incorporating cellulose-decomposing microorganisms, thereby avoiding the very slow process of evolving novel efficient enzyme systems and regulatory elements by themselves.

Key Points

- Life, in the form of prokaryotes, first appeared on earth about 3.8 billion years ago. The abiogenesis theory of the origin of life begins with the experimentally supported concept that simple organic compounds would have been produced spontaneously in the earth's primitive reducing atmosphere. These molecules could have been concentrated into a "prebiotic organic soup," which by chemical evolution gave rise to polymers and eventually protocells. The Panspermia Theory considers that once the earth cooled it was infected with extremophile organisms from other celestial bodies.
- The "RNA world" hypothesis assumes an early phase of life, wherein catalytic biopolymers consisted exclusively of ribozymes. Subsequently, prototypical ribosomes began the translation of RNA sequences into protein sequences. Much later, RNA as a replicating informational molecule was replaced by the chemically more stable DNA.
- Single cell eukaryotes first appeared ca. 1.8 billion years ago, so that prokaryotes were the only cellular form of life for 2.1 billion years. During this time, prokaryotes evolved most of the biochemistry present in all forms of life and split into two groups, or domains, termed Bacteria (or eubacteria) and Archaea (or archaebacteria).
- The formation of eukaryotes involved endosymbiosis of prokaryotes, mitochondria being derived from a *Rickettsia*-like bacterium and chloroplasts from cyanobacteria. The origin of the membrane-bound nucleus that characterizes eukaryotic cells is highly speculative. Hypotheses include an invagination of the prokaryote cell membrane to enclose the DNA, endosymbiosis of an archaebacterium within a eubacterium, with the archaebacterium becoming a nucleus, and a membrane-bound virus becoming established in a prokaryote and evolving into the eukaryotic nucleus by acquiring genes from the host.
- Although the origin of viruses remains an unsolved mystery, it is highly likely that virus-like entities predated the appearance of modern cells.
- The data suggest that eukaryotes arose from prokaryotes by fusion, aggregated into multicellular complexes, initially using prokaryotic genetic information, and differentiated into animals and plants, always in close

association with microorganisms. The earliest multicellular animal that still exists is the sponge which has certain similarities to bacterial biofilms because both lack tissues and organs, but are composed of a three-dimensional matrix that allows for the flow of water, nutrients, metabolites, and oxygen. The fossil record of sponges demonstrates their ancient association with bacteria, further indicating that prokaryotic symbionts were essential components of animals from their very beginning.

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Chapter 3
Abundance and Diversity of Microbiota

> *Mutually beneficial relationships between microbes and animals are a pervasive feature of life on our microbe-dominated planet. We are no exception: the total number of microbes that colonize our body surfaces exceeds our total number of somatic and germ cells by 10-fold, and the total number of microbial genes in our aggregate microbial communities is >100-fold greater than the number of genes in our human genome.*
>
> —Jeffrey Gordon.

There are no natural germ-free animals or plants. All are holobionts and contain associated microorganisms, including viruses. Moreover, as we have shown in the previous chapter, eukaryotic organisms have evolved together with microorganisms from the beginning. The hologenome concept emphasizes the importance not only of the intracellular symbionts but also and especially of the cooperation that takes place between all of the diverse and dynamic extracellular microbial symbionts that is present in all the animal and plant holobionts.

When asking fundamental questions as to the complex interactions between hosts and their microbiota and the evolution of a specific holobiont, it is important first to define the players within the holobiont and establish their numbers and their types. This is the subject of the present chapter.

Abundance of Microbiota

In most animals, including man, the largest numbers of symbionts are found in the digestive tract. Often, the number of symbiont cells exceeds that of the host, e.g., humans contain ca. 10^{14} bacteria and 10^{13} host cells (Berg 1996). In general, animals contain ca. 10^9 bacteria per g wet weight, which interestingly is similar to rich soil. Especially high concentrations of symbiotic bacteria are found in certain marine sponges, 10^{10} per g wet weight (Hentshel et al. 2006). Regarding plants, bacteria are by far the most numerous colonists of plant leaves, being found in numbers up to 10^8 cells per g, sufficiently numerous to contribute to the behavior of the individual plants on which they live (Lindow and Brandt 2003). The rhizosphere (close root area) of plants contains 10^5–10^6 fungi and 10^7–10^9 bacteria per g soil (Sylvia et al. 2005), the highest concentration being attached to the root epidermis.

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The number of microbial symbionts of various hosts has traditionally been estimated by total (microscopic) and/or viable counts. We now know that the viable counts of most environmental samples, such as water and soil, are generally one to three orders of magnitude lower than the total counts. The same is true for many determinations of microbiota associated with animals and plants. For example, the whole body human skin contains ca. 2×10^{10} and 1×10^{12} viable and total counts, respectively (Grice et al. 2009), and coral tissues contain 1×10^{6} and 2×10^{8} viable and total counts per cm^2, respectively (Koren and Rosenberg 2006). Exceptions to this generality are human feces (van Houte and Gibbons 1966) and cow rumen (Grub and Dehority 1976). In these systems, using optimized media and anaerobic culturing techniques, Eller et al. (1971) achieved similar total and viable counts, namely, about 1×10^{11} bacterial cells per g wet weight of feces or rumen content.

The large difference between total and viable count in environmental samples is generally attributed to the viable-but-not-culturable (VBNC) state of many bacteria. A bacterium in the VBNC state has been defined "as a cell which can be demonstrated to be viable, while being incapable of undergoing the sustained cellular division required for growth in or on a medium normally supporting growth of that cell" (Oliver 1993). In the cases of human gut and cow rumen bacteria, we suggest that the rapid turnover of bacteria in the gut and rumen would eliminate cells in the VBNC state, leaving only growing bacteria that can form colonies under appropriate conditions. Another possibility, not excluding the first, is the special effort put into growing bacteria in these two well-studied important systems.

Abundance of specific groups of bacteria can be determined by combining microscopy with 16S rRNA gene-targeted fluorescent probes (Levsky and Singer 2003). For example, the number of *Escherichia coli* in a sample was determined by using an oligonucleotide probe that is complimentary to that part of the 16S rRNA gene that is unique to *E. coli* (Smati et al. 2013). Choosing appropriate probes, it is possible to determine the abundance of specific strains, species, genera, phyla, or domains. This technology has been used widely, including samples from human feces (Franks et al. 1998; Weickert et al. 2011), chickens (Zhu and Joerser 2003), pigs (Hein et al. 2008), white flies (Gottlieb et al. 2008), and rice roots (Lu et al. 2006).

Diversity of Microbiota

Current research on the diversity of Bacteria and Archaea associated with a particular organism relies primarily on culture-independent DNA-based technology (Hamady and Knight 2009). The most popular approach relies on extracting the total DNA from a tissue, amplifying the 16S rRNA bacterial genes by polymerase chain reaction (PCR) technology and sequencing these genes. Sequences with greater than 97 % identity are typically assigned to the same species, those with >95 % identity are typically assigned to the same genus, and those with

>80 % identity are typically assigned to the same phylum (Kamfer and Glaeser 2013), although these distinctions are arbitrary and controversial (Schloss and Handelsman 2005). In sexually reproducing animals and plants, the ability of two organisms to interbreed and produce fertile offspring of both sexes is generally accepted as a simple indicator that the organisms share enough genes to be considered members of the same species. Thus a "species" is a group of interbreeding or potentially interbreeding organisms (Mayr 1942). Because this criterion cannot generally be applied to prokaryotes, the 97 % identity of 16S rRNA genes is routinely used to define a species. However, it is interesting to consider that the conserved region of the 18S rRNA genes of man and chimpanzee show 99.2 % identity (Laudien et al. 1985), so it is likely that different bacterial species may share more than 98 % identity. Thus, a higher resolution should probably be used to differentiate between bacterial species, though it is impossible at this time because of technical reasons.

The PCR method has been widely used for the amplification, detection, and quantification of DNA targets since its introduction (Olsen et al. 1986; Schmidt et al. 1991), resulting in a greatly increased knowledge of microbiota of animals and plants. However, the efficiency and accuracy of PCR can be diminished by many factors including primer–template mismatches, reactant concentrations, the number of PCR cycles, annealing temperature, the complexity of the DNA template, and others (van Wintzingerode et al. 1997). Primer–template mismatches are the most important because they can lead to selective amplification which prevents the correct assessment of microbial diversity (Polz and Cavanaugh 1998). Target sequences that cannot match the primers precisely will be amplified to a lesser extent, possibly even below the detection limit. The relative content of the sequences achieved is therefore changed, resulting in a deviation from the true community composition.

As things stand to date, if a microbial species is present, but rare, it is likely not to be detected with current technology, and lead to an underestimation of diversity. For example, if a particular bacterial species was present in 10^6 copies in the human colon (total bacteria = 10^{14}), it would be necessary to sequence 10^8 16S rRNA genes to detect it. This is clearly not possible with the existing technology. In an attempt to overcome this problem, microbial ecologists estimate diversity by extrapolations, assuming that low abundant species are present in a statistically predictable frequency. However, there is no evidence to support this assumption (Dunbar et al. 2002). We hasten to point out that rare bacterial species may amplify in numbers when conditions change and allow the holobiont to adapt better to the new condition.

Although censuses of microorganisms associated with different animal and plant species are rather sporadic and biased toward subjects currently being investigated for different reasons, there is already enough data (Table 3.1) to derive certain interesting generalizations: (1) Diversity of microbial species and strains associated with particular animals or plants is high, although diversity of phyla is usually small. (2) In spite of the high species diversity, one often finds metabolic conservation, i.e., different species perform the same metabolic

Table 3.1 Numbers of microbial species associated with different holobionts (animals and plants)

Host	Number of microbial species	Major microbial groups
Invertebrates		
Marine sponge (Schmidt et al. 1991; Webster et al. 2010)	2,996	Proteobacteria, Chloroflexi, Poribacteria
Coral (Sunagawa et al. 2009, 2010; Gates and Ainsworth 2011)	2,050	*Symbiodnium*, Proteobacteria, Planctomycetes
Hydra (Franzenburg et al. 2013; Bosch 2012; Fraune and Bosch 2007)	350	Bacteroidetes, Betaproteobacteria
Termite gut (He et al. 2013; Ohkuma 2008; Hongoh et al. 2005)	800	*Treponema*, Fibrobacteres, Spirochaetes, Clostridiales, Bacteroidales
Drosophila melanogaster (Cox and Gilmore 2007; Wong et al. 2013)	209	*Wolbachia*, Firmicutes, Proteobacteria
Vertebrates		
Human gut (Qin et al. 2010; Dethlefsen et al. 2008; Ley et al. 2006; Eckburg et al. 2005; Nam et al. 2011; Mariat et al. 2009; Huttenhower et al. 2012; Frank and Pace 2008)	5,700	Bacteroidetes, Firmicutes
Human mouth (Jenkinson 2011; Zarco et al. 2012)	1,500	*Lactobacillus*, *Staphylococcus*, *Corynebacterium*, and various anaerobes
Human skin (Grice et al. 2009; Blaser et al. 2013)	1,000	Actinobacteria, Firmicutes, Proteobacteria
Great ape gut (Ochman et al. 2010; Yildrim et al. 2010)	8,914	Firmicutes, Proteobacteria, Bacteroidetes
Black howler monkey gut (Amato et al. 2013)	7,000	Firmicutes, Bacteroides
Reindeer rumen (Sundset et al. 2007)	700	Clostridiales, Bacteriodales
Bovine rumen (Kim et al. 2011; Jami and Mizrahi 2012)	5,271	Prevotella, *Butyrivibrio*, *Fibrobacter*
Rat (Brooks et al. 2003)	338	Bacteroides, *Cytophaga*, *Lactobacillus*
Chicken (Zhu et al. 2002)	243	*Clostridium*, *Sporomusa*, Enterobacteriaceae
Zebrafish (Roeselers et al. 2011)	178	γ-Proteobacteria, *Fusobacterium*
Burmese python (Costello et al. 2010)	500	Bacteroidetes, Firmicutes
Plants		
Phylosphere (Bulgarelli et al. 2013; Redford et al. 2010; Ikeda et al. 2010; Delmontte et al. 2009)	252	Proteobacteria, Bacteroidetes, Firmicutes Actinobacteria
Rhizosphere (Schuler et al. 2001; Mendes et al. 2011; Uroz et al. 2010; Berendsen et al. 2012)	30,000	Fungi (Glomeromycota), Acidobacteria, Proteobacteria

(continued)

Table 3.1 (continued)

Host	Number of microbial species	Major microbial groups
Endophytes (Cankar et al. 2005; Sun et al. 2007; Sessitch et al. 2012; van der Heijden et al. 2008; Whipps et al. 2008)	77	Proteobacteria, Archaea, Fungi (*Neotyphodium*)
Marine green alga *Ulva australis* (Burke et al. 2011)	1,061	Alphaproteobacteria, Gammaproteobacteria, Bacteroidetes
Pitcher plant *Sarracenia* (Koopman et al. 2010)	1,000	Enterobacteriaceae

function. (3) The host-associated microbial community is generally different from the community in the surrounding environment. (4) In some cases it has been shown that similar, but not identical, microbial populations are found on the same animal or plant species that are geographically separated, while different populations are found on different animal or plant species at the same location. (5) Different microbial communities generally dominate different tissues of the same organism. (6) In several cases where a large diversity of associated bacterial species exists, certain bacterial groups dominate.

When analyzing the number of microbial species associated with a specific host, it should be kept in mind that the estimated number of bacterial species associated with various invertebrate and vertebrate animals and plants, such as those presented in Table 3.1, are minimum numbers. The reason is that species representing less than 0.01 % of the total bacterial population would generally not be detected with current methods. This reservation can have far-reaching implications since as will be discussed in Chap. 6, amplification of minor species can play an important role in the adaptation of holobionts to changing conditions and also in their evolution.

Microbiota of Invertebrates

Sponges range in size from a few millimeters to more than a meter in diameter. It is interesting that these filter-feeding sessile organisms, which may be the most ancient animal that still exists, contain the highest concentration of microbial symbionts. Microbial associates can comprise as much as 40 % of sponge tissue volume, with densities in excess of 10^9 microbial cells per ml of sponge tissue. Using 97 % sequence similarity of 16S rRNA genes to define operational taxonomic units, it has been reported that sponges contain over 2,567 bacterial species, assigned to 25 bacterial phyla (Schmitt et al. 2012).

Corals are composed of two layers of cells, the epidermis and gastrodermis, which are covered by a surface mucus layer and are connected to a large, porous

calcium carbonate skeleton. These structures interact with diverse forms of microbial life; It has been shown that the coral holobiont contains microbial representatives from all three kingdoms—Bacteria, Archaea, and Eucarya as well as numerous viruses (Rosenberg et al. 2007). The primary symbionts are microscopic algae of the genus *Symbiodinium* (commonly referred to as zooxanthellae), which reside inside the gastrodermal cells (Gates and Ainsworth 2011). The mucus, skeleton, and gastrovascular cavity of corals are populated with tissue-specific abundant and diverse species of bacterial exosymbionts (Koren and Rosenberg 2006).

Hydra is a well-established cnidarian animal model for studying host–microbe interaction (Franzenburg et al. 2013). Hydra can be grown clonally under constant laboratory conditions. Hydra's tube-like body resembles in several aspects the anatomy of the vertebrate intestine with the endodermal epithelium lining the gastric cavity and the ectodermal epithelium providing a permanent protective barrier to the environment (Bosch 2012). It has been shown that freshly collected Hydra from nature contains species-specific bacteria and that these can be retained for many generations also in laboratory cultivation (Fraune and Bosch 2007).

Termites harbor abundant and diverse gut bacteria, which are thought to play essential roles in the carbon and nitrogen metabolism of their host termites. He et al. (2013) found 800 phylotypes, defined by 97 % sequence identity of 16S rRNA genes from 20,000 analyzed clones. The hindgut paunch microbiota was dominated by Spirochaetes, particularly members of the genus *Treponema*, followed by Fibrobacteres, together accounting for ~90 % of the bacterial community. Many of the phylotypes were found for the first time. Some of them constituted novel lineages in several bacterial phyla, including the candidate phylum termite group I (TG1) (Hongoh et al. 2005).

Drosophila melanogaster is a genetically tractable model for studying host–microbe interactions. *Drosophila* possesses a diverticulated GI tract consisting of an acidic crop and midgut, and a neutral to acidic hindgut. First, *Wolbachia*, a genus of Gram-negative bacterium of the alphaproteobacteria group, is a common obligate intracellular symbiont of *D. melanogaster* and other insects. It is probably the most ubiquitous endosymbiont on the planet. Second, *Drosophila* also harbors exosymbionts which have been studied by a number of groups. The abundant and diverse exosymbionts fall into two major phyla (Cox and Gilmore 2007; Wong et al. 2013): Firmicutes (37 %, primarily the genus *Enterococcus*) and Proteobacteria (61 %, primarily the genus *Acetobacter*).

Microbiota of Vertebrates

Plant abundance, diversity, and activities are essential for life on the planet, and microorganisms play a central role in all three phenomena (Bulgarelli et al. 2013). Microorganisms supply plants with nutrients, play a role in establishment of plants and in their development of root systems, and in protection against pathogens and

other environmental stress conditions. Studies on the microbiology of plants have been performed with microorganisms found in three main locations: (1) the phyllosphere, namely the upper parts of the plant (Redford et al. 2010; Ikeda et al. 2010; Delmontte et al. 2009), (2) the rhizosphere, i.e., around the roots (Schuler et al. 2001; Mendes et al. 2011; Uroz et al. 2010; Berendsen et al. 2012), and (3) the endophytic, i.e., inside plant cells (Cankar et al. 2005; Sun et al. 2007; Sessitch et al. 2012). The great majority of microorganisms have different degrees of beneficial relationships with plants, whereas only a small minority is parasitic. It is estimated that about 20,000 species of plants are obligatorily dependent on microbial cooperation for development, growth, and survival (van der Heijdener et al. 2008). As with other eukaryotes, the close cooperation between plants and microorganisms necessitates overcoming the plant's immune response. Often this occurs by interactions between factors of the plant immune system together with other plant components and elements of the microbiota (Akira et al. 2006).

In addition to Bacteria, the human gut contains several species of Archaea and a large diversity of fungi (Hoffmann et al. 2013). A single fecal sample from a healthy African male was reported to contain 16 fungal species and several other micro-eukaryotes (Hamad et al. 2012).

There is considerable species variation in the gut microbiota between healthy individuals. Each person possesses their own personalized fingerprint of gut microbiota (Eckburg et al. 2005; Faith et al. 2013), which includes a core microbiota of ca. 100 species which are common to all humans and hundreds to thousands of microbial species that are present in a combination unique to each individual. Human microbial communities are affected by factors such as lifestyle, dietary patterns, antibiotic usage, and by the host genotype, age (Nam et al. 2011), and gender (Mueller et al. 2006; Markle et al. 2013). Furthermore, the human intestinal microbiota undergoes maturation from birth to adulthood and is further altered with aging (Mariat et al. 2009; Dominguez-Bello et al. 2011). The ratio of Firmicutes to Bacteroidetes evolves during different life stages. For infants, adults, and elderly individuals the reported ratios were 0.4, 10.9, and 0.6, respectively (Mariat et al. 2009).

In addition to the gastrointestinal tract, there is a high abundance and diversity of microbes on all surfaces of the human body, including the oral cavity (Jenkinson enkinson 2011; Zarco et al. 2012), skin (Grice et al. 2009; Blaser et al. 2013), nasal cavity, pharynx, esophagus, and urogenital tract (Human Microbiome Project Consortium 2012). The total number of bacteria on the surface of an average human (ca. 2 m^2) has been estimated to be 10^{12}. Most are found in the superficial layers of the epidermis and the upper parts of hair follicles. The skin provides three very different ecological areas, moist, dry, and sebaceous, each with their distinct bacterial community. Use of 16S rRNA gene analyses of skin samples has revealed 19 bacterial phyla and about 1,000 species (Grice et al. 2009). The most abundant phyla are Actinobacteria (51.8 %), Firmicutes (24.4 %), Proteobacteria (16.5 %), and Bacteroidetes (6.3 %). A comparison of the skin microbiota of South American Amerindians and residents of the United States indicated that ethnicity, lifestyle, and geography can influence the structure of human cutaneous bacterial communities (Grice et al. 2009).

In 2008, the United States National Institutes of Health (NIH) launched a $157 million, 5-year human microbiome project (HMP) to begin determination of the genomes and proteomes of 300 healthy individuals, sampled at 15 body sites. In 2010, the first report of the HMP was published. It included an analysis of 178 genomes and 29,693 unique proteins from microbes that live in or on the human body (www.nih.gov/news/health/may2010/nhgri-20.htm). With 24 % (375) of projects complete, over 1,500 bacterial species have been identified and 178 genomes have been sequenced from more than 285 individuals. Almost half of the organisms identified are members of the Firmicutes (46 %), followed by Actinobacteria (20 %), Proteobacteria (16 %), and Bacteroidetes (12 %). In 2012, the examination of the microbiome of 242 healthy adults was published (Huttenhower et al. 2012), each of whom were sampled at 15 (male) to 18 (female) body sites. This large consortium effort led to the sequencing of microbial eukaryotes, archaea, bacteria, and viruses (both mammalian and bacterial). Hundreds of complete genomes from the human microbiome were published. Although the analysis of these data will take many years, a few conclusions are already evident. As expected, different parts of the body harbor different microbiota. Although there are large differences in the bacterial species of individuals, there is conservation of function. For example, if a particular polysaccharide is broken down by one or more bacterial species in one individual, in another individual, the polysaccharide is also metabolized, but often by another bacterial species. The HMP study revealed that even healthy individuals differ remarkably in the microbes that occupy habitats such as the gut, skin, and vagina. Much of this diversity remains unexplained, although diet, environment, host genetics, and early microbial exposure have all been implicated.

In an interesting comparative study of the distal gut microbiotas of *primates* (including *Homo sapiens*), it was found that the branching order of host-species phylogenies based on the composition of these microbial communities is completely congruent with the known relationships of the hosts (Ochman et al. 2010; Yildrim et al. 2010). Although the gut is initially and continuously seeded by bacteria that are acquired from external sources and influenced by diet, the study established that over evolutionary timescales, the composition of the gut microbiota among great ape species is phylogenetically conserved and has diverged in a manner consistent with vertical inheritance.

The gut microbiota of the *black howler monkey* (*Alouatta pigra*) varied greatly with host habitat in relation to diet (Amato et al. 2013). Howlers occupying suboptimal habitats (captive) consumed less diverse diets and correspondingly had less diverse microbiota (1,300 species out of 30,000 reads) compared to forest howlers (5,000 species out of 30,000 reads). Also, howlers in suboptimal habitats had a reduction in the number of genes related to butyrate production in their microbiomes, which may impact host health.

As a model system to study responses to feeding and fasting, Costello et al. (2010) used microbiota of the *Burmese python*. The snake normally consumes large prey at intervals greater than 1 month. The phyla Bacteroidetes and Firmicutes numerically dominated the python gut. Fasting was associated with increased

abundances of Bacteroides, Synergistes, and the genera *Akkermansia* and *Rikenella*, and with reduced overall diversity. After eating a meal, Firmicutes increased in relative abundance and gradually outnumbered the Bacteroides, and the species-level diversity increased, reaching 500 species (out of 2,000 16S rRNA genes sequenced) in 3 days.

Microbiota of Plants

Plant abundance, diversity, and activities are essential for life on the planet, and microorganisms play a central role in all three phenomena (Bulgarelli et al. 2013). Microorganisms supply plants with nutrients, play a role in establishment of plants and in their development of root systems, and in protection against pathogens and other environmental stress conditions. Studies on the microbiology of plants have been performed with microorganisms found in three main locations: (1) the phyllosphere, namely the upper parts of the plant (Redford et al. 2010; Ikeda et al. 2010; Delmontte et al. 2009), (2) the rhizosphere, i.e., around the roots (Schuler et al. 2001; Mendes et al. 2011; Uroz et al. 2010; Berendsen et al. 2012), and (3) the endophytic, i.e., inside plant cells (Cankar et al. 2005; Sun et al. 2007; Sessitch et al. 2012). The great majority of microorganisms have different degrees of beneficial relationships with plants, whereas only a small minority is parasitic. It is estimated that about 20,000 species of plants are obligatorily dependent on microbial cooperation for development, growth, and survival (van der Heijdener et al. 2008). As with other eukaryotes, the close cooperation between plants and microorganisms necessitates overcoming the plant's immune response. Often this occurs by interactions between factors of the plant immune system together with other plant components and elements of the microbiota (Akira et al. 2006).

Large populations of microorganisms live in the *phyllosphere* (leaves, stems, flowers, and fruit). Although Archaea, filamentous fungi, and yeast are present in the phyllosphere, bacteria are considered to be the dominant microbial inhabitants present on the plant surface and within the plant tissue (Lindow and Brand 2003). Stressful conditions on the leaves, such as extreme temperatures, dryness, irradiation, and oxidative stress, in addition to poor nutrient availability determine the kinds of bacteria, their mode of growth, and their activities (Bulgarelli et al. 2013). The global surface area of the phyllosphere, estimated to be 4×10^8 km^2, harbors a bacterial population in the region of 10^{26} cells including $2–3 \times 10^6$ species (Whipps et al. 2008). Interestingly, culture-independent techniques have revealed that similarly to the human gut, these species fall within a relatively small number of dominant phyla, the Proteobacteria being the most abundant on leaves (Bulgarelli et al. 2013). Microorganisms are unevenly distributed on leaves, mainly on the lower part, as single cells or in aggregates. Bacterial leaf communities differ between seasons: similar ones are found on leaves sampled during the same season, this pattern being predictable from year to year (Redford et al. 2010).

Most of the higher plant species enter into a mutualistic *root* endosymbiosis with arbuscular mycorrhiza. The fungi involved are obligate symbionts all grouped into a single phylum the Glomeromycota (Schuler et al. 2001). Development of the mycorrhiza begins with invasion of the plant root by the soil fungus; growth of the fungus toward the root is stimulated by plant excretion into the soil of certain compounds, including flavonoids and strigolactones (Koltai 2013). The fungal mycelium penetrates the root cells and develops intracellularly, forming structures called arbuscules (Allen 1991). However, a large fraction of the mycelium remains in the soil rhizosphere contributing to its assembly. Development of this highly compatible association requires the coordinated molecular and cellular differentiation of both symbiont and host to form specialized interfaces over which bidirectional nutrient transfer occurs (Smith and Smith 1990). In addition to fungi, many bacterial species interact with plant roots (Uroz et al. 2010; Berendsen et al. 2012). The rhizosphere, which is the narrow zone of soil that is influenced by root secretions, can contain up to 10^{11} microbial cells per gram root and more than 30,000 prokaryotic species (Mendes et al. 2011). The collective genome of this microbial community is much larger than that of the plant. Rhizosphere microbial communities differ between plant species, between ecotypes within species, between different developmental stages of a given plant, and also from those present in bulk soil.

Bacterial and fungal *endophytes* live within a plant for at least part of their life without causing apparent disease. Endophytes are ubiquitous and have been found in all the species of plants studied to date. While many endophytes are known to colonize multiple species of plants, some are host specific. Endophytic species are very diverse; a single leaf of a plant can harbor many different species of endophytes, both bacterial and fungal. As an example, 16S rRNA gene sequences representing 77 bacterial species have been recovered from inside rice plants (Sun et al. 2007).

Marine *macro-algae* play a critical role in the structuring of coastal communities. Apart from comprising a major component of primary production, macroalgae are habitat-defining organisms. They provide nurseries and protective environments for many invertebrate species. The surfaces of marine algae also provide a habitat for microbial communities. 16S rRNA gene libraries of the green alga *Ulva australis* exhibited more than 1,000 bacterial species (Burke et al. 2011), predominantly Alphaproteobacteria (54.4 %), Bacteroidetes (27.6 %), and Gammaproteobacteria (8.4 %).

The ability of American *carnivorous pitcher plants* (*Sarracenia*) to digest insect prey is facilitated by microbial associations (Koopman et al. 2010). Each pitcher (a modified leaf) of the plant contains a microcosm composed of larval insects, fungi, algae, rotifers, nematodes, and bacteria that, together, ultimately break down nutrients from insect prey for the plant. Although 5 phyla, 9 classes, 15 orders, 23 families, 29 genera, and ca. 1,000 bacterial species are represented in pitchers, one family (Enterobacteriaceae) represents approximately 72 % of the total bacterial 16S rRNA gene sequences (Kroopman et al. 2010).

Factors Affecting Abundance and Diversity of Microbiota

The basis of the total abundance of microorganisms associated with any particular animal or plant is not well understood. Most likely, the fitness of the holobiont, its immune system and other characteristics defined by genetics of the host are major factors. Other factors are nutrient availability, usable surface area, available volume, and surrounding conditions. For example, in one study, the population size of epiphytic bacteria on plants was limited by the abundance of carbon sources on the leaf surface (Mercier and Lindow 2000). A different effect was obtained with the number of coliform bacteria in the rat gut. When rats were starved for 48 h, the number of coliforms increased 100-fold (Nettelbladt et al. 1997). It is well known that fasting leads to an increase in available mucins in the colon (Deplancke and Gaskins 2001), and those mucins are good nutrient sources for some gut bacteria (Larson et al. 1988).

Diversity, as opposed to abundance, generally refers to the number of different species that are present in a particular area, such as the human colon. However, the ability to accurately determine bacterial diversity depends on the experimental capability of detecting rare species, as discussed at the beginning of this chapter.

Let us analyze and summarize some factors that determine the diversity of microorganisms associated with holobionts that were documented in this chapter. The factors determining diversity are mostly similar to those determining abundance, namely genetics of the host and microbes, the immune system, the gender of the host, and external factors such as nutrients and surrounding conditions. Along with these factors, and most importantly, each offspring receives, to begin with, a diverse set of microbes from their parents. This initial input as we think today is probably the most important imprint of diversity in an offspring (Dominguez-Bello et al. 2010). Later on, this first basic microbiota can be supplemented with microorganisms from the environment throughout life.

In addition to these determinants of diversity let us consider some internal forces that would maintain a high diversity. Many microorganisms are specialists. Given that hosts provide a variety of different niches that can change with the developmental stage of the host (Palmer et al. 2007; Crielaard et al. 2011; Tang et al. 2012), the diet (De Filippo et al. 2010; Claesson et al. 2012), temperature (Kuzmina and Pervushina 2003), and other environmental factors, a diverse microbial community is established, with different microbial strains filling the different niches. This microbial diversity, and therefore its versatility, may allow the holobiont as a whole to function more optimally and adapt more rapidly to changing conditions.

The idea that microbial diversity can play a critical role under conditions of fluctuating environments has been referred to as the insurance policy hypothesis (Yachi and Loreau 1999). There are two parts to this hypothesis: (1) a single holobiont can contain in its microbiome a reservoir of rare microbes which have the potential to amplify when conditions change and assist that specific holobiont to adapt and survive in a new environment. (2) the presence of rare microbes in a

population of holobionts of the same species, but not necessarily in all members of the species, can help insure the long-term survival of the species. In essence, the insurance hypothesis offers another layer to biodiversity.

Another factor that may contribute to bacterial diversity is bacteriophage (Mills et al. 2012). High concentrations of bacteriophages are present in animal and plant tissues. The human gut, for example, contains 1,200 viral genotypes (Breitbart et al. 2003). If any bacterial strain becomes abundant, it has a high probability of being lysed by bacteriophages because the collision frequency of a bacteriophage and its bacterial host is directly proportional to the host concentration. This concept, referred to as the 'kill the winners' hypothesis (Thingstad and Lignell 1997), is supported by experimental data (Middeboe et al. 2001; Jardillier et al. 2005) and mathematical models of the bacteria: bacteriophage dynamics (Weitz et al. 2005). This mechanism selects for rare types and thus maintains high diversity (Weeks and Hoffman 2008).

On the other hand, there exist opposing forces that limit the number of species that can survive and become established in the animal and plant holobiont. Microorganisms can be eliminated because of changing internal conditions such as life cycle or developmental stage (larva, seed, aging etc.) or changing external conditions such as nutrition, chemicals, and changing locations. But probably the most important is the immune system (i.e., innate and adaptive) in addition to general protection mechanisms. The first line of discrimination of microorganisms includes physical barriers, enzymes, acid, and other excretions. These are coupled with the players of the innate, nonspecific immune system namely antimicrobial molecules, specific binding proteins for microbial attachment (e.g. the peptidoglycan-binding protein and lectin complement systems), and production of reactive oxygen species and phagocytes. Interestingly, resident symbiotic bacteria are part of the first line of discrimination in plants and animals, and as such may be considered part of the innate immune system—by occupying potential adhesion sites and by producing antimicrobial materials. The adaptive or specific immune system in vertebrates includes specific recognition of foreign microorganisms, generation of responses to eliminate these microorganisms, and development of immunological memory to hasten the response to subsequent infections with the same microbe. In essence, the immune system of the host, animal or plant, is responsible for both limiting the types of microorganisms that can survive within the host and recognizing and accommodating the normal microbiota, thereby regulating the kinds of microorganisms that can reside in the holobiont. One should bear in mind that also plants have evolved an immune system that include myriad phytochemicals that prevent infection by harmful microorganisms and enable coexistence with beneficial ones.

The human gut is a well-studied example through which we can summarize this section. Although one finds an enormous diversity of bacterial species and strains in the human gut, they belong by and large in two main bacterial phyla (Sekirov et al. 2010), out of more than 75 phyla identified (Konstantinidis and Stackebrandt 2013), the Firmicutes and the Bacteroidetes. The Archaea are represented mainly by only one species, the anaerobic *Methanobrevibacter smithii* (Samuel et al.

2007). *M. smithii* is well equipped to persist in the mammalian gut through production of surface glycans resembling those found in the gut mucosa, expression of adhesin-like proteins, consumption of a variety of fermentation products produced by saccharolytic bacteria, and effective competition for nitrogenous nutrient pools. The gut, in spite of providing a diversity of niches for microbial colonization and thus possibility for diverse species and strains, imposes, as discussed above, strict requirements for their survival, and in doing so probably limits the kinds of microbes manifested as a small number of phyla. The important survival forces imposed on human gut microbial residents are adaptation to digestive enzymes, evading the potent innate and adaptive immune systems, escaping washout from the gut and the ability to live anaerobically. These strict requirements force a narrowing of the variability of microorganisms, leaving only those that are able to create a viable and well-adapted holobiont with their host.

Key Points

- All animals and plants are holobionts, containing abundant and diverse symbiotic microorganisms, including viruses.
- Different tissues of animals and plants contain different populations of symbionts. The most abundant and diverse animal and plant symbionts are found in the digestive tract and associated with roots, respectively.
- In general, animals contain ca. 10^9 bacteria per gram wet weight, which in humans corresponds to ten times more bacterial cells than human cells and contains several hundred times more genes than are present in the human genome.
- A number of factors affect the diversity and abundance of microbial symbionts in holobionts, including the genetics, gender, and the innate and adaptive immune systems of the host, transfer from parent, space, host diet, developmental stage and aging, temperature, dampness, and bacteriophages.

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Chapter 4
Microbiotas are Transmitted Between Holobiont Generations

> *As is well known, the gastrointestinal tract is sterile in the normal fetus up to the time of birth. During normal birth, however, the baby picks up microbes from the vagina and external genitalia of the mother and any other environmental source to which it is exposed.*
>
> —Dwayne C. Savage (Savage 1977)

The hologenome concept of evolution relies on ensuring the continuity of partnerships between holobiont generations. Accordingly, both host and symbiont genomes must be transmitted with accuracy from one generation to the next. The precise modes of vertical transmission of host genomes are well understood and need not be discussed here. However, in recent years, it has become clear that microbial symbionts can also be transmitted from parent to offspring by a variety of methods. In an insightful review on transmission of microbial symbionts, Bright and Bulgheresi (2010) divide the modes for maintaining symbionts faithfully between generations into two categories, vertical—from parent and horizontal—from environment, while correctly acknowledging that mixed modes also occur. We would like to take this approach one step further by suggesting that numerous mixed and intermediate cases, many of which are discussed in this chapter, best describe the large variety in modes of transmission which are known at present to reconstitute plant and animal holobionts. It is this continuum of modes of transmission from vertical to horizontal that makes it impractical to often place them in any specific category.

Table 4.1 presents some of the diverse modes of transmission of symbionts in animals and plants. The first two cases listed in the table are *mitochondria* and *chloroplasts*, which can be considered "extreme" symbionts that are transmitted by the most direct mode, namely, cytoplasmic inheritance. Nearly, all eukaryotes examined to date inherit their mitochondria from only one parent (DeLucca and Farrell 2012). This uniparental inheritance is a keystone principle used in tracing evolutionary lineages and population migrations, and is a fundamental attribute of numerous heritable diseases. Although chloroplasts and mitochondria are transmitted primarily by maternal inheritance, rare cases of paternal inheritance have been well documented in plants (Ellis et al. 2008), mice (Gyllenstein et al. 1991), and man (Schwartz and Vissing 2002).

Some animals and most plants can develop from cells other than gametes, namely, from somatic cells (Buss 1987). The most striking example is *vegetative*

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Table 4.1 Examples of modes of symbiont transmission

Holobiont: Microbiota	Mode of transmission	Reference
General		
Eukaryote: Mitochondria		
	Cytoplasmic inheritance	DeLuca and O'Farrell (2012)
Plants: Chloroplasts		
	Cytoplasmic inheritance	Ellis et al. (2008)
Plants/some animals: Microbiota	Vegetative reproduction	Buss (1987), Fell (1993), Bosch (2009), Winston (1983)
Invertebrates		
Sponge: Microbiota		
	Enter oocytes by endocytosis	Ereskovsky et al. (2006), Schmidt et al. (2012)
Hydra: Microbiota		
	Sexually and budding	Fraune and Bosch (2007)
Coral: Microbiota		
	Sexually and via seawater	Sharp et al. (2010, 2011), Rohwer et al. (2002)
Termite: Microbiota		
	Feces of adult termites fed to newly hatched juveniles	Brune (2011), Nalepa (2011)
Aphids: Buchnera		
	Transferred to ova	Wilkinson et al. (2003)
Drosophila: Wolbachia		
	Permanently in ova	Fuller and Spradling (2007)
Drosophila: Microbiota		
	Fly lays eggs in its feces	Bakula (1969), Sharon et al. (2010)
Squid: Vibrio		
	Via seawater	Nyholm and McFall-Ngai (2004), Nyholm et al. (2008)
Vertebrates		
Human gut: Microbiota		
	Physical contact starting at birth, mother's milk and from the environment	Penders et al. (2006), Zoetendal et al. (2001), Mueller et al. (2006), Dominguez-Bello et al. (2010), Faith et al. (2013), Turnbaugh et al. (2009), Martín et al. 2004, Fernandez et al. 2013, Jost et al. 2013
Great apes: Microbiota		
	Physical contact starting at birth and from the environment	Ochman et al. (2010), Yildirim et al. (2010)
Cow rumen: Microbiota		
	Physical contact with parents and via food contaminated with feces	Dehority (2003), Russell and Rychlik (2001), Jami and Mizrahi (2012)

(continued)

Table 4.1 (continued)

Holobiont: Microbiota	Mode of transmission	Reference
Rabbits, iguanas, horses, elephants, hippos: Microbiota	Coprophagy	Linaje et al. (2004), Kovacs et al. (2006)
Koala: Microbiota	Joey eats mother's pap	Osawa et al. (1993), Brown (1986)
Birds: Microbiota	Parents regurgitate food to chicks	van Dongen et al. (2013)
Plants		
Most plants: Microbiota	Vegetative reproduction = vertical transmission	Harada and Iwasa (1994)
Land plants: Mycorrhiza fungi	Via seeds on ground and by vegetative reproduction	Wilkinson (2001), Wang and Qui (2006)
Plant: Endophytes	Insect vectors	Purcell and Hopkins (1996)
Legume plants: Rhizobia	Through root hairs	Jones et al. (2007), Heath and Tiffin (2008)

reproduction in plants. When a fragment of a plant falls to the earth, it may root and grow into a fully developed plant. In such cases, it will clearly contain many of the symbionts of the original plant (direct transfer). The same is true for some animals, where vegetative reproduction is achieved by budding, for example, in sponges (Fell 1993), coelenterates (Bosch 2009), and bryozoans (Winston 1983).

Invertebrates

Vertical transmission from parent to offspring occurs with several invertebrate endosymbionts, where the microorganisms are present in or on the reproductive cells. For example, in the aphid–Buchnera symbiosis, bacteria are intracellularly located in bacteriocytes and are transferred to and transmitted via the eggs (Wilkinson et al. 2003). Another well-studied example of vertical transmission is in the Drosophila-Wolbachia endosymbiosis (Fuller and Spradling 2007). Wolbachia is a genus of bacteria which infects arthropod species, including a high proportion of insects ($\sim$90 % of species), as well as some nematodes. In this case, the endosymbiont is present permanently in the female germ line stem cells, so that no symbiont translocation is necessary. The exosymbionts of Drosophila are transmitted to offspring by the female laying her eggs in her feces and subsequently the young larva eating the outer shells of the eggs (Bakula 1969; Sharon et al. 2010).

An example of a horizontally transferred symbiont is the *squid* light organ-*Vibrio fischeri* symbiosis (Nyholm and McFall-Ngai 2004). Following fertilization of the eggs within the female, the embryos develop an immature light organ which is free of bacteria but has three pores leading to separate epithial-lined crypts. The female host lays clutches of hundreds of eggs, which hatch almost synchronously at dusk. Adult squid release large amounts of *V. fischeri* into the water at dawn every day. The result is that sufficient symbionts are available to colonize the hatchlings. Furthermore, the squid provides a habitat in which only *V. fischeri* that emits light is able to maintain a stable association (Nyholm et al. 2008). Thus, even in horizontal (environmental) transmission, the holobiont is reconstituted faithfully.

Corals may reproduce both sexually and asexually. An individual polyp may use both reproductive modes within its lifetime. Asexual reproduction occurs when some polyps or portions of colonies from the parent colony are dislodged and become deposited on another part of the reef. During this type of vegetative reproduction, the original microbiota is conserved (Sharp et al. 2011). Sexual reproduction in corals occurs by either internal or external fertilization. Internally fertilized eggs are brooded by the polyp for days to weeks, during which time they acquire internal and external microbiota from their parent. Free-swimming larvae are also released into the water and settle within hours. Synchronous spawning occurs in many corals. Polyps release eggs and sperm into the water at the same time. This spawning method disperses eggs over a larger area. The fertilized eggs develop into larvae and acquire their specific microbiota from the seawater (Sharp et al. 2010). Species-specific associations of bacteria with corals are maintained over space and time, providing additional evidence for the accurate transfer of microbiota from parent to offspring (Rohwer et al. 2002). Recently, it was shown that adult corals release bacteria with their offspring to benefit the fitness in early coral life stages (Ceh et al. 2013).

An indirect but reliable mode of transmission of gut symbionts is employed in the *termite* hindgut–microbiota symbiosis, practicing a behavioral trait unique to termites and some cockroaches, referred to as proctodeal trophallaxis (Brune 2011). It involves the transfer of a drop of hindgut fluid from the rectum of one individual to the mouth of a nestmate, which distributes the symbionts among all colony members. Proctodeal trophallaxis is considered a key element in the evolution of the wood-digestive capacity and eusociality in termites (Brune 2011). More generally, it has been suggested that microbes have been powerful selective agents in the development of social behavior in insects, such as ants, bees, wasps, and termites (Nalepa 2011), as well as in herbivore vertebrates (Lombardo 2008).

It should be born in mind that on one hand, close contact ensures that beneficial microorganisms are transmitted from one generation to the next; on the other hand, it provides ideal conditions for transfer of contagious diseases. To help solve this problem, many social insect holobionts harbor symbiotic bacteria, which protect the host against pathogens by producing antibiotics (Stow and Beattie 2008). The ability of pathogens to develop resistance to specific antibiotics requires that the antibiotic defenses be continually updated. This “arms race” helps drive bacterial diversity, with different symbionts producing different antibiotics.

In *sponges*, transmission of symbiotic bacteria between generations may occur in different ways (Ereskovsky et al. 2006). In the case of the sponge *Halisarca dujardini*, the bacteria penetrate growing oocytes by endocytosis. One part of the bacteria plays a trophic role for oocytes and the other part remains undigested in membrane-bound vacuoles within the cytoplasm. In the state of early embryo development, bacteria are situated between the blastomeres or in membrane-bound vacuoles within the cytoplasm. In the blastula all bacteria are disposed in the blastocoels, and in the free-swimming larvae the symbionts are located in intercellular spaces. The species-specific bacterial community of sponges is probably mainly vertically transmitted (Schmidtt et al. 2012).

Symbionts of the metazoan *Hydra* demonstrate both specificity and accuracy of transmission. The Hydra reproduces similarly to corals and plants, namely, vegetatively (by budding) and sexually. Fraune and Bosch (2007) showed, first, that two different species of Hydra were colonized by different communities of microorganisms and, second, in both cases members of the same two species of Hydra were populated with similar microorganisms both in the laboratory and in nature, even after more than 30 years of maintaining the animals in the laboratory. Using Hydra as a model system, Bosch (2013) have challenged the prevailing hypothesis that immune systems evolved exclusively to control invading pathogens. Because the major factors of innate immunity systems, such as antimicrobial peptides, shape the microbiota, Bosch argues that immune systems evolved because of the need to control the resident beneficial microbes.

Vertebrates

Although it is widely accepted that fetuses are sterile, and that initial bacterial colonization of the newborn gut occurs only when the baby initiates transit through the labor channel via contamination by maternal vaginal and fecal bacteria (Mackie et al. 1999), there are now data that indicate that term fetuses are not completely sterile and that a prenatal mother-to-child efflux of commensal bacteria occurs (Moles et al. 2013; Satokari et al. 2009; Jiménez et al. 2008). Nevertheless, the mode of delivery sets the pattern of gastrointestinal tract colonization; Human infants born by vaginal delivery will be colonized by microbes resident in the birth canal and the mother's own GI tract, whereas infants born by cesarean section are initially colonized primarily by skin microbiota (Penders et al. 2006). Subsequent colonization occurs by close physical contact with parent or family and community members. If the transmission is primarily parent–child, we should detect kinship patterns in microbial communities of the hosts.

In the last years, human breast milk has been shown to be a continuous source of commensal, mutualistic, and/or probiotic bacteria to the infant gut; including staphylococci, streptococci, bifidobacteria, lactic acid bacteria, and a wide variety of gut-associated obligate and facultative anaerobes (Martin et al. 2004; Fernandez et al. 2013; Jost et al. 2013). From the point of view of the hologenome concept, it is

reassuring to realize that babies acquire much of their microbial diversity from their mother's milk. Also, viruses can be transmitted via human milk (Mofenson 2010).

Support for vertical transmission of microbiota in humans comes from studies that showed greater similarity of microbiota within family members when compared with between families (Zoetandal et al. 2001) and within the same European population as compared with between different European populations (Mueller et al. 2006; Fallani et al. 2010). Historically, it was shown, by using dry-preserved feces (coprolites) that geographically separated pre-Columbian humans contained culture-specific microbiota (Santiago-Rodriquez et al. 2013). Recently, it was shown that bacterial species, defined as isolates sharing >96 % of their genome content, were maintained over time within an individual and between family members but not between unrelated individuals. Thus, early gut colonizers, such as those acquired from our parents and siblings, have the potential to exert their physiologic, metabolic, and immunologic effects for most, and perhaps all of our lives (Faith et al. 2013). However, it should be born in mind that there are also clusters of gut bacteria that are present in all humans, so-called core microbiota, regardless of where they live (Arumugam et al. 2011).

The similarity of microbiota within family or group can evolve as a result of genetic relatedness and/or because of early physical contact and transmission from the parent in addition to similar surrounding conditions. The report that the overall similarity of gut bacterial community composition is the same in adult mono and dizygotic twin pairs supports the notion that physical contact during and after birth is more dominant than genetic relatedness (Turnbaugh et al. 2009). Moreover, each individual contains a distinct microbiota, even if they are members of a monozygotic twin pair.

Early environmental exposures as delivery mode (vaginal delivery vs. delivery by cesarean section) and administration of antibiotics in infancy have been found to affect the establishment and diversity of the infants' intestinal microbiota (Ajslev et al. 2011). Vaginally delivered children are colonized with bacterial strains from the mothers' vaginal—and gastrointestinal tract during delivery in contrast to children delivered by cesarean section (Dominguez-Bello et al. 2010), and these differences seem to persist throughout infancy (Bennet and Nord 1987). Infants delivered by cesarean section have a higher risk of asthma (Thavagnanam et al. 2008; Almquist and Rejnö 2013) and twofold higher odds of childhood obesity than vaginally delivered infants (Huh et al. 2012). It has also been observed that a correlation exists among mother's BMI, weight, and weight gain during pregnancy and infant's microbiota, implying a possible effect on fetal and child metabolic development (Collado et al. 2010). Furthermore and very important, exposure to broad spectrum antibiotics in infancy may have long-term implications on the gut microbial composition (Phillips 2009).

Because some human symbionts are transmitted with great accuracy from mother to offspring for many generations, they can be used as a window into human migration (Yamaoka et al. 2009). In particular, the bacterium *Helicobacter pylori* has been used as a conserved marker of ancestry and migration (Dominguez-Bello and Blaser 2011). For example, the reduction of genetic diversity

among humans as distance from East Africa is mirrored by the genetic distances between *H. pylori* strains circulating among human populations. Such parallelism is consistent with coevolution of bacteria and their human hosts since their exodus from Africa.

It also has been established that over evolutionary timescales, the composition of the gut microbiota among great ape species is phylogenetically conserved and has diverged in a manner consistent with vertical inheritance (Ochman et al. 2010; Yildrim et al. 2010). The close match between the gut microbial phylogeny and the great ape species phylogeny cannot be due to factors other than the evolutionary divergence of the hosts. Different species in the same location had different microbiota, while the same species living at different locales had the same microbiota. These data provide strong support for the fidelity of transmission of microbiota over long-time periods.

Funkhouser and Bordenstein (2013) have argued for the universality of maternal transmission of microbiota in animals. Recent studies suggest that infants incorporate an initial microbiome before birth and receive copious supplementation of maternal microbes through birth and breastfeeding: Bacteria have been detected in umbilical cord blood (Jimenez et al. 2005), amniotic fluid (Rautava et al. 2012), and fetal membranes (Steel et al. 2005) from babies without any indication of inflammation. Furthermore, an infant's first postpartum bowel movement harbors a complex community of microbes (Gosalbes et al. 2013).

Many young animals, including iguanas, rabbits, horses, elephants, pandas, koalas, and hippos eat the feces (*coprophagy*) of their mothers, thereby obtaining the bacteria required to properly digest vegetation found in their environment (Linaje et al. 2004; Kovacs et al. 2006). When they are born, their intestines do not contain these bacteria and without them, they would be unable to extract sufficient nutritional value from plants. Foals engage in coprophagy specifically with maternal feces (Crowell-Davis and Caudle 1989). Koalas use a special adaptation of coprophagy (Osawa et al. 1993; Brown 1986); development of the young in the pouch is very slow with the joey remaining in the pouch for 5–6 months and relying only on the mothers' milk. When the joey is approximately 5 months of age, the mother produces a second type of feces (known as pap) which the joey eats over several days up to a week. This facilitates introduction of the appropriate gut microbiotas into the developing juveniles' stomach and caecum and the subsequent digestion of the eucalyptus leaves and enables eventual weaning from the mother.

Interestingly, the idea of sharing gut microbiota via feces is now finding its way into human medicine. A review of data from more than 300 patients concluded that fecal transplants can cure 92 % of people with recurring *Clostridium difficile* infections for which antibiotics proved ineffective (Gough et al. 2011; van Nood et al. 2013).

Although mammals acquire important maternal microbiota during birth, *birds* are more likely to acquire microbiota after hatching from other sources, such as the nesting environment and food (van Dongen et al. 2013). Many bird species regurgitate food to their young, thus permitting a mode of transmission that most mammals lack.

Plants

Many plants can *reproduce vegetatively* by generating runners, rhizomes, root sprouts, etc. The offshoots remain connected to the parent for a while, but if the segments can live independently after separation it results in vegetative propagation (Harada and Iwasa 1994). Symbiotic microorganisms that are present in or on the parent tissue will be transferred to the offshoot. These phenomena are most apparent in dense forests and jungles where little light reaches the floor. In addition to transmitting microbes attached to the parent plant, these modes of asexual reproduction will most likely transfer rhizosphere fungi (mycorrhiza) and other microorganisms from the soil adjacent to the parent (Wilkinson 2001; Wang and Qui 2006).

Endophytes, such as *Pseudomonas syzygii* (Purcell and Hopkins 1996) and *Xylella fastidiosa* (Chatterjee et al. 2008), are transmitted from plant to plant by sucking insects that feed on xylem sap. There are three essential steps on *X. fastidiosa*'s vector transmission to plants. First, the endophyte must be acquired from an infected plant. Retention is the second step, in which *X. fastidiosa* must attach to the cuticle of insects, followed by colonization of that surface. Lastly, vectors inoculate the bacteria into a susceptible host generating a new infection. Vectors can acquire *X. fastidiosa* from an infected plant and immediately inoculate it into a new host (Purcell et al. 1979); thus, colonization is not a requirement for transmission and there is no latent period. The insects are able to transmit the bacteria to plants for months after acquisition from an infected plant (Hill and Purcell 1995). *Airborne transmission* of microbiota from plant to plant also occurs (Fahlgren et al. 2010).

Legume-Rhizobia symbioses have been studied extensively because of their agricultural importance and potential to reduce the impact of fertilizers on the environment (Stougaard 2000). The symbiosis begins when soil rhizobia are attracted to and attach to susceptible root hairs. The nodulation process requires molecular communication between both symbiotic partners and involves the induction and repression of a large number of bacterial and plant genes. It has been suggested that plants have evolved to recognize beneficial rhizobial signals during the early stages of symbiosis, and that signaling between plants and rhizobia may be subject to coevolutionary pressures (Jones et al. 2007; Heath and Tiffin 2008).

Transmission of Microbiota and Social Behavior

In an interesting review, Lombardo (2008) argued that animal sociality is linked to transmission of mutualistic microorganisms in both herbivores and nonherbivores. The author posits that individuals benefit from group living by gaining access to symbionts and the complexity of social behavior is associated with the mode of acquisition. He explains that animals need mutualistic symbionts for two main

reasons: (a) if they have diets that contain plant materials or other items that are indigestible without the aid of symbionts, or (b) to protect them from pathogens. In fact, based on the information that will be presented in Chap. 5, the argument should be extended to all of the useful properties that have been outsourced to the microbiota. Lombardo suggests that the associations between transmission of mutualistic symbionts and the group living of their hosts are ancient and that the start of these associations coincided with the evolution of host sociality: Host–symbiont mutualisms coevolved with host sociality. He even contemplates the possibility that kissing, which is used by primates, especially humans, to communicate affection, reassurance, and reconciliation initially arose as a way for parents to transmit required symbionts along with food to their offspring. Transmission of beneficial gut bacteria has been suggested to represent an important benefit of sociality in bumble bees (Koch and Schmid 2011) and other social insects (Engel et al. 2012).

Transmission and the Holobiont Concept

Though it seems in some cases that transmission of microbiota is not as accurate as is that of the genetic material of the host, it appears that in those cases, where the fitness of the holobiont is absolutely dependant on the microbiome, the symbiont transfer is not less precise than host genes. Even in ruminant animals in which not a single microorganism is needed, but rather a whole orchestra, the transmission is such that the physiological functions are conserved. As yet, little is known about the importance of species transmission between generations versus transfer of functions which need not be delivered by specific microorganism of the same species. We wish to emphasize that the small variations in microorganisms that may occur between individuals and between generations of the same species can give rise to genetic variation and evolution as we shall discuss in Chaps. 6 and 8.

It therefore becomes clear, in spite of the great variation in holobionts and in means of transmission discussed in this chapter, that the microbiome together with its microbiota and its functions are transferred from one generation of holobionts to the next with fidelity. The fidelity of transmission of the microbiome provides a strong basis for each holobiont to be considered a unique biological entity, largely maintaining the uniqueness of the entity and conserving the species from one generation to the next. The large variations in modes of transmission have an interesting implication: individuals can acquire and transfer symbionts throughout their lives, and not just during their reproductive phase. Furthermore, this implies that the environment can have an influence on the composition of the hologenome.

Key Points

- Microbiotas are transferred from parent to offspring by a variety of methods, including cytoplasmic inheritance, via eggs, coprophagy (consumption of feces), direct contact during and after birth, via insect vectors, and via the environment. In the numerous cases of vegetative (asexual) reproduction, the microbiota is automatically transferred to offspring. Regardless of the mechanism used, there is now good evidence that the microbial component of the holobiont is transferred with fidelity from generation to generation.
- It has been suggested that transmission of mutualistic symbionts and group living (animal sociality) coevolved.
- The large varieties in modes of transmission have an interesting implication: individuals can acquire and transfer symbionts throughout their lives, and not just during their reproductive phase.
- The fidelity of transmission of the microbiome lends a strong basis for each holobiont to be a unique biological entity, largely maintaining the uniqueness of the entity and conserving the species, from one generation to the next.
- The fact that transmission of microbiota from mother to offspring is not as precise as the transfer of host genes, can contribute to variation and evolution of holobionts (e.g., differences between identical twins).

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Chapter 5
Microbiotas are Part of Holobiont Fitness

It is not the strongest of the species that survive, nor the most intelligent, but the one most responsive to change.

—Charles Darwin

The concept of fitness is central to the Darwinian theory of natural selection—the fittest survive and spread their advantageous traits through populations, but there are many definitions of biological fitness. The following qualitative definition of fitness will suffice for the purposes of this chapter: *The propensity of a holobiont to survive and reproduce in a specified environment and population*. The phrase "in a particularly specified environment and population" should be emphasized because there is no absolute fitness, only relative fitness within a particular organic and inorganic environment. For example, remote islands contain a high proportion of wingless insects (Gillespie and Roderick 2002), presumably because periodic high winds blow the winged insects out to sea. Thus, wings are a positive fitness trait on the continents from whence the insects migrated, but a negative fitness trait on the islands. A microbial example of environmentally dependent fitness is antibiotic resistance. Bacteria are selected for antibiotic resistance in the presence of the antibiotic, but when the antibiotic is not present in the immediate environment, antibiotic-sensitive strains will be selected because they have a slightly faster multiplication rate than antibiotic-resistant strains (Pettibone et al. 1987).

In the case of a holobiont, fitness includes beneficial interactions between the host and its symbionts, between the symbionts themselves, as well as between the holobiont and other holobionts and the environment. Considering the holobiont as a unique biological entity and a level of selection in evolution, we argue that the cooperation between the normal microbiota and the host generally leads to improved fitness. The host contribution to its microbiota is assumed to be mainly a protected environment (constant temperature, predators) and abundant nutrition; however, symbionts may also benefit from living together with their host for a number of other reasons, including, higher probability of sexual recombination, efficient mechanisms for being transmitted to new hosts and cell density-dependent activities.

The concept that microbes contribute to the health of humans dates back at least to Elie Metchnikoff who in 1907 wrote, "The regular consumption of lactic acid bacteria in fermented dairy products, such as yogurt, was associated with enhanced health and longevity in Bulgarian peasant populations." He linked this to the Bulgarian bacillus (Metchnikoff 1908).

E. Rosenberg and I. Zilber-Rosenberg, *The Hologenome Concept: Human, Animal and Plant Microbiota*, DOI: 10.1007/978-3-319-04241-1_5,

Although the idea that resident microbial communities are important contributors to fitness of their hosts is not new, we are now developing a broader and deeper appreciation of the wide range of host functions that are insourced to symbiotic microorganisms and regulated by them, including the general concept of the cooperation of the different players within the holobiont. Although the term outsourcing has been applied to describe functions that microbiota contribute to its host (Bevins and Salzman 2011), the term insourcing is more appropriate because microbiota are an integral part of the holobiont. It should be emphasized that the known contributions of microbiota to holobionts are probably only the tip of the iceberg, because microbiota were there from the very beginning and all multicellular organisms evolved together with them, such that some functions were transferred from the microbiota to the host and others remained with the microbiota. Sometimes the contribution was made with one specific microbe, sometimes by a few and more often by a combination of many. Although we will review in this chapter data on contributions of specific microbiotas to their hosts, one should keep in mind that the situation is more complex and the holobiont should be considered a single entity with interacting parts. Bosch and McFall-Ngai (2011) have emphasized the need for multidisciplinary approaches to the study of metaorganisms (another name for holobionts), and McFall-Ngai et al. (2013) have provided a stimulating review focused on animal-bacterial interactions demonstrating the complexity of these many holobiont systems.

Table 5.1 summarizes some of the different contributions of microbial symbionts to their hosts. The first two examples listed are mitochondria and chloroplasts, which can be considered "extreme symbionts" that are transmitted by the most direct mode, namely, cytoplasmic inheritance. In both cases, the prokaryotic precursor to the organelle lost many genes required for independent growth during the course of evolution (Gillespie and Roderick 2002; Andersson et al. 1998). Some of these genes can now be found in the nucleus of their host cell. In the next sections, we shall discuss separately the different contributions of microbiotas and how they reveal themselves in different organisms.

Microbiotas Protect Against Pathogens

Protection against pathogens is one of the most general and important contributions of the resident microbiota to the health of the holobiont. Most bacterial pathogens infect their animal hosts predominantly via mucosal surfaces. In addition to mechanical and immunological barriers, mucosal surfaces are protected against pathogen infection by the high concentration of microbiota colonizing the mucosa. The exact mechanism is unknown in most cases, but it has been suggested (Innerebner et al. 2011) that resident bacteria occupy binding sites needed by pathogens for adhesion and release antibacterials active against pathogens. In addition, it was shown that DNA derived from gut commensal bacteria of mice

Table 5.1 Examples of microbial contributions to the fitness of holobionts

Contribution of microbiota	Examples
Respiration and ATP production	Mitochondria (*Rickettsia*) in all animals and plants (Andersson et al. 1998)
Photosynthesis	Chloroplasts (cyanobacteria) in all plants (Martin et al. 2002)
Protection against pathogens	Guinea pigs (Fomal et al. 1961)
	Mice (Butterton et al. 1996; Hall et al. 2008; Leatham et al. 2009)
	Rabbits (Shanmugam et al. 2005; Silva et al. 2004)
	Humans (Huppert & Cazin1955; Guarino et al. 2009; Witkin et al. 2013)
	Plants (Innerebner et al. 2011; Cytryn and Kolton 2011)
	Corals (Krediet et al. 2013; Mills et al. 2013)
	Mosquito (Dong et al. 2009)
Providing nutrients to hosts	Corals (Fallowski et al. 1984)
	Sea slugs (Rumpho et al. 2011)
	Chemosynthetic symbioses (Ponsard et al. 2013; Dubilier et al. 2008)
	Insects (Akman et al. 2002; Nogge 1981; Wilson et al. 2010; Brune 2011)
	Bovine rumen (Mizrahi 2011)
	Humans (Sekirov et al. 2010; Pluznicka et al. 2013; Sela et al. 2008)
	Plants (Remy et al. 1994; Bidartondo 2005; Lugtenberg and Kamilova 2009; Bloemberg and Lugtenberg 2001)
Development	Hydra (Rahat and Dimentman 1982; Fraune and Bosch 2010)
	Zebrafish (Rawis et al. 2004)
	Squid eye organ (Nyholm and McFall-Ngai 2004)
	Human and mouse immune system (Clarke et al. 2010; Lee and Mazmanian 2010)
	Human angiogenesis (Stappenbeck et al. 2002)
	Human pregnancy (Koren et al. 2012)
	Algae (Provasoli and Pintner 1980)
	Plants (Ott et al. 2009; Patten and Glick 2002; Tsavkelova et al. 2006; Wesemann et al. 2013)
	Tsetse fly (Weiss et al. 2012)
	Mosquito immune system (Dong et al. 2009)
Obesity	Mice (Turnbaugh et al. 2006; Everard et al. 2013)
	Humans (Tremaroli and Bäckhed 2012; Vijay-Kumar et al. 2010; Fang and Evans 2013; Le Chatelier 2013; Ridaura et al. 2013; Leung et al. 2013)
Behavior	Human and mice brain function (Heijtz et al. 2011; Bravo et al. 2011; Morgan and Curan 1991; Sudo et al. 2004; Neufeld et al. 2011)
	Human metabolites (Shaw 2010) and hormones (Bercik et al. 2011)
	Human and mice stress (Gonzalez et al. 2011; Foster and Neufeld 2013)
	Human autism (Cryan and Dinan 2012; Ramirez et al. 2013; Kang et al. 2013)
	Social behavior (Lombardo 2008; Lizé et al. 2013)

(continued)

Table 5.1 (continued)

Contribution of microbiota	Examples
Mating selection and speciation	Drosophila (Sharon et al. 2010, 2011)
	Wasps (Brucker and Bordenstein 2012, 2003)
	Mammals (Gorman 1976; Archie and Theis 2011; Singh et al. 1990)
	Humans (Chaix et al. 2008)
	Birds (Shawkey et al. 2007, 2009)
Detoxification of toxicants	Ruminants (Chaix et al. 2008)
	Mice and humans (Craig 1995; Swann et al. 2009; Monachesea et al. 2012; Srinath et al. 2002; Ibrahim et al. 2006; Michalke et al. 2008)
	Insects (Senderovich and Halpern 2013)
Temperature adaptation	Fish (Kuz'mina and Pervushina 2003)
	Desert plants (Rodriguez and Redman 2008; McLellan et al. 2007; Turbyville et al. 2006)
	Grass (Redman et al. 2002)
Providing warmth	Humans (James 1987)
	Cows (Russel 1986)
	Mice (Bäckhed et al. 2004)
	Winter-blooming herb *Helleborus foetidus* (Herrera and Pozo 2010)

respond to foreign antigens, e.g., pathogens, and activate the immune system (Hall et al. 2008).

Invertebrates: In corals, ca. 8 % of the culturable coral commensal bacteria produce inhibitors of a pathogen glycosidase which is responsible for a 10–100-fold reduction in the ability of the pathogen to grow on coral mucus (Krediet et al. 2013). Also in corals, which lack an adaptive immune system and do not produce antibodies, resident bacteria protect against the bleaching pathogen *Vibrio shiloi* (Mills et al. 2013). In mosquitoes, intestinal bacteria inhibit infection of the mosquito with the human malaria parasite *Plasmodium falciparum* through a mechanism that involves the mosquito's immune system (Dong et al. 2009).

Vertebrates: Several experiments have been reported demonstrating that germ-free animals, born and grown under sterile conditions, are considerably more sensitive to infection and death following oral administration of a pathogen than conventional animals (those containing the normal microbiota). These experiments include infection of guinea pigs with *Shigella flexneri* (Fomal et al. 1961), mice with Vibrio cholera (Butterton et al. 1996) or Influenza A (Dolowy and Muldoon 1964), and rabbits with *Bacteroides vulgatus* (Shanmugam et al. 2005). As an experimental example of bacterial protection against infection, mice were treated with *Bifidobacterium longum*, part of the normal microbiota, and then infected with the pathogen *Salmonella typhimurium*. The mice that received *B. longum* survived, whereas the control group (*S. typhimurium* alone) all died within a few days (Silva et al. 2004). Interestingly, it has been demonstrated that mice

precolonized with human commensal *Escherichia coli* strains are resistant to infection with pathogenic *E. coli* (Leatham et al. 2009). It should be mentioned that immune response to integral microbiota via IgM differs from its reaction to pathogenic microorganisms (Hapfelmeier et al. 2010).

In **humans**, the normal microbiota has been shown to protect against infection by pathogens in the oral cavity, the intestine, the skin, and the vaginal epithelium. The fact that there is an increased frequency of infection by the yeast pathogen *Candida albicans* following antibiotic therapy (Huppert & Cazin 1995) is consistent with this concept. Also, vaginal lactic acid bacteria protect against upper genital tract infections (Witkin et al. 2013). One of the strongest arguments that beneficial bacteria prevent disease in humans comes from recent probiotic experiments. Several randomized controlled trials and meta-analyses indicate that probiotics are effective in primary and secondary prevention of gastroenteritis and its treatment, respectively. Selected Lactobacillus strains had a significant effect in primary prevention. *Saccharomyces boulardii* was effective in antibiotic associated and in *Clostridium difficile* diarrhea (Guarino et al. 2009). The subjects of probiotics and prebiotics will be discussed in detail in Chap. 9.

The newborn infant not only tolerates but requires colonization by commensal microbes for development and health. Human milk provides not only all the nutrients needed to satisfy the neonate physiological needs, but also specialized complex oligosaccharides that stimulate the growth of select bifidobacteria (Sela et al. 2008). This three-way relationship among mother, child, and gut microbes protects the infant against infection by hostile pathogens at a time when the infant has an untrained immune system and produces low amounts of caustic stomach acid, which in adults kills most bacteria.

Plants: Diseases of plants can be caused by a multitude of diverse organisms, including fungi, bacteria, and viruses. Plant-associated microorganisms can protect plants from phytopathogens either through direct interaction with the pathogen or by elicitating induced systemic resistance in plant hosts (Innerebner et al. 2011; Cytryn and Kolton 2011). The beneficial microbes include bacterial strains from the genera Pseudomonas, Flavobacteria, and Bacillus in addition to fungi from the genus Trichoderma. Interestingly, beneficial bacteria applied to the roots of plants can prevent leaf disease (Cytryn and Kolton 2011). The most likely explanation is that these root bacteria produce antimicrobial products that can be transported to upper parts of the plant.

Microbiotas Provide Nutrients to Their Hosts

The contribution of microbiota to the nutrition of their hosts has been known for many years in a number of host-symbiotic systems. In this section, we shall discuss some classical systems and some new ones in different plants and animals, including man.

Invertebrates: **Dinoflagellate endosymbionts** of the genus Symbiodinium, commonly known as zooxanthellae, are found in many marine holobiont invertebrates, including corals, mollusks, and sponges. These photosynthetic endosymbionts provide their hosts with nutrients. In the case of corals, the zooxanthellae provide more than 50 % of the energy requirements of their hosts by transferring photosynthetically fixed carbon to the coral (Fallowski et al. 1984). Thus, the symbiosis of corals and Symbiodinium makes possible densely populated coral reefs in oligotrophic (nutrient-poor) marine environments. In addition, coral-associated bacteria perform nitrogen fixation that contributes to the growth of coral holobionts in their nitrogen-limited environment (Shashar et al. 1994).

Symbiotic animals containing green photobionts challenge the common perception that only plants are capable of capturing the sun's rays and converting them into biological energy through photosynthesis (Rumpho et al. 2011). The sea slug, *Elysia chlorotica*, lives as a "plant" when provided with only light and air as a result of acquiring plastids (chloroplasts) during feeding on its algal prey *Vaucheria litorea*. The captured plastids are retained intracellularly in cells lining the digestive tract of the sea slug. Photosynthesis by the plastids provides the sea slug with energy and fixed carbon for its entire lifespan of $\sim$10 months.

Chemosynthetic symbioses between bacteria and marine invertebrates were discovered 35 years ago at hydrothermal vents on the Galapagos Rift. Remarkably, it took the discovery of these symbioses in the deep sea for scientists to realize that chemosynthetic symbioses occur worldwide in a wide range of habitats, including cold seeps, whale and wood falls, shallow-water coastal sediments, and continental margins (Ponsard et al. 2013). In chemosynthetic symbioses, chemoautotrophic bacterial endosymbionts synthesize organic matter from CO_2 and are the primary source of nutrition for their animal host (Dubilier et al. 2008). In turn, the host provides its symbionts a habitat in which they have access to the substrates of chemoautotrophy (O_2, CO_2, and reduced inorganic compounds such as H_2S). Together, these partners create holobionts with novel metabolic capabilities. The evolutionary success of these symbioses is evident from the wide range of animal groups that have established associations with chemosynthetic bacteria; at least seven animal phyla are known to host these symbionts. The diversity of the bacterial symbionts is equally high, and phylogenetic analyses have shown that these holobiont associations have evolved on multiple occasions by the acquisition by animals of chemosynthetic symbionts.

Insects are the most diverse animal group on earth, embracing several million species. Insect–microbe symbioses take many forms: some are endocellular and many more are extracellular symbionts. Most insects live on an unbalanced diet that requires supplementation from their microbiota. For example, aphids and other sap-feeding insects feed on a diet rich in sugar but poor in essential amino acids and vitamins. Other insects feed solely on blood, such as the tsetse fly. Wigglesworthia, for example, the endosymbiont of tsetse flies, is able to synthesize pantothenate, biotin, thiazole, thiamine, flavin adenine dinucleotide, lipoic

acid, pyridoxine, protoheme, nicotinamide, and folate (Akman et al. 2002). If female flies are symbiont-free, they cannot reproduce.

In several well-studied cases, neither the host nor the primary symbiont can survive without the other (absolute mutualism). In the **aphid**–*Buchnera aphidicola* symbiosis, for example, the primary endosymbiotic bacterium is harbored in specialized host cells called bacteriocytes in the abdominal body cavity of almost all aphids and provides essential amino acids that are lacking in the phloem sap diet of the insects (Wilson et al. 2010). The aphid partner depends on these essential amino acids that are synthesized and furnished by the symbiont.

Termites are among a few animal species that are able to utilize wood as an energy source due to a highly specialized hindgut (Brune 2011). The hind gut of wood-feeding termites is a tiny but astonishingly efficient bioreactor, in which diverse microbes catalyze the conversion of plant cell walls (lignified cellulose/hemicellulose) to fermentation products that drive the metabolism of their host. Molecular phylogenetic data have revealed the presence of hundreds of microbial species in this one microlitre-sized environment (Warnecke et al. 2007), Furthermore, the gut microbiota also provides essential nitrogen to the termite, which is low in wood, by fixing atmospheric nitrogen and assimilating ammonia. These activities provide essential amino acids and vitamins, and efficiently recycle nitrogenous wastes.

Vertebrates: The **bovine rumen** symbiosis has been studied extensively for many years (Mizrahi 2011), largely because of its obvious commercial importance. The rumen acts as a temperature-controlled anaerobic fermenter that mainly processes ground cellulose-containing material together with saliva from the cow's mouth. In the rumen, bacterial enzymes convert the cellulose into its glucose subunits, which are then fermented by different groups of bacteria to produce short-chain fatty acids. These fatty acids are absorbed through the wall of the rumen into the bloodstream and then circulated via the blood to the various tissues of the body where they are respired. The microbial population of the rumen grows rapidly, and a mass of the microbial cells passes periodically out of the rumen with undigested plant material into the lower stomachs. There, the microbial mass is digested by secreted enzymes of the host, providing nitrogenous compounds and vitamins that are absorbed into the blood and used by the animal. The bovine holobiont benefits from the cooperation by being able to grow and reproduce on a simple and abundant diet of cellulose, water, and inorganic salts, in spite of the fact that the host lacks the ability to synthesize cellulases and some vitamins and essential amino acids. The concept that symbiotic microorganisms benefit their hosts by allowing them to derive energy from complex compounds and by providing essential nutrients is a general phenomenon in animals, as we shall see in the next examples.

In **humans**, the gut microbiota is a complex ecosystem that plays an essential role in the catabolism of dietary fiber, production of vitamins and amino acids, and detoxification of harmful chemicals (Sekirov et al. 2010). Certain bacteria in the gut catabolize dietary fiber, that part of plant polysaccharides in our diet that is not metabolized in the upper digestive tract, because the human genome does not

encode adequate enzymes. In general, many herbivores and omnivores would not be able to extract energy from plant fiber if it were not for their gut microbiota. During times of food shortage, symbioses with fiber-degrading bacteria provided a clear fitness advantage. For a long time it has been known that a high fiber diet is correlated with a reduction in blood pressure, blood cholesterol, and cardiovascular diseases (CVDs). One of the factors that plays a role in achieving this effect is short-chain fatty acids, produced by gut bacteria by fermentation of nondigestible carbohydrates (fiber, resistant starch) (Satija and Hu 2012; Pluznicka et al. 2013).

Bacteria found in the gut synthesize and excrete vitamins in excess of their own needs, which can be absorbed as nutrients by their host. For example, in humans, enteric bacteria secrete Vitamin K and Vitamin B12, and lactic acid bacteria produce other B-vitamins. Moreover, germ-free animals and the human newborn are deficient in Vitamin K to the extent that it is necessary to supplement their diets or administer an injection at birth, respectively.

Plants: Most of the vascular plant species enter into a mutualistic root endosymbiosis with arbuscular mycorrhiza, in which plant sugar, primarily glucose, is traded for fungal minerals, mainly phosphorus and water, thereby protecting the plant against environmental stress such as drought. This is an ancient symbiosis, which has been detected in fossils of early land plants (Remy et al. 1994), and the fungi involved are obligate symbionts all grouped into a single phylum the Glomeromycota. Mutations in plant (Kistner et al. 2005) or fungal (Javot et al. 2007) genes involved in the symbiosis leads to inhibition of mycorrhiza development and stunted plant growth. In addition, mycorrhizal fungi are widely thought to have shaped plant characteristics of great evolutionary importance, such as root structure, seed morphology, and seedling physiology (Bidartondo 2005). In addition to fungi, many bacterial species interact with plant roots and contribute to carbon transfer to soil, nitrogen fixation, nitrate reduction, mineralization of organic materials, solubilization of minerals in rocks by excretion of organic acids, maintenance of soil structure, and water cycling, all of which promote plant growth directly or indirectly (Lugtenberg and Kamilova 2009; Bloemberg and Lugtenberg 2001). Beneficial plant bacteria are often referred to as plant-growth promoting bacteria (PGPB). Several specialized kinds of bacteria, including the most studied, *Rhizobium*, engage in symbiotic relationships with peas, soybeans, and other legumes to convert nitrogen (N_2) gas into ammonia (NH_3) and further into organic nitrogen-containing compounds (see next section for more details). This fixed nitrogen is subsequently assimilated by the host plant, resulting in improved growth and productivity, even under N-limiting environmental conditions. (Symbiotically fixed N accounts for 90 million metric tons per year. This nitrogen is important not only to plants but also for the animal world and for the general turn-over of matter on our planet.)

Microbiotas Influence Animal and Plant Development

Evolutionary developmental biology is based on the principle that evolution arises from hereditable changes in development. In the past, the focus of these changes has been on the host genome (genetic and epigenetic) and occasionally on the genome of a specific primary symbiont. However, in recent years a number of experiments have demonstrated that microbial symbionts also contribute to development programming of a variety of tissues, functions, and organs. Here, we will provide some examples demonstrating the concept that holobionts (host plus all of their symbionts) have developed and evolved together.

Invertebrates: Hydra have been simple model systems in developmental biology since the 1980s (Fraune and Bosch 2010). Rahat and Dimentman (1982) showed that germ-free hydra were unable to produce buds and multiply asexually. Development of budding and asexual reproduction could be rescued by inoculation with bacteria isolated from hydra.

Studies with germ-free zebrafish have also demonstrated that bacteria play a role in development (Rawis et al. 2004). Because zebrafish are transparent until adulthood, the researchers were able to observe that the germ-free fish were compromised in their ability to process nutrients and develop a proper immune system, compared to conventional fish. In addition, DNA microarray comparisons of gene expression in the digestive tracts of 6 days post-fertilization germ-free and conventionally raised zebrafish revealed 212 genes regulated by the microbiota, including those involved in stimulation of epithelial proliferation, promotion of nutrient metabolism, and innate immune responses. Bacteria also contribute to the development of the immune system in tsetse flies (Weiss et al. 2012).

The symbiosis between the Hawaiian bobtail squid *Euprymna scolopes* and the luminous bacterium *Vibrio fischeri* is one of the best studied systems that demonstrate how a bacterial symbiont can play a role in the development of an animal organ (Nyholm and McFall-Ngai 2004). Both partners can be cultured independently in the laboratory, which has allowed experimental manipulation of the partners both as individuals and as dual participants in the association. Following fertilization of the eggs within the female, the embryos develop an immature light organ that is free of bacteria but has three pores leading to separate epithial-lined crypts. The female host lays clutches of hundreds of fertilized eggs, which hatch almost synchronously at dusk. Within hours after hatching, the juvenile squid becomes colonized by *V. fischeri*, which triggers morphogenesis of the light organ. The cells lining the crypts differentiate, becoming more cubical and swelling to four times their original size, and the microvilli of the crypt epithelium (Lamarcq and McFall-Ngai 1998) become lobate and branching, surrounding, and supporting the symbionts. Over the next 4 days, the crypt spaces enlarge and the ciliated, microvillous epithelial structure regresses as a result of bacteria-induced cell death. This modification of squid tissue by a specific bacterium is a remarkable example of interspecies signaling leading to morphogenesis. Recently, it has been

shown that initiation of the symbiosis between *V. fischeri* involves aggregation of the bacteria along the mucociliary membranes of a superficial epithelium prior to entering host tissues (Kremersend et al. 2013). These few early host-associated symbionts specifically induce robust changes in host gene expression that are critical to subsequent colonization steps. This exquisitely sensitive response to the host's specific symbiotic partner includes the upregulation of a host endochitinase, whose activity hydrolyzes polymeric chitin in the mucus into chitobiose, thereby priming the symbiont and also producing a chemoattractant gradient that promotes *V. fischeri* migration into host tissues. Thus, the host responds transcriptionally upon initial symbiont contact, which facilitates subsequent colonization. The presence of as little as five *V. fischeri* cells in the crypts for 12 h is sufficient to induce the 4-day morphogenetic program. In the absence of the specific symbiont, no morphogenesis takes place (Kremersend et al. 2013).

It has been shown in mosquitoes that intestinal microbiota contributes to the development of the innate immune system (Dong et al. 2009). The same immune factors that are needed to control the mosquito's microbiota are also defending against the malaria parasite *Plasmodium*. In general, it is likely that the immune system first evolved to preserve and protect its own microbiota and subsequently was used to kill dangerous foreign microorganisms (Pamer 2007; Lee and Mazmanian 2010).

Vertebrates: Development of an efficient immune system is crucial for the health and survival of all organisms. It is accepted to subdivide the immune system into two categories—innate and adaptive. Innate immunity refers to nonspecific defense mechanisms, such as skin, cytokines, and antibacterial peptides in the blood and tissues, and immune cells that nonspecifically attack foreign cells and materials in the body. We suggest that the normal microbiota of all animals and plants should be considered part of the innate immune system because they are a first line of defense against pathogens as described above. For example, corals which lack an adaptive immune system (they do not produce antibodies), become sensitive to bleaching pathogens when treated with antibiotics (Mills et al. 2013). Similarly, humans become more sensitive to yeast infections when treated with antibiotics (Huppert et al. 1995). A further role for the microbiota in innate immunity is enhancing killing of pathogens by neutrophils. This is accomplished by translocation of peptidoglycan from the gut microbiota to neutrophils at their production site in the bone marrow (Clarke et al. 2010).

The microbiota also primes the adaptive or antigen-specific immune system. During development and into adulthood, gut bacteria shape the tissues, cells, and molecular profile of the mammalian gastrointestinal immune system. This partnership is based on a molecular exchange involving bacterial signals that are recognized by host receptors to mediate beneficial outcomes for both microbes and hosts. Evidence shows that the commensal microbiota "programs" many aspects of T-cell differentiation (Lee et al. 2009), thus augmenting the developmental instructions of the host genome to engender the full function of the adaptive immune system. Recently, it has been demonstrated that antibody-producing B-cell development occurs in the intestinal mucosa, where it is regulated by

extracellular signals from commensal microbes that influence gut immunoglobulin repertoires (Wesemann et al. 2013).

Germ-free animals, born and grown under sterile conditions, are a useful tool for studying the relationship between host and its microbiota (Lee and Mazmanian 2010). Germ-free mice exhibit significant differences in gut development and function as compared with mice grown conventionally, i.e., possessing normal gut microbiota. The germ-free mice demonstrate enlarged caeca (Juhr and Ladeburg 1986) a slow digested food transit time (Abrams and Bishop 1967), and altered kinetics of epithelia turn-over in the small intestine (Savage et al. 1981). The immune system distinguishes between self and foreign antigens and mounts an appropriate response to clear invading pathogens by recognizing non-self-molecules (Lee and Mazmanian 2010). The fact that the immune system recognizes the microbiota as "self" is further support for the hologenome concept.

The adult intestine of mammals contains an intricate vascular network. Adult germ-free mice have arrested capillary network formation, which can be restarted and completed within 10 days after colonization with a complete microbiota harvested from conventionally raised mice, or with *Bacteroides thetaiotaomicron*, a prominent inhabitant of the normal mouse/human gut (Stappenbeck et al. 2002). This microbial regulation of postnatal angiogenesis depends on Paneth cells, where microbes colonizing a mucosal surface are assigned responsibility for regulating elaboration of the underlying microvasculature by signaling through a bacteria-sensing epithelial cell.

Recently, it has been shown that pregnancy is associated with a profound alteration of the gut microbiota (Koren et al. 2012). By the third trimester, the structure and composition of the bacterial community resemble a disease-associated dysbiosis that differs among women. Dysbiosis, inflammation, and weight gain are features of metabolic syndrome, which increases the risk of type 2 diabetes in nonpregnant individuals. These same changes are, however, part of normal pregnancy, where they may be highly beneficial, as they promote energy storage in fat tissue and provide for the growth and development of the fetus, which is central to the fitness of a mammalian species. The origins of host-microbial interactions that skew host metabolism toward greater insulin resistance, and which underlie much of the present-day obesity epidemic, may lie in reproductive biology and survival mechanisms correlated with food shortage.

Plants: An illustration of bacteria-dependent plant development can be seen in many green algae which cannot develop normally when they are grown in the absence of bacteria (Provasoli and Pintner 1980). For example, the marine green alga, *Ulva lactuca*, loses its typical leafy morphology in an axenic culture and develops into pincushion-like colonies consisting of uniseriate branching filaments. However, these abnormal algal colonies can be restored to their typical leafy morphology by reinfection with appropriate marine bacteria.

Several specialized kinds of bacteria, including the most studied, *Rhizobium,* engage in symbiotic relationships with peas, soybeans, and other legumes to convert nitrogen gas into ammonia and further into organic nitrogen-containing

compounds. Rhizobia are highly specific for their plant host. Their specificity arises, in part, from chemical ''cross-talk'' between the bacteria and plant. The interaction begins when legumes secrete flavonoids into the rhizosphere. When a bacterium recognizes this signal, it responds by synthesizing a specific oligosaccharide (Nod factor), which is responsible for host specificity. The bacteria invade tiny hairs on the roots of the legume, penetrating into the root tissue. There, the bacteria differentiate into larger cells referred to as bacteroids. The appropriate Nod factor triggers the developmental nodulation program in the plant that ultimately leads to the formation of bacteroid-filled root nodules, where nitrogen fixation takes place. The bacterial enzyme responsible for nitrogen fixation, nitrogenase, is extremely sensitive to inactivation by oxygen. Low partial pressures of oxygen are maintained in the nodule by synthesis of leghemoglobin, which becomes concentrated in the root cytoplasm surrounding the vacuoles that enclose the bacteroids. (This imparts the characteristic pink color to the actively nitrogen-fixing nodules.). Interestingly, neither the plant nor the bacterium can synthesize leghemoglobin individually; the apoprotein is encoded by a plant gene, and the heme moiety is synthesized by bacterial enzymes (Ott et al. 2009). Thus, a two-way conversation between the bacterium and its plant host is responsible for the development of the nodule and its nitrogen-fixing capability.

Indoleacetic acid (IAA) is the most common as well as the most studied plant hormone, or auxin. IAA affects plant cell division, extension, and differentiation, stimulates seed and tuber germination, increases the rate of xylem and root development, controls processes of vegetative growth, and initiates lateral and adventitious root formation (Tsavkelova et al. 2006). Most plant-stimulating bacteria produce IAA, including those that form specific symbiotic relationships with plants. IAA synthesized by bacteria may be involved at different levels in plant-bacterial interactions. In particular, plant-growth promotion and root nodulation are both affected by IAA. The role of IAA, synthesized by the *Pseudomonas putida*, in the development of canola roots, was studied following the construction of an IAA-deficient mutant of this strain (Patten and Glick 2002): Seed inoculation with wild-type *P. putida* induced the formation of roots that were 35–50 % longer than the roots from seeds treated with the IAA-deficient mutant and the roots from uninoculated seeds.

The development of symbiosis between bacteria and plants often involves quorum sensing. As the bacteria move toward their plant host, they cluster around the roots and the cell population density rises. This increase in numbers leads to the coordinated regulation of bacterial genes in a process known as quorum sensing (Whitehead et al. 2001). The ability to detect quorum is a widespread phenomenon in bacteria (Waters and Bassler 2005). It relies on the production of specific signal molecules called autoinducers, which are used to detect the presence of other self-like bacteria in the environment and adjust the expression of specific genes accordingly. The best characterized of these signal molecules are N-acyl homoserine lactones (AHLs) produced by many gram-negative bacteria. AHLs accumulate with a rise of cell population density and, once they reach a particular threshold level, bind to their cognate transcriptional regulators. These active complexes then interact with specific DNA sequences to facilitate the

activation or repression of target genes. A variety of bacterial functions such as exopolysaccharide synthesis, motility, biofilm formation, symbiosis, and virulence are tightly controlled by quorum sensing in an array of symbiotic and pathogenic organisms. For example, quorum sensing in the plant pathogen *Erwinia carotovora* controls the population density-dependent expression of pathogenicity factors, such as extracellular enzymes and secretion systems, as well as antibiotic production (von Bodman et al. 2003).

Obesity

The twin epidemics of obesity and type 2 diabetes mellitus have generated a wealth of literature regarding the mechanisms of human metabolism in insulin resistance. It has been shown in mice (Turnbaugh et al. 2006) and humans (Tremaroli and Bäckhed 2012) that obesity is correlated with different bacterial communities and that a gradual transition occurs in humans from the obese microbiota to the lean microbiota during a course of a restrictive energy intake. Obese animals, including humans, have a 50 % reduction in the abundance of Bacteroidetes and a proportional increase in Firmicutes. Obese mice also harbored more methanogenic Archaea, which may increase the efficiency of bacterial fermentation of polysaccharide fibers to short-chain fatty acids. The increased extraction of energy from dietary fibers may partly contribute to the excessive weight gain of animals. These data suggest a relationship between obesity and the diversity of intestinal microbiota and open the future possibility of treating obesity by gut microbiota manipulation. People whose guts contain a low diversity of bacteria and bacterial genes were found to contain higher levels of body fat and inflammation than those with high gut-microbial richness (Fang and Evans 2013; Le Chatelier et al. 2013). Moreover, obese microbiota have been implicated in obesity-related metabolic disorders, such as type 2 diabetes, inflammation, disordered lipid metabolism, atherosclerosis, and fatty liver, primarily via bacterial gram-negative lipopolysaccharide (LPS) metabolic effects (Cani et al. 2007; Vijay-Kumar et al. 2010). Obesity may also be associated with *Clostridium difficile* infections (Leung et al. 2013). Regarding "obese bacteria," as noted above, they are also associated with weight gain during the third trimester of pregnancy (Koren et al. 2012), so that caution should be used in considering obese bacteria as harmful microbes.

Everard et al. (2013) claim that the abundance of the mucin-degrading bacterium, *Akkermansia muciniphila,* is decreased in obese and type 2 diabetic mice. Moreover, feeding the mice with this bacterium normalized *A. muciniphila* abundance, and was correlated with an improved metabolic profile. In addition, *A. muciniphila* treatment reversed high-fat diet-induced metabolic disorders, including fat-mass gain, metabolic endotoxemia, adipose tissue inflammation, and insulin resistance.

A recent elegant experiment by the Gordon group (Ridaura et al. 2013) demonstrates both microbiota and diet influence obesity. Separate groups of germ-free mice were infected with microbiota from obese and lean human twins discordant

for obesity. Bacteria from the feces of the obese twin caused significantly greater increase in body mass and adiposity than bacteria from the lean twin. Differences in body composition were correlated with differences in fermentation of short-chain fatty acids (increased in lean) and metabolism of branched chain amino acids (increased in obese). Placing the obese and lean mice in the same cage (mice are coprohagic animals) prevented development of increased body mass in the obese mice—only when they were fed a diet low in saturated fatty acids and high in fruit and vegetables. The data show that diet and microbiota interact to influence the biology of the host.

Microbiotas Influence Animal Behavior

Mice experiments have demonstrated that gut microbiota affect the brain and behavior (Heijtz et al. 2011). Germ-free mice are more active and spend more time scurrying around their enclosures than conventional mice. They are also less anxious and more likely to take risks, such as spending long periods of time in bright light or open spaces, compared to the normal mice. Inoculating the gut microbiota from healthy mice into germ-free baby mice caused them to behave in the "normal" cautious way. If sterile adults were inoculated with the gut bacteria, their behavior did not change, suggesting that the microbiota affect the early development of the brain that subsequently influences adult behavior (Foster and Neufeld 2013). There appears to be a critical window during development when microbiota influence the central nervous system wiring related to stress-related behaviors. Furthermore, there is over a two-fold difference in gene expression in more than 100 genes in the brain between germ-free and conventional mice (Wikoff et al. 2009). Some of these genes are involved in providing cells with energy, others in chemical communications across the brain, and yet others in strengthening the connections between nerve cells. The data suggest that during evolution, the colonization of gut microbiota has become integrated into the programming of brain development, affecting motor control and anxiety-like behavior. For example, a specific *Lactobacillus* strain regulates emotional behavior in mice (Bravo et al. 2011).

How do gut bacteria affect the brain? To begin with, the long branching vagus nerve transmits information about what happens in the gut (and other organs) to the brain. But the bacteria also signal the brain via changing levels of dietary metabolites (Shaw 2010) and hormones. The latter, by definition, can affect parts of the body over long distances. For example, plasma levels of the neurotransmitter serotonin were 2.8-fold higher in conventional mice than germ-free animals (Bercik et al. 2011). Since bacteria do not synthesize serotonin, it is likely that the increased level of plasma serotonin results from an as yet undefined host microbe interaction. Altered behavior in germ-free mice was also accompanied by a decrease in the N-methyl-D-aspartate receptor subunit mRNA expression in the central amygdala, increased brain-derived neurotrophic factor expression and decreased serotonin receptor expression in the dentate granule layer of the hippocampus (Neufeld et al. 2011).

With regard to physical and psychological stress, the interaction of gut bacteria with the brain is bidirectional. Stress can affect the composition of intestinal microbiota in rodents and primates, and as was discussed above commensal microbes affect the neural network responsible for controlling stress responsiveness (Sudo et al. 2004). There is also growing evidence that gut microbiota affects sleep and autism (Gonzalez et al. 2011). Interestingly, autism and accompanying GI symptoms were characterized by distinct and less diverse gut-microbial compositions with lower abundances of the genera *Prevotella*, *Coprococcus*, and *Veillonellaceae* (Kang et al. 2013). One case report describes the benefits of antimicrobial therapy on behavior in a 14-year-old boy with autism (Ramirez et al. 2013). Over the course of treatment, the boy exhibited a reduction in aberrant behaviors, increased gastrointestinal function, and improved quality of life.

The emerging concept of a microbiota–gut–brain axis has led to the suggestion that modulation of the gut microbiota may be a tractable strategy for developing novel therapeutics for complex central nervous system disorders (Cryan and Dinan 2012).

Lombardo (2008) has argued that access to mutualistic endosymbiotic microbes is an underappreciated benefit of group living and was probably a strong selective force in the evolution of social behavior. Kissing, hugging, and touching ensure that offsprings acquire the beneficial microbiota of the group. Lizé et al. (2013) expanded on this concept by suggesting that gut microbiota play a role in kin recognition, the cognitive process by which animals distinguish kin and non-kin. This is an important biological property because the ability to recognize one's relatives provides a mechanism allowing for the emergence of sociality. Not only kin, but nestmates or group members, who share microbiota, can be recognized by their common microbially determined odors.

Bacteria Play a Role in Mating Preference and Speciation

Diet-induced mating preference in *Drosophila* was reported many years ago; however, the mechanism was unknown until it was reported in 2010 that changing the diet caused an amplification of a particular bacterial symbiont, *Lactobacillus plantarum*, and that this bacterium was responsible for the mating preference (Sharon et al. 2010). Analytical data suggested that the symbiotic bacteria influence mating preference by changing the levels of cuticular hydrocarbon sex pheromones (Sharon et al. 2011). The combination of partial geographic separation and bacterial-induced mating preference could reduce interbreeding of the populations. Slower changes in the host genome would further enhance the mating preference. The stronger the mating preference, the greater the chance that two populations will become sexually isolated, and evolutionary biologists have argued that the emergence of sexual isolation is the central event in the origin of species (Coyne 1992; Schluter 2009).

Since microbes are largely responsible for the odor of animals, it is likely they play a general role in mating preference. It has been established that commensal bacteria play an essential role in determining the unique odors of several animals

(Archie and Theis 2011), including fish (Landry et al. 2001), rats (Singh et al. 1990) the monoose *Herpestes auropunctatus* (Gorman 1976), deer (Alexy et al. 2003), bats (Voigt et al. 2005), and humans (Natsch et al. 2006). Microorganisms contribute to the odor of animals by two mechanisms: (1) anaerobic microbiotas produce volatile short-chain fatty acids, alcohols, and ketones which are prominent and active components of mammalian scent (Müller-Schwarze 2006). (2) Odorless organic products of sebaceous and apocrine glands are metabolized by microbiota to generate a variety of compounds, such as steroids, sulfanyl alkanols, and branched fatty acids that yield the characteristic odors of specific individuals (Gower et al. 1994). The process of axillary odor production is complex, and requires multiple, metabolically complementary bacterial species (Austin and Ellis 2003). The link between odor and mating preference has been demonstrated in some human populations (Chaix et al. 2008).

In some birds, feather color influences sexual preference. Interestingly, it has been shown that bacteria on the surface of certain bird feathers can affect the color brightness (Shawkey et al. 2007). Male finches (*Carpodacus mexicanus*) with redder plumage preferred by females had lower feather-degrading bacteria than males with less red plumage (Shawkey et al. 2009). Thus, plumage color can signal abundance of feather-degrading bacteria to potential mates and suggests that these bacteria may play a role in sexual selection.

In a recent publication, Brucker and Bordenstein (2013) demonstrate that microbiota play an important role in *Nasonia* wasp speciation. The researchers found that the gut microbiota of two recently diverged wasp species act as a barrier that prevents their evolutionary paths from reuniting. The wasps have significantly different collections of gut microbes (Brucker and Bordenstein 2012), and when they cross-breed, the hybrids develop a distorted microbiome that causes their death during the larval stage. Antibiotic curing of the gut bacteria significantly rescued hybrid survival. Moreover, feeding bacteria to germ-free hybrids reinstated lethality. The authors conclude, “In this animal complex, the gut microbiome and host genome represent a coadapted hologenome that breaks down during hybridization, promoting hybrid lethality and assisting speciation.”

Microbiotas Detoxify Toxicants

Animals, including man, are exposed to environmental toxic materials that are known to cause disease and death. A number of microorganisms associated with higher organisms have the ability to bind and detoxify some of these substances. For example, plant toxins, such as pyrrolizidine, polycyclic diterpene alkaloids (larkspur) and glycoalkaloids, and fungal toxins, such as zearalenone (Mycotoxin F2), are commonly found on maize and can be detoxified by microorganisms in the rumen of cows and sheep (Craig 1995). Highly toxic hydrazine is metabolized by bacteria in the human gut (Swann et al. 2009). Also, toxic metals can be removed by microbiota. Studies in mice showed that gut microbiotas provided the first line of

defense to the body by converting toxic Cr(VI) to a less-toxic Cr(III) (Monachesea et al. 2012). Human fecal bacteria can bind and sequester chromium (Srinath et al. 2002). Two common probiotics, *Lactobacillus rhamnosus* and *Propionibacterium freudenreichii*, bind and absorb lead and cadmium (Ibrahim et al. 2006). Human and mice gut microbiota are able to transform metals and metalloids into volatile derivatives ex situ under conditions mimicking those present in the host gut, i.e., anaerobic incubation, 37 °C, and neutral pH (Michalke et al. 2008).

A good example of detoxification by bacteria in the human colon is the breakdown of oxalic acid by *Oxalobacter formigenes* (Stewart et al. 2004). Oxalate is ingested in a wide range of animal feeds and human foods and beverages, including coffee, chocolate, rhubarb, spinach, nuts, and other fruits and vegetables, and is formed endogenously as a waste product of metabolism. The ingestion of gram quantities of oxalate can result in precipitation of calcium oxalate in the kidneys (kidney stone disease). The ability of *O. formigenes* to degrade dietary oxalates prompted its successful use in clinical trials as a therapeutic and prophylactic option in calcium oxalate nephrolithiasis and associated renal failure.

Recently acquired gut bacteria protect the corn rootworm (*Diabrotica virgifera*) against toxic cysteine protease inhibitors expressed in soybean foliage (Chua et al. 2013), allowing the worm to adapt to brief soybean herbivory. Microbiotas have also been shown to protect insects from toxic metals, allowing their survival in polluted environments (Senderovich and Halpern 2013). Koch-like postulates were applied to demonstrate that chironomid endogenous bacterial species protect the insect from toxic heavy metals, such as lead, chromium, and mercury.

In general, gut microbiota can prove invaluable in preventing adverse outcomes following inadvertent environmental exposure to toxic compounds.

Temperature Adaptation

Plants and ectothermic animals which exhibit a wide body temperature variation face a biochemical challenge—how to carry out cellular metabolism at both the high and low temperatures they encounter. Enzymes that have a high temperature optimum for activity are inefficient at low temperatures and vice versa. Microbiota can help overcome this problem by continuously providing enzymes optimized for the ambient temperature. When the temperature rises, those microorganisms which grow faster at the higher temperature become more abundant and secrete enzymes with high temperature optima. At lower temperatures, microbes which are adapted to these temperatures will become dominant and release enzymes with the same substrate specificities but with lower temperature optima. An example of this phenomenon is temperature adaption of pike fish (Kuz'mina and Pervushina 2003). Proteinases produced both by the mucous membrane of the intestine of the host fish and the intestinal microbiota play a role in digestion. The proteinase activity of the membrane enzyme is only 7 % at 10 °C compared to 30 °C, whereas the microbiota proteinase activity is 83 % at 10 °C compared to 30 °C.

Fungus-conferred heat tolerance in desert plants is another interesting example of how microbiotas extend the environments in which plants can survive (Rodriguez and Redman 2008). All plants in natural ecosystems are thought to be symbiotic with mycorrhizal and/or endophytic fungi. Fitness benefits conferred by fungi expressing mutualistic lifestyles include temperature stress tolerance. Without the habitat-adapted fungal endophytes, the plants are unable to survive in their native habitats. The mechanism appears to involve heat shock proteins (Turbyville et al. 2006). Fungi isolated from cacti express inhibitors of heat shock protein HSP-90. When one of these inhibitors was added to the model plant *Arabidopsis thaliana*, the plant became more thermotolerant (McLellan et al. 2007).

It is interesting that the stress tolerance conferred by some endophytes involves habitat-specific fungal adaptations. For example, within the geothermal soils of Yellowstone National Park, a small number of plant species reside. One plant species, *Dichanthelium lanuginosum* (Panic grass), has been studied and found to be colonized by one dominant endophyte, *Curvularia protuberata*. This endophyte confers heat tolerance to the host plant, and neither the fungus nor the plant can survive separate from one another when exposed to heat stress >38 °C (Redman et al. 2002).

Microbiotas Warm Their Hosts

We suggest that the exothermal metabolism of microbiota contribute to maintaining the temperature of warm-blooded animals, such as mammals and birds, and to raising the temperature of ectothermic animal and plant holobionts. Biological growth depends on the catabolism of nutrients and the transfer of energy from catabolic to anabolic processes. At each step, energy is dissipated into the environment in the form of heat. Using microcalorimeter measurements, it was reported that a typical single anaerobic bacterium produces ca. 0.2×10^{-12} cal/s (Russel 1986; James 1987). Considering this, the human colon's 10^{14} resident bacteria would produce 20 cal/s or 72 kcal/h. Assuming that the heat was spread over a 72 kg person, it would raise the temperature 1 °C/h. This theoretical argument is based on the assumption that gut bacteria produce heat at a rate similar to anaerobic bacteria growing slowly in a chemostat. Regardless of the exact magnitude of the temperature rise, it is clear that microbially generated heat in holobionts might under certain conditions be advantageous to both warm- and cold-blooded animals. The heat output by gut microbiota may help explain that germ-free mice had 40 % less total body fat than conventionally raised mice, even if their caloric intake was 29 % higher than that of conventionally raised animals (Bäckhed et al. 2004). In general, heat loss is greater in small animals than larger ones because of their higher ratio of surface area to mass. Also, heat loss is a much greater problem for aquatic animals than terrestrial ones, owing to the increased conductivity of water compared with air (Bullard and Rapp 1970).

To our knowledge the only published report that has considered the warming effect of microbiota in plants involved flowers (Herrera and Pozo 2010). Heat

produced by the sugar catabolism of yeast populations inhabiting floral nectar increased the temperature of floral nectar and, more generally, modified the within-flower thermal microenvironment. It was suggested that the warming effect of nectar-dwelling yeast would introduce novel ecological mechanisms potentially linking nectarivorous microbes with winter-blooming plants and their insect pollinators.

The Holobiont as a Unique Biological Entity

Initially, summarizing the functions and interactions of the microbes within different holobionts was important for the synthesis of the hologenome concept. However, it is now clear that the microbiota, living in close contact with their hosts from the beginning, and playing an essential role in their evolutionary development, are an integral part of all holobionts. Moreover, it is erroneous to separate the host, animal, or plant, from its microbiota, functionally or from an evolutionary stand-point. Thus, we conclude that it is logical to consider each holobiont, host, and microbiota, as an authentic, unique biological entity whatever the interactions between the players within the holobiont may be.

Nowhere are the interactions between the microbiota and host more clearly demonstrated than on metabolomic studies on mammalian blood metabolites, Comparison of plasma extracts from germ-free and conventional animals showed that approximately 10 % of all features observed showed significant changes (Wikoff et al. 2009). Amino acid metabolites were particularly affected. For example, the bacterial-mediated production of bioactive indole-containing metabolites derived from tryptophan was largely impacted. The serum metabolome of conventional mice was also characterized by increased levels of energy metabolites, e.g., pyruvic acid, citric acid, fumaric acid, and malic acid, while levels of cholesterol and fatty acids were reduced (Velagapudi et al. 2010). Further, it was shown that changes in gut microbiota controls metabolic endotoxemia, inflammation, and associated disorders by a mechanism that could increase intestinal permeability (Cani et al. 2008). These data suggest a significant interplay between bacterial and mammalian metabolism and demonstrate the inseparable interactions between the players within the holobiont (Hussa and Goodrich-Blair 2013).

Key Points

- The vast contribution of the microbiota to the fitness of its host and to the holobiont has been unraveled mainly in the last decade.
- Protection against pathogens is one of the most general and important contributions of resident microbiota to the health of the holobiont. Provision

of essential nutrients is another general benefaction of microbiota to their hosts.

- In humans, the gut microbiota is a complex ecosystem that plays an essential role in the catabolism of dietary fibers, production of vitamins and amino acids, detoxification of harmful chemicals, regulation of angiogenesis and blood pressure, and development of the innate and antigen-specific immune systems. Although the mechanisms are not clear, gut bacteria in man and mice influence obesity and affect the brain.
- The holobiont is an authentic, unique, biological entity encompassing within it mutual and inseparable interactions among the players within the holobiont, namely the microbiota and the host.

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Chapter 6
Variation in Holobionts

The new genetic system—a merger between microbe and animal cell or microbe and plant cell—is really different from the ancestral cell that lacks the microbe. Analogous to improvements in computer technology, instead of starting from scratch to make all new modules again, the symbiosis idea is an interfacing of preexisting modules. Mergers result in the emergence of new and more complex beings. I doubt new species form just from random mutation.

—Lynn Margulis

Introduction: Darwinism and Lamarckism

Variation is the raw material for evolution. Without genetic variation, evolution cannot occur—no genetic variation = no evolution. To begin with, variation is a readily observable feature of the biological world—no two sisters or brothers are identical (even identical twins are not identical). Plant and animal breeders have for hundreds of years used variation to develop species with desirable traits. Darwin used his knowledge of biological variation to derive the theory of evolution by natural selection. But what is the origin of variation? Darwin's ideas on variation differ significantly from more recent views. As can be seen from the following quotation, Darwin (1859) tended to accept the Lamarckian view that variations arose as a result of the conditions of life:

> I have hitherto sometimes spoken as if the variations so common and multiform in organic beings under domestication, and in a lesser degree in those in a state of nature had been due to chance. This, of course, is a wholly incorrect expression, but it serves to acknowledge plainly our ignorance of the cause of each particular variation. Some authors believe it to be as much the function of the reproductive system to produce individual differences, or very slight deviations of structure, as to make the child like its parents. But the much greater variability, as well as the greater frequency of monstrosities, under domestication or cultivation, than under nature, leads me to believe that deviations of structure are in some way due to the nature of the conditions of life, to which the parents and their more remote ancestors have been exposed during several generations.

The only methodical evolution theory preceding Darwin was the one presented by Jean-Baptiste Lamarck, a renowned French botanist, zoologist, and philosopher of science, published in 1809 in his book *Philosophie Zoologique* (discussed in Burkhardt 1972). Lamarck believed in spontaneous creation of living creatures and in their slow evolution—a linear continuous evolvement from simple to complex

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creatures. He also proposed that environmental forces lead to change in organisms and branching off from the basic linear pattern of evolution. Lamarck believed that these environmentally induced changes were then passed on to future generations. Thus, the main features of his theory of acquired characteristics, referred to today as "Lamarckism", are:

(1) Use and disuse—individuals lose characteristics they do not use and develop characteristics that are useful.
(2) Inheritances of acquired characteristics—individuals transmit acquired characteristics to offspring.

Lamarckism was discredited and largely ignored throughout most of the last century. There were two major scientific arguments for rejecting Lamarckism. First, the evolutionary theorist August Weismann argued that inheritance only takes place by means of germ cells and that germ cells cannot be affected by anything somatic cells of the body acquire during their lifetime (Weismann 1893). Weismann proposed that it was the sexual process, reduction division, which brought together different combinations of the parents' determinants, which was responsible for variation. Second, during the first decade of the twentieth century, Hugo de Vries in Holland recognized that a lot of variation in nature is discontinuous, big jumps with no intermediates. This led him to conclude that variation was the result of "mutation," a process that suddenly and without apparent cause irreversibly changed the germ plasma (Stamhuis et al. 1999). Mutation produces a biological variant in a single step. In the Modern Synthesis version of Darwinian evolution, beginning in the 1930s, germ plasma became genes, units of heredity, and then genes became DNA sequences. Mutations, the ultimate source of variation, are equivalent to changes in DNA sequence, which arise through rare and random errors during DNA replication or are caused by physical or chemical mutagens.

Apparently, the final nail in the coffin of the Lamarckian concept of variation came from the now classic Luria and Delbrück (1943) experiment. It was known at the time that plating a culture of *Escherichia coli* on a nutrient agar medium containing bacteriophage T1 killed most of the bacteria, but a few appeared as colonies on the agar plate. These colonies contained phage-resistant *E. coli* mutants. Luria and Delbrück asked the question: Did the mutants arise as a result of contact with the phage (Lamarckian concept) or where they already present in the culture fluid as a result of random mutation (neo-Darwinian concept)? To distinguish between these two possibilities, they inoculated several tubes containing broth with *E. coli* and allowed the bacteria to multiply overnight. They then plated aliquots from each tube on agar medium containing the T1 phage. If the *E. coli*-resistant variants arose as a result of contact with the phage, then each of plates should have about the same number of resistant colonies. However, if the phage-resistant variants were already present in the different tubes as a result of spontaneous mutation, then there would be a large difference in the number of colonies on the different plates. In other words, if the rare mutation occurred

during the early phase of the growth in the tube, the mutant would multiply for several hours and give rise to many resistant colonies on the plate, whereas if the mutation occurred late in the growth period, there would be only a few resistant colonies. The result of the experiment was a large variation in the number of colonies, with the variance considerably greater than the mean. Thus, the conclusion was that mutations in bacteria, as in other organisms, are random rather than directed.

The general conclusion of the Luria–Delbrück experiment has been challenged by experiments that have shown that certain spontaneous mutations in *E. coli* seem to occur at a higher frequency when they are beneficial (Shapiro 1984; Hall 1990; Cairns and Foster 1991). Since it is a tenet of neo-Darwinism that the frequency of mutational events should be independent of their immediate utility, the interpretation of these experiments are controversial and are unlikely to gain wide acceptance until the underlying molecular biology of adaptive mutation is understood.

Since the 1980s, Lamarckism is being considered with growing interest by at least some evolutionary biologists (Gould 1999). Jablonka and Lamb (2005) comprehensively discussed inheritance of acquired characteristics via epigenetics, behavioral, and symbolic (language) modes. The epigenetic inheritance systems include DNA methylation, self-sustaining feedback loops, prions, chromatin-marking, and RNA interference. Taken together these mechanisms indicate that variation can occur without changes in the host DNA sequence. As we will discuss in this chapter, the hologenome concept brings forth novel modes of variation, some of which contain Lamarckian principles within a Darwinian framework (Rosenberg et al. 2009a, b).

Modes of Variation Within Holobionts

According to the hologenome concept of evolution, genetic variation can arise from changes in either the host or the symbiotic microbiota genomes. In host genomes, variation occurs during sexual reproduction, chromosome rearrangements, epigenetic changes and ultimately by mutation. These same processes occur in microorganisms with the noteworthy difference that in haploid bacteria recombination occurs, within the same species, by conjugation, transduction, and DNA transformation. In addition to recombination and mutation, changes in the microbial component of the hologenome (the microbiome) can occur by three additional processes that will be discussed below:

1. microbial amplification;
2. acquisition of novel strains from the environment;
3. horizontal gene transfer (HGT) between different species and between different strains of the same species.

These three processes can occur rapidly and may be important elements in the evolution of animals and plants. Let us also bear in mind that acquisition of novel strains and HGT are modes of bacterial variation that can be driven stochastically by random events, whereas microbial amplification results from deterministic (environmental) effects such as change of nutrients, phage infection, or temperature change (Dinsdale et al. 2008).

Microbial Amplification

Microbial amplification is the most rapid and easy to understand mode of variation in holobionts. It involves changes in the relative numbers of the diverse types of associated microorganisms that can occur as a result of nutrient availability, changing temperatures (for plants and cold blooded animals), and exposure to antibiotics or other environmental factors.

Increases and decreases of different microorganisms as function of changes in conditions have an effect on the microbiome gene pool. An increase in the number of a particular microbe is equivalent to variation by gene amplification. Considering the large amount of genetic information encoded in the diverse microbial population of holobionts, microbial amplification is a powerful mechanism for adapting to changing conditions. Summing up the theoretical aspect of this phenomenon one could say that microbial amplification at the level of the microbe is pure Darwinian selection (the result of favorable conditions), and at the level of the holobiont, selection, and amplification of a microbe is a genetic variation.

Diet. Numerous studies with humans, mice, and other animals have demonstrated that changes in diet bring about predictable and rapid changes in the gut microbiota (Muegge et al. 2011). For example, children on a high fiber diet have a high abundance of bacteria from the genera *Prevotella* and *Xylanibacter*, known to contain a set of bacterial genes for cellulose and xylan hydrolysis, whereas children on a high carbohydrate diet have abundant *Shigella* and *Escherichia* (De-Filippo et al. 2010).

16S rRNA gene analysis showed a dramatic and sustained increase in the abundance of Bacteroidetes in infants immediately after the introduction of peas and other table foods to their diet (Koenig et al. 2010). The Bacteroidetes are specialized in the breakdown of complex plant polysaccharides, so that the introduction of plant-derived carbohydrates into the diet would predictably boost their populations (Claesson et al. 2012). In another study, adult cats fed a diet high in protein had elevated levels of *Clostridium perfringens* and lower *Bifidobacterium* populations than cats fed a moderate protein diet (Lubbs et al. 2009). It is well-known that *C. perfringens* has a highly active proteolytic enzyme system. In general, adaptation of the microbiota to diet is similar across different mammalian lineages and functional repertoires of microbiome genes, such as those encoding carbohydrate-degrading enzymes and proteases, and can be predicted from bacterial species assemblages (Muegge et al. 2011). The host genome also affects how

diet changes the bacterial diversity of the human gut. For example, it was shown in germ free mice that differences in host genetype affecting the mucus glycan composition of the distal gut interacted with carbohydrate from diet (simple vs. complex carbohydrate) to alter the composition of the microbiota (Kashyap et al. 2013). Diet-induced changes in microbiota have been reported in many other animals, including fruit flies (Sharon et al. 2010; Fink et al. 2013), earthworms (Knapp et al. 2009), fish (Ringo et al. 2006), birds (Sun et al. 2013), piglets (Rist et al. 2013), and cows (Jami and Mizrahi 2012).

Temperature. Temperature-induced amplification of specific groups of symbionts can be an important mechanism for adaptation in ectothermic animals and also in plants. Each microbe has a specific temperature optimum for growth, so that different species and strains amplify and become abundant in their hosts during medium and long-term temperature shifts. For example, the microbiota of the mucus of the Mediterranean coral *Oculina patagonica* contains 8.4 % *Vibrio splendidus* biovar II and 3.8 % *V. splendidus* B17 in the winter (15–20 °C), but less than 0.2 % of each of these strains in the summer (25–30 °C) (Koren and Rosenberg 2006). By contrast, *Prosthecochloris* sp. AJ888467 and *Vibrio harveyi* were not detected in the winter, but represented 7.0 and 4.2 %, respectively, of the microbiota in the summer.

In recent years, temperature-induced coral bleaching has been the subject of numerous articles in the scientific and public press. At the global scale, coral bleaching is the most serious disease threatening coral reefs. Coral bleaching is caused by the disruption of the symbiotic interaction between coral hosts and endosymbiotic algae of the genus *Symbiodinium* (Brown 1997). The loss of the algae and/or its photosynthetic pigments causes the coral to lose color (this is the bleaching process). In general, coral bleaching coincides with the hottest period of the year, and is most severe at times of warmer-than-normal conditions, suggesting a connection to global warming. In fact some coral biologists have predicted the complete demise of coral reefs in 50 years if temperatures will rise according to most current models (Hoegh-Guldberg 1999). These predictions are based on the assumption that coral adaptation is too slow to keep up with global warming. Although this assumption is probably correct for genetic changes in the coral host, it may likely be incorrect at the level of the holobiont and its microbiota.

Previously thought to be a single species, molecular phylogenetic evidence over the past couple decades has shown there to be great diversity in *Symbiodinium* (Rowan 1998). The adaptive hypothesis of coral bleaching puts forth the concept that expulsion of the algae allows more temperature-resistant clades of *Symbiodinium* to infect the coral and establish a more favorable symbiosis (Buddemeier et al. 2004). An alternative to this mode of adaptation is based on the report that corals often contain more than one clade of *Symbiodinium* (Silverstein et al. 2012). When the temperature rises, the clade that is more temperature resistant will amplify, thus preventing bleaching.

Another adaptive process of bleached corals to increased temperature is the amplification of cyanobacteria in the coral skeleton. The photosynthetic products of these bacteria are transferred to the host and help them survive the temperature-

induced bleaching episode (Fine and Loya 2002). The acquisition of cyanobacteria and heat-resistant *Symbiodinium* fits within the hologenome concept of evolution. One can conclude that rapid changes in coral microbiota can assist the holobiont in adaptation and evolution and may help explain the evolutionary success of corals and moderate predictions of their demise. Many of the arguments presented to support this hypothesis of adaptation and evolution of corals are relevant to other invertebrates, including insects (Huang and Zhang 2013), as well as to higher animals and plants. Indeed, extrapolating from this hypothesis led us to propose a higher order of postulation, namely, the hologenome concept of evolution (Rosenberg et al. 2007; Zilber-Rosenberg and Rosenberg 2008).

Antibiotics and other environmental factors. Antibiotics are administered to target a specific pathogenic population. However, most antibiotics in clinical use have a broad-spectrum of activity, so they also affect the nonpathogenic microbial community. It has been shown that antibiotic treatments result in drastic changes in the microbial community in mice (Sekirov et al. 2011) and humans (Dethlefsen et al. 2006, 2008). In mice, exposure to antibiotics did not significantly alter the total numbers of intestinal bacteria but altered the composition of the microbiota. These perturbations in the microbiota resulted in increased mouse susceptibility to subsequent infections with pathogens and more severe intestinal pathology. The mice data suggest that antibiotic treatment alters the balance of the microbial community, which predisposes the host to infection, demonstrating the importance of a healthy microbiota in host response to enteric pathogens.

In humans, antibiotic treatment influenced the abundance of about a third of the bacterial taxa in the gut, decreasing the taxonomic richness, diversity, and evenness of the community (Dethlefsen et al. 2008). In this study, the taxonomic composition of the bacterial community closely resembled its pretreatment state by 4 weeks after the end of treatment, but several taxa failed to recover within 6 months. Further emphasizing the long-term effects of antibiotics, piglets given antibiotics 1 day after birth had substantially altered microbiota for at least 5 weeks (Janczyk et al. 2007). Willing et al. (2011) have reviewed the effects of different antibiotics on host-microbiota mutualism.

With humans and other warm-blooded animals, temperature, humidity, and ultraviolet light can affect the number and type of microorganisms on the skin. For example, low-temperature and high-humidity conditions are associated with a higher frequency of Gram-negative bacteria on the back and feet (Grice and Segre 2011).

Acquisition of Novel Symbionts and the "Hygiene Hypothesis"

Acquiring new symbionts from the environment is another mechanism for introducing variation into holobionts. However, it is sometimes difficult to distinguish novel symbionts from amplification of minor symbionts. Animals (including man) come in contact with billions of microorganisms during their lifetime in the food

they eat, water they drink, air they breathe, and by direct interaction with other animals. Plants contact numerous microorganisms through their roots, the surrounding air, and also by insect vectors. It is reasonable to assume that occasionally, as a random event, one of these microorganisms will overcome the immune system, find a niche and become established in the host. Under the appropriate conditions, the novel symbiont may become more abundant and affect the phenotype of the holobiont. Unlike microbial amplification, acquiring new symbionts can introduce entirely new sets of genes into the holobiont.

Research on acquisition of microbes from the environment has been focused during the past century mainly on pathogens because these harmful infections represent a key challenge to agriculture and to human health. However, many of the principles derived from studies on the transmission of pathogens should also apply to beneficial microorganisms. For the acquired microbe to survive in the host it must overcome the immune system and multiply. This is true for both pathogens and mutualists. Epidemics occur when there is an unusual increase in the infection rate above baseline for a specific organism. If it is possible to have epidemics of pathogens, why is it not possible (even more likely) to have epidemics of beneficial bacteria? It is likely that epidemics of beneficial microbes occur frequently but go unnoticed. This could be a mechanism for bringing about rapid variation in a population. An applied aspect of the principle of variation by acquiring beneficial bacteria is probiotics, which will be discussed subsequently in Chap. 10.

Interestingly, our accidental discovery that the Mediterranean coral *O. patagonica* became resistant to the coral pathogen *Vibrio shiloi* (Rosenberg and Falkovitz 2004) provided the stimulus for the hologenome concept of evolution. Because corals possess a restricted adaptive immune system and do not produce antibodies, we presented the coral probiotic hypothesis (Reshef et al. 2006) to explain the coral development of resistance to infection by *V. shiloi*. The hypothesis posits that the corals acquired from the marine environment "beneficial" bacteria that prevent infection by the pathogen. Recently, we showed that treatment of the corals with antibiotics to kill the beneficial bacteria made the coral sensitive to *V. shiloi*, thus providing support for the coral probiotic hypothesis (Mills et al. 2013).

Another example of the acquisition of a beneficial bacterium is the uptake of *Burkholderia* from soil by stinkbugs (Kikuchi et al. 2012). These bacteria established a specific and beneficial symbiosis with stinkbugs that conferred resistance to the host insects against the organophosphorus insecticide fenitrothion. The data suggest that the symbiont-mediated insecticide resistance became established in the stinkbug population within a single insect generation and was rapidly transferred horizontally to different pest insects and other organisms. Another study shows that the bacterium *Rickettsia* swept into a population of an invasive agricultural pest, the sweet potato whitefly, *Bemisia tabaci*, in just 6 years (Himler et al. 2011). Compared with uninfected whiteflies, *Rickettsia*-infected whiteflies produced more offspring, had higher survival to adulthood, developed faster, and produced a higher proportion of daughters. The symbiont thus functions as both a mutualist and a reproductive manipulator. Symbiont invasions such as these

represent sudden evolutionary shifts for the holobiont, with potentially large impacts on their fitness under specific ecological circumstances.

The hygiene hypothesis was originally put forth to explain both the increased risk of allergy in small families and in Western countries (Strachan 1989). Subsequently, the hypothesis posited that "the overly hygienic Western lifestyle limits general microbial exposure and alters the colonization of the infant gut, which in turn disrupts normal development of the immune system and ultimately leads to allergic disease" (Wold 1998). In support of the hypothesis, it has been shown that reduced diversity of gut microbiota during infancy is associated with allergic disease later in childhood (Azad et al. 2013).

The hygiene hypothesis has more recently been expanded to help explain the rise in obesity and related syndromes (Blaser and Falkow 2009; Musso et al. 2010). Data suggest that improved Westernized sanitation and living conditions, overzealous antimicrobial therapy, delivery by cesarean section, and formula-feeding infants, all of which are widely practiced in developed countries, may predispose individuals to metabolic diseases just as improved hygiene was shown to increase the susceptibility to allergic and autoimmune diseases (Penders et al. 2006). In essence, reducing exposure to microorganisms can inhibit acquisition of beneficial symbionts, which have evolved to participate in the metabolism and health of human holobionts.

Horizontal Gene Transfer

HGT, also known as lateral gene transfer, refers to the movement of genetic information across normal mating barriers, between more or less distantly related organisms, and thus stands in distinction to the standard vertical transmission of genes from parent to offspring. HGT is an additional potent mechanism for generating variability in holobionts. Most prokaryotes possess different classes of mobile genetic elements that allow for the acquisition, loss or rearrangement of sometimes large regions of the bacterial genome. HGT is mediated by transposons, plasmids, genomic islands, and viruses, including bacteriophages, which can be either on bacterial chromosomes or on plasmids (Frost et al. 2005). Interestingly, genomic islands encode many functions necessary for bacteria–host interactions and are found in both pathogens, where they are referred to as pathogenicity islands (Hacker and Kaper 2000; Schmidt and Hensel 2004), and in beneficial symbionts, where they are called symbiosis islands (Finan 2002; MacLean et al. 2007). The proximity and, in many cases, the high density of bacteria within a holobiont would accelerate the rate of HGT. The evolutionary significance of HGT is that a large block of DNA, e.g., a symbiosis island can be transferred from one bacterium to another in a single event. This has resulted, for example, in rapid evolution of diverse strains of nitrogen-fixing mesorhizobia in legumes (Nandasena et al. 2007). HGT may also be a mechanism by which genetic information can be exchanged between pathogens and symbionts (Hacker et al. 2005).

An interesting example of variation in the human gut microbiome by HGT is the transfer of genes coding for porphyranases, agarases, and associated proteins from the marine bacterium *Zobellia galactanivorans* to the human gut bacterium *Bacteroides plebeius* in the Japanese population (Hehemann et al. 2010). The enzyme porphyranase breaks down a polysaccharide that makes up around 40 % of the cell walls of *Porphyra*, a red alga used to make the nori sheets that wrap around sushi. Seaweeds, rich in the complex polysaccharide agar, also make an important contribution to the daily diet in Japan. Comparative gut metagenome analyses showed that porphyranases and agarases are frequent in the Japanese population but absent in North American individuals. Data indicate that seaweeds and sushi with associated marine bacteria were the routes by which the genes for agarase and porphyranase were acquired by the Japanese population. Thus, contact with nonsterile food may be a general mechanism for diversity in human gut microbiota.

The distal colon has been regarded an ecologically suitable site for HGT between microorganisms due to its high microbial cell density (Ley et al. 2006). Consistent with this hypothesis is the report that a Tn1549-like conjugative transposon family is explosively amplified in human gut microbiomes (Kurokawa et al. 2007). It seems reasonable that such conjugal elements, which mediate genetic exchanges and transmittance through cell–cell contact, are key players in HGT in the colon.

Although more rare, HGT can also take place from microorganisms to animals and plants and vice versa. Examples include transfer of carotenoid biosynthetic genes from a fungus to aphids (Moran and Jarvik 2010), evolution of glyoxylate cycle enzymes in metazoans (Kondrashov et al. 2006), transfer of alpha- and beta-tubulin genes from eukaryotes to the bacterium *Prosthecobacter* (Schlieper et al. 2005), the fructose bisphosphate aldolase gene from red algae to the closely related cyanobacteria, *Prochlorococcus* and *Synechococcus* (Rogers et al. 2007), and the transfer of functional cellulase genes from bacteria to the necromenic nematode *Pristionchus pacificus* (Danchin and Rosso 2012).

Large tracts of *Wolbachia* DNA have been horizontally transferred, often quite recently, from these common intracellular bacterial endosymbionts to the nuclear genome of their insect and nematode hosts (Dunning-Hotopp et al. 2007; Nikoh et al. 2008). In the most extreme case, an entire copy of the *Wolbachia* genome was found in the genome of a fruit fly, and 2 % of the transferred genes were shown to be transcribed. It has been suggested that the introduction of transposable elements by horizontal transfer into eukaryotic genomes has been a major force propelling genomic variation and biological innovation (Schaack et al. 2010). In Chap. 8, we will further discuss HGT as it relates directly to evolution of holobionts.

It should be noted that variation in holobiont offspring (including identical twins) also arises as a result of acquisition of different microbiota from the mother during birth and breast feeding. This initial seeding will influence the microbiota of the offspring for the rest of his life as discussed in Chap. 4.

The Inheritance of Acquired Characteristics (Lamarckism) Revisited

We would like to reiterate that the hologenome concept of genetic variation by acquisition of novel microbes and amplification contains Lamarckian aspects within a Darwinian framework (Rosenberg et al. 2009a, b). Lamarck, of course, was unaware of microbiota and proposed his theory of inheritance of acquired characteristics to explain animal variation and evolution (discussed in Burkhardt 1972). Lamarckism was discredited largely because Weismann pointed out, as mentioned above, that inheritance only takes place by means of germ cells and that germ cells cannot be affected by anything somatic cells of the body acquire during their lifetime (Weismann 1893). However, we now know that inheritance in holobionts also occurs by transmission of microbiota (discussed in Chap. 4). Thus, combining the demonstrated mechanism of variation in holobionts by acquisition of novel strains and amplification together with the realization that microbes are transmitted between generations leads to the conclusion that evolution can occur by inheritance of acquired characteristics (Lamarckism), in that: (1) they are regulated by "use and disuse" (of microbes) and (2) the variations in the hologenome can be transmitted to offspring. These Lamarckian aspects exist within a Darwinian framework in that genetic variations within holobionts when occurring in either the host or microbiota are selected for or stochastically increase in numbers.

Key Points

- Evolution by natural selection relies on variation in a population. According to the hologenome concept of evolution, genetic variation can arise from changes in either the host or the symbiotic microbiota genomes.
- Holobionts give rise to several underappreciated mechanisms of variation, including acquisition of novel microbes from the environment, microbial amplification, and HGT (between microbes and between microbes and host).
- The proximity and, in many cases, the high density of bacteria within a holobiont would accelerate the rate of horizontal gene transfer.
- Variations in symbiotic microbiota may allow the holobiont to adapt and survive under rapidly changing environmental conditions, thus providing the time necessary for the host genome to evolve.
- In addition to constructing new genes by mutations, animals, and plants have the potential to acquire preevolved genetic information and functions from microorganisms, including viruses.

- The hologenome concept of genetic variation by acquisition of novel microbes and amplification contains Lamarckian aspects within a Darwinian framework.

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Chapter 7
Viruses are Part of the Holobiont's Fitness and Evolution

An inefficient virus kills its host. A clever virus stays with it.

—James Lovelock

Since viruses cannot be observed in the light microscope and the electron microscope was not invented until 1931, their existence was first recognized when certain pathogens were found to pass through filters that generally stop bacteria. For example, Pasteur showed in 1884 that the agent responsible for rabies could be transmitted through a Chamberland-Pasteur filter (Bordenave 2003), and Iwanovsky (1892) made a similar observation with the pathogen of tobacco mosaic disease. However, both Pasteur and Iwanovsky assumed that the infectious agent was a small bacterium. Beijerinck (1898) studied the infectious agent of tobacco mosaic disease in more detail and concluded that the agent of the disease is not a bacterium but a virus—*contagium vivum fluidum*. His idea that a pathogen can be a soluble molecule that proliferates when it is part of the protoplasm of a living cell was novel and revolutionary. This new concept laid the foundation of virus research and directed further studies on the nature of viruses.

Throughout the twentieth century, research was carried out on pathogenic viruses that attack a variety of host organisms, including animals, plants, algae, fungi, protozoa, and bacteria, the latter being referred to as bacteriophages, or phages for short. Although most of these studies dealt with disease processes, there is now a growing literature on the importance of viruses to the global environment (Suttle 2007; Brussaard et al. 2008; Rohwer and Thurber 2009; Danovaro et al. 2011; Weitz and Wilhelm 2012) and the health of all organisms (Shen 2009; Rohwer and Youle 2011). Viruses have been defined as noncellular obligate intracellular parasites, using RNA or DNA as their genetic material, that are capable of directing their own replication (Roossinck 2011a). This chapter will focus on viruses as integral parts of the holobiont and the hologenome of animals and plants, and as such, their role in the fitness and evolution of animals, plants, and microbes. As will be discussed in the next sections, some of these integrated viral genes code for essential proteins.

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Abundance and Diversity of Viruses in Holobionts

Viruses are the most abundant "lifeforms" on the planet and are a huge reservoir of genetic information. It has been estimated that there are over 10^{30} viruses in the ocean, approximately 15 times the number of bacteria (Suttle 2007). In terms of genetic diversity, the viral pangenome is probably more complex than the pangenome of all cellular life forms (Kristensen et al. 2010). Determination of virus abundance in animal and plant tissues is considerably more difficult than in water bodies. Most of what we know about viruses has been derived from studies on viral diseases of man and domesticated plants and animals. However, since metagenomic analysis of DNA from apparently healthy humans, animals, and plants contain large numbers of viral genomes, it is likely that most viruses of multicellular eukaryotes are commensal or mutualistic. Apparently, these non-pathogenic viruses are tolerated by the immune system and are an integral part of the holobiont. Accordingly, there have been relatively little quantitative estimation of viruses associated with different tissues of animals and plants, the data that do exist suggest they are abundant. For example, viruses in the human gut have been estimated to be approximately 10^{13} (Leruste et al. 2012), and in coral mucus, $3-11 \times 10^7$ per cm^3 (Kim et al. 2011). Metagenomic analysis of DNA from viruses isolated from human fecal samples yielded 7,175 different viral genomes, 80 % of which had not been seen previously (Minot et al. 2011). Examination of the nonredundant viral database indicated that 73 % were eukaryotic viral genomes and 27 % were phages and prophages; 77 % of the phages and prophages were double-stranded-DNA phages, mostly members of the order Caudovirales. The bacterial hosts of these known bacteriophages are principally members of Proteobacteria (54 %), Firmicutes (32 %), and Actinobacteria (7 %). Samples taken at different times showed that viral communities in the human gut were stable (Minota et al. 2013). Apparently, these viral populations (the "human virome") coexist with the rest of the human hologenome. Understanding the role of the human virome in health and disease requires a much deeper understanding of their composition and dynamics.

Metagenomic analyses have also been used to characterize the viromes in pigs (Shan et al. 2011), wild rodents (Phan et al. 2011), bats (Smith and Wang 2013), sea lions (Li et al. 2011), badgers (van den Brand et al. 2012), grapevine plants (Rwahnih et al. 2011), cow rumen (Berg-Miller et al. 2012), and corals (Correa et al. 2013). In corals, dsDNA and ssRNA viruses were associated with the dinoflagellate endosymbionts (Correa et al. 2013). Random pyrosequencing of virus-enriched metagenomes (viromes) isolated from bovine rumen fluid yielded up to 28,000 different viral genotypes obtained from each cow (Berg-Miller et al. 2012). It has recently been estimated that there are a minimum of 320,000 mammalian viruses awaiting discovery (Anthony et al. 2013).

It should be pointed out that the above summary of abundance and diversity of viruses in holobionts only includes data obtained from isolated viruses. In fact, considerable viral DNA information is present in animal (Reyes et al. 2010) and

plant genomes (Chiba et al. 2011). For example, at least 8 % of the human genome is of viral origin, including retroviruses and bornaviruses (Lander et al. 2001).

Beyond the ubiquity and high abundance of viruses, the viromes of multicellular eukaryotes show dramatic differences from those of Bacteria and Archaea. In contrast to prokaryotes whose viromes are heavily dominated by viruses with double-stranded DNA genomes (Hatful 2008), plants and animals harbor enormously diverse viromes enriched for RNA viruses and reverse-transcribing (retroid) viruses (Lang et al. 2009), which are absent from prokaryotes. The implication of these findings is that the shift from DNA- to RNA-dominated viromes occurred at the earliest stages of eukaryote evolution, which might be related to the emergence of the cytosol, an "RNA compartment" well suited for propagation of RNA viruses (Koonin et al. 2008).

There are numerous examples of viral gene sequences and gene order that are conserved in animal and plant viruses. Dolja and Koonin (2011) suggested that there are three distinct scenarios for the origin of related viruses of plants and animals: (1) evolution from a common ancestral virus predating the divergence of plants and animals, (2) horizontal transfer of viruses, for example, through insect vectors, and (3) parallel origin from related genetic elements. There is evidence that each of these scenarios contributed, to a varying extent, to the evolution of different groups of viruses.

Transmission of Viruses

Similar to bacteria and other microorganisms, viruses can be transferred horizontally between hosts of the same generation or vertically from parent to offspring. One way in which viruses can be vertically transmitted is by integrating into the host genome and becoming part of the genetic material of their host species, a process that is called endogenization (Katzourakis and Gifford 2010). Such endogenous viral elements result from the chromosomal integration of viral DNA (or DNA copies of viral RNA) in the host germ cells, which allows for vertical transmission and potential fixation in the host population. Although endogenization of eukaryotes was for a long time thought to be limited to retroviruses, it is now accepted that all major types of eukaryotic viruses can give rise to endogenous viral elements (Feschotte and Gilbert 2012). In Chap. 8, we will discuss how endogenization of viruses has contributed to the evolution of the host genome by introducing genetic variation and innovation, leading to new protein-coding genes with cellular functions.

Another mechanism of vertical transmission of viruses to the next generation is via infection of germ cells with virions (Terzian et al. 2009; Yu et al. 2013). To establish chronic infections, viruses must develop strategies to evade the host's immune responses. Many viruses are transmitted most efficiently through mucosal surfaces rich in microbiota. By using germ-free mice or antibiotic-treated mice it was shown that virus transmission to offspring was greatly reduced (Kane et al. 2011;

Kuss et al. 2011). These findings reveal the fundamental importance of commensal microbiota in viral transmission.

Viruses can occasionally be horizontally transmitted from one host species to another. When this occurs it can initiate major disease epidemics in their new host. For example, the acquired immune deficiency syndrome (AIDS) pandemic can be traced back approximately one century ago by the transfer of HIV-1 from chimpanzees to humans in southeastern Cameroon (Sharp and Hahn 2010). Regarding horizontal transmission, it has been suggested that acquisition of viruses and insertion of their genes contributed to the divergence between humans and chimpanzees through changes in gene expression and development (Van Blerkom 2003).

Many animal and plant viruses are transmitted by vectors from one host to another (Andret and Fuchs 2005). Since plants do not move, plant-to-plant transmission of viruses requires motile vectors, such as arthropods, nematodes, fungi, and plasmodiophorids (soil-borne, plant-associated organisms with a multistage life cycle). Vector–virus transmission consists of several successive steps: acquisition of virions from an infected source, stable retention of acquired virions at specific sites through binding of virions to ligands, release of virions from the retention sites upon salivation or regurgitation, and delivery of virions to a site of infection in a viable plant cell. Each step of this sequence is needed for the transmission to be successful.

Viruses are Part of the Fitness and Evolution of Holobionts

Although it is clear that viruses have caused extensive disease and suffering in humans and domesticated plants and animals, there are many viruses that are clearly beneficial to their hosts. Beneficial viruses have been discovered in many different hosts, including bacteria, insects, plants, fungi, animals, and man (Horie et al. 2010). Examples of some of the better studied cases of beneficial viruses are described below:

Bacteriophages: The earliest studies on beneficial viruses focused on the role of lysogenic phages in providing a selective advantage for bacteria harboring these phages (Lehnherr et al. 1993). Some phages can exist for many generations integrated into the genomes of their bacterial hosts, a condition known as lysogeny. Bacteria possessing lysogenic phages are immune to infection by virulent forms of the virus. Periodically, the lysogenic phage excises from the genome and reproduces rapidly, producing hundreds of progeny and killing the host cell in the process. The death and lysis of the host cell releases the viruses into the extracellular environment, where they can bind to and kill competing bacteria that are not lysogenic for the virus. While the bacterial cell that releases the phage is sacrificed it benefits the remaining population of bacteria that retains the lysogenic phage, allowing for the invasion of new territory previously occupied by non-lysogenic bacteria.

An interesting example of a beneficial bacteriophage involves the interaction of an insect, a bacterium, and a phage. The aphid *Acyrthosiphon pisum* hosts the endosymbiotic bacterium *Hamiltonella defensa* which protects the aphid from attack by the parasitoid wasp *Aphidius ervi* by killing wasp larva (Oliver et al. 2009). The toxin responsible for killing the wasp is encoded by a bacteriophage that is present in the endosymbiotic bacterium. When the phage is lost, the aphid becomes more susceptible to parasitism.

Recently it has been shown that phages on the mucosal surface of metazoans provide defense against bacterial pathogens (Barra et al. 2013). Enrichment of phage in mucus occurs via binding interactions between mucin glycoproteins and Ig-like protein domains exposed on phage capsids. In particular, phage Ig-like domains bind variable glycan residues that coat the mucin glycoprotein component of mucus. Metagenomic analysis found these Ig-like proteins are present in phages sampled from many environments, particularly from locations adjacent to mucosal surfaces. The ability of phages to kill pathogenic bacteria has been exploited in phage therapy and will be discussed in Chap. 10.

Bacterial genetic variation and bacterial diversity, both contribute to the ability of holobionts to adapt to changing environmental conditions, as discussed in previous chapters. One of the ways phages can contribute to bacterial variation is by transduction. Transducing phages play a key role in sexual recombination in bacteria. The main advantages of sexual recombination in bacteria, as well as with other organisms, are that it reduces the accumulation of deleterious mutations and makes it possible to recombine beneficial mutations that arise in different lineages to be united in the same genome (Rice 2002). Experiments with *Chlamydomonas reinhardtii* have generally supported the idea that sex can accelerate adaptation to novel environments (Colegrave et al. 2002).

Probably the most important contribution of phages to holobionts is to drive bacterial diversity by the "kill the winner" mechanisms (Winter et al. 2010). The rate at which a lytic phage kills its specific bacterial host is proportional to the concentration of the host. Thus, when a particular bacterial strain becomes abundant, it is rapidly killed, providing a niche for different bacteria to proliferate. This mechanism for stimulating bacterial diversity has been well described for marine bacteria in the sea (Winter et al. 2010). Similar data have been reported for the horse gut, where the diversity and abundance of *Escherichia coli* strains have been shown to be directly linked to the relative abundance of specific coliphages (Golomidova et al. 2007), representing an experimental demonstration of phage-driven generation of functional diversity of bacteria within a holobiont.

Recently, a gnotobiotic mouse model of phage–bacterial host dynamics in the human gut has been constructed and studies (Reyes et al. 2013). A consortium of sequenced human gut bacteria was introduced into germ-free mice followed by infection with phages purified from the fecal microbiota of healthy adults. A variable pattern of phage attack and multiplication was observed over a 25 day period. This study is the first defined community-wide view of phage/bacterial dynamics in the human gut and may contribute to the understanding of the part phage play in the holobiont.

Marine Invertebrates: Reproduction of the sea slug *Elysia chlorotica* requires attachment of the larva to a specific algal species (Pierce et al. 1999; Cruz et al. 2013). Once attachment takes place, the juvenile slug feeds on the algae and acquires its chloroplasts. However, chloroplasts do not encode the entire set of genes essential to carry out photosynthesis, since many of the necessary genes are encoded in the algal nuclear DNA (Eberhard et al. 2008). Thus, it is surprising that the chloroplasts remain functional in the adult slugs for several months, providing energy to the slug. The explanation for this remarkable finding is horizontal gene transfer of genes for chloroplast functions, from the algal nuclear DNA to the slug, mediated by slug retroviruses (Rumpho et al. 2008). The slug contains both endogenous and exogenous forms of retrovirus. At 9-months, the adult slugs lay eggs and, in a highly synchronous manner, the whole adult population dies. At this synchronous end of life, all of the adult slugs have a high concentration of the exogenous version of the virus, which is transferred to the eggs and can subsequently be used as vectors for horizontal gene transfer of genes necessary for photosynthesis from the algae to the juvenile slug.

Over the past 30 years there has been an approximately 30 % worldwide decline in the coral population, largely due to emerging diseases (Hughes et al. 2003). The most serious coral disease in the Gulf of Eilat, Israel, is the white plague-like disease caused by the bacterial pathogen *Thalassomonas loyana* (Barash et al. 2005; Thompson et al. 2006). During a successful field trial of phage therapy of the disease it was observed that some control corals (no added phage) were naturally resistant to infection with *T. loyana* (Atad et al. 2012). Examination of these resistant colonies indicated that they contained lytic phages specific to the pathogen. It was suggested that natural phages play a role in limiting bacterial disease in the marine environment, thereby preventing extinctions.

Insect viruses: The most well-studied mutualistic viruses are the polydnaviruses (Webb 1988). The polydnaviruses are obligate symbionts of their parasitoid wasp hosts. The viruses are required for the successful development of the wasp eggs in the caterpillar hosts they parasitize (Soltz and Whitfield 2009). The polydnaviruses have evolved with wasps for so long that some question exists if they should still be considered viruses (Thézé et al. 2011; Renault et al. 2005). Most of the viral genes reside in the nuclear genome of the wasp, while the virions package wasp genes for delivery into the caterpillar where the wasp deposits its eggs. Many parasitoid wasps lay their eggs in living insect larvae. The innate immune system of the larva would normally wall off the egg, forming an encapsulation structure that prevents the egg from developing, but the wasp genes carried by the polydnavirus virions suppress this response. Without this suppression, the wasp eggs would not survive. It has also been shown that several wasp species harbor reoviruses and that at least two of them suppress caterpillar defense, allowing the development of the parasitoid wasp eggs (Renault et al. 2005).

Viruses contribute to the survival of aphids by inducing wing development and thereby promoting dispersal (Ryabov et al. 2009). Production of the winged morphology in asexual clones of the apple aphid, *Dysaphis plantaginea*, is dependent on their infection with a DNA densovirus. Virus-free clones of the

aphid do not produce the winged morphology in response to crowding and poor plant quality. On the other hand, an infection with densovirus results in a significant reduction in aphid reproduction rate (Guerra 2011), together with the production of the winged morphology (even at low insect density). These aphids can now fly and colonize neighboring plants. The latter data suggest a mutualistic relationship between the apple aphid and its viruses.

Beneficial mammalian viruses: Most research dealing with mammalian viruses has been concerned with viruses that cause diseases. Isolating and characterizing viruses that inhibit disease in mammals is technically more of a challenge (Shen 2009). However, there have been a number of reports of viruses that have potentially beneficial functions in combatting disease in mammals: Adeno-associated virus reduces the number of cancerous tumors in newborn hamsters infected by human adenovirus type 12 (de la Maza and Carter 1981). Patients infected with HIV-1 progress to full-blown AIDS much more slowly if they are also infected with hepatitis G virus, a nonpathogenic hepatitis virus that is common in humans (Tillman et al. 2001). Human cytomegalovirus has also been reported to suppress infection with HIV-1 (King et al. 2006), and hepatitis A virus can suppress infection with hepatitis C virus (Deterding et al. 2006). Apparently, the protecting viruses interfere with various functions of the pathogenic viruses, including replication. Viruses can also protect against nonviral diseases. For example, lymphotropic viruses prevented type 1 diabetes in nonobese mice (Oldstone 1988), and herpes virus protects mice from infection by the bacterial pathogens *Listeria* and *Yersinia* (Barton et al. 2009). In general, viruses modulate the host immune system by stimulating innate immunity.

Occasionally, infection of a germ line cell by a retrovirus may lead to an integrated provirus that is passed on to the offspring and inherited as a Mendelian gene; this is known as an endogenous retrovirus. Retroviruses have incorporated themselves into the genomes of our ancestors for hundreds of millions of years. Retroviruses are found in all mammals and a wide range of other vertebrates. The human genome contains about 100,000 fragments of retroviruses, making up over 8 % of our DNA (Barton et al. 2009). Although for most of this viral DNA there is no known function, there are some viral genes that clearly code for essential proteins in our bodies. One particular viral-encoded protein, syncytin, is required for the development of the placental syncytium, an essential part of the barrier that prevents maternal antigens and antibodies getting into the fetal bloodstream. The indispensable nature of the syncytin gene was demonstrated in sheep, where this viral gene is expressed at high levels in the genital tract of ewes. When the virus gene was suppressed by antisense oligonucleotides, pregnant sheep aborted (Dunlap et al. 2006). It is thought that originally, syncytin allowed viruses to fuse host cells together so they could spread from one cell to another. Now the viral protein allows babies to fuse to their mothers. Research on syncytin genes has led to the suggestion that integration of viral genes into host genomes led to a major evolutionary leap, the formation of placental mammals (Dupressoir et al. 2009).

Plant viruses: The ability of internal viruses to prevent infection by virulent viruses is also prevalent in plants. Plants harbor numerous endogenous

pararetroviruses. These viruses package DNA rather than RNA (Staginnus et al. 2007). Tomato endogenous pararetrovirus sequences generate small interfering RNAs (siRNAs) that are important in plant defense against infection by other related viruses (Han et al. 2012). In petunia plants, an endogenous virus contributes to immunity by preventing infectious viruses from entering the petunia meristem (Noreen et al. 2007).

Several viruses confer drought or cold tolerance to their plant hosts. For example, when beet, cucumber, pepper, watermelon, squash, or tomato plants were infected with cucumber mosaic virus, they survived longer after periods of drought than uninfected plants (Xu et al. 2008). Also, these viral-infected plants exhibited significantly improved tolerance to freezing. Although the mechanisms for these observations are not yet known, the viral-infected plants showed an increase in several osmoprotectants and antioxidants during drought stress, including trehalose, other sugars, putrescine, proline anthocyanins, tocopherols, and ascorbic acid. Trehalose is a disaccharide that is known to confer drought and heat tolerance in fungi (Hottiger et al. 1987). These results indicate that virus infection can improve plant tolerance to abiotic stress.

Plant viral nucleic acid can persist inside plant cells either as free viruses (Roossinck 2011b) or integrated into the plant genome (Squires et al. 2011). Persistent viruses are vertically transmitted and remain with their host indefinitely (i.e., through many generations). One persistent plant virus, white clover cryptic virus, encodes a gene for its legume plant host that can affect nodulation (Nakatsukasa-Akune et al. 2005). Maintaining the persistent state of the virus would then be of prime importance to the plant, establishing a mutualistic symbiosis. This has also been observed in a plant–fungus–virus interaction, where a persistent virus in an endophytic fungus is required for thermal tolerance of plants growing in geothermal soils (Márquez et al. 2007). The mechanism of this thermotolerance may involve viral control of fungal gene products that are involved in stress tolerance. A comparison of the transcriptomes of fungi with and without the virus under mild heat stress implicated genes involved in the synthesis of trehalose and melanin, a pigment that is associated with abiotic stress tolerance in fungi (Dadachova and Casadevall 2008).

Key Points

- Viruses are noncellular, obligate intracellular parasites that are an active part of all animal and plant holobionts. They can be transmitted horizontally (from the surrounding) or vertically (to progeny).
- Bacterial viruses (phages) in holobionts contribute to bacterial diversity.
- Endogenous viruses (present in the host genome) are transmitted vertically from one generation to the next with fidelity and thus help propagate the unique properties of the holobiont.

- The human genome contains about 100,000 fragments of retroviruses, making up over 8 % of the DNA.
- Viruses contribute to the immunity of their hosts by preventing infection of bacterial and viral pathogens. They also add metabolic functions and facilitate development of offsprings in different ways. These insourced tasks enable a better adaptation of the holobiont.
- New viral genes are continuously created during viral replication by recombination; occasionally these novel genes find their way into host genomes and help drive evolution. One particular viral-encoded protein, syncytin, led to a major evolutionary leap, the formation of placental mammals.

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Chapter 8
The Evolution of Holobionts

So, like it or not, microbiology is going to be in the center of evolutionary study in the future—and vice versa.

—Carl R. Woese

Introduction

In the previous chapters, we have presented published experimental data in support of the principles upon which the hologenome concept are based. We also pointed out special aspects of the concept, such as the additional modes of genetic variation within the holobiont. In this chapter, we will discuss the hologenome concept of evolution and how the concept relates to other ideas in evolutionary biology. The key point that we would like to stress is that microorganisms were fundamental in the formation and evolution of complexity in animals and plants.

Following Darwin's lead and evolutionists before him, biologists have studied evolutionary processes of animals and plants for more than 150 years—without knowledge of the vast and dynamic genetic information that is present in their microbiotas. The neo-Darwinian evolutionary synthesis that emerged in the 1930s and 1940s was thus a concept void of any microbial foundation. It was founded on a world of two kingdoms: plants and animals. Microbiology largely remained a world apart from evolutionary biology until the development of new concepts and molecular methods for determining phylogenetic relationships. Molecular phylogenetics is the branch of phylogeny that analyzes hereditary molecular differences, mainly in DNA sequences, enabling construction of evolutionary relationships. The results of these molecular phylogenetic analyses, most often obtained by using sequences of rRNA genes, are usually expressed in phylogenetic/evolutionary trees. rRNA gene sequencing has been used to study phylogeny and evolution for a number of reasons, the main ones being: (1) its presence in almost all cells; (2) the function of the rRNA gene over time has not changed, suggesting that random sequence changes are an accurate measure of time (evolution); and (3) the rRNA gene is large enough for informatics purposes (Janda and Abbott 2007).

Approximately 40 years ago, rRNA gene sequence-based phylogenetic analyses changed fundamentally our understanding of natural relationships among organisms, establishing three domains of life, Archaea, Bacteria, and Eukaryota (Woese and Fox 1977), and placing the microbes (Archaea and Bacteria) at the base of the universal tree of life (Woese 1994). In the last two decades, these

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concepts and improved sequencing technologies were applied to microbes associated with Eukaryota. The outcome of these studies has been a better understanding of the role of microbes in the fitness and evolution of animals and plants.

In this chapter, we will summarize recent data describing how microbiotas, including bacteria, archaea, protists, and viruses, have played and continue to play major roles in the evolution of higher organisms. We suggest that consideration of the hologenome concept requires a paradigm shift in evolutionary biology, and realizes that replacement of one paradigm by another is not an easy process. The fact that microbiomes and viromes comprise the vast majority of genetic information in animals and plants (99.3 % in humans) and that they can change more rapidly and by more processes than the host genome alone (Chap. 6), should encourage evolutionary biologists in the future to consider hologenomes.

Levels of Selection and Drift in Evolution

The debate on units of selection has been a central issue within the field of evolutionary biology from its beginnings. Darwin considered the individual organism as the primary unit of selection in evolution (Ruse 1980), as did and do many evolutionists till today. However, the co-discoverer of evolution, Alfred R. Wallace, argued that a characteristic can evolve also because it benefits the group even though it may be harmful to the individual possessing it (letter from Wallace to Darwin quoted by Ruse 1980). Subsequently, evolutionary biologists referred to this latter concept as group selection and to such traits as altruistic (Wynne-Edwards 1963). For many years, group selection, in addition to individual selection, was accepted by evolutionary biologists. Then, George C. Williams published his book *Adaptation and Natural Selection* (Williams 1966) that claimed biologists had unnecessarily discussed traits existing for the good of the group or species because explanations of the phenomena exist at lower levels. The real unit of selection, according to Williams, is neither the group nor the individual, but the gene. Subsequently, Richard Dawkins popularized this concept in his book *The Selfish Gene* (Dawkins 1976).

More recently, a number of prominent evolutionary theorists have come to see the body of ideas known loosely as "multi-level selection theory" as a potent explanatory principle, though each in a slightly different way (Kerr and Godfrey-Smith 2002; Okasha 2006). Multilevel selection theory takes as its starting point the notion that natural selection can operate simultaneously at different levels of the biological hierarchy. So the evolution of a given trait can be affected by selection at more than one level.

An interesting and basic aspect of multilevel selection theory is the idea that the direction of selection may be different at different hierarchical levels; a trait may be selectively disadvantageous at the individual level, but selectively advantageous at the group level. Interactions among individuals (social behavior) may create substantial heritable variation, which is hidden to classical individual analyses (Bijma

et al. 2007; Goodnight and Stevens 1997; Wolf et al. 1999). Selection acting on higher levels of organization (groups) captures this hidden variation. For example, Muir et al. (2013) have shown that quails housed in kin groups had a reduced mortality and increased weight compared to quails raised in random groups. Thus, kin group selection was effective in reducing detrimental social interactions (fighting and cannibalism), which contributed to improved weight gain.

We argue that the holobiont (host + microbiota) with its hologenome (host genome + microbiome, including virome) is a unique biological entity and therefore an important and underappreciated level of selection. The major reasons for considering the holobiont over the individual animal or plant as a unique biological entity and thus a unit of selection in evolution are the following:

1. There has never been any natural animal or plant free of microorganisms (Zilber-Rosenberg and Rosenberg 2008; Chap. 3 of this book). In a recent review, Gilbert et al. (2012) have reiterated this point by claiming, "We have never been individuals."
2. The holobiont has its own specific properties that are not necessarily the sum of the host + its microbiota. In other words, one can consider the synergistic interactions that occur in the holobiont as a special type of selection resulting from close interactions (Goodnight and Stevens 1997; Wolf et al. 1999). A specific example is nitrogen fixation by legumes that depends upon leghemoglobin, where the apoprotein is encoded by a plant gene (Ott et al. 2009), and the heme moiety is synthesized by bacterial enzymes (Hardison 1996). A more general and comprehensive example of cooperation between microbiota and host comes from metabolomics studies of mammalian blood, which show that production of plasma metabolites involves interactions between microbial and host genes (Wikoff et al. 2009; Velagapudi et al. 2010).
3. In many cases, holobionts have their specific structures that result from the interactions between hosts and microbiotas, e.g., squid light organ (Nyholm and McFall-Ngai 2004), legume nodules (Monaham-Giovanelli et al. 2006), the cow rumen (Mizrahi 2011), and the termite pouch (Breznak and Pankratz 1977). Additional details can be found in Chap. 5 of this book.
4. Each holobiont stands by itself, facing its environment, competing as a whole with other holobionts (whereas the smaller units of selection, namely, the separate genomes and individual genes, are selected for within the holobiont).
5. The hologenome is transmitted from one generation to the next with reasonable accuracy (see Chap. 4).

What does the hologenome concept contribute to the debate over levels of selection and our understanding of adaptation and evolution?

- The holobiont consisting of the host and hundreds or thousands of different microbial species should be considered a group. Thus, the concept of group selection should be extended to all animals and plants because each one of them is a holobiont, namely a group. Each one is a complex system that includes several functional levels: the holobiont, the individual host organism, each of

the diverse microorganisms, and the multitude of genes present in the hologenome—all cooperating and competing continuously. Variation, selection, (or drift, see below), and evolution within the holobiont can occur individually in all participants of the holobiont, namely, in the host as well as in the individual microorganisms (West et al. 2006; Dethlefsen et al 2007). We suggest that variation, selection, (or drift), and evolution can also occur via group selection of the holobiont with its hologenome.

- One of the important conclusions arising from the above is that the only truly independent individuals that can be considered in evolution are free-living microorganisms. Each animal and plant is a group, a holobiont.
- As discussed in Chap. 6, consideration of the holobiont as a level of selection brings forth several novel modes of variation, such as microbial amplification, acquisition of microbes from the environment and horizontal gene transfer, which may contribute to our understanding of evolution.
- Microbially driven variation and heritability within the holobiont (or hologenome) can be considered another mode of epigenetic inheritance.

An additional concept that was ascribed to the holobiont is the concept of the superorganism (Gordon et al. 2013). This term was coined first by Wheeler (1928) and then further developed primarily to explain the behavior of social insects (Wilson and Sober 1989; Hölldobler and Wilson 2008). A superorganism has been defined as a "collection of single creatures that together possess the functional organization implicit in the formal definition of organism" (Hölldobler and Wilson 2008). We suggest that the term superorganism be retained to describe the behavior of certain social organisms, with the understanding that a superorganism is, in fact, a population of holobionts, which interact closely with each other. Accordingly, the original meaning of a superorganism will be preserved and is consistent with the argument of Wilson and Sober (1989) that "only some groups and communities qualify as superorganisms." In contrast, one of the fundamental principles of the hologenome concept is that all animals and plants are holobionts.

Random Drift and Evolution of Holobionts

Random drift of microbes and genes can occur in holobionts as it does in other populations, adding another dimension to the possibilities of evolving and spreading genetic variants. Random drift differs from selection in that the change of the genetic composition of a population is a result of random genetic events rather than a result of a non-random selection process. Random drift usually expresses itself in small populations while its effect in large populations is small (Ridley 2004). Holobionts include tens to thousands of microbial species, some of which are abundant and clearly selected for under favorable conditions, whereas others are selected against and may be lost or remain in very small numbers. Amongst the many microbial species in the holobiont it is likely that some may

also be neutral microbes that were acquired randomly from parents or from the environment and multiply within the holobiont with little or no selection pressure. For example, identical twins have been shown to have similar but not identical microbiota, demonstrating, at least partially, neutral drift of certain microbes during birth and from a similar environment (Turnbaugh et al. 2009, 2010). These neutral microorganisms may be in a minority because of strong competition within the holobiont, but conditions may arise in which these randomly drifting microbes will be selected for and become abundant species and even may contribute to the holobiont and its evolution by aiding it in changing environments. There also is a possibility that a microbe drifting randomly into a holobiont will multiply and amplify with no selective pressure and remain present for long periods. When a rare microbial species is amplified, and becomes more abundant, by drift alone or by selection, it has an increased chance of being transferred to the next generation. We would like to stress that hologenomic drift can occur at all the different levels of the holobiont from single genes of the microbes or the host to the holobiont itself. Thus, genetic drift could contribute significantly to the evolution of holobionts.

Cooperation and Cheating

Mutually beneficial symbioses (mutualisms) between microorganisms and their hosts evolved millions of years ago, from the start of eukaryotic existence, and have persisted to this day (Chap. 2). Well-documented, straightforward classical examples present till today include arbuscular mycorrhizal fungi and roots of vascular plants (ca. 400 million years ago) (Redecker et al. 2002), endosymbiotic algae and corals (ca. 240 million years ago) (Woods 1999), ruminants and their microbiota (ca. 60 million years ago) (Collinson and Hooker 1991), and the great apes and gut microbiota (15–20 million years ago) (Ochman et al. 2010; Yildirim et al. 2010).

In Chap. 5, we presented many examples of how cooperation between microbiotas and their hosts benefits the holobiont. Not only is there cooperation between microbiota and host, but there are numerous cases of cooperative interactions between different species of microorganisms within the host, for example, cross-feeding between microbes in the mucus of coral holobionts (Ben-Dov et al. 2009). The close associations of different microorganisms within a holobiont encourage cooperation between them because extracellular products excreted by one species will readily come into contact with other species. This is true for microbial biofilms on the outer surface of animals or plants as well as the digestive tract of animals (Bäckhed et al. 2005). In fact, cooperation is the key element of the holobiont.

Without suggesting mechanisms, Wilson (1989) claimed that an organism is considered altruistic when its behavior benefits the group, at an immediate cost to itself; however, it may itself benefit in the long run by being part of the group.

Cooperation and altruism pose a problem to evolutionary biologists because organisms that confer benefit to others are often considered to be at a disadvantage relative to "selfish" organisms. Selfish organisms in the case of the holobiont are microbes that provide no benefit (cheaters) while growing on the expense of the holobiont. Regarding this problem, we would like to discuss the different ways in which cooperation could be carried out within a holobiont, its benefits in addition to how holobionts may overcome cheating.

First, as has been pointed out by Douglas (2008), some cooperative interactions are cost-free. For example, bacteria in the large intestine of mammals produce short-chain fatty acids (SCFAs), which benefit the host (Bugaut 1987). SCFAs are necessary byproducts of the normal anaerobic metabolism of these bacteria. These types of cost-free symbioses are also called byproduct mutualistic interactions. Moreover, in some cases, removal of the end product of the metabolism of one organism by another removes feedback inhibition and/or thermodynamically drives the metabolism of the end product-producing organism. In such cases both organisms benefit from the cooperation.

A second driving force for the evolution of cooperation could be that it provides a fitness benefit to the holobiont that outweighs the cost of providing the benefit (West et al. 2007). Consider the well-studied aphid/*Buchnera* mutualistic symbiosis (Wilson et al. 2010), in which the bacterium overproduces and excretes amino acids that are essential to the host insect. Overproduction of the amino acids is costly to the bacterium but benefits the holobiont (aphid plus bacteria), and by being part of the holobiont, the bacterium benefits also. Thus, the connection between the bacterium overproducing the amino acids and receiving nutrients from the aphid in return is so close that the behavior might better be described as reciprocal cooperation, or mutualism, or tit-for-tat according to game theory (Axelrod and Hamilton 1981; Axelrod 1984; Nowak 2006), rather than altruism. What would cheating look like in such a system? One might speculate that *Buchnera*, which did not overproduce amino acids (cheaters) would emerge and soon dominate, since the cheaters would initially multiply faster than the noncheaters. However, this is unlikely because bacteria clone themselves, and consequently, aphids which contain too many cheaters (unable to produce amino acids) probably could not reproduce or alternatively they would die, eliminating the cheating bacteria. In the case of the aphid/*Buchnera* symbiosis, the situation is even more extreme since *Buchnera* has lost many of its genes during coevolution with aphids and could not survive outside its host (Pérez-Brocal et al. 2006).

Another topic that has received considerable attention regarding cooperation and cheating is the production of what is referred to as "public-goods" (Frank 1998). For example, bacteria growing on polymers must produce extracellular enzymes that can breakdown the polymers into small molecules which then enter the cells. The bacteria cooperate in a cell density-dependent manner to produce enough enzymes to efficiently degrade the polymer (Rosenberg et al. 1977). The central problem is that while investment in a public good (extracellular enzymes) is costly to individuals that produce it, the public good may also be used by others; thus, *all else being equal*, cheats that reap the rewards of cooperation without

making any investment should be able to invade a population of cooperators. However, *all else is not equal*. Large molecules diffuse slowly, so there is a higher concentration of enzymes around the producing cells compared to the bulk medium, and as a result also a higher concentration of the monomeric and oligomeric breakdown products. Thus, the cheater actually is at a disadvantage when nutrients are limited (Wakano et al. 2009).

In holobionts that contain many different microbial species, such as in the mammalian gut, understanding the problem of cheaters is complex. It appears that cheating in such systems can be tolerated to some extent as can be deducted from the very complex and quite stable microbiota existing within holobionts (Faith et al. 2013). On the other hand, it is also clear that a high abundance of cheaters is a disadvantage to a holobiont. Therefore, one can reasonably assume that in addition to the specific examples discussed above, holobionts evolved systems that overcome or suppress cheating: one is functional redundancy (Mahowald et al. 2009; Lozupone et al. 2012), and probably the most important one is the immune system (Dethefsen et al. 2007). Functional redundancy enables overcoming exploitation by cheaters; if the production of a beneficial product by one bacterial species is greatly reduced because of cheaters in its population, another bacterial species will often supply the product. The immune system is probably the main force that suppresses cheating to a level that can be accepted by the holobiont, enabling it to be fit, survive, and reproduce. Both innate and adaptive immune systems in animals and plants can play important roles preventing overgrowth of cheaters; a delicate equilibrium is achieved between the host immune system and its microbiota, the immune system constantly monitoring its microbiota. Any change above a certain background level can be detected by the immune system which reacts with an inflammation, or with simpler local mechanisms such as antimicrobials, and eliminates the cheater.

Evolution by Cooperation

We argue that higher organisms evolved by utilizing the wealth of existing prokaryotic and viral genetic material, in addition to inventing or reinventing genes from scratch. The hologenome concept places emphasis on cooperation between microbiota and their hosts in the evolution of holobionts.

How can animals and plants evolve using microbial genes? The first holobiont was probably a eukaryotic microorganism formed by the endocytosis of one or more prokaryotes into another (see Chap. 2). This is consistent with the evolutionary point of view laid out by Nowak (2006) who argued, in general, that cooperation is needed in evolution for the construction of more complex levels of organization. After assembling eukaryotes, higher organisms continued to evolve, in large part, by acquiring and subsequently modifying by mutation and rearrangement microbial genetic material, in addition to constructing entirely new genes (Parfreya et al. 2011). Beyond the acknowledged origin of mitochondria and chloroplasts by

endosymbiosis of bacteria (Andersson et al. 1998; Martin et al. 2002), application of modern DNA technology has taught us that animals and plant holobionts contain a wide variety of microbial and viral genetic material, both in their microbiome and in the host genome. These techniques have shown us that the collective genomes of human gut bacteria contain at least 150 times as many genes as our own genome (Gill et al. 2006; Qin et al. 2010). Also, of the approximately 22,000 human genes that code for proteins (Intl. Human Gen. Seq. Consort. 2004), 60% of them are homologs of bacterial genes (Domazet-Loso and Tautz 2008), primarily those involved in intermediate metabolism. In addition, 100,000 pieces of retrovirus DNA have been identified in our genes, making up another 8% of the total human genome (Belshaw et al. 2004). Similar results have been obtained with plants: Approximately 18% of Arabidopsis protein-coding genes were acquired from the cyanobacterial ancestor of chloroplasts (Martin et al. 2002) and another 25% from other prokaryotes (Gerdes et al. 2011). Let us now speculate on how these microbial genes were acquired and how complex holobionts may have evolved, and are still evolving, using microbial and viral genes.

Higher organisms were and still are constantly exposed to environmental microbes. A small fraction of these microbes enter cells, while a larger fraction attach to the outer surfaces of tissues. If these microbes can find a niche, overcome the immune system and amplify, they become resident endosymbionts or exosymbionts, respectively. These symbionts contain whole sets of genes, which become part of the hologenome (insourcing), and consequently the holobionts, which contain these novel genes are variants upon which selection can act, namely, that can be selected for or against.

Potentially, newly acquired, useful microbes can either (1) remain intact and express their beneficial genetic potential in the holobiont as part of the microbiota (acquisition of microbiota as endo or exosymbionts), or (2) have some of their genetic material transferred to host chromosomes (acquisition of microbial genes). The latter must be more common with endosymbionts and viruses than with exosymbionts.

Evolution by acquisition of microbiota: An obvious example of microbial insourcing is cellulose degradation in ruminants and termites; in fact, all of fitness traits attributed to symbionts described in Chap. 5 fit into this category. In the case of cellulose degradation by termites, it has been suggested that the evolution of a sophisticated community of hindgut microorganisms may be viewed as a gradual process of internalizing consortia of external anaerobic microbes that digest plant litter in the soil (Nalepa et al. 2001). Instead of plant debris decaying to varying degrees in the external environment prior to ingestion, it "rots" primarily in the hindgut after ingestion. The primary difference between many termites and other invertebrate decomposers is that in termites freshly dead plant materials (wood, grass, leaf litter) may be consumed before these substrates have been significantly degraded by microorganisms outside (Wood 1976). Similar arguments have been put forth for the origin of herbivorous dinosaurs (Mackie 2002) and the first plant-eating mammals (Collinson and Hooker 1991). Most intriguing is the

additional fact that in all these cases, cellulose decomposition by gut microbiota has been selected for over integrating the appropriate genes into the host genome.

One of the major advantages of maintaining the symbiotic genetic information in exosymbiont microbes, rather than transferring it to the host genome, is that it allows for rapid adaptation to new environments. For example, when a previously rare nutrient (or toxic chemical) becomes prevalent, a symbiont which has the genetic capacity to process the material will multiply and become more abundant (amplification). Not only can this provide a fitness advantage to the holobiont in the new environment, but the process of microbial amplification is equivalent to gene multiplication and, as such, is a variation that can be inherited. A good example of such a possibility is the acquisition from soil of the bacterium *Burkholderia* resistant to an insecticide by stinkbugs in areas where this insecticide was applied. The acquisition resulted in a resistance of the host insect to the insecticide which spread to populations of other stinkbugs in the surrounding (Kikuchi et al. 2012). Another example is the horizontal gene transfer of genes coding for porphyranases and agarases from a marine bacterium to a human gut bacteria from the genus *Bacteroides* (Hehemann et al. 2010; see also Chap. 6 regarding horizontal gene transfer).

It appears that in invertebrate animals and also in plants endosymbionts are prevalent. However, as one reaches higher in the evolutionary tree and the immune system becomes more complex and aggressive, it appears that the microbiota is carefully kept extracellular and even out of the reach of most internal liquids (blood) and organs, though they still can be found in those internal organs that are open to the outside world (gut, vagina, respiratory system). This leads to the probable conclusion that higher animals no longer required endosymbionts. Instead, they found ways of interacting with exosymbionts by integrating certain microbial metabolites into host metabolism, benefiting from several functions carried out by the microbiota, and adapting to their presence in different ways, while giving them protection and livelihood. This kind of cooperation probably created more resistant and versatile holobionts.

What happened in plants? The first photosynthetic eukaryotes were most likely formed about 1.6 billion years ago by the endosymbiosis of a cyanobacterium by a single-celled Protist (Price et al. 2012). The captured cyanobacterium was retained and over time evolved into a double membrane-bound chloroplast. This primary endosymbiosis event gave rise to three autotrophic clades with chloroplasts: the land plants, the red algae, and the glaucophytes (De Clerck et al. 2012). Land plants evolved from green algae (Zhong et al. 2013), but the initial invasion of dry and rocky land was probably possible only through an alliance of ancient algae, fungi, and bacteria (Bidartondo et al. 2011), forming lichen-like holobionts. Bacteria and filamentous fungi were essential for the chemical weathering of minerals, which was also a crucial prerequisite for the appearance and evolutionary transition of the first ancient land plants into highly specialized modern higher plants (Dolan 2009). These alliances between bacteria, filamentous fungi, and plants are also obvious in the current plants (Denison and Kiers 2011), where mycorrhizal fungi and associated microbial communities fulfill important functions in the ecology of plants

such as accessing recalcitrant forms of mineral nutrients, and production of signaling and other compounds in the vicinity of the plant roots (Jansa et al. 2013).

In the course of plant evolution, there is an obvious trend toward an increased complexity of plant morphology, as well as an increased sophistication of plant metabolism, behavior, and communication. Similar to animals, not only the emergence of plants but also their ongoing evolution was shaped and driven by symbiotic microorganisms (Baluška and Mancuso 2013). One of the interesting contributions of microbiota to plants is the synthesis of the hormone auxin. Spatial auxin distribution, produced both by the plant and symbiotic bacteria, underpins most of plant growth responses to its environment in addition to plant growth and developmental changes in general (Moriguchet al. 2001). Interestingly, auxin is not only a crucial signaling molecule for plants, but it is also an ancient signaling molecule produced and used by microorganisms (Mazhar et al. 2013). Auxins of today synthesized by bacteria and filamentous fungi living in the rhizosphere and attached to roots initiate several growth and developmental processes in plants such as root hair initiation and tip growth, lateral root formation, and the plasticity of root system architecture (Zamioudis et al. 2013). These auxin-producing microorganisms were and probably still are the driving force behind the evolution of plant synapses and other "neuronal" aspects of higher plants; this is especially pronounced in the evolution of the apical meristem of roots. Plant synapses allow synaptic cell–cell communication and coordination, as well as sensory-motor integration in roots searching for water and mineral nutrition.

As a concluding remark for this section, it should be pointed out that acquisitions of beneficial microbes have the potential to be amplified and spread rapidly to other holobionts, in a manner similar to epidemics of pathogens. This would accelerate the rate of holobiont adaptation and evolution.

Evolution of holobionts by acquiring microbial genes: Microbial symbionts not only provide holobionts with an enormous amount of genetic information, but they also have the potential to transfer some of their microbial genes to host chromosomes (Keeling and Palmer 2008). This probably occurs more frequently via viruses and endosymbionts than by exosymbionts. Let us take the mitochondria and chloroplasts as a good example: in addition to the radical DNA changes in these organelles, many mitochondrial and chloroplast genes, originating in bacteria, have been translocated into nuclear genomes (Sorenson and Fleischer 1996; Martin et al. 2002). In fact, mitochondrial genome evolution has taken radically different pathways in diverse eukaryotic lineages, and the mitochondria itself is increasingly viewed as a genetic and functional mosaic, with a large portion of the mitochondrial genome having an evolutionary origin outside of its original Alphaproteobacterium (Gray 2012).

There are numerous other known examples of horizontal gene transfer (HGT) between symbionts and their hosts some of which were discussed in detail in Chap. 6 regarding variation in holobionts. With regard to the evolution of holobionts, it has been shown that biosynthetic fungal genes for carotenoid production were horizontally transferred from a fungus to aphids (Moran and Jarvik 2010), many

genes from the endosymbiont bacterium Wolbachia have been inserted into the chromosomes of their arthropod hosts (Dunning-Hotoop et al. 2007), and the long interspersed nuclear element L1 has been transferred from humans to the genome of some strains of the bacterial pathogen *Neisseria gonorrhoeae* (Anderson and Seifort 2011). On a global scale, ca. 10 % of the human genome can be recognized as of viral origin, which indicates that the DNA must have been transferred by HGT from viruses to the host genome (Horie et al. 2010).

One particularly interesting example of viral HGT in evolution is the gene coding for the protein syncytin in mammals, which is required for the development of the placenta (placentation) (Dupressoir et al. 2009). Interestingly, the transfer of the retroviral syncytin gene occurred independently in different mammals. It is thought that syncytin originally allowed retroviruses to fuse host cells together, which enabled spreading of the virus from one cell to another. Now the viral protein allows babies to fuse to their mothers, enabling the tolerance of the fetus by the maternal immune system. Research on syncytin genes has generated the suggestion that integration of viral genes into host genomes generated a major evolutionary leap, the formation of placental mammals (Dupressoir et al. 2012).

Insightful evidence for HGT playing a role in evolution also comes from a proteomic analysis of sponge-associated bacteria indicating that 139 proteins of the sponge microbiota are homologous to eukaryotic proteins (Liu et al. 2012). It is not clear if the eukaryotes receive the genes coding for these proteins from bacteria or vice versa. However, interkingdom gene transfer must have occurred. It should be noted that sponges are considered the simplest and oldest existing multicellular eukaryotes.

Recently, it was shown that HGT occurred frequently in the early evolution of land plants (Yue et al. 2013). Genome analyses of the moss *Physcomitrella patens* identified 57 families of nuclear genes that were acquired from prokaryotes, fungi, or viruses. Many of these gene families were transferred to the ancestors of green or land plants. These anciently acquired genes are involved in some essential or plant-specific activities such as xylem formation, plant defense, nitrogen recycling as well as the biosynthesis of starch, polyamines, hormones, and glutathione (Yue et al. 2012). These findings suggest that HGT had a critical role in the transition of plants from aquatic to terrestrial environments.

We suggest that once microbes or their genes become established in the holobiont, a slow and random process of mutation and selection of microbiota and host genes takes place optimizing the fitness of the holobiont. This process has occurred throughout the evolution of animals and plants and continues to this day; the more recent the event, the greater the chance to detect it and establish its origin.

Hologenomes and Speciation

Species are one of the fundamental units of comparison in virtually all subfields of biology, especially systematics and evolution. The central role of species in evolutionary biology is evidenced by the titles of two of the most important

publications in the period of the Modern Evolutionary Synthesis, Dobzhansky's *Genetics and the Origin of Species* (1937) and Mayr's *Systematics and the Origin of Species* (1942). Mayr (1996) articulated the biological species definition: "Species are groups of interbreeding natural populations that are reproductively isolated from other such groups. The isolating mechanisms by which reproductive isolation is effected are properties of individuals. Geographic isolation therefore does not qualify as an isolating mechanism." Dobzhansky is usually credited with introducing the view that speciation results from hybrid sterility caused by sets of interacting complementary genes. Hybrids between closely related species are often inviable or, if they live, they are sterile. This hybrid inviability and sterility is collectively known as hybrid incompatibility. It is usually attributed to alleles at different genetic loci that do not function well together (Johnson 2000).

By considering the hologenome concept together with the biological species (Mayr 1996) and the hybrid incompatibility (Dobzhansky 1936) concepts, Brucker and Bordenstein (2012) reasoned that negative interaction (or negative epistasis) between host genes and host microbiome can accelerate the evolution of hybrid lethality and sterility. Brucker and Bordenstein (2013) then demonstrated that microbiota play an important role in Nasonia wasp speciation. The researchers found that the gut microbiota of two recently diverged wasp species act as a barrier that prevents their evolutionary paths from reuniting. The wasps have significantly different collections of gut microbes, and when they cross-breed the hybrids develop a distorted microbiome that causes their death during the larval stage. Eliminating gut bacteria via antibiotic treatment significantly rescued hybrid survival. Moreover, feeding bacteria to germ-free hybrids reinstated lethality. The authors conclude: "In this animal complex, the gut microbiome and host genome represent a co-adapted hologenome that breaks down during hybridization, promoting hybrid lethality and assisting speciation." These experiments provide powerful support for the hologenome concept of evolution. Although the precise mechanism for the microbial-based hybrid lethality was not established, the authors suggest it results from negative epistasis, mismatched gene–gene interactions that occur between chromosomal genes and the microbiome.

Another proposed mechanism for the origin of new species is mating preference (Coyne and Orr 2004; McKinnon et al. 2004). Mating preference in the fruit fly *Drosophila melanogaster* is known to include courtship behaviors, such as patterned acoustic signals or "songs" (Kyriacou and Hall 1980), rhythmic movements or "dances" (Ejima and Griffith 2007), chemical (cuticular hydrocarbons) signals (Kim et al. 2004), and finally physical contact. Experimentally, mating preference in flies has been shown to occur when populations were divided and reared separately for many generations under different environmental conditions (Rice and Hostert 1993). In one of these studies, Dodd (1989) reared *Drosophila pseudoobscura* separately on starch-based and on maltose-based media for more than 25 generations and discovered that "starch flies" preferred to mate with other starch flies and that "maltose flies" preferred to mate with other maltose flies (i.e., positive assortative mating). These data were unexpected because there was no selection for the observed mating preference. In addition, 25 generations seemed to

be insufficient for the host genomes to diverge sufficiently to explain the data. Somehow mating preference developed as a correlated response when selection favored novel adaptation to the different food sources. Recently, it was reported that the "correlated response" driving mating preference is the emergence of different bacterial communities associated with the two fly populations (Sharon et al. 2010). The researchers reported also that the starch diet caused an amplification of a particular bacterial symbiont, *Lactobacillus plantarum*, and that this bacterium was responsible for the mating preference. Addition of antibiotics prior to mating abolished the mating preference and infection with *L. plantarum* reestablished the mating preference. Analytical data suggested that the symbiotic bacteria influence mating preference by changing the levels of cuticular hydrocarbon sex pheromones (Sharon et al. 2011). These findings fit within the hologenome concept positing that variability would be expected to occur in the microbiota, where changes in diet can cause rapid alterations in the bacterial community by amplification of strains that prefer starch in one population and disaccharides in the other.

How can bacterially induced mating preference, as described here, contribute to speciation and evolution in nature? One possibility is that, in the natural world, multiple environmental factors act synergistically to differentiate the microbiota and strengthen the homogamic mating preference, i.e., mating of the same kind (including similar microbiota). For example, it is reasonable to assume that fly populations living on different nutrients will also be, at least to some extent, geographically separated. The combination of partial geographic separation and diet-induced mating preference would reduce interbreeding of the populations. Slower changes in the host genome could further enhance the mating preference. The stronger the mating preference, the greater the chance that two populations will become sexually isolated, and many evolutionary biologists have argued that the emergence of sexual isolation is the central event in the evolution of species (Coyne 1992; Schluter 2009). Since microbes are largely responsible for the odor of animals, it is likely they play a general role in mating preference. It has been established that commensal bacteria play an essential role in determining the unique odors of several mammals (Archie and Theis 2011), including rat (Singh et al. 1990), deer (Alexy et al. 2003), bat (Voigt et al. 2005), mongoose (Gorman 1974), and human (Austin and Ellis 2003).

Competition and Cooperation in the Biological Evolution of Complexity

Darwin's explanation of how evolution works is explicitly competitive ("survival of the fittest"). It may be the most important explanatory principle in biology, and is extremely powerful in many other fields. In such a struggle for existence, cooperation (working with another for a mutual benefit) appears to be opposed to self-interest and as such to be the very kind of behavior that should be selected

against. Yet what we see in the biological world is that many types of cooperation have evolved and persisted within holobionts. As Benkler (2011) has pointed out, cooperation triumphs over pure self-interest in many complex structures from families to collective farming up to the world's richest corporations. *The data presented in this book clearly demonstrate that evolution of animal and plant complexity was achieved primarily by cooperation and then selected for by competition. Thus, competition and cooperation are complimentary mechanisms for evolution.*

The hologenome concept places emphasis on cooperation between microbes and between microbes and their hosts. Probably, one of the most important changes in life on this planet was brought about by cooperation between prokaryotes to give rise to eukaryotes containing mitochondria and chloroplasts (see Chap. 2). These evolutionary changes by cooperation were quantum leaps in the development of biological complexity. Cooperation continued to play a key role in the evolution of multicellular animals and plants. As discussed in Chap. 5, the fitness of holobionts relies to a great extent on the cooperation of hosts and their microbiota, as it does on the cooperation between all cells belonging to the host. This cooperation does not contradict the competition existing within the holobiont between its participants (microbes and host) and also between different holobionts.

Cooperation can be observed also at the level of morphology. Paleontology traditionally investigates evolution of humans, animal, and plants using morphology of dated fossils, deriving from them evolutionary trees. However, what is generally not appreciated is that microbiota can affect not only the metabolism of animals and plants but also their morphology. Examples include the rumen of a cow (Jami and Mizrahi 2012), the nodule of legumes (Ott et al. 2009), and the squid eye organ (Nyholm and McFall-Ngai 2004). In each of these examples, the morphology of the holobiont has undergone an evolutionary change while optimizing the interaction of the host with its microbiota for the benefit of the holobiont.

Based solely on the host genome, animals and plants would evolve extremely slowly because of (1) their relatively long generation times, (2) the fact that only changes in the DNA of the germ line are transmitted to the next generation, (3) mutation occurs at a low frequency and are mostly detrimental, and (4) often a whole set of new genes is required to introduce a beneficial phenotypic change. If the environment changes relatively rapidly, the host genome alone may not evolve quickly enough and the organism may lose competitiveness and become extinct. We argue that rapid changes in the symbiotic microbiota by amplification, acquisition of novel microbes and HGT could allow the holobiont to adapt faster and survive under changing environmental conditions, thus providing the time necessary for the host genome to evolve. Moreover, a large fraction of host genome evolution was driven by the acquisition of novel bacterial and viral genes and clusters of genes by HGT, giving rise to quantum leaps in the evolution of complexity of animals and plants. Rather than "reinventing the wheel," animals and plants have the potential to acquire pre-evolved genetic information from microorganisms and viruses.

Let us summarize this chapter by indicating that the Darwinian "survival of the fittest" as a synonym for natural selection (Darwin 1859) is a harsh conviction for the biological world when it comes to individuals and even more so for human society. The hologenome concept of evolution suggests a less abrasive mode of evolutionary change, emphasizing cooperation hand in hand with competition. Cooperation has been selected at every level in evolution, from genes to holobionts, groups of holobionts, and human societies. The historian Yuval Harari has argued that the ability to cooperate effectively in large groups has made *Homo sapiens* the masters of planet Earth (Harari 2012). Thousands of humans working together created trade routes, mass celebrations, political institutions, and technology. A modern jet aircraft is produced through the cooperation of thousands of strangers all over the world: from workers who mine the metals to engineers who test the aerodynamics. To ignore cooperation in human behavior, as well as in the rest of the biology world, is to neglect one of the greatest attributes of living organisms. Hopefully, recognition of the hologenome concept of evolution by cooperation will moderate the selfish view of Social Darwinism.

Key Points

- Evolution by natural selection can proceed at different levels, from genes to ecosystems. Most biologists focus on the individual as the level of selection. However, there are no individual animals or plants in the natural world—all are groups of organisms (holobionts) consisting of the host and many species of microorganisms and viruses.
- The hologenome concept considers the holobiont with its hologenome as a unique biological entity and therefore a natural level of selection in the evolution of organisms. Evolution of the holobiont, the host, and the microbiota, individually and by groups, can also occur via random drift and not only by natural selection.
- Cooperation within holobionts, between microbes and between microbes and hosts, is a stable property that has persisted in some cases for millions of years. One of the important means of achieving this was and is by preventing potential cheaters from becoming abundant by a variety of mechanisms, the main one being—the immune system.
- Evolution by cooperation can occur by acquisition of microbiota (e.g., cellulose-degrading microbes in termites and ruminants) and/or by acquiring microbial and viral genes by HGT (e.g., synctin genes in placental mammals).
- Evolution can proceed not only by mutation and selection, but most significantly by selection following the acquisition of pre-evolved information from microorganisms and viruses.
- Microbiota have also been shown to contribute to the origin of species
- The hologenome concept leads to the conclusion that evolution of animals and plants occurred primarily by natural selection for cooperation.

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Chapter 9
Pathogens as Symbionts

> *Pathogenicity is not the rule. Indeed, it occurs so infrequently and involves such a relatively small number of species, considering the huge population of bacteria on earth, that it has a freakish aspect. Disease usually results from an inconclusive negotiation for symbiosis, an overstepping of the line by one side or the other, a biological misinterpretation of borders.*
>
> —Lewis Thomas (1974)

The generally accepted broad definition of symbiosis is "the living together of different species" (de Bary 1879). In the case of microbial pathogens, some are clearly symbionts in the sense that they remain with their hosts for exceptionally long periods. For example, *Mycobacterium tuberculosis*, the etiological agent of tuberculosis (TB), has the ability to persist in its human host for decades or even throughout life (Neyrolles et al. 2006). At the other extreme, *Vibrio cholerae* produces such a potent endotoxin that it either kills its human host or is eliminated within 2–7 days (Finkelstein 1973). In addition to time spent in close contact with their hosts, pathogens share a number of characteristics with commensal and mutualistic symbionts. These pathogen versus symbiont relationships as part of the holobiont will be discussed in this chapter within the framework of the hologenome concept.

It is well known that pathogens comprise a very small fraction of the microbial world. It is less known that amongst the few bacterial species that contain pathogens, most strains are not pathogenic. In order to better appreciate the interesting parallelism between pathogenicity and symbiosis, we will discuss the circumstances that lead to pathogenicity and compare the properties of pathogens with closely related nonpathogenic strains. Since it is beyond the scope of this book to discuss pathogens comprehensively, we will present only a few representative types of infectious human (representative of the animal world) and plant bacterial pathogens (Table 9.1) and some important noncommunicable diseases in humans affected by commensal microbiota. Through these examples we wish to demonstrate the broad types of pathogens and also the similar traits they share with commensal symbionts.

Infectious Human Diseases

Tetanus: This is an example of an **accidental disease**. The causative agent of tetanus, the spore-forming, soil bacillus, *Clostridium tetani,* derives no benefit from the infection and has no properties characteristic of a symbiont. Spores of the

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Table 9.1 Bacterial pathogens of humans and plants

Disease	Pathogen	Mode of transmission	Reference
Human			
Tetanus	*Clostridium tetani*	Accidental	Brüggemann et al. (2003)
Tuberculosis	*Mycobacterium tuberculosis*	Aerosol droplets	Smith (2003), Cole et al. (1998), Dye and Williams (2010)
Urinary tract infection	Uropathogenic *Escherichia coli*	Fecal–urinary tract	Nicolle (2008), Hacker and Kaper (2000), Schmidt and Hensel (2004)
Ulcers	*Helicobacter pylori*	Fecal–oral and oral–oral	Blaser et al. (2008), Blaser (2008), Marshall and Warren (1983, 1984)
Cholera	*Vibrio cholerae*	Water initially, then fecal–oral spread	Nelson et al. (2009), Vezzulli et al. (2008), Abd et al. (2007), Lipp et al. (2002), Sánchez and Holmgren (2011)
Plants			
Frost damage	*Pseudomonas syringae*	Aerosals	Lindow (1987), Hirano and Upper (1990), Scholz-Schroeder et al. (2003), Dudnik and Dudler (2013)
Crown gall	*Agrobacterium tumefaciens*	From soil	Lee et al. (2009), Kumar et al. (2005), Flores-Mireles et al. (2012), Sauerwein and Wink (1993)

bacterium enter the body through open wounds and germinate once inside. They then produce a potent 150 kDa protein toxin, tetanospasmin (Brüggemann et al. 2003), that travels through the body via the nervous system and reaches the spinal cord. The toxin interferes with neurotransmitter synthesis and thus blocks messages between neuronal cells. This leads to unwanted muscle contractions and spasms, and often death. It is the only vaccine-preventable disease that is infectious, but not contagious, meaning it cannot be transferred in any way from person to person. The bacterium is unable to proliferate in host tissue or colonize a surface. The gene coding for tetanospasmin resides on a plasmid in *C. tetani*, and since the plasmid is nonconjugative, it cannot be transferred to other bacteria (Eisel et al. 1986). Tetanospasmin has no known function for *C. tetani* in the soil environment where they are normally encountered. The natural microbiota may play some role in protecting adult humans against food born *C. tetani* that may germinate in the gut. Infants, on the other hand, who have not yet developed a mature and active gut microbiota are highly susceptible to the *C. tetani* infection and the disease (infant botulism) and therefore are immunized against the toxin.

Tuberculosis: TB, an ancient disease caused by the aerobic bacillus *M. tuberculosis*, remains one of the deadliest infectious diseases of our time. A closely related bacterium, *Mycobacterium bovis*, is the causative agent of TB in cattle

(known as bovine TB) and many other mammals. *M. bovis* can jump the species barrier and cause TB in humans (Cosivi et al. 1998). The World Health Organization (WHO) estimates that one-third of the world's population is infected with *M. tuberculosis*, and two million people die each year from this malady (Vilchèze et al. 2013). Based on Assyrian clay tablets describing patients coughing blood, TB was already well entrenched in the seventh century BCE (Smith 2003). In modern times, most TB infections are initiated by the respiratory route of exposure, usually arising from dissemination of the bacilli from lungs of infected humans. During the primary stage of the disease, mycobacteria grow in the lungs. They are initially surrounded by lymphocytes and macrophages, and then subsequently enclosed by connective tissue that forms a firm structure termed tubercle. The mechanism by which virulent mycobacteria prevent phagosomes from maturating and killing them is unknown. At this stage, the disease is usually arrested, but the mycobacteria can remain alive in the tubercle for decades, maintaining a constant number by growing and dying at an equally slow rate. Although most humans who are infected with TB do not exhibit progression of the disease, sometimes the mycobacterial cells escape from the tubercle, usually as a result of weakening of the host cellular immune system, and spread to other sites where, if untreated, they often kill their human host.

The *M. tuberculosis* genome contains approximately 4,000 genes (Cole et al. 1998). Annotation of the genome shows that this bacterium has some unique features. Over 200 genes are annotated as encoding enzymes for the metabolism of fatty acids, comprising 6 % of the total. By comparison, a related actinomycete has a total of 115 enzymes involved in fatty acid metabolism, corresponding to a little more than 1 % of the proteins. This large number of *M. tuberculosis* enzymes that putatively use fatty acids may be related to the ability of this pathogen to grow in the tissues of the infected host, where fatty acids maybe the major carbon source.

Specially timed horizontal transmission of *M. tuberculosis* is a key to its evolutionary success. The pathogen is dormant and not contagious as long as the host remains healthy and has a strong immune system. However, when the host immune system suffers, as a result of aging, malnutrition, diabetes, tobacco use, alcohol abuse, or HIV/AIDS infection, the bacterium converts into an active pathogen (Dye and Williams 2010). When people with active TB cough, sneeze, speak, or spit, they expel infectious aerosol droplets. A single sneeze releases thousands of droplets, each one of which may transmit the disease, since the infectious dose of TB is very low (fewer than 10 bacteria). Family members and others in close contact with people with active TB are at particularly high risk of becoming infected. Thus, like other symbionts, *M. tuberculosis* can be transmitted to offspring. Interestingly, the major disease symptom, coughing, provides a mechanism for *M. tuberculosis* transmission.

Urinary tract infection: Most *Escherichia coli* strains are harmless and are part of the normal microbiota of the gut, and can benefit their hosts by producing vitamin K (Bentley and Meganathan 1982), and by preventing the establishment of pathogenic bacteria within the intestine (Leatham et al. 2009). However, some strains are pathogenic, especially when they colonize areas outside the gut. *E. coli* pathogens

are characterized by their ability to cross anatomical barriers, breach host defenses, and multiply in hostile environments, such as the urinary tract (Johnson 1991). These invasive properties are essential to the pathogen's survival in nature. Uropathogenic *Escherichia coli* (UPEC) are responsible for approximately 90 % of urinary tract infections (UTI). UPEC strains initiate infection by binding to bladder epithelial cells. In the majority of cases, this is achieved by type 1 pili. This adherence prevents the pathogen from being washed out by the urine flow. These pili adhesins specifically bind D-galactose-D-galactose moieties on the P-blood group antigen of erythrocytes and uroepithelial cells. UPEC produce alpha- and beta-hemolysins, which cause lysis of urinary tract cells. They also have the ability to form capsular polysaccharides that contribute to biofilm formation. Biofilm-producing *E. coli* are recalcitrant to immune factors and antibiotic therapy, and are often responsible for chronic urinary tract infections (Ehrlich et al. 2005).

What characterize the pathogenic strains of *E. coli* (and many other animal and plant pathogens) are large blocks of DNA (10–200 kb) that carry virulence genes, referred to as **pathogenicity islands** (PAI) (Hacker and Kaper 2000). In their review of the properties of PAI, Schmidt and Hensel (2004) point out:

1. PAI are present in the genomes of pathogenic bacteria but are absent from the genomes of a nonpathogenic representative of the same species or closely related species.
2. Virulence factors located within PAI include: (a) pili, fimbriae, and other proteins that allow pathogens to adhere to host cells, (b) toxins, (c) iron uptake systems, (d) secretion systems that are required for defense against, and interaction with, host cells during pathogenesis, and (e) proteins causing apoptosis.
3. PAI often differ from the core genome in their base composition and also show a different codon usage, suggesting they are acquired by horizontal gene transfer from different bacterial species.
4. PAI are frequently located adjacent to tRNA genes. tRNA genes serve as anchor points for insertion of foreign DNA that has been acquired by horizontal gene transfer because genes encoding tRNAs are highly conserved between various bacterial species. Thus, a DNA fragment that contains a tRNA gene can insert into the recipient's genome by recombination between the tRNA genes.
5. PAI are frequently associated with mobile genetic elements. They are often flanked by direct repeats of DNA sequences of 16–20 bp with a perfect or nearly perfect sequence repetition.
6. PAI are intrinsically unstable and delete with frequencies that are higher than the normal rate of mutation. The same genetic mechanisms that allow for the uptake of PAI by horizontal gene transfer also determine their genetic instability.
7. Deletions of PAI lead to nonpathogenic phenotypes.

Extensive sequencing of bacterial genomes has revealed that blocks of DNA with properties similar to PAI are also present in most nonpathogenic bacteria (Dhillon et al. 2013), where they are referred to as fitness islands (Dobrindt et al. 2004), or in the case of symbionts as **symbiosis islands** (MacLean et al. 2007). Symbiosis islands differ from PAI in that they lack genes for pathogen-associated "offensive" virulence proteins, such as toxins and host contact-dependent secretion mechanisms, which typically elicit a strong inflammatory response (Brown et al. 2006; Ho Sui et al. 2009). Symbiosis islands are highly relevant to the hologenome concept because they accelerate the rate at which environmental microbes can become commensal or mutualistic symbionts. For example, a symbiosis island present in a *Mesorhizobium loti* strain was found capable of transforming, by a single horizontal gene transfer event, nonsymbiotic strains of *M. loti* into symbiotic counterparts (Sullivan and Ronson 1998). Not surprisingly, some genes, such as those coding for iron uptake systems, are found in both pathogenicity and symbiosis islands, where they assist the microbe to multiply in iron-limited host environment (Anisimov et al. 2005).

Ulcers: The Nobel Prize in Physiology or Medicine in 2005 was awarded to Barry Marshall and Robin Warren "for their discovery of the bacterium *Helicobacter pylori* and its role in gastritis and peptic ulcer disease" (Marshall and Warren 1983, 1984). To prove that *H. pylori* was a causative agent of ulcers, Marshall drank a culture of *H. pylori*, developed the disease in 2 weeks, and re-isolated the pathogen from his stomach, thus fulfilling Koch's postulates. Finally, he cured himself of the disease with antibiotics. Today, peptic ulcers are treated with a combination of antibiotics, as well as additional medications to reduce stomach acid.

Subsequent research on *H. pylori* has shown that it is not only a common chronic infection in humans, linked to gastritis, gastric/duodenal ulcers, and gastric cancer, but also part of the beneficial microbiota of the human holobiont. There is evidence that *H. pylori* is protective against the development of conditions such as inflammatory bowel disease, childhood-onset asthma, allergy, esophageal reflux, and possibly obesity (Blaser et al. 2008). This example demonstrates that some bacteria belonging to host microbiota can exhibit both pathogenic and beneficial characteristics, depending upon specific strains and circumstances. An important property of *H. pylori* that allows it to be a symbiont in the harsh, acidic environment of the stomach is its ability to secrete urease, which converts urea into ammonia (Kusters et al. 2006). The production of ammonia around *H. pylori* neutralizes the acidity of the stomach, making it more hospitable for the bacterium. In addition, the helical shape of *H. pylori* allows it to burrow into the mucus layer, which is less acidic than the inside space, or lumen, of the stomach.

Most mammals contain species-specific *Helicobacter* in their stomachs. The human species, *H. pylori*, has been observed only in humans and other primates. In humans a high genetic variability was observed from unrelated people (Clover and Peek 2013), so much so that it was used as a means to determine human migration (Dominguez-Bello and Blaser 2011). Only strains of *Helicobacter* that contain the cagA gene on a 40 kb pathogenicity island (PI) are pathogenic (Censini et al.

1996), which suggests that the acquisition of this PI by HGT is an important event in the evolution of *H. pylori* virulence.

Clone libraries of 16S rRNA genes show that *H. pylori* is the dominant microbe colonizing the human stomach. *H. pylori* is acquired mostly in young childhood, primarily by close contact with parents and family members; it persists in the stomach essentially for life unless eradicated by antibiotics. However, *H. pylori* has been progressively disappearing over the course of the last 50 years in developed countries as a result of increased antibiotic exposure during childhood and improved hygienic conditions (Perez-Perez et al. 2002). Clearly, *H. pylori* is part of the human holobiont that evolved in the gastric niche. Its elimination has consequences that we still do not fully appreciate (Blaser 2008).

Cholera: The causative agent of cholera, the Gram-negative bacterium *V. cholerae,* is a facultative pathogen that has both human and environmental stages in its life cycle (Nelson et al. 2009). Vibrios spp, including *V. cholerae*, are natural inhabitants of the marine coastal environment throughout the world, and they are equipped with a battery of adaptive response mechanisms that allow them to persist in hostile environments, such as those with limited nutrients (Vezzulli et al. 2008). The main reservoir of *V. cholerae* is aquatic sources such as brackish water and estuaries, often in association with copepods or other zooplankton, shellfish, and aquatic plants. *V. cholerae* strains are also facultative intracellular bacteria (i.e., endosymbionts), able to survive and multiply symbiotically inside aquatic free-living amoeba (Abd et al. 2007). *V. cholerae* can be found in large numbers as commensal bacteria associated with copepods attached to chitin surfaces and in the gut. Although it has not been reported, *V. cholerae* may provide nutrients to their invertebrate hosts by degrading complex compounds, such as chitin. The copepod serves both as a natural reservoir and a vector for *V. cholerae* (Lipp et al. 2002).

Although more than 200 serogroups of *V. cholerae* have been identified, only serogroups O1 and O139 are associated with epidemic cholera. Both serogroups cause clinical disease by producing an enterotoxin that promotes the secretion of fluid and electrolytes into the lumen of the small intestine by a well-established mechanism (Sánchez and Holmgren 2011). The result is a large volume of fluid produced in the upper intestine that overwhelms the absorptive capacity of the lower bowel, causing severe diarrhea. Unless the lost fluid and electrolytes are replaced adequately, the infected person may develop shock from profound dehydration and acidosis from loss of bicarbonate. Interestingly, the gene encoding the cholera toxin is present on a prophage and was introduced into *V. cholerae* by horizontal gene transfer (Li et al. 2003).

The growth style of *V. cholerae* in its natural aquatic hosts contrasts sharply with that in humans. Attached to copepods, the bacterium grows slowly and is more resistant to high temperature, ultraviolet radiation, and predation than when living freely. As a commensal bacterium, it does not apparently cause any harm to its host. However, when a toxin-producing *V. cholerae* infects a human, its growth is explosive and it quickly damages the host and gets released as a result of the diarrhea. It can then either infect another human, via the contaminated drinking water, or find its way back to its natural aquatic host. It is interesting to consider

the possibility that the selection of the toxin and resulting explosive diarrhea enables the bacteria to escape the human host and return to its natural marine habitat. Its extreme pathogenicity to humans today correlates with a recent acquisition of the toxin gene by *V. cholera* and the short time the human immune system has had to adapt to this pathogen.

Cholera, like many infectious diseases, can spread, causing epidemics and pandemics (Faruque et al. 1998). We suggest that **epidemics of commensal and mutualistic microbes** may also occur periodically, but generally go unnoticed, though some examples are available. Apparently, a bacterium which prevents bleaching of corals by a specific pathogen spread to corals in the Eastern Mediterranean over a period of 3 years (Reshef et al. 2006; Mills et al. 2013). Another example of the acquisition of a beneficial bacterium is the uptake of *Burkholderia* from soil by stinkbugs (Kikuchi et al. 2012). These bacteria established a specific and beneficial symbiosis with stinkbugs that conferred resistance of the host insects against an insecticide. Symbiont-mediated insecticide resistance became established in the stinkbug population within a single insect generation and was rapidly transferred horizontally to other organisms. Thus, the ability to cause epidemics is another example of a similarity between pathogens and beneficial microbes.

Infectious Plant Diseases

Frost damage and lesion formation by *Pseudomonas syringae:* *P. syringae* is a Gram-negative bacterium with polar flagella. It is a plant pathogen that can infect a wide range of plant species, and exists as over 50 different pathovars (Hirano and Upper 1990). Some strains of *P. syringae* produce proteins which cause water to freeze at fairly high temperatures, resulting in injury to plants (Dudnik and Dudler 2013). For plants without antifreeze proteins, frost damage usually occurs between −4 and −12 °C as the water in plant tissue can remain in a supercooled liquid state. P. syringae can cause water to freeze at temperatures as high as −1.8 °C (Lindow 1987). The freezing causes injuries in the epithelia and makes the nutrients in the underlying plant tissues available to the bacteria.

P. syringae, typical of other members of the genus *Pseudomonas*, is characterized by a large genome (6.1–6.5 Mb) containing 14 genomic islands (Feil et al. 2005). These genomic islands contain numerous genes that provide information for the versatile lifestyle of *Pseudomonas,* including growth on a wide variety of carbon substrates in soil, plants, and animals. The opportunistic pathogen *P. syringae* can be found in association with healthy leaves, growing and surviving for many generations on the surfaces of leaves as an epiphyte. Although studied mainly as a pathogen, it is now clear that *P. syringae* has evolved to live in association with leaves, and incidentally, some strains can cause lesions on some plants (Hirano and Upper 2000). Owing to early availability of the genome sequence for *P. syringae* strains and the ability of selected strains to cause disease on well-characterized host plants including *Arabidopsis thaliana*, *Nicotiana*

benthamiana, tomato and wheat, *P. syringae* has come to represent an important model system for experimental characterization of the molecular dynamics of plant-opportunistic pathogen interactions (Dudnik and Dudler 2013).

One of the key questions regarding opportunism is: When and how does a commensal microbe become a pathogen? *P. syringae* has two ways to destroy its own habitat, frost injury, and lesion formation. Frost injury was described above; lesions may provide a place for the bacteria to survive during unfavorable weather conditions but little other advantage. As an example, population sizes of the bacteria are highly variable in lesions but may remain quite large, even after several weeks of dry weather, when population sizes associated with asymptomatic leaves decline markedly. There is no clear selection for destroying the leaf habitat. For most organisms, habitat destruction is regarded as highly unfavorable. Both of these events, lesion formation and frost injury, become highly likely only when the population sizes of the bacteria are relatively large, that is, when the bacteria have been particularly successful on or in their habitat. Is it not paradoxical that bacteria reward themselves for success by destroying their habitat? Hirano and Upper (2000) proposed a model in which frost damage and lesion formation are unfortunate accidents of overpopulation that benefit neither the bacteria nor the plant. Thus the actual survival mechanism of *P. syringae* is to live on healthy leaves.

Crown-gall disease: *Agrobacterium tumefaciens* is a Gram-negative soil bacterium in the family of Rhizobiaceae, which includes the nitrogen-fixing legume symbionts. Unlike the nitrogen-fixing symbionts, the crown-gall tumor producing *Agrobacterium* is pathogenic though it benefits the plant in some ways. There are a wide variety of plants affected by *Agrobacterium* that include walnuts, grape vines, stone fruits, nut trees, sugar beets, horse radish, and rhubarb. The extent of crown-gall disease depends largely on the physiological conditions of the host plants. When the plants are in a good state of health, tumors are limited and do not influence the viability of the hosts. However, affected plants may be stunted and sensitive to environmental stress. Severally infected plants may decline and die (Schroth et al. 1988).

The mode of infection by *A. tumefaciens* is interesting not only biologically but because it has provided the basis for genetic engineering of plants (Toki et al. 2006). The infectious cycle begins when bacteria in the soil sense chemical exudates from plants, such as sugars and acetosyringone, and accumulate around the roots. Crown galls develop upon transfer of a portion of the tumor-inducing (Ti) plasmid, the T-DNA, in a semi-random location into the genome of the plant (Lee et al. 2009). T-DNA transfer is initiated when *Agrobacterium* detects phenolic molecules released from actively growing cells in a plant wound. These phenolics induce expression of multiple virulence genes in the bacterial DNA, encoding products responsible for processing and transferring the single-stranded T-DNA across the bacterial membrane system into the plant cell, where it becomes integrated into the plant genome. Genes encoded by the T-DNA are expressed and subsequently alter plant hormone levels, leading to uncontrolled cell division and tumor formation.

Plants that are transformed by *Agrobacterium* produce and excrete an unusual class of low molecular weight secondary amines called **opines**. Opines are formed

by the condensation of an amino acid with a ketoacid or a sugar. The opines are a major source of carbon, nitrogen, sulfur, and energy for *Agrobacterium*, whereas most other bacteria cannot utilize opines. Accordingly, *Agrobacterium* strains that harbor the Ti plasmid and transform plants have a selective advantage over strains that cannot produce the plant tumors (Flores-Mireles et al. 2012). Interestingly, the excreted opines also benefit infected plants because opines inhibit growth of insect larva and prevent seed germination of potentially competing plants (Sauerwein and Wink 1993). Thus, *Agrobacterium* can be considered both a pathogen and a beneficial microbe.

Based on 16S rRNA gene analyses, *Agrobacterium* strains have been reclassified as members of the genus *Rhizobium* (Young et al. 2001). Interestingly, pathogenic *Agrobacterium* and beneficial nitrogen-fixing *Rhizobium* share a number of important characteristics. Both display chemotaxis and adhere to plant roots, exchange signals with the plant that leads to their endocytosis, and produce molecules that encourage tumor-like growth on plants. In addition, both benefit the plant by the nutrients resulting from the symbiosis: opines in the case of *Agrobacterium* and fixed nitrogen in the case of *Rhizobium.* This comparison highlights the similarity between beneficial and pathogenic symbionts in their interactions with hosts.

Viral Pathogens

Similar to bacterial pathogens, some viral pathogens are clearly symbionts in the sense that they can establish permanent infection and persist indefinitely within the host, whereas others are self-limiting, resulting in either rapid clearance of the pathogen or death of the host (Kane and Golovkina 2010). Examples of the former are herpes simplex virus, which persists through establishment of latency (Efstathiou and Preston 2005), and retroviruses which integrate into the host genome (Bushman et al. 2005). Flu viruses are typical of most viruses that reside in their hosts for only a few days or weeks (Hilleman 2002).

To understand the relationship of viral pathogens with the holobiont and its hologenome, and how it is incorporated within the hologenome concept, it is instructive to consider their evolutionary origins. Many human viral pathogens have their origin in animal hosts (zoonosis) where, similarly to bacteria, they appear to be adapted and cause minor or no disease signs. A well-studied example is the viruses that are the causative agents of AIDS (Sharp and Hahn 2001; Moss 2013). It appears highly likely that chimpanzees were the source of HIV-1 and HIV-2 (Huet et al. 1990), and that the viruses at some point, probably during the late nineteenth or early twentieth century (Korber et al. 2000), crossed species from chimpanzees to humans. A virus similar to the one isolated from chimpanzees was found to cause AIDS in patients in western Africa (Clavel et al. 1986). Soon thereafter, viruses, collectively termed simian immunodeficiency viruses (SIVs) were found in various different primates from sub-Saharan Africa, including African green monkeys, sooty mangabeys, mandrills, chimpanzees, and

others. These viruses appeared to be largely nonpathogenic in their natural hosts. These discoveries provide evidence that AIDS had emerged in humans as a consequence of cross-species infections with viruses from different primate species demonstrating again the narrow boundary between commensalism and pathogenicity where host species determine pathogenicity.

Experimental Evolution of a Bacterial Pathogen into a Nodulating Symbiont

Ralstonia solanacearum is a typical root-infecting pathogen of over 200 host plant species. It invades root tissues and heavily colonizes the vascular system, where excessive production of extracellular polysaccharides blocks water traffic, causing wilting (Genin and Boucher 2004). Rhizobia on the other hand are mutualistic endosymbiotic bacteria of major ecological importance that together with their legume hosts contribute ca. 25 % of global nitrogen fixation. Rhizobia induce the formation of root nodules on legumes that they colonize and multiply intracellularly (Batut et al. 2004). The rhizobia and legume host cooperate to fix nitrogen for the benefit of the plant holobiont. Rhizobia do not form a homogeneous taxon but are a phylogenetically dispersed group of bacteria (Masson-Boivin et al. 2009). The occurrence of rhizobia in several distant genera is thought to have originated from repeated and independent events of horizontal gene transfer of key symbiotic functions into nonsymbiotic bacterial genomes (Martinez-Romero 2009). Horizontal gene transfer alone, however, cannot solely account for the wide biodiversity of rhizobia, since only a few recipient bacteria, phylogenetically close to existing rhizobia, have turned into nitrogen-fixing legume symbionts (Sullivan and Ronson 1998).

Can one turn a pathogen into a symbiont by genetic manipulations? Marchetti et al. (2010) converted the pathogen *R. solanacearum* into a legume symbiont. This process required two steps. As the first step, they transferred a symbiotic plasmid into *R. solanacearum* that carried all the genes required for nitrogen fixation and the nodulation genes required to trigger the plant developmental program of nodule formation. Although the constructed strain contained all the genetic information for nitrogen fixation and nodule formation, the strain was still unable to nodulate the legume host and retained the pathogenic properties of *R. solanacearum*. Apparently, the symbiotic potential could not be expressed.

To isolate strains with symbiotic potential, the researchers introduced a second step that took advantage of specific traits of rhizobium–legume symbioses: Legume plants act as a trap by selecting rare, nodulation-proficient mutants from a population of bacteria (Long et al. 1982). From several hundred plants that were inoculated with transformed *R. solanacearum*, they obtained three strains that caused nodulation in the legume. The genomes of the nodulating *R. solanacearum* strains were then sequenced and compared to the nonnodulating strains. Two types of adaptive mutations in the virulence pathway of *R. solanacearum* were identified that were crucial together for the transition from pathogenicity toward mutualism:

inactivation of a type III secretion structural gene allowed nodulation and early infection to take place, whereas inactivation of the master virulence regulator hrpG allowed intracellular infection of nodule cells. Thus, inactivation of two regulatory genes allowed the transition from legume pathogenesis to symbiosis. The authors (Marchetti et al. 2010) suggest: "natural selection of adaptive changes in the legume environment following horizontal gene transfer has been a major driving force in rhizobia evolution and diversification and show the potential of experimental evolution to decipher the mechanisms leading to symbiosis."

Rabbit Myxomatosis: A Lesson in the Evolution of Host–Parasite Relationships

Domestic rabbits of European origin were introduced into Australia by European settlers. The rabbits proliferated unchecked by any natural predators, reaching 600 million by 1950, and competing with sheep for pasture lands, causing great economic losses to Australian sheepherders and farmers. To overcome the problem, the Commonwealth Scientific and Industrial Organization (CSIRO) introduced into Australia the myxoma virus, a member of the pox family of DNA viruses. The virus naturally infects wild hares that are native to North and South America, causing a very mild skin reaction. The disease is propagated by mosquitoes and fleas biting an infected hare in the region of the skin lesion and then transmitting the virus to the next hare they bite. In European rabbits (the ones present in Australia), the same virus causes the lethal disease myxomatosis.

The introduction of myxoma virus into the European rabbit population in Australia in 1950 initiated the best-known example of what happens when a novel pathogen jumps into a completely naïve host. The strain of virus originally released in Australia killed 99.8 % of infected rabbits. The relatively short generation time of the rabbit and their vast numbers in Australia meant evolution could be studied in real time. The carefully documented emergence of attenuated strains of virus that were more effectively transmitted by the mosquito vector and the subsequent selection of rabbits with genetic resistance to myxomatosis is the paradigm for pathogen virulence and host-pathogen coevolution (Kerr 2012). Through natural selection, myxoma virus initially became more attenuated (less virulent) since the more attenuated viruses were more effectively transmitted. This attenuation allowed some infected rabbits to survive, which in turn led to natural selection for resistance to myxomatosis in the wild rabbit population. This ongoing coevolution of myxoma virus and rabbits means that today many wild rabbits are quite resistant and survive the attenuated myxomatosis.

Microbiota and Noncommunicable Diseases in Humans

The human resident or commensal microbes are involved not only in contributing to health and fitness of the holobiont as described in detail in Chap. 5, but are also implicated in playing a role in a number of human chronic noncommunicable diseases. The main diseases that have been associated with the human resident microbiota include inflammatory bowel diseases, colon cancer, cardiovascular diseases, and diabetes mellitus. The effect of the microbiota in these diseases is manifested in some cases directly on the tissue and in other cases indirectly, e.g., via the blood. Interestingly, and in accord with the "hygiene hypothesis" (Okada et al. 2010), the incidence of these diseases has risen in the last 40 years in all parts of the world that moved from a traditional to a modern way of life. This kind of involvement of the microbiota reflects the ambivalence of the interaction with the human host. On one hand, the microbiota is kept at bay by the immune system in spite of being an integral part of the holobiont. On the other hand, in essence the human microbiota contributes to the health and survival of the human holobiont.

Inflammatory bowel diseases (IBD): IBD include ulcerative colitis (UC) and Crohn's disease (CD). Both are characterized by chronic relapsing inflammation: UC in the colon alone and CD in the entire alimentary tract in addition to extra gut symptoms. These diseases are believed to result from the interaction between the host genes and immune system, the microbiota, and environmental factors. Of the human genes known to contribute to IBD (close to 100), many are involved in the interaction with the microbiota such as NOD2 and those of the IL-23/Th-17 pathway (Khor et al. 2011; Cheon 2013). Additional contributors to IBD are the combination of immune response disregulation (expressed as relapsing inflammation in the presence of resident gut microbiota) together with microbial dysbiosis. The dysbiosis is characterized mainly by reduction in microbial diversity of resident bacteria and increase in Enterobacteriaceae and other species with low abundance in the healthy human holobiont (Shanahan 2012; Yu and Huang 2013).

Colon cancer: Most cases of colon cancer are sporadic and there are wide geographic variations in incidence of this disease pointing to environment, especially the diet, as being the dominant effecter (Doll and Peto 1981). There are a number of observations that point at the microbiota as being an additional causative agent of colon cancer (Sobhani et al. 2013): (1) The bacterial density in the colon is $\sim 10^5$ times higher than in the small intestine and in parallel the risk of cancer is 12-fold greater in the colon. (2) The phylogenetic core of microbiota is significantly different in the stools of individuals with colon cancer as compared to healthy individuals (Marchesi et al. 2011). (3) Certain types of bacteria found adhering to colon adenomas, (e.g., *Fusobacterium sp*.) are implicated as players in colon cancer development (McCoy et al. 2013). (4) Certain bacteria from the resident microbiota (e.g., *Bacteroides fragilis*) can cause tumor formation in germ-free mice. The mechanisms suggested include bacterial-induced DNA changes, surface changes induced by microbial binding, cytokine induction, and carcinogen production.

The human microbiota is also implicated in other types of cancers such as liver, bladder, prostate, and breast, but evidence in these cancer diseases is sparse and the connection evidently is not direct.

Cardiovascular diseases (CVD): Since the nineteenth century, infections were considered one of the causes of atherosclerosis, the pathological phenomenon at the basis of all CVD. About 25 years ago, a possible connection between oral microbiota and CVD was suggested when positive correlations between periodontal diseases and CVD emerged (Mattila et al. 1989). This hypothesis was reinforced by the finding of bacterial DNA, primarily from oral microbiota (*Chryseomonas*, *Veillonella* and *Streptococcus*), but also gut bacteria, in atherosclerotic plaques (Koren et al. 2011). It was suggested that the bacterial burden in the plaque is correlated with the level of inflammation occurring in the atherosclerotic plaque. Another possible mechanism connecting CVD and microbiota is trimethylamine-N-oxide—a microbial atherogenic breakdown product of the nutrients choline and L-carnitine, which is also implicated in fatty liver disease (Howitt and Garrett 2012; Koeth et al. 2013). L-carnitine is abundant in red meat and it was suggested that its atherogenic break down product may be also the connection between red meat consumption and CVD. Other mechanisms suggested being involved in the positive association between microbiota and CVD include induction of obesity, inflammation, and dyslipidemia (Caesar et al. 2010; Erridge 2011).

Diabetes mellitus: Both type 1 (T1D, once termed childhood) and type 2 (T2D, once termed adult) diabetes are correlated with the microbiota, and their incidence has been growing in recent decades. T1D, usually a childhood disease, is an autoimmune disease resulting in destruction of the insulin secreting pancreatic Langerhans islets beta cells. The factors triggering T1D have not been identified, but human data and animal models indicate that genetic and environmental factors, especially viruses and bacteria, contribute to the disease. Among the viruses implicated in T1D are cytomegalovirus, coxsackie B, rubella, mumps, Epstein–Barr, and rotavirus. Regarding bacteria, human studies, though scarce, have shown reduced Firmicutes and increased Bacteroidetes in gut microbiota in addition to changes of species within the phyla in children progressing to T1D. Animal models indicate that the resident microbiota is involved in the initial stages of T1D development via defective cross talk with the innate immune system (Naoko et al. 2013).

Genetics and environment are also involved in the development of T2D but in different ways. T2D is a slowly developing disease, mainly in adults, and is correlated with the metabolic syndrome that includes obesity, insulin resistance, and the metabolic manifestations of CVD such as dyslipidemia, hypertension, and low-grade inflammation. The gut microbiota is implicated in contributing to the T2D and other related disorders via mechanisms involving gut barrier dysfunctions. A faulty gut barrier enables the endotoxic pro-inflammatory lipopolysaccharides (LPS) from the cell wall of gram-negative bacteria in the gut to reach the blood and contribute to the development of low-grade inflammation (Everard and Cani 2013). LPS has been shown by these authors and others, in mice and humans, to be correlated with the development of T2D. Bacteremia has also (potentially) been implicated in this process (Amar et al. 2011).

Evolutionary Considerations on the Role of Pathogens in Holobionts

Pathogens, which by definition harm their hosts, appear to be in contradiction to the hologenome concept of cooperation in holobionts. Although only a very small minority of the vast numbers of viruses and species of bacteria that colonize animals and plants cause disease, it is still incumbent on us to attempt to explain the emergence of these pathogens. Microbial (in addition to viral) pathogens vary greatly in many respects, including mechanisms of infection, extent to which they damage their host, modes of transmission and if they are naturally part of the resident microbiota in the affected holobiont. Nevertheless, there are a few general principles that may help to explain the evolution of pathogens.

Pathogens were acquired from other host species in the not-so-distant past and have not yet reached equilibrium: It is generally accepted that as the relationship between a parasite and its host matures, natural selection in either the parasite or host population or both will lead to the extinction of one or the other species or the evolution of commensalism or mutualism. Eventually, or as it was once referred to, on "equilibrium day" (Levin et al. 2000), mutualism will prevail and the immune over response will be tempered. The above discussion of rabbit viral myxomatosis in Australia is a well-documented demonstration of this phenomenon.

The most important infectious diseases of modern humans were acquired from other animals only within the past 11,000 years, following the rise of agriculture (Diamond 2002). Because these diseases are horizontally transmitted they can only be sustained in large dense human populations that did not exist anywhere in the world before agriculture. Some of these diseases are continuously acquired from animals, such as plague (rats via fleas), tuberculosis (cattle), Legionnaires' disease (amoebae), anthrax (herbivorous mammals), brucellosis (cattle), tularemia (rabbits), Rocky Mountain spotted fever (ticks), and cholera (marine invertebrates). The bacteria responsible for some of these infections, such as *M. tuberculosis*, are transmitted between humans and can be maintained without the animal source, the disease is thus termed contagious. Others such as *Legionella pneumophila* are not. As long as the transfer to new hosts requires viable hosts, selection will favor bacteria or viruses that are not only infectiously transmitted but also persist longer in colonized hosts without killing the host. Selection will favor ever-more-benign, symbiotic bacteria or viruses. Evolution in the host population will also be toward reduced microbial/viral virulence (Levin 1996).

Accidental pathogenesis: There are several examples of microbially induced diseases in which the pathogen gains no advantage in harming the host. Virulence is simply a consequence of the bacteria being in the wrong host or in a wrong site in the right host (Levin and Svanborg 1990). The bacterial products responsible for the morbidity and/or mortality of the host, virulence determinants, evolved in response to selection for some function other than virulence. A clear example of this is tetanus, where the pathogen, *C. tetani*, is a soil bacterium that derives no

benefit from the infection and has no properties characteristic of a symbiont. The bacterium is neither able to proliferate in host tissue nor to be transferred from person to person.

Many important pathogens have humans as their normal ecological niche where healthy carriage dominates over disease (Henriques-Normark and Normark 2010). These diseases may have originated early in human evolution and have equilibrated partially with the human holobiont. The ability of human **commensal pathogens**, such as *Streptococcus pneumoniae*, *Staphylococcus aureus*, *Haemophilus influenzae*, *Neisseria meningitides* and *H. pylori* to cause disease depends on a series of microbial factors as well as of genetic and environmental factors in the human host affecting the clearing capacity mediated by the innate and adaptive immune system. This delicate interplay between microbe and host affects not only the likelihood for a commensal pathogen to cause disease, but also disease type and severity. Similar arguments are relevant to some plant commensal pathogens, such as *Erwinia carotovora*. Several of the noncommunicable diseases in humans described above are due, at least in part, to commensal pathogens.

A good example is ulcers where the causative agent, *H. pylori*, colonizes and maintains populations in the stomachs of the majority of humans for most of their lives without generating symptoms. *H. pylori* colonization can also result in a chronic inflammatory state that is generated when the host response fails to clear the bacteria and lymphoid aggregates form in the lamina propria of the stomach and duodenum. This continued stimulation of the immune and inflammatory cells results in the destruction of the gastric epithelium, formation of peptic ulcers, and increased risk for gastric cancers (Moss and Blaser 2005). The inflammatory response and the subsequent diseases are caused only by strains that contain the cagA gene on a pathogenicity island (Censini et al. 1996), and provide no advantage to *H. pylori* in a colonized host.

Selection for virulence determinants: Some pathogens appear to remain virulent in spite of the general argument that natural selection favors a decrease in pathogen virulence and an increase in host resistance. A counterargument is that selection will favor whatever level of virulence maximizes the rate of increase and transmission of the pathogen, thus leading to increased virulence. For example, the *V. cholerae* enterotoxin virulence protein damages its human host by causing a large volume of fluid to be produced in the intestine, resulting in severe diarrhea and release of *V. cholera*. The pathogen can then either infect another human, via the contaminated drinking water, or find its way back to its natural aquatic host. Infectious pathogens must be transmitted horizontally, whereas resident microbiotas are transmitted from parent to offspring, as described in Chap. 4.

Lenski and May (1996) suggested that the optimum level of virulence will be a compromise between the short-term interests of the pathogen and its effect on host mortality, with selection often favoring an intermediate degree of virulence. As a consequence of successive adaptations by the pathogen to increased virulence, the density of susceptible hosts is reduced, thereby altering the balance between selective forces so as to favor reduced virulence. However, the evolutionarily stable strategy that is achieved is bounded away from complete avirulence. Models

in which intermediate virulence is favored do not necessarily contradict the conventional wisdom that evolution favors reduced virulence in the long run; in fact, these models provide a simple mechanistic explanation for the development of reduced virulence.

Selection for genome size: As discussed above, acquisition of genomic islands is a common and important aspect in the evolution of both beneficial and pathogenic microbial symbionts. On the other hand, genome reduction is also frequently found in symbiotic bacteria that live in close association with their hosts (Ochman 2005). Generally, obligate bacterial endosymbionts and intracellular pathogens have smaller genomes than their free-living ancestors, probably because the hosts provide the bacteria with a constant supply of nutrients, thereby rendering unnecessary many genes that were previously needed by free-living bacteria. The evolution experiments of Nilsson et al. (2005) using the pathogenic bacterium *Salmonella enterica* show that deletions occur frequently, so their impact on evolution is likely to be enormous. The rapidity of these large-scale changes in genome content in laboratory experiments substantiates the view that some gene loss can occur quickly in bacterial lineages that adopt to intracellular pathogenic or symbiotic lifestyles.

Key Points

- Only a small fraction of microbial species contain pathogens, and amongst these rare species only a few strains are known to cause illness.
- Pathogens vary greatly with respect to host specificity, mode of transmission, mechanism of infection, and type and extent of damage they do to their host. Some pathogens spend a long time within the holobiont with no deleterious effect, behaving as part of the resident microbiota.
- Bacterial pathogens contain PAI, blocks of DNA that carry virulence genes, such as toxins. Many beneficial bacteria contain symbiosis islands which are similar to PAI but lack virulence genes.
- The ability of commensal microbes to cause disease depends on microbial factors as well as on host factors, especially on the innate and adaptive immune system. This delicate interplay between microbe and host affects not only the likelihood of a commensal pathogen to cause disease, but affects also the disease type and disease severity, including the cases of chronic noncommunicable diseases, such as inflammatory bowel diseases and cardiovascular diseases.
- Pathogens tend to evolve reduced virulence to their hosts; this is a result of the probability that more virulent pathogens are more likely to drive their hosts, and themselves, to extinction. Since many human pathogens were acquired from other host species in the not-so-distant past, they may have not yet reached equilibrium.

- From an evolutionary point of view the differences between commensal residents and pathogens are not great. Their interactions with hosts are best described as a range of possibilities from considerable benefit to extreme pathogenicity. Thus, it seems that eukaryotes will continue to do what they did from the start, namely adapt to the ever-evolving microorganisms, and continuously face novel microbes that can cause illness or provide benefits. Evolution generally selects for an equilibrium that will maximize the survival of the holobiont.

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Chapter 10
Prebiotics, Probiotics, Synbiotics, and Phage Therapy

> *A reader who has little knowledge of such matters may be surprised by my recommendation to absorb large quantities of microbes as a general belief is that microbes are harmful. This belief is erroneous. There are many useful microbes amongst which the lactic bacilli have an honorable place.*
>
> —Metchnikoff (1907)

Introduction

As will be described in this chapter, prebiotics, probiotics, synbiotics, and phage therapy can be considered applied aspects of the hologenome concept. While the popularity of probiotics has surged in recent years, the idea that living organisms in food can be salutatory is not a new concept. The Old Testament (Genesis 18:8) states that "Abraham owed his longevity to the consumption of sour milk" and In 76 BC the Roman historian Plinius advocated fermented milk products for the treatment of gastroenteritis (Bottazzi 1983). Modern interest in the natural microbiota and claims of its advantage to human health has paralleled the development of microbiological research since the last half of the nineteenth century. Henry Tissier and the Nobel laureate Elie Metchnikoff are considered the first to suggest that it is possible to modify the gut microbiota and to replace harmful microbes with useful microbes (Tissier 1906; Metchnikoff 1907). Tissier recommended the administration of *Bifidobacteria* to infants suffering from diarrhea, claiming that *Bifidobacteria* were predominant in the gut microbiota of breast-fed infants (Tissier 1906). Evidence for the beneficial effects of intestinal microbiota came from studies in the 1950s showing that animals treated with antibiotics were much more sensitive to infection with pathogens (Bohnhoff et al. 1954; Freter 1955). The first definition of probiotics as "substances secreted by one microorganism which stimulate the growth of another," was introduced by Lilly and Stillwell (1965). As will be discussed later in this chapter, this definition is far from the one currently accepted.

During the last two decades, the development of molecular tools for analyzing microbial communities without having to culture the microorganisms has inspired numerous studies dealing with the human microbiota. This has brought together many disciplines concerned with health and disease of humans, animals, and plants, including prebiotic, probiotic, and synbiotic research. The latter three fields

E. Rosenberg and I. Zilber-Rosenberg, *The Hologenome Concept: Human, Animal and Plant Microbiota*, DOI: 10.1007/978-3-319-04241-1_10,

have remained applied, essentially testing different formulations for their health benefits. The goal of this chapter is to show how consideration of the complex interactions between the species that comprise a holobiont can stimulate new and interesting research on prebiotics, probiotics, and synbiotics. As an example of how probiotics has gained recognition in the scientific community in the last few years, only five-indexed publications with "probiotic" as topic were published in 1990, while more than 1,500 publications were recorded in 2012 (NIH PubMed database).

Prebiotics: Variation of Holobionts by Amplification of Indigenous Bacteria

The classical definition of prebiotics was given by Gibson and Roberfroid (1995) who defined prebiotics as "non-digestible food components, usually oligosaccharides, which evade digestion by mammalian enzymes, in the upper regions of the gastrointestinal tract, reach the colon in an intact state and are metabolized by the beneficial members of the indigenous microbiota." According to the International Scientific Association for Probiotics and Prebiotics "a dietary prebiotic is a selectively fermented carbohydrate ingredient that results in specific changes, in the composition and/or activity of the gastrointestinal microbiota, thus conferring benefit(s) upon host health (Roberfroid et al. 2010)." The implication of this definition is that prebiotics in the form of functional carbohydrates change the gut microbiota and its metabolic activity similarly to natural fiber present in the normal human diet, except that prebiotics are targeted at specific bacteria and are degraded in a specific form and therefore are more controlled. The most extensively studied prebiotics are inulins (fructans), fructo-oligosaccharides (FOS), and galacto-oligosaccharides (GOS), all of which are indigestible in the stomach and the small intestine and are anaerobically fermented by bacteria in the colon (Mitsuoak et al. 1987; Pachikian et al. 2011). Both fiber and prebiotics have been shown to benefit the human body and metabolism as a whole and the gastrointestinal tract in particular, principally by increasing the concentration of short-chain fatty acids (Pan et al. 2009). Short-chain fatty acids, mainly acetate, propionate, and butyrate, stimulate colonic blood flow and fluid and electrolyte uptake. Butyrate is a preferred substrate for colonocytes and appears to promote a normal phenotype in these cells while propionate affects the lipid metabolism in the liver and may play a role in liver health. Other important health benefits to the gastrointestinal tract related to fiber and prebiotics are improving or stabilizing microbiota, improving intestinal function, modulating gastrointestinal excretions, energy metabolism and satiety, modulating intestinal immune functions, and reducing the risk of intestinal infection (Roberfroid et al. 2010). Additional postulated general health benefits include enhanced resistance to invading pathogens (Bosscher et al. 2006), anti-colon cancer properties (Liong 2008), lipid lowering

action (Ooi and Liong 2010), improved calcium bioavailability and osteoporosis management (Cashman 2003), alleviation of menopausal symptoms (Smejkal et al. 2003), improvement of vitamin supply, and possibly protection against type 2 diabetes (Roberfroid 2000).

During the last few years, there have been numerous studies demonstrating that change in the diet results in change in the gut microbiota (Turnbaugh et al. 2009; De Filippo et al. 2010; Claesson et al. 2012). This is precisely the concept of variation in holobionts by amplification. Certain bacterial species are amplified, whereas others decrease in number depending on their metabolic potential. This leads to a change in the hologenome, which can either be beneficial, neutral, or disadvantageous to the holobiont (Whelan 2011; Tzounis et al. 2011; Zimmermann et al. 2010).

The classical definition of prebiotics, including carbohydrates alone, seems today to be inadequate since any material reaching the human gastrointestinal tract can change the microbiota, its own metabolic fate and its effects on the body. A good example is flavanols. These compounds are found in cocoa and chocolate and a wide variety of foods and beverages, including cranberries, apples, peanuts, onions, tea, and red wine, and can serve as prebiotics. In a randomized, controlled, double-blind, crossover intervention study with healthy humans, it was shown that cocoa-derived flavanols significantly increased the populations of beneficial bacteria (*Bifidobacteria* and *Lactobacillus*), while decreasing the number of pathogenic *Clostridium* (Tzounis et al. 2011). These microbial changes were paralleled by significant reductions in plasma triglyceride concentration and proteins associated with inflammatory bowel disease. Since these effects were not dependent on the dose of polyphenol present in the diets, other compounds present in cocoa raw material used for the diets could be key factors in this effect (Massot-Cladera et al. 2013).

A recent review discusses commercially available prebiotics, trends in prebiotic production from novel food sources and food industrial wastes, and future perspectives (Patel and Goya 2012).

Probiotics: Variation of Holobionts by Ingestion of Bacteria

As discussed in Chaps. 6 and 8, animals are constantly being exposed to microorganisms (sometimes also novel microorganisms) in the food they eat, the water they drink, and the air they breathe. A small group of these microbes are pathogens. For over 100 years, infectious disease has been a major area of microbiological research, including modes of infection, virulence, and transmission. However, to the best of our knowledge, natural epidemics of beneficial bacteria have not been considered. Although not easy to detect, it is reasonable to assume that such beneficial epidemics do take place and contribute to the evolution of holobionts. We presented the "coral probiotic hypothesis" to explain how certain

corals became resistant to a specific pathogen, *Vibrio shiloi*, even though corals do not produce antibodies (Reshef et al. 2006). It was shown that the natural population of corals acquired a bacterium that lyses *V. shiloi* and prevents the infection (Mills et al. 2013).

Probiotics has been defined in different ways since the term was initially proposed in 1965. The following general accepted definition of probiotics appears to capture our current knowledge of the subject: "Live microorganisms which, when administered in adequate amounts, confer a health benefit on the host" (Hoffman et al. 2008). Unlike variation of holobionts by acquisition of microbes from the environment, probiotic technology involves the nonrandom introduction of specific bacteria to improve the health of the host. The effects of probiotics can even be transferred to the next generation as was shown in a study where pregnant women treated with *Lactobacillus rhamnosus* and *Bifidobacterium lactis* had reduced frequency of gestational diabetes mellitus and diminished risk of larger birth size in affected cases (Luoto et al. 2010). In view of the fact that large birth size is a risk factor for later obesity, the results are of significance for public health in demonstrating that this risk is not only modifiable by probiotic treatment but can also be transferred from one generation to the next.

Probiotics have been effective in treating recurrent diarrhea caused by the pathogen *Clostridium difficile*. In initial human trials, patients who had not responded to antibiotic treatment were successfully treated with a nasogastrically administered liquefied blend of feces obtained from healthy related donors (MacConnachie et al. 2009). The assumption is that the feces contained one or more bacterial strains that reduced the concentration of *C. difficile* in the recipient's gut, but it is also possible that bacteriophages or other materials in the crude stool preparation were responsible for the cure. In a similar clinical trial, in a cohort of 159 individuals, the overall reported success rate for restoring normal homeostasis to the gut microbiota was 91 % (Khoruts and Sadowsky 2011). In a patient who was infused with her husband's microbiota through a colonoscope, there was a rapid and prolonged change in the bacterial composition of her gut microbiota, with Bacteroides spp. becoming dominant, *C. difficile* disappearing, and defecation frequency returning to normal (Khoruts et al. 2010). In a review article, summing up data from more than 300 patients it was concluded that fecal transplants can cure 92 % of people with recurring *C. difficile* infections for which antibiotics proved ineffective (Gough et al. 2011). Moreover, a controlled clinical trial recently published in the New England Journal of Medicine led to the conclusion that fecal transplant should be considered for first-line therapy among patients with recurrent diarrhea caused by *C. difficile* (van Nood et al. 2013). Standard probiotics (*Bifidobacterium*, *Lactobacillus*, *Saccharomyces*, or *Streptococcus* species) were also effective in treating *C. difficile*-associated diarrhea (Johnston et al. 2012; Ki et al. 2012). Regarding *C. difficile*, an interesting positive association was observed between obesity and *C. difficile*-associated diarrhea (Leung et al. 2013). Thus, it would be interesting to examine if *C. difficile*-associated diarrhea patients treated with fecal transplants or standard probiotics—subsequently lost weight.

It is important to note that in spite of accumulating data on the positive effects and many uses of probiotics, it has been realized that not all situations and diseases can be treated the same way and that different species, strains, and substrains should be used on different occasions, as demonstrated in the review on probiotics and inflammatory bowel disease and irritable bowel syndrome (Whelan and Quigley 2013). It is thus reasonable to assume that in the near future probiotics will employ more and more isolated microorganisms rather than crude mixtures to treat specific diseases, such as obesity (Gøbel et al. 2012), diabetes (Panwar et al. 2013), coronary heart disease (Saini et al. 2010), psoriasis (Groeger et al. 2013), and behavioral diseases (Cryan and O'Mahoney 2011). Going one step further we speculate that, since probiotics can be transmitted to future generations, they could, in principle, be used to treat genetic diseases, such as galactosaemia (Fensom et al. 1974), phenylketonuria (Enns et al. 2010), and celiac (van der Windt et al. 2010) diseases.

Probiotics has also been used in agriculture to combat diseases and improve yields and achieve specific characteristics in plants (Picard et al. 2008; Berlec 2012) and animals (Fuller 1989). Antifungal and chitinolytic *Bacillus circulans* and *Serratia marcescens* were effective in preventing leaf spot disease in peanut plants in greenhouse and field experiments (Kishore et al. 2005). When chitin (as prebiotic) was included in the administration of these probiotics to plants, there was a further improvement in the biological control of the disease. Chitinases produced by *B. circulans* and *S. marcescens* attack fungal and insect pathogens (Nagpure et al. 2013). Probiotics (*Enterococcus faecium* and *Saccharomyces cerevisiae*) raised the rumen pH of dairy cows suffering from subacute ruminal acidosis from 4.4 to 5.0 (Chiquette 2009). It was shown that the effectiveness of probiotics depends on the acidosis type. Certain bacterial probiotics were useful in treating butyric and propionic acidosis, but were ineffective in lactic acidosis of rumen (Lettat et al. 2012). It was suggested that administration of microbial mixtures of beneficial microbes, rather than individual species, should be considered in future agricultural applications (Chaucheyras-Durand and Durand 2010).

What are the mechanisms postulated to explain the effects of probiotics? In addition to modulating local and whole body metabolism by short-chain fatty acids mentioned above, the positive effects of probiotics, and to some extent also prebiotics, on human health have frequently been attributed also to their indirect and direct immunomodulating capacity (Klaenhammer et al. 2012). Probiotics may elicit immunomodulatory effects through direct interactions with the intestinal epithelium, especially in the small intestine, which is less densely populated by the commensal microbiota compared to the colon (Zoetendal et al. 2012). On the other hand, probiotic immunomodulatory effects in the densely populated colon are more likely to occur via modulation of the endogenous microbiota (Reid et al. 2011). Probiotics may also have indirect immunomodulatory functions through their actions on nonimmune cells such as epithelial cells, and may even exert their effect independently of the immune system by inhibiting the colonization of the intestinal mucosa by pathogenic microorganisms and/or by inducing the release of

antimicrobial peptides. Interestingly and of considerable practical significance is the fact that completely different effects may be observed depending on the species and the strain of the microorganism used. Some strains have a pro-inflammatory effect, whereas others are more anti-inflammatory (Foligne et al. 2007).

Synbiotics

Synbiotics refers to nutritional supplements combining prebiotics and probiotics in a synergistic manner, hence synbiotics. The goal of the prebiotic addition is to alter the nutritional environment in order to increase the probability that the probiotic will colonize the recipient, compared to the use of the probiotic alone. The use of synbiotics is powerful because it combines the hologenome concepts of variation by acquisition of bacteria (probiotics) and amplification (prebiotics).

Manipulation of the microbiota of healthy adults: A pioneering study on synbiotics was performed by Tanaka et al. (1983). A combination of the prebiotic galacto-oligosaccharides (GOS) and the probiotic *Bifidobacterium breve* was administered to 16 healthy adult men. The selective fermentation of GOS by bifidobacteria and the ability of a specific strain of *B. breve* to utilize it were established in vitro, prior to the in vivo study. The synbiotic treatment resulted in an increase in commensal bifidobacterial levels, whereas the probiotic alone did not mediate the same effect. Although this was a very early study and gut microbiota changes were evaluated using only culture techniques, the selection of a probiotic and its complementary prebiotic, as well as the inclusion of the proper controls, allows us to draw conclusions about the mechanism of synbiotic efficacy.

More recently, several controlled clinical trials using healthy volunteers have shown that synbiotics are effective in establishing and maintaining beneficial bacteria in the colon. For example, Casiraghi et al. (2007) investigated the effect of a synbiotic milk product containing the probiotics *Lactobacillus acidophilus* and *Bifidobacterium lactis* and the prebiotic inulin on 26 healthy adults, and Ouwehand et al. (2009) studied the effect of *Lactobacillus acidophilus* and Lactitol on 51 elderly people. In both cases, significant increases in probiotic bacteria were observed in the digestive tract following synbiotic ingestion. After the intervention, stool frequency was higher in the synbiotic group than in the placebo group. These results put together demonstrate the significant effect of synbiotics on gut microbiota and improvement of some markers of intestinal mucosal functions, refuting past claims that probiotics cannot actually change natural microbiota.

Treatment and prevention of disease: Synbiotics has shown promising results in altering the microbiota and reducing disease symptoms for inflammatory bowel disease (Dughera et al. 2007; Whorwell et al. 2006), methicillin-resistant enteritis (Kanamori et al. 2003), ulcers (Gotteland et al. 2005), atopic dermatitis in children (Farid et al. 2011), and constipation (Waltzberg et al. 2013). Synbiotics also reduced the risk factors and progression of colon cancer in animals (Le Leu et al. 2005, 2009) and humans (Rafter et al. 2007). In the animal studies, it was

demonstrated that the synbiotics reduced exposure to genotoxins, which correlated with tumor incidence in these animals (Kolida and Gibson 2011).

Ingestion of synbiotics in patients prior to and after operations has significantly reduced postoperative infections (Rayes et al. 2007) by maintaining and repairing the gut microbiota and gut environment. In the critically ill, such as patients with major abdominal surgery, trauma, and in the ICU, synbiotic therapy has been shown to significantly reduce septic complications (Shimizu et al. 2013). In a small clinical trial, synbiotics were shown to be helpful in reducing structural damage to the intestinal mucosa in chronic HIV patients (Schunter et al. 2012).

Recently it has been shown in human clinical trials and in vitro that a combination of the yeast *S. cerevisiae* and its fermentation products, referred to as EpiCor, have immunomodulation properties (Possemiers et al. 2013). These include gradually changing the gut microbial community structure, reducing potential pathogens, quantitatively increasing lactobacilli, and qualitatively modulating bifidobacteria. Thus, EpiCor can be considered a synbiotic where the probiotic is *S. cerevisiae* and the undefined dried fermentation products are the prebiotics. These kinds of data demonstrate the importance of using specific probiotics and prebiotics for specific purposes.

Improved molecular techniques for analysis of the gut microbiota, new manufacturing biotechnologies for prebiotics, and increased understanding of the metabolism of prebiotics will lead to a rational development of synbiotics. One of the areas that should be considered is the possibility that administered probiotics are transferred to offspring, i.e., directed evolution of microbiota. On the other hand, to date, probiotics, prebiotics, and synbiotics, to be effective, have to be added continuously since the natural microbiota has the potential to revert to its pretreatment state when the therapy ceases.

Phage Therapy

There is a certain similarity between phage therapy and probiotic therapy because both utilize live biological entities to treat pathogenic bacterial infections. However, in the case of probiotic therapy, beneficial microorganisms are introduced to improve health, whereas with phage therapy, specific bacterial viruses (bacteriophages or phages for short) are employed to kill specific bacterial pathogens. Phage therapy is based on the natural process in which bacteria and phages in natural environments are in dynamic equilibrium. The concentration of any single bacterial strain is often limited by density-dependent mortality from phage predation (Williams 2013). In essence, phage therapy simply pushes the equilibrium of the "arms race" temporarily in favor of the phage. We shall elaborate on this subject for two reasons: (1) mainly because of its important medical potential in treating infectious diseases in light of the multiantibiotic resistance developing amongst pathogens in the world and (2) because of the unrecognized importance phages may have in holobionts discussed in Chap. 7.

The history of phage therapy is rather interesting. Phages were discovered in 1915 by the British microbiologist Felix Twort (1915), and, independently in 1917, by the French–Canadian microbiologist Felix d'Hérelle (1917). Shortly after their discovery, phages were successfully used to treat several animal diseases (Summers 1999), including avian typhosis (*Salmonella gallinarum*), *Shigella dysenteriae* infection of rabbits, dysentery (Bruynoghe and Maisin 1921), and human staphylococcal skin disease (d'Herelle 1926).

Extensive clinical trials of phage therapy on human diseases were carried out by d'Hérelle in the 1920s (d'Herelle 1926; Summers 1993). Before undertaking the trials, d'Hérelle and his coworkers ingested large quantities of the phage preparations. d'Hérelle wrote "After being assured that no harmful effects attended the ingestion of the Shiga-bacteriophage, this treatment was applied for therapeutic purposes to patients afflicted with [culture-confirmed] bacillary dysentery." In a highly publicized experiment, d'Hérelle treated four bubonic plague patients on a ship passing through the Suez Canal. All four patients recovered in what was considered a remarkable fashion (Summers 1993).

d'Hérelle was then invited by the British government to go to India to work on phage therapy of the plague disease. This project carried out in India, examined the application of phage therapy, especially for cholera epidemics that occurred regularly in association with religious festivals and pilgrimages. From the initial reports arriving from India in the 1920s and 1930s, it was consistently observed that the severity and duration of cholera symptoms and the overall mortality from the disease were reduced in patients given cholera-specific phage by mouth. d'Hérelle established phage therapy centers in several countries, including the United States, France, and Soviet Georgia. A fictionalized account of his work was depicted in the Pulitzer Prize-winning novel *Arrowsmith* by Sinclair Lewis (1931).

In spite of the initial enthusiasm and successful trials, phage therapy was essentially abandoned in the West with the discovery and development of antibiotics in the 1940s. However, phage therapy continued to be used in the Soviet Union. The advantages that antibiotics had over phage therapy included their ease of large-scale production, relatively broad spectrum of action, and the stability of the preparations. In an excellent review on phage therapy, Summers (2001) has suggested that "in the postwar period, maintaining a distance from anything Soviet, be it ideas, politics, or even medicine, was important in the United States. Thus, to some extent, phage therapy became politically tainted as well."

Phage and antibiotic therapies—advantages and disadvantages: The major advantages of phage therapy over antibiotic treatment are host specificity and self-replication. In general, phages are highly specific for a particular bacterial species and often for only certain strains of that species. Thus, phages that infect pathogens do not attack beneficial bacteria, whereas antibiotics have a broader host range and kill many beneficial bacteria (Blaser 2011). Phages do not cause negative side effects to patients, as demonstrated in a study in which human volunteers drank water containing the *Escherichia coli* phage T4 (Bruttin and Brüssow 2005). The biological advantage of host specificity is potentially a commercial disadvantage

because it will be necessary to isolate and store separate phages for each bacterial pathogen.

The fact that phage multiply on their bacterial host indicates that a small number of phages are effective in treating a disease. For example, 10^3 phages per ml were sufficient to prevent the infection of corals with 10^6 bacterial pathogens per ml (Efrony et al. 2007). A few hours after the phage were added their concentration increased to 10^8 per ml and the concentration of the bacterial pathogen decreased to $<10^2$ per ml. Only when the concentration of the specific bacterial pathogen is greatly reduced, will the concentration of phage begin to diminish. In contrast, antibiotics decay rapidly by both excretion and metabolism (Levin and Bull 1996).

Bacteria can mutate both to antibiotic resistance and phage resistance. In recent years, the development of multiantibiotic resistance has become a serious problem in the treatment of infectious disease. Multidrug resistance in bacteria may be generated by one of two mechanisms (Nikaido 2009). First, these bacteria may accumulate multiple genes, each coding for resistance to a single drug, within a single cell. This accumulation occurs typically on resistance (R) plasmids. Second, multidrug resistance may also occur by the increased expression of genes that code for multidrug efflux pumps, extruding a wide range of drugs. Phage therapy has shown promise in treating infections of multidrug resistance pathogens (Filippov et al. 2012). Not enough in vivo studies of phage therapy have been carried out to evaluate if phage resistance would become a problem if the technology were employed on a large scale. It has been suggested that the use of "phage cocktails" (mixtures of phages) will greatly decrease the development of phage-resistant pathogens (Tanji et al. 2004).

Recent studies and future perspectives: The development of antibiotic resistance in hospitals has been a major driving force in re-evaluating phage therapy for the treatment of infectious diseases in the West. In a well-controlled experiment, Smith et al. (1987) showed that a single dose of phage was more effective than multiple doses of several antibiotics in treating calves that had been infected with a pathogenic strain of *E. coli*. As expected, phage-resistant bacteria occurred in vivo, but interestingly the resistant strains were less virulent than the parent pathogen.

Animals: Recently published animal laboratory studies on model systems using experimental designs that meet the current scientific standards have generally been encouraging. Barrow et al. (1998) confirmed some of the earlier observations on the effectiveness of phage treatment in calves and extended the results to *E. coli* infections of chickens. A single dose of a *Pseudomonas aeruginosa* phage cocktail increased the mortality of thermally injured, *P. aeruginosa*-infected mice (from 6 % survival without phage treatment to 87 % survival with treatment (Trigo et al. 2013). *P. aeruginosa* phages were also shown to be effective against infection in *Drosophila melanogaster* (Heo et al. 2009). The lytic mycobacteriophage D29 was effective against infection by *Mycobacterium ulcerans* in a murine footpad model (McVay et al. 2007).

The use of phages to control infectious diseases of marine animals seems particularly promising because marine natural phages have evolved to be successful in liquid media. The host organisms, i.e., fish, mollusks, crustaceans, or corals, live in aqueous media and hence the therapeutic phage can have continuous and intimate physiological contact with the pathogens in their natural environment. Phage therapy has been successfully used to protect fish (Nakai and Park 2002) and corals (Atad et al. 2012) against experimentally induced bacterial infections in the sea. In the coral experiments, the phage both inhibited the progression of the disease in infected corals and prevented the transmission of the disease to adjacent coral colonies. Interestingly, phage therapy appears to reflect a natural phenomenon in which a small fraction of corals naturally contain phages against specific bacterial pathogens (Atad et al. 2012). Corals that harbor appropriate phages may help explain the often reported occurrence of a few corals in the population that are naturally resistance to disease.

Plants: Phage therapy of plant disease has also been studied (Frampton et al. 2012; Salifu et al. 2013). *Ralstonia solanacearum* is a Gram-negative bacterium and the causative agent of bacterial wilt in many important crops. Pretreatment of tomato seedlings with phage RSL1 drastically limited penetration, growth, and movement of root-inoculated *R. solanacearum* cells. All phage-treated tomato plants showed no symptoms of wilting during the experimental period, whereas all untreated plants had wilted by 18 days postinfection (Fujiwara et al. 2011). In another experiment, a highly virulent *Streptomyces* phage was used to disinfest seed potato tubers artificially inoculated with a common scab-causing streptomycete (McKenna et al. 2001). Phage-treated scab-affected seed potatoes planted into field soil-produced tuber progeny with reduced levels of surface lesions of scab (1.2 %) compared with tubers harvested from nonphage-treated tubers (23 %). Negishi and Maeda (1990) introduced a novel technique of phage therapy by inoculating *Pseudomonas solanacearum*-infected tobacco plants with an avirulent strain of *P. solanacearum* together with the phage. Using the avirulent strain to serve as a host, the effective concentration of phage was greatly increased. Inoculation of the phage alone reduced incidence and severity of tobacco wilt, compared with the untreated control but treatment with both phage and the avirulent strain was more effective than either treatment alone. This novel technique should be tested in other animal and plant models.

Humans: In addition to controlled animal and plant studies of phage therapy, there is a large body of the postWorld War II clinical literature on phage therapy of humans that has been carried out primarily in Poland (reviewed by Abedon et al. 2011). Thousands of patients have been treated with phages and the results were favorable, especially if one considers that most of the patients had failed to respond to antibiotic treatment and were treated as a last resort only in “hopeless” cases. In the years 1981–1986, phage therapy was applied to 550 cases of suppurative infections of the skin, middle ear, and veins. Positive results were obtained in 508 cases (92 %). As mentioned above, there has been some Western political antagonism to these claims in addition to scientific skepticism because studies were not blinded and not all cases were presented in sufficient detail. Since

Poland is now a member of the European Union, one would expect that the future clinical trials will be conducted according to Western regulatory standards.

The prospect for bacteriophage therapy in Western medicine was discussed by Merril et al. (2003). Since that time, several developments suggest that phage therapy will eventually find a place in Western medicine: In August 2006, the U.S. Food and Drug Administration approved spraying meat with phages (targeting against *Listeria monocytogenes).* Phase I and II clinical trials of phage therapy were successfully completed at the Royal National Throat, Nose, and Ear Hospital, London for chronic otitis due to antibiotic-resistant *P. aeruginosa* (Wright et al. 2009). Phage therapy was reported to be effective also in treating antibiotic resistant *P. aeruginosa* urinary tract infections (Khawaldeh et al. 2011). No bacteriophage-resistant bacteria arose, and the kinetics of bacteriophage and bacteria in urine suggest self-sustaining and self-limiting infection. The synergistic action of antibiotics and bacteriophage has also been studied (Kirby 2012). In light of the growing problem of antibiotic resistance, it is likely that phage therapy will gain further interest as an alternative antibacterial approach.

Key Points

- Prebiotics, probiotics, synbiotics, and phage therapy can all be considered applied aspects of the hologenome concept. Prebiotics are dietary supplements that result in specific changes in the composition and/or activities of the natural gastrointestinal microbiota, thus conferring benefits upon host health. This is precisely the concept of variation in holobionts by amplification; beneficial bacterial species are amplified, whereas others decrease in number.
- Probiotics are live microorganisms which, when administered in adequate amounts confer a health benefit on the host. Unlike variation of holobionts by acquisition of microbes from the environment, probiotic technology involves the nonrandom introduction of specific bacteria to improve the health of the host. Some of these may be novel microorganisms adding new genetic material to the hologenome.
- Synbiotics, the wedding of prebiotics and probiotics, are a powerful tool because they combine the hologenome concepts of variation by acquisition of bacteria (probiotics) and amplification (prebiotics).
- Phage therapy—the eradication of specific bacterial pathogens by lytic phages—was shown to be successful in the treatment of certain human and animal diseases as early as the beginning of the twentieth century. It was abandoned in the West with the discovery and development of antibiotics, but continued to be used in the former Soviet Union and Poland primarily for the treatment of antibiotic-resistant infections of the skin, middle ear, and veins.

- The major advantage of phage therapy is its specificity; the selected phage attacks the pathogen but not beneficial bacteria. Antibiotics, on the other hand, though easier to handle and possessing a broader host range, also kill many beneficial bacteria and generate the development of broad-range resistance. In light of the growing problem of antibiotic resistance, phage therapy is now gaining interest as an alternative antibacterial approach in Western medicine.

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Chapter 11
Epilogue

As the area of light increases, so does the circumference of darkness.

—Albert Einstein

Summary

We are in the midst of a paradigm change in biology: animals and plants can no longer be considered individuals, but rather holobionts, each of which is an independent biological entity and an independent level of selection in evolution. These eukaryote holobionts arose from prokaryotes by fusion and aggregated into multicellular complexes, utilizing prokaryotic genetic information, while always remaining in close contact with microorganisms. Thus, the holobiont of today includes the host, or more precisely the large participant, and the microbiota that can include bacteria, archaea, viruses, yeast, fungi, algae, and other protists. This conglomerate of creatures interacts via competition and cooperation to achieve the best possible survival and propagation of the complex holobiont. Within the holobiont, the genome of the host remains constant throughout the life of the holobiont and to a large extent so does the core microbiome of the microbiota. Both the host genome and the microbiome are transferred with reasonable accuracy from generation to generation. The less precise transfer of the microbiome than the host genome provides an additional source of variability to offspring. Furthermore, the relative numbers of specific microorganisms comprising the microbiota (and its microbiome) can change as a function of conditions, such as diet. In addition, from time to time a new microorganism (commensal or pathogenic) can be acquired by the holobiont, remaining an undetectable minority until conditions change and enable its amplification. In the case of commensals, amplification can be for the benefit of the holobiont, whereas in the case of pathogenic organisms amplification can cause disease and death. Variation in the holobiont can also occur by HGT between microbes and between microbe and host. This latter type of variation in the holobiont has the potential of bringing upon it a genetic change that can generate a quantum evolutionary leap (e.g., the syncytin gene). Thus, two major conclusions can be derived from the hologenome concept: (1) evolution of animals and plants was driven primarily by natural selection for cooperation between and with microorganisms, and (2) microorganisms have played a key role in the origin of new animal and plant species. Finally and hopefully, the hologenome concept will inspire new approaches to evolutionary biology and future research on many new and unresolved questions that have emerged.

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Need for Further Analyses of Microbiota Diversity

Many ecological studies begin with a survey of the various species that occupy an experimental site. With microorganisms this is no simple task for several reasons. To begin with, the concept of species in prokaryotes is vague. The common definition of a biological species (Mayr 1970) "as a group of organisms that can interbreed to produce fertile progeny" is not applicable to prokaryotes. Bacterial genetic exchange via transformation, transduction, conjugation, and horizontal gene transfer has been demonstrated for several bacteria, but it is not confined to species: It can extend to genera and, in some cases, even domains. Another widely used species concept is based on the structural features of an organism. While this morphological concept of species has proven useful to zoologists, botanists, and paleontologists, it is of less practical value to bacteriologists because prokaryotic cell shapes are too simple and indistinguishable to be used as the basis for species determination. Until the advent of DNA sequencing methods, bacterial classification was based largely on biochemical tests and colony morphology, rather than organismal morphology, and not surprising, more often than not those divisions fit with current classification schemes. What is now generally accepted as a pragmatic basis for placing groups of bacteria into a species is DNA–DNA hybridization (Wayne et al. 1987; Stackebrandt and Goebel 1994). If two bacterial strains show a value of 70 % or more by DNA–DNA hybridization, they are considered to be in the same species. Unfortunately, the test is still time-consuming and cannot be applied to the majority of bacteria which have not been cultured. The most widely used current method for assigning bacteria to a taxon is based on the sequence of its 16S rRNA gene. The method is excellent for placing a bacterium in a genus and higher taxonomic ranks, but is inaccurate at the species level because bacteria which are evidently different species by DNA–DNA hybridization and physiological properties can share a very similar or identical 16S rRNA gene sequence (Chan et al. 2012). Clearly, the bacterial species concept needs further clarification, both theoretically and experimentally. James Staley has proposed a phylogenomic species concept, which combines phylogenetic and genomic analyses, to circumscribe species, using less highly conserved genes than the 16S rRNA gene (Staley 2009). The reason why it is important to be able to place a bacterium in a species or subspecies is that at higher taxons it is not possible to ascribe specific functions to the microbe. Even describing microbiota at the species level may be insufficient because different strains of the same species may show different phenotypic and genetic characteristics (Welch et al. 2002). Thus, although genome analysis is a powerful tool to describe microbial community structure, to fully understand the effect of microbiotas on holobiont fitness it will be necessary to revert to culturing procedures to study their physiological properties in vitro and in vivo as has been performed with gnotobiotic mice.

Another problem in describing all of the diverse bacterial species associated with any particular animal or plant is that some may be present in relatively small numbers and go unnoticed. Considering the human gut, for example, the total

number of bacteria is approximately 100 trillion (10^{14}). If one million (10^6) copies of a particular bacterial species is present in the gut, it would be necessary to sequence one hundred million 16S rRNA genes to detect it. Although the technology for detecting such rare bacteria is not yet available; it should be available in the near future (Loman et al. 2012). The significance of rare bacteria is that in spite of being undetected at a particular time, they may be part of the core microbiome and could be amplified under appropriate conditions. Knowledge of the absence or presence of rare bacteria will allow for a better prediction of the outcome of a particular holobiont when conditions change. The same argument may also apply at the species level. The future of a particular animal or plant species may depend on the presence of certain microbes in the population.

Other important areas of future research are analyses of host-associated viruses (Reyes et al. 2010; Hofer 2013) and eukaryotic organisms, such as yeast, fungi, and other protists (Andersen et al. 2013) in animals and plants. Until more data are available, it will be difficult to evaluate the extent that viruses and microbial eukaryotes affect the health and evolution of higher organisms. A small human clinical trial suggested that yeast can change the gut community structure, reduce potential pathogens, and increase lactobacilli and bifidobacteria (Possemiers et al. 2013).

Little is also known about how host genetics, diet, long-term drug administration, pollution, and temperature in plants and cold-blooded animals may contribute to individual differences. Microbes often produce unique states through cooperative associations. Important future challenges include understanding cooperative activities rather than the activity of single taxa or species in addition to what kind of forces select for certain microbes in holobionts. In these regards, for example, gut microbiota studies using a model artificial gut may prove valuable (McNulty et al. 2013).

Physiologic and Other Functions of Microbiota Within Holobionts

In Chap. 5, we presented examples of activities attributed to microbiota that contribute to holobiont fitness. However, those were just the tip of the iceberg. Every month there are new reports of previously unknown important functions carried out by microbiota in animals and plants. The full extent of the microbial activity in holobionts remains to be determined and will probably require a combination of culture-independent and -dependent techniques.

Many physiologic experiments on animals (including humans) and plants were performed in the past without consideration of the possible contribution of the microbiota and should be reinterpreted and/or reexamined. Just to give one example, Davies (1980) reported on respiration rates of Caribbean corals as a function of species, coral depth, and surface-to-volume ratio. Respiration was measured by the standard method of oxygen consumption in the dark. How much

of the oxygen was consumed by the microbiota was not determined and would probably affect the interpretation of the experiments. In general, animals and plants contain large numbers of symbiotic microbes that have high metabolic rates. One way to assess the relative contributions of microbiotas and hosts is to compare the activities of germ-free with conventional animals and plants. In the cases where these kinds of experiments have been performed, including pioneering studies in the 1960s (Sprinz et al. 1961; Abrams and Bishop 1966, 1967), large differences in physiologic and biochemical parameters have been reported (Sommer and Bäckhed 2013; Young et al. 2013; Zamioudis et al. 2013; Marcobal et al. 2013).

Interaction of the Immune System and Microbial Symbionts

The interaction of microbiota with both the innate and adaptive immune system plays an essential role in the health of animal and plant holobionts. The interaction goes both ways: microbiota contributes to the development of the immune system and the immune system regulates the concentration and types of microbes that are tolerated by the holobiont. Fitness of a holobiont depends to a large extent on the ability of the immune system to distinguish between beneficial and harmful microbes, tolerating the beneficial ones, and removing the pathogens. It has been proposed but not demonstrated that the immune system first evolved to preserve and protect its own microbiota and subsequently was used to kill dangerous foreign microorganisms (Pamer 2007; Lee and Mazmanian 2010; Bosch 2013).

Although how bacterial colonization of the mammalian gut influences the development and specificity of the immune system has become a major focus of interest (e.g., Lee and Mazmanian 2010; Lathrop et al. 2011; Hansen et al. 2012; Hooper et al. 2012), understanding host–microbial immune mutualism remains a future challenge. Attention should be paid to determining how transmission of microbiota to offspring shapes the education of the immune system and how failure to adapt leads to diseases, such as inflammatory bowel disease in humans. Little is known about the interaction of microbiota and plant immunity (Jones and Dangl 2006).

Microorganism's Contribution to the Health of Human, Animal, and Plant Holobionts

During the last decade there has been an explosion of publications in top journals describing previously unknown roles that microbes play in adaptation, behavior, development, and health of plants, animals, and especially humans. It is reasonable

to assume that these discoveries will continue and more attention will be paid to defining and culturing the microbes responsible for the phenomena and the detailed biochemical mechanisms. It will then be possible to begin examining the complexity of these interactions in an integrated approach. The more that is known about how microbes affect fitness, the easier it will be to utilize them to combat disease and encourage health. We see a future where well-defined prebiotics, probiotics, synbiotics, and viral therapy will become accepted as alternative technologies for preventing and treating human, domestic animal and agricultural diseases. Preliminary data suggest that beneficial microbiota can potentially be used to treat allergy (Bjorksten 2009), obesity (Turnbaugh et al. 2009), autism (Finegold et al. 2010), inflammatory bowel disease (Jonkers et al. 2012), diabetes (Qin et al. 2012; Vrieze et al. 2012), celiac disease (Ray 2012), and severe diarrhea (Khoruts and Sadowsky 2011; Petrof et al. 2013). Since novel microbes can be established in the human gut and be transmitted between generations, the potential also exists for introducing selected strains to combat genetic diseases.

Manipulation of hologenomes in humans and domestic animals and plants will require safety and ethical considerations, which should include both our growing knowledge and our still existing ignorance of hologenome biology in addition to social and legal acceptance. An organism that is generated through genetic engineering is considered to be a genetically modified organism (GMO). The first GMOs were bacteria in 1973; insulin-producing GM-bacteria were commercialized in 1982 (Walsh 2005), and genetically modified food has been sold since 1994. Today, 12 % of arable land worldwide is planted with genetically modified (GM) crops. GM-animals, such as zebrafish, have been developed for research purposes (Ekker 2008), as pets (Stewart 2006), and food (Ledford 2013).

It has recently been suggested that genetic engineering could be used to help save endangered species (Thomas et al. 2013). Biodiversity is threatened by global warming, which has already altered species distributions, changed phenology, and caused the extinction of populations. The loss of biodiversity has the potential to contribute to a decline in ecosystem services. Conservative estimates predict that 15–40 % of living species will be effectively extinct by 2050 as a result of climate change, habitat loss, and other consequences of human activities (Thomas and Williamson 2012). In the case of corals, Hoegh-Guldberg (1999) has predicted that most coral species will be extinct by 2050 as a result of temperature-induced coral bleaching. Since microbiota have been shown to assist animal and plant adaptation to stress (e.g., Rodriguez and Redman2008; McLellan et al. 2007; Kuz'mina and Pervushina 2003). We suggest that infection of corals, as well as other threatened animals and plants, with temperature-resistant microorganisms, and other resistant traits, should be tested as an alternative potential technology to conserve species.

We suggest that genetically engineering the human microbiome should also be explored. It should be easier and safer to modify the microbiome than the human genome. An interesting example of a natural HGT that took place in the human microbiome was the acquisition of genes coding for porphyranases, agarases, and associated proteins from the marine bacterium *Zobellia galactanivorans* to the human gut bacterium *Bacteroides plebeius* in the Japanese population (Hehemann

et al. 2010). In principle, human gut bacteria could be cultured, modified under defined conditions by genetic engineering to produce desirable proteins to combat disease, and then reintroduced to their original hosts as probiotics. A growing field that is taking the microbiota to this direction is the field of nutrigenomics/nutrigenetics, the science that explores the interaction between diet and the human genome (Peregrin 2001; Müller and Kersten 2003). The overall goal of this field is to uncover causal relationships between genes, nutrients, and molecular processes that underline health and the development of diseases such as chronic diseases in which nutrition is known to be deeply involved (Dimitrov 2011). In recent years, the field has expanded to include the gut microbiota (Faith et al. 2011; Kang 2013). Variation in gut microbiota within and between individuals is large (Turnbaugh and Gordon 2009). Adjusting gut microbiota, thereby modulating the hologenome, could become a key component of personalized nutrition in the future, as gut microbiota and their metabolic products have been shown to alter host metabolism and health status (Lewis and Burton-Freeman 2010).

Variation and Evolution of Holobionts

The experimental data presented in previous chapters led us to the conclusion that evolution of animals and plants has proceeded primarily by cooperation between microorganisms and their hosts. Microbiotas have contributed to the evolution of animals and plants, initially in the creation of eukaryotes by fusion between them and since then both as symbionts that provide novel functions and as reservoirs of genes that were horizontally transferred to host chromosomes. However, it is not clear how much of the variation and evolution of higher organisms occurred as a result of acquisition of microbiota (insourcing) and microbial genes (HGT) and how much via mutation and rearrangement of host genomes. With the rapidly developing powerful tools of sequencing genomes and bioinformatics it should be possible to provide some answers to these questions in the near future. It is likely that in the early evolution of animals and plants much of the genetic information for metabolic pathways was obtained from microorganisms, but this happened so long ago that subsequent mutations and rearrangements may make it difficult to retrace these early events.

Considering more recent evolution, it should be possible to determine by comparative hologenome analyses how the host genome and microbiome changed over time. In many cases, changes in diet appear to have been the major driving force for evolution. Utilization of novel nutrients frequently requires a change in the microbiota. For example, it is difficult to understand the evolution of ruminants and termites with their unique physiologies and morphologies without the prior acquisition of anaerobic cellulose-degrading bacteria. With regard to the evolution of humans and chimpanzees from a common ancestor, humans rely on processed starch for a large part of their energy (Gibson et al. 1996), while the chimpanzees diet consists mainly of fructose-rich fruit, supplemented with insects, bird eggs,

honey, and small to medium-sized mammals (Watts 2008). Simultaneously there are significant differences in the microbiota of humans and chimpanzees (Moeller et al. 2012). It will be interesting to examine if acquisition of different bacteria played a role in the evolution of humans. In general, tracing the changes in host genomes and microbiomes during evolution will enrich the field of evolutionary biology and open new possibilities.

Teaching Biology/Microbiology

At colleges and universities, microbiology is traditionally taught after the students have studied zoology, botany, physiology, genetics, and ecology. This appears irrational since it is contrary to evolutionary development, microorganisms being the earliest and simplest forms of life and responsible for most of the turnover of matter on this planet. Further, it has been known for more than 20 years that the natural relationships between organisms place microbes at the base of the universal tree of life (Woese 1994). Why then is microbiology relegated to a minor part of the biology curriculum with its main function to serve as a tool to study molecular biology and infectious disease? Besides the obvious answer of tradition, most biologists, including microbiologists, do not appreciate the evolutionary relationships and diversity of microbes that comprise two of the three domains of life. Just at the time that microbiology is turning into a fundamental science, many institutions are closing their microbiology departments and turning them into applied programs. One of the ironic aspects of this development is that molecular microbiologists, who have developed the tools for studying evolutionary biology and ecology, need to cooperate with general biologists and medical researchers to fully appreciate their own discoveries.

The hologenome concept is an additional rational for bringing microbiology into mainstream biology. All animals and plants contain abundant and diverse microbes which contribute significantly to their well-being and evolution. It is impossible to fully understand an animal or plant without considering their symbiotic microbes. On the other hand, it is possible to study microbes without knowledge of animals and plants. The late C. B. van Niel of Stanford University taught a highly successful course in microbiology to students who had little or no previous knowledge of general biology (Spath 2004).

One logical way to begin the study of biology is with a discussion of hypotheses that have been put forth to explain the origins of life. This would lead to an introduction of the key molecules of life, initially amino acids, sugars, purines, and pyrimidines, followed by their polymerization products, proteins, polysaccharides, and nucleic acids. Such a discussion can emphasize the importance of hypotheses and the power of experimentation to disprove a hypothesis and offer an alternative one, i.e., the scientific method. The next stage should be the study of the simplest evolved prokaryote and viruses and finally the study of biology should reach eukaryotes and their evolution.

Microbiology is finally a complete biological discipline, resting on a firm evolutionary base, and is finding its rightful place in the biological sciences. This will eventually be reflected also in the teaching of biology. As the paleontologist and evolutionary biologist Stephen Jay Gould (1993) wrote, "On any possible, reasonable or fair criterion, bacteria are—and always have been—the dominant forms of life on Earth."

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