THE YEAST HANDBOOK

Carlos A. Rosa
Gábor Péter (Eds.)

Biodiversity and Ecophysiology of Yeasts

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With 40 Figures and 43 Tables

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Preface

Biological diversity or biodiversity, according to the United Nations Convention on Biological Diversity, "means the variability among living organisms from all sources including, inter alia, terrestrial, marine and other aquatic ecosystems and the ecological complexes of which they are part" and includes genetic diversity, species diversity, and ecological diversity. Authors trying to estimate the number of extant yeast species usually come to the conclusion that, similarly to other groups of microorganisms, a very small proportion of the yeasts, possibly only 1% of the species, have been described.

Studies on "yeast biodiversity" are more focused on taxonomic inventories, with emphasis on the description of novel species. Approximately 30% of known yeast species were described from a single strain, and information about the ecology and the genetic and physiological variability of these yeasts is missing or incomplete.

In the last few decades an increasing number of new yeast habitats have been explored. As a result, a large body of ecological information has been accumulated and the number of known yeast species has increased rapidly. The volume "Biodiversity and ecophysiology of yeasts" provides a comprehensive and up-to-date overview of several areas in the field of yeast biodiversity and ecology. The chapters are written by respected experts in various fields. The first chapters approach yeast biodiversity from different points of view, including phylogenetics and genomics. Some aspects of sugar and nitrogen metabolism are also discussed. Separate chapters are devoted to stress responses of yeasts, to environmental factors influencing them, to antagonistic interactions among them, to methods used for investigating yeast biodiversity, and to the role of culture collections in handling the ever-increasing number of yeast strains and relevant data.

The chapters dealing with yeast communities from different habitats include reviews on yeasts from invertebrates, the phylloplane, soil, freshwater and marine ecosystems, cactophilic communities, as well as Antarctic and tropical forest ecosystems. In some chapters the effect of human activity on yeast communities is also considered. The black yeasts are treated in a separate chapter, and finally the role of yeast biodiversity in biotechnology is reviewed.

We gratefully acknowledge the contributors to this book. We hope that it will provide a useful overview of the biodiversity and ecophysiology of yeasts, and that it will stimulate increasing efforts in yeast biodiversity research.

Carlos Rosa
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Contents

Chapter 1

Yeast Biodiversity: How Many and How Much?

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1.1 Introduction

Biodiversity is now a common word. Harper and Hawksworth (1995) tabulated the frequency of use of the term in *Biosis* and reported its first occurrence in 1988 followed by an increase to approximately 900 by 1994. A similar search of the PubMed database yielded a cumulative total of 1,361 hits by the end of 2003. By comparison, the number of articles using the word "yeast" is approaching 100,000. If the present trend continues, by the year 2016 searches for either word will produce in excess of 36,000 hits for that year only. The task at hand is to make similar predictions about yeast biodiversity.

Biodiversity means different things to different individuals. Gaston (1996) reviewed several definitions and concluded that the concept is an abstract expression of all aspects of the variety of life. Recent publications dealing with yeast *diversity*, had they appeared only 15 years earlier, might have used instead such terms as *taxonomy*, *ecology*, or *survey* (Nout et al. 1997; Buzzini and Martini 2000; Fell et al. 2000; Poliakova et al. 2001; Gadanho et al. 2003; Granchi et al. 2003; Lachance et al. 2003a; Ganga and Martinez 2004; Renker et al. 2004) or even *enzymology* (Lamb et al. 1999). The Convention on Biological Diversity (Anonymous 1992) defines biological diversity as "the variability among living organisms from all sources including, inter alia, terrestrial, marine and other aquatic ecosystems and the ecological complexes of which they are part; this includes diversity within species, between species and of ecosystems." As with most things in our society, biodiversity became a tangible reality when it could be assigned a significant economic value. And as with most things in science, the recognition of biodiversity as a worthy research topic is predicated on measurability and the generation of testable hypotheses. The current urgency of the scientific study of biodiversity stems from the realization that only a small fraction (approximately 8%) of the total diversity of life is known (Stork 1999) and that species extinction is occurring at a measurable and increasing rate (Purvis and Hector 2000).

1.2 Measurement and Significance of Biodiversity

1.2.1 Levels of Diversity

The inclusion of any level of biological variation in the definition of biodiversity could lead to a trivialization of the concept, as variation is the very essence of biology. A more restrictive circumscription should limit the term to ecological and evolutionary variation. Theoretical ecologists, following the model of Whittaker (1960), often subdivide species diversity into three hierarchical components, namely within-sample (α), between-samples (β), and global (γ) diversity. These components may be considered additive ($\gamma = \alpha + \beta$, Crist et al. 2003). The main units of measurement are richness (simple species count) and heterogeneity (relative abundance of each species in a community). The two measures can be examined simultaneously in relative abundance plots, which contrast the number of species in a sample as a function of the number of individuals representing each species. Considerable interest in the underlying causes of such distributions was stimulated by the pivotal publication of MacArthur and Wilson's (1967) treatise on island biogeography. A recent model (Hubbell 2001) attributes a large portion of the species composition of a community to chance. Implicit to this view (but perhaps not sufficiently explicit) is that membership of a species in a community depends initially on its fundamental niche, in other words, the sum of its intrinsic properties. For example, the community of floral nectar rarely contains basidiomycetous yeasts. This is not due to chance, but to the fact that such a habitat favours fermentative, osmotolerant, copiotrophic species, which are found most often in the Saccharomycetales. In the neutral model, a community is seen as an assemblage of ecologically equivalent species, where the abundance of each species within a local community is not so dependent on the fundamental niche. Instead, species composition is affected by speciation in the metacommunity, the rate of influx of species, the size of the local community, and the local rate of extinction. This is almost entirely analogous to Kimura's (1983) neutral model of evolution. Natural selection remains the preliminary screen that causes rapid elimination of deleterious mutations and rapid fixation of adaptive alleles, just as the environment determines whether or not a species can enter a community. The majority of species in the community have already "passed the test" of selection, and are equally adapted. As is the case for selectively neutral mutations, the relative abundance of a species will be due not so much to some intrinsic property, but to chance.

Application of such theories to yeast communities is not yet completely practical. Most models of community ecology were developed for communities where members can be identified and enumerated rapidly, e.g., forest trees and insect biota. The recent development of identification methods based on DNA sequencing (Kurtzman and Robnett 1998; Fell et al. 2000) has not yet resulted in practical means of identifying yeasts instantly, in the field, but such technologies are no doubt forthcoming. An attempt to explore the factors that underlie community structure was made recently (Lachance et al. 2003a). The yeast biota of morning glory flowers and associated nitidulid beetles was characterized in a "forest island" (*kīpuka*) on the slope of the Mauna Loa volcano in Hawaii. The yeast community is highly

specialized, consisting almost entirely of members of two clades with affinities to *Metschnikowia* and *Wickerhamiella*: the former clade is vectored primarily by the beetles, and the latter by drosophilid flies. Although the resulting community is a mixture, each clade can be studied separately with selective media. The *Metschnikowia* clade members in the community consists of six species and whose frequencies follow the expected log series distribution, from abundant to rare. Two of the species (*Metschnikowia hawaiiensis* and *Candida kipukae*) are probably Hawaiian endemics. The others have also been found in Central America and are thought to have reached Hawaii in recent history. The six species are similar physiologically, suggesting that they might be mutually neutral with respect to niche. However, their distribution within the *kīpuka* is not random and follows closely the distribution of the host beetles. The latter consist of two major species, one Hawaiian endemic and one that was introduced in the early twentieth century. Maximum growth temperature and insect choice may be important factors in the local distribution, such that a completely neutral model would have to be ruled out. The study is in progress, and increased sampling is hoped to provide a test of the neutral hypothesis.

1.2.2 Diversity Within Species

Even if one agrees that species abundance is central to the characterization of biodiversity, genetic diversity is an essential feature of the species itself. Even orthodox proponents of the phylogenetic/autapomorphic species concept would have to agree that a "species" that is completely devoid of variation can hardly be regarded as a species (Wheeler and Meier 2000). Variation among members of a species has long plagued pragmatic systematists in their search for stable diagnostic (autapomorphic) characters (Lodder and Kreger-van Rij 1952). As DNA sequence analysis took the study of yeast diversity by storm, the recurring dream of an invariant species trait was temporarily rekindled (Kurtzman and Robnett 1998). However, the sequencing approach has in some instances brought to light considerable variability among individuals that share a common gene pool and thus are members of the same biological species. One response might be to denounce the biological species concept as antiquated and inoperable (Wheeler and Meier 2000). Another would be to accept that the genes that are most amenable to phylogenetic construction are not necessarily involved in conferring a common evolutionary destiny to members of a species, and that species cannot be defined on the basis of invariance in gene sequences.

In a study of the distribution of yeasts in seawater, Gadanho et al. (2003) subjected 234 isolates to microsatellite-primed PCR fingerprinting and demonstrated that in most cases multiple isolates of various basidiomycetous yeast species contain a substantial amount of genomic variation. Ascomycetous species recovered in that habitat exhibited less variation.

Intraspecific variability has been examined in the two species in the genus *Clavispora*, both of which occur in nature as heterothallic, haploid mating types. This offers the advantage that species boundaries can be assessed by mixing of compatible strains and observation of ascospores. *Clavispora opuntiae* has so far

been isolated exclusively from necrotic tissue or tunnels of moth larvae found in cacti. Hundreds of specimens have been recovered globally and preserved for study. Although the growth responses of most isolates are generally constant, polymorphisms have been detected at the level of the ribosomal DNA (rDNA) gene cluster (Lachance et al. 2000b). In some 500 isolates examined by restriction mapping, over 40 variants were recognized. These correlated to a large degree with geography, host plant species, and insect vectors. Most of the variation was shown by sequencing to be located in the intergenic spacer region, although a small amount of polymorphism was also detected in the large subunit rRNA gene. Strains representing the extremes of that variation had been shown previously to exhibit a lower degree of interfertility (Lachance et al. 1994) and perhaps represent the beginning of a speciation event.

C. lusitaniae is similar morphologically and physiologically to *C. opuntiae*, but exhibits much less habitat specificity, having been recovered in cactus fruit, agave rots, industrial wastes, clinical specimens, and several other sources. Mating compatibility and large subunit rDNA sequences were determined in 37 strains (Lachance et al. 2003c). The sequences could be assigned to ten types belonging to two families that differed by as much as 32 substitutions in the D2 domain. The variation was not correlated with mating intensity or abundance of mature asci.

Although these studies do not allow generalizations about the evolutionary or ecological significance of genetic diversity within yeast species, they would seem to support the view that variability is an intrinsic property of species.

1.2.3 Species Diversity

From the first to the current edition of the *The yeasts, a taxonomic study*, the number of species described has grown from 164 in 1952, to 349 in 1970, to 500 in 1984, and to 700 in 1998 (Lodder 1970; Kurtzman and Fell 1998). Extrapolation of these numbers leads to the prediction that an eventual 2016 edition would contain approximately 1,000 species. However, this number may very well be exceeded in the forthcoming fifth edition, planned for 2005. The increase is due to several factors, including methodology and species concepts. In the first edition (Lodder and Kreger-van Rij 1952), species were circumscribed on the basis of morphology and a small number of growth tests. The doubling in the number of species found in the second edition was due in part to the use of a much larger battery of nutritional properties. Early application of molecular approaches had a considerable impact on the third edition, but was not entirely accountable for species proliferation, as the shift to a genomic basis for species delineation also caused the merger of physiological or morphological variants into larger and more diverse species. The publication of the fourth edition coincided with early application of DNA sequencing in yeast identification and phylogenetic reconstruction, although the full impact of this approach came later. Again, the result is a mixture of species fusions and subdivisions.

The definition of species is fundamental in the generation of meaningful estimations of biodiversity, which accounts in part for the heartiness of the debate on that subject (Wheeler and Meier 2000). The species problem as it applies to bacterial and

fungal diversity has been discussed by O'Donnell et al. (1995), who pointed out the lack of a common standard. Although species concept controversies are not alien to yeast systematics, many practitioners agree that species should, whenever possible, represent cohesive evolutionary units. Individual researchers may disagree on how best to document the boundaries of such units, but the result is nonetheless a relatively stable consensus. As the issue is far too complex to be examined here in detail, it will be expedient to assume, rightly or wrongly, that taxa which are recognized at any given time constitute genuine and meaningful species.

Sequence analysis resulted in an enormous increase in the ease and speed of identification, making intense biodiversity surveys almost manageable. Many species descriptions currently being published come from material collected in the past and stored in collections in the hope that new technologies would eventually facilitate meaningful species assignments. The sequencing approach has fulfilled this need. Unfortunately, the clarifications brought forward by sequencing have done little to improve our understanding of the natural history or ecology of the species being described. Unless the ecological context of species is also documented, Linnean binomials will remain no more than mere labels of little relevance to biodiversity. By their very nature as unicellular heterotrophs, yeasts are inexorably dependent on other fungi, bacteria, animals, and plants for their existence, and ideally species descriptions should include data on these interactions. The old precept, "everything is everywhere", although no longer tenable, sadly continues to influence yeast taxonomy. An inordinate amount of energy is devoted to transforming sequence data into "correct" trees, at the expense of the yeasts themselves and their biology. Another important consequence of the dependence of yeasts on other life forms is the urgency of documenting their natural history before their very habitats disappear. Unless conservation efforts are intensified, it will become easier to determine the rate of extinction of yeasts than to estimate the number of extant species. The fact that the construction of a comprehensive inventory of life on Earth is seen as a priority by an increasing number of researchers, governments, and granting agencies (Mulongoy et al. 1999) should be viewed with optimism. Equally encouraging is the emergence of more frequent studies aimed at characterising whole yeast communities in relation to their insect vectors. A case in point is a recent description of 16 closely related species originating from fungivorous beetles and their habitats (Suh et al. 2004). Members of the Coleoptera associated with tree decay have long been known to harbour numerous yeast species, as evidenced by the work of pioneers such as L.J. Wickerham, J.P. van der Walt, and H.J. Phaff. These yeasts are suspected to engage in intimate symbiotic relationships with insects, although the nature of the interaction remains elusive in most cases. Recent studies of yeasts found in tropical bees led to the discovery of the genus *Starmerella* (Rosa and Lachance 1998), the nucleus being a growing clade whose membership has increased from 12 described species in 1998 to 29 putative species at the last published count (Rosa et al. 2003). Studies of nitidulid beetles associated with ephemeral flowers have resulted in the near doubling of described species of *Metschnikowia* (Lachance et al. 2003b) and a significant expansion of the formerly monotypic genera *Kodamaea* (Lachance et al. 1999) and *Wickerhamiella* (Lachance et al. 2000a).

1.3 Predicting the Number of Yeast Species

Hawksworth (1991) attempted to predict the number of fungal species on the basis of an estimated 69,000 described species and the ratio of fungi to other life forms. By reference to vascular plants, he proposed a conservative estimate of 1.5 million fungal species, and a comparison with insect species extended the range to three million. The proportion of fungi described (in 1991) was thus thought to be approximately 5% of the total fungal biota. A hasty transposition of Hawksworth's reasoning to the yeasts generates a forecast of approximately 12,000 species.

Hughes et al. (2001) pointed out that microbes may be too diverse to enumerate exhaustively and argued for a statistical approach. One tool used in reaching this objective is the accumulation curve, where species abundance is plotted as a function of sampling intensity. Species sampling follows a rarefaction pattern in which the rate of increase in the detection of species obeys the law of diminishing returns. Well-sampled habitats produce curves that can be fitted to saturation models such as the Michaelis–Menten equation or the negative-exponential function, characterized by growth towards an asymptotic maximum. Poorly sampled habitats produce nearly linear curves. Gadanho et al. (2003) applied this approached to yeasts in seawater and estimated that the 31 species recovered represented approximately 60% of the existing species in their study site. In order to extrapolate beyond a single site, Lachance (2000a) used random internal sampling of collection records to generate accumulation curves. The data were fitted to trend line functions available in Microsoft Excel and the curves were extrapolated to large sample sizes. For insects associated with ephemeral flowers, data from eight localities worldwide (26 yeast species in total) led to the prediction that sampling from 50 localities would raise the number to 42 species. Simulations based on yeasts from tree fluxes were validated by predicting, from eight samples, the total number of yeast species (45) present in 47 actual samples. Extrapolation to 1,000 samples predicted that the number of species would rise to approximately 500. In Fig. 1.1, the same data were analysed using the Michaelis–Menten model. Linear regressions of the double-reciprocal plots of species richness as a function of sampling intensity predict asymptotic maxima of 40 species for floricolous insects and 182 species for tree fluxes, which is not entirely inconsistent with the previous predictions. Using these numbers and an estimate of 1,000 currently described yeast species, a simple rule of 3 predicts that the number of yeast species on Earth is in the order of 1,500 on the basis of the insect model, and 15,000 on the basis of the flux model. The lower value comes from a highly specific yeast community, whereas the upper boundary is characteristic of a more generalistic community. Other well-sampled, highly specific communities follow a conservative pattern similar to that of flower insects. Calderone (2002) recognised 13 *Candida* species as human pathogens compared with eight in 1988. The highly specialized nature of yeasts associated with humans, combined with the extremely high sampling intensity makes the current number of species a good approximation of the saturation point. In the case of the moderately specific community of yeasts associated with necrotic cacti, 3,701 samples yielded fewer than 80 species (Starmer et al. 1990).

The broad range of predictions from 1,500 to 15,000 yeast species in total reflects the fact that the average degree of specificity for all yeast communities is not known.

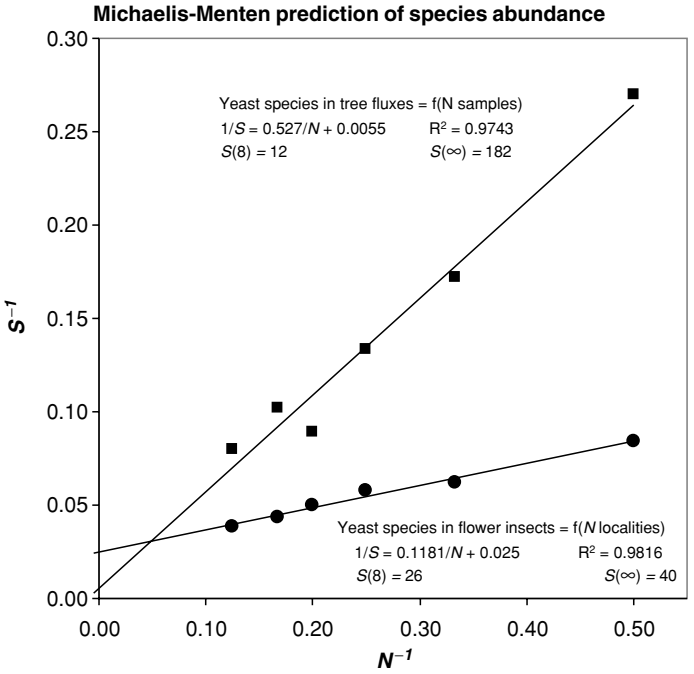

Fig. 1.1. Prediction of yeast species abundance in tree sap fluxes and in insects associated with morning glories based on pseudoreplicated collections from N=8 localities. As the data follow Michaelis–Menten kinetics, double reciprocal plots were used to predict $S(\infty)$, the number of species that would be found in similar habitats in an infinite number of localities. The corresponding linear equations and their regression coefficients are given. The equation S (total) = 1000($S(\infty)$/$S(8)$) was used to estimate of the total number of extant yeast species assuming that the global depletion curve is similar, which of course may not be the case

Furthermore, the numbers and kinds of habitats remaining to be studied are unknown. As floricolous insect and tree flux communities are greatly affected by the activity of members of Coleoptera, whose number of described species is in the order of 350,000, one might predict similar numbers for yeasts; however, the proportion of beetles that harbour yeasts remains to be established. Although tree-boring species and floricolous nitidulids frequently contain yeasts, the very speciose family Chrysomelidae seldom does. It is therefore not reasonable to assume that the number of yeasts is commensurate to that of beetles as a whole.

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Yeast Systematics and Phylogeny – Implications of Molecular Identification Methods for Studies in Ecology

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2.1 Introduction

A major factor that determines the validity of studies in yeast ecology is the correct identification of species in the ecosystem. Before the present era of yeast taxonomy, which uses gene sequences and other molecular criteria, identifications were of necessity based on phenotypic tests. Although phenotype can sometimes be used to correctly identify species, molecular comparisons have shown that many earlier identifications based on phenotype have been incorrect. While this does not mean that earlier work in yeast ecology is invalid, it does say that conclusions drawn from this work may need to be reexamined following more accurate identification of species. In particular, the often-asked question "Is everything everywhere?" cannot be adequately addressed until taxa are correctly identified. In this chapter, we will discuss molecular methods now used for identification of yeasts, what we perceive of their genetic resolution, their impact on systematics, and finally a description of some of the rapid molecular methods that are applicable to the large species populations often examined in ecological studies.

2.2 Molecular Identification of Species

The transition from phenotypic identification of yeasts to molecular identification began with determination of the mole percent guanine (G) plus cytosine (C) ratios of nuclear DNA. These analyses demonstrated that ascomycetous yeasts range from approximately 28 to 50 mol% G+ C, whereas basidiomycetous yeasts range from approximately 50 to 70 mol% G+ C. Depending on the analytical methods

used, strains differing by 1–2 mol% are recognized as separate species (Price et al. 1978; Kurtzman and Phaff 1987). The need for quantitative assessment of genetic similarity between strains and species was satisfied, in part, by the technique of nuclear DNA reassociation or hybridization. DNA from the species pair of interest is sheared, mixed, made single-stranded, and the degree of relatedness determined from the extent of reassociation. Many different methods are used to measure this process, which can be done spectrophotometrically or through use of radioisotopes or other markers (Kurtzman 1993a).

A major question has been how to interpret DNA reassociation data. Measurements of DNA complementarity are commonly expressed as percent relatedness. This usage can be misleading because DNA strands must show at least 75–80% base sequence similarity before duplexing can occur and a reading is registered on the scale of percent relatedness (Bonner et al. 1973; Britten et al. 1974). Experimental conditions can greatly influence the extent of duplex formation, but under optimum conditions, different methods of assessing DNA relatedness do give essentially the same result (Kurtzman 1993a). Percent DNA relatedness provides an approximation of overall genome similarity between two organisms, but the technique does not detect single gene differences or exact multiples of ploidy, although aneuploidy can sometimes be detected (Vaughan-Martini and Kurtzman 1985).

On the basis of shared phenotype, strains that showed 80% or greater nuclear DNA relatedness were believed to represent members of the same yeast species (Martini and Phaff 1973; Price et al. 1978). This issue was also examined on the basis of the biological species concept (Dobzhansky 1976), asking what is the fertility between strains showing varying degrees of DNA relatedness (Kurtzman 1984a, b, 1987; Kurtzman et al. 1980a, b). In one of these studies, the heterothallic species *Pichia amylophila* and *P. mississippiensis*, which showed 25% DNA relatedness, gave abundant interspecific mating, but ascus formation was limited and no ascospores were formed. Similar results were found for crosses between *P. americana* and *P. bimundalis* (21% DNA relatedness) and between *P. alni* and *P. canadensis* (*Hansenula wingei*), the latter pair showing just 6% DNA relatedness. The varieties of *Issatchenkia scutulata*, which exhibit 25% DNA relatedness, behaved somewhat differently. Crosses between *I. scutulata* var. *scutulata* and *I. scutulata* var. *exigua* gave an extent of mating and ascospore formation comparable to that of intravarietal crosses. Ascospore viability from these intervarietal crosses was about 5%, but sib-matings of the progeny had 17% ascospore viability. However, backcrosses to the parentals gave poor ascosporulation and very low viability, which suggests that these two varieties represent separate species. *Williopsis saturnus* is a homothallic species with five varieties that range in DNA relatedness from 37 to 79% (Kurtzman 1987). Intervarietal fertility is reduced and varies depending on the strains crossed. Consequently, the preceding studies show that mating among heterothallic as well as homothallic taxa can occur over a wide range of DNA relatedness values, but that highly fertile crosses, which demonstrate conspecificity, seem to require 70–80% or greater DNA relatedness between strains. Because species barriers are complex and involve a number of factors, the numerical range of 70–100% DNA relatedness as indicative of conspecificity should be viewed as a prediction.

Nuclear DNA reassociation studies have had a marked impact on recognizing yeast species, but the method is time consuming and the extent of genetic resolution goes no further than that of closely related species. Gene sequencing offers a rapid method for recognizing species and resolution is not limited to closely related taxa. Peterson and Kurtzman (1991) determined that domain 2 of large subunit (26S) ribosomal RNA (rRNA) was sufficiently variable to resolve individual species. Kurtzman and Robnett (1998) expanded the preceding work by sequencing both domains 1 and 2 (approximately 600 nucleotides) of 26S ribosomal DNA (rDNA) for all known ascomycetous yeasts, thus providing a universally available database for rapid identification of known species, the detection of new species, and initial phylogenetic placement of the species. Fell et al. (2000) published the D1/D2 sequences of known basidiomycetous yeasts, thus completing the database for all known yeasts. Resolution provided by the D1/D2 domain was estimated from comparisons of taxa determined to be closely related from genetic crosses and from DNA reassociations. In general, strains of a species show no more than zero to three nucleotide differences (0–0.5%), and strains showing six or more noncontiguous substitutions (1%) are separate species. Strains with intermediate nucleotide substitutions are also likely to be separate species. One impact of the D1/D2 database has been to permit detection of a large number of new species, which has resulted in a near doubling of known species since publication of the most recent edition (fourth) of *The yeasts, a taxonomic study* (Kurtzman and Fell 1998). Another use is that the nontaxonomist can now quickly and accurately identify most known species, as well as recognize new species, by sequencing approximately 600 nucleotides and doing a BLAST search in GenBank.

The internal transcribed spacer regions ITS1 and ITS2, which are separated by the 5.8S gene of rDNA, are also highly substituted and often used for species identification, but for many species, ITS sequences give no greater resolution than that obtained from 26S domains D1/D2 (James et al. 1996; Kurtzman and Robnett 2003). However, Fell and Blatt (1999) were able to resolve cryptic species in the *Xanthophyllomyces dendrorhous* species complex that had been unresolved from D1/D2 sequence analysis, and Scorzetti et al. (2002) reported ITS sequences to provide somewhat greater resolution among many basidiomycetous species than was found for D1/D2, although, a few species were less well resolved by ITS than by D1/D2. Consequently, it appears useful to sequence both D1/D2 and ITS when comparing closely related species. The intergenic spacer (IGS) region of rDNA tends to be highly substituted and sequences of this region have been used with good success to separate closely related lineages of *Cryptococcus* (Fan et al. 1995; Diaz et al. 2000), *Xanthophyllomyces* (Fell and Blatt 1999), *Mrakia* (Diaz and Fell 2000) and *Saccharomyces* (Kurtzman et al., unpublished). Because of the occurrence of repetitive sequences and homopolymeric regions, the IGS region tends to be difficult to sequence for some species. Small subunit (18S) rDNA, which has been extremely important in broad-based phylogenetic analyses, is generally too conserved to allow separation of individual species (James et al. 1996; Kurtzman and Robnett 2003).

The focus of our discussion on species identification from gene sequences has been on rDNA. A major advantage of rDNA is that it is present in all living organisms, has a common evolutionary origin, occurs as multiple copies and is easy

to sequence because primer pairs for conserved regions can generally be used for all organisms. However, gene sequences other than those of the rDNA repeat have been used for separation of species from many kinds of fungi (Geiser et al. 1998; O'Donnell et al. 2000), including the yeasts. Belloch et al. (2000) demonstrated the utility of cytochrome oxidase II for resolution of *Kluyveromyces* species, Daniel et al. (2001) successfully used actin-1 for species of *Candida*, and Kurtzman and Robnett (2003) showed the usefulness of elongation factor 1-α and RNA polymerase II for resolution of *Saccharomyces* species. At present, the main impediment to widespread use of gene sequences other than rDNA is developing sequencing primers that are effective for essentially all species, and construction of databases that include sequences from all known species. Daniel et al. (2001) and Daniel and Meyer (2003) have made considerable progress in development of an actin sequence database for species identification, although no primer set has been effective for all species, thus requiring additional primers to obtain these sequences. The need for multiple primers seems to be a problem common to sequencing of protein encoding genes because of frequent nucleotide substitutions. Resolution of taxa from actin is somewhat greater than from D1/D2, but not surprisingly, clear separation of closely related species is not always certain.

Separation of species using single gene sequences is not always reliable. Different lineages may vary in their rates of nucleotide substitution for the diagnostic gene being used, thus confusing interpretation of genetic separation, and hybrids are common and appear to be part of the speciation process. For example, Vaughan-Martini and Kurtzman (1985) proposed from DNA reassociation studies that *Saccharomyces pastorianus* is a natural hybrid of *S. cerevisiae* and *S. bayanus*. Peterson and Kurtzman (1991) confirmed the proposal by showing that the D2 domain rRNA sequence of *S. pastorianus* is identical to that of *S. bayanus,* but divergent from *S. cerevisiae*. The three varieties of *Candida shehatae* may also represent hybrids, or are examples of a lineage with a slow rate of nucleotide substitution in the rDNA. From DNA reassociation, the varieties show approximately 50% relatedness, but they have essentially identical domain 2 large subunit sequences (Kurtzman 1990). Groth et al. (1999) discovered a natural chimeric isolate of *Saccharomyces* with genetic material from three species, and Nilsson-Tillgren et al. (1981) presented evidence that several natural and industrial yeast strains are hybrids. Kurtzman et al. (2005) reported that *Kazachstania heterogenica* appears to be a natural hybrid that shares an RNA polymerase II gene with *K. pintolopesii*. In an additional study, Lachance et al. (2003) found interfertile strains of *Clavispora lusitaniae* that are highly polymorphic in the D1/D2 domain. Detection of unexpected divergence in a gene sequence should be possible from its lack of congruence with other gene sequences. Single gene sequences are extremely useful for rapid species identification, but from the foregoing examples, caution in interpretation of species identity is required.

Other molecular-based methods commonly used for species identification include species-specific primer pairs and probes, randomly amplified polymorphic DNA (RAPD), amplified fragment length polymorphisms (AFLP), restriction fragment length polymorphisms (RFLP), and karyotyping. Species-specific primers are effective when used for PCR-based identifications involving a small number of species or

when a particular species is the subject of the search (Fell 1993; Mannarelli and Kurtzman 1998). Otherwise, there is the likelihood that PCR mixtures containing large numbers of species-specific primer pairs will lead to uncertain banding patterns. Microsatellite-primed RAPDs (Gadanho et al. 2003) and AFLP fingerprints (de Barros Lopes et al. 1999) have been effectively used in some laboratories. One concern in using these latter two techniques is reproducibility between laboratories because small differences in PCR conditions may impact the species-specific patterns that serve as a reference. Karyotyping with pulsed-field electrophoresis and RAPD on mitochondrial DNA can serve in the initial characterization and identification of yeast species. However, the interpretation of the chromosome band patterns and mitochondrial restriction fragments for taxonomic purposes is complicated by the high degree of polymorphism, such as chromosomal rearrangements, within some yeast taxa (Spírek et al. 2003).

2.3 Molecular Phylogeny and Systematics of the Yeasts – an Overview

In the previous section, we discussed various molecular methods for species identification. In addition, many phylogenetic relationships among the yeasts and other fungi have been resolved from analysis of gene sequence divergence. These studies presume that horizontal gene transfer among different lineages has been limited, which can be tested by comparing the congruence of phylogenies derived from different genes. Most of the analyses have used rDNA sequences, but there are generally no major differences in tree topologies whether the analyses are from rDNA sequences or from those of other genes (Geiser et al. 1998; Liu et al. 1999; O'Donnell et al. 2000; Kurtzman and Robnett 2003). Although phylogenetic trees derived from analyses of various genes are generally congruent, support for basal lineages from single gene analyses is often weak (Kurtzman and Robnett 2003; Rokas et al. 2003). Because of this weak support, branching order is uncertain, leading to ambiguity of what constitutes a genus, a family or an order. Hawksworth et al. (1995) addressed this issue in part by stating "there are no universally applicable criteria by which genera are distinguished, but in general the emphasis is now on there being several discontinuities in fundamental characters ...". Many systematists now regard these fundamental characters as gene sequences. However, a number of factors impact our recognition of genera and higher levels of classification. Phylogenetic trees determined from single genes are seldom robust, leading to uncertainty whether neighboring species groups are a separate genus or members of a more broadly based genus. Multigene analyses generally strengthen support for basal lineages. Kurtzman and Robnett (2003) examined relationships among the approximately 80 species of the "*Saccharomyces* complex" from multiple genes. Combined analysis of 18S, 26S, 5.8S/conserved ITS and mitochondrial small subunit rDNAs with elongation factor 1-α and cytochrome oxidase II gave high bootstrap support for moderately deep lineages, which were interpreted as genus-level, but not for more basal lineages.

Rokas et al. (2003) screened the published genome sequences from seven *Saccharomyces* species and that of *Candida albicans* and selected 106 widely distributed

orthologous genes for phylogenetic analysis. The resulting analyses showed that a dataset comprising a concatenation of a minimum of nearly any 20 genes gave well-supported trees that were comparable to those of a dataset comprising 106 genes. This work clearly illustrates that a much larger number of genes is required for reconstructing phylogenies than is currently being analyzed in most laboratories. Whether 20 gene sequences will strongly resolve species clades larger than *Saccharomyces* needs to be determined. However, partial genome sequence analysis appears sufficient to resolve phylogenetic relationships within different groups of yeasts. Another factor that impacts resolution, as well as circumscription of genera, is the issue of missing taxa. It seems likely that fewer than 1% of extant species are known, which can be inferred from the high frequency of long single-species branches in phylogenetic trees. Consequently, the majority of the yeasts are yet to be discovered and characterized, and their addition to future phylogenetic analyses is likely to influence our perception of genera, even those that are presently circumscribed from multigene analyses.

2.4 Ascomycetous Yeasts

The distinction between yeasts and dimorphic filamentous fungi has often been uncertain. Some authorities have viewed the yeasts as primitive fungi, whereas others perceived them to be reduced forms of more evolved taxa (Cain 1972; Redhead and Malloch 1977). Phylogenetic analyses of rDNA sequences demonstrated the ascomycetous yeasts, as well as yeast-like genera such as *Ascoidea* and *Cephaloascus*, to comprise a clade that is a sister group to the "filamentous" ascomycetes (euascomycetes). *Schizosaccharomyces*, *Taphrina*, *Protomyces*, *Saitoella, Pneumocystis,* and *Neolecta*, a mushroom-like fungus, form a divergent clade basal to the yeast-euascomycete branch (Hausner et al. 1992; Hendriks et al. 1992; Kurtzman 1993b; Nishida and Sugiyama 1993; Wilmotte et al. 1993; Kurtzman and Robnett 1994, 1995, 1998; Landvik 1996; Sjamsuridzal et al. 1997; Sugiyama 1998; Kurtzman and Sugiyama 2001). Nishida and Sugiyama (1994) have termed the basal ascomycete clade the "archiascomycetes." Some members of the yeast clade, such as certain species of *Ascoidea* and *Eremothecium,* show no typical budding, whereas budding is common among the so-called black yeasts in the genera *Aureobasidium* and *Phialophora*, as well as in certain other dimorphic euascomycete genera. Similarly, vegetative reproduction by fission is shared by *Dipodascus* and *Galactomyces*, members of the yeast clade, as well as by the distantly related genus *Schizosaccharomyces*. Consequently, yeasts cannot be recognized solely on the basis of the presence or the absence of budding, but with a few exceptions, ascomycetous yeasts can be separated phenotypically from euascomycetes by the presence of budding or fission and the formation of sexual states unenclosed in a fruiting body.

During the past 10 years, the widespread use of molecular taxonomic methods has resulted in the discovery and description of a large number of new taxa, bringing the total of ascomycetous species to nearly 1,000. Many of these new species are readily detected by sequencing a single species-resolving gene, such as domains D1/D2 of large subunit rDNA, keeping in mind the exceptions discussed in the previous section. If we accept that fewer than 1% of extant species are known and that current sequencing technologies allow rapid detection of new species, the limiting

factor for presenting new species is the time required for formal description, which includes information on vegetative and sexual states, fermentation and assimilation reactions, and ecology, where known.

From single gene analyses, such as the D1/D2 phylogenetic trees presented by Kurtzman and Robnett (1998), it is apparent that many of the ascomycetous yeast genera are not well circumscribed, but actual boundaries are often not clear. Multigene sequence analyses have been applied to just a few genera, such as those of the "*Saccharomyces* complex", which includes *Saccharomyces*, *Kluyveromyces*, *Tetrapisispora*, *Torulaspora*, and *Zygosaccharomyces*, as well as the neighboring genera *Eremothecium*, *Hanseniaspora*, and *Saccharomycodes* (Kurtzman and Robnett 2003). In this multigene study, approximately 80 species were compared from the combined signal of seven genes. The analysis gave 14 phylogenetically defined clades, most of which had strong bootstrap support. From this study, the major genera *Saccharomyces*, *Kluyveromyces*, and *Zygosaccharomyces* were shown to be polyphyletic, leading to reclassification of certain of the species in the new genera *Naumovia*, *Nakaseomyces*, *Vanderwaltozyma*, *Zygotorulaspora*, and *Lachancea*, and expansion of the earlier described genus *Kazachstania* (Kurtzman 2003) (Fig. 2.1). Lineages basal to the branches supporting the 14 clades generally had low bootstrap support, leaving uncertain the genetic relationships among the genera. The genus *Eremothecium* appears separate from the family *Saccharomycetaceae* and was maintained in the *Eremotheciaceae*. Similarly, the sister genera *Hanseniaspora* and *Saccharomycodes*, which reproduce by bipolar budding rather than multilateral budding typical of the *Saccharomycetaceae*, were retained in the family *Saccharomycodaceae*. As demonstrated from this analysis, as well as that of Rokas et al. (2003), a relatively large number of gene sequences will be required to understand phylogenetic relationships among the yeasts. Currently accepted ascomycetous yeast genera are listed in Table 2.1 with their proposed assignments to orders and families. Because of weak basal resolution in phylogenetic trees, many of the genera cannot be reliably assigned to families. Furthermore, on the basis of the large amount of phylogenetic divergence conveyed by present datasets, it seems likely that many new families will need to be described.

From D1/D2 sequence analysis, the greater than 100 species assigned to the genus *Pichia* are seen to be distributed across the *Saccharomycetales* (Kurtzman and Robnett 1998). Major species groups in *Pichia* are centered on *P. membranifaciens*, *P. anomala*, and *P. angusta* (*Hansenula polymorpha*), the latter species representing the majority of methanol-assimilating taxa. Some of the species will be maintained in *Pichia* and some will need to be placed in new genera as stronger datasets become available. A few of the outlying species have already been assigned to new genera. *P. pastoris*, the outlying member of the methanol-assimilating yeasts, was transferred to *Komagataella* (Yamada et al. 1995a), and support for this genus as a distinct clade recently increased with the discovery of two additional species of *Komagataella* (Dlauchy et al. 2003; Kurtzman 2005). *P. burtonii*, now transferred to *Hyphopichia*, is phylogenetically distant from the three main clades of *Pichia*, as are the D-xylose-fermenting species *P. stipitis* and *P. segobiensis*. An additional change was the assignment of *P. ohmeri* to the genus *Kodamaea* (Yamada et al. 1995b). Support for this genus has increased with the discovery of additional species closely related to

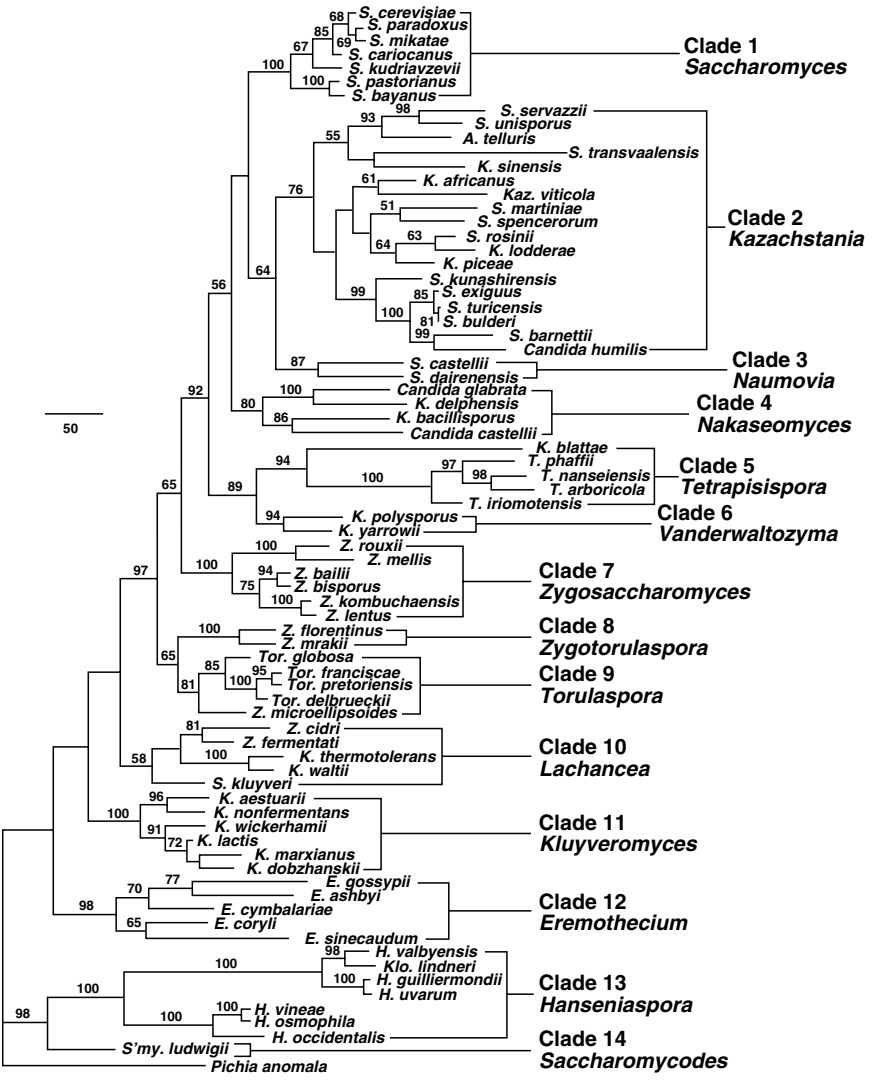

Fig. 2.1. Maximum parsimony tree resolving species of the "*Saccharomyces* complex" into 14 clades, which are interpreted as phylogenetically circumscribed genera. The analysis resulted in the description of five new genera. Earlier generic assignments are given for each species. This phylogenetic tree was derived from analysis of a dataset comprised of nucleotide sequences from 18S, 5.8S/alignable ITS, and 26S (three regions) *rDNAs*, elongation factor 1-α, mitochondrial small subunit rDNA and COXII. Branch lengths are based on nucleotide substitutions as indicated by the *bar*, and bootstrap values under 50% are not given. *Pichia anomala* is the outgroup species in the analysis. (Modified from Kurtzman 2003; Kurtzman and Robnett 2003)

Table 2.1 Classes, orders and families of yeasts and yeast-like genera of the *Ascomycota*

Neolectomycetes
Neolectales Landvik, O.E. Eriksson, Gargas & P. Gustafsson
Neolectaceae Redhead
Neolecta Spegazzini (T)

Pneumocystidomycetes
Pneumocystidales O.E. Eriksson
Pneumocystidaceae O.E. Eriksson
Pneumocystis P. Delanöe & Delanöe (A)

Schizosaccharomycetes
Schizosaccharomycetales Prillinger, Dörfler, Laaser, Eckerlein & Lehle ex Kurtzman
Schizosaccharomycetaceae Beijerinck ex Klöcker
Schizosaccharomyces Lindner (T)

Taphrinomycetes
Taphrinales Gäumann & C.W. Dodge
Protomycetaceae Gray
Burenia M.S. Reddy & C.L. Kramer (T)
Protomyces Unger (T)
Protomycopsis Magnus (T)
Saitoella S. Goto, Sugiyama, Hamamoto & Komagata (A)
Taphridium Lagerheim & Juel ex Juel (T)
Volkartia Maire (T)
Taphrinaceae Gäumann & C.W. Dodge
Lalaria R.T. Moore (A)
Taphrina Fries (T)

Saccharomycetes
Saccharomycetales Kudryavtsev
Ascoideaceae J. Schröter
Ascoidea Brefeld & Lindau (T)
Cephaloascaceae L.R. Batra
Cephaloascus Hanawa (T)
Dipodascaceae Engler & E. Gilg
Dipodascus Lagerheim (T)
Galactomyces Redhead & Malloch (T)
Geotrichum Link:Fries (A)
Endomycetaceae J. Schröter
Endomyces Reess (T)
Helicogonium W.L. White (T)
Myriogonium Cain (T)
Phialoascus Redhead & Malloch (T)
Eremotheciaceae Kurtzman
Coccidiascus Chatton emend. Lushbaugh, Rowton & McGhee (T)
Eremothecium Borzi emend. Kurtzman (T)
Lipomycetaceae E.K. Novak & Zsolt
Babjevia van der Walt & M.Th. Smith (T)
Dipodascopsis Batra & Millner (T)
Lipomyces Lodder & Kreger van Rij (T)
Myxozyma van der Walt, Weijman & von Arx (A)
Zygozyma van der Walt & von Arx (T)
Metschnikowiaceae T. Kamienski
Clavispora Rodrigues de Miranda (T)
Metschnikowia T. Kamienski (T)

Continues

Table 2.1 Classes, orders and families of yeasts and yeast-like genera of the *Ascomycota—cont'd*

Pichiaceae Zender
Brettanomyces Kufferath & van Laer (A)
Dekkera van der Walt (T)
Pichia Hansen (*pro parte*) (T)
Saturnispora Liu & Kurtzman (T)
Saccharomycetaceae G. Winter
Kazachstania Zubkova (T)
Kluyveromyces Kurtzman, Lachance, Nguyen & Prillinger (T)
Lachancea Kurtzman (T)
Nakaseomyces Kurtzman (T)
Naumovia Kurtzman (T)
Saccharomyces Meyen ex Reess (T)
Tetrapisispora Ueda-Nishimura & Mikata (T)
Torulaspora Lindner (T)
Vanderwaltozyma Kurtzman (T)
Zygosaccharomyces Barker (T)
Zygotorulaspora Kurtzman (T)
Saccharomycodaceae Kudryavtsev
Hanseniaspora Zikes (T)
Kloeckera Janke (A)
Saccharomycodes Hansen (T)
Saccharomycopsidaceae von Arx & van der Walt
Saccharomycopsis Schiönning (T)

Saccharomycetales incertae sedis
Aciculoconidium King & Jong (A)
Ambrosiozyma van der Walt (T)
Arxula van der Walt, M.Th. Smith & Y. Yamada (A)
Ascobotryozyma J. Kerrigan, M.Th. Smith & J.D. Rogers (T)
Blastobotrys von Klopotek (A)
Botryozyma Shann & M.Th. Smith (A)
Candida Berkhout (A)
Citeromyces Santa María (T)
Cyniclomyces van der Walt & Scott (T)
Debaryomyces Lodder & Kreger-van Rij (T)
Hyphopichia von Arx & van der Walt (T)
Kodamaea Y. Yamada, T. Suzuki, Matsuda & Mikata emend. Rosa, Lachance, Starmer, Barker, Bowles & Schlag-Edler (T)
Komagataella Y. Yamada, Matsuda, Maeda & Mikata (T)
Kuraishia Y. Yamada, Maeda & Mikata (T)
Lodderomyces van der Walt (T)
Macrorhabdus Tomaszewski, Logan, Snowden, Kurtzman & Phalen (A)
Nadsonia Sydow (T)
Nakazawaea Y. Yamada, Maeda & Mikata (T)
Ogataea Y. Yamada, Maeda & Mikata (T)
Pachysolen Boidin & Adzet (T)
Phaffomyces Y. Yamada, Higashi, S. Ando & Mikata (T)
Schizoblastosporion Ciferri (A)
Sporopachydermia Rodrigues de Miranda (T)
Starmerella Rosa & Lachance (T)
Starmera Y. Yamada, Higashi, S. Ando & Mikata (T)
Stephanoascus M. Th. Smith, van der Walt & Johannsen (T)
Sympodiomyces Fell & Statzell (A)

Table 2.1 Classes, orders and families of yeasts and yeast-like genera of the *Ascomycota—cont'd*

Trichomonascus Jackson (T)
Trigonopsis Schachner (A)
Wickerhamia Soneda (T)
Wickerhamiella van der Walt (T)
Yamadazyma Billon-Grand emend. M. Suzuki, Prasad & Kurtzman (T)
Yarrowia van der Walt & von Arx (T)
Zygoascus M.Th. Smith (T)

[1](A) = Anamorphic genus, (T) = Telemorphic genus.
[2]Anamorphic and teleomorphic genera are placed together in the same family when relationships are known. For many anamorphic and teleomorphic genera, phylogenetic relationships are unclear and the genera are placed in Saccharomycetales *incertae sedis* until family relationships become known.

K. ohmeri (Rosa et al. 1999). On the basis of single gene analyses, species of the *Lipomycetaceae* and such genera such as *Yarrowia*, *Citeromyces*, and *Saccharomycopsis* appear to be natural groups. *Metschnikowia*, which is characterized by elongated, needlelike ascospores, is represented by a large number of phylogenetically divergent species, but molecular data are insufficient to determine if the genus is monophyletic. Consequently, multigene sequence analysis will be required to resolve relationships between the preceding genera as well as for determining relationships within the genera.

2.5 Basidiomycetous Yeasts

The division *Basidiomycota* is a group of approximately 30,000 described species, with a distinct sexual cycle that includes the production of spores on a clublike structure (basidium). The majority of the species, which are easily recognized as mushrooms, bracket fungi, rusts, and smuts, produce filamentous hyphae and do not have a yeast phase. The recognition of a phylogenetic connection between yeasts and basidiomycetes was slow to evolve. An initial observation of the presence of ballistoconidia led Kluyver and van Niel (1924, 1927) to suggest that *Sporobolomyces* was related to the basidiomycetes. An often overlooked basidiomycete connection was provided by Nyland's (1949) description of the teliosporic genus *Sporidiobolus*. Subsequently, Banno's (1967) description of a teliosporic life cycle in *Rhodosporidium toruloides* gave a solid recognition to the presence of basidiomycetes among the yeasts. That discovery was followed by descriptions of several teleomorphic genera, including *Filobasidium* (Olive 1968), *Leucosporidium* (Fell et al. 1969), *Filobasidiella* (Kwon-Chung 1975), *Cystofilobasidium* (Oberwinkler et al. 1983) and *Bulleromyces* (Boekhout et al. 1991). The phylogenetic relationship between the genera and to the anamorphic species remained open to conjecture until sequence analyses became readily available.

Many researchers explored basidiomycete phylogeny, and a particularly significant report (Swann and Taylor 1995) of 18S rDNA analysis found that basidiomycetous

yeasts occur in three classes: *Uredinomycetes*, *Hymenomycetes*, and *Ustilagenomycetes*. The *Hymenomycetes* are generally associated with the jelly fungi (*Tremellales*). Yeasts are found within four major clades of the *Hymenomycetes*: *Tremellales*, *Trichosporonales*, *Filobasidiales*, and *Cystofilobasidiales*. The *Uredinomycetes*, which are often linked with the rust fungi, include four major clades of yeasts and related genera: *Agaracostilbales*, *Microbotryales*, *Sporidiobolales*, and the *Naohidea* clade. The majority of the *Ustilaginales* are plant and fungal parasites, with the smuts as well-known examples. Sampaio (2004) reported three major groups in the Ustilaginales: Entorrhizomycetidae, Exobasidiomycetidae, and Ustilaginomycetidae. Yeasts are found in the latter two subclasses.

A list of the genera assigned to the three classes (Table 2.2) was modified from the information provided by Scorzetti et al. (2002) and Sampaio (2004). An observation of note is the presence of anamorphic genera *Cryptococcus*, *Rhodotorula*, and *Sporobolomyces* in more than one phylogenetic group. Historically, anamorphic genera were described on phenotypic characteristics. For example, the genus *Rhodotorula* was originally delineated by the characteristic red color of the colony, although species with white and cream colonies were subsequently included in the genus (Weijman et al. 1988). A cursory identification of a red yeast as *Rhodotorula* has a high probability of being correct, however, the color is not phylogenetically specific. These phenotypic names are temporarily being maintained, with conversion to teleomorphic nomenclature as sexual cycles and species relationships are determined. For example, Sampaio et al. (2004) found the complete sexual cycle of

Table 2.2 Classes and orders of yeasts and yeast-like genera of the *Basidiomycota*

Hymenomycetes
Cystofilobasidiales Boekhout & Fell
Cystofilobasidium Oberwinkler & Bandoni (T)
Cryptococcus Vuillemin (A)
Guehomyces Fell & Scorzetti (A)
Itersonilia Derx (A)
Mrakia Y. Yamada & Komagata (T)
Phaffia Miller, Yoneyama & Soneda (A)
Tausonia Bab'eva (A)
Udeniomyces Nakase & Takematsu (A)
Xanthophyllomyces Golubev (T)
Filobasidiales Julich
Cryptococcus Vuillemin (A)
Filobasidium Olive (T)
Trichsporonales Boekhout & Fell
Cryptococcus Vuillemin (A)
Trichosporon Behrend (A)
Tremellales Rea emend. Bandoni
Auriculibuller Sampaio (T)
Bullera Derx (A)
Bulleribasidium Sampaio, Weiss & Bauer (T)
Bulleromyces Boekhout & Fonseca (T)
Cryptococcus Vuillemin (A)

Table 2.2 Classes and orders of yeasts and yeast-like genera of the *Basidiomycota—cont'd*

Cuniculitrema Sampaio & Kirschner (T)
Dioszegia Zsolt emend.Takashima, Deak & Nakase (A)
Fellomyces Y. Yamada & Banno (A)
Filobasidiella Kwon-Chung (T)
Holtermannia Saccardo & Traverso (T)
Kockovaella Nakase, Banno & Y. Yamada (A)
Sirobasidium Lagerheim & Patouillard (T)
Sterigmatosporidium Kraepelin & Schulze (T)
Tremella Persoon (T)
Trimorphomyces Bandoni & Oberwinkler (T)
Tsuchiyaea Y. Yamada, Kawasaki, M. Itoh, Banno & Nakase (A)

Uredinomycetes

Agaricostilbales Oberwinkler & Bauer
Agaricostilbum Wright emend. Wright, Bandoni & Oberwinkler (T)
Bensingtonia Ingold emend. Nakase & Boekhout (A)
Chionosphaera Cox (T)
Kondoa Y. Yamada, Nakagawa & Banno emend. Fonseca et al. (T)
Kurtzmanomyces Y. Yamada, M. Itoh, Kawasaki, Banno & Nakase emend. Sampaio (A)
Sporobolomyces Kluyver & van Niel (A)
Sterigmatomyces Fell emend. Y. Yamada & Banno (A)
Microbotryales
Bensingtonia Ingold emend. Nakase & Boekhout (A)
Curvibasidium Sampaio & Golubev (T)
Leucosporidiella Sampaio (A)
Leucosporidium Fell, Statzell, Hunter & Phaff (T)
Mastigobasidium Golubev (T)
Reniforma Pore & Sorenson (A)
Rhodotorula Harrison (A)
Rhodosporidium Banno (T)
Sporobolomyces Kluyver & van Niel (A)
Naohidea clade
Bannoa Hamamoto (T)
Erythrobasidium Hamamoto, Sugiyama & Komagata (T)
Naohidea Oberwinkler (T)
Rhodotorula Harrison (A)
Sakaguchia Y. Yamada, Maeda & Mikata (T)
Sporobolomyces Kluyver & van Niel (A)
Sporidiobolales Sampaio,Weiss & Bauer
Rhodotorula Harrison (A)
Rhodosporidium Banno (T)
Sporidiobolus Nyland (T)

Ustilaginomycetes

Rhodotorula Harrison (A)
Sympodiomycopsis Sugiyama, Tokuoka & Komagata (A)
Malassezia Baillon (A)
Pseudozyma Bandoni emend. Boekhout (A)
Tilletiopsis Derx ex Derx (A)

[1](A) = Anamorphic genus, (T) = Telemorphic genus.
[2]Some genera, such as the anamorphic genus *Cryptococcus*, are presently polyphyletic as defined, and members of the genus are found in more than one teleomorphic order.

Rhodotorula fujisanensis, for which they described the genus and species *Curvibasidium cygneicollum*. Wholesale description of new anamorphic genera on the basis of clade relationships should be avoided. These nomenclatural changes would result in temporary taxonomic fixes that would be confusing and potentially phylogenetically incorrect.

The development of extensive basidiomycetous rDNA (ITS and D1/D2) databases (Fell et al. 2000; Scorzetti et al. 2002) provided a springboard for sizeable expansion in the rate of the descriptions of new species and genera. The number of genera increased from 33 (Kurtzman and Fell 1998) to approximately 55 (Sampaio 2004). The increase in number of species can be exemplified by the genus *Trichosporon*: 19 (Guého et al. 1998) to 36 (Fell and Scorzetti 2004). Importantly, the resulting phylogenetic trees indicate the extent of genetic diversity and the extent of relationships between species, including the anamorphic and teleomorphic species.

The definition of a basidiomycetous species, based on sequence analysis, needs considerable attention. As previously discussed, zero to three nucleotide differences among ascomycetous yeasts in the D1/D2 region generally signifies strains within a single species. This general concept is not always applicable among basidiomycetes. Several significant examples exist, which demonstrate that other genetic regions must be examined to distinguish taxa. *Mrakia gelida* and *M. frigida* are identical in the D1/D2 and significantly different in the ITS and IGS regions (Diaz and Fell 2000). Similarly, the pairs *Filobasidiella neoformans*: *F. bacillispora* and *Phaffia rhodozyma*:*Xanthophyllomyces* type strains differ by one base pair in the D1/D2 domains and significantly in the ITS and IGS regions (Fell and Blatt 1999; Scorzetti et al. 2002).

2.6 Rapid Identification of Yeasts from Ecological Studies

Prior to the existence of molecular phylogeny, ecological research on basidiomycetous yeasts was hampered by reliance on phenotypic characteristics. As a consequence, there was a generalized concept that many of the species have worldwide distributions in diverse environments. This concept was particularly true for species such as *Cryptococcus albidus* and *Rhodotorula glutinis*. Fonseca et al. (2000) dispelled this concept by demonstrating that *C. albidus* is a complex of 12 species. This concept is further dispelled as established species and their phenotypic synonyms are being examined, e.g., *Rhodosporidium* (Sampaio et al. 2001), and as new species are being described that are phenotypically indistinguishable from related and unrelated species, e.g., *Trichosporon* (Middelhoven et al. 2004).

A major ecological problem is that estimates indicate that only 1% of the yeast species in nature have been described. Yeast ecology, therefore, is at a stage of discovery. The ability to undertake biocomplexity studies, viz., environmental/population interactions, is difficult, if the individual players (species) are unknown. A case in point is an ongoing study of yeast populations in the Florida Everglades (Fell and Statzell-Tallman, unpublished). This study involves quarterly (seasonal) sampling in a subtropical Everglades watershed that ranges from freshwater marshes to seawater mangrove habitats. The number of cells ranges from 100 to 2,700 per liter of water. These variations in density correlate with sample location and season of the year.

Preliminary data demonstrate that the biodiversity in the water is comprises 23 genera and 120 species of basidiomycetes and ascomycetes. The undescribed species in these collections represented 54% of the taxa. More than 800 strains were sequenced during this study. In order to examine a large number of strains, ecological studies are most efficiently undertaken with the assistance of a high throughput sequencer. These instruments can sequence approximately 1,000 strains/day. Throughputs of this magnitude are beyond the capabilities of the average single researcher to manipulate and analyze on a daily basis; however, large numbers of strains can be sequenced on a weekly and monthly basis.

Studies of complex unknown communities, such as illustrated with the Florida Everglades program, are most efficiently studied by direct sequence analysis of the species. In contrast, monitoring of ecological niches with known community structure can employ a variety of techniques, including temperature and denaturing gradient gel electrophoreses (TGGE and DGGE), single-strand conformation polymorphism (SSCP), terminal RFLP (T-RFLP), amplified rDNA restriction analysis (ARDRA) and amplified ribosomal intergenic spacer analysis (ARISA). The application of these techniques to fungal ecology was reviewed by Anderson and Cairney (2004). By necessity, all of these techniques must be confirmed by sequence analysis. Therefore, the bias and research concentration in our laboratories has been directed to sequence-based identification methods.

PCR-based species-specific primers represent the least expensive and easiest method for identification of small numbers (one to ten) of taxa (Chapman et al. 2003). Multiplex PCR of larger numbers of primers in a single reaction is difficult owing to various factors, such as formation of primer dimers and differences in temperature requirements for primer hybridizations. Our laboratories have been exploring a high-throughput probe hybridization method for detection of multiple species in multiple samples. The method (Diaz and Fell 2004; Page and Kurtzman, 2005) is an adaptation of the Luminex xMAP technology (Luminex Corporation), which consists of a combination of 100 different sets of fluorescent beads covalently bound to species-specific capture probes. Upon hybridization, the beads bearing the target amplicons are classified in a flow cytometer by their spectral addresses with a 635-nm laser. The hybridized biotinylated amplicon is quantitated by fluorescence detection with a 532-nm laser. The multiplex assay is specific and fast: species that differ by one nucleotide can be discriminated and the assay can be performed, after amplification, in less than 50 min in a 96-well format with as many as 100 different species-specific probes per well. The advantage of this method for ecological research is that multiple species can be identified from multiple samples, such as water and soil. In practice, DNA is extracted followed by the Luminex xMAP procedures. This technique is applicable to a variety of ecological monitoring strategies. For example, we have been successfully employing this method for the analysis of sewage-associated bacteria in waters adjacent to marine recreational beaches (Fell et al., unpublished).

In summary, rapid detection and accurate identification of yeasts is now possible through use of a variety of molecular methods. Application of these methods will bring a greater degree of clarity to studies in yeast ecology, which previously was not possible when yeasts were identified from the phenotype.

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Chapter 3

Yeast Biodiversity and Culture Collections

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3.1 Introduction

It is in the nature of humans to collect, to classify, to organize and to summarize their findings. From the early days of taxonomic surveys and collecting fieldtrips, scientists and amateurs have been gathering samples and evidence related to their discoveries. In those days, the only way to keep track of previous findings was to describe the morphology, the ecology and some basic physiological properties. Species of yeasts were then often described on the basis of one or several specimens but the latter were not always mentioned and/or properly described, analyzed and preserved. When it became mandatory to designate type specimens, for every new species to be described and to keep them available for future reexaminations, the need for proper storage of the material was raised. The first and most obvious way of doing this was by the drying of living specimens and the creation of herbaria. Dried, nonliving material is of great importance in mycology but of limited use for yeasts, since the physiological, biochemical, genetic and ecological properties cannot be properly studied or reexamined. In order to perform more advanced investigations and analyses on previously collected specimens, they had to be kept alive and maintained in a condition as close as possible to their "original" state. This is where the culture collections (CCs) came into the picture, which still play a key role in the preservation of the yeast biodiversity (Agerer et al. 2000; Hawksworth 2004). Proper preservation methods have been mainly developed by scientific staff working for CCs (Mikata and Banno 1989; Tan et al. 1991, 1994, 1995, 1998; Tan 1997; Tan and van Ingen 2004). CCs often host a pool of scientists who study the strains of the collection and produce a wealth of new and valuable data on these strains. CCs are not only large biological storage and distribution facilities, they are also information and references centers that provide advice, courses, training and valuable information through their catalogues and more recently the Internet. Consequently, other researchers working in academic institutions, clinics or industry benefit greatly from the services of CCs.

3.2 Strains and Species

In the past, most members of the scientific community considered the species as the basis of the exchange of information. Nowadays, researchers are increasingly using information at the strain level. The definition of a yeast species is a summary made by systematicians and is based on the observation of taxonomically informative characteristics of one or several strains, thought to belong to the same biological, phenetic and/or phylogenetic entity. These entities can be large, like *Saccharomyces cerevisiae*, or small when a species is known from a single strain only. The size or the volume of species clusters does not only depend on the number and the heterogeneity of the strains used for its circumscription, but also on the variability of the set of characteristics that is considered. Known taxonomic information associated with the species is not always relevant or sufficient for workers in other disciplines. For example, people working in industry, in ecology, in biotechnology or in clinical settings may not be satisfied with a species name. They may require data related to the resistance to heat in sterilization procedures that can be strain- and not species-dependent, or they may want to know the transformation potential of strains in complex ecological cycles. Clinicians are usually interested in the pathogenicity and the resistance of the strains against antibiotics. As many properties can be variable within a given species, it is of the utmost importance to record and keep data related to the strains and to be able to dynamically create homogeneous groups of strains that share the same phenotypic profiles. Species are often subjective entities and their circumscriptions, definitions and volumes are variable and may evolve with time, the methods used to study them and the understanding of the taxonomists. When properly preserved, strains may keep the same properties and can therefore be used and reused in many different types of studies, such as physiology, genetics or biotechnology. All previously recorded data associated with a given strain can be complemented with new ones. This allows continuous updating and improvement of our understanding of the mechanisms underlying the basal processes connected with metabolism, pathogenicity and many other strain-related characteristics and properties. It also permits some cross-character correlations to be performed that would not be possible or desirable otherwise when working with species only.

Strain preservation, characterization, identification, analysis and distribution are the core business of CCs. Therefore, and even more than herbaria and museums, CCs remain the ideal place for taxonomic endeavors. In the past, systematic work was usually the main task of CCs, but this is not the case anymore in many cases, as applied research is becoming more and more important. In addition, many CCs tend to specialize (viz., mutant collections, specialized applications in fermentation industries, screening for new biologically active compounds). However, several major yeasts CCs have hosted most of the editors of major monographs on yeasts [e.g., Lodder 1934, 1970; Diddens and Lodder 1942; Wickerham 1951; Lodder and Kreger-van Rij 1952; Kreger-van Rij 1984; Yarrow (in Barnett et al. 1983, 1990a, 2000a); Kurtzman and Fell 1998]. This made sense since those researchers had access to all type strains and had the possibility to review them by

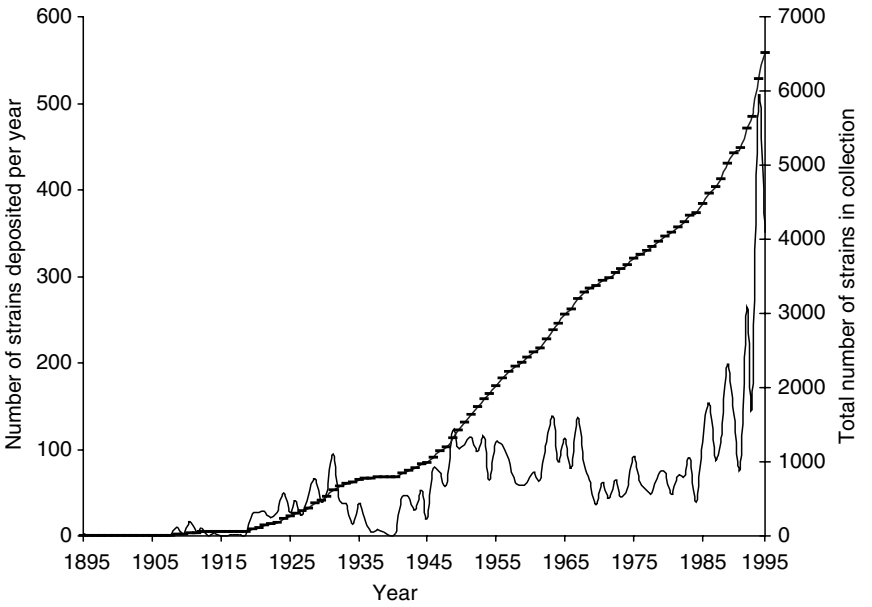

Fig. 3.1 Number of strains deposited in the yeast collection of the Centraalbureau voor Schimmelcultures (CBS) between 1895 and 2004. The *smooth line* represents the annual deposit, while the *line with bars* represents the cumulative curve

applying the new methods that became available. Since the early days of yeast taxonomy, the methods employed for the characterization of yeasts have constantly evolved. In the early days, only microscopically and macroscopically visible morphological features were recorded. Later, some basic physiological tests were introduced and a number of them are still used in today's standard testing panels. More recently, biochemical data were added (viz., cell wall carbohydrates, coenzyme Q, GC contents), followed by sequence data of the ribosomal genes. Certainly, one can expect more new features to be included in the descriptions panels, such as transcriptome, proteome and metabolome data. Modern CCs are not only able to keep strains in the original condition, but they also archive all previously recorded data at the unit level (strains); therefore, they allow researchers to build on existing knowledge and to concentrate their efforts on new developments. Since species are usually or ideally based on several units (strains, specimens), the addition of new data cannot be linked to them in a straightforward manner, as previously collected data should be reviewed again. With the growing number of species described and accepted, namely182 in 1952 (Lodder and Kreger-van Rij), 341 in 1970 (Lodder), 518 in 1984 (Kreger-van Rij), 597 in 1990 (Barnett et al. 1990a), 678 in 2000

(Barnett et al. 2000a) and more than 900 in 2004 (Robert et al. 2004), it has become almost impossible to reanalyze all these species and their related strains, thus stressing the value of maintaining strain-related data.

Recently, a trend emerged to isolate more strains from the environment and to explore new niches (see the annual and cumulative deposit at the Centraalbureau voor Schimmelcultures,CBS, CC in Fig. 3.1).

There are three good reasons for this. The first one is that new methods such as DNA sequencing are now relatively cheap, easy, reliable and, thanks to the pioneering work of some yeast taxonomists, complete databases are available for identification. The second reason is that the increased rate of destruction of our environment makes it important to investigate "unconventional" niches. The third one relates to the work of biotech companies that are isolating and screening large numbers of strains for biologically interesting compounds. Figure 3.2 shows the geographic origin of the currently available strains in the CBS CC. Although the analysis is obviously biased since only 66% of the exact geographic coordinates of the strains are available, it can easily be seen that most activity has been performed in some "hot spots" such as western Europe, Japan, the USA, South Africa and some coastal regions.

Countries in dark shades have been poorly investigated until now, usually because they lack the facilities needed, research labs and CCs. The presence of active researchers working in previously nonsampled areas can be very "rewarding", and result in the discovery of many species new to science. The example of J. van der Walt in South Africa is very striking. Before he started collecting, very few strains, if any, were isolated from South Africa. During his long and fruitful career, he and his colleagues collected, described and published many new findings that were all deposited in CCs and are still available now. H.J. Phaff and M.-A. Lachance, among many others, also did pioneering work by collecting previously ignored or less sampled areas or substrates like cacti or insects. From our experience of fieldtrips to South America, Africa and Asia, we estimate that roughly 50% of the material collected, which mainly originated from flowers, represented new species. Strikingly, a number of isolates originating from different continents belonged to the exact same new species (not yet published but available from the CBS CC). Suh et al. (2004) and Suh and Blackwell (2005) found that almost one third of their 650 isolates from beetles represented new species. The same kinds of findings were obtained by other researchers working on beetles who found the same species distributed all over the American continent (Lachance et al. 1998; Rosa et al. 1999). This suggests that even with a statistically nonrepresentative number of isolates, we can still discover new species that have a pandemic distribution (Robert et al. 1998; Hawksworth 2004; Suh et al. 2004; Suh and Blackwell 2005). This means that our understanding of yeast biodiversity is still extremely poor, which is in agreement with a number of publications in which the importance of fungal biodiversity, particularly in tropical and threatened or fragile environments, was discussed (Hawksworth 1991, 2001, 2004; Bills and Polishook 1994; Hawksworth and Rossman 1997; Hyde and Hawksworth 1997; Rossman 1997; Lachance and Starmer 1998; Prance et al. 2000).

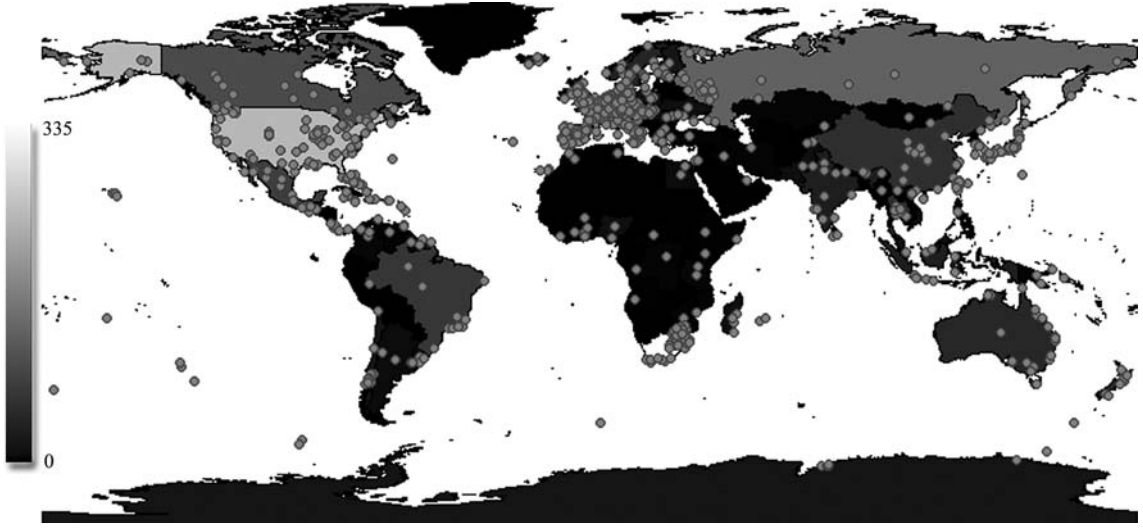

Fig. 3.2. Geographical distribution of the isolation localities of yeast strains deposited at the CBS collection. *Dots* represent localities where the strains were isolated. Countries from where many strains have been isolated are in *white* (South Africa, Japan and many countries in western Europe) although they are not always visible owing to the large number of strain-related dots. Countries in *black* have been sampled less

3.3 Culture Collections, Diversity and Expertise

CCs are hosting a wide range of activities and scientific disciplines. Statistics maintained by the World Data Centre for Microorganisms (WDCM, at http://wdcm.nig.ac.jp) show this diversity of objectives and focus points amongst the various CCs. Four hundred and eighty-nine CCs from over 65 countries are registered in the WDCM system. Most of them are governmental bodies (33%), semigovernmental bodies (7%) or linked to universities (27%). Only a small proportion of the CCs are supported by private institutions or industry (5%). There was no information available for the other 28%. A total of 2,700 researchers, technicians and administrative staff are employed by the CCs and about one third of them produce a catalog. Slightly fewer than half of the CCs provide storage, distribution, identification, training and consulting services. As can be seen from Tables 3.1 and 3.2, most of the collections are located in Europe, Asia or America. Very few are situated in Africa, although this continent is likely to hold a large portion of the world's biodiversity of yeasts. The geographic distribution of the CCs can be directly correlated to the origin of the yeast strains present in the CBS CCs (Fig. 3.2). This implies means that huge effort should be invested to set up CCs in Africa and other less developed parts of the world and to properly train the staff to enable them to collect the many yeast strains that are available in their countries. However, the setup and maintenance of new CCs is costly, and it is unlikely that many African governments will be able or tempted to support such infrastructures, especially in times where struggles against poverty, hunger and disease are legitimate priorities. Presently, existing well-established CCs can help in the training of scientists from less developed countries and can provide them with the facilities they need to store and study their strains. It is also the duty of CCs to initiate and support forays in those unexplored areas, which is in agreement with the Rio Convention on Biodiversity (CBD).

In Table 3.2, some of the largest CCs that accept, maintain and/or work with yeasts are listed with their URLs. CCs that are not members of the World

Table 3.1 Geographic origins and type of services provided by the culture collections (*CCs*). (Data from World Data Centre for Microorganisms)

Region	Patent deposit	Storage service	Distribution service	Identification service	Training offered	Consultancy service	No. of CCs/ no. of cultures
Africa	1	4	4	6	23	8	10/8,540,
Asia	13	54	52	59	168	121	161/248,845
Europe	47	86	93	92	243	232	161/487,405
America	7	42	56	53	164	132	114/319,179
Pacific	1	14	17	22	28	36	43/88,206
Total	69	200	222	232	626	529	489/1,152,175

Table 3.2 Some of the major CCs working with yeasts strains ordered by descending number of publicly available strains.

Acronym	Name	Country	No. of yeasts	Internet address
NRRL	National Center For Agricultural Utilization Research	USA	14,500	http://nrrl.ncaur.usda.gov
CBS	Centraalbureau voor Schimmelcultures	Netherlands	6,600	http://www.cbs.knaw.nl
UCD	University of California Davis, Herman J. Phaff Collection	USA	6,000	http://www.phaffcollection.org
UWO-PS	Department of Biology, University of Western Ontario	Canada	>5,000	
DBVPG	Dipartimento di Biologia Vegetale e Biotecnologia Agroambientale	Italy	4,500	http://www.agr.unipg.it/dbvpg/home.html
CCY	Culture Collection of Yeasts	Slovakia	3,800	http://www.chem.sk/activities/yeast/ccy/
BCCM/ IHEM	Scientific Institute of Public Health – Louis Pasteur	Belgium	3,500	http://www.belspo.be/bccm
AMFC	Australian Medical Fungal Collection, Westmead Hospital in Sydney	Australia	3,500	http://www.mmrl.med.usyd.edu.au
NBRC (former IFO)	NITE Biological Resource Center	Japan	>3,150	http://www.nbrc.nite.go.jp
NCYC	National Collection of Yeast Cultures	UK	3,100	http://www.ncyc.co.uk
CECT	Coleccion Espanola de Cultivos Tipo	Spain	2,500	http://www.uv.es/cect
JCM	Japan Collection of Microorganisms, Bioresource Center	Japan	2,400	http://www.jcm.riken.jp
VKM	All-Russian Collection of Microorganisms	Russia	2,300	http://www.vkm.ru
BCCM/ MUCL	Mycotheque de l'Universite catholique de Louvain	Belgium	2,200	http://www.belspo.be/bccm
ATCC	American Type Culture Collection	USA	>2,000	http://www.lgcpromochem.com
LCC	Labatt Culture Collection, Technology Development	Canada	2,000	http://www.labatt.com
ZIM	Culture Collection of Industrial Microorganisms	Slovenia	1,740	http://www.bf.uni-lj.si/zt/bioteh/chair/index.html

Continues

Table 3.2 Some of the major CCs working with yeasts strains ordered by descending number of publicly available strains.—*cont'd*

Acronym	Name	Country	No. of yeasts	Internet address
BCRC	Bioresource Collection and Research Center	Taiwan	1,564	http://www.ccrc.firdi.org.tw
PYCC	Portuguese Yeast Culture Collection	Portugal	>1,500	
NCAIM	National Collection of Agricultural and Industrial Microorganisms	Hungary	>1,200	http://ncaim.uni-corvinus.hu
VTT	VTT Biotechnology, Culture Collection	Finland	1,120	http://www.vtt.fi/bel/services/culture_collection.htm
CLIB	Collection de Levures d'Interet Biotechnologique Collection of Yeasts of Biotechnological Interest	France	1,000	http://www.inra.fr/clib/
TISTR	TISTR Culture Collection Bangkok	Thailand	650	http://www.tistr.or.th
AWRI	Institute of Molecular and Cellular Biosciences, The University of Tokyo	Japan	600	http://www.awri.com.au
CCOC	Coleção de Culturas Oswaldo Cruz	Brazil	>500	http://sicol.cria.org.br/crb?INCQS
DSMZ	Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH	Germany	500	http://www.dsmz.de
IAM	Institute of Molecular and Cellular Biosciences, The University of Tokyo	Japan	450	http://www.iam.u-tokyo.ac.jp/misyst/ColleBOX/en/about.html
KCTC	Korean Collection for Type Cultures	South Korea	225	http://www.brc.re.kr
KACC	Korean Agricultural Culture Collection	South Korea	62	http://kacc.rda.go.kr
CBTC-CCAS	The Cell Bank of Type Culture Collection of Chinese Academy of Sciences	China	?	http://www1.im.ac.cn/typecc/en.html

Federation of Culture Collections (WFCC) have been omitted, as they usually do not have the required structure for distribution, and indeed they rarely distribute strains on a regular basis to third parties. There are also large collections outside the public domain (e.g., pharmaceutical or biotech companies) that are not included in the statistics displayed in Tables 3.1 and 3.2.

Out of the 1,152,175 microbial strains that are maintained in public CCs worldwide, about 400,000 are fungi, including some 80,000 yeasts. The largest yeast CCs are biodiversity collections that maintain living-type specimens and related strains. The North American collections, with the NRRL, the UCD, the UWO and the ATCC, maintain almost 30,000 strains of yeasts. The ATCC is a generalist collection with a strong focus on *Saccharomyces* mutants. The others are general biodiversity collections but are also developing projects with industry. In South America some large institutions, like the Fiocruz in Brazil, the Corpoica in Colombia and several others in Mexico, Cuba, Chile and Argentina, have CCs. Europe hosts various yeast CCs. The CBS is the largest among them and focuses, like BCCM/MUCL, IGC, DSMZ and VKM, mainly on type strains and general yeast biodiversity, but they also maintain industrially important strains, notably under the Budapest Treaty. The NCYC, DBVPG, CECT, NCAIM, ZIM and CLIB, as well as some others, are more specialized CCs and work in close connections with food and agriculture related industries, e.g., the wine and beer industries. In Asia, several major CCs have a long history in zymology and many taxonomists have been active in both the NRBC (former IFO) and the JCM in Japan. In addition to industry-associated research, they remain active in the field of biodiversity studies in general and develop projects linking several Asian institutions through bilateral and/or multilateral projects (e.g., through the MIRCEN Network). Institutions like Biotech and TISTR in Thailand host growing collections of yeasts that are used to screen the biodiversity for potential industrial and pharmaceutical applications. Researchers associated with the Chinese Academy of Science in Beijing are involved in studies on China's vast yeast biodiversity, which resulted in the discovery of many new species. As said before and with the notable and important exception of South Africa, Africa lacks important yeast-associated CCs.

3.4 Preservation and Stability

Proper preservation of yeast cultures is essential if one wants to maintain them for future investigations. As the cultures need to keep the same morphological and physiological properties, preservation studies and media optimization have received considerable attention at CCs. The early-used methods consisted of regular subculturing of the strains, thus allowing for important stability and mutational problems to occur. The introduction of lyophilization and cryopreservation techniques permitted major enhancements of the stability of the strains. A better understanding of the freeze-drying (lyophilization) techniques, especially provoked by the development of cryomicroscopes, has resulted in the possibility to dry recalcitrant organisms and a prolonged shelf life of freeze-dried specimens as well.

Freeze-drying is a rather complicated series of processes, aimed at the drying of organisms to such an extent that the metabolic activity is strongly reduced.

The result should allow for the storage of these organisms at a practical temperature and guarantee a successful revival after a prolonged period. The process starts with a suspension of living cells in a lyoprotectant. This suspension is frozen and subsequently dried by providing an open connection to a colder condenser under a relatively low vacuum. The drying occurs in two steps: primary drying, in which the frozen free water (crystalline) is removed, and secondary drying at higher temperatures. During primary drying the cells dehydrate by exposure to an eutectic solution with a high osmotic value, which freezes later to become a glass. The aim of secondary drying is to reduce the residual moisture content of the primary dried material in order to obtain a stable glass at preservation temperature. Residual moisture content is expressed as a percentage of the absolute dry weight. For fungi the tolerance of residual moisture content is between 1 and 2.5%. More severe drying will result in irreversible damage to the cells owing to denaturation of proteins and lipids, while higher moisture contents will reduce the shelf life of the material. Most yeast strains can now be lyophilized reliably.

Although for distribution freeze-drying has advantages over freezing, the method of choice for the preservation of yeasts is freezing at ultralow temperatures. It is the stablest and most reliable technique and it also allows for a better preservation compared with periodical subculturing. Yeast researchers can concentrate on their research without worrying about the stability of the strains that are provided by CCs.

The usage of the latter methods is of the utmost importance, not only for the quality of the stored microbiological materials, but also because they allowed for a drastic reduction of the time spent in subculturing.

3.5 Distribution

One of the major functions of CCs is the distribution of strains. Nowadays, the quality of the material, such as correct identity and physiological condition, is only a part of the task of the CCs. More and more governmental and postal regulations have important implications for CCs. For example, quarantine organisms, which can cause harm to humans, animals or local crops, or organisms that potentially can be used by terrorists are not easily distributable anymore and are subject to restrictions. There are now a number of obligations that are imposed by international treaties such as the Convention on Biodiversity, the Budapest Treaty (patents) or by transport regulations (IATA, road transport). Because of all these elements, qualified personnel are needed and special protocols have to be followed, which has considerable implications and costs.

The Convention on Biological Diversity (CBD), now ratified by over 185 countries, was established to support the conservation and utilization of biodiversity, ensuring fair and equitable sharing of benefits arising from the latter and was implemented in 1993. It has three major objectives, namely, conservation of biodiversity, sustainable development of genetic resources and fair and equitable sharing of resultant benefits. The CBD assigns sovereign rights to the country of origin and requires that prior informed consent (PIC) be received from the country in which access to organisms is requested. Mutually agreed terms (MAT) on the conditions

under which access is granted and on which benefits will be shared, should they occur from the use of the organisms, must be put in place. Benefit sharing may include monetary elements, but may also include information, technology transfer and training. The supply of organisms must also be under agreed terms under material transfer agreements (MTA) between supplier and recipient to ensure benefit sharing with, at least, the country of origin.

CCs must put in place mechanisms to comply with the provisions of the CBD, but they cannot completely police the distribution of strains. What they can do is to restrict further distribution, to allow traceability, to raise the attention of the client to the obligations related to the CBD and to provide the means to do so. When authorized they can also negotiate between the client and the country of origin.

Key developments for the endorsement of a voluntary code of conduct for access and benefit sharing have been made thanks to the Microorganisms Sustainable Use and Access Regulation International Code of Conduct (MOSAIC) project and the Bonn Guidelines.

3.6 Underpinning Science and Industry

Preserving strains under good and stable conditions at CCs contributes largely to scientific research in general by providing valuable quality material for the technologists to develop new or better products. However, CCs are not just biological stores, as the research staff of CCs also contribute to the value of the collections by bringing new methodological data (Barnett et al. 1985, 1987, 1990b, 1994, 1996, 2000b; Mikata and Banno 1989; Tan et al. 1991, 1994, 1995, 1998; Deak 1992, 1995; Robert et al. 1997, 2004; Tan 1997; Esteve-Zarzoso et al. 1999; Boekhout et al. 2002; Robert and Szoke 2003; Tan and van Ingen 2004), expertise and new isolates (Suh et al. 2004; Suh and Blackwell 2005). These interests have sparked the development of research programs at leading collections, which include investigations into phylogeny, taxonomy and nomenclature, strain preservation techniques, functional genomics, screening and bioinformatics.

In order to make CCs ever more useful and to share the important information that is kept at the strain and species levels, CCs have developed software and Internet websites. As an example, the CBS codeveloped the BioloMICS software (Robert and Szoke 2003) for the management of strains and species data, the statistical analysis of those data and for identification and classification of the isolates. Detailed information about more than 900 yeast species and 6,600 yeast strains is available online (http://www.cbs.knaw.nl/yeast.htm). Hundreds of characters are annotated for each record, which include morphological, physiological and molecular data. This information is freely accessible for each record, as is textual, bibliographic, geographic and taxonomic information. The database includes thousands of macroscopic and microscopic images and a bibliography of almost 10,000 references. The taxonomic database features 23,500 scientific names (including anamorph and teleomorph names, as well as synonyms). It is also possible for users to align their own sequences with a database containing up to 450,000 fungal sequences. The BioloMICS software also allows for online polyphasic similarity-based identifications, which can be performed using any combination of geographic, morphological, physiological or

molecular data. The CBS in The Netherlands, CABI in the UK and Landcare Research in New Zealand have launched the Mycobank website (Crous et al. 2004; www.mycobank.org) that allows the online registration of new fungal names as well as associated data. For the yeasts, this website is coupled to the yeast BioloMICS database and new species are made available online directly. We hope that this will be a highly valuable tool for all yeast researchers, especially those working on biodiversity and ecology.

3.7 Conclusions

The results obtained from biodiversity and functional studies of yeasts should be investigated in a more global and comprehensive approach, allowing a better understanding of the roles of these organisms in nature and their relationships to other living beings. Attaining this seemingly idealistic goal necessitates the use of collaborative teams of scientists as well as the training of additional taxonomists. Ecologists, taxonomists, botanists, zoologists and chemists must prioritize the niches that must be explored in the light of endangered habitats and ecosystems. These scientists should work with statisticians to define sampling plans, with field technicians to assist in the collection, isolation and the characterization of the isolates, with biochemists to search for interesting properties, and with computer scientists to construct useful databases. They must also convince politicians of the importance of long-term, comprehensive studies in order to finance the development and continued existence of large, multidisciplinary scientific teams and CCs. International collaboration will be crucial, not only for financing these scientifically difficult studies, but also because scientific expertise and technology and financial and ecological resources are scattered over different countries throughout the world. CCs are at the heart of this problem and should act and be seen as the core basic instrument allowing us to reach the previously mentioned targets.

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Chapter 4

Genomics and Biodiversity in Yeasts

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4.1 Introduction

The first complete nucleotide sequence of a yeast – *Saccharomyces cerevisiae* – genome was published in 1997 (The Yeast Genome Consortium 1997). It was also the first case of complete genome sequencing of a eukaryotic genome. The analysis of this yeast genome has revealed a set of conceptually new and unexpected features whose meanings were not fully understood for quite a while (number of genes, gene duplications, segmental duplications, etc.). By a concerted effort of the yeast research community the sequencing program was then developed to a systematic functional analysis, achieving a functional description of a large part of the genetic information.

The full eukaryotic genome sequences which followed this first attempt (*Caenorhabditis elegans*, by the *C. elegans* Sequencing Consortium 1998; *Drosophila melanogaster*, by Adams et al. 2000; *Arabidopsis thaliana*, by the *Arabidopsis* Genome Initiative 2000; *Homo sapiens*, by Lander et al. 2001 and Venter et al. 2001; *Anopheles gambiae* by Holt et al. 2002; *Mus musculus* by Waterston et al. 2002) belonged to organisms dispersed in the evolutionary tree of life. It is only recently that a set of ascomycetes complete genome sequences appeared in the literature and their availability is a real bonus to undertake comparative genomics studies. Yeasts share a common way of life as unicellular eukaryotic organisms (developmental steps are minimum, cell-to-cell communications is restricted to specific pathways such as sexual conjugation, killer system, etc.), but are largely dispersed along the evolutionary tree. The five yeasts analyzed by Dujon et al. (2004) appear molecularly more diverse than the whole phylum of chordates. During this long period of evolution and speciation, they also found their specific ecological niches and lifestyle. Several yeast genome sequences are now available (see Fig. 4.1 for their phylogenetic relationship). This information can be used to identify the molecular events that drove their evolution, and also to analyze the process of environmental adaptation. For the past few years, biologists have studied evolutionary developmental mechanisms also called "Evo-Devo" (reviewed by Hall 2003). A focus of this complex problem has been the mechanisms by which the transcriptional regulatory systems

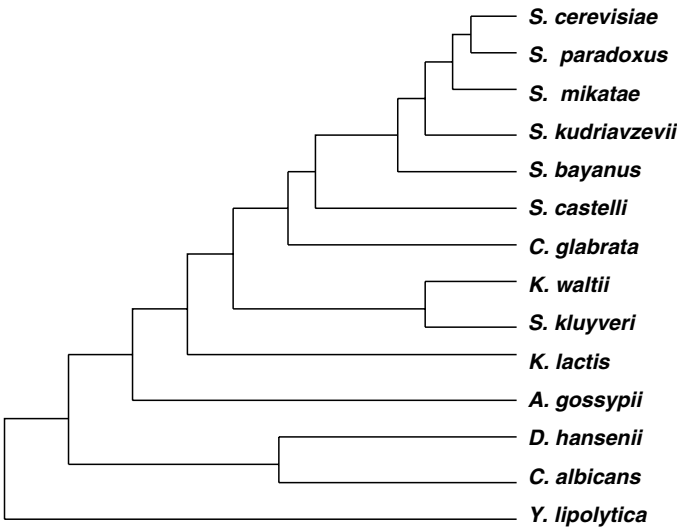

Fig. 4.1. Schematic phylogenetic relationships of the genome-sequenced yeasts. (Taken from Wolfe 2004)

evolved (published works are largely based on the studies of homeotic genes), and the contributions of these regulatory changes in the evolution of phenotype (Wray 2003). Such studies can now be undertaken with yeasts, based this time on complete genome sequence, in order to understand what could be called "Evo-Adapt" mechanisms. There are several recent reviews which deal with yeast genome evolution (Herrero et al. 2003; Piskur and Langkjaer 2004; Wolfe 2004). This short review is not exhaustive but intends to present our personal, perhaps biased, view of the present developments and perspectives of this new exciting field.

4.2 The Which and Why of Complete Yeast Genome Sequencing

The *S. cerevisiae* genome sequencing project was proposed 2 decades ago by Goffeau to the European Community and received strong support from the yeast research community. *S. cerevisiae* was not only an essential industrial yeast, but also "the" model yeast for basic research at that time. Sequencing of other yeast genomes started later, but the choice of species and the work calendar were not coordinated, and were rather dictated by circumstances and specific interests of different research groups. The genome sequencing of *Schizosaccharomyces pombe*, another model yeast for some aspects of cell biology, such as "cell cycle", and of the medically important *Candida albicans* (Tzung et al. 2001) were the first to be undertaken. The complete analysis of the *S. pombe* genome came out in 2002 (Wood et al. 2002). The determination of the complete nucleotide sequence of several *sensu stricto* and *sensu lato Saccharomyces* was undertaken with particular interest focused on intergenic regions. Since these regions diverge much faster than coding sequences, the

detection of conserved features in such regions required a comparative study of closely related yeasts (Cliften et al. 2003; Kellis et al. 2003; see Sect. 5.1 for more details). The *Ashbya gossypii* (*Eremothecium gossypii*) project also started a few years ago and the results were published recently (Dietrich et al. 2004). The choice of this yeast was based on several features of particular interest, including the very small size of its genome, the high degree of open reading frame (ORF) conservation with respect to *S. cerevisiae* and its filamentous form. More recently the genome sequence of *Kluyveromyces waltii* (= *Lachancea waltii*) was reported (Kellis et al. 2004). Sequence analysis of *K. waltii* and *A. gossypii* confirmed the view that the complete genome duplication occurred before the *S. cerevisiae* speciation, a hypothesis proposed earlier by Wolfe and Shields (1997). In the meantime, the French consortium "Génolevures" explored the hemiascomyceteous phylum (that includes most of the commonly known yeast species) by very partial shotgun sequencing of 13 different species well dispersed along the evolutionary tree of hemiascomycetes (The Génolevures Consortium 2000). This project was later completed by total genome sequencing of four selected species chosen on the basis of their adaptation to different environmental conditions as well as their supposed positions in the evolutionary tree (*C. glabrata*, *K. lactis*, *Yarrowia lipolytica*, and *Debaryomyces hansenii*) (Génolevures II, Dujon et al. 2004). Finally, the genome of *Hansenula polymorpha* (= *Pichia angusta* = *Ogataea polymorpha*) was sequenced and analyzed because of the well-known industrial interest in this yeast (Ramezani-Rad et al. 2003). In the near future, many more yeast sequences are expected to be released, increasing our repertoire and the possibilities of comparative genomic analysis.

An important fallout of all these projects was the improvement of annotation of the *S. cerevisiae* genome based on genome sequence comparison. This allowed the number of coding sequences to be reduced from the initial 6,200 to about 5,700–5,800 (Blandin et al. 2000; Brachat et al. 2003; Cliften et al. 2003; Kellis et al. 2003).

Speaking of a complete nucleotide sequence, the published data are not of the same level of completeness. In most cases, sequences are based on shotgun sequencing (usually between 5 and 8 times genome coverage), which leaves unfinished pieces and makes assembly difficult, resulting in a collection of large contigs. For the moment only a few genomes have been fully assembled as was done for *S. cerevisiae*: such is the case for *A. gossypii, S. pombe*, and the Génolevures II program (*C. glabrata, K. lactis, Y. lipolytica*). According to the type of analysis to be done, this (in)completeness has some importance as will be discussed in Sect. 4.1.

A comparison of genomes revealed first that all ascomycetes genomes were in the range 9–20 Mb, a small variation compared with the size of most eukaryotic genomes. Compared with higher eucaryotes, these small genomes also share common features. The number of ORFs varies around 6,000 (*A. gossypii*, the smallest one, contains 4,718 ORFs, while *Y. lipolytica* is supposed to encode 6,703 ORFs). Goffeau's description for *S. cerevisiae*, i.e., "life with 6,000 genes" (Goffeau et al. 1996) seems to apply to other yeasts. In addition, intergenic regions are generally short, and there are few introns, or repetitive elements, even if the genome of *Y. lipolytica* tends to be slightly more expanded than the others. Transposons are scarce and only a few cases of horizontal transfer have been detected.

Despite these common characteristics of their genome, each yeast species shows a distinct physiology. Their niches are diverse: trees and fruits (*S. cerevisiae*), dairy products (*K. lactis*), decaying organic compounds (*Y. lipolytica*), seawater (*K. aestuari*), high-salt food (*D. hansenii*) or highly acidic media (*Zygosaccharomyces bailii*). Some can be pathogens (*C. glabrata, C. albicans*). Even within the sole *Saccharomyces* group a quantitative difference of phenotype is evident, such as the low level of glucose repression typically observed in *S. kluyveri* (= *Lachancea kluyveri*) (Moller et al. 2002). Sugar assimilation patterns also illustrate the diversity, offering a convenient key for yeast classification. A general description of sugar assimilation patterns of yeasts can be found in Kurtzman and Fell (1998).

To summarize, the hemiascomycetous yeasts share many basic features of the genome, and contain roughly the same number of ORFs, whereas their physiology varies greatly. We may ask how yeast generates this diversity from the largely similar genomic components. The question can now be approached by both bioinformatics and experimental tests. We will consider here three possible mechanisms, not mutually exclusive, that may be at the basis of this adaptation process. The observed phenotypic variations could involve (1) large chromosomal rearrangements (such as translocations for example), (2) gene duplication/gene loss, possibly associated to functional differentiation, and (3) modified expression of the same gene subsets under the control of different regulatory mechanisms (variation of regulatory networks). Many earlier studies focused on the second mechanism, but more recent works have analyzed the two other possibilities. The respective weights of these different mechanisms in the functional evolution of yeasts are for the moment difficult to estimate. The aim of this review is to sum up the recent approaches dealing with these mechanisms.

4.3 Gross Chromosomal Rearrangements

In 2000, Fischer et al. analyzed the genomes of six closely related *Saccharomyces* species belonging to the *sensu stricto* complex for the possible presence of gross chromosomal rearrangements and identified several translocations (mostly reciprocal). These rearrangements happened at specific points ("hot spots") and were associated to ectopic recombination between Ty elements or duplicated pairs of genes. Highly polymorphic chromosomes appear to be a common characteristic of industrial *Saccharomyces* strains (Codon et al. 1998; for a review, see Mortimer 2000) where Ty-mediated reciprocal translocations have been identified (Codon et al. 1997; Rachidi et al. 1999; Infante et al. 2003). In some cases, these rearrangements have been shown to increase adaptative evolution. During long-term cultivation experiments in glucose-limited medium, sequence alterations occurred which have been correlated with adaptive changes of physiology (Dunham et al. 2002). In another observation on a wine yeast strain, a reciprocal translocation led to a promoter change in the *SSU1* gene, coding for a sulfite transporter involved in the resistance to sulfite, a preservative used in winemaking (Perez-Ortin et al. 2002).

Such gross rearrangements have been postulated as a potential mechanism of speciation, since these strains can mate, but interspecific pairings produce a sterile hybrid. A closer examination of the chromosome structure of different *sensu stricto*

yeast strains (Fischer et al. 2000) revealed that gross rearrangements were not present in all the species since the chromosomal maps of *S. paradoxus* and *S. kudriavzevii* were collinear with the *S. cerevisiae* map. Such a result showed that gross rearrangements such as translocations did not drive alone the speciation process. Taking a direct experimental approach, Delneri et al. (2003) engineered a *S. cerevisiae* strain by modifying its chromosomes to make them collinear with two *S. mikatae* strains. When interspecific hybrids were produced from these strains, their progeny were mostly viable, but they were extensively aneuploid, while the crosses between the noncollinear version of the otherwise isogenic strains indicated that reciprocal translocations caused a marked reduction in hybrid fertility. This confirms that translocations can contribute to the reproductive isolation between species, in combination with other mechanisms such as mismatch repair (Hunter et al. 1996).

4.4 Gene Duplications Leading to Adaptation and Biodiversity

Gene duplications have long been thought to be a major driving force in biological evolution. This mechanism provides an extra copy which can later be modified to produce a "novel function" (Ohno 1970) without losing – initially at least – the original function and therefore without risk. The importance of this mechanism in evolution has already been evidenced in many cases (Papp et al. 2003; Gu et al. 2004; Zhang and Kishino 2004, references therein).

4.4.1 Gene Duplication in Functional Evolution

Wolfe and Shields (1997) have proposed that a global genome duplication occurred prior to the speciation of *Saccharomyces* (*sensu stricto*). This seems to be confirmed by the analysis of the complete sequence of *K. waltii* (Kellis et al. 2004). Further analyses of the various genomes now at our disposal revealed a large variety of events which may superpose their effects prior or posterior to the global duplication event. Rearrangements and gene loss followed the complete genome duplication (Wong et al. 2002). Spontaneous local duplications have also been shown experimentally to happen quite frequently (Koszul et al. 2004) and could play an important role in reshaping the genome. Paralogous genes thus generated can follow separate fates. The two copies can evolve to different functions as mentioned before, or keep the same function. Alternatively one of the copies can be lost by deletion or inactivated by mutation leaving in place a pseudogene (also called a "relic" when highly degenerate). Some relic genes have been identified recently (Lafontaine et al. 2004).

There are no general rules that allow us to predict the functional fate of duplicated genes or the proportion of duplicates which are functionally differentiated. In addition, the notion of functional differentiation is loosely defined since it may mean the same function with a different expression (as for example the pair of anaerobic/aerobic genes of *S. cerevisiae* which play the same function but are expressed in two different physiological conditions), or a duplicate gene can actually change to exert a function different from that of the original gene. This question was

approached experimentally by examining the function of pairs (or more) of gene families as described, for example, in Llorente et al. (1999). In this case the two most closely related pairs of three genes were shown to have the same function with no differences in expression, while the third one, which acquired another domain (gene fusion), seemed to encode a protein with a different (and yet unknown) function. Delneri et al. (1999) analyzed the family of aryl alcohol dehydrogenase and showed that in terms of response to oxidative stress, the apparent genetic redundancy is more apparent than real since only one gene seems to be the key player; nevertheless, the real function of these genes has still to be established. In another report on the *THI5* gene family (involved in thiamine biosynthesis), only very subtle differences in the regulation were revealed (Wightman and Meacock 2003). In the last two cases, the genes are located within the subtelomeric regions, which may act as a "nest" for the formation of newly duplicated sequences, as proposed by Piskur and Langkjaer (2004) in their review. Such (evolutionary) very young copies did not have enough time to differentiate. With a more theoretical approach, Langkjaer et al. (2003) tried to assess the initial assumption that "rarely paralogues are preserved because they differentiate and become functionally specialized" but they could only deduce by phylogenetic studies whether the gene duplicated before or after the divergence of two yeast lineages. Using a different approach, Zhang and Kishino (2004) tried to predict the fate of a duplicate with respect to the recombination rate of the genomic region around this gene. An alternative way of functionally classifying proteins is to rely on their capacity of protein–protein interactions independently of sequence data. Baudot et al. (2004) developed a bioinformatic method called PRODISTIN which was applied to 899 duplicated genes. Their main conclusion is that the fate of duplicate genes is distributed quite unequally between three categories: the majority (63%) of protein pairs are involved in the same biological process, 7.5% may be involved in different aspects of the same biological process. Only a limited fraction (22%) acquire new biological functions. If this is confirmed by other methods, one may consider that the role of duplication in functional evolution, based on acquisition of new functions, has probably been quantitatively overestimated.

Despite these attempts, most of our means to understand functional evolution of a biological pathway are restricted to a systematic comparative analysis in different species of the presence/absence/copy number of the genes involved in that pathway. This approach has been successfully applied to several biological processes, such as mating type genes, silencing and subtelomeric gene families (Fabre et al. 2004), the MAT locus and HO endonuclease (Butler et al. 2004) or DNA replication, repair and recombination (Richard et al. 2005), allowing stimulating hypotheses to be formulated for the acquisition of new functions. This comparison also led to the observation that the simultaneous loss of genes of the GAL pathway in some species was a way to their adaptation to specific ecological "niches" (Hittinger et al. 2004). In our laboratory we are examining carbon and oxygen metabolism on the same basis (Bolotin-Fukuhara et al. 2005; Bolotin-Fukuhara and Bao, unpublished data) and some of the data which complete the analysis on anaerobiosis by Gojkovic et al. (2004) are presented later.

Before any conclusions are drawn however, one should be aware that this type of analysis (presence/absence of genes/copy number) relies entirely upon the quality of

the sequence in terms of assembly and polishing. When the genome coverage is low, the absence of the homologue may only mean that either the corresponding sequence is absent, or that the partial sequence did not allow detection of the homologue. The same holds true for the detection of duplicates. This problem was encountered in the Génolevures I program, for which functional analysis was not very informative (Gaillardin et al. 2000). Only completely finished sequences can lead to precise and definitive conclusions. Therefore, the recent works described in the preceding discussion relied to a great part on the completely sequenced and assembled genomes from the Génolevures II project. This point has been discussed in detail in Bolotin-Fukuhara et al. (2005).

4.4.2 Central Metabolism

The sugar sources which can be assimilated vary greatly according to yeast species (Barnett 1976), but glucose remains a universal carbon source of yeasts. Glucose is directed to pyruvate through glycolysis and most yeasts metabolize pyruvate partly though fermentation and partly through respiration (such is the case of *K. lactis* or *P. stipitis)* or use it exclusively for respiration as does *Y. lipolytica* and other typically respiratory species. *S. cerevisiae* and closely related species are, in contrast, predominantly fermentative yeast in which respiratory pathways are strongly repressed by glucose (Crabtree effect positive). Very complete carbon source assimilation spectra are listed in Kurtzman and Fell (1998). A more recent review addressing carbohydrate and energy-metabolism in nonconventional yeasts (Flores et al. 2000) concluded that "basic knowledge is missing on many components of these pathways and that studies on regulation of critical steps are scarce." The existence of genomic data offers, therefore, an opportunity to reinvestigate these important pathways.

In a previous review (Bolotin-Fukuhara et al. 2005), we compared the presence/absence of genes involved in glycolysis, the tricarboxylic acid cycle and related pathways. We took advantage of the fact that the three yeasts which were analyzed, *S. cerevisiae*, *K. lactis* and *Y. lipolytica* represent three different levels of balance between fermentation and respiration. A decade ago the glycolytic pathway was studied in *K. lactis* by screening for Rag mutants (RAG means resistance to antimycin on glucose and allows mutants of the glycolytic pathways to be selected). Fourteen complementation groups have been identified in this screen (Wesolowski-Louvel et al. 1992) and later molecular analysis revealed a low level of gene redundancy as compared with that in *S. cerevisiae*. In the latter most of the glycolysis genes are duplicated or even multiplicated (such as multiple hexose transporters). The systematic analysis made on the three genomes confirmed this trend (Bolotin-Fukuhara et al. 2005). Extending this type of analysis to the yeasts whose genome is sequenced reinforces this idea since the genes of the upper part of the glycolysis pathway (Table 4.1) are generally not redundant in these yeasts, as opposed to *S. cerevisiae*. This may be explained – among other hypotheses – if we assume that storage reactions have to be more active in fermentative yeasts like *S. cerevisiae* since the energetic yield of glycolysis is smaller than in respiratory metabolism. It is interesting to note that this low redundancy of the genes involved is not restricted to the species placed in the phylogenetic tree before the general duplication of the genome.

Table 4.1 Distribution of some genes involved in carbon metabolism in yeast. Yeast species are placed in the order of the phylogenetic tree presented in Fig. 4.1. The yeast species abbreviations are *Sc* for *Saccharomyces cerevisiae*, *Sa* for *S. castellii*, *Cg* for *Candida glabrata*, *Kw* for *Kluyveromyces waltii*, *Sy* for *S. kluyveri*, *Kl* for *K. lactis*, *Ag* for *Ashbya gossypii*, *Dh* for *Debaryomyces hansenii*, *Ca* for *C. albicans* and *Yl* for *Yarrowia lipolytica*. Genes encoding enzymes involved in carbohydrate storage and the pyruvate decarboxylase genes. A *question mark* indicates that the data are unclear (partial sequence only, ambiguity in allele attribution, weak similarity which does not discriminate between the pairs, etc., see also the discussion in Sect. 2)

	Part A: carbohydrate storage									
Enzyme activity	**Sc**	**Sa**	**Cg**	**Kw**	**Sy**	**Kl**	**Ag**	**Dh**	**Ca**	**Yl**
Phospho-glucomutase	2: *PGM1 PGM2*	2	2	1	1	1	1	1	1	1
Glycogen synthase	2: *GSY1 GSY2*	2	2	1	1	1	1	1	1	1
Glycogenin glucotransferase	2: *GLG1 GLG2*	1?	1	1	1	1	1	2	1?	1
alpha, alpha-trehalose-phosphate synthase (regulatory activity)	2: *TPS3 TSL1*	1	2	1	1	1	1	1	1	1
alpha, alpha-trehalase	2: *NTH1 NTH2*	1	2	1	1	1	1	1	1	1
	Part B: pyruvate decarboxylase									
	3: *PDC1 PDC5 PDC6*	3?	2	1	3	1	2	3	2	1

Therefore, the situation may reflect particular physiological requirements of each species. A species such as *S. castellii* does not maintain the complete set of duplicate genes perhaps because its metabolism is less fermentation-oriented than that of *S. cerevisiae* and its *sensu stricto* relatives. This absence of redundancy may also be linked to the reduced glucose repression in *S. kluyveri*, a *sensu lato Saccharomyces* like *S. castellii*.

The general redundancy of glycolysis genes in *S. cerevisiae* may suggest that it is a way of increasing the metabolic flux of this pathway. However, no direct evidence has been provided yet for actually increased levels of most enzymes. A few attempts at genetic engineering have been made on key metabolic points which are often critical in industrial processes. Such is the case of pyruvate decarboxylase (PDC) genes. PDC is a key enzyme that directs pyruvate to fermentation rather than respiratory pathways. Indeed, construction of *S. cerevisiae* strains with reduced specific Pdcp activity has been attempted (Flikweert et al. 1999; Remize et al. 2000). PDC is encoded by three genes in *S. cerevisiae* (a major form, *PDC1*, and a minor form, *PDC5*, Hohmann and Cederberg 1990; the third enzyme, *PDC6*, is inducible by sulfur limitation, Fauchon et al. 2002), while in *K. lactis*

there is only one single gene (Bianchi et al. 1996). In this case, the reduced copy number of *PDC* genes is probably not associated with increased respiratory activity. In other species (Table 4.1), the number of PDC genes is variable and is not correlated with the intensity of the respiratory metabolism of each species. Experimental data also point to this conclusion. Moller et al. (2004) identified three PDC genes in the yeast *S. kluyveri* and measured the enzyme activity under various conditions. They could show that *S. kluyveri* and *S. cerevisiae*, grown under comparable conditions, had different flux distributions at the pyruvate branch point, and hypothesized that factors other than the Pdcp activities are responsible for this difference.

4.4.3 The Case of Aerobiosis/Anaerobiosis

Yeasts cannot grow on strict anaerobiosis, with the exception of some *Saccharomyces* yeasts. Among the yeasts for which the genome has been completely sequenced, there are two types of physiology: *S. cerevisiae* and the *Saccharomyces sensu stricto* species (*S. bayanus*, *S. kudriavzevii*, *S. Paradoxus* and *S. mikatae*) and *sensu lato* species (*S. kluyveri* and *S. castellii*) are able to grow anaerobically, while *K. lactis*, *D. hansenii*, *C. glabrata*, *A. gossypii*, *C. albicans* and *Y. lipolytica* are not. As an example of functional evolution, the study of these genomes may shed light on the question of how some yeasts have acquired (or lost) the capacity to grow anaerobically. For *S. cerevisiae*, a systematic study has been done to compare the genes expressed in anaerobiosis versus aerobiosis by a genome-wide transcriptional analysis (Ter-Linde et al. 1999). Only a small number of genes responded to these different physiological conditions. The study revealed *ROX1* (the repressor of hypoxic and specific anaerobiosis genes) as a main site of response, mediating the other targets. This was further confirmed by transcriptome studies of Rox1p targets (Ter Linde and Steensma 2002). Some pathways are usually dependent upon oxygen and anaerobic yeasts developed a way to circumvent oxygen limitation. Such is the case of the fourth step of the pyrimidine biosynthesis pathway, the dihydroorotate dehydrogenase (DHODase) encoded by the *URA1* gene in *S. cerevisiae*. In relation with the DHODase, Gojkovic et al. (2004) recently proposed an explanation why some yeasts are obligate aerobe. In most species this enzyme activity is dependent upon oxygen, because they possess only a mitochondrial form of the enzyme. In *S. cerevisiae* (and in other *Saccharomyces* yeasts) there is a cytoplasmic form of the enzyme. This cytoplasmic enzyme is phylogenetically related to a bacterial DHODase and Gojkovic et al. postulated that yeast has acquired the corresponding gene by horizontal transfer. An elegant confirmation of the model was presented on the basis of an analysis of the *S. kluyveri* genome, which possesses both mitochondrial and cytoplasmic forms of DHODase. Each of the corresponding genes was separately disrupted and the effects were analyzed (Gojkovic et al. 2004) to substantiate the proposal.

Another oxygen-dependent pathway is the sterol biosynthesis pathway. When it is functional, yeast does not take up a significant amount of exogenous sterols (a phenomenon called aerobic sterol exclusion, Lewis et al. 1985). In anaerobiosis however, *S. cerevisiae* has to rely on external sterol supply for growth. Only if ergosterol is added to the medium *S. cerevisiae* grows in the absence of oxygen. Wilcox et al.

(2002) have shown that a few genes are essential for sterol influx: the two paralogous sterol transporters Aus1p and PdrIIp, as well as the cell-wall protein called Dan1p and the transcription factor Upc2p. Another hypoxic transcription regulator, Sut1p, is probably also involved in the process (Alimardani et al. 2004). In a previous comparison (Bolotin-Fukuhara et al. 2005), we came to the conclusion that the sterol uptake pathway was probably absent in the two aerobic yeasts *K. lactis* and *Y. lypolitica*. In order to generalize the role of sterol transport in anaerobic growth of yeasts, this line of study has been extended to other yeasts. Table 4.2 indicates the presence/absence of the corresponding genes in the complete panel of yeasts whose complete genome has been sequenced. It seems reasonable to conclude that all *sensu stricto Saccharomyces* yeasts have the complete set of genes for sterol uptake as well as the cytoplasmic form of *URA1*, which account for their ability to grow anaerobically, although this conclusion has restrictions inherent to the analysis of incomplete genomes as discussed before (see Sect. 4.1). Interestingly, other yeasts that are less closely related to *S. cerevisiae* (*S. castellii, S. kluyveri* and *C. glabrata*) have only one copy of the sterol transporters (Aus1p/PdrIIp), suggesting that the duplication of sterol transporter gene is probably a late event in evolution. The presence of the special cell-wall protein Dan1p is considered to be essential for anaerobic growth (Alimardani et al. 2004). Indeed, the corresponding gene has been detected only in *sensu stricto Saccharomyces* and in *S. castellii* (this yeast can grow anaerobically according to Langkjaer et al. 2003) and, surprisingly, in *K. waltii* (but only as a fragment; the capacity of anaerobic growth of this species is not known). *C. glabrata* seems to possess most of the genes necessary for sterol import despite its inability to grow anaerobically. The absence of the *DAN1* gene was a possible explanation, but a closer examination revealed that this species did not contain the cytoplasmic *URA1* gene form which, according to the Gojkovic hypothesis (Gojkovic et al. 2004), should be present. A general scheme of introduction of new anaerobic traits during the evolution of yeasts is represented Fig. 4.2. This complexity only confirms that many events superimposed their effect on lineage evolution and took place during the general evolution of yeasts as was discussed previously. While information gained from the sequence data and their bioinformatic analysis provides ideas about the process of acquisition of the capacity of anaerobic growth, experimental confirmation is required to confirm/invalidate the different hypotheses proposed.

4.5 Changes in Regulatory Circuits for Adaptation to New Environments and Physiology

As described before, modification of expression of a subset of genes by changing their (coordinated) regulation is one of the possibilities of adaptation of a species to a different environment. On *S. cerevisiae*, much effort has been made in the past year to unravel the regulatory circuitries through either theoretical or experimental approaches with the aim to predict the behavior of *S. cerevisiae* in different conditions. Even though global pictures of the regulatory circuitry of *S. cerevisiae* are emerging (Lee et al. 2002), we have still a long way to go to reach this goal. Extending these approaches to the other yeasts further requires identification of the *cis* and *trans* elements involved in transcriptional control, followed by a comparative

Table 4.2 Presence/absence of genes involved in key pathways required for anaerobiosis in yeasts. Same as in Table 4.1. *Sp* is *S. paradoxus*, *Sm* is *S. mikatae*, *Sk* is *S. kudriavzevii* and *Sb* is *S. bayanus*. The presence/absence of putative orthologues of *AUS1*/*PDRII* was concluded after examining the whole family of transporters. The *numbers* in *parentheses* indicate the number of copies which were found. An *asterisk* indicates that the information was obtained from fragments of genes and not complete sequences. A *question mark* means that the conclusion is ambiguous (usually because of the presence of only a fragment of the sequence)

Genes (as in Sc)	Sc	Sp	Sm	Sk	Sb	Sa	Cg	Kw	Sy	Kl	Ag	Dh	Ca	Yl
UPC2	Yes	Yes	Yes	Yes*	Yes*	Yes	Yes?	No	Yes?	No	No	No	No	No
DAN1	Yes	Yes	Yes	Yes	Yes	Yes?[a]	No	Yes	No	No	No	No	No	No
AUS1/*PDRII*	Yes (2)	Yes (2)	Yes (2)	Yes (2)	Yes (2)	Yes (1)	Yes (1)	No	Yes (1)	No	No	No	No	No
SUT1	Yes	Yes	Yes	Yes	Yes	No	Yes?	No	No	No	No	No	No	No
URA1 (cytoplasmic)	Yes	Yes	Yes	Yes	Yes	Yes	No	Yes	Yes	Yes	No	No	No	No
URA1 (mitochondrial)	No	No	No	No	No	No	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes

[a]Two putative proteins were identified which match with Dan1p but not with other Dan proteins, nor between themselves.

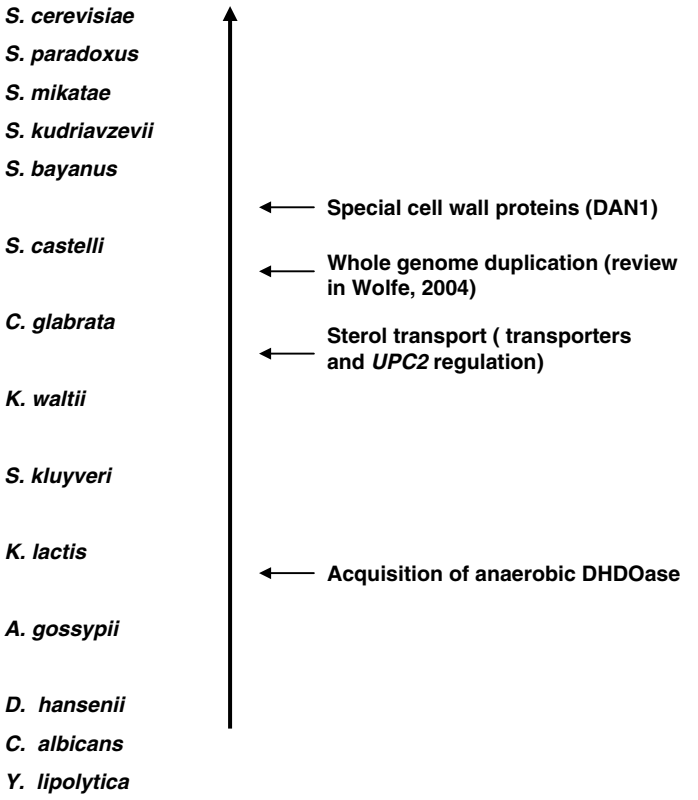

Fig. 4.2. Introduction of new anaerobic traits during the evolution of yeasts. The acquisition of traits related to anaerobiosis such as a new cytoplasmic form of the dihydroorotate dehydrogenase, or different functions related to sterol uptake, are indicated along the evolutionary tree of yeasts as represented Fig. 4.1

analysis of their regulatory networks. The availability of several complete genomic sequences of yeasts now offers the necessary tools to reach this ultimate goal.

4.5.1 Identification of *cis*-Regulatory Elements by a Comparative Genome Approach

It is not an easy task to systematically identify the *cis*-regulatory elements in a genome. Several methods have been developed for this purpose. One may wonder if comparison of genomic sequences can help to find out functionally conserved intergenic sequences, in the way we are searching for orthologous genes. The difficulty comes from the facts that (1) intergenic regions diverge rapidly as compared with coding sequences and (2) the regulatory sequences are generally short and often tolerant to some sequence variation. Comparison between species for which coding sequences are already quite different will not be helpful because the signal-to-noise

ratio of these short sequences is not significant enough. In contrast, exploiting sequences of closely related yeasts may be less informative for functional domain analysis of proteins but should allow the detection of conserved signals ("phylogenetic footprints") in intergenic regions. Previous work (Moses et al. 2003) has indeed shown that binding sites for transactivating elements evolve slower than their surrounding sequences. Therefore, we expect that an improved analysis of intergenic sequences may detect regulatory elements.

This was the rationale followed by two groups who chose to analyze *Saccharomyces sensu stricto* species *S. paradoxus*, *S. mikatae* and *S. bayanus* in one case (Kellis et al. 2003) and several yeasts of the *sensu stricto* and *sensu lato Saccharomyces* groups (*S. mikatae*, *S. kudriavzevii*, *S. bayanus*, *S. castellii* and *S. kluyveri*) in the other (Cliften et al. 2003). The *sensu lato* strains were included here in order to help pinpoint some sequences when the background intergenic sequences are not divergent enough.

The two groups developed different algorithms to identify the binding sites of transcription factors and proposed 72 and 79 *cis*-binding sites respectively. However, while Kellis et al. (2003) emphasized that "such an approach has the power to identify key functional elements without previous biological knowledge," many biological results (functional significance of neighboring genes, chromatin immunoprecipitation, ChIP-chip assays, coregulation, etc.) were in fact used in this work to select the best candidates. The regulatory sequences thus identified are thought to be of biological significance, because the result of the search included the already known, functionally characterized elements. Further experimental data may still be necessary to substantiate this approach.

4.5.2 The Repertoire of Transactivators

Transcriptional regulators have been well described in *S. cerevisiae* and for many of them their binding sites and (at least some of) their cellular functions have been identified. Homologues of these regulators can be searched for in the genomes of other yeast species. This systematic work has been achieved on the basis of the Génolevures I data (Bussereau et al. 2004). From these data it was inferred that if the DNA-binding motifs are well conserved, the rest of the sequences diverge very rapidly. This means that functional homologues could probably be identified rather easily in the species close to *S. cerevisiae* as was the case for the *cis* sequences reported in Sect. 5.1. For more distant species the task may be more difficult, but such systematic analysis, combined with phylogenetic studies, is now in progress (Bolotin-Fukuhara et al., unpublished data). The number and distribution of transactivators between the different DNA-binding classes is also of interest, and in particular for the group which is only found in ascomycetes, such as the zinc-binuclear cluster group (Todd and Andrianopoulos 1997). A few questions may be of particular interest: is the number of transactivators belonging to each class more or less constant along the phylogenetic tree? Are these proteins evolving by domains (disappearance or addition of new motifs) to produce new proteins not directly recognizable anymore but able to function in higher eucaryotes? Along this evolution what functions tend to be conserved more than others? Are they specific of ascomycetes?

The transcriptional repressor Rox1p is a good example of the problems raised in transcriptional regulator analysis. In *S. cerevisiae,* this repressor has an essential regulatory role as a function of oxygen (see Sect. 4.3). Several genes expressed in anaerobiosis are repressed by Rox1p in aerobiosis. Searching for Rox1p homologues in the *Saccharomyces* species allows the identification of a putative protein which is highly conserved in the *sensu stricto* group (Fig. 4.3a). However, this conservation becomes less obvious in *S. castellii* and *S. kluyveri*, with the exception of the readily recognizable HMG box motif (Fig. 4.3b). This illustrates the difficulty to identify transactivator homologues along the evolutionary tree (even in related species). Yet, all the yeasts mentioned previously can grow anaerobically and we expect the presence of a functional homologue of Rox1p. In other yeast species which cannot grow in anaerobiosis, the presence of a Rox1p-like protein may be questioned. Sequence analysis reveals in all cases the presence of a protein which contains a HMG motif but has no other sequence similarities (Bolotin-Fukuhara et al. 2005). One may wonder what is the biological meaning of such a finding. Obviously, experiments have to be done to decide if these proteins are functional equivalents of Rox1p. A similar question has been asked about the Hap4p protein, which is the regulatory and transactivator part of a transcriptional regulation complex (the HAP complex). For a long time this protein has been known only in *S. cerevisiae*. This seemed to make sense because its role in the cell is to allow the transcriptional reprogramming when the cell goes from a fermentative to a respiratory metabolism (DeRisi et al. 1997; Buschlen et al. 2003; Lascaris et al. 2003). However, we were able to isolate a *HAP4* functional homologue from the respiratory yeast *K. lactis* (Bourgarel et al. 1999) and more recently identified such proteins from all ascomycetes whose genome is sequenced using two short conserved motifs detected by comparing the *K. lactis* and *S. cerevisiae* sequences. A *HAP4* homologue from *H. polymorpha*, a respiratory yeast species more distant from *S. cerevisiae* than *K. lactis* in the phylogenetic tree, was shown to be fully functional despite the absence of one of the conserved motifs (Sybirna et al. 2005). The questions raised for *ROX1* can consequently also be raised for *HAP4* and probably many other transactivators.

4.5.3 Comparison of Regulatory Networks in Different Yeasts

As discussed before, it is possible to identify conserved *cis*-binding motifs specific for transcriptional regulators which themselves seem to be conserved in most cases. The next step is therefore to know to what extent the regulatory network controlled by a given transactivator is conserved in related species. Two possibilities can be considered: (1) either the same set of genes are coregulated or (2) only the global function of the network (such as energy control and production of specific metabolites) is conserved but is achieved through the regulation of a modified set of genes. To discriminate between these two possibilities, experiments are needed and are awaiting the necessary experimental genome-wide tools. Some information comes from the studies of *K. lactis*. From this yeast, several transactivators homologous to *S. cerevisiae MIG1* (Cassart et al. 1995), *HAP4* (Bourgarel et al. 1999) and *CAT8* (Georis et al. 2000) have been cloned and analyzed. While *CAT8* controls the key enzyme of gluconeogenesis in *S. cerevisiae*, the *K. lactis* homologue did not show this function.

```
S. cerevisiae
S. paradoxus       MNPKSSTPKIPRPKNAFILFRQHYHRILIDEWTAQGVEIPHNSNISKIIGTKWKGLQPED
S. mikatae          MNPKSSTPKIPRPKNAFILFRQHYHRILIDEWTAQGVEIPHNSNISKIIGTKWKGLQPEE
S. kudriavzevii   ------------------------------------------------------------
S. bayanus        -------------------MWWNSDEWTTQGVEIPHNSNISKIIGTKWKGLQPEE
                  ********************     **** *************************

S. cerevisiae     KAHWENLAEKEKLEHERKYPEYKYKPVRKSKKKQLLLKEIEQQQQQQQKEQQQ-------
S. paradoxus      KAHWESLAKKEKLEHERKYPEYKYKPIRKSKKKQLLLKEIEQQQQQQQQQQQQ-------
S. mikatae        KAHWEDLAKKEKLEHERKYPEYKYKPIRKSKKKQLLLKEVEEQQQQQ---TQQ-------
S. kudriavzevii   ------------------------------GDLILALWWN-------------------
S. bayanus        KSHWESLAKKEKLEHERKYPEYKYKPIRKSKKRQLLLKEMEQQQQAQQQQAQQPLQQQQP
                  * *** **  ****************** *****  ***      *** *    **

S. cerevisiae      ----------QKQSQPQLQQPFNNNIVLMKRAHSLSPSSSVSSSNSYQFQLNNDLKRLPIP
S. paradoxus       ---------PQPQPQSQLQQPFNNNIILMKRAHSLSPSSSVSSSNSYQFQLNNDFKRLPIP
S. mikatae         ----------AQTQPQPQLQQPFNNNIVLMKRAHSLSPSSSVSSSNSYQFQLNNDFRRLPIP
S. kudriavzevii    ------------------------------------SSSISSSSSYQYQLNNDFRRLPNP
S. bayanus         LQQQQPLQQQQQQQPQLQPLNSNNIILMKKAHSLSPSSSVSSSNSYQYQLNNDFRRLPNP
                                        *** *** ********* *** *** ***** **** *

S. cerevisiae       SVNTS--NYMVSRSLSGLPLTHDKTARDLPQLSSQLNSIPYYSAPHDPSTRHHYLNVAQA
S. paradoxus        SVNSP--NYMVSRSLSGLPLSHEKTARDLPQLSSQLNSIPYYSAQHDRSTRHHYLNVAQA
S. mikatae          SVSSP--NYMVSRPLNGLPLAHDKAVRDLPQLSSQFHSIPHYSAPHDSATRHHYLNIAQA
S. kudriavzevii     SVNSP—NYMASRPLNGLPLAHEKRAGDLPQLSSQLHAIPYFPATHDSTARHH-FLNVAQ-
S. bayanus          SINSPAGNYLVSRPSNGLPLTHDRTARDLPQISSQLHPMPYYSSTYDSTTRNHFLNIAQA
                    *      *  **   **** *     *** ***    *       *    *  ** **

S. cerevisiae       QPRANSTPQLPFISSIINNSS-QTPVT-TTTTSTTTATSSPGKFSSSPNSSVLENNRLNS
S. paradoxus        QPRANSTPQLPLISSIINNNS-QTPVT-TTTTTTTTATSSPSKFSSSPNSSILENNRLNS
S. mikatae          QPRANSTPQLPLISSIINSSN-QTSVTNSTATTTTTATSSPSKLSSSPNSSIFENNRLNS
S. kudriavzevii    -PRANSTPQLPLISSIIENSNNQTPVT-NTTTASTTAASSPNKVSSSPNSSISENNRLNG
S. bayanus          QPRANSTPQLPLISSIINNNNNNNNQT-PATTTTTTETSSPGKFTSSPNSSVSENNRLNS
                   ***************** *                  **  ***    ******  ******

S. cerevisiae       INNSNQYLPPPLLPSLQDFQLDQYQQLKQMGPTYIVKPLSHTRNNLLSTTTPT-HHHIPH
S. paradoxus        INISNQYLPPPLLPSLQDFQLDQYQQLKQMGPTYIVKPLSHTRNNLLSTTTPT-HHHIPH
S. mikatae          INNSNQYLPPPLLPSLQDFQLDQYQQLKQMGPTYIVKPLSHTRNNLLSTTTPT-HHHIPH
S. kudriavzevii    INSLNQYLPPPLLPSLQDFQLDQYQQLKQMGPTYIVKPLSHARNNLLSTTTPT-HHHIPH
S. bayanus          INNSNQYLPPPLLPSLQDFQLDQYHQLKQLGPTYIVKPLSHTRGNLLSTAAPTHHHHIPH

S. cerevisiae       IPNQNIPLHQIINSSNTEVTAKTSLVSPK
S. paradoxus        IPNQNIPLHQIMNSSNTEITAKTSLVSPK
S. mikatae          IPNQNIPLHQIINS-NNEVTAKTSLVSPK
S. kudriavzevii    ISNQNISLHQIINANNNEVTAKTSLVSPK
S. bayanus          IPNQNIPLHQLMNASNNEVTAKTGLISPK
                   * **** ***  *    * **** * ***
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(Continued)

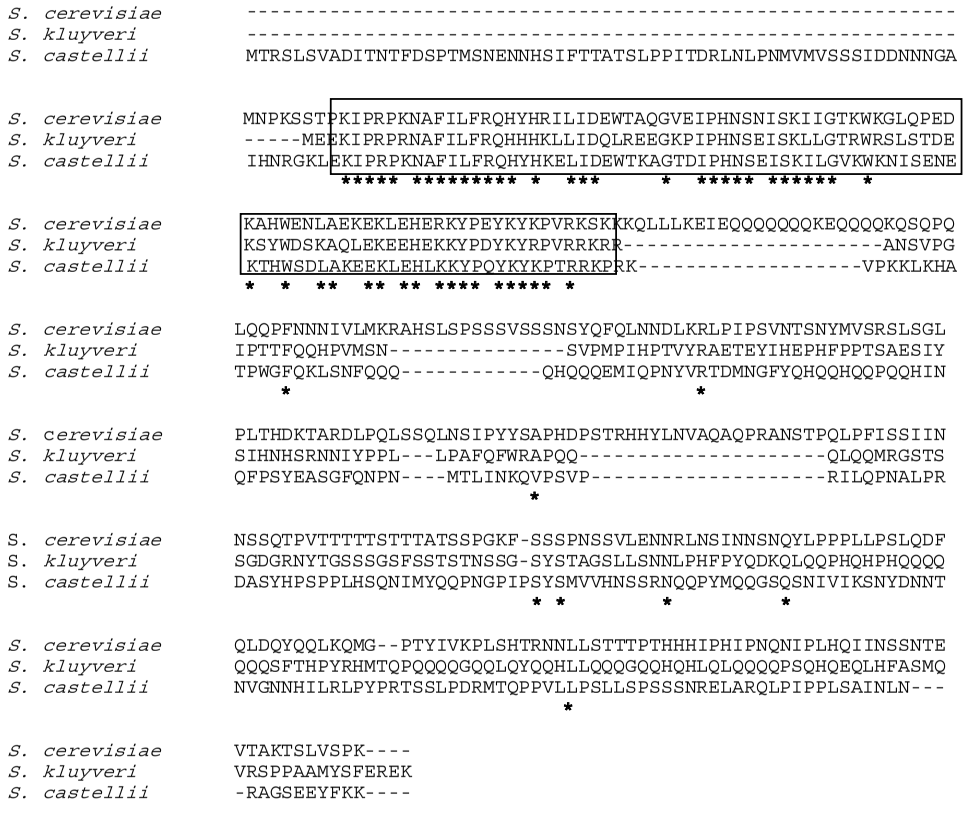

Fig. 4.3. (Cond't) Clustal W alignments of Rox1p putative proteins from different yeast species. **a** Rox1p homologues alignment in the *Saccharomyces sensu stricto* species. An *asterisk* indicates identities. The 5′ part of the Rox1p homologues of *S. kudriavzevii* and *S. bayanus* could not be identified from the sequences available. The box underscores the HMG motif contained in the Rox1p sequence. **b** Alignment of the *S. cerevisiae* protein with homologues from *S. castellii* and *S. kluyveri*. An *asterisk* indicates identities. The 5′ part of the Rox1p homologues of *S. kudriavzevii* and *S. bayanus* could not be identified from the sequences available. The box underscores the HMG motif contained in the Rox1p sequence

Further analyses have revealed some functional conservations in the pathway (in particular the relation *SNF1*/*CAT8*) as well as differences in specific gene regulation (Lodi et al. 2001; Charbon et al. 2004). Clearly the range of function of *CAT8* is different in *K. lactis*. The same holds true for the other transactivators. The invertase gene is tightly regulated by *MIG1* in *S. cerevisiae*, but is not regulated in *K. lactis* (Georis et al. 1999). Disruption of the components of the *K. lactis* HAP complex (Nguyen et al. 1995; Bourgarel et al. 1999) does not lead to any growth defect on respiratory carbon sources, and the *CYC1* gene regulated by the HAP complex in *S. cerevisiae* is not regulated in *K. lactis* (Ramil et al. 1998). The possibility to study global gene expression with DNA arrays in the different yeasts should help us to unravel this complexity and better understand the evolution of regulatory circuits.

4.6 Conclusions

Yeast species live in different ecological niches, but their genomes reveal many conserved characteristics. This short review has tried to analyze and evaluate how the species exploit this basic genomic information to adapt to their new environment. Function evolution can be acquired by different mechanisms, based on gross chromosomal rearrangements, gene gain or loss or differences in gene expression. Much of the information we now have is predictive and is based mostly on bioinformatics analysis. While these data are very helpful for analyzing the mass of information now available, this new research field badly needs experimental data to confirm/invalidate those predictions. Fortunately, the "omics" tools necessary to obtain such data are or will soon be available for these so-called nonconventional yeasts (complete sequence, transcriptomics, proteomics, metabolomics, etc.). Some of these are even amenable to experimentation by classical and molecular genetics. These possibilities open the way to many comparative functional studies and will certainly change the respective importance of the different yeasts, building up new model yeasts for specific studies.

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Chapter 5

Methods for Investigating Yeast Biodiversity

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Abbreviations

ASBC	American Society of Brewing Chemists
CFU	Colony forming units
CLEN	Cadaverine lysine ethylamine nitrate
CLPP	Community level physiological profiling
DG18	Dichloran 18% glycerol agar
DGGE	Denaturing gradient gel electrophoresis
DIC	Differential interference contrast
FAME	Fatty acid methyl ester
FISH	Fluorescence in situ hybridization
G+C	Guanine plus cytosine
GPS	Global Positioning System
IGS	Intergenic spacer
ITS	Internal transcribed spacer
MPN	Most probable number
mRNA	Messenger RNA
NSA	Niger seed agar
OD_{600}	Optical density at 600 nm
OIV	Office International de la Vigne et du Vin
PCR	Polymerase chain reaction
PDA	Potato dextrose agar
Q-PCR	Quantitative PCR
RFLP	Restriction fragment length polymorphism
rRNA	Ribosomal RNA
SGA	Sabouraud's glucose agar
SSCP	Single-strand conformation polymorphism
TGGE	Temperature gradient gel electrophoresis
TGYA	Tryptone–glucose–yeast extract agar
VNC	Viable nonculturable
X-α-gal	5-Bromo-4-chloro-3-indolyl-α-galactoside
YEPD	Yeast extract peptone dextrose
YM	Yeast extract–malt extract medium

5.1 Introduction

Much can be learned from a comparison of the methods used for microbial analysis of various materials by different specialists. Methods that are reproducible, specific, and accurate for one substrate can be appropriately modified for a new substrate. For this reason, in this chapter, some aspects of standardized industrial and clinical methods will be compared with those used for sampling natural ecosystems. Microbial methods developed for enumeration and detection of bacteria that can be or are being applied to yeast are also discussed. This chapter is not intended to be a complete description of all yeast sampling, detection, and enumeration methods that have been used. Rather it is a cross-section of methods used in a variety of applications, chosen to illustrate the many factors to consider when designing your own experimental protocol. Topics in this chapter include sampling, plating, microscopy, and culture-independent methods. The sampling, detection, and enumeration methods most appropriate for a specific application depend on what question is being asked. Different methods would be used for general surveys of the most common species present in a habitat than for detection of a low-abundance pathogen, for example. Details of detection and enumeration methods developed for sampling of specific habitats such as the phylloplane, marine and fresh water, and cacti can be found in other chapters of this book.

In recent decades, increasing numbers of researchers have discovered the rich field of yeast ecology. Yeasts interact in fascinating ways with other organisms and with their environment, whether in a wine fermentation, a mycosis, or decaying plants. Much of our knowledge of yeast ecology has been gleaned through culture-dependent enumeration and detection methods; however, inherent restrictions placed by culture-dependent methodologies have limited our progress in understanding the ecology of yeast habitats. While they have limitations of their own, recently developed culture-independent methods are providing new insights into yeast ecology.

A broad diversity of microbial species including yeast play crucial roles in biogeothermal cycles and cycling of organic compounds, as well as human endeavors, including the food, biotechnology, and pharmaceutical industries. The global production of fermented beverages, involving primarily the yeast species *Saccharomyces cerevisiae*, amounts to billions of dollars annually. Non-*Saccharomyces* yeast species (nonconventional yeast) are being used in an increasing array of applications, including chemicals, enzymes, feed additives, fuels, flavors, and neutraceutical and pharmaceutical compounds (reviewed in Abbas 2003). Furthermore, the growing population of immunocompromised individuals has resulted in an alarming increase in the occurrence of mycoses. Pathogens and opportunistic pathogens are wreaking havoc on the medical care system. The influence of yeasts on human society is not a recent development. *S. cerevisiae* has been called the "oldest cultivated plant" (Braidwood 1953; Rose 1960) owing to its role in beverage fermentations. Moreover, it has been proposed that humans relinquished their nomadic lifestyle in favor of an agricultural one primarily to cultivate grain for fermented beverages (Braidwood 1953). Although this may be pressing the point, few would deny that yeast-fermented grain beverages are far more enticing than nonfermented ones.

Despite the huge influence of yeasts on natural ecosystems and human society, it is estimated that less than 5% of yeast species have been described (Staley et al. 1997). It is not known if this small percentage is representative of overall yeast diversity. The number of yeast species described to date (over 800) (Barnett et al. 2000; Kurtzman and Fell 2000 and subsequent publications) has been limited by several factors, including the limited number of yeast ecologists, systematists, and taxonomists. Another contributing factor is the constraints placed by the culture-dependent methods used to detect and characterize yeasts. The similar rich media and aerobic incubation conditions used for yeast surveys may not allow growth of many yeast species. For example, the large budding yeast species *Cyniclomyces guttulatus* was observed in the intestinal tract of rabbits in 1845 (Remak 1845), but was not successfully cultured for over 100 years. Repeated attempts to culture this organism on commonly used media such as malt extract were not successful, until the correct growth conditions were determined, which include humidity, temperature, pH, and nutritional requirements (Shifrine and Phaff 1958). Similarly, the yeast species *Coccidioascus legeri* was observed long ago within the cells of the intestinal epithelium of *Drosophila funebris* (Chatton 1913), and can be observed in Geimsa-stained gut smears of live *Drosophila* (Ebbert et al. 2003), but has eluded cultivation and characterization.

A revolution is currently under way in microbial ecology, owing to several culture-independent molecular, biochemical, and microscopy methods developed for the study of microbial ecology in various fields ranging from natural ecology to pathology to food spoilage to fermentation science. For example, unprecedented biological diversity is being revealed in many ecosystems through molecular methods such as the polymerase chain reaction (PCR) followed by hybridization or sequencing. Huge numbers of undescribed species are being detected solely on the basis of ribosomal DNA sequences. Unfortunately, while a ribosomal sequence may allow presumptive phylogenetic placement of a species, proper characterization of these uncultivable (or more properly, "not yet cultivated"), low-incidence, fastidious, or otherwise recalcitrant species must await further technological developments, including cultivation methods.

5.2 Sampling Methods

The sampling method used in any given application depends on what question is being asked. Similar questions are asked in a number of natural and anthropogenic contexts, including:

- What are the dominant yeast species in a habitat, and what is their relative and absolute abundance at various times?
- What are the nondominant species or strains?
- What vectors deliver these yeasts to this habitat?
- Is a specific species present, and in what abundance?

As fungi, yeasts are saprophytic, and thrive in habitats containing a simple carbon source. A host of yeast habitats have been surveyed by yeast ecologists, as described in other chapters of this book: the phylloplane, cactus, marine and fresh

water, insects, soil, forests, and extreme environments. These surveys have revealed that many yeast species are specialists, meaning they are found almost exclusively in specific habitats. New species, and new habitats of known species, have recently been discovered from sampling of habitats as diverse as leaves (Wang and Bai 2004), bees (Rosa et al. 2003; Brysch-Herzberg 2004), beetles (Lachance and Bowles 2002), cork (Villa-Carvajal et al. 2004), pickles (Tominaga 2004), and soft drinks (Stratford et al. 2002). Future analysis of unexplored habitats, such as insects and other invertebrates, will undoubtedly uncover additional species. Even frequently studied habitats such as soil contain undescribed species (Renker et al. 2004).

Certain food and beverage industries have developed standardized methods for microbial sampling, detection, and enumeration for quality control purposes, such as to confirm sanitation of equipment, or to follow the course of fermentation. These methods are more critical in some industries than in others. For example, the temporal and regional variation of product in the wine industry is not only accepted but celebrated, and ascribed such terms as vintage or *terroir*. Consumers enjoy the result of variation in fermentation conditions. On the other hand, the brewing industry, particularly any internationally marketed brand, depends on consistency of their product across the globe and over the years, and sanitation of the facility is more crucial for product quality and safety. The American Society of Brewing Chemists (ASBC) has, since 1945, produced a regularly updated handbook (ASBC 2003) with detailed protocols for the detection and enumeration of yeasts and bacteria from equipment, ingredients, and at various stages of the production process. Specific methods for microbial analyses of process water and compressed air supplies are even described. Internationally standardized methods for microbial analysis of wine have also been published, such as those by the Office International de la Vigne et du Vin (OIV) (OIV 2004). Owing to high risks of spoilage, methods for microbial analysis of dairy products (IDF 1987; APHA 2001) are particularly stringent. In the 1930s and 1940s, the International Association of Milk and Food Sanitarians and the American Public Health Association tested various media formulations to find one that could be used for aerobic plate counts of the broadest variety of fresh foods. "Standard methods agar" (now called plate count agar) was designated as the official medium for aerobic plate counts (Walter 1967). Detailed sampling, dilution, and plating methods for meats, produce, dairy products, water, food ingredients, and processed foods have been compiled by the International Commission on Microbial Specifications for Foods (ICMSF 1978, 1986). Despite publication of these recommended protocols, a review of 100 studies of plate counts of fresh meat and poultry products since 1985 found that 15 different plating media were used, as well as many different incubation temperatures and times (Jay 2002).

Studies of biodiversity in natural ecosystems must consider many of the same issues as industrial analyses, namely, the heterogeneity of samples, and temporal and spatial variability of populations within the substrate. Sampling, detection, and enumeration techniques must be carefully designed to take these factors into consideration. For example, when describing a new species, it has been recommended that the description be based on the isolation and characterization of a number of strains gathered at different times from different locations in order to obtain a clear view of the habitat preference and the biogeography of the species (Phaff et al. 1978). In

addition, the biogeography of a species has larger ramifications. The effect of geographic isolation of populations on the speciation of larger organisms has long been known. The effect of spatial separation of two or more populations on microbial speciation has recently been recognized (Papke and Ward 2004).

5.2.1 Surface Sterilization and Aseptic Sample Collection

A variety of methods have been used for gathering a sample for microbial analysis. In cases where the sample is inherently of very small volume and homogenous, such as the nectar in a flower, the liquid can be withdrawn with a sterile capillary pipet, and plated directly on isolation medium (Herzberg et al. 2002). Substances such as tree sap flux (Lachance et al. 2001) or insect frass (Phaff and do Carmo-Sousa 1962) have been directly placed or streaked onto the surface of plates. When determining the microbial flora present on plant surfaces, the plant material such as a leaf or flower petal can be pressed to the surface of a plate (Brysch-Herzberg 2004). Yeasts on the tarsi and other outer surfaces of insects have been isolated using a "walk plate," in which an insect such as a fruit fly (Lachance 1995) or bee (Rosa et al. 2003) is allowed to walk on the surface of an agar plate for 15-60 min, then removed. The material deposited by the insect may be spread over the surface of the plate with a sterile loop (Lachance et al. 1999). Yeasts that produce forcefully ejected ballistospores can be isolated from substances such as leaves using the ballistospore-fall method, described by Derx (1930). A leaf or other specimen is suspended above an agar plate, and the ballistospores are deposited onto the surface of the plate.

Other sampling methods must be used when sampling a larger specimen. With the exception of a laboratory fermenter, with its aerator aerating and impeller impelling, few microbial microcosms of significant size exhibit a uniform distribution of oxygen, nutrients, pH, moisture, or cell density. Furthermore, yeast are immobile, and thus the distribution of microorganisms is most likely not homogenous. For example, yeasts are most abundant on the surface of a grape in areas where juice might escape, such as around the stem (Belin 1972).

Thus it is important to select a sampling method that addresses the question at hand. If one is interested in determining what microbes may be deposited by or consumed by certain insects, the surface of a rot should be sampled. Identification of yeasts consumed by larvae feeding within the rot would require sampling of deeper tissues. Collection of several samples over a period of time would reveal the temporal succession of microflora. A survey of all yeasts present in the rot would require homogenization of a cross-section of rot, including sections both near the surface and within the rot.

This concept is reflected in recommended methods for obtaining a representative sample of brewing yeast. Yeast inocula used in brewing, whether a slurry or a compressed cake, "can easily become stratified" (ASBC 2003). Combination of a series of samples gathered aseptically from various sections of the yeast cake or yeast slurry is therefore recommended. Sampling techniques used in the brewery include letting several liters of liquid flow through the tap before collecting a sample (ASBC 2003). Similarly, in a clinical setting, patients are told to collect mid-stream urine. Examples of recommended methods for sampling various food products include

drilling to the center of a container of frozen eggs using a sterile bit, slicing a sector of Gouda cheese with a sterile blade, or aseptically collecting one center and one peripheral sample from canned meats (ICMSF 1986).

Sampling must also consider the differentiation between surface contaminants and autochthonous inhabitants. Often samples are surface-sterilized prior to removal of a sample for microbial analysis. Brewers may disinfect the zwickel cock with 70% ethanol, followed by flaming with a propane torch (C. Wallin, personal communication). Quality control technicians in a dairy packaging facility, on the other hand, may use an iodine solution to sanitize the sampling spigot. Certain clinical specimens are collected after swabbing the area with iodine, ethanol, or 2-propanol solutions to remove surface bacterial and other contaminants (ALA 1985). Similar methods can be used for surface sterilization of natural hosts of yeasts, but selection of a method requires careful consideration of the biology of the specimen being sampled. For example, sampling the intestinal microflora of small insects such as *Drosophila* must be performed very soon after collecting the specimen, because the ingested yeast are digested quickly (Shehata and Mrak 1951). Soaking certain insects in 70% ethanol is best preceded by immobilization by freezing for a few minutes to suppress the insect's regurgitation and ingestion reflex. Alternatively, the insect can be anesthetized with carbon dioxide prior to pressing the surface of the insect to an agar plate (Brysch-Herzberg 2004). Discussions with specialists with knowledge of the biology of the host organisms are highly valuable. Botanists can provide advice on the season and time of day when certain flowers will be in bloom and open, as well as where they may be found. Entomologists can be asked what season, weather conditions, and times of day are optimal for finding certain insect species, where they may be feeding, what luring and trapping methods are best used for that insect species, what body cavities may most likely harbor microbes, and how they can be dissected.

Different researchers may use different methods for surface sterilization of the same substance. For example, Rogers et al. (2004) compared several methods of surface sterilization of glacial ice cores prior to plating or PCR analysis, including exposure to bleach, ethanol, UV radiation, acid and base, and hydrogen peroxide. Treatment with bleach was found to be most effective in killing surface contaminants with the least loss of viability of interior microorganisms.

Aseptic sampling can involve a variety of implements, such as a sterile spatula, an inoculation loop, a needle, a swab, a pipet, or the collection of a liquid sample. The sample volume depends on the concentration of cells and the detection method to be used. Yeast in liquids with low concentrations of particulate matter, such as beverages or marine water, can be visualized and enumerated directly by microscopy, if present in sufficient abundance.

One of the most extensively studied yeast habitats is the wine fermentation. The resident yeast community has been examined from the grape in the vineyard through the stages of fermentation to the final product (Amerine and Kunkee 1968; Kunkee and Amerine 1977; Martini et al. 1980; Kunkee and Bisson 1993; Fleet et al. 2002; Ganga and Martinez 2004). As has been reviewed extensively, it is clear that the abundance and identity of yeast species shifts as the biochemical composition of the medium changes. Apiculate yeasts are common on the surface of the

grape, and in the early stages of fermentation. As the sugar level drops, and the ethanol level rises, ethanol-sensitive species subside, and *S. cerevisiae*, the champion of ethanol tolerance, predominantes. Successional stages of microbial flora have been observed in a number of other habitats, including, for example, a single slime flux over the course of a year (Phaff et al. 1964), a commercial malt whisky fermentation (van Beek and Priest 2002), degrading wood (Gonzalez et al. 1989), amapa fruit (Morais et al. 1995), and cocoa fermentation (Schwan and Wheals 2004). The biochemical and physiological environment of a microorganism is altered by the metabolic activity of the microbes themselves, which can affect several factors, including macronutients and micronutrients, pH, temperature, metabolites, and killer toxins. The community development in many cases "can be thought of as an orderly sequence of chemical changes each catalyzed by species waiting their turn in an imaginary eco-queue" (Giraffa 2004). Careful observation of the physiological state of a substrate is necessary to determine the optimal sampling strategy for enumeration and detection of yeasts. If the temporal course is of interest, samples should be collected before or shortly after the introduction of a yeast to a substrate, and at several points during and after the development of the yeast population.

Carefully recorded sampling details will not only allow future reisolation of specific species, but will also reveal details of the preferred habitat of a yeast species, successional stages, and geographic distribution. It has been shown that the yeast community present on grapes varies with factors such as climate, including temperature, rainfall, and geographic location (Parish and Carroll 1985; Longo et al. 1991); application of antifungal compounds (Monteil et al. 1986); grape variety and vineyard age (Martini et al. 1980; Rosini 1982; Pretorius et al. 1999); and soil type (Farris et al. 1990). Data that should be recorded when sampling any substance for yeast include geographic location (Global Positioning System, GPS, coordinates if possible), habitat sampled, state of the hosts and vectors present (whether flowers are in bloom, whether cactus flesh is rotting, presence of any insects), composition of any diluent used before plating or microscopy, surface sterilization method, medium formulation of plates used, and the length of any time delay between sampling and plating. Metadata that may be noted include references for host species identification, such as a field guide, and the reference datum used for GPS coordinates.

5.2.2 Sample Homogenization

The importance of collecting a representative sample, or a statistically significant number of samples, cannot be overstated. Yeasts and other microbes are not often evenly distributed in a substance. Scanning microscopy studies of leaves (Beech and Davenport 1970) and grapes (Belin 1972) have shown that yeasts adhere tightly to surfaces in the form of microcolonies. Bacteria are known to cluster in the rhizosphere around the roots of plants (Curl and Truelove 1986); yeasts may behave similarly. In certain foods, yeasts are also highly localized at high densities in reticulate structures (Fleet 1999). Geostatistical methods have been developed to describe spatial distribution of soil microorganisms, and power analyses can be used to determine the optimal sample size (Klironomos et al. 1999).

Where appropriate, a representative sample can be homogenized before analysis. This will give an estimate of the overall abundance of various species throughout the sample. Various methods have been used to homogenize specimens before enumeration by plating or other methods. The sample can be ground if necessary, then combined with a known volume of one of the diluents described later. The sample can be shaken manually or in a wrist-action shaker, pummeled in a peristaltic agitator such as a Stomacher, stirred on a magnetic stirrer, swirled in an orbital shaker, or pureed in a blender. Low-tech methods are also used: sterile gloves were used to hand-squeeze aseptically collected grapes in a study of *S. cerevisiae* isolated from indigenously fermenting musts (Cappello et al. 2004). Homogenization methods must be carefully selected. Fleet (1999) has summarized in detail the implications of erroneous enumeration of cell density owing to improper maceration and dilution steps when analyzing microbes in foods.

Fortes et al. (2001) have detected the pathogen *Cryptococcus neoformans* in the hollows of trees in Brazil. In cases such as this in which homogenization of a large sample, such as a tree, is not practical, a number of samples must be collected from different parts of the same rot and analyzed. Spatial heterogeneity results from inherent variability in the structure and composition of organic matter, differences in the external environment, and also results from microbial activity.

5.2.3 Sample Concentration

Many methods of enumeration of yeasts require a particular cell concentration range. Dilute liquid specimens can be concentrated by centrifuging at 3,000*g* or higher for several minutes, removing the supernatant, and resuspending the pellet in the remaining liquid (OIV 2004).

Yeast are known to be present in both freshwater (van Uden and Ahearn 1963; Spencer et al. 1964) and marine environments (Fisher and Brebeck 1894; van Uden and Fell 1968), and are known to be the dominant fungi in oceans (Sieburth 1979). However, direct plating is difficult owing to low cell counts, ranging from ten to 1,000 colony forming units (CFU) per liter (van Uden and Fell 1968). For enumeration of yeasts in liquids with low cell counts, whether marine water or beverages after bottling, membrane filtration is used to concentrate the cells to a detectable level. For most plating methods including membrane filtration, a target of 30–300 CFU per plate (Fugelsang 1997) or 20–200 CFU for wine (OIV 2004) or wastewater (Greenberg et al. 1992) or 25–250 CFU for various prepared foods (Zipkes et al. 1981) has been recommended as a statistically significant and countable range. A measured volume of liquid is passed through a sterile filter. Cellulose acetate or cellulose nitrate membranes with 0.45-μm porosity are recommended for beer samples (ASBC 2003). Antifoam can be placed in the receiving flask when filtering beer samples (ASBC 2003); this may be helpful for filtration of other liquids with the potential of foam formation. In the brewery, saline solution consisting of 0.85% NaCl is used to wash the membrane before transfer to an agar plate (ASBC 2003). Membrane filtration has also been used in the analysis of marine yeasts (van Uden and Fell 1968; Gadanho et al. 2003). After filtration, the membrane is placed on the surface of an appropriate agar medium, and colony formation on the surface of the membrane is monitored.

5.2.4 Sample Dilution

Desiccated substances, such as tree exudates, have been rehydrated with sterile water (Phaff and Starmer 1987). Samples with high concentrations of yeasts, such as decaying fruits, must be diluted before plating in order to obtain a countable and significant number of colonies upon plating. Serial dilution involves preparation of a series of tenfold dilutions in sterile diluent. Aliquots of each dilution are plated on appropriate media, and the number of colonies is used to calculate the concentration of yeasts in the original sample, expressed as CFU per milliliter.

Various diluents have been utilized for enumeration of yeasts in different contexts. Distilled water is not recommended in most cases owing to osmotic shock effects. Saline solution (0.85% NaCl) is used by brewers (ASBC 2003); 0.85% NaCl or Ringer's solution have been recommended for wine (OIV 2004); 0.1% peptone solution is used in analysis of foods (Mian et al. 1997); milk or Butterfield's phosphate-buffered water are used as diluents in analysis of diary products (Maturin and Peeler 1998). A low concentration of surfactant such as Tween 80 (0.01–0.05%) can be included in the diluent to aid in separation of cell clumps and filamentous structures (Deak 2003). Diluents with lower water activity must be used with osmotolerant yeast, such as *Zygosaccharomyces rouxii*, which may be encountered in very high sugar environments. Glucose concentrations of 20–30%, or glycerol concentrations of 18–26%, have given good recovery of this species from fruit juice concentrates and syrups (Hernandez and Beauchat 1995; Hocking et al. 1992).

Yeast in diluent must be plated promptly, particularly in saline solutions, which have been shown to have an adverse effect on viability, even for salt-tolerant yeast such as *Debaryomyces hansenii* (Andrews et al. 1997). Using the traditional plating method, 50–100 μL of a series of tenfold dilutions is placed on the surface of agar plates and spread with a flame-sterilized bent-glass rod while spinning the plate. Alternatively, a pour plate can be prepared by adding 15–20 mL of molten agar to up to 1 mL of liquid sample, and swirling to distribute the sample in the medium. These procedures involve a considerable expenditure in disposable plastics and media. The "track-dilution technique" is a modification of this method that decreases the time and expense involved (Jett et al. 1997). One 10-μL aliquot of each of six tenfold dilutions is spotted along one edge of a square agar plate. The plate is tipped so that the dilutions migrate in parallel tracks down the plate. After incubation, the dilutions with a countable numbers of colonies are selected and enumerated. Use of this method with wine-associated yeast cultures appears effective (D. Mills, personal communication).

Spiral plate counting is used in the food industry for enumeration of bacteria, and could also be used for enumeration of yeasts. Four logs of dilution of a sample are delivered on a single plate using a spiral plater. The dispensing stylus deposits decreasing amounts of a sample onto the surface of a rotating agar plate as it moves from the center of the plate outward. A sector with a reasonable number of colonies is selected for counting. The number of colonies in that sector is divided by the volume of sample dispensed on that sector to obtain the cell density, expressed as CFU per milliliter.

5.3 Direct Observation Methods

Antonie van Leeuwenhoek first viewed "animalcules," including brewing yeast, in the 1680s using his meticulously ground glass lenses. The microscope continues to be an extremely valuable tool in the microbiologist's laboratory. Direct observation of yeasts in a variety of substrates is used for detection and enumeration.

5.3.1 Microscopy

Light microscopy is useful for detection and enumeration of yeasts when the concentration of yeasts in the sample exceeds 5×10^5 cells/mL. Cell concentrations can be adjusted by concentration or dilution as described before. A detailed summary of yeast specimen preparation and photomicrography methods is presented in Barnett et al. (2000). The four optical systems commonly available and used to visualize yeast are bright field, dark field, phase contrast and differential interference contrast (DIC). Barnett et al. (2000) prefer the use of DIC for visualization of nonfilamentous vegetative cells, bright field for ascospores, and phase contrast for filaments.

In a clinical setting, a presumptive identification of certain fungal genera can be made on the basis of microscopic examination of clinical specimens (summarized in Koneman et al. 1997). For example, *Candida* spp. are seen as pseudohyphae, and sometimes as budding yeast forms. *Cryptococcus* spp. are seen as spherical and irregular-sized yeast forms, some with a thick polysaccharide capsule, and buds attached by a narrow constriction. In the winery, apiculate yeast species and *Brettanomyces*/*Dekkera* species also have characteristic cell morphologies. Some processing of specimens may be required to reveal the presence of yeasts. Potassium hydroxide is added to clinical specimens to dissolve epithelial and bacterial cells, allowing visualization of alkali-resistant yeast spores and hyphae by microscopy (Reilly 1991).

Electron microscopy has also been used to detect yeast cells in substrates such as ancient wine (McGovern 2003).

5.3.2 Direct Enumeration

Direct microscopy is used for the enumeration of many types of liquid suspensions of cells including yeast in various substrates. Brewers (ASBC 2003) and enologists (Fugelsang 1997; OIV 2004) commonly use a hemocytometer with Neubauer ruling, which has two etched 1-mm^2 grids of 400 squares each in a 0.1-mm-deep well, giving a volume of 0.1 μL. A sample of appropriate cell concentration is prepared. After homogenization and degassing, a sample is placed in the counting chamber and visualized under bright field illumination. The ASBC manual describes detailed protocols for reproducible counting, including whether to count cells touching the boundary lines, and when to count buds as additional cells. The number of cells per grid is multiplied by 10^4 and any dilution factor to obtain the number of cells per milliliter in the original sample.

The cell density of pure cultures in liquid medium can be estimated spectrophotometrically by measuring the optical density at 600 nm (OD_{600}). Cultures should be

diluted so that the OD_{600} is less than 1. The OD_{600} should be calibrated against another method of determining cell density, such as direct counting in a hemocytometer or plating for viable colonies. For the species *S. cerevisiae*, for example, an OD_{600} of 1 corresponds to roughly 3×10^7 cells/mL (Treco and Winston 2001).

5.3.3 Viability Staining

Brewers, enologists, histologists, and others use a variety of stains such as methylene blue, Ponceau-S, and Walford's stain to distinguish between viable and nonviable yeast cells. Of the various staining procedures available, methylene blue is preferred in both the wine and the brewing industries (Smart et al. 1999; ASBC 2003; OIV 2004). Methylene blue stains dead cells blue, while viable cells remain unstained. Various formulations are used, with and without buffers, but the preferred method for brewing yeast is the Fink–Kühles phosphate-buffered methylene blue method (Fink and Kühles 1933).

5.3.4 Fluorescent Labeling

DNA or RNA hybridization to probes specific for a species or group of species is an important tool for microbial ecology. Originally, radioactive labels were required, but more recently probes have been tagged at the 5′ end with markers such as fluroescein or rhodamine. After dot blotting, the cells are lysed to release nucleic acids, and the probes are hybridized. Changes in the relative amount of hybridization to ribosomal RNA (rRNA) reflect changes in the abundance and/or the rRNA content of the population being probed (Theron and Cloete 2000). Hybridization of monoclonal antibodies or nucleotide probes can also be performed in situ on environmental samples, which provides information on the spatial distribution of the species of interest. This method, called fluorescent in situ hybridization (FISH), has been used to study the spatial distribution of bacteria in minimally disturbed habitats such as in seafood (Connil et al. 1998), wine (Sohier and Laonvaud-Funel 1998), and cheese (Kolloffel et al. 1999), and yeast in tissue sections (Lischewski et al. 1996, 1997), *Aureobasidium pullulans* on leaf surfaces (Li et al. 1997), and *Brettanomyces* in wine (Stender et al. 2001). Use of FISH to analyze yeast on the phylloplane is described elsewhere in this book (see Chap. 13).

5.4 Culture-Dependent Methods

In the past, surveys of the yeast species resident in a habitat, host, or vector such as plants, insects, and soil were only possible through plating a specimen, incubating to allow for colony formation, noting the number and type of distinguishable colony morphologies, and identifying one or more isolate of each colony morphology (Phaff et al. 1978; Phaff and Starmer 1987). These types of plating methods involving selective plating and direct viable counts are relatively inexpensive, and provide information on the active, heterotrophic organisms in a population. These methods have given us innumerable insights into the life of yeasts, such as the range of species present, the substrates that support growth of certain yeast species, and the vectors

that distribute yeast to new habitats. We have learned that many yeast species are associated with a specific habitat and geographic range, which allows the isolation of a particular species at will from its known habitat. Culture-dependent methods have also resulted in the isolation of thousands of pure cultures that can be further characterized and utilized by future generations. In cases where the spectrum of resident species is well known, such as beverage fermentations, culture-dependent detection and enumeration techniques are preferred because they are reproducible, inexpensive, and accurately reflect the prevalence of the species of interest.

However, culture-dependent methods have limitations. Oftentimes different yeast species have indistinguishable colony morphologies, or one species may exhibit two or more colony morphologies, leading to inaccuracies in determining relative species abundances. Furthermore, there are very likely many species that do not grow under the growth conditions used, including temperature, pH, and a host of macronutrients and micronutrients. Cells growing in microcolonies or biofilms must be dislodged from their support medium in order to be enumerated accurately. Plate growth favors species that grow quickly. Taken together, these limitations influence the apparent diversity of the microbial community being analyzed. It may be impossible to estimate how many species are *not* detected using these methods.

It is now widely accepted that plate culturing techniques reveal only a portion of the true yeast diversity of a natural or man-made ecosystem: those individuals that are viable, and culturable. Organisms that are stressed, are in the so-called viable nonculturable (VNC) state, or that cannot grow under the conditions used are not detected. This in part explains why only a small fraction of the yeast species believed to exist have been described. A sublethally injured cell, including cells in the VNC state, may not be detected, but can survive until conditions improve. This has been observed in the case of lactic acid bacteria during wine storage and ageing (Millet and Lonvaud-Funel 2000), and has also been demonstrated for non-*Saccharomyces* yeast in wine fermentation (Cocolin et al. 2000a). Yeast cells that have been sublethally injured by heat or osmotic shock may grow poorly on plates, but can be revived by various resuscitation techniques (Fleet 1992; Deak and Beauchat 1996), indicating that they still influence the ecosystem.

5.4.1 Liquid and Solid Media

Being fungi, yeasts are saprophytic. Yeasts cannot perform photosynthesis or nitrogen fixation, and thus require carbon and nitrogen sources for growth. Yeasts also require a range of vitamins, minerals, and other growth factors, the range of which depends on the yeast species. Several rich media formulations have all the necessary factors required for the growth of many known yeast species. Media used for isolation and enumeration are generally complex and nutritionally rich. Common ingredients include a carbon source (e.g., glucose, fructose, sucrose), a nitrogen source, such as a digested protein (e.g., peptone, tryptone, casitone), and a complex supplement (e.g., yeast extract, malt extract). Selective and differential media are used to detect specific species or groups of yeasts. Some of the more commonly used rich media are described later. A sample of the selective and differential media that can be used in detection of specific species is also presented.

Malt extract medium, an early yeast medium formulation, was developed for the benefit of the brewing industry. This medium was made from diastatic malt, and was used either in a solid (malt extract agar) or in a liquid form. Wickerham modified this recipe by including yeast extract and peptone, resulting in yeast extract–malt extract (YM) medium still commonly used for maintaining and storing yeast cultures (Wickerham 1951). YM and many other standardized isolation and selection media formulations are commercially available in premixed form.

Several formulations of rich media are used by researchers in various fields. Clinical yeast isolations are often performed using Sabouraud's glucose agar (SGA) (Odds 1991). Brewers often use universal beer agar (ASBC 2003). In addition to malt extract and YM media, commercially available rich media useful for the cultivation of yeasts include yeast extract, peptone, and dextrose (YEPD), potato dextrose agar (PDA), and tryptone–glucose–yeast extract agar (TGYA). The development of these and other media has been described (King et al. 1986; Jarvis and Williams 1987; Fleet 1990; Deak 1991). Variants on TGYA have been developed, with differences in glucose concentration, or supplemented with chloramphenicol to retard growth of bacteria, or acidified to pH 3.5. One variant, yeast extract glucose chloramphenicol agar, lacking tryptone, has been recommended by the International Organization for Standardization as an international standard medium (ISO 1987) for use in food analysis. Yeasts from high-sugar environments such as nectar (Herzberg et al. 2002) have been grown on a medium containing 40% sugar (Hautman 1924).

The presence of bacteria and spreading molds in certain foods, decaying fruits, and soil complicates plating-based enumeration of yeasts. Supplements added to media include antibiotics such as oxytetracycline or chloramphenicol to decrease the growth of bacteria. Propionic acid or calcium propionate decrease mold growth significantly, but also limit growth of some aerobic yeast (Buhagiar and Barnett 1971). Rose Bengal (Martin 1950), ox gall (Miller and Webb 1954), eugenol, dichloran (Bell and Crawford 1967), or oligomycin can be added to inhibit rapidly spreading molds. Deak et al. (1998) have recommend that media containing Rose Bengal should be kept in the dark, to prevent degradation of Rose Bengal to a mold-inhibiting derivative. Studies of the recovery of yeasts from food products, including cheese and other foods, have indicated that media containing these additives perform more favorably than previous media acidified with organic or inorganic acids (Beauchat 1993). Researchers in five countries recently evaluated 11 selective media for the enumeration of yeasts from blue-veined cheese (Viljoen et al. 2004), which contains high levels of *Penicillium* species, bacteria, and often yeasts as natural contaminants, causing spoilage or affecting the final flavor. The tested media contained various additives such as biphenyl, molybdate, oxytetracycline, gentamycin, chloramphenicol, and eugenol to control bacteria or molds. Biphenyl was superior to Rose Bengal in controlling mold growth. The results indicated that dichloran 18% glycerol agar (DG18) and malt extract with biphenyl were superior in mold inhibition and yeast recovery from blue cheese, while Rose Bengal chloramphenicol agar, dichloran Rose Bengal chloramphenicol (King et al. 1979), and malt extract agar supplemented with sodium chloride and oxytetracycline were also acceptable for sampling this substrate. The use of plates containing Rose Bengal and/or chloramphenicol in recent

ecological studies (Lachance et al. 2001; Rodrigues et al. 2001; Rosa et al. 2003; Villa-Carvajal et al. 2004) marks a shift from the long-standing use of acidified media for control of bacteria.

When the spectrum of yeast species commonly found in a particular substrate has been extensively studied, it is possible to design a selective minimal medium in which only one or a few of the resident species are able to grow, based on the carbon and nitrogen assimilation patterns or other growth characteristics. Positive selection can be used to enumerate only yeast that can grow on a particular carbon or nitrogen source. For example, the cactus-specific yeast *Candida sonorensis* has been enumerated on yeast nitrogen base agar with 0.5% methanol as the sole source of carbon (Miller et al. 1976). Negative selection can be used to eliminate the growth of yeast species that can only grow in the presence of a particular nutrient, such as the sulfur-containing amino acids required by *Pichia amethionina* (Starmer et al. 1978). Media and growth conditions have been developed for selection of psychrotrophic, acid-resistant, or xerotolerant yeasts (Fleet 1992; Deak and Beauchat 1996). Several selective media are used in the brewery to detect wild *Saccharomyces* and non-*Saccharomyces* species. Detection of contaminating wild yeast is particularly crucial in brewing because pitching yeast is often reused for several fermentations. Several selective media have been designed for the detection of wild yeast contaminants in the presence of brewing yeast (ASBC 2003). Lysine medium contains L-lysine as the sole nitrogen source (Heard and Fleet 1986; Walters and Thiselton 1953). Wild non-*Saccharomyces* species grow on this medium, while brewing and wine strains of *S. cerevisiae* do not. Lin's wild yeast medium contains fuchsin sulfite and crystal violet, and restricts growth of brewery yeasts and permits growth of wild yeasts. Medium containing dextrin as the sole carbon source can be used to detect superattenuating yeast (Ingledew and Casey 1982). CLEN medium contains cadaverine, lysine, ethylamine, and nitrate as nitrogen sources, and allows some wild yeast to grow, while brewing yeasts are unable to grow. Additional differential media include Schwarz differential medium (Brenner et al. 1970), and MYGP plus copper sulfate medium (Taylor and Marsh 1984) (MYGP is another name for YM medium, so named because it contains malt extract, yeast extract, glucose and bacto peptone.) Certain contaminating yeast can also be detected by incubation at 37°C. A comparison of several of these methods for detection of nonbrewing yeasts in pitching yeast was performed by van der Aa Kuhle and Jespersen (1998). In an analysis of 101 pitching yeast samples from 45 breweries, contaminating yeast were most often detected on MYGP plus copper sulfate medium. Because different contaminants grow on different media, analysis on multiple media has been recommended (van der Aa Kuhle and Jespersen 1998).

Molybdate agar containing 0.125% propionate can be used to distinguish several yeast species found on tropical fruit (Rale and Vakil 1984). Kish et al. (1983) developed ethanol sulfite agar, containing 12% v/v ethanol and 150 mg/L sulfite to detect *S. cerevisiae* when outnumbered by apiculate yeasts. Acid-resistant yeasts can be enumerated on *Z. bailii* agar (Erickson 1993), or TGYA. Xerotolerant yeast can be enumerated on DG18 (Deak and Beauchat 1996). *Zygosaccahromyces* differential medium was developed to detect the spoilage yeast *Z. bailii* in wine (Schuller et al. 2000).

Agar medium can be supplemented with indicator compounds to distinguish between yeast species, or strains within a species. For example, acid-producing yeast species can be identified on plates containing a pH indicator such as bromocresol green or bromophenol blue. 5-Bromo-4-chloro-3-indolyl-α-galactoside (X-α-gal) is used to differentiate between melibiase-secreting lager yeast and nonsecreting ale yeast. Ale yeast colonies grown on medium containing X-α-gal remain white, while lager yeast colonies turn blue-green. The pathogen *Cr. neoformans* is identified in clinical specimens by formation of a black pigment on medium containing 3,4-dihydroxyphenylalanine (Chaskes and Tyndall 1975). *Dekkera*/*Brettanomyces* differential medium was developed to detect low levels of *Brettanomyces* and *Dekkera* species, which can cause phenolic taint in wine (Rodrigues et al. 2001). This medium is partially selective for *Brettanomyces* and *Dekkera* owing to the presence of ethanol as the sole carbon source and inclusion of cycloheximide, and is differential for the detection of these genera based on colony morphology, the time required for colony growth, the color change of a pH indicator, and the distinctive odor released by metabolism of *p*-coumaric acid to phenolic compounds. Another example of a differential medium is Niger seed agar (NSA), which was developed for testing environmental samples for the presence of *Cr. neoformans*, which forms brown colonies on this medium (Staib 1962). NSA has been used to detect *Cr. neoformans* in various trees and bird droppings (Pfeiffer and Ellis 1992; Sorrell et al. 1996; Lazera et al. 1998, 2000; Fortes et al. 2001). This medium can also be used to screen clinical specimens (Sukroongreung et al. 2001).

In the analysis of clinical specimens, SGA is frequently used as a primary isolation medium. However, differentiation of *Candida* species on the basis of colony morphology is not possible on this medium. Different yeast species plated on CHROMAgar Candida (CHROMAgar Co., Paris, France), which contains a proprietary chromogenic substrate, produce distinctive colony colors. Because this medium contains chloramphenicol to suppress bacterial growth, it can be used as both a primary isolation plate and a differential screening plate. Of 22 clinically relevant species screened, only *C. albicans* formed green colonies on this medium, allowing a presumptive identification of this species from clinical samples (Odds and Bernaerts 1994). This medium has been particularly useful to identify mixed infections, allowing appropriate antifungal treatment (Yera et al. 2004). The range of colony colors obtained with different yeast species may make this medium useful as a primary isolation plate for ecological surveys. Molds, however, are able to spread on this medium.

Variations on the standard Petri plate include Petrifilm (3M, St. Paul, MN, USA) and SimPlate (BioControl Systems, Bellevue, WA, USA), which are reported to produce comparable enumeration results to standard plate counts in the analysis of various foods (Beauchat et al. 1990, 1991, 1998). The thin, flexible Petrifilm yeast and mold count plate may prove convenient in remote locations for isolation of yeasts from certain natural habitats. A thin, clear film is peeled back to expose a water-soluble gel matrix. After addition of a liquid specimen, the film is replaced and the plate is incubated. After growth, colonies can be removed for purification and analysis. This film compares favorably to conventional plate counts in the enumeration of yeasts from various foods (Beauchat et al. 1990). Limitations include the inability to

streak for separation of colonies, and differences in colony morphology from those seen on standard Petri plates owing to the contact of the cover membrane with the growth medium.

5.4.2 Growth Conditions

In general, yeasts are grown in the laboratory under conditions similar to those of their natural habitat. Nutrients and other growth conditions can be manipulated to select for growth of particular species or groups of species. Enrichment cultures are used to isolate and characterize any number of niche-specific microbes, ranging from pesticide-degrading bacteria to thermophilic bacteria to halotolerant yeast. Simply stated, a specimen is placed in media with restrictive growth conditions, and passaged for several generations until the organisms best able to grow predominate. The restrictive growth conditions can be the presence of a certain compound as the sole carbon and/or nitrogen source, the presence of a growth-inhibiting compound, low water activity, or high or low temperature. The fermentation of wine can be considered one of the earliest enrichment culture techniques used by man. While *S. cerevisiae* is present in very low numbers on grapes (Rosini et al. 1982; Martini et al. 1996), the proportion of this ethanol-tolerant species relative to that of other yeast species rises dramatically as the ethanol concentration increases. Indigenous fermentation has been used as an enrichment technique to isolate wild strains of *S. cerevisiae* from aseptically gathered grapes (Cappello et al. 2004; Versavaud et al. 1995). An enrichment technique was also used to isolate novel limonene-degrading yeast species (Ngyuen Thanh et al. 2004).

The growth of most yeast species is not inhibited by a pH value as low as 3.0, although the optimal pH range for growth of most species is 4.5–6.5 (Phaff et al. 1978). Low pH media have been used in yeast surveys for decades to inhibit growth of bacteria (Miller et al. 1962).

Yeast can generally be grown at temperatures close to that of their natural habitat. This is particularly important for yeasts isolated from low temperatures, such as the *Cr. vishniacii* isolated from soil in Antarctica (Vishniac and Hempfling 1979), the type strain of which grows well at 10°C but poorly at 20°C. Many species isolated in temperate zones grow well at 20–25°C, and poorly at 30°C. A few psychrophobic yeasts, isolated from warm-blooded animals, require incubation temperatures above 30°C (Travassos and Cury 1971).

Aerobic plating conditions are used predominantly for quality control in the food industry (APHA 2001) and also in most surveys of yeast biodiversity. However, the presence of oxygen severely inhibits growth of many microbial species. The use of anaerobic in addition to aerobic conditions may improve yeast surveys of potentially anaerobic or microaerobic environments such as deep water, vertebrate and invertebrate guts, and the interior of plant rots. Under field conditions where anaerobic incubators are impractical, convenient anaerobic devices such as a candle jar or the Gas Pak chamber or bag (BD Biosciences) can be used for generation of anaerobic, microaerophilic, or CO_2-enriched conditions. Alternatively, a novel paraffin wax overlay method has been used to exclude oxygen from pour plates, allowing the isolation and enumeration of purple non-sulfur bacteria from flooded paddy soil (Archana et al. 2004).

Many yeast species form colonies on agar plates within 2–3 days. However, when exposed to a variety of stresses (including lyophilization and cryopreservation), colony formation by even fast-growers may be delayed. Slow-growing yeast species such as *Z. bailii* or *Dekkera bruxellensis* may require up to 14 days to form colonies (Millet and Lonvaud-Funel 2000; Rodrigues et al. 2001). An appropriate incubation time must be selected to allow growth of yeasts of interest.

Manipulation of incubation conditions may reveal the presence of rare or slow-growing yeasts. A decrease in incubation temperature from 25 to 17°C, an increase in humidity, and periodic replacement of the plates used in a ballistospore-fall plate (Nakase and Takashima 1993) resulted in the isolation of four novel slow-growing *Sporobolomyces* yeast species from plant leaves in Japan (Wang and Bai 2004).

Some yeast species require medium amendments for satisfactory growth on agar media. For example, *Schizosaccharomyces octosporus* will grow poorly on minimal medium unless it is supplemented with 15 mg adenine/L (Northam and Norris 1951). The yeast *Debaryomyces mycophilus*, associated with wood lice, is only able to grow on media supplemented with chelated iron (Thanh et al. 2002). While most halotolerant yeast species can grow well on media lacking sodium chloride, strains of the species *Metschnikowia bicuspidata* will not grow on media with salt concentrations less than 2% (Lachance et al. 1976). In contrast, sugar-tolerant yeast species such as *Z. bisporus* or *Z. rouxii*, found in high-sugar environments such as honey, do not grow well when placed directly on standard media containing much lower sugar concentrations. It has been recommended to use media with 30–40% sugar for primary isolation of yeasts from these environments, followed by transfer to lower-sugar media (Phaff and Starmer 1987). The yeast-like organism *Eremascus albus*, however, does not have the ability to adapt to lower-sugar media (Phaff and Starmer 1987).

5.4.3 Liquid Culture Methods

The most probable number (MPN) method was developed for the enumeration of microorganisms. This method is particularly useful for enumeration of yeasts in a specimen containing a high particulate content which would preclude membrane filtration or microscopy, such as must or solid food. The specimen is homogenized, and a series of tenfold dilutions are prepared. When inoculated in culture medium, the more concentrated cell suspensions will result in growth, while the more dilute suspensions will not. Using a table based on McCrady's probability calculations, the positive and negative growth results from the dilutions are used to extrapolate the concentration of cells in the original sample (Greenberg et al. 1992). This method is used in the analysis of food (Harrigan 1998), wine (Fugelsang 1997; OIV 2004) and wastewater (Greenberg et al. 1992). When used in conjunction with selective media, the MPN technique can be used to enumerate minority spoilage or fermenting yeast (Loureiro and Malfeito-Ferreira 2003).

Just as a pure microbial culture displays a distinct pattern of carbon source utilization, a mixed population of microbes also exhibits a distinct pattern (Garland and Mills 1991). Changes in microbial populations are reflected in changes in carbon utilization patterns, which are measured using a technique called community level physiological profiling (CLPP). Biolog (Hayward, CA, USA) distributes

fungal-specific microtiter plates, SFN2 and SFP2, with three replicates of 31 environmentally relevant carbon sources in liquid media (Classen et al. 2003). After inoculation with environmental samples, the ability of the resident microbial community to utilize the various substrates is monitored. Multivariate analysis is used to determine functional diversity of environmental populations. This method has been used to assess metabolic diversity in environmental substrates including plant rhizospheres (Ellis et al. 1995; Garland 1996; Grayston and Campbell 1996; Grayston et al. 1998). The API system (bioMerieux, France), including strips for identification of yeast species, could be used to measure functional diversity of yeasts and other microbial communities (Torsvik et al. 1990). The advantages of this method are that it is fast, reproducible, relatively inexpensive, and can distinguish differences in microbial communities. Because CLPP is a culture-dependent method, however, it has the limitations common to these methods: the profile only represents the culturable portion of the community, and it favors fast-growing organisms (Kirk et al. 2004). Moreover, the in vivo metabolic diversity may not reflect in situ diversity.

5.5 Indirect Detection Methods

Although brewing yeast were some of the first microorganisms visualized by van Leeuwenhoek, many of the recent advances in microbiology first emerged in studies of prokaryotes and were then applied to eukaryotes. Yeast researchers have applied these new methods, from DNA–DNA hybridization to ribosomal sequencing, resulting in new knowledge of yeast biodiversity. Some recent advances in detection and enumeration of mixed microbial populations have also been based on methods used to characterize pure cultures.

5.5.1 Biochemical-Based Methods

Signature fatty acids are indicative of specific taxonomic groups, and form a relatively constant proportion of cell biomass when standardized culture conditions are used. Just as species can be differentiated by fatty acid methyl ester (FAME) analysis, a change in the fatty acid profile of a community can be detected. Zelles (1999) has reviewed the use of FAME analysis to characterize microbial communities. FAME analysis does not require culturing, and the detection of signature fatty acids indicates the presence of certain taxonomic groups. However, this method has limitations, including the fact that the cellular fatty acid composition can vary under different growth conditions, such as temperature and nutrition.

5.5.2 Molecular Methods

Molecular, DNA-based methods of detection and enumeration have gained popularity in recent years owing to the ease of use, specificity, and, unlike biochemical methods such as FAME analysis, independence of the metabolic state of the cell. PCR-based methods are particularly powerful owing to the sensitivity of these methods, as will be discussed later. Many methods are based on PCR amplification of DNA from a mixed population, followed by separation by a variety of techniques.

5.5.2.1 Community Profiling Methods

Two DNA-based methods developed for the analysis of pure cultures have recently been modified for profiling the genetic diversity of communities of microorganisms: guanine plus cytosine (G+C) content and nucleic acid reassociation. While they do not provide detailed information on the identity of individual species present, they can be used as a measure of the genetic diversity of a microbial community, and can detect shifts in microbial community profiles.

The G+C content of DNA is characteristic of a yeast species, with ascomycetous yeast species having G+C contents of 27–50%, and basidiomycetous species having contents of 48–70%. The overall G+C content of mixed populations, as well as other methods, has been used to study the differences in microbial diversity between forest cover and pasture in Hawaiian soil (Nusslein and Tiedje 1999). This method has the advantage in that it is not subject to PCR amplification biases, but it does require large amounts of DNA.

The rate of DNA–DNA reassociation of two yeast strains is an extremely valuable measure of genetic similarity of closely related species and strains. DNA reassociation rates have also been used to assess the genetic complexity of DNA from an environmental sample, which reflects the biodiversity of the resident microbial community (Torsvik et al. 1990). The rate of reassociation of DNA extracted from an environmental sample depends on the complexity of the DNA sequences present: as the complexity increases, the rate of DNA reassociation decreases (Theron and Cloete 2000). The time needed for half the DNA to reassociate ($C_o t1/2$) is considered a diversity index, which can be compared with that of other environmental samples. Moreover, the rate of reassociation of DNA isolated from two different environmental samples has been used to measure the similarity of their microbial communities (Griffiths et al. 1999).

5.5.2.2 Detection of Species or Groups of Species

Hybridization-based molecular methods can be used to detect specific species or groups of species in environmental or other samples. While these methods can provide detailed information on the species present in a sample, they suffer from lack of sensitivity: nondominant species may not be detected. PCR amplification of target sequences prior to hybridization can often be used to eliminate this problem. DNA can be amplified directly to detect all species present, whether active or dormant, and complementary DNA from reverse transcription of messenger RNA (mRNA) can be amplified to detect active microbial communities.

Flow cytometry has been used to detect, enumerate, and characterize liquid suspensions ranging from bacteria to mammalian cells (reviewed in Davey 2002), and has been used extensively for analysis and sorting of cells, including yeast. For example, Jespersen et al. (1993) used an enrichment technique followed by flow cytometry to detect as few as one wild yeast cell in a background of 106 brewer's yeast cells. The Luminex 100 is a flow-cytometer system utilizing a set of color-coded fluorescent latex beads, each of which can be tagged with a different nucleotide probe. Using species-specific capture probes based on ribosomal sequences, this method

has been used for detection and enumeration of yeasts of the genus *Trichosporon* in a mixed population (Diaz and Fell 2004). The Luminex system is extremely rapid, sensitive, and can distinguish between sequences differing by a single nucleotide. The range of species being detected must be determined in advance.

PCR methods have been developed for the detection and identification of a single species or group of species. Species-specific primers have been developed for the identification of pure cultures, as discussed elsewhere in this book. These primers have been useful in detecting specific species in mixed population and environmental samples. For instance, *S. cerevisiae* was detected in an ancient wine jar from the tomb of King Scoprion I in Egypt, dated to 3,000 B.C. (Cavalieri et al. 2003) using primers that amplify the *S. cerevisiae* ITS1, 5.8S, and ITS2 region (Guillamon et al. 1998). A mixed microbial community can be profiled by amplifying a target sequence, then separating the amplicons by a variety of methods.

Variations on PCR-based detection methods introduce even more sensitivity. A nested PCR method developed for detection of *Dekkera/Brettanomyces* strains was sensitive to as few as ten cells in sherry (Ibeas et al. 1996). In multiplex PCR, primers to several target genes are combined in one PCR amplification. This has been used, for example, to detect and discriminate between pathogenic *Escherichia coli* strains on the basis of the presence of six strain-specific toxins and virulence factors (Watterworth et al. 2004).

Quantitative PCR (Q-PCR) can be used to detect the presence of specific organisms in environmental samples. An extremely sensitive and accurate Q-PCR method has recently been developed for the detection of six pathogenic *Candida* species in drinking water (Brinkman et al. 2003). Species-specific PCR primers were designed to amplify a region of the D1/D2 domain of the large (26S) ribosomal subunit. This filter-based method could detect as few as 1–3 cells per filter. Another Q-PCR assay using species-specific primers to the D1/D2 domain of the 26S rRNA gene was developed to detect and enumerate the spoilage yeast *D. bruxellensis* from wine (Phister and Mills 2003).

Ribosomal intergenic spacer (IGS) analysis and automated ribosomal IGS analysis have been used to profile bacterial communities. In these methods, the IGS between the 16S and 23S ribosomal subunits is amplified by PCR, denatured, and analyzed on a polyacrylamide gel under denaturing conditions. The IGS region of different species differs in length and sequence. These differences are detected directly by silver staining of the gel, or by automated detection of a fluorescently labeled primer (Fisher and Triplett 1999). These methods have been used to compare microbial diversity in soil (Borneman and Triplett 1997; Ranjard et al. 2000) and the rhizosphere of plants (Borneman and Triplett 1997).

Molecular detection methods have the distinct advantages of being culture-independent, and, if DNA sequencing is involved, can also provide information on the phylogenetic placement of species detected. These molecular methods have revealed the presence of previously undetected and uncharacterized species. However, DNA-based methods have limitations and potential biases at each step, including cell lysis, DNA extraction, and purification. The efficiency at which cells or mycelia are lysed can vary within and between microbial groups (Prosser 2002): spores and mycelia lyse differently, and mycelia of different ages lyse differently. DNA and RNA extrac-

tion methods that result in DNA shearing, such as bead beating, can also lead to biases (Wintzingerode et al. 1997). Certain contaminants in DNA preparations such as humic acids from soil can interfere with PCR amplification. Subsequent purification steps can lead to loss of DNA or RNA. Furthermore, detection of cells that have been stressed or injured can be difficult.

Wintzingerode et al. (1997) have summarized factors that can result in differential PCR amplification of templates within a mixed population, including different affinities of primers to various templates, different copy number of templates, and varying levels of hybridization efficiency and primer specificity. Furthermore, sequences with lower G+C content may separate more easily, and therefore be preferentially amplified (Wintzingerode et al. 1997). This could conceivably lead to biases in amplification in mixed populations of ascomycetous and basidiomycetous yeasts, which differ in G+C content.

DNA microarrays bring a new level of specificity to DNA–DNA hybridization studies. Because a single array can contain thousands of DNA sequences, a large number of target sequences can be detected in an environmental sample simultaneously. These target sequences can be either species-specific probes to detect and quantify particular species, or they can be function-specific probes, such as metabolic genes, to detect functional diversity. Microarrays have been used to detect and quantify several bacterial species in a sample with high specificity (Cho and Tiedje 2001). Direct profiling of microbial communities in sediment samples was performed by hybridization of extracted rRNA to microarrays containing oligonucleotides specific for major microbial groups (El Fantroussi et al. 2003).

Target regions to be amplified are often rRNA genes or internal transcribed spacer (ITS) regions, because these are present in all organisms, they are not subject to horizontal transfer, and sequence databases are publicly available. Although only a small proportion of yeast species have been described, certain DNA regions such as the D1/D2 region of the large (26S) ribosomal subunit as well as the ITS1 region have been sequenced in the majority of known yeast species. This information is extremely useful for identification of yeast species, as discussed elsewhere in this book, as well as for the detection of specific species within a mixed population. Moreover, community-level fingerprints representative of microbial diversity can be obtained using PCR amplification of target DNA such as D1/D2 or ITS regions, followed by separation of amplicons by two similar methods, denaturing gradient gel electrophoresis (DGGE) (Muyzer et al. 1993) or temperature gradient gel electrophoresis (TGGE). The difference between these two methods is the nature of the gradient: denaturing chemicals (urea and formamide) in the former, and temperature in the latter. In both techniques, the forward primer contains a 35–40 base pair GC clamp, which ensures that part of the amplified DNA fragment remains double-stranded as it passes through the denaturant. Amplicons are separated on a polyacrylamide gel. As they pass through an increasing concentration of denaturants, domains melt in sequence-specific manner, with AT-rich domains denaturing before GC-rich ones. The bands can be excised, reamplified, and sequenced to identify the species. The number and intensity of the bands is indicative of the genetic diversity of the sample. Theoretically, a single base-pair difference can be detected. DGGE and TGGE are reliable, reproducible, rapid, and multiple samples can be analyzed

simultaneously. DGGE and TGGE have been used extensively to detect bacterial and/or fungal diversity in a range of habitats, such as the guts of pigs (Simpson et al. 2000) and chickens (van der Wielen et al. 2002), soil (Gomes et al. 2003), and rhizosphere (Duineveld et al. 1998, 2001; Smalla et al. 2001). Cocolin et al. (2000a) used this method to analyze the yeast species present at successional stages in wine fermentations. Nonculturable yeast were detected late in the fermentation of a *Botrytis*-affected wine (Mills et al. 2002). Prakitchaiwattana et al. (2004) compared DGGE with traditional plating methods to study the yeast ecology of wine grapes. DGGE was less sensitive than agar plating methods, detecting only populations of more than 104 cells/mL, but a greater diversity of species was detected. DGGE has also been used to detect yeast in the fermentation of *Coffea arabica* (Masoud et al. 2004) and sourdough (Meroth et al. 2003a).

Theoretically, the PCR primers selected should amplify all species of interest in the microbial community. Universal or group-specific ribosomal DNA regions are often used. For instance, primers to the bacterial nitrite reductase gene (*nirK*) were used to detect denitrifying bacteria in soils. The sequences of most of the of 56 PCR products analyzed were similar, but not identical, to those of known species, indicating the presence of uncultivated denitrifying species (Henry et al. 2004). In some cases, reverse-transcriptase PCR (RT-PCR) of short-lived mRNA is used to preferentially detect metabolically active cells (Sheridan et al. 1998). For example, an RT-PCR method was recently published for the detection of *Cr. neoformans* in clinical specimens, based on the amplification of the capsular *CAP10* gene mRNA (Amjad et al. 2004). Very little target DNA is required.

Single-strand conformation polymorphism (SSCP) is also based on the electrophoretic separation of DNA molecules on the basis of small differences in DNA sequence. Polyacrimide gel electrophoresis is used to separate single-stranded PCR-amplified DNA on the basis of differences in their secondary structure (Lee et al. 1996) caused by differences in sequence. In addition to several bacterial studies, this method has been used to study the arbuscular mycorrhizal fungi species present in roots (Simon et al. 1993; Kjoller and Rosendahl 2000). SSCP has also been used to detect polymorphisms in clinical isolates of *C. albicans* (Graser et al. 1996). Limitations of this method are similar to those of DGGE/TGGE. Unlike DGGE/TGGE, however, SSCP does not require a GC clamp or the use of gradient gels.

Restriction fragment length polymorphism (RFLP) patterns, in addition to amplified rDNA restriction analysis, are very useful to identify pure microbial cultures, including yeasts (Esteve-Zarzoso et al. 1999). RFLP patterns have also been used to detect changes in bacterial community structure (Massol-Deya et al. 1995), but cannot be used to quantify diversity or detect specific phylogenetic groups (Liu et al. 1997). The use of a 6-base rather than a 4-base recognition restriction enzyme would result in a less complex pattern when mixed microbial communities are analyzed.

The RFLP method has been modified to address some of these limitations. In terminal RFLP, one of the PCR primers is labeled with a fluorescent dye, which allows the detection of only the terminal restriction fragment. This results in a simplified banding pattern, with one labeled fragment representing each sequence

present (Tiedje et al. 1999). A similar method, terminal fragment length polymorphism analysis, has been developed for the differentiation of *Malassezia* yeast species (Gemmer et al. 2002). This nested PCR approach utilizes a second PCR reaction rather than restriction digestion, and could be useful for the detection and discrimination of other fungal species.

5.6 Concluding Remarks

Both culture-based and culture-independent detection and enumeration methods have great utility in the detection and enumeration of yeasts, but they also have limitations. A polyphasic approach is recommended to draw on the strengths of both these methods. As a case in point, in a study of the microbes responsible for the traditional fermentation of cassava dough, some *Lactobacillus* species detected by DGGE were not recovered on plating, while some species recovered from enrichments were not detected by DGGE (Miambi et al. 2003). A combination of DGGE/TGGE and plating methods was more informative than either method alone for the identification of dominant populations within the community in studies of sausage (Cocolin et al. 2000b), cheese (Ercolini et al. 2002; Ogier et al. 2002; Randazzo et al. 2002), and doughs (Meroth et al. 2003b). Prakitchaiwattana et al. (2004) compared DGGE with cultural isolation of yeasts from wine grapes, and found that DGGE was less sensitive to low-concentration organisms, but detected a greater diversity than plating. The use of a combination of culture-dependent and molecular methods is recommended in detecting the spectrum of yeast species that may be present in a substrate.

When examining a substrate that may contain nonculturable organisms, microscopy methods such as FISH in addition to plating have been successful in the study of the microflora of cheese (Kolloffel et al. 1999) and wine (Millet and Lonvaud-Funel 2000). However, FISH has a low level of sensitivity, making it difficult to enumerate nondominant populations. Furthermore, cells with low rRNA levels, or damaged or stressed cells, may be difficult to detect by FISH. DNA-based methods that do not require cultivation of microorganisms, such as shotgun metagenome sequencing, are revealing aspects of the metagenome of mixed microbial populations such as soil (Rondon et al. 2000) and marine water (Venter et al. 2004). While information on individual species is difficult to obtain by shotgun sequencing, particularly for organisms such as yeast that have multiple chromosomes, a billion base pairs of nonredundant sequence revealed a plethora of information on the gene content, diversity, and relative abundance of organisms present in sea water (Venter et al. 2004), including the presence of 148 previously unknown bacterial phylotypes and 782 new rhodopsin-like photoreceptors. While these studies do not result in isolation of type cultures, amazing insights into the lives of yeasts can be obtained by these methods.

While PCR-based methods are powerful and useful methods, limitations of these methods include PCR biases (Ercolini et al. 2002), labor, and the difficulty in detecting organisms representing less than 1–2% of the population (MacNaughton et al. 1999). Also, one band may represent more than one species, and one species may give multiple bands on the gel. Intragenomic sequence heterogeneity in the

ribosomal region has been demonstrated with many bacterial species (Coenye and Vandamme 2003) and also in the yeast species *Clavispora lusitaniae* (Lachance et al. 2003). This sequence heterogeneity within a strain could result in multiple bands.

Some methods introduced for the analysis of bacterial communities hold great promise for use in detection and enumeration of pure and mixed communities of yeasts. These include microarrays containing species-specific probes to a number of species, and the many variants of PCR analysis. However, the use of culture-dependent methods is far from obsolete. Culture-based methods using new selective and differential media and variations in growth conditions will continue to be valuable tools of the yeast ecologist.

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Sugar Metabolism in Yeasts: an Overview of Aerobic and Anaerobic Glucose Catabolism

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6.1 Introduction

Yeasts are ubiquitous unicellular fungi widespread in natural environments colonizing from terrestrial, to aerial to aquatic environments, where the successful colonization is intimately related to their physiological adaptability to a highly variable environment. The metabolic pathways of the central carbon metabolism are basically identical between different yeast species, suggesting that these microorganisms might constitute a metabolic homogenous group. Nonetheless, the mechanisms for nutrient uptake, the number of different isoenzymes and most importantly the regulation of fermentation and respiration differ substantially (Flores et al. 2000) and make yeasts a highly heterogeneous and complex metabolic group.

In yeasts, like other heterotrophic organisms, the energy and carbon metabolism are intimately interconnected, i.e., anabolism is coupled with catabolism. ATP is provided by the oxidation of organic molecules that also act as carbon sources for biosynthesis, and ultimately it is used as energetic currency for all kinds of cellular work.

In the natural environment yeast species have a broad set of carbon sources (e.g., polyols, alcohols, organic acids and amino acids) that can support their growth but preferentially they metabolize sugars. The information related to the metabolism of different carbon sources is huge, the most widely studied being sugars such as hexoses (glucose, fructose, galactose or mannose) and disaccharides (maltose or sucrose) as well as compounds with two carbons (ethanol or acetate). The metabolic networks employed for the metabolism of hexoses and disaccharides share the same pathways (most metabolic building blocks are derived from intermediaries of glycolysis, the tricarboxylic acid cycle (TCA), and the pentose phosphate pathway) and differ only in the initial basic steps of metabolism. However, significant changes could be observed when the metabolism of sugars is compared with that of the

two-carbon compounds. In this case, the TCA, the pentose phosphate pathway together with gluconeogenesis and the glyoxylate cycle are essential for the provision of anabolic precursors.

Yeast environmental diversity mostly leads to a vast metabolic complexity driven by carbon and the energy available in environmental habitats. It is the scope of this chapter to contribute a comprehensible analysis of yeast metabolism specifically associated with glucose catabolism in *Saccharomyces cerevisiae*, under both aerobic and anaerobic environments. A brief introduction to glycolysis together with the most relevant effects triggered by oxygen and glucose are presented in order to center the reader in the problems discussed later. Most of our attention is given to the metabolic flux on the pyruvate branch point, with reference to alcoholic fermentation and respiration. As a last issue we address the most pertinent features of anaerobic metabolism, culminating with the hitherto unexplained metabolic requirements for fully anaerobic growth.

6.2 A Brief Comment on Pasteur, Crabtree and Custer Effects

Yeasts may be physiologically classified with respect to the type of energy-generating process involved in sugar metabolism, namely non-, facultative- or obligate-fermentative (van Dijken and Scheffers 1986). The nonfermentative yeasts have exclusively a respiratory metabolism and are not capable of alcoholic fermentation from glucose (e.g., *Rhodotorula glutinis*), while the obligate-fermentative yeasts – "natural respiratory mutants" – are only capable of metabolizing glucose through alcoholic fermentation (e.g., *Candida slooffii* = *Kazachstania slooffiae*). Most of the yeasts identified are facultative-fermentative ones, and depending on the growth conditions, the type and concentration of sugars and/or oxygen availability, may display either a fully respiratory or a fermentative metabolism or even both in a mixed respiratory-fermentative metabolism (e.g., *S. cerevisiae* or *Pichia jadinii* – the latter is herein always referred to as *C. utilis*).

The sugar composition of the media and oxygen availability are the two main environmental conditions that have a strong impact on yeast metabolic physiology. There are three frequently observed effects associated with the type of energy-generating processes involved in sugar metabolism and/or oxygen availability: Pasteur, Crabtree and Custer. The known Kluyver effect is beyond the scope of the metabolic overview of this chapter (see the review in Fukuhara 2003 for more details).

6.2.1 Pasteur Effect

In modern terms the Pasteur effect refers to an activation of anaerobic glycolysis in order to meet cellular ATP demands owing to the lower efficiency of ATP production by fermentation compared with respiration. In 1861 Pasteur observed that *S. cerevisiae* consume much more glucose in the absence of oxygen than in its presence. However, there are several misinterpretations concerning the results presented by Pasteur that were reviewed in depth by Lagunas (1981, 1986, and references therein). This author gathered all the information referred to *S. cerevisiae* and showed that the initial descriptions of Pasteur were an artefact due to anaerobic growth impairment

by the lack of sterols and fatty acids. In fact, *S. cerevisiae* only shows a Pasteur effect under special experimental conditions, specifically at low growth rates (sugar-limiting continuous culturing) and at resting-cell conditions, where a high contribution of respiration to sugar catabolism occurs owing to the loss of fermentative capacity.

6.2.2 Crabtree Effect

The Crabtree effect is currently defined as the occurrence of alcoholic fermentation under aerobic conditions (for a review see Pronk et al. 1996, and references therein). After the initial descriptions of Crabtree, it was shown that *S. cerevisiae* catabolyzes glucose mainly by a fermentative process, and this effect was presented as the Crabtree effect (Swanson and Clifton 1948). From all the theories presented in the literature one should stress that the Crabtree effect has received special attention from several research teams and, even today, a lack of consensus in terms of its definition is observed. De Deken (1966) described the Crabtree effect as "...the phenotypic expression of a regulatory system involved in the synthesis of cytochromes..." Glucose repression became accepted as synonymous of the Crabtree effect until the 1980s with the emergence of the theory involving an "overflow/limited respiratory capacities" in the branching point of pyruvate metabolism (Kappeli 1986). The Crabtree effect was divided into short- and long-term effects, mainly based on continuous culturing studies where under steady-state conditions the growth rate can be experimentally manipulated (Petrik et al. 1983). The short-term effect is defined as the capability of triggering alcoholic fermentation upon the sudden condition of glucose excess, whereas the long-term effect is characterized as the respiration-fermentative metabolism observed with batch cultivations or with continuous culturing above critical dilution rates.

6.2.3 Custer Effect

The Custer effect is known as the inhibition of alcoholic fermentation by the absence of oxygen. A clear example of the Custer effect is found in yeasts belonging to the *Brettanomyces* and *Dekkera* genera that ferment glucose into ethanol and acetic acid under aerobic conditions (Wijsman et al. 1984; van Dijken and Scheffers 1986). However, upon a shift to an anaerobic condition, fermentation is strongly inhibited. This phenotype can be fully rescued by the reintroduction of oxygen into the culture media or by the addition of H^+ acceptors such as acetoin (which is reduced to 2,3-butanediol) or other aliphatic carbonyl compounds (Sheffers 1966; Wijsman et al. 1984). From all the previous considerations it is thought that the Custer effect is caused by reductive stress. It seems that yeasts displaying this effect are somehow incapable of closing the redox balance through the production of glycerol or other highly reduced compounds.

6.3 Glycolytic Metabolic Central Block: a Brief Summary

After glucose uptake, intracellular glucose is fated to be dissimilated and/or assimilated by metabolic processes (see reviews in Lagunas 1993; Boles and Hollenberg

1997; Forsberg and Ljungdahl 2001; Jeffries and Jin 2004). In this section we will briefly summarize the glycolytic pathway that constitutes the central block of hexose and disaccharide metabolism and that has been extensively revised during the last few years (for reviews see Gancedo and Serrano 1989; Richard 2003; Kruckeberg and Dickinson 2004).

Once inside the cell, glucose is phosphorylated by kinases to glucose 6-phosphate and then isomerized to fructose 6-phosphate, by phosphoglucose isomerase. The next enzyme is phosphofructokinase, which is subject to regulation by several metabolites, and further phosphorilates fructose 6-phosphate to fructose 1,6-bisphosphate. These steps are the first part of glycolysis that requires energy in the form of ATP.

The subsequently acting enzymes are aldolase, triosephosphate isomerase, glyceraldehyde 3-phosphate dehydrogenase, phosphoglycerate kinase, phosphoglycerate mutase, enolase and pyruvate kinase. The last block of glycolysis leads to pyruvate formation associated with a net production of energy and reducing equivalents.

Essentially the glycolytic pathway is common to all yeast species, the carbon flux regulation being done at the level of the pentose phosphate pathway. Several flux analyses have shown that approximately 50% of glucose 6-phosphate is metabolized via glycolysis and 30% via the pentose phosphate pathway in Crabtree negative yeasts. However, about 90% of the carbon going through the pentose phosphate pathway reentered glycolysis at the level of fructose 6-phosphate or glyceraldehyde 3-phosphate. The distribution of carbon flux between glycolysis and the pentose phosphate pathway seems to play a more important role in glucose dissimilation in Crabtree negative yeasts than in Crabtree positive ones (Bruinenberg et al. 1983; González-Siso et al. 2000). Specifically, *S. cerevisiae*, a Crabtree positive yeast, was shown to have low catabolic fluxes through the pentose phosphate pathway (Blank and Sauer 2004). These observations indicate that the pentose phosphate pathway in Crabtree positive yeasts is predominantly used for NADPH production but not for biomass production or catabolic reactions, as will be discussed later for redox balances.

6.4 From Pyruvate to Acetyl–Cofactor A

At the pyruvate (the end product of glycolysis) branching point, pyruvate can follow three different metabolic fates depending on the yeast species and the environmental conditions (Pronk et al. 1996). On the other hand, the carbon flux may be distributed between the respiratory and fermentative pathways.

Pyruvate might be directly converted to acetyl–cofactor A (CoA) by the mitochondrial multienzyme complex pyruvate dehydrogenase (PDH) after its transport into the mitochondria by the mitochondrial pyruvate carrier. Alternatively, pyruvate can also be converted to acetyl–CoA in the cytosol via acetaldehyde and to acetate by the so-called PDH-bypass pathway (reviewed in Pronk et al. 1996). The PDH-bypass pathway requires the activity of three different enzymes: (1) pyruvate decarboxylase, which converts pyruvate to acetaldehyde; (2) acetaldehyde dehydrogenase (ALD), which converts acetaldehyde to acetate; and (3) acetyl-CoA synthetase (ACS), which converts acetate to cytosolic acetyl–CoA that can then be transported

unidirectionally into the mitochondria via the carnitine acetyltransferase system (Kispal et al. 1991; Pronk et al. 1996) (Fig. 6.1). It is still matter of debate if the flux distribution during pyruvate metabolism determines whether the pyruvate flows inward through the PDH complex or through the PDH-bypass pathway and thereby the split between respiration and fermentation (Pronk et al. 1996).

One of the mechanisms underlying the regulation of pyruvate flux through the different routes is the regulation of the enzymes involved and their kinetic properties (Pronk et al. 1996). Compared with cytosolic pyruvate decarboxylase, the mitochondrial PDH complex has a higher affinity for pyruvate and therefore most of the pyruvate will flow through the PDH complex at low glycolytic rates. However, at increasing glucose concentrations, the glycolytic rate will increase and more pyruvate is formed, saturating the PDH bypass and shifting the carbon flux through ethanol production and beginning the fermentation (Fig. 6.1).

In addition to the different enzyme affinities for pyruvate, the enzymatic activities may also play a role in the regulation of pyruvate flux. In *S. cerevisiae*, high glucose concentrations induce an increase of 3–4 times the pyruvate decarboxylase activity and a decrease of acetaldehyde dehydrogenase activity, favoring the alcoholic fermentation. On the other hand, at low glucose concentrations, pyruvate is mainly converted to acetyl–CoA by the PDH complex. From an energetic point of view PDH-bypass is less efficient owing to the consumption of an ATP molecule, which is converted into AMP by the acetyl-CoA synthetase (Steensma 1997).

As already discussed and particularly in the yeast *S. cerevisiae*, the external glucose level controls the switch between respiration and fermentation. Although

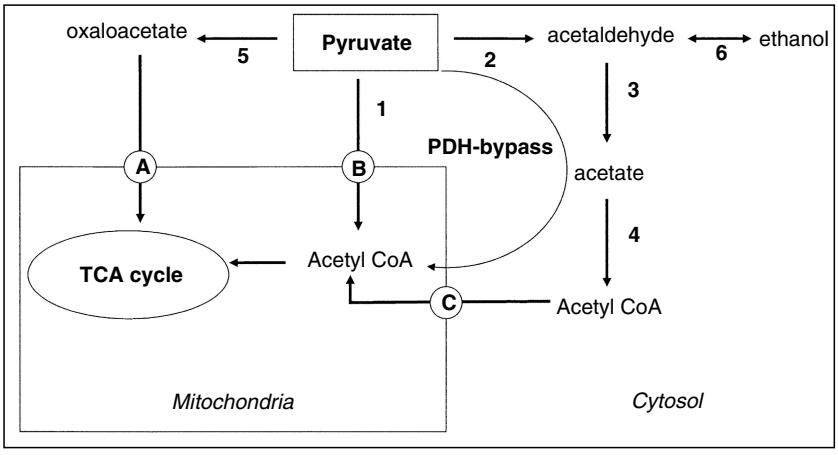

Fig. 6.1. Scheme of pyruvate branch point pathways. Pyruvate formed in glycolysis is converted to acetyl–cofactor A (*CoA*) and/or oxaloacetate, both intermediates of the trycarboxylic acid cycle. *1* pyruvate dehydrogenase complex, *2* pyruvate decarboxylase, *3* acetaldehyde dehydrogenase, *4* acetyl–CoA synthetase, *5* pyruvate carboxylase and *6* alcohol dehydrogenase. *A* mitochondrial oxaloacetate carrier; *B* mitochondrial pyruvate carrier and *C* carnitine acetyltransferase

significant ethanol production is generally absent in Crabtree negative yeasts during aerobic conditions (de Deken 1966; González-Siso et al. 1996), yeast species such as *Pichia anomala, C utilis* and *Kluyveromyces lactis*, during aerobic conditions and high glucose concentration, temporarily (during the exponential growth phase) produce ethanol. However, it has been shown that this transient ethanol production is mainly due to inadequate aeration (Kiers et al. 1998).

6.5 Tricarboxylic Acid Pathway

Acetyl–CoA generated either by the PDH complex or by PDH bypass is the link between glycolysis and the tricarboxylic acid (TCA) cycle. The main catalytic function of the TCA cycle is to provide reducing equivalents to the respiratory chain through the oxidative decarboxylation of acetyl–CoA. However, the TCA cycle also functions in furnishing biosynthetic metabolism and, with the exception of isocitrate, every TCA cycle intermediate is commonly used by other metabolic reactions. The eight enzymes from the TCA cycle are encoded by 15 nuclear genes in *S. cerevisiae* (McAlister-Henn and Small 1997; Przybyla-Zawislak et al. 1999). The first reaction of TCA is catalyzed by citrate synthase (encoded by *CIT1*, *CIT2* and *CIT3*) and it is the condensation of acetyl–CoA and oxaloacetate resulting in the formation of citrate (McAlister-Henn and Small 1997). Only Ct2p is a nonmitochondrial protein peroxisomally localized contributing to the efficiency by which the cells use two-carbon compounds in anaplerotic pathways especially in the glyoxylate cycle.

The second reaction of the TCA cycle is catalyzed by aconitase, leading to the conversion of citrate into isocitrate. Aconitase (encoded by *ACO1*) is located both in mitochondria and in cytosol.

The next step of the TCA cycle is the oxidative decarboxylation of isocitrate to α-ketoglutarate. There are three known isoenzymes of isocitrate dehydrogenase, a mitochondrial NAD^+-specific one and two $NADP^+$-dependent ones (one mitochondrial and the other cytosolic). A number of pieces of evidence point to the role of mitochondrial NAD^+-specific isocitrate dehydrogenase in the regulation of the rate of mitochondrial assembly besides its specific role in the TCA cycle (Kruckeberg and Dickinson 2004).

The formation of succinate is catalyzed by α-ketoglutarate dehydrogenase, which promotes the oxidative decarboxylation of α-ketoglutarate via succinil–CoA to succinate, which is then converted to fumarate by succinate dehydrogenase. The next step of the TCA cycle is the conversion of fumarate to malate by the enzyme fumarase, which exists as separate cytosolic and mitochondrial forms. There is not yet a clear explanation for the existence of these two forms; however, the localization and distribution of fumarase appears to be unique because there is only one translation product which is targeted to mitochondria (Sass et al. 2001).

Malate dehydrogenase catalyzes the last step of the TCA cycle and leads to the oxidation of malate to oxaloacetate. There are three isoenzymes of malate dehydrogenase: a cytosolic, a mitochondrial and a peroxissomal one; however, the mitochondrial one accounts for 90% of malate dehydrogenase activity when glucose is being metabolized (Steffan and McAlister-Henn 1992).

TCA cycle flux appears to be constricted at two steps on the basis of the limited availability of the substrates oxaloacetate and α-ketoglutarate. As represented in Fig. 6.1, in *S. cerevisiae*, the synthesis of oxaloacetate from cytosolic pyruvate catalyzed by pyruvate carboxylase constitutes the anaplerotic pathway for the replenishment of this TCA-cycle intermediate. The TCA cycle works in a two-minicycles model interconnecting these two substrates (oxaloacetate and α-ketoglutarate) and their transamination products (glutamate and aspartate) (Yudkoff et al. 1994; Rustin et al. 1997). This model is consistent with the unique regulation of the first three enzymes of the TCA cycle in yeasts (McCammon et al. 2003).

The genes encoding TCA cycle proteins might also be regulated by glucose levels. In *S. cerevisiae* the depletion of glucose increases 3–10 times the TCA messenger RNAs (DeRisi et al. 1997). Oxygen limitation could also induce a shift in the TCA pathway, which operates as a cycle during aerobic growth and as a two-branched pathway under oxygen limitation, sustaining only the synthesis of the biomass precursor's oxaloacetate and α-ketoglutarate as will be discussed hereafter. However, TCA operating as a two-branched pathway was also identified in *S. cerevisiae* during aerobic fermentation on glucose (Gombert et al. 2001).

6.6 Aerobic Metabolism: Oxidative Phosphorilation and Redox Balance

6.6.1 Oxidative Phosphorilation

The mitochondrial oxidative phosphorylation is a complex and highly controlled network through which ATP synthesis must be continuously adapted to changes in the cell energy demand to sustain growth and/or homeostasis. During respiratory metabolism, both cytosolic and mitochondrial NADH are reoxidized by the respiratory chain. However, *S. cerevisiae*, in contrast to many eukaryotic cells including other yeast species, lacks the multi-subunit complex-I-type NADH dehydrogenase (Nosek and Fukuhara 1994). Instead, *S. cerevisiae* contains a single-subunit NADH:ubiquinone oxidoreductase, which couples the oxidation of intramitochondrial NADH to the respiratory chain. This enzyme (encoded by *NDI1*), referred to as the "internal NADH dehydrogenase," catalyzes the transfer of two electrons from intramitochondrial NADH to ubiquinone (de Vries and Grivell 1988; Marres et al. 1991).

Nonetheless, yeast mitochondria, like those of plants (Moller et al. 1993), not only contain the internal mitochondrial NADH dehydrogenase, but also a mitochondrial external NADH dehydrogenase activity (von Jagow and Klingenberg 1970). *S. cerevisiae* has two genes encoding external NADH dehydrogenase isoenzymes, *NDE1* and *NDE2*, both of them typical aerobic expressed genes (Luttik et al. 1998; Small and McAlister-Henn 1998). Like the internal NADH dehydrogenase, the external isoenzymes do not pump protons (von Jagow and Klingenberg 1970). Therefore, *S. cerevisiae* has a low ATP stoichiometry of oxidative phosphorylation. Notwithstanding this low stoichiometry of oxidative phosphorylation, complete respiratory dissimilation of a glucose molecule yields approximately 16 ATP molecules (four ATP molecules from substrate-level phosphorylation – two from glycolysis and

two from GTP formed in the TCA cycle – and about 12 ATP molecules from oxidative phosphorylation). This is eightfold higher than the maximum ATP yield from glucose dissimilation via alcoholic fermentation. The much higher ATP yield from respiratory sugar dissimilation is reflected in the biomass yields of sugar-limited cultures: the typical biomass yield on glucose of respiring cell cultures is five-fold higher than that obtained in fermenting cell cultures (Verduyn 1991).

Another peculiar feature of the *S. cerevisiae* respiratory chain is that the complexes bc1 (III) and cytochrome c oxidase (IV) were shown to assemble into large supercomplexes (Schagger and Pfeiffer 2000). The respiratory chain of *S. cerevisiae* differs from that of other fungi and of plants not only in the presence of the NADH dehydrogenase not coupled to the proton pump but also owing to the absence of a cyanide-insensitive alternative oxidase which catalyzes the direct oxidation of ubiquinone by molecular oxygen without generating a proton motive force (Vanlerberghe and McIntosh 1997).

Resting cells of the yeast *S. cerevisiae* have shown that the overall rate of oxidative phosphorylation is short-term controlled (1) downstream from the ATP synthase by the cytosolic ATP turnover, (2) upstream from the respiratory chain by reducing equivalent availability and allosteric activation of dehydrogenases and (3) by the ionic permeability of the inner mitochondrial membrane (Beauvoit et al. 1993).

Dejean et al. (2000) have shown that mitochondria are the major energy dissipative system in a fully aerobic metabolism and that energy dissipation can be regulated by the decrease in mitochondrial enzyme content to maintain the oxidative phosphorylation regime.

6.6.2 Redox Balances

The pyridine-nucleotide cofactors NAD^+/NADH and $NADP^+$/NADPH play a central role in yeast metabolism. NADH is preferentially used in dissimilatory metabolism, whereas NADPH is generally required for assimilatory reactions (van Dijken and Scheffers 1986). In *S. cerevisiae*, *C. utilis*, and probably in the yeasts in general, NADH and NADPH cannot be interconverted owing to the absence of a transhydrogenase activity (Bruinenberg et al. 1983). The maintenance of a redox balance is a prerequisite for living cells in order to sustain the regular metabolic activity and enable growth. Hence, since biological membranes are impermeable to pyridine-nucleotides, to maintain the redox balances, the reduced coenzymes must be reoxidized in the compartment in which they are produced.

Cytosolic NADPH is produced by the oxidative part of the pentose phosphate pathway, which branches off from glycolysis at the level of glucose 6-phosphate. Also, $NADP^+$-dependent isocitrate dehydrogenase and $NADP^+$-dependent acetaldehyde dehydrogenase can contribute to NADPH production (Bruinenberg et al. 1983; Minard et al. 1998). *S. cerevisiae* cannot directly couple the oxidation of NADPH to the respiratory chain (Bruinenberg 1986) and therefore is unable to directly oxidize the surplus of cytosolic NADPH. Consequently, the pentose phosphate pathway, which produces the NADPH required for biosynthesis, cannot function as a dissimilatory route in *S. cerevisiae* (González-Siso et al. 1996). Also in *S. cerevisiae* fermentative sugar dissimilation, the role of NADPH is limited, as the major alcohol

dehydrogenases are strictly NAD^+-dependent (Ciriacy 1979). In contrast, *K. lactis* uses the pentose phosphate pathway for glucose dissimilation when glycolysis is blocked, suggesting that, in this yeast species, oxidation of the cytosolic NADPH generated in the pentose phosphate pathway can be efficiently coupled to the mitochondrial respiratory chain (González-Siso et al. 1996). On the other hand, mitochondrial NADPH dehydrogenases, which couple the oxidation of cytosolic NADPH to the mitochondrial respiratory chain, are common in plants, and this mitochondrial NADPH oxidizing activity has also been reported in *C. utilis* (Bruinenberg 1986; van Urk et al. 1989) but it is absent from *S. cerevisiae* (de Vries and Marres 1987; van Urk et al. 1989; Small and McAlister-Henn 1998).

S. cerevisiae has several mechanisms to reoxidize NADH allowing the metabolism to proceed (Bakker et al. 2001). The reduction of NAD^+ occurs both in the cytosol by glycolysis and in the mitochondria by the PDH complex and dehydrogenases of the TCA cycle. Both pools of NADH can be oxidized by the mitochondrial respiratory chain with oxygen as the terminal electron acceptor (de Vries and Marres 1987; Luttik et al. 1998; Overkamp et al. 2000; Bakker et al. 2001).

Aerobically, several systems for conveying excess cytosolic NADH to the mitochondrial electron transport chain exist in *S. cerevisiae* (Bakker et al. 2001). The two most important systems in this respect seem to be the external NADH dehydrogenase (Nde1p/Nde2p) (Luttik et al. 1998; Small and McAlister-Henn 1998) and the glycerol 3-phosphate shuttle (Larsson et al. 1998) although other shuttles could in particular situations play some role (Bakker et al. 2001) (Fig. 6.2). While the external NADH dehydrogenase is suggested as the main system employed for oxidation of cytosolic NADH, the glycerol 3-phosphate shuttle is proposed to be of some importance at low growth rates and perhaps its real significance is only expressed during starvation conditions. The relative importance of these two systems under different conditions is still to a large extent an unsolved matter. Since the external NADH dehydrogenase and the glycerol 3-phosphate shuttle fulfill the same physiological function some kind of regulatory interactions between the two systems would be expected. However, it was shown that the glycerol 3-phosphate shuttle system, which involves the flavin adenine dinucleotide (FAD) dependent Gut2p (Pahlman et al. 2001), is a more efficient system used under conditions where the availability of energy is limited and in fact this system has a higher ATP/O ratio compared with the external NADH dehydrogenase (Larsson et al. 1998). On the other hand, the external NADH dehydrogenase is superior in terms of producing ATP at a high rate and it is therefore the preferred alternative under most other conditions. Nonetheless, all known pathways of respiratory NADH oxidation in *S. cerevisiae* converge at the ubiquinone pool of the respiratory chain.

Kinetic interactions between Nde1p/Nde2p and Gut2p have been demonstrated since the deletion of either of the external dehydrogenases causes an increase in the efficiency of the remaining enzyme (Pahlman et al. 2001). Moreover, the same authors showed that the activation of NADH dehydrogenase inhibited Gut2p, although this inhibition was not a consequence of the direct action of NADH on Gut2p (Pahlman et al. 2001). Most interestingly is the recent work of Bunoust et al. (2005) where they described that electrons coming from Nde1p have the right of way over those coming from either Gut2p or Ndi1p. Therefore, they proposed that the

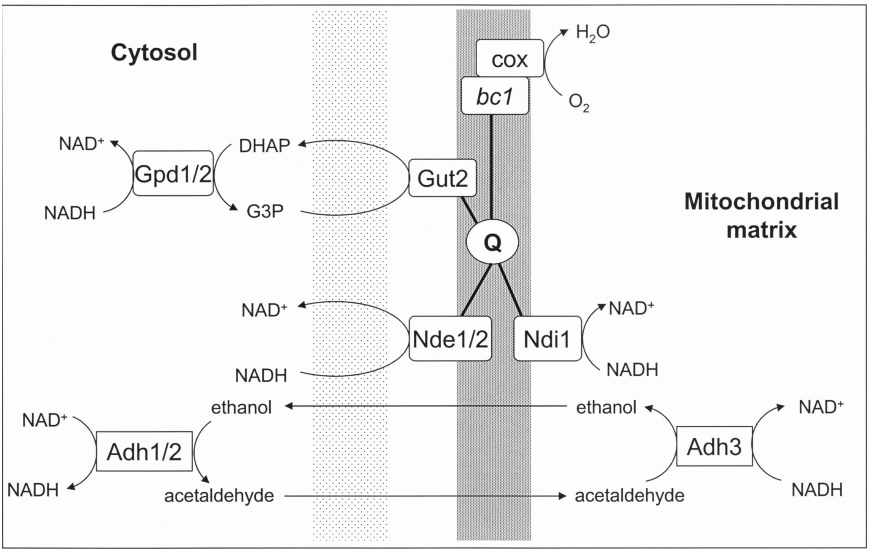

Fig. 6.2. Scheme of redox balance systems in *Saccharomyces cerevisiae*. *Adh* alcohol dehydrogenase; *bc1* bc1 complex; *cox* cytochrome c oxidase; *Gpd* soluble glycerol 3-phosphate dehydrogenase; *Gut2* membrane-bound glycerol 3-phosphate dehydrogenase; *Nde* external NADH dehydrogenase; *Ndi1* internal NADH dehydrogenase; *Q* ubiquinone (Adapted from Bakker et al. 2001)

metabolic organization of the respiratory chain is such that it allows a selection and a priority in electron supply, pointing to a new mechanism of regulation of the yeast oxidative metabolism.

The regulation between the two NADH reoxidation systems has also to contemplate the glucose catabolic repression especially when we are talking about *S. cerevisiae*. At high glucose concentrations respiration is only partly repressed (Blomberg et al. 1988; Larsson et al. 1998). These traits will render the glycerol 3-phosphate shuttle inactive since one of its components, Gut2p, is subject to glucose repression (Sprague and Cronan 1977). To a large extent, under these conditions, cytosolic redox balance is restored by ethanol and glycerol formation, though the activity of the external NADH dehydrogenase might explain the lower glycerol formation obtained during aerobic compared with anaerobic batch cultures of *S. cerevisiae* (Rigoulet et al. 2004). On the other hand, during aerobic growth at low glucose concentrations in chemostat cultures, both the external NADH dehydrogenase and the glycerol 3-phosphate shuttle seem operative simultaneously (Rigoulet et al. 2004) (Fig. 6.2). However, the NADH dehydrogenase activity appears more important under these conditions.

6.7 Anaerobic Metabolism

As referred to before, yeasts may be physiologically classified with respect to the type of energy-generating process involved in sugar metabolism, namely non-,

facultative- or obligate-fermentative. Within these classes only the last two have a fully operative pathway that can provide free-energy-transduction under anaerobic growth. Alcoholic fermentation is acknowledged as the catabolism of glucose to ethanol. According to the stoichiometry of alcoholic fermentation two molecules of ATP are produced per molecule of glucose converted into ethanol functioning as the main energy supply for maintenance and growth. Additionally, the alcoholic fermentation is a redox-neutral process, since the NADH produced during the oxidation of glyceraldehyde 3-phosphate by glyceraldehyde 3-phosphate dehydrogenase is subsequently reoxidized in the reduction of acetaldehyde to ethanol by alcohol dehydrogenase (van Dijken and Scheffers 1986) (Fig. 6.2). However, one cannot overlook that growth is associated with anabolic processes and yeasts have an overall biomass composition that is more oxidized than that of compounds like glucose, which implies that anabolic processes lead to a surplus of reducing equivalents. In contrast to aerobic conditions, the absence of oxygen abolishes the possible oxidation of reduced pyridine nucleotides in the respiratory chain coupled to oxidative phosphorilation. One common way to satisfy the redox balances during growth under anaerobic conditions is the split of glucose metabolism towards glycerol. Glycerol is produced by reduction of the glycolytic intermediate dihydroxyacetone phosphate to glycerol 3-phosphate followed by a dephosphorylation of glycerol 3-phosphate to glycerol. The first step is catalyzed by NAD^+-dependent glycerol 3-phosphate dehydrogenase (encoded by the two isogenes *GPD1* and *GPD2*), whereas the second reaction is catalyzed by the activity of glycerol 3-phosphatase (encoded by *GPP1* and *GPP2*) (Eriksson et al. 1995; Larsson et al. 1993; Norbeck et al. 1996).

Additionally, the up-regulation of *GPD2* associated with an anaerobic growth impairment of *S. cerevisiae* gpd2 mutant clearly establishes the metabolic relevance of this isoenzyme (Ansell et al. 1997; Bjorkqvist et al. 1997; Nissen et al. 2000). In the same line of thought, whereas the Gpp2p seems not to play a relevant role in glycerol production under anaerobic conditions, mutants lacking *GPP1* display a growth defect under identical conditions. One should stress that the anaerobic growth defects presented by the latter mutant are not due to redox balance impairments but are mainly due to growth-inhibiting levels of intracellular glycerol 3-phosphate (Pahlman et al. 2001). Moreover, anaerobic conditions do not affect expression of *GPP2*, while *GPP1* is induced (ter Linde et al. 1999). Hence, the glycerol biosynthesis has defined physiological roles in the metabolic adaptation of *S. cerevisiae*. The correlation between glycerol production and growth under anaerobic conditions is clearly illustrated by the restoring of anaerobic growth defects of mutants with impaired glycerol production by the presence of the electron acceptors acetoin (3-hydroxy-2-butanone) and acetaldehyde. These two compounds alleviate the imposed cellular reductive stress since they enzymatically oxidize intracellular NADH to NAD^+. Furthermore, the increasing flux towards glycerol biosynthesis with the increasing of growth rate is well established. This metabolic shift is the result of an increasing drain from the catabolic pathways to support the higher level of biosynthetic molecules such as RNA and proteins (Nissen et al. 1997).

On the other hand, several yeast species, including *S. cerevisiae*, are well known by their capability of producing acetic acid under both aerobic and anaerobic/

oxygen-limiting conditions. In *S. cerevisiae*, the further metabolism of acetic acid through acetyl–CoA synthetase (encoded by *ACS1* and *ACS2* genes) is the only source of cytosolic acetyl–CoA, an imperative building block of fatty acid biosynthesis (van den Berg and Steensma 1995; Flikweert et al. 1996). When acetaldehyde is converted into acetic acid a surplus of two reducing equivalents is produced. The formation of such a product poses an additional problem in the redox balances, once again circumvented by the production of glycerol. However, if the yeasts cannot cope with the surplus of reducing equivalents the anaerobic activity will come to a standstill, as discussed hereafter. From an anabolic point of view, when acetaldehyde is converted into acetate, NADPH is generated. From the five currently known isogenes of acetaldehyde dehydrogenases, only *ALD4*, *ALD5* and *ALD6* gene products have a role in acetate production during glucose fermentation under both aerobic and anaerobic/oxygen limiting conditions (Remize et al. 2000; Saint-Prix et al. 2004). While the last one encodes a cytoplasmic isoform of acetaldehyde dehydrogenase, the first two encode mitochondrial proteins (Meaden et al. 1997; Tessier et al. 1998; Wang et al. 1998). Moreover, this reaction together with the ones catalyzed by malic enzyme (encoded by *MAE1*) and $NADP^+$-dependent isocitrate dehydrogenase (encoded by *IDP1*) are currently known to generate mitochondrial NADPH, necessary for biosynthetic purposes.

TCA pathway activity is maintained during fermentation for primary fuel biosynthetic reactions supplying cells with four and five carbon compounds, namely oxaloacetate and 2-oxoglutarate, the precursors of aspartate and glutamate. The TCA pathway is known to operate in a branched fashion under anaerobiosis or conditions of glucose repression (Gombert et al. 2001) where succinate dehydrogenase is nonfunctional (Camarasa et al. 2003). Hence, the flux from 2-oxoglutarate to oxaloacetate is zero. Therefore, the split of the carbon flux in the TCA cycle results in the formation of one oxidative branch leading to the formation of 2-oxoglutarate (Nunez de Castro et al. 1970) and one reductive branch that culminates with the formation of fumarate (Atzpodien et al. 1968). The key enzyme of the redutive branch is the fumarate reductase encoded by *FRDS* and *OSM1* genes (Arikawa et al. 1998). The relevant role of this reaction in the reoxidation of intracellular NADH is highlighted by the inability to grow under anaerobic conditions of fumarate reductase null mutant that is restored by the addition of the oxidized form of methylene blue or phenazine methosulfate, which nonenzymatically oxidizes cellular NADH to NAD^+ (Enomoto et al. 2002). It may perhaps be considered that the role of the enzyme pyruvate carboxylase is to catalyze the reaction of pyruvate to oxaloacetate at the expenses of one molecule of ATP and carbon dioxide (de Jong-Gubbels et al. 1998). This anaplerotic reaction functions to further replenish the TCA pathway for biosynthetic purposes or organic acid production as referred to previously.

The synthesis of precursors for biomass building blocks actively results in the net formation of NADH in the mitochondrial matrix (Visser et al. 1994). The reductive branch, thermodynamically favorable, and the *ADH1*/*ADH3* shuttle (Fig. 6.2) are two feasible routes for the oxidation of the surplus of NADH produced in mitochondria by both the oxidative TCA branch and the synthesis of amino acids. It should be noted that, stoichiometrically, the formation of succinate by the reductive pathway or by the oxidative pathway results, respectively, in two molecules of FAD

oxidized or in five molecules of reduced NADH per molecule of glucose consumed. The surplus of reducing equivalents may be reoxidized by the *ADH1*/*ADH3* shuttle involving the mitochondrial alcohol dehydrogenase, adh3p, that results in a net production of NADH in the cytosol and that can be circumvallated by glycerol production (Nissen et al. 1997; Bakker et al. 2000). In fact, Nissen et al. (1997) showed that the *ADH1*/*ADH3* shuttle is responsible for the regeneration of NAD^+, whereas the reductive branch is associated with succinate production. Nevertheless, the *ADH1*/*ADH3* shuttle does not strictly control the redox balance under anaerobic conditions, since a null *ADH3* mutant still shows anaerobic growth, albeit slower than that of the wild type (Bakker et al. 2000). The TCA pathway during fermentation also leads to the formation of organic acids, mainly citrate, malate and succinate (Heerde and Radler 1978), and depending on the different yield products there are differences in the way that cells have to cope with the redox balance. As yet, however, the nitrogen source strongly influences cellular metabolism and product formation in *S. cerevisiae*, especially owing to redox contour-balancing under anaerobic conditions (Albers et al. 1996). A high demand of NADPH is required for glutamate formation when cells are using ammonium as the only nitrogen source. In contrast, when glutamate is used as a nitrogen source the reduced synthesis of 2-oxoglutarate from glucose causes fewer reducing equivalents to be formed, which reduces the glycerol yield and hence increases the ethanol yield (Albers et al. 1996; Camarasa et al. 2003).

The regulatory pattern of malic enzyme (encoded by *MAE1*) suggests the specific physiological function in the provision of intramitochondrial NADPH or pyruvate in anaerobic metabolism; however, this enzyme does not have a strict function in redox metabolism, since null mutant in *MAE1* shows identical glycerol production under anaerobic conditions (Boles et al. 1998).

6.8 Yeast Growth Ability under Anaerobic Conditions: Is There a Common Prerequirement?

Of the 678 recognized species, around 60% are considered to be fermentative on the basis of taxonomic tests such as gas production in Durham tubes (Barnett et al. 2000). However, this number is even higher since, under certain conditions, some of those species considered as nonfermentative are also able to ferment glucose (van Dijken et al. 1986; Visser et al. 1990). While the ability to ferment glucose under oxygen limitation turns out to be a common feature of the different yeast species, apparently the capability of growth under anaerobic conditions is not widespread among these microorganisms (Visser et al. 1990). In fact, only very few yeast species are capable of fast growth under those conditions and *S. cerevisiae* stands out as the yeast generally acknowledged as a facultative anaerobe (Verduyn et al. 1990; Visser et al. 1990). It is commonly accepted that facultative anaerobes have the ability to grow both under aerobic and anaerobic conditions using, respectively, molecular oxygen or another compound as the final electron acceptor of the reducing equivalents that overflow from anabolic processes. Owing to the fact that anaerobic growth is associated with a low energy yield compared with that observed under complete oxidative processes, these microorganisms display two common characteristics:

(1) the rate of sugar-substrate consumption is higher under anaerobic processes than that observed under aerobic conditions and (2) oxygen is used as a preferential source of the final electron acceptor.

As clearly pointed out by Lagunas (1979, 1981, 1986), *S. cerevisiae* can roughly meet the previously described criteria for the reasons given hereafter. Even though *S. cerevisiae* is capable of rapid anaerobic growth it requires an external supply of sterols and fatty acids (Verduyn et al. 1990). The biosynthetic pathways involved in the production of these compounds, essential for membrane turnover, require molecular oxygen (Andreasen and Stier 1953, 1954). Besides the expected benefits that would be produced from a shift from an anaerobic (where energy is the limiting factor) to an aerobic environment (with the putative ability to produce 1.4–5.4 times more ATP per mole of sugar) they are almost irrelevant in *S. cerevisiae* (Lagunas 1979, 1986, and references therein; Rodrigues et al. 2001). It should be stressed that in this species only around 5-10% of glucose, maltose or fructose is not metabolized via alcoholic fermentation under aerobic batch growth. The ratios between the flux of those sugar consumptions under aerobic and anaerobic conditions are, respectively, 1.05, 0.90 and 1.08 (Lagunas 1979). Ultimately, a rate value close to 1 means that no Pasteur effect occurs for the metabolism of the sugars described before.

Nevertheless, the biosynthetic oxygen requirements of facultative-fermentative yeasts are extremely small. Therefore, for reliable and meaningful identification of yeast species with the ability to grow under strictly anaerobic conditions special precautions (e.g., use of oxygen-resistant tubing and ultrapure nitrogen gas for sparking) are needed during the setup process of growth to minimize oxygen entry to the extent that these small oxygen requirements become apparent (Visser et al. 1990; Rodrigues et al. 2001). Albeit, in some cases the requirements of molecular oxygen may be substantial, whereas, for instance, an insignificant leakage of oxygen between 0.3 and 6 μmol of O2 h^{-1}, corresponding to normal strict anaerobic conditions, is sufficient to allow the yeasts *Zygosaccharomyces bailii* and *C. utilis* to grow (Rodrigues et al. 2001). In spite of the linearity of the growth kinetics characteristics of oxygen limitation, *C. utilis* requires 1.5-fold more time than *Z. bailii* to finish cell proliferation, highlighting the differences in oxygen demand of both species for growth. This raises the general question, what are the further limitations that explain the anaerobic growth incapability of "non-*Saccharomyces*" yeasts?

During the last few years, different research groups have raised several hypotheses, most of them related to the inability to fulfill the biosynthetic requirements under anaerobic conditions. Ergosterol and unsaturated fatty acids were shown to be essential for *S. cerevisiae* and *S. kluyveri* (Moller et al. 2001). Strengthening the specific requirements of *S. cerevisiae* for ergosterol and unsaturated fatty acids, Wilcox et al. (2002) showed that the ATP-binding transporters Aus1p and Pdr11p, associated with sterol uptake, are essential for anaerobic growth. However, it seems that *Schizosaccharomyces japonicus* does not require ergosterol for growth (Bulder 1971). On the other hand, the discovery by Nagy et al. (1992) that *S. cerevisiae* has one dihydroorotate dehydrogenase (DHODase) (encoded by the gene *URA1*), which catalyzes the single redox reaction converting dihidroorotate into orotate, not dependent on the functionality of the respiratory chain, opened a new line of research into the ability of yeasts to grow under anaerobic conditions. In this line, it was possible

to turn the oxygen-dependent yeast *Pichia stipitis* into an anaerobic culturable yeast just by expressing the *URA1* gene from *S. cerevisiae* (Shi and Jeffries 1998). More recently, it was experimentally shown that the genome of *S. kluyveri* also encodes a DHODase, functionally equivalent to that of *S. cerevisiae* (Gojkovic et al. 2004). The nice study of Gojkovic et al. (2004) illustrated that while aerobic yeasts, such as *S. pombe* and *C. albicans* contain just a mitochondrial DHODase, with a quinine-type electron acceptor, the facultative anaerobes yeasts, such as *S. cerevisiae*, *S. bayanus* and *S. castellii* contain a cytosolic DHODase, with a fumarate-type electron acceptor, whereas *S. kluyveri* contains both. This study pointed to horizontal gene transfer from bacteria to the progenitor of these lineages, and raised the hypothesis that a remodeling of the biochemical pathways during yeast diversification took place, allowing the progressive reduction of oxygen requirements for growth. As yet, however, the capability of *Z. bailii* to grow under anaerobic conditions in yeast–peptone–glucose opposed to defined media is still unexplained since it is not due to uracil auxotrophy (Rodrigues et al. 2001). The involvement of additional biosynthetic requirements may perhaps be considered in other cases.

Another even more generalized limitation to anaerobic growth may be related to either/both a sufficiently high rate of ATP formation of a given substrate or/and an impaired mitochondrial ADP/ATP translocation, as well as constraints on redox metabolism (van Dijken and Scheffers 1986; Verduyn et al. 1990; Visser et al. 1994; Trezeguet et al. 1999). Under anaerobic conditions and in contrast to aerobic conditions, the TCA cycle has only an anabolic function and the cell growth is completely dependent on ethanol formation for the provision of energy. In theory it is conceivable that, for certain yeast species, the glycolytic flux is not sufficient to drive the free energy for cellular processes. Verduyn et al. (1992) showed that the *S. cerevisiae* fermentation rate limits the supply of ATP for maintenance purposes above that required for anabolic reactions, to a value of approximately 17 mmol ATP Eq g^{-1} h^{-1}. However, when the maintenance requirement increases to a threshold value (e.g., triggered by the uncoupling effect of weak carboxylic acids or by the decrease of the pH of the culture medium below 2.8), glycolytic flux can no longer provide further ATP, leading to cytosolic acidification and subsequently cell death (Verduyn et al. 1990). It is to be stressed that the previous considerations are not related to the well-known Custers effect on yeast which is characterized by fermentation inhibition upon the transition to an anaerobic environment. In *Torulaspora delbrueckii*, it seems that the increase of glycolytic flux under anaerobic conditions is not sufficient to cope with growth demands (Hanl et al. 2004). On the other hand, an impairment of mitochondrial ADP/ATP translocation may lead to growth arrest in the absence of oxygen. In *S. cerevisiae* it was shown that the blockage of ADP/ATP translocators by bongkrekic acid induced a reduction of approximately 50% in the growth rate under anaerobic conditions (Visser et al. 1994). This dependence was further established by the inability of *S. cerevisiae* null mutant in *AAC* genes (*AAC1*, *AAC2*, *AAC3*; coding ADP/ATP translocators) to grow under those conditions. One should emphasize that the same authors showed that this triple mutant was still able to grow on fermentable sources in the presence of oxygen and that *AAC3* was induced under anaerobic conditions (Kolarov et al. 1990; Lawson et al. 1990; Drgon et al. 1991). Furthermore, in *S. cerevisiae* batch cultivations, the

surplus of reducing equivalents formed in anabolic reactions has to be balanced by the formation of glycerol estimated in approximately 5% of glucose breakdown. In this species, the null mutant in glycerol 3-phosphate dehydrogenase is incapable of growth under anaerobic conditions, and the simple deletion of the *GPD1* gene leads to a decrease of 5 times the growth rate (Nissen et al. 2000). More surprisingly, the same authors showed that the expression of a cytoplasmic transhydrogenase from *Azotobacter vinelandii* could not rescue the phenotype, showing that the NAD^+ pool that limited growth was lower than the threshold value favoring the transhydrogenase reaction. Therefore, an impaired redox metabolism may, in some cases, be behind the inability to grow anaerobically.

From everything discussed here, the hitherto unexplained inability of most yeast species to grow under anaerobic conditions is still an unsolved puzzle. Scientific efforts must, therefore, focus on an integrated approach keeping the essential strait balance between the two complementary approaches – the yeast physiology and the yeast molecular biology – and thus driving the expected increase in knowledge of yeast performance.

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Diversity of Nitrogen Metabolism Among Yeast Species: Regulatory and Evolutionary Aspects

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7.1 Introduction

Yeasts are capable of utilizing a wide diversity of nitrogen compounds as nitrogen sources, and the ability or inability to utilize one or several of these compounds can be used in classifying yeasts. It is worth noting that nitrogen compounds can also be used as a carbon source or be incorporated into proteins, but these metabolisms will not be considered in the present chapter.

More than 50 different nitrogen sources are known to be utilizable by yeast species, including L-amino acids, D-amino acids, pyrimidines, purines, polyamines, amines, nitrate and nitrite. However, the nitrogen utilization profiles markedly vary according to yeast species, many of which are much more versatile than *Saccharomyces cerevisiae*. The utilization of nitrogen compounds as a nitrogen source has been well studied in *S. cerevisiae*, and less extensively in other yeasts. In 1986, Large (1986) presented an extensive survey of organic-nitrogen-compound utilization by yeast strains from various genera. This article focused on the diversity of nitrogen compound degradation, especially in its chemical and metabolic aspects. Since 1986, mainly for *S. cerevisiae*, many new data have become available concerning the levels of transport and the control of nitrogen metabolisms allowing the establishment of metabolic and regulatory networks and an integration of nitrogen metabolism. In contrast, for other yeast species the study of nitrogen metabolism and its regulation did not raise the same interest, and only dispersed data with no integration are available in the literature, with the exception of nitrate/nitrite metabolism in *Hansenula*. However, since the genome sequence of 24 yeasts (as stated by the consortium Génolevures) was recently performed, the comparison between genes from *S. cerevisiae* and related hemiascomycetous yeasts or a more distant yeast species such as *Schizosaccharomyces pombe* could lead to the development of new approaches to study the diversity of nitrogen metabolism among yeasts. Such

comparisons could bring to light the reason for nonutilization of some nitrogen compounds, and could also provide new data on molecular evolution.

Degradation of nitrogen-containing compounds as the sole source of nitrogen for growth leads to the formation of three key nitrogen compounds in the cell, ammonia, glutamate and glutamine. These are interlinked by three enzyme systems: NADP-dependent glutamate dehydrogenase (GDH) (ammonia→glutamate, reaction 1), NAD-dependent GDH (glutamate→ammonia, reaction 2) and the glutamine synthetase/glutamate synthase system (reactions 3, 4) as illustrated in Fig. 7.1. The major pathway for glutamate synthesis is through combination of ammonia with α-ketoglutarate. Glutamine is synthesized by the combination of ammonia with glutamate by glutamine synthetase. The glutamine synthetase coupled to the glutamate synthase system is an alternative pathway to produce glutamate, which is common in bacteria (Tempest et al. 1970), but is present only in a small number of yeasts. Although glutamine synthetase is widespread in yeasts, glutamate synthase activity has only been detected in *S. cerevisiae* (Roon et al. 1974) and in certain *Schizosaccharomyces* species (Brown et al. 1973). Comparison among five yeast genomes that have been completely sequenced revealed the presence of ortholog genes to *GLT1* (coding for glutamate synthase in *S. cerevisiae*) in *Candida glabrata, Yarrowia lipolytica*, and *Kluyveromyces lactis*. It is probably also present in *Debaryomyces hansenii*, because there are two contiguous open reading frames whose products share strong similarity to *S. cerevisiae* glutamate synthase. The importance of this alternative pathway in *S. cerevisiae* is supported by the fact that a mutant lacking NADP–GDH grows on ammonia at approximately half the rate of the wild type. However a double *gdh1*, *glt1* mutant still presents residual growth with ammonia as the sole nitrogen source, indicating the existence of another alternative pathway. A computer search identified a new gene, *GDH3*, whose product shows similarity to *GDH1* product, and the triple mutant strain was strict glutamate auxotroph (Avendano et al. 1997). In contrast, in *S. pombe, C. glabrata, Y. lipolytica, K. lactis*, and *D. hansenii,* there is only one putative gene encoding the NADP-dependent GDH.

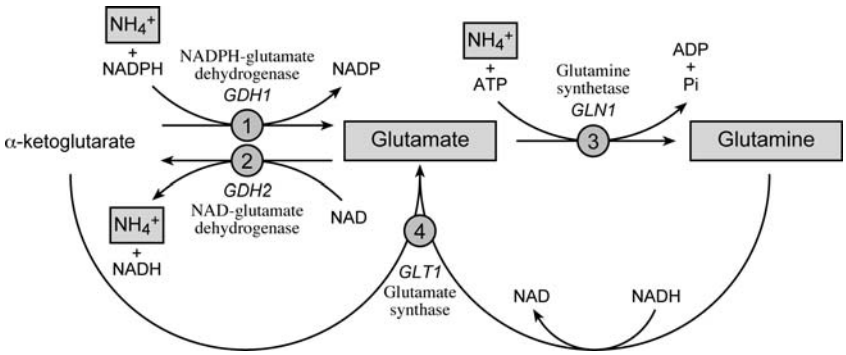


Fig. 7.1. Central pathways for nitrogen metabolism. The *Saccharomyces cerevisiae* gene for each of the enzymatic steps is designated in *italics*

7.2 Nitrogen Transporters

Transport of amino acids for breakdown to supply nitrogen for the synthesis of other molecules involves transporters, such as Gap1 and Agp1. The general amino acid permease (Gap1) (Grenson et al. 1970; Jauniaux and Grenson 1990) can recognize most if not all amino acids, including ones not present in proteins (e.g., citrulline, ornithine, γ-aminobutyric acid), D-isomers, β-alanine, and many toxic amino acid analogs. Contrary to a widely held belief, Gap1 displays a very high affinity for most of its substrates (apparent K_m in the micromolar range). This permease is most active under limiting nitrogen supply conditions, e.g., when the sole nitrogen source is ammonia at low concentration, urea, or proline. The main function of Gap1 under these conditions is probably to scavenge traces of amino acids to be used as a source of nitrogen. When amino acids (other than proline) are present at relatively high concentrations, Gap1 is inactive and broad-range-specificity amino acid permeases are induced. Among the latter is Agp1, an amino acid permease able to recognize most neutral amino acids. The affinity of Agp1 for these amino acids is lower (K_m about 100–200 μM) than the affinity of Gap1 (Iraqui et al. 1999b; reviewed in Boles and Andre 2004). Proline uptake is also mediated by Put4, also able to recognize γ-aminobutyric acid (GABA) (Vandenbol et al. 1989), alanine and glycine (Regenberg et al. 1999). Other amino acid permeases first believed to recognize only a single or a few amino acids (Bap2, Bap3, Gnp1, Tat1, Tat2, Dip5) actually recognize larger sets of amino acids, although their specificity ranges do seem narrow as compared with Agp1. Bap2 preferentially transports branch-chained amino acids, and glutamine and asparagine are efficiently transported into the cell by the products of *GNP1* and *AGP1* genes (Zhu et al. 1996; Schreve et al. 1998). Tat1 transports at least leucine and tyrosine, Tat2 mainly transports tryptophan, and Dip5 transports dicarboxylic amino acids (Schmidt et al. 1994; Regenberg et al. 1998). GABA is transported into the cell by the inducible Uga4 high affinity permease, and urea entry is mediated by the inducible permease Dur3. *DAL4* encodes the allantoin permease, a member of the uracil/allantoin permease family, and *DAL5* the allantoate and ureidosuccinate permease (Andre 1995). Besides these transporters, *S. cerevisiae* also synthesizes specific permeases mediating high-affinity uptake of arginine (Can1, Alp1), lysine (Lyp1), histidine (Hip1), and methionine (Mup1, Mup2) (reviewed in Boles and Andre 2004). Ammonia transport requires three permeases encoded by the *MEP1*, *MEP2*, and *MEP3* genes (Marini et al. 1997).

Genes encoding amino acid transporters have also been reported in yeasts other than *S. cerevisiae*. The gene *CaGAP1* of *C. albicans* can functionally complement a *S. cerevisiae gap1* mutant. Mutation of the *CaGAP1* gene has an effect on citrulline uptake in *C. albicans*. Transcription of *CaGAP1* is regulated by the quality of the nitrogen source, and is dependent on the Cph1-mediated Ras1 signaling pathway. Defective filamentation or abnormal colony morphology in homozygous and heterozygous *CaGAP1* disruptants is found under certain conditions (Biswas et al. 2003). The *C. albicans CaCAN1* gene, encoding a high-affinity permease for arginine, lysine, and histidine, can complement a *S. cerevisiae* strain lacking basic amino acid permeases. The CaCan1 protein sequence is strongly homologous to both permeases for basic amino acids, Can1 and Lyp1 of *S. cerevisiae* (Sychrova and

Souciet 1994). The expression of CaCan1 was influenced by the available nitrogen source, being almost negligible when cells were grown in the presence of ammonia (Matijekova and Sychrova 1997). In *S. pombe*, there is only a single high-affinity system for the transport of basic amino acids (Sychrova et al. 1992).

Tight regulation at the level of transporter synthesis or at the level of transporter activity and stability in response to nitrogen availability allows fine-tuning of nitrogen import. These aspects will be discussed in a further section.

7.3 Nitrogen Sources and Their Degradative Pathways

Yeasts are capable of utilizing the diversity of nitrogenous compounds that they find in their rich natural environment. Figure 7.2 summarizes the nitrogen compounds known to serve as a source of nitrogen for yeasts. An exhaustive list of these nitrogen compounds is presented in Large (1986). All these degradative pathways lead to ammonia or glutamate or both. Ornithine, GABA, tryptophan, tyrosine, phenylalanine, isoleucine, leucine, valine, alanine, methionine, and aspartate provide by transamination, glutamate or the α-ketoacid analog of the corresponding amino acid, whereas glutamine, asparagine, threonine, cytosine, adenine, cytidine, guanine, and urea lead to ammonia production by deamination. In *S. cerevisiae,* two asparaginases have been implicated in asparagine metabolism (Jones 1977), one of these, asparaginase I (*ASP1*) is cytosolic and acts only on intracellular L-asparagine.

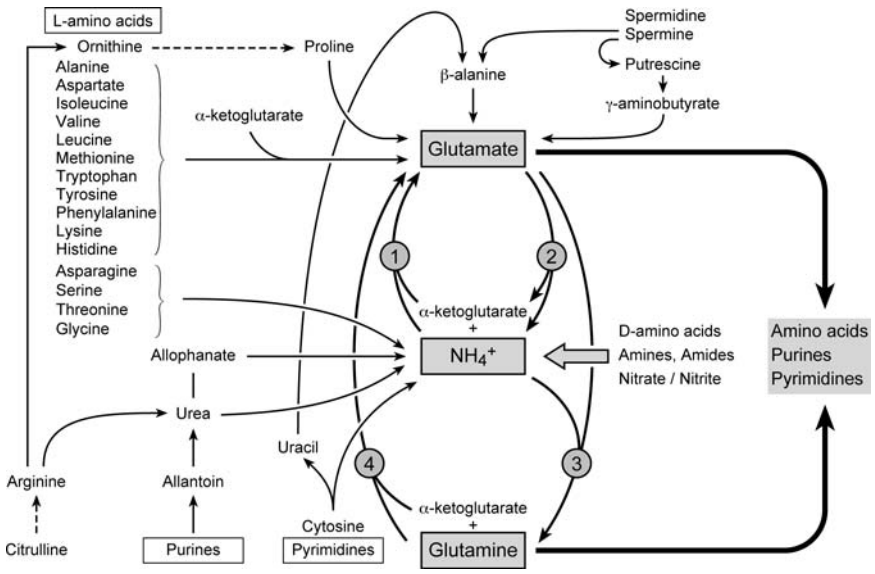


Fig. 7.2. Schematic representation of the main reactions involved in nitrogen utilization in yeasts grown on various nitrogenous compounds. See the text for specific pathways used by the different yeasts

Asparaginase II is produced extracellularly and can hydrolyze both D-asparagine and L-asparagine in the growth medium. Some strains of *S. cerevisiae* lack asparaginase II (*ASP3*) (Dunlop et al. 1976), but this enzyme occurs in a wide range of yeasts (Imada et al. 1973). Degradation of serine and threonine is catalyzed by the catabolic L-serine (L-threonine) dehydratase (*CHA1*), which converts serine in one step into pyruvate and ammonia (Ramos and Wiame 1982; Petersen et al. 1988). GABA is degraded in two steps to glutamate. Gene *UGA1* encodes 4-aminobutyrate aminotransferase, and gene *UGA2* encodes succinate semialdehyde dehydrogenase (Ramos et al. 1985). Arginine is cleaved by arginase (*CAR1*) to ornithine and urea. The ornithine so formed is converted via transamination (*CAR2*) to glutamate and glutamate semialdehyde. *S. pombe* genome contains two putative orthologs of gene *CAR1*. One of these genes (SPB26C9.02c) has been cloned by functional complementation of a *car1* mutant strain of *S. cerevisiae* (van Huffel et al. 1994). Further catabolism of ornithine proceeds via the amino acid proline, whose catabolism occurs in the mitochondria (Brandriss and Magasanik 1980). Proline is oxydized by proline oxydase (*PUT1*) to Δ-pyrroline 5-carboxylate, which is then oxydized to glutamate by the mitochondrial pyrroline 5-carboxylate dehydrogenase (*PUT2*). Citrulline is used as a nitrogen source by conversion to arginine by the two last arginine biosynthetic enzymes, argininosuccinate synthetase (*ARG1*) and argininosuccinase (*ARG4*). Urea produced by the degradation of arginine or purines, or present in the medium, can be processed via two different routes depending on the yeasts. Schizosaccharomycetacae are the only urease-positive ascomycetous yeasts reported (Booth and Vishniac 1987), but basidiomycetous yeasts such as *Cryptococcus neoformans* and *Rhodotorula* spp., also present urease activity (Seeliger 1956; Sen and Komagata 1979). In the other yeasts (listed in Fig. 7.7), including *Y. lipolytica,* the reaction proceeds via the intermediate formation of allophanic acid and the process requires ATP. The two enzymes, urea carboxylase and allophanate hydrolase (*DUR1*,*2*) are tightly associated in *S. cerevisiae* in a multienzyme complex (Sumrada and Cooper 1982; Whitney and Cooper 1972). *DUR1,2* orthologs can be found in *D. hansenii, Y. lipolytica, C. glabrata* and *K. lactis*.

The catabolism of the branched-chain (leucine, isoleucine, valine) and aromatic amino acids (tryptophan, phenylalanine, tyrosine) in *S. cerevisiae* proceeds through the Erlich pathway involving three enzymatic steps (Dickinson 2000; Dickinson et al. 2003). A first transamination produces the α-ketoacid analogs of the amino acids, a decarboxylation step yields the corresponding aldehydes, and in a third step, the resulting aldehydes are reduced to alcohols, collectively referred to as fusel oils. This general scheme of amino acid degradation has long been proposed from studies of metabolic intermediates and end-product formation. But only in recent years, the enzymes involved in these degradative pathways and their genetic determinants have been studied. Degradation of the branched-chain amino acids starts with the reversible transfer of the α-amino group to α-ketoglutarate to form glutamate and the respective branched-chain α-keto acids (α-ketoisocaproic from leucine, α-ketoisovaleric acid from valine, and α-keto-β-methylvaleric acid from isoleucine). These reactions are mainly catalyzed by the branched-chain aminotransferase isozymes, mitochondrial Bat1 and cytosolic Bat2; *bat1 bat2* double mutants are indeed auxotrophic for isoleucine, valine, and leucine (Eden et al. 1996; Kispal et al.

1996; Prohl et al. 2000). Bat1 and especially Bat2 diversely contribute also to the formation of higher alcohols from branched-chain amino acids (Eden et al. 2001; Yoshimoto et al. 2002), but their respective contribution to branched-chain amino acid utilization as nitrogen sources remains to be investigated. There are significant differences in the way each α-ketoacid is subsequently decarboxylated. In the leucine degradation pathway, the major decarboxylase is encoded by *KID1*/*YDL080c* (Dickinson et al. 1997). In valine degradation, any one of the three isozymes of pyruvate decarboxylase encoded by *PDC1*, *PDC5*, and *PDC6* can decarboxylate α-ketoisovalerate (Dickinson et al. 1998), and in isoleucine catabolism, any member of the family of decarboxylases encoded by *PDC1*, *PDC5*, *PDC6*, *KID1*, and *ARO10*/*YDR380w* is apparently sufficient for the conversion of isoleucine to active amyl alcohol (Dickinson et al. 2000). In aromatic amino acid metabolism also, two isozymes can catalyze in vitro the reversible conversion of tryptophan, phenylalanine, and tyrosine to indol-3-pyruvate, phenylpyruvate, and hydroxyphenylpyruvate, respectively: the constitutive aromatic amino acid aminotranferase I, encoded by *ARO8,* and the inducible aromatic aminotranferase II, encoded by *ARO9* (Kradolfer et al. 1982; Iraqui et al. 1998; Urrestarazu et al. 1998). Both catalyze the last step of phenylalanine and tyrosine biosynthesis, as *aro8* or *aro9* single mutants are prototrophs, whereas *aro8 aro9* double mutants are phenylalanine and tyrosine auxotrophs (Urrestarazu et al. 1998). Aro9 catalyzes the first step of tryptophan degradation as *aro9* mutant strains grow poorly on tryptophan, whereas the enzyme is dispensable for growth on phenylalanine or tyrosine, indicating that Aro8 or other transaminases can also ensure their degradation (Iraqui et al. 1999a). In both phenylalanine and tryptophan catabolism the decarboxylation step can be carried out by any one of the gene products of *PDC1*, *PDC5*, *PDC6*, or *ARO10* (Dickinson et al. 2003). In fact, it has been shown for phenylalanine that the inducible Aro10 is the physiologically relevant 6-phenylpyruvate decarboxylase in wild-type cells; in *aro10* mutants, an alternative activity was observed requiring the combined presence of Kid1 and at least one of the three pyruvate decarboxylases, Pdc5, Pdc1, or Pdc6 (Vuralhan et al. 2003). According to Dickinson et al. (2003), any one of six alcohol dehydrogenases (encoded by *ADH1*, *ADH2*, *ADH3*, *ADH4*, *ADH5*, or *SFA1*) is sufficient for the final stage of aromatic and branched-chain amino acid catabolism that converts an aldehyde to a long-chain or complex alcohol. This preliminary work, however, does not determine what are the physiologically relevant alcohol dehydrogenases that contribute to fusel oil formation in wild-type cells.

Glycine can serve as the sole nitrogen source, but only very poorly (Sinclair and Dawes 1995) (Fig. 7.3). This degradation involves a mitochondrial glycine decarboxylase complex, which catalyzes the breakdown of glycine to CO_2 and ammonia, yielding the activated one-carbon unit 5,10-methylenetetrahydrofolate and NADH. In *S. cerevisiae* the GCV complex comprises four subunits: the T-protein (*GCV1*), the P-protein (*GCV2*), the H-protein (*GCV3*) and the L-protein (*LPD1*) (Ross et al. 1988; Sinclair et al. 1996; McNeil et al. 1997; Nagarajan and Storms 1997). Possibly the physiological role of the GCV complex is more attuned to one-carbon metabolism rather than for provision of nitrogen. Nonetheless it is possible to show a non-growth phenotype of the *gcv1*, *gcv2*, *gcv3*, or *lpd1* mutants on a medium with glycine as the sole nitrogen source.

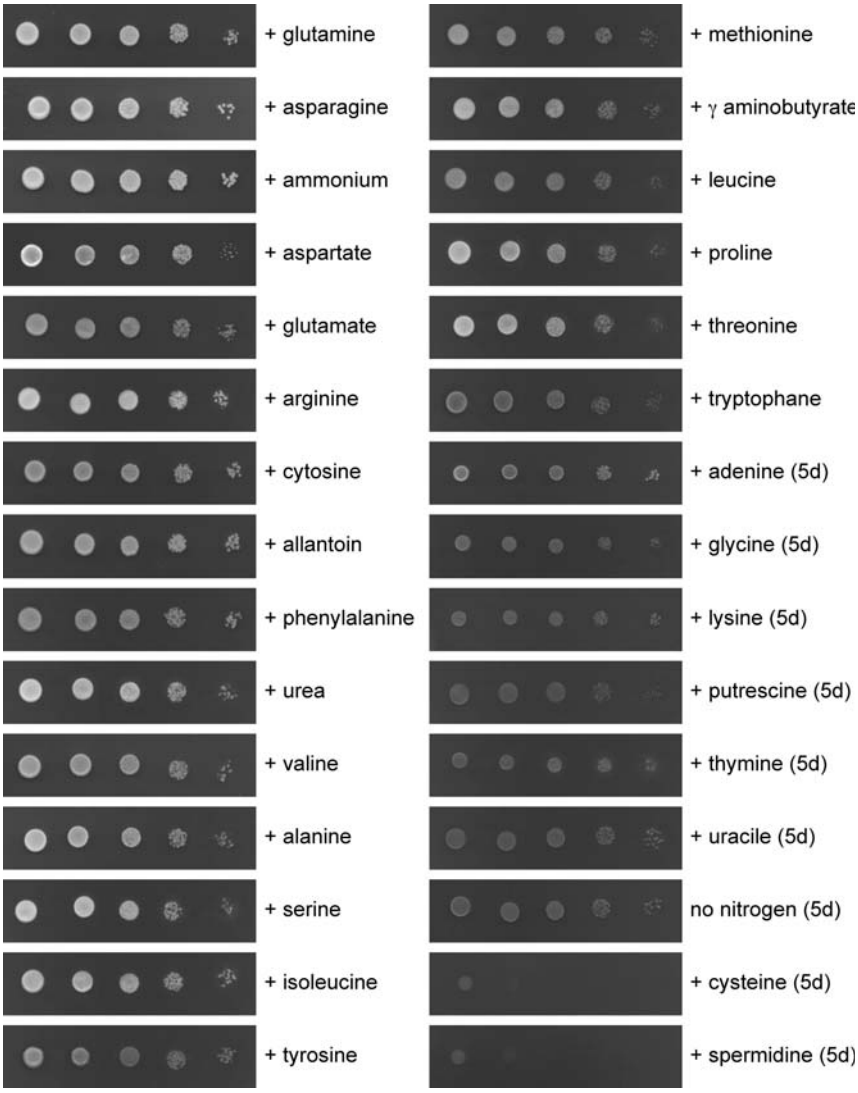


Fig. 7.3. Growth tests of a wild-type *S. cerevisiae* strain on various nitrogen sources. Tenfold serial dilutions of cells from the haploid strain ∑1278b were plated and incubated at 30°C for 3 days (except when otherwise indicated) on minimal medium with 2% glucose and the different nitrogen sources as indicated (1mg/ml). The growth tests are classified according to the growth rates on each nitrogen source. The growth reference is the same minimal medium without nitrogen

Many yeasts, but not *S. cerevisiae*, are able to utilize histidine or lysine as the sole nitrogen source. The histidine degradative pathway remains uncharacterized, whereas the lysine degradative pathway has been studied. Among the archiascomycete and hemiascomycete yeasts *S. pombe*, *Y. lipolytica*, *Debaryomyces*, and different *Candida*, *Pichia*, and *Kluyveromyces* are lysine utilizers. The lysine degradation pathway has been investigated in *Y. lipolytica* by the group of Gaillardin (1976). The first enzyme – lysine N6-acetyltransferase – is encoded by the *LYC1* gene, which has been cloned and sequenced (Beckerich et al. 1994). The second step involves a N6-acetyllysine aminotransferase providing glutamate by transamination of α-ketoglutarate. Lysine N6-acetyltransferase activity was found in *C. tropicalis* (Large 1986), which grows on lysine as the sole nitrogen source. For other yeasts no information about enzyme activity is available. Recently a comparison of genomic sequences revealed in *S. cerevisiae* the presence of a sequence YGR111w presenting a very weak similarity to *S. pombe* Lyc1. This sequence is also present in *C. glabrata* and *K. lactis*. The gene encoding N6-acetyllysine aminotransferase has not yet been characterized. It is thus still unclear why some yeasts grow on lysine and others do not. The ability to degrade purines to provide nitrogen is widespread. Adenine deamination by adenine deaminase (*AAH1*) leads to the formation of hypoxanthine, which is then oxidized to uric acid via xanthine dehydrogenase (gene uncharacterized in any yeast). Guanine deaminase (*GUD1*) converts guanine to xanthine. Uric acid is degraded via allantoin and allantoate to urea. These different steps are catalyzed by urate oxydase (gene uncharacterized in any yeast), allantoinase (*DAL1*), and allantoicase (*DAL2*). Gene *DAL3* encodes ureidoglycolate hydrolase, which converts ureidoglycolate to glyoxylate, ammonia, and CO2.

Many yeasts can use cytosine and uracil as good nitrogen sources, but thymine is less frequently used (LaRue and Spencer 1968). However *S. cerevisiae* grows well on cytosine but not on uracil. Cytosine deaminase activity (*FCY1*) provides ammonia and uracil. When uracil is used as a nitrogen source, it is degraded in three steps to β-alanine and ammonia (as shown in Fig. 7.2).

Since all nitrogen sources are not used with equal efficiency, the quality of different nitrogen sources has been evaluated by growth tests (serial dilutions) of a wild-type *S. cerevisiae* strain (Σ1278b background) on different nitrogen compounds compared with the residual growth without nitrogen in the medium (Fig. 7.3). This residual growth could result from the storage of nitrogen compounds in the cell, but the presence of nitrogen traces in the agar cannot be excluded. The best nitrogen sources are glutamine, asparagine, and ammonia (doubling time about 2 h), followed by aspartate, glutamate, arginine, cytosine, allantoin, and phenylalanine. A significant reduction in growth rate is observed on urea, valine, alanine, serine, isoleucine, tyrosine, methionine, and GABA. On leucine, proline, threonine, or tryptophan the doubling time is at least 3 h. Adenine and glycine are very poor nitrogen sources, and lysine, putrescine, thymine, and uracil cannot serve as a nitrogen source. Cysteine and spermidine are even inhibitors of residual growth. Growth on histidine was not tested.

Although the genera *Saccharomyces* and *Schizosaccharomyces* are unable to use nitrate or nitrite as nitrogen sources, more than 150 species of different genera can grow on these compounds (Fig. 7.4). Curiously some yeasts are able to use nitrite but not nitrate. Recently, the availability of classical genetics and molecular biology tools for the yeast *Hansenula polymorpha* (renamed *Pichia angusta* = *Ogataea poly-*

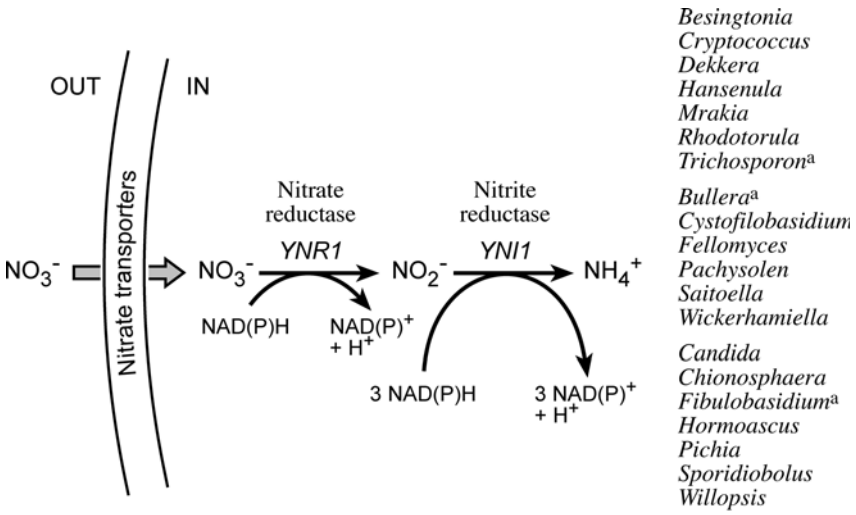


Fig. 7.4. Nitrate/nitrite utilization pathway and yeast utilizers. Some of the species of this genera utilize nitrite but not nitrate. The genes for each enzymatic step are in *italics*

morpha) has shed some light on this metabolic pathway in yeast (reviewed in Siverio 2002). Nitrate is transported in the cell by Ynt1, a high-affinity nitrate transporter, which is also able to transport nitrite. Ynt1 belongs to the major facilitator superfamily (MFS). In addition, nitrite uptake takes place by a nitrite-specific transporter (Machin et al. 2004). Nitrate assimilation in yeast follows the same pathway as that described for plants and filamentous fungi. Nitrate is converted to ammonia by two successive reductions catalyzed, respectively, by nitrate and nitrite reductase. *YNR1* gene is the only one that encodes nitrate reductase, and *YNI1* gene encodes nitrite reductase. These genes lie closely together in a cluster also containing two other genes encoding transcriptional regulatory factors. Regulation of this pathway will be addressed in the following section.

7.4 Regulation and Coordination of Nitrogen Catabolism

7.4.1 Control of Gene Expression in Response to the Quality of the Nitrogen Source

Selective nitrogen source utilization in *S. cerevisiae* is accomplished through a physiological process designated nitrogen catabolite repression (NCR) (Cooper 1982; Wiame et al. 1985). NCR consists in the specific inhibition of transcriptional

activation systems of genes encoding the permeases and catabolic enzymes needed to degrade poor nitrogen sources (e.g., allantoin, proline, GABA). When readily used nitrogen sources (e.g., Asn, Gln or, ammonia in some strains) are available, NCR-sensitive genes are expressed at low levels. Upon depletion of these repressive nitrogen sources, NCR is relieved and transcription of NCR-sensitive genes is increased. There are two different criteria that can be used to judge the quality of a particular nitrogen source. The growth rate that can be supported by a source of nitrogen would seem to be the simplest criterion for quality. As shown in Fig. 7.3 the differences in the growth rate are often small and it is therefore difficult to use the growth rate to make clear distinctions between the qualities of different nitrogen sources. A second criterion is based on the level to which systems for use of alternative nitrogen sources are derepressed during growth on a particular nitrogen source. The relation between the quality of the nitrogen source and the level of gene activation is illustrated in Fig. 7.5. On glutamine, the level of NCR-sensitive genes such as *MEP2* and *DAL5* is very low, compared with the level on proline, which is the nitrogen source leading to optimal derepression of these genes. Arginine and glutamate lead to a moderate increase of expression of NCR-sensitive genes, independently of the genetic background of the strain. Some observations (see later) provide evidence that intracellular concentration of glutamine would be the signal responsible for NCR establishment. However, although wild-type strains of S288c or Σ1278b background grow on ammonia as efficiently as on glutamine, there seems

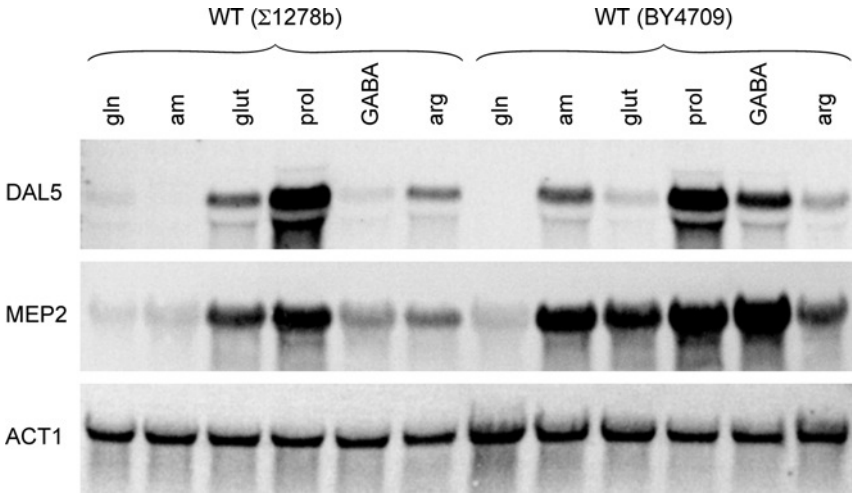


Fig. 7.5. Expression of nitrogen catabolite repression (*NCR*) sensitive genes of two wild-type *S. cerevisiae* strains on different nitrogen sources by Northern blot analysis. Total RNAs were extracted from strains ∑1278b and S288c and 30 μg was analysed by Northern blotting with *DAL5*, *MEP2*, and *ACT1* (encoding actine as a control) DNA probes. These strains were grown on the following nitrogen sources: glutamine (*gln*), ammonia (*am*), glutamate (*glut*), proline (*prol*), γ-aminobutyrate (*GABA*), and arginine (*arg*)

to be a difference in regulation between the two strains. In the Σ1278b background, ammonia exerts a strong repression on *MEP2* and *DAL5* genes, whereas in the S288c background, expression of these genes is partially derepressed on that medium (Fig. 7.5). In the Σ1278b genetic background, it was reported that ammonia could regulate nitrogen repression without being converted into glutamine (Dubois et al. 1977; ter Schure et al. 1998).

Modulation of NCR gene expression results from interplay between four GATA factors, each containing a GATA binding zinc-finger motif that has been conserved in organisms from yeast to metazoans. Gln3 and Nil1/Gat1 act as transcriptional activators, and for some NCR genes their action is counteracted by two negative GATA factors, Dal80/Uga43, and Nil2/Gzf3. The *NIL1, DAL80*, and *GZF3* genes are subject to nitrogen regulation, whereas *GLN3* is expressed constitutively regardless of the nitrogen source (Coffman et al. 1997; Soussi-Boudekou et al. 1997). There is thus a network of cross-regulating- and autoregulating GATA factors. A major role of this regulation might be to ensure a proper balance between the concentrations of the four GATA factors, this balance being tightly adapted according to nitrogen supply. It is noteworthy that NCR-sensitive genes are activated either by Gln3, or by Nil1, or by both factors, but the organization of the promoters does not provide a clue to predict by which factor(s) the gene will be regulated. Indeed all the genes responding to Gln3 and Nil1 activation contain a sequence named UASNTR, consisting of at least two GATAAG or GATTAG sequences. Some promoters require an auxiliary binding site, TTGT/GT, or a binding site for Abf1 or Rap1. The amplitude of response for each NCR-sensitive gene depends on the number and the position of these regulatory elements (reviewed in Magasanik and Kaiser 2002). The nitrogen-utilizing genes, encoding either transporters or degradative enzymes and responding to NCR regulation, are listed in Fig. 7.6 (shaded in gray). In addition to the four GATA family transcription factors, nitrogen catabolic gene expression is negatively regulated by Ure2. In a *ure2* mutant strain, repression no longer occurs (Drillien et al. 1973; Dubois and Grenson 1974; Grenson et al. 1974; Courchesne and Magasanik 1988).

It has been shown that the TOR kinases have an essential role in preventing the expression of nitrogen-regulated genes in cells using glutamine as a source of nitrogen (Beck and Hall 1999). Treatment of yeasts with rapamycin, an immunosuppressant drug, results in the activation of the expression of nitrogen-sensitive genes. Tor1/2 proteins inhibit expression of NCR genes by sequestering the GATA binding transcription factors Gln3 and Nil1 in the cytoplasm. In the presence of a good nitrogen source, Gln3 is phosphorylated in a TOR-dependent manner and is thereby tethered to the cytoplasmic Ure2 protein. Upon rapamycin treatment, Gln3 is dephosphorylated by the type-2A-related phosphatase Sit4, released from Ure2 inhibition, and translocated into the nucleus, where it activates target genes (Bertram et al. 2000). Recent data suggest that the TOR pathway senses glutamine (Crespo et al. 2002). This signaling mechanism had been extrapolated to the cell response to nitrogen starvation and to nitrogen availability. However, recently the Cooper group showed that on a poor nitrogen source Gln3 is localized in the nucleus despite its hyperphosphorylation (Cox et al. 2004). These data suggest that rapamycin treatment, a short-term response, and growth on poor nitrogen sources,

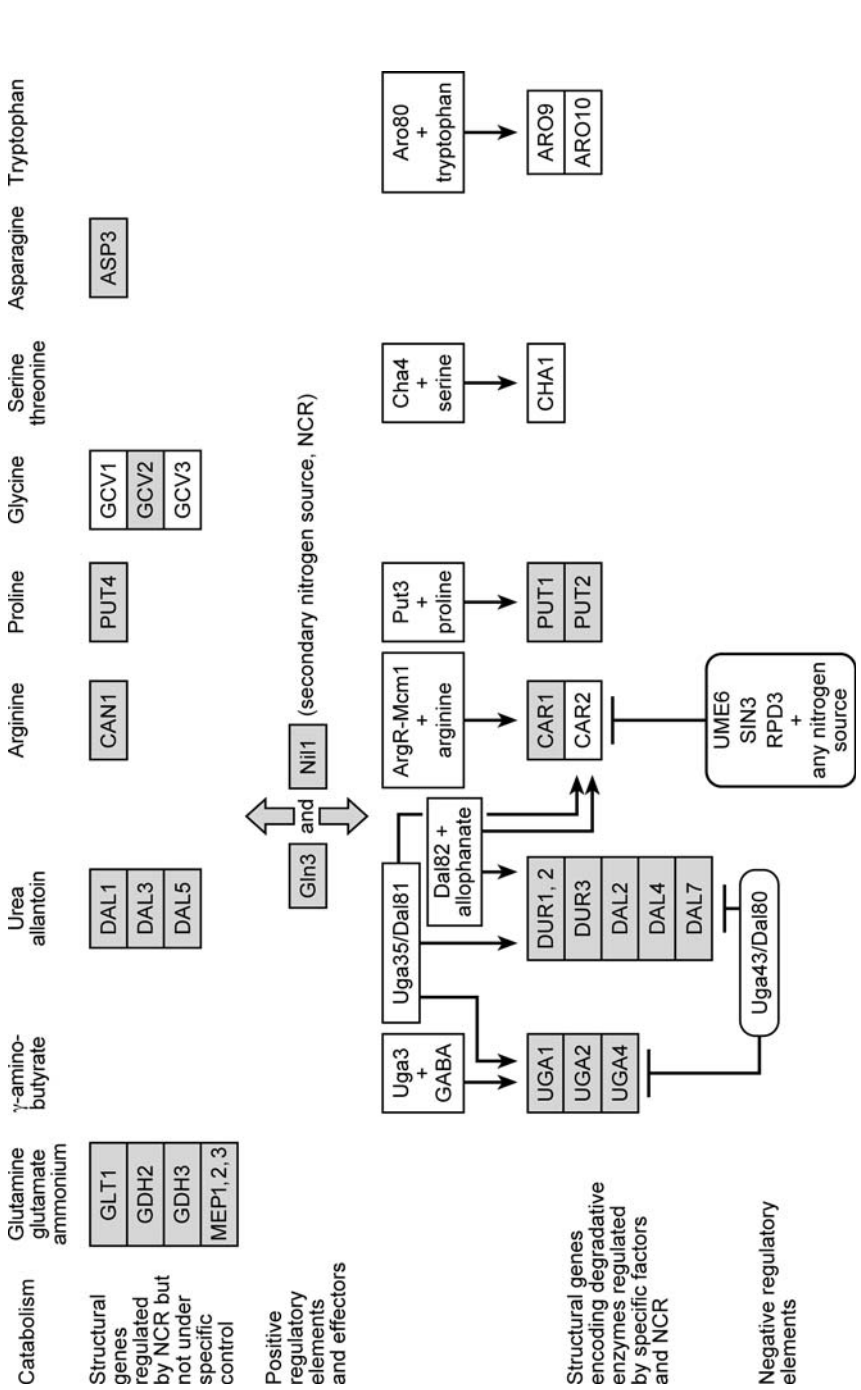


Fig. 7.6. Regulatory network controlling structural genes involved in the catabolism of various nitrogen sources. The products of the genes present in the figure are described in the text. The genes regulated by NCR are shaded in *gray*

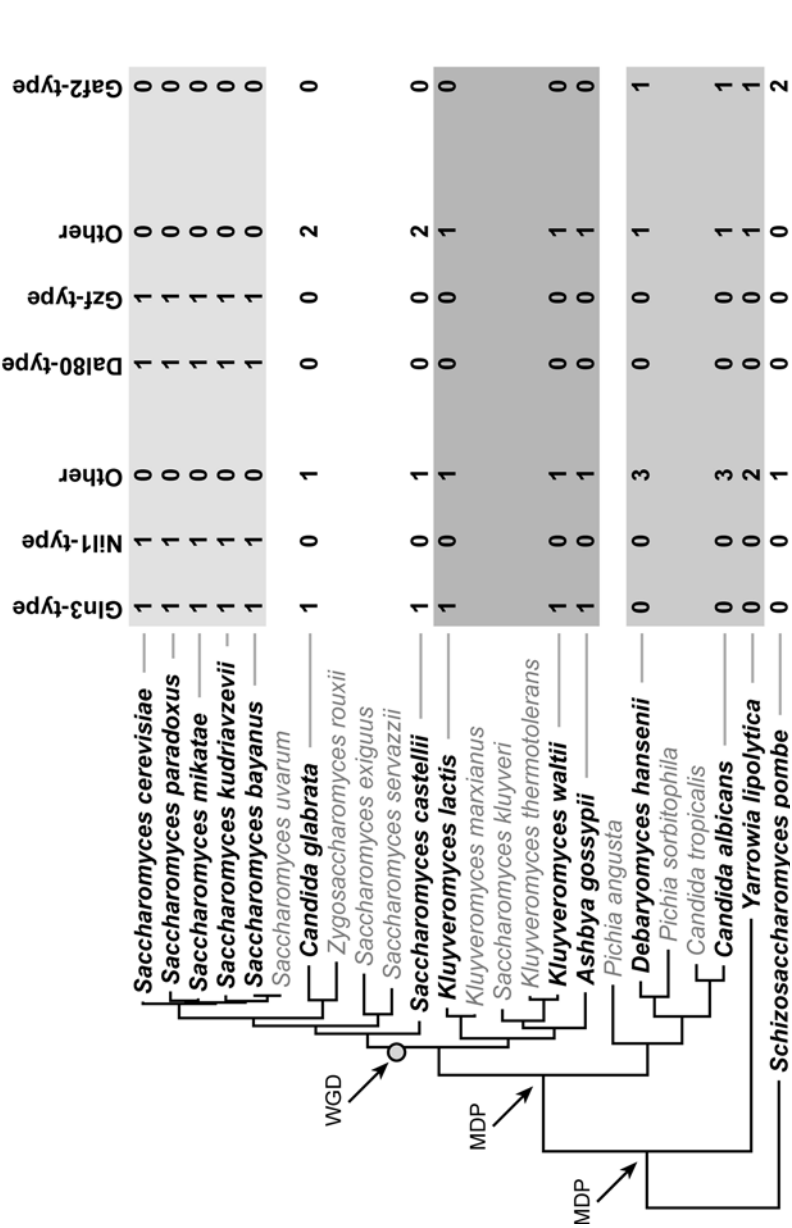
a long-term response, generate the same cellular response but likely do so by different mechanistic pathways (reviewed in Cooper 2002; Crespo and Hall 2002; Magasanik and Kaiser 2002; Rohde and Cardenas 2004).

Interestingly, Ure2 does not only inhibit Gln3 or Nil1, but could fulfill other cellular functions. Ure2 exhibits primary sequence and three-dimensional homologies to known glutathione S-transferases. Rai et al. (2003) showed that Ure2 is required for detoxification of glutathione S-transferase substrates and cellular oxydants. *ure2* mutants are hypersensitive to cadmium and nickel ions and hydrogen peroxide. *ure2* mutations possess the same phenotypes as mutations in known *S. cerevisiae* and *S. pombe* glutathione S-transferase genes, suggesting that Ure2 could serve as glutathione S-transferase in yeast. Whether this enzyme activity of Ure2 is involved in NCR regulation is still unknown. In addition the *ure2* mutation prevents the inactivation of glutamine synthetase when glutamine is added to wild-type cells grown with glutamate as a nitrogen source (Legrain et al. 1982; Coschigano and Magasanik 1991), suggesting a broader role for Ure2 in the control of nitrogen metabolism.

The presence of Ure2 orthologs in ascomycetous yeast species was searched for by comparative sequence analysis. A single Ure2 protein with typical structural features allowing clear distinction with classical glutathione S-transferase enzymes was found in all the yeast species analyzed and presented in Fig. 7.7, except in *S. pombe*. There is thus no clear coevolution between Ure2 and Gln3, i.e., yeast species like *D. hansenii* without any true Gln3 ortholog have a Ure2 protein. This is consistent with the ability of Ure2 proteins to control the subcellular location of GATA factors other than Gln3, e.g., Nil1 in *S. cerevisiae* (see later). Interestingly, *S. pombe* has one protein with a single GATA zinc finger (GZF) domain but no Ure2 ortholog.

GATA factors have been studied in other yeast species, e.g., *S. pombe* (Gaf2) and *C. albicans* (Gat1) as well as in *Ustilago maydis* (Urbs1). The Gaf2 and Urbs1 factors have two GZF domains, a property shared by several other proteins found in *D. hansenii*, *C. albicans*, and *Y. lipolytica* (Fig. 7.7). The *S. pombe* Gaf2 is transcribed constitutively, irrespective of the nitrogen source in the medium and up till now there is no evidence of a role of Gaf2 in the control of nitrogen-utilizing genes (Hoe et al. 1996). In contrast, Gat1 of *C. albicans* seems to be implicated in nitrogen regulation. The growth of mutants lacking Gat1 is reduced when isoleucine, tyrosine, or tryptophan are the sole sources of nitrogen, and *gat1* mutants are unable to activate expression of *GAP1, UGA4*, or *DAL5*, nitrogen-responsive genes in *C. albicans*. This regulatory defect does not prevent filamentation of *gat1* mutants in nitrogen repressing or nonrepressing conditions, demonstrating that NCR does not influence dimorphism. However, the mutants are highly attenuated in a murine model of disseminated candidiasis, suggesting an important role for nitrogen regulation in the virulence of *C. albicans* (Limjindaporn et al. 2003).

All yeast species analyzed shown in Fig. 7.7 contain three to five proteins of the GZF domain family. These proteins may be classified in three categories: (1) some factors (hereby named GZF) contain a single GZF domain, like the positive factors Gln3 and Nil1 of *S. cerevisiae*; (2) some (named GZF–GZF) contain a duplicated GZF domain, e.g., the *S. pombe* Gaf2 protein; (3) others (named GZF–LZ) contain a single GZF domain associated with a leucine zipperlike (LZ) domain likely


Single-GZF factor
Single-GZF-LZ factor
Double-GZF factor
Gln3-type
Nil1-type
Other
Dal80-type
Gzf-type
Other
Gaf2-type
Saccharomyces cerevisiae 1 1 0 1 1 0 0
Saccharomyces paradoxus 1 1 0 1 1 0 0
Saccharomyces mikatae 1 1 0 1 1 0 0
Saccharomyces kudriavzevii 1 1 0 1 1 0 0
Saccharomyces bayanus 1 1 0 1 1 0 0
Saccharomyces uvarum
Candida glabrata 1 0 1 0 0 2 0
Zygosaccharomyces rouxii
Saccharomyces exiguus
Saccharomyces servazzii
Saccharomyces castellii 1 0 1 0 0 2 0
Kluyveromyces lactis 1 0 1 0 0 1 0
Kluyveromyces marxianus
Saccharomyces kluyveri
Kluyveromyces thermotolerans
Kluyveromyces waltii 1 0 1 0 0 1 0
Ashbya gossypii 1 0 1 0 0 1 0
Pichia angusta
Debaryomyces hansenii 0 0 3 0 0 1 1
Pichia sorbitophila
Candida tropicalis
Candida albicans 0 0 3 0 0 1 1
Yarrowia lipolytica 0 0 2 0 0 1 1
Schizosaccharomyces pombe 0 0 1 0 0 0 2
WGD
MDP
MDP

involved in dimerization, e.g., the negative factors Dal80 and Gzf3 of *S. cerevisiae* (Fig. 7.7). Yeast species closely related to *S. cerevisiae* (*sensu stricto*) have a Gln3 and a Nil1 ortholog, with the Nil1 orthologs displaying much higher divergence than the Gln3 orthologs. More distant species (down to *A. gossypii* in Fig. 7.7) have a Gln3 ortholog plus a second GZF factor that may not be considered as a true Nil1 ortholog. Still more distant species have one to three GZF factors which do not correspond to true Gln3 or Nil1 orthologs (Fig. 7.7). These distant species also contain a single GZF–GZF protein. In contrast, the *S. pombe* proteome contains only one GZF and two GZF–GZF factors. This yeast species does not contain any member of the GZF–LZ family. All other yeast species indicated in Fig. 7.7 have at least one GZF–LZ factor. In species deriving from the one that has undergone whole genome duplication (WGD) (Wolfe and Shields 1997; Kellis et al. 2004), two genes encoding a GZF–LZ protein are present. In the *Saccharomyces sensu stricto* species, a clear divergence between Dal80-type and Gzf3-type GZF–LZ proteins is observable, with a much higher divergence among Gzf3 than Dal80 orthologs. These results suggest the following evolutionary scenario. Ancestral yeast species likely contained a single GZF factor gene. Duplication events probably led to the appearance of GZF paralogs and/or to one or several genes encoding GZF–GZF proteins. After the separation of lineages leading to *S. cerevisiae* and *S. pombe*, one of the GZF-encoding genes likely evolved into one coding for a GZF–LZ factor that has acquired a negative regulatory function. After separation of the lineages leading to *S. cerevisiae* and *C. albicans*, one of the GZF proteins evolved to the main regulator Gln3 likely involved in transcriptional activation of the other genes encoding GZF or GZF–LZ proteins (as is the case in *S. cerevisiae*). Finally, after WGD, the duplicated GZF–LZ factor genes were maintained, while the extra copies of each of the two GZF-encoding genes disappeared. The duplicated GZF–LZ factors finally evolved to generate the Dal80 and Gzf3 proteins known in *S. cerevisiae* and the *sensu stricto* species.

The *S. pombe* homologs of the TOR genes have been characterized. The *S. pombe TOR2* gene is essential for growth, whereas *TOR1* is required only under starvation, osmotic, and oxydative stresses. So far, no role for the Tor proteins in the control of nitrogen-gene expression has been reported (Weisman and Choder 2001). It was found from a comparison of yeast genomes that Tor1 and Tor2 proteins are present

Fig. 7.7. Phylogenetic distribution of GATA zinc-finger (*GZF*) domain proteins among diverse yeast species. The tree represents yeast species whose genome has been completely sequenced (indicated in *bold*) (Goffeau et al. 1996; Wood et al. 2002; Cliften et al. 2003; Kellis et al. 2003; Dietrich et al. 2004; Dujon et al. 2004; Kellis et al. 2004; Jones et al. 2004) plus several others whose genome sequencing is in progress by the Genolevure consortium (http://cbi.labri.fr/Genolevures/). The estimated position of the whole genome duplication (*WGD*) event is indicated, as well those of the main divergence points (*MDP*). The *columns* on the *right* represent the number of (1) proteins with a single GZF domain which are highly related to Gln3 or Nil1 and those which are not highly similar to Gln3 nor to Nil1 outside of their GZF domain, (2) proteins with a single GZF plus a leucine zipperlike (*LZ*) domain which are highly similar to Dal80 or Gzf3 and those which are not highly related to Dal80 nor to Gzf3 outside of their GZF and LZ domains, and (3) proteins with a double GZF domain similar to that of *S. pombe* Gaf2 protein

in *C. glabrata,* whereas in *K. lactis* and *D. hansenii* only the Tor2 ortholog was found, and in *Y. lipolytica* there is only one Tor protein with equivalent similarity to *S. cerevisiae* Tor1 and Tor2 proteins.

7.4.2 Control of Gene Expression by Specific Effectors: Arginine, Proline, Allantoin/Urea, γ-Aminobutyrate, Serine, Tryptophan, Amino acids, and Nitrate/Nitrite

In addition to general nitrogen control, some degradative pathways respond to specific inducers. Figure 7.6 summarizes the network of catabolic pathways regulated by NCR and by specific factors and effectors.

The metabolism of arginine presents the unique feature of possessing a regulatory mechanism coordinating its biosynthesis and its degradation. This coordination is achieved through the involvement of the same regulatory elements in the control of both pathways. In the presence of arginine, Arg80/ArgRI, Arg81/ArgRII, Mcm1, and Arg82/ArgRIII are required to repress the synthesis of five anabolic enzymes and to induce the synthesis of two catabolic enzymes. Arg81, a zinc cluster protein, is the sensor of arginine and interacts with the two MADS box proteins Arg80 and Mcm1 to form a complex (called ArgR–Mcm1) at the "arginine boxes" present in all the arginine coregulated genes. Arg82, an inositol polyphosphate kinase, acts as a chaperone for Arg80 and Mcm1 by stabilizing them in the nucleus. The inositol polyphosphate kinase activity of Arg82 is not required for arginine control (Messenguy et al. 1991; Amar et al. 2000; Dubois et al. 2000; El Bakkoury et al. 2000; Messenguy and Dubois 2000). Expression of *CAR1* and *CAR2* genes is not only regulated specifically by arginine and by the quality of the nitrogen source, but also in response to nitrogen availability by the histone deacetylase complex Ume6–Sin3–Rpd3. Ume6 is a DNA-binding protein belonging to the Zn2C6 family of transcription factors, interacting with DNA at a sequence named URS1 (Strich et al. 1994). Ume6 recruits the Rpd3–histone deacetylase complex by interacting with Sin3 (Kadosh and Struhl 1997). The role of the Ume6–Sin3–Rpd3 complex in the control of arginine catabolism is to block the expression of *CAR1* and *CAR2* promoters as long as exogenous nitrogen is available (Messenguy et al. 2000).

Vissers et al. (1982) investigated the repression of arginine biosynthesis (ornithine carbamoyltransferase, OTCase) and induction of the catabolism (arginase) in response to arginine, in the presence or absence of ammonia in the growth medium, in 28 yeast species. These species were obligate aerobes, such as *D. hansenii,* facultative anaerobes with a strong Pasteur effect, such as *K. lactis*, and facultative anaerobes with a weak Pasteur effect, such as *S. cerevisiae* and *S. pombe*. Most yeasts showed the classical repression of anabolic OTCase and induction of catabolic arginase, when arginine was added to the growth medium. In addition, they were subject to NCR, as judged by the increase in arginase synthesis when arginine was the only source of nitrogen compared with ammonia plus arginine as the nitrogen nutrient. No specific regulators involved in arginine regulation have been identified so far in these yeast species. Moreover, the promoter of the *S. pombe CAR1* gene does not contain the arginine boxes defined as the targets of the ArgR–Mcm1 proteins in the promoters of the arginine coregulated genes in *S. cerevisiae*. In addition,

the heterologous expression of *S. pombe CAR1* gene in *S. cerevisiae* is independent of the Arg81 gene product (van Huffel et al. 1994).

The expression of *PUT* genes involved in proline degradation is regulated by the Put3 activator protein, which responds to the presence of proline in the medium and increases the transcription of the *PUT1* and *PUT2* genes (Wang and Brandriss 1987). Put3 appears to respond to two signals, the presence of proline and the absence of preferred nitrogen sources, which are the conditions for maximal activation of *PUT1* and *PUT2*. Hyperphosphorylation of Put3 is correlated with growth on nonpreferred nitrogen sources (Huang and Brandriss 2000; Saxena et al. 2003). Put3 containing a six-cysteine, two-zinc domain (Zn2Cys6) constitutively binds to the upstream activation sequences in the promoters of both *PUT1* and *PUT2* genes in vitro and in vivo but activates transcription only in the presence of proline (Axelrod et al. 1991). The presence of the specific inducer causes a conformational change in the Put3 protein, allowing Put3 to shift from an inactive to an active state (des Etages et al. 1996, 2001).

Among the genes involved in allantoin and urea degradation, some, such as *DAL5*, are only NCR-sensitive, some, such as *DAL3*, are also down-regulated by Dal80, and others, such as *DAL7* and *DUR80*, are in addition regulated in response to the inducer, allophanate or its analog oxalurate (Yoo and Cooper 1991). Induction requires the dodecanucleotide sequence, UISall, and two transactive factors, Dal82/DurM and Dal81/DurL/Uga35. The precise function of Dal81 is still unknown, although this protein contains a zinc cluster motif, whereas Dal82, despite the absence of any known DNA-binding motif, binds UISall, present in the promoters of some *DAL* genes and also in the *CAR2* promoter (Dorrington and Cooper 1993; Park et al. 1999). Dal82 was recently shown to bind to UISall, and in vitro the 85 N-terminal amino acids of Dal82 are sufficient to bind UISall (Scott et al. 2000). In the absence of inducer, even on a poor nitrogen source, Dal80-mediated repression prevails and allantoin pathway genes are expressed at a low basal level (Rai et al. 1999). In the presence of an inducer, Gln3–Nil1-mediated transcription prevails and the expression of these genes occurs at a high level. This suggests that the role of the inducer would be to release repression by Dal80 at the GATAA sequences, allowing the binding of the two GATA activators, although this has never been demonstrated.

GABA induces transcription of the *UGA* genes required for its utilization as a nitrogen source. These genes contain in their promoters a conserved GC-rich sequence (UASgaba) essential to induction by GABA. To be effective, UASgaba requires two positive-acting proteins that both contain a zinc cluster motif, namely pathway-specific Uga3 and pleiotropic Dal81/Uga35 (Andre et al. 1995; Talibi et al. 1995). Alone the UASgaba element is sufficient to enable transcription of a reporter gene in the presence of GABA, even on a good nitrogen source; however, in the full-length promoters of *UGA* genes, the regulation is more complex, because another UASgata element, located just upstream from UASgaba, plays a determining role in producing a high level of induced expression. In the absence of an inducer, Dal80/Uga43 would prevent Gln3 or Nil1 from binding, whereas in the presence of the inducer Uga3 and Uga35/Dal81 factors acting through UASgaba promote some levels of transcriptional activation on their own, but could also facilitate binding of

Gln3 or Nil1 to the adjacent UASgata. This synergistic action leads to the optimal expression of *UGA* genes, only when GABA is the unique nitrogen source.

The transcription of *CHA1,* the gene encoding the catabolic L-serine (L-threonine) deaminase responsible for utilization of serine/threonine as nitrogen sources is activated by Cha4, a zinc cluster protein. Two serine–threonine response elements have been identified in the *CHA1* promoter, to which Cha4 is bound constitutively (Holmberg and Schjerling 1996). Regulation of *CHA1* basal expression involves assembly of a positioned nucleosome over the TATA element which requires the RSC complex (Moreira and Holmberg 1998, 1999) Upon activation, this nucleosome is removed, but so far no role for Cha4 in this chromatin remodeling has been reported.

Transcription of *ARO9* and *YDR380w*/*ARO10* is induced by aromatic amino acids by the transacting factor Aro80, a zinc cluster protein (Iraqui et al. 1999a). *aro80* cells are unable to grow on tryptophan medium, but their growth is only slowed down on a medium containing tyrosine or phenylalanine as a nitrogen source. A 36-base-pair element present in *ARO9* and *ARO10* 5′ regulatory regions is necessary and sufficient to mediate transcriptional activation of a reporter gene in response to aromatic amino acids. UASaro could thus be the DNA element to which Aro9 binds.

In response to external amino acids, *S. cerevisiae* induces the expression of amino acid permease genes, such as *AGP1*, *BAP2*, *BAP3*, *TAT1*, *TAT2*, and *GNP1*, and the dipeptide and tripeptide permease gene *PTR2* (reviewed in Boles and Andre 2004). The presence of external amino acids is transmitted to the transcription machinery through the Ssy1 amino acid sensor (de Boer et al. 1998; Didion et al. 1998; Jorgensen et al. 1998; Iraqui et al. 1999b; Klasson et al. 1999; Forsberg et al. 2001) Ssy1 is part of a multicomponent membrane-associated signaling complex (SPS), which includes at least two other proteins, Ptr3 and Ssy5 (Forsberg et al. 2001). Ssy1 contains 12 transmembrane spans (TM1–TM12), and its long N-terminal cytosolic tail likely plays a crucial role in signaling (Bernard and Andre 2001) together with Ptr3. The signal generated by Ssy1 and Ptr3 would be transmitted to Ssy5, which appears to interact with Ptr3. A next step in the signaling pathway is the endoproteolytic processing of transcription factors Stp1 and Stp2 (Andreasson and Ljungdahl 2002), requiring the SCFGrr1 ubiquitin–ligase complex. Recent data indicate that Ssy5 would be the endoprotease cleaving Stp1 (Abdel-Sater et al. 2004a) After processing, truncated Stp1 is translocated into the nucleus, and acts via an upstream sequence (UASaa) to activate gene expression. UASaa consists of at least two copies of the 5′-CGGC-3′ tetranucleotide separated by four to nine nucleotides (de Boer et al. 1998; Abdel-Sater et al. 2004b). Another transcription factor, Uga35/Dal80, is also important for induction of Ssy1-regulated genes.

Ssy1, Ptr3, and Ssy5 orthologs are present in all yeast species shown in Fig. 7.7, but *S. pombe* (Souciet et al. 2000; Cliften et al. 2003; Kellis et al. 2003). Hence, yeast species always have the three or none of the Ssy1–Ptr3–Ssy5 proteins, a result compatible with the three proteins being associated into a complex (SPS) (Forsberg et al. 2001). The Ssy5 orthologs share a well-conserved C-terminal protease domain and a rather divergent N-terminal domain possibly involved in the interaction with Ssy1 and Ptr3. The Ptr3 orthologs exhibits structural similarities with WD40 proteins and

they are also more closely related in their C-terminal regions. Comparison of the Ssy1 orthologs revealed that the large N-terminal tails and the extrapeptide regions between TM7 and TM8 have poorly similarity, while the extrapeptide regions between TM5 and TM6 are highly conserved. As shown in Fig. 7.8, the Ssy1 N-terminal end from *D. hansenii, C. albicans* and *Y. lipolytica,* present some degree of conservation despite their difference in length. The *C. albicans* Ssy1 end-terminal tail is composed of two regions, one present in *Y. lipolytica* Ssy1 (dark gray) and the other in *D. hansenii* Ssy1 (medium gray). Furthermore, all Ssy1 proteins contain an invariant triad (L/IFP) at their N-terminal end. Recently, the *SSY1* ortholog of *C. albicans* was functionally characterized (Brega et al. 2004). This protein (Cys1) is required for induction at the transcriptional level of several amino-acid permease genes as well as for filamentation in serum and amino acid based media. Interestingly, the range of amino acids to which Cys1 responds markedly differs from that of Ssy1.

The genes, *YNT1, YNR1*, and *YNI1*, encoding the enzymes for the nitrate-assimilation pathway are clustered in *H. polymorpha*. The genes encoding the transcriptional factors, *YNA1* and *YNA2*, two zinc cluster proteins, are located in the same cluster (Avila et al. 1998; Siverio 2002), but these genes are independently transcribed. Yna1 and Yna2 present similarity to the transcriptional factors nirA and nit4, involved in the nitrate induction in *Aspergillus nidulans* and *Neurospora crassa*, respectively. Deletion of *YNA1* or *YNA2* impairs growth on nitrate and nitrite, and leads to the absence of expression of *YNT1, YNR1*, and *YNI1*. A similar specific regulation occurs in *Hansenula* (*Pichia*) *anomala* (Garcia-Lugo et al. 2000). It has been shown that nitrate reductase has no role in the transcriptional induction of the nitrate-assimilatory genes in *H. polymorpha*, in contrast to data reported for filamentous fungi (Hawker et al. 1992; Siverio 2002). Expression of these genes is also subject to NCR, but only in *H. polymorpha*. Indeed there is a striking drop in nitrate-utilizing-enzyme activities when the cells are grown on glutamine plus nitrate compared with the levels on nitrate, and the fact that rapamycin abolishes NCR reveals the involvement of a TOR signaling pathway in the control of nitrate-assimilation genes (Navarro et al. 2003). It was reported that *NMR1* gene could be important in mediating the negative effect of the optimal nitrogen source on the

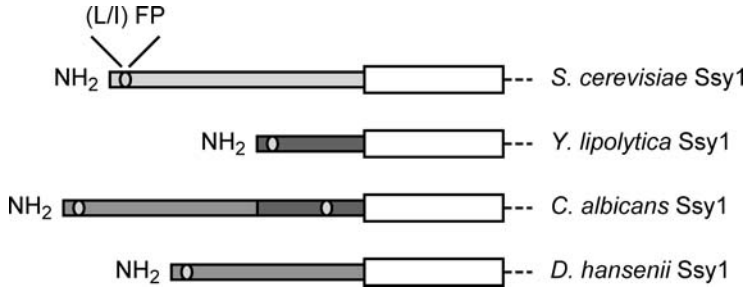


Fig. 7.8. Organization of Ssy1 transporters in different yeast species and comparison of their N-terminal tails

nitrate-assimilation pathway, since an *nmr1* mutant exhibits activation of these genes in the presence of nitrate plus ammonia (Serrani et al. 2001).

7.4.3 Control at the Level of Enzyme Activity and Enzyme Localization

S. cerevisiae has developed several regulatory mechanisms which exclude interference between biosynthesis and degradation of arginine. In addition to coordinated repression of biosynthesis and induction of catabolism by arginine and the ArgR–Mcm1 complex, this yeast has a peculiar mechanism to avoid the formation of a futile urea cycle when yeast is growing on arginine as the sole nitrogen source (Messenguy and Wiame 1969) Arginase, the first enzyme of the arginine degradative pathway, converts this amino acid into ornithine and urea. This enzyme is able to inhibit OTCase, the biosynthetic enzyme which converts ornithine and carbamoylphosphate to citrulline and phosphate. In the presence of ornithine and arginine, the respective substrates of the two enzymes, OTCase and arginase, both trimeric enzymes, form a one-to-one enzyme complex in which the activity of OTCase is inhibited, whereas arginase remains catalytically active. This regulation prevents the recycling by OTCase of ornithine produced by arginase, thus further excluding interference between anabolism and catabolism of arginine, since both enzymes are cytosolic in *S. cerevisiae*. This regulation was found to occur in vivo in eight out of 32 yeast species tested (Vissers et al. 1982). Obligate aerobes were devoid of this regulation (such as *D. hansenii*). Among fermenting species, *Schizosaccharomyces* and budding genera had different properties: all *Schizosaccharomyces* species were devoid of this regulation, whereas all species of budding yeasts tested showing a weak or absent Pasteur effect had this regulation (*Saccharomyces fermentati* = *Torulaspora delbrueckii*, *Saccharomyces kloekerianus* = *T. globosa*). Strains showing a strong Pasteur effect and taxonomically related to *Saccharomyces* (*Kluyveromyces*) had the regulation. It was also reported that *S. pombe* arginase is a hexamer instead of being a trimer, which can explain why *S. cerevisiae* OTCase is not inhibited by *S. pombe* arginase in vitro (El Alami et al. 2003). The absence of this epiarginasic regulation in some species can be linked to a mitochondrial localization of OTCase, whereas arginase is cytosolic, as for example in *D hansenii*, *H. anomala*, and *S. pombe* (Jauniaux et al. 1978). So yeasts can use different methods to control efficiently the flux of metabolites (in this case arginine and ornithine), and an efficient compartmentation of enzymes and metabolites can be sufficient to replace the sophisticated regulatory mechanisms operating in *S. cerevisiae*. Metabolites such as amino acids can be sequestered in the vacuole, so allowing them to function as a nitrogen reserve even under conditions when enzymes that are capable of catabolizing them are present at high levels in the cell. When growing on ammonia as a source of nitrogen, basic amino acids (arginine, lysine, histidine) are 90% vacuolar, threonine, serine, glycine, alanine, valine, tyrosine, and phenyalanine are 75% vacuolar, leucine and isoleucine are equally distributed between the vacuole and the cytoplasm, whereas glutamate and aspartate are mainly cytosolic (Messenguy et al. 1980). Since most of these amino acids can supply nitrogen, their release from the vacuole to the cytoplasm under conditions of starvation would then allow their nitrogen to be immediately available. However, when the cells are shifted

from a minimal medium containing ammonia to a minimal medium devoid of a nitrogen source, not all amino acids are released immediately from the vacuole (Messenguy et al. 2000). Interestingly, nitrogen deprivation leads to a rapid consumption of cytosolic glutamate without release from the vacuole. Glutamine is 80–90% vacuolar when the cells are grown on ammonia medium but is quickly released in the cytoplasm and immediately utilized as a nitrogen source upon nitrogen starvation. In contrast, the vacuolar arginine, whose pool concentration is much higher than that of glutamine, is released more slowly. After 40 min of starvation only 15% of the vacuolar arginine is consumed without accumulation in the cytosol. In contrast, when cells growing on arginine as the sole nitrogen source are shifted to a medium without nitrogen, the very high arginine vacuolar pool decreases rapidly because arginine is consumed very efficiently by the highly induced cytosolic arginase (Kitamoto et al. 1988). Thus, mobilization of vacuolar reserves may depend on environmental growth conditions.

7.4.4 Control at the Level of Protein Stability

In addition to control at a transcriptional level, many plasma membrane transporters are subject to tight control at the membrane trafficking level and modulation of intrinsic activity. Typical physiological conditions inducing these controls include changes of substrate concentration and availability of alternative nutrients. These changes of conditions often provoke the downregulation of specific transporters eventually accompanied by upregulation of others which are more appropriate to the new conditions. Downregulation involves the onset or acceleration of endocytosis of the transporters and subsequent targeting to the vacuole where it is degraded. The same physiological signal can also induce diversion of the neosynthesized transporter from the Golgi apparatus to the endosomal/vacuolar degradation pathway without passing through the plasma membrane (reviewed in Haguenauer-Tsapis and Andre 2004).

S. cerevisiae possesses about 20 different amino acid permeases, the substrate specificities of which overlap in many cases. These proteins are not simultaneously present in the cell, most are differentially regulated according to the nitrogen and/or amino acid content of the growth medium. In conditions of poor nitrogen supply the synthesized Gap1 permease accumulates at the plasma membrane in an active and stable form (Grenson 1983a; de Craene et al. 2001; Soetens et al. 2001). Upon the addition of ammonia, Gap1 is internalized by endocytosis and targeted to the vacuole for degradation (Nikko et al. 2003). Ubiquitination of Gap1 involving the ubiquitin ligase (Npi1/Rsp5) is essential to this ammonia-induced downregulation (Hein et al. 1995; Springael and Andre 1998). This downregulation may also be triggered by addition of amino acids (Stanbrough and Magasanik 1995). *NPR1* encoding a protein kinase is another gene involved in the posttranscriptional control of Gap1. In *npr1* mutants growing on a poor nitrogen source, the amount of *GAP1* transcript is unaltered but Gap1 is inactive (Grenson 1983a). Other ammonia-sensitive permeases, including the proline permease Put4 (Vandenbol et al. 1990), the ureidosuccinate, and allantoate permease Dal5 (Rai et al. 1988), and the inducible GABA permease Uga4 (Andre et al. 1993) are also dependent on Npr1 to be active.

The negative effect of an *npr1* mutation on Gap1 activity is suppressed by mutations inhibiting Gap1 downregulation such as *npi1*. This suggests a role of Npr1 in the control of Gap1 trafficking (Grenson 1983b). Recently it was shown that Npr1 phosphorylation is regulated by nitrogen through the TOR signaling pathway. Nitrogen starvation or growth on proline results in Npr1 dephosphorylation (Schmidt et al. 1998). During the downregulation of Gap1 following the addition of high concentration of amino acids, other broad-specificity amino acid permeases such as Agp1, Tat2, Tat1, and Bap2 are induced (Iraqui et al. 1999b). These permeases, therefore, take on the functions of Gap1. Unlike Gap1, these permeases are synthesized and active in cells grown on YPD medium. Shifting cells from YPD to nitrogen-starvation conditions results in the derepression of Gap1 and the rapid downregulation of Tat2 and Bap2. This downregulation process may also be induced by adding rapamycin to the medium. The requirements for ubiquitin, Rsp5, and lysines in the downregulation of Tat2 and Bap2 have been extensively studied (Beck et al. 1999; Omura et al. 2001; Umebayashi and Nakano 2003; reviewed in Boles and Andre 2004). The fate of newly synthesized transporters present in the secretory pathway may also depend on environmental conditions: the protein may be targeted to the cell surface, or directly diverted to the multivesicular bodies/vacuole for degradation without passing through the plasma membrane. For example, in cells grown on glutamate as the sole nitrogen source Gap1 is synthesized but is inactive because it is diverted from the secretory pathway to the vacuole without passing through the plasma membrane (Roberg et al. 1997a, b). Bul1 and Bul2 are redundant proteins required for the direct sorting of Gap1 to the vacuole and for its polyubiquitylation, suggesting that polyubiquitylation is required for direct sorting of the permease to the vacuole (Helliwell et al. 2001). The sorting of Tat2 is also regulated by cellular nitrogen status (Beck et al. 1999). Tryptophan availability in the medium plays an important role in controlling the fate of newly synthesized Tat2 (Umebayashi and Nakano 2003). In the presence of low concentrations of tryptophan Tat2 is targeted to the cell surface, whereas on media containing a high concentration of tryptophan Tat2 is sorted from the secretory pathway to the vacuole. Lipid rafts and ubiquitin play key roles in this regulated permease sorting.

7.5 Concluding Remarks

In the last decade biochemical and genetic studies allowed us to get deeper insight into nitrogen metabolic pathways, mainly in *S. cerevisiae*. Genes encoding enzymes catabolyzing aromatic and branched-chain amino acids have been characterized and new regulators involved in their control have been identified. In contrast, in other yeast species little new information has been provided about nitrogen degradative pathways, with the exception of nitrate/nitrite utilization and its regulation in *H. polymorpha*. It seems that most efforts were aimed at elucidating the complex and multiple regulations controlling nitrogen utilization in *S. cerevisiae*, and even for this yeast we still have a long way to go. Now that the genome sequences of many yeasts are known, it is rather easy to identify orthologs of genes encoding nitrogen-utilizing enzymes and their regulatory factors. However, biochemical and genetic studies are still necessary to test their functionality. This is especially true for the regulatory

factors, in which the best conserved sequences are the DNA-binding motifs, suggesting a putative regulatory function but not necessary involved in the control of the same pathway. Moreover, this comparative genomic analysis will not lead to an understanding of why yeasts use a particular nitrogenous compound or not, unless the genes and gene products involved in this degradation are characterized at least in one species, and this is far from being the case for many pathways. Hopefully, the novel approach to "evolutionary genomics" proposed by the Génolevures consortium will provide a rationale and better criteria to further unravel the full diversity of nitrogen utilization among yeast species.

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Chapter 8

Environmental Factors Influencing Yeasts

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8.1 Introduction

Yeasts are ubiquitous microorganisms that form part of the microbiota of most if not all natural ecosystems. A wide variety of yeast species occur in soil, fresh water and marine water, they are normal inhabitants on plants, are commonly associated with animals, and are also found frequently in man-made habitats such as foods. The conditions prevailing in these natural and artificial habitats determine the metabolic activity, growth and survival of yeasts. A variety of abiotic and biotic factors influence the life of yeasts, and exert stress conditions which the cells must withstand and adapt to or otherwise they die. Death of individual cells may not bring about the extinction of a whole population as there is a large variation in resistance to stress factors among cells. Also, these factors change with time and show spatial heterogeneity; their effect is manifested differently in microenvironments. So, when looking at the environmental relations of yeasts, we should deal with a very complex interrelation of factors that are only partially understood. Nevertheless, basic knowledge of these is important for understanding the ecology and biodiversity of yeasts as well as to control the environmental factors in order to enhance the exploitation of yeasts or to inhibit or stop their harmful and deleterious activity.

In this chapter our current knowledge of the environmental relations of yeasts is summarized. First, the most relevant physical, chemical and biotic factors and their interactions are described in a changing environment. Further on, responses of yeast cells to stress conditions are discussed at single cell and population level. In-depth treatment of the genetic background of variation, and the mechanism of adaptation to stress factors would be beyond the limits of this chapter. Further chapters in this book are devoted to their discussion. Reference is made to relevant literature and to the large amount of data covered and summarized in previous works and reviews (Phaff and Starmer 1987; Rose 1987; Watson 1987; Boddy and Wimpenny 1992; Fleet 1992; Deak and Beuchat 1996; Deak 2004).

8.2 Physical Factors

The most important physical factor influencing the life of yeasts is temperature. Other factors exerting less definite, and also less studied effects are light, radiation and pressure. In a wider sense, geography, locality and climate can be considered as ecological factors.

8.2.1 Temperature

The temperature limits and range for growth of yeasts vary with species. Most yeasts are mesophilic, and grow best at temperatures between 20 and 30°C. In a study covering nearly 600 strains of more than 100 species including genera of *Saccharomyces*, *Kluyveromyces*, *Debaryomyces*, *Pichia*, *Candida* and others (Vidal-Leira et al. 1979), the upper limit for growth of 98% of yeasts fell between 24 and 48°C, for a few it was below 24°C but for none was it above 50°C (Table 8.1). Yeasts such as *Leucosporidium scottii*, *Mrakia frigida* and a few others can be considered psychrophilic, having a minimum growth temperature as low as −1 to 4°C and a maximum at about 20°C. At 37°C only a limited number of species can grow, mostly those associated with warm-blooded animals, at least temporarily, such as *Candida albicans* and a number of other opportunistic pathogenic yeasts. Most strains of *Saccharomyces cerevisiae* occurring widely in industrial fermentation can grow at 37°C, whereas growth of *S. bayanus* in a similar environment is limited up to 30–35°C. As to the lower limit of growth temperature, it may extend a few degrees below 0°C for psychrophiles provided the water suspension remains fluid, such as the salty seawater in arctic regions. A number of yeasts can be considered psychrotrophic, possessing a range of growth temperatures between 0 and 25°C, whereas some mesophilic species may also grow at 5–10°C as the lowest limit. Even those yeasts isolated from the water and marine animals of the Arctic Ocean, as well as from frozen foods, grow faster at higher temperatures, and develop colonies in 1 week at 20°C but in 2 weeks at 5°C. Some yeasts are characterized with a rather wide

Table 8.1 Maximum growth temperatures of some yeast species

Species	T_{max} (°C)
Kluyveromyces marxianus	44–47
Candida glabrata	43–46
Candida albicans	42–46
Pichia guilliermondii	38–43
Pichia anomala	35–37
Yarrowia lipolytica	33–39
Metchnikowia pulcherrima	31–39
Candida zeylanoides	32–34
Candida vini	27–31
Leucosporidium scottii	22–24

Adapted from Vidal-Leira et al. (1979)

range of growth temperature, e.g., for *Pichia membranifaciens* it extends from 5 to 45°C, whereas others have much narrower limits, e.g., 28–42°C for *Candida sloofii*. Few yeasts only can grow above 40°C and none can be considered termophilic. *Kluyveromyces marxianus* is rather thermoduric; many strains of this species can grow up to 45–47°C; a few strains of exceptional thermotolerance were found to be able to grow and ferment at 52°C (Banat and Marchant 1995). Temperatures above 50°C are lethal for yeast cells and not even the sexual spores show much higher heat resistance.

The minimum, optimum and maximum limits of the growth temperatures are not absolute degrees as they are influenced by the physiological state of the cells as well as other environmental conditions. Inhibitory factors such as low water activity, low pH and the presence of antimicrobial substances, e.g., ethanol, will limit the range of the growth temperature. In turn, temperatures below or above the optimum would decrease the resistance of yeasts to other stress factors.

Temperature would not only determine whether or not a yeast strain is able to grow but it also influences the rate of growth (or its reciprocal, the generation time) once it started. Within the range of the growth temperature, changing of the growth rate can be described by the equation developed by Arrhenius according to which the growth rate increases linearly with temperature in the optimum range, approaching the minimum it decreases steadily, whereas moving from the maximum it decreases abruptly.

8.2.2 Light and Solar Radiation

Yeasts are not photosynthetic organisms; hence, illumination is not vital for their existence. Few observations refer to any effect of light on them, but they point to the possible killing effect of UV wavelengths of sunlight. This may explain the relative abundance of pigmented species (e.g., *Cryptococcus, Rhodotorula*) on surfaces of plant leaves. A systematic study on a phylloplane microbial community suggested that the position of a leaf within an apple tree canopy substantially affects the resident population (Andrews et al. 1980). Differences in the density of phylloplane microbiota could be attributed to a variation in the exposure to light and rainfall and also to the accessibility of leaves to airborne microbes (see Chap. 13).

8.2.3 Pressure

Under natural land conditions, atmospheric pressure does not affect the life of yeasts. When they occur in deep sea, the cells should withstand high hydrostatic pressure. Yeasts are frequently found in shallow marine environments but only recently have isolates been obtained from sediments and benthic microorganisms from deep sea of 2,000–6,500 m. Red yeasts (*Rhodotorula* and *Sporobolomyces*) were commonest among the isolates, some of them represented new species, such as *Kluyveromyces nonfermentans* (Nagahama et al. 1999, 2001). Unlike bacteria, however, no data refer to the physiology of yeasts occurring in the deep sea. Our knowledge of baroresistance of yeast cells comes from studies on the possible application of high hydrostatic pressure in food preservation (Smelt 1998). High

pressure exerts a strong effect on cell structures, and the viability of yeasts decreases with increasing pressures above 100 MPa; between 200 and 300 MPa cells are killed (Palhano et al. 2004). Unexpectedly, when cells were exposed to mild stress (hydrogen peroxide, ethanol or cold shock), it induced higher resistance to pressure. This hints to the function of a general mechanism of stress response in yeast cells, similar to that protecting them against other stress factors (see Sect. 8.5).

8.2.4 Geography, Climate

By and large, geography, locality and climate act as ecological determinants on the biodiversity and communities of yeasts. These large-scale phenomena manifest themselves through changes of temperature, rainfall, wind, solar radiation, drought, soil composition, vegetation, insect vectors and the like. Several studies have shown the impact of these factors on the ecology of yeasts at the microhabitat level (Andrews et al. 1980; Barker et al. 1987; Starmer et al. 1992; Chand-Goyal and Spotts 1996; Slávikovᡠand Vadkertiová 1997).

8.3 Chemical Factors

Among these factors are included the availability of nutrients and water, the acidity and pH, the oxygen relations, as well as the effect of inhibitory and antimicrobial substances. Mutual interactions between these factors as well as the physical environment bring about very complex relations. Their manifestation is hardly known in natural habitats and has been mostly studied under laboratory conditions.

8.3.1 Nutrients

Yeasts require for maintenance and growth some sources of carbon, nitrogen, mineral salts and certain vitamins and growth factors. The different ability of yeasts to utilize various nutrients is one of the most important ecological factors to determine habitat specificity.

The most important carbon and energy sources are carbohydrates, mostly sugars such as hexoses and oligosaccharides. Glucose and a number of simple sugars can be utilized by many yeasts both fermentatively and oxidatively. A wide range of other carbon sources (e.g., alcohols, organic acids) can be metabolized only by aerobic respiration. A faulty generalization arising from the vigorously fermentative group of *Saccharomyces* species is that all yeasts are capable of fermenting. In reality, about half of the currently known yeast species lack the ability to ferment and carry out aerobic oxidation exclusively. Not even the fermentative species can permanently survive under anaerobic conditions because some constituents of membrane lipids can be synthesized only aerobically. Both the aerobic and the anaerobic utilization of various carbon sources has been traditionally used for the physiological characterization and identification of yeasts; the list of these compounds extends to over 40 (Yarrow 1998; Barnett et al. 2000).

Polysaccharides and other macromolecules such as proteins and lipids are not commonly used nutrients of yeasts. Few species produces a substantial amount of

hydrolytic enzymes extracellularly. The number of amylolytic yeasts is small and none are known to hydrolyze intact cellulose. It seems, however, that protease, peptidase or lipase activities are widespread in yeasts. In studies on tropical yeast communities, 40–60% of isolates was found to possess proteolytic enzymes and around 30% produced pectinases (Abranches et al. 1997; Trindade et al. 2002). This empowers the yeasts to expand their ecological niche and make better use of the available carbon sources.

All yeasts can utilize inorganic nitrogen sources such as ammonium salts, many of them also nitrates. Amino acids, urea and other organic nitrogen sources can be utilized by various species. Some yeasts can grow without any external vitamin sources, others may require biotin, thiamin, nicotinic acid or some other growth factors. Inorganic phosphate salts satisfy the need of all yeasts; sulfate, sulfite and thiosulfate can serve as a sulfur source. A number of other inorganic elements, e.g., potassium, magnesium, iron and zinc, are required in low concentration. These requirements are generally met in most natural habitats of yeasts. Under laboratory conditions care should taken to satisfy specific needs of certain yeasts, but most of them can be cultivated on relatively simple growth media. If an essential micronutrient is lacking some members of the natural community may be lost in laboratory culture.

8.3.2 Water

Nutrients are taken into yeast cells in the form of water solutions and water itself is an essential requirement for life. Water should be fluid and free (not chemically bound) in order to be absorbed. The availability of water is usually expressed in terms of water activity (a_w); the more exact physicochemical term water potential (ψ) is used less frequently in microbial ecology (Marechal et al. 1995).

Yeasts living in marine habitats and large bodies of fresh water must cope with the highly diluted nutrients, and are rarely exposed to the osmotic pressure exerted by concentrated solutes that more frequently occur in habitats of solid matrices, such as soil or foodstuffs. Most yeasts can develop well at water activities around 0.95–0.90 (Table 8.2). A particular group of yeasts, however, is notable of being able to grow at much lower a_w values. In the earlier literature, an a_w value of 0.61 was given as the minimum value for growth. This was not confirmed in recent reinvestigations that resulted in values around 0.70 (Jermini and Schmidt-Lorenz 1987). This group of yeasts is often called osmophilic and halophilic depending on whether the solutes are sugars or salts, respectively, bringing about low water activities, corresponding to 55–65% sugar or 15–25% salt concentrations. A more general and appropriate term is xerotolerant for most strains of the species in question can grow in dilute solutions and do not require increased solute concentrations (Silva-Graca et al. 2003). *Zygosaccharomyces rouxii*, *Z. mellis, Z. bisporus*, *Debaryomyces hansenii*, *Candida versatilis*, *C. lactiscondensi* and *C. halonitratophila* are the species most notable for being xerotolerant; several other yeasts, among them certain strains of *S. cerevisiae* as well as *Schizosaccharomyces pombe*, *Torulaspora delbrueckii*, *Pichia anomala* and others possess less tolerance to a_w lower than 0.80.

Xerotolerant yeasts are of special importance to the food industry because they are able to cause spoilage of foods preserved by added sugar or salt. It was found

Table 8.2 Minimum a_w for growth of yeasts in media adjusted by different solutes

Yeast species	Minimum a_w for growth controlled by			
	Glucose	Fructose	Sucrose	NaCl
Candida lactiscondensi	0.79	0.78	0.70	0.92
Candida versatilis	0.79	0.80	0.79	0.84
Debaryomyces hansenii	0.84	0.86	0.81	0.84
Hanseniaspora uvarum	0.90	0.93	0.90	0.95
Pichia membranifaciens	0.90	0.92	0.90	0.94
Rhodotorula mucilaginosa	0.90	0.92	0.90	0.90
Saccharomyces cerevisiae	0.89	0.91	0.91	0.92
Torulaspora delbrueckii	0.86	0.89	0.87	0.90
Zygosaccharomyces bisporus	0.85	0.85	0.79	0.95
Zygosaccharomyces rouxii	0.79	0.82	0.79	0.86

Data from Tokouka and Ishitani (1991)

that various processing factors such as temperature, pH and the composition of the food interact with water activity with regard to the inhibitory effect of growth (Tokouka 1993); it can be supposed that these factors interact whenever low water activity is encountered in the environment.

The physiological background of resistance to low a_w stress conditions has also been the subject of a wide range of studies. Production of compatible solutes (glycerol, mannitol), active pumping out of sodium ions or their exchange for K^+, induction and differential expression of stress-responsive genes have been supposed as protective mechanisms; however, different species show contrasting reactions (Ramos 1999; Hohman 2002). Extreme osmotic stress can exceed the osmoregulatory capacity of cells and cause loss of viability (Table 8.3). Beyond industrial concern, understanding the phenomenon of xerotolerance is of relevance for the biodiversity of yeasts in natural habitats such as plant nectars and saps or salt ponds, where ecological factors are exerted in high solute concentrations.

Table 8.3 Viability of *Saccharomyces cerevisiae* in relation to osmotic stress

Water activity (a_w)	Water potential (MPa)	Cell viability (% related to control)	
		Glycerol	Poly(ethylene glycol) 600
0.90	–14.5	92	59
0.80	–30.8	65	58
0.70	–49.2	55	40
0.60	–70.5	20	28
0.50	–95.7	10	0

Data from Marechal et al. (1995)

8.3.3 Oxygen Relations

Yeasts are basically aerobic organisms although they are most noted for the vigorous fermentation of sugars – a remarkable characteristics of *Saccharomyces* species and a number of other genera but this certainly does not hold good for all yeasts as pointed out in Sect. 8.3.1. Most basidiomycetous yeasts, *Cryptococcus*, *Rhodotorula* and others, are strictly aerobic and not able to ferment. Even the fermentative yeasts are facultatively anaerobic and under aerobic conditions they switch to respiration under the well-known metabolic regulation, the Pasteur effect. This regulation is more complex, however, because in addition to oxygen the concentration of glucose is also an effector in that at high glucose concentrations yeasts start alcoholic fermentation even under aerobic conditions (Crabtree effect) (Gancedo 1998) (see also Chap. 6).

In most natural habitats normal atmospheric conditions prevail, with high oxygen and low carbon dioxide concentrations. In an aquatic environment the dissolved oxygen in water may be a factor influencing metabolism and growth. The degree of saturation depends on the climate, in particular temperature, and other factors, e.g., the stirring of water.

Carbon dioxide is a metabolic product of various microorganisms, including alcoholic fermentation of yeasts. Under natural conditions, CO_2 accumulates rarely in inhibitory concentrations. Yeasts living in the intestinal tract of insects may be subjected to high CO_2 concentrations. More often, being easily soluble in water but depending on the pH, carbon dioxide forms bicarbonate ions, which inhibit growth of yeasts (Curran and Montville 1989; Dixon and Kell 1989).

8.3.4 Acidity and pH

In general, yeasts prefer a slightly acidic medium and have an optimum pH between 4.5 and 5.5 (Table 8.4). However, they tolerate a wide range of pH and grow readily at pH values between 3 and 10. Moreover, yeasts show a remarkable tolerance to pH, and several species can grow at strong acidic pH values as low as 1.5. The actual pH range of growth for a given species depends upon the kind of acid dissociating in the medium. Acetic acid is generally more inhibitory than lactic, propionic, citric and other organic as well as inorganic acids. As in the case of each environmental factor, the impact of pH on growth is influenced by other factors. For instance, at

Table 8.4 Effect of pH and a_w on the specific growth rates of *Zygosaccharomyces rouxii*

pH	Specific growth rate (μh^{-1}) at a_w values				
	0.957	0.923	0.904	0.880	0.843
2.5	0.21	0.15	0.12	0.08	0.05
3.5	0.33	0.23	0.18	0.14	0.08
4.5	0.34	0.24	0.19	0.14	0.09
5.5	0.30	0.21	0.17	0.12	0.07

a_w values adjusted with 300, 500, 600, 700 and 800 g l^{-1} final sugar concentrations obtained by mixing 30% glucose and 70% fructose; temperature 25°C. (Adapted from Membré et al. 1999)

low temperatures the minimum pH permitting growth is higher. In this respect, the interaction of yeasts with other organisms in microbial communities should be considered. Yeasts and lactic acid bacteria occur together in many natural habitats because they possess many common ecological requirements. The concentration of lactic acid produced by bacteria and the corresponding low pH could be inhibitory for fermentative yeasts; on the other hand, yeasts pursuing aerobic metabolism can utilize lactic acid oxidatively. A particular group of yeasts, genus *Dekkera* (anamorph *Brettanomyces*) is noted for a peculiar metabolic regulation that increases alcoholic fermentation under aerobic conditions and also results in the production of a high amount of acetic acid. In narrow habitats (e.g., in laboratory culture) the concentration of acetate may accumulate up to a level that kills the cells of the producing yeast.

8.3.5 Antimicrobial Compounds

In addition to acetate, lactate and others, some weak organic acids exert specific inhibitory effects towards yeasts, such as benzoic and sorbic acid. These are widely used preservatives in the food industry, but are rarely encountered in natural habitats. Plant and animal tissues, however, contain a variety of compounds that may inhibit yeast growth. Spices and herbs are particularly rich in phenolic and aromatic compounds, essential oils, volatile fatty acids, oleoresins and other constituents that have antifungal activity (Kim et al. 2004). Antibiotics are well known, and some of them are strong fungicides; these and other metabolites of microorganisms, among them yeast products, are discussed in the next paragraph.

The main product of alcoholic fermentation of yeasts, ethanol, exerts a toxic effect on various organisms (not to mention humans), among them yeasts and the producing strain itself. The ethanol tolerance of yeasts has been the subject of extensive studies especially concerning the production of wine (Fleet and Heard 1993). Natural residents on grapes such as *Hanseniaspora* (*Kloeckera*) and *Candida* species that start the spontaneous fermentation of grape juice are relatively sensitive to ethanol, and die out soon at concentrations around 5–8%. Most strains of the true wine yeast, *S. cerevisiae,* can tolerate 13–15% ethanol, some strains up to 18% or somewhat higher. As in the case with many inhibitors, tolerance to ethanol is affected by other environmental factors, in particular temperature and pH (Fleet 2003), and according to recent results some strains of *Candida stellata* and *Hanseniaspora* spp. may show resistance to ethanol comparable to that of *S. cerevisiae* (Table 8.5).

8.3.6 Interactions Between Environmental Factors

Under natural conditions the effect of no environmental factor manifests itself alone isolated from other factors. Many of these come into force together and simultaneously, mutually influencing the effect of the others; moreover, their interaction is dynamic and changes in time and space. The outcome of interaction is hardly predictable when several factors come into play. For practical applications, the food industry is very interested in the combination of physical and chemical treatments

Table 8.5 Minimum inhibitory concentration of ethanol on yeast growth

Species	Ethanol % (v/v)
Candida utilis	6.1–6.5
Kluyveromyces marxianus	8.6–9.4
Pichia anomala	10.0–10.9
Schizosaccharomyces pombe	11.8–12.5
Hanseniaspora valbyensis	11.9–13.2
Saccharomyces cerevisiae	11.3–13.7

Glucose-peptone broth, pH 5.6, 30°C. (Adapted from Antoce et al. 1997)

in order to better retain the safety, quality and stability of minimally processed foods (Tapia de Daza et al. 1996). Extensive experiments have been carried out and complex statistical methods as well as predictive mathematical models have been developed for their evaluation (Kalathenos et al. 1995; Table 8.6). The interaction between temperature, water activity, pH, salt, sugar and preservatives has been studied in various combinations with different types of food. Reference is made only to some examples relating to the growth inhibition of spoilage yeasts (Praphailong and Fleet 1997; Charoenchai et al. 1998; Betts et al. 2000; Battey et al. 2002). Although these studies have greatly increased our understanding of the interaction between environmental factors on the spoilage yeasts from the point of view of how to inhibit their activity, they bear little on what reactions and interactions really occur in natural habitats (Fleet 1998). Nevertheless, the concerted action of the very same

Table 8.6 Combinations of ethanol, fructose, pH and a_w on the doubling time of *Saccharomyces cerevisiae*

Ethanol (% v/v)	Fructose (% w/v)	pH	a_w	Doubling time (h)
0	2.5	3.5	0.991	1.69
4	2.5	5.5	0.985	2.19
8	2.5	5.5	0.973	2.34
12	2.0	3.2	0.962	8.49
0	4.0	2.5	0.996	2.49
0	16.0	2.5	0.984	2.68
0	32.0	2.5	0.969	2.93
0	50.0	2.5	0.952	6.73
0	50.0	5.5	0.952	5.72
0	50.0	8.0	0.952	5.71
3	8.0	4.0	0.983	1.88
3	8.0	7.0	0.983	2.64
3	40.0	4.0	0.953	4.29
3	40.0	7.0	0.953	6.60

Selected values from a multifactorial response surface experiment conducted at 25°C in Bacto yeast nitrogen base broth adjusted to various treatment combinations. (Adapted from Kalathenos et al. 1995)

and similar factors plays a prominent role in determining the biodiversity and life of yeast communities in natural habitats as well, but they have been studied and explored less intensively until now. For example, it has been shown that the composition of yeast communities associated with sugarcane is under the influence of temperature, pH and the concentration of sugars as they change with various parts of the plant and the time passed since plantation (de Azeredo et al. 1998). Specific yeast communities have also been described for a great variety of plant microhabitats (fruits, flowers, leaves, tree saps, decaying tissues, etc; Spencer et al. 1992; Rosa et al. 1995; Santos et al. 1996), and these studies revealed habitat specialization being influenced by a variety of the respective biotic and abiotic factors. From these studies it also emerged that a biotic factor, the insect vectors, is of particular significance in shaping yeast communities. This will be discussed in more detail in the next section.

8.4 Biotic Factors

In natural and artificial ecosystems yeasts are always subjected to interactions with other organisms. These effects can be mutual or unidirectional, neutral, synergistic or antagonistic, and involve interactions of yeasts with themselves, bacteria, filamentous fungi and higher organisms (Lachance and Starmer 1998).

8.4.1 Yeasts and Yeasts

Abundant literature deals with the products of yeasts lethal to other yeasts, called killer toxins. These polypeptides are genetically determined on plasmids or chromosomes, and about a dozen types of them have been described (Magliani et al. 1997; Marquina et al. 2002; Schmitt and Breinig 2002). Growth inhibitory or lethal action of killer toxins impacts mainly on yeasts; earlier claims to extend it to bacteria and eukaryotes other than yeasts have been verified with certain plant pathogenic and wood decaying fungi (Walker et al. 1995). A killer positive property is widespread among yeasts. Strains of the producing species are resistant, while other species can be sensitive or neutral to the toxin. In natural communities 9–27% of species were shown to produce toxin, in some cases, e.g., in fermenting grape juice, toxigenic strains reached 50–75%, whereas the ratio of sensitive yeasts varied between 10–40% (Starmer et al. 1992; Vagnoli et al. 1993). Indigenous species are less sensitive than members of different communities; within the same habitat 3–10% of killer sensitive species occur but 20–40% among yeasts from different localities and habitats (Abranches et al. 1997; Trindade et al. 2002). The ecological role of killer yeasts in natural communities can be attributed to the competition with sensitive species leading to their exclusion from sources of nutrients (Starmer et al. 1987) (see also Chap. 10).

Killer yeasts often play a role in the competitive interaction between yeast species associated with fruits. Initially, ripening fruits are always colonized by apiculate yeasts belonging to the genera *Hanseniaspora* and *Kloeckera*. These fermentative species have a narrow assimilative profile and are replaced, after a few days, by yeasts utilizing a broader range of substrates and/or fermenting more strongly (Abranches at al. 2001). Killer strains may also facilitate the dominance of wine

yeasts during the spontaneous fermentation of grape juice. In other fermentation this may not be the case (Lachance 1995), and the succession of yeast species in the course of fermentation is governed by the competition for nutrients and the tolerance to ethanol. The successional development in grape juice of *Hanseniaspora* (*Kloeckera*), *Metschnikowia*, *Pichia* and *Candida* species and finally the true wine yeast *S. cerevisiae* has been thoroughly demonstrated and generally attributed to the degree of ethanol tolerance of the respective yeasts (Fleet and Heard 1993). In commercial wine production, however, the spontaneous course of events is disturbed by human intervention. This takes place by the treatment of grape juice with sulfur dioxide and the inoculation of selected wine yeast starter in order to control fermentation (Fugelsang 1997).

Predation among yeasts has been considered as a unique and rare phenomenon, but recent findings show that it may be a widespread property of filamentous species of *Saccharomycopsis* and related yeasts (Lachance and Pang 1997). Yeast and moulds may serve for prey and they are attacked by haustoria-like outgrowths that penetrate and kill other cells. The ecological impact of predacious yeasts remains to be assessed. As a peculiar property, predation has also been demonstrated in several yeast species. These belong mostly to *Saccharomycopsis* and related hyphal genera (Lachance and Pang 1997). Predatory species were found to be sulfur auxotrophs, and it is believed that the ecological significance of predation lies in obtaining nutrients.

8.4.2 Yeasts and Molds

Yeasts may rely on nutrients produced by molds, e.g., take up simple sugars liberated by the polysaccharide-splitting enzymes of molds. Recently, strict dependence of a yeast, *Debaryomyces mycophilus*, was shown on the iron-containing siderophore, a metabolic product of some common soil fungi such as *Cladosporium cladosporoides*, *Aspergillus alliaceus*, and *Penicillium* spp. (Than et al. 2002).

Some yeast species, in particular *Pichia guilliermondii* and *P. anomala*, inhibit the growth of certain moulds attacking fruits during postharvest storage (Wisniewski and Wilson 1992; Suzzi et al. 1995). Conversely, a large number of mycelial fungi can attack yeasts. Parasitism is commoner among the basidiomycetes than in other fungal groups; nearly 50% of the basidiomycete fungi tested positive (Hutchinson and Barron 1996). Mycoparasitic fungi utilize yeasts as a nutrient source either by lysing yeast cells or by penetrating the cell wall, similar to the way they attack plants and nematodes.

8.4.3 Yeasts and Bacteria

Reference has been made (Sect. 8.3.4) to the association of yeasts with lactic acid bacteria in a number of spontaneous and controlled fermentations. Further examples for mutualistic and synergistic interactions are given here. In kefyr grains their interaction is synergistic; the vitamins provided by yeasts and the lactate produced by bacteria are mutually utilized (Leroi and Courcoux 1996). A similar association develops in sour dough between the maltose fermenting lactobacilli and glucose

fermenting yeasts (Gobetti et al. 1994). In the fermentation of sauerkrauts and pickles, both fermentative and oxidative yeasts live together with lactic acid bacteria; the yeasts often form films on the surface of salt brine where the aerobic decomposition of lactic acid may open a way to spoilage (Buckenhüskes 1997). In red wine, the malolactic fermentation by *Oenococcus* (*Leuconostoc*) *oenos* is facilitated by vitamins and amino acids produced by yeasts (Alexandre et al. 2004). In oriental fermentation of rice, soy, vegetables and even fishes, mixed communities of molds, yeasts, lactic acid and other bacteria and bacilli participate with manifold interactions among them (Nout 2003). In the ripening of sausages, cheeses and other dairy products, yeasts develop interactive associations with bacteria and molds alike (Viljoen 2001).

Lactic acid bacteria are known for the production of various bacteriocins, the direct effect of which on yeasts is not verified (Magnusson et al. 2003). Hydrogen peroxide, often liberated by catalase-negative lactic acid bacteria, may have a lethal effect on yeasts. In turn, disregarding the inhibitory effect of ethanol, no specific compounds are produced by yeasts that are antagonistic to bacteria.

Special and strong metabolic interactions, mutualistic relations and competition take place in biofilms developing on solid surfaces where mixed communities of cells get in close spatial contact. Biofilms are formed mostly by bacteria, with yeasts often contributing to them. Yeasts producing extracellular slime and capsules play an important role in the adhesion to sites. A complex structure of biofilms occurs on the mucosal membranes covering the gastrointestinal tract and other body cavities of macroscopic organisms and they have become the subject of intensive studies from the medical point of view (Costerson and Lewandowski 1997; Watnick and Kolter 2000; El-Azizi et al. 2004). On soil particles, living and inanimate underwater objects and other natural habitat biofilms are common life forms of microorganisms. The formation of biofilms facilitates the colonization of habitats and offers protection to stress reaction caused by the changing physical and chemical conditions of the environment.

8.4.4 Yeasts, Plants and Animals

Yeasts are generally saprotrophic organisms, but a few species are parasitic on plants or pathogenic to animals. Typical examples are, respectively, *Nematospora coryli* and *Metschnikowia bicuspidata*. For humans, *Cryptococcus neoformans* and *Candida albicans* are the most threatening pathogenic yeasts, although in recent years the number of opportunistic pathogen species has greatly increased (*C. glabrata*, *C. tropicalis*, *C. krusei* and others). Adaptive evolution of tolerance to antifungal agents may be responsible for the emergence of these yeasts as epidemiological agents (Ahearn 1998). Pathogenicity of yeasts to warm-blooded animals is beyond the scope of the present chapter. Other manifold relations between yeasts and invertebrates will not be discussed either; this topic is covered extensively by Phaff and Starmer (1987) and Ganter in this Volume (see Chap. 14). However, one aspect of this interrelation is of prominent significance from an ecological point of view, i.e., the role of insect vectors in transmitting and distributing yeasts in different habitats.

Insects serve not only for vectors but also feed on yeasts. In both ways they contribute actively in structuring yeast communities (Morais et al. 1995; Lachance et al.

2001). Association between yeasts, bees and flies, in particular *Drosophila* species, may be highly specialized and results in coadaptation of the partners (Starmer and Fogelman 1986; Rosa et al. 2003). Extensive studies carried out by Phaff's group on yeasts associated with cacti also revealed the intimate relations with drosophilas and other insects visiting the plant (Phaff and Starmer 1987; Starmer et al. 1991). Similar close relations have been detected between yeasts, flowers and insects that can be considered as natural ecosystems (Lachance et al. 2001, 2003).

8.5 Effect of Environmental Factors on Populations

Much of our knowledge of the effect of environmental factors on yeasts comes from observations obtained with populations. In natural conditions communities are formed from mixed populations of species, whereas the laboratory pure cultures represent in the best-case clones of a single species. Investigations provide average responses of all cells in a population but no information on individual cells or subpopulations. It has become possible only recently, with the development of the technique of fluorescent flow cytometry, to sort individual cells and get information on the heterogeneity of the population (Attfield et al. 2001). Long before, it was recognized that heterogeneity is a natural attribute of a cell population. For instance, studies on lethal stress factors often resulted in survival curves with a shoulder and/or a tail, revealing the different resistance among cells in a culture. S-shaped survival curves are obtained frequently after heat treatment or irradiation. Heterogeneity may be due to mutation and other genetic changes, and this forms the basis of natural selection, adaptation and evolution (Zeyl 2000). However, heterogeneity also occurs in isogenic populations and manifests itself in differential sensitivity to stress conditions which can be fundamental to the fitness and persistence of an organism in its habitat (Booth 2002). The reasons for phenotypic heterogeneity are manifold. Cell-to-cell variations come from differences in cell cycle stages, growth phases and growth rate, the age of cells and other properties that show a stochastic distribution (Sumner and Avery 2002). Yeast cells have a definite life span which lasts until the cessation of cell division. The age of a cell is marked by the number of bud scars (in the case of *S. cerevisiae* the average is about 20). Aging is genetically determined, and with aging changes take place in physiological processes, metabolic regulation and stress responses (Sinclair et al. 1998; Jazwinski 1999). The practical significance of heterogeneity among cells is the ability of a fraction of the population to survive exposure to stress factors that kill the majority of the cells. When the survivors outgrow into a new population, it will have the same degree of variation as the original culture.

In addition to phenotypic heterogeneity, yeast populations are capable of developing an adaptive response to stress factors. Starvation is one of the most common stresses encountered in natural habitats. Starving yeast cells enter into a stationary phase and maintain viability until they resume growth again (Werner-Wasburne et al. 1993). Sublethal stress imposed by heat, osmotic shock, toxic chemicals or other inhibitors confers yeasts with resistance to withstand subsequent higher doses of stress. In recent years several studies have focused on the elucidation of the mechanism of adaptation. Induction of adaptive responses include the synthesis of

heat-shock proteins, activation of plasma membrane H^+-ATPase and the accumulation of trehalose conferring cells to maintain intracellular pH homeostasis and protecting membrane integrity and functional proteins (Mager and Ferreira 1993; Ribeiro et al. 1999; Piper et al. 2001; Cabral et al. 2004). In-depth studies have revealed a common pattern of stress sensor system and signal transduction pathways which activate the genetic transcription of many genes in response to environmental stress (Bauer and Pretorius 2000; Rossignol et al. 2003; Garay-Arroyo et al. 2004; Zuzuarregui and del Olmo 2004). It is beyond the scope of this chapter to go into more detail, and we conclude that physiological adaptation of yeast cells is crucial to maintain viability and is essential for the cells to survive stressful environmental transitions (see also Chap. 9 and Chap. 15).

Despite the armory of protective responses to stress factors, yeast cells suffer sublethal injury as a result of exposure to adverse conditions. Plasma membrane, enzyme proteins, DNA and gene transcription may be damaged rendering cells to lose the ability to grow and retain viability. Injured cells may be able to repair damage and resume growth under appropriate conditions (Fleet and Mian 1998). The viable but nonculturable state of cells has been the subject of numerous studies. It may draw a false picture in the assessment of pathogens after clinical treatment or food preservation, and also of the real composition of microbial communities occurring in a natural habitat. If selective media are used without resuscitation, injured cells would not be able to form colonies; hence, plate counts less then real are obtained (Beuchat 1984).

8.6 Concluding Remarks

Major advances have been made in understanding the ecology of yeasts in various habitats and ecosystems. However, the majority of yeast ecological studies focused on the identification of species, often determining the most frequent isolates only. Moreover, much of our knowledge is derived from laboratory studies on isolated strains about how the various growth factors regulate growth and survival. Despite enormous progress, much has to be learned on the responses to environmental stresses and interactions of individual cells, their population and mixed communities of different species occurring in most natural habitats.

According to an excellent analysis by Fleet (1998), to understand the growth and activities of yeasts in their habitats further information and data are required on:

- The physiological properties of species that permit their growth and activity in the ecosystem
- Quantitative data on the limits for growth of environmental factors regarding populations and communities
- The specific interactions between different strains and species of yeasts and between other microorganisms occupying a common habitat
- The dynamics and changing of activity, growth and survival of species and populations in time and space in response to the changing environment

Rarely does a single species occur in any habitat, rather populations of different species, yeasts and other microorganisms are assembled in communities; therefore,

the activity and growth of any one strain or species is influenced by the presence of other microorganisms. Little is known about how cells sense and communicate with each other and the mechanism regulating population growth.

In colonizing the habitat yeasts grow in spatial heterogeneity and form microcolonies and biofilms, also with other microorganisms. In the complex structure of the biofilm spatially organized mixed populations develop where metabolic interactions and interspecies competition and cooperation manifest themselves. Almost nothing is known about yeast biofilms, apart from the special cases of dental plaques and human body cavities (El-Azizi et al. 2004).

Species communities in an ecosystem are not static in time. The sequential development of strains and species lead to a continuously changing association both qualitatively and quantitatively in response to the changing environment. Moreover, the cells are simultaneously exposed to a combination of stress factors, and their effect may be additive or synergistic. The changing microenvironment and interactions between biotic and abiotic factors will determine the actual niche of an organism in the habitat.

Recent advances in the techniques of molecular biology will allow the assessment of the composition of species in mixed populations and their localization in space and the change with time. Culture-independent methods (e.g., the direct epifluorescent filter technique, DEFT, flow cytometry, fluorescent in situ hybridization, FISH) as well as refined PCR-based methods (denaturing gradient gel electrophoresis, DGGE, thermal gradient gel electrophoresis, TGGE, amplified fragment length polymorphism, AFLP) have recently been applied to study bacterial biodiversity in natural ecosystems (Schloter et al. 2000; Giraffa and Neviani 2001; Giraffa 2004), but their use for investigating yeasts has just started (Cocolin et al. 2000; Brul et al. 2002; Zuzuarregui and del Olmo 2004).

In microbial populations a large genetic variation is always present, which is the prerequisite for biological evolution. New molecular analytical approaches will allow us to understand the underlying genetic mechanisms and the impact of the microenvironment on the diversification of genes and their expression (Gibson 2002; Rodriguez-Valera 2004). The increasing number of completely sequenced yeast species ranks these organisms in the frontiers of research on environmental genomics (Zeyl 2000, 2004; Kellis et al. 2003; Querol et al. 2003). The interplay between genetic microdiversity and the influences of the microenvironment guarantees the creation, existence and maintenance of rich biodiversity in populations and the development of new evolutionary lines.

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Yeast Responses to Stresses

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9.1 Introduction

In their natural habitat, yeasts are continuously exposed to a myriad of changes in environmental conditions. These changes may occur suddenly or may take place over an extended period of time and they can constitute a single specific change or a combination of changes. It is clear that extreme changes, e.g. in physical and chemical conditions, will always represent a stress to the cells and will require specific response mechanisms in order to protect and adapt the cells to the new condition. The same is true for starvation and any dramatic change in the nutrient supply. Such conditions are generally referred to as stress conditions and the adaptation mechanisms they evoke as stress responses (Hohmann and Mager 2003). On the other hand, in the case of moderate changes in the growth conditions the amount of stress experienced by the cells is not always clear. The difference between response to stress and adaptation to a new growth condition is not clear-cut and probably in reality also represents a gradual transition. Yeast cells must always be on guard to protect themselves in order to continue to survive, grow and multiply. For example, yeasts found on the surface of sugar-containing plant material can expect to be exposed to high sugar concentrations under dry conditions that might change rapidly with rainfall. Similarly on the same plant material, fermentation of the sugar can result in an elevated ethanol concentration and a reduced pH that also impose stress on the yeast. Once the nutrients are exhausted, a prolonged nutrient starvation period usually follows. In order to survive and multiply under dramatically variable conditions, microorganisms like yeasts have developed a complex set of sensing and signalling mechanisms that enable them to rapidly adapt their physiology to the new conditions.

9.2 Types of Stress

A large number of stress-inducing conditions have been described and may be either physical or chemical or biological in nature. They include changes in temperature, osmotic pressure, pH and concentration of water, ions and solutes, as well as exposure to extremes of radiation, pressure and toxic chemicals, to oxidative conditions and to nutrient starvation. Stress is also imposed on pathogenic yeasts by the host defence mechanisms. For example, the high levels of reactive oxygen species produced by neutrophil cells called an oxidative burst are an important host defence mechanism used to eliminate pathogenic yeasts (Moye-Rowley 2003).

Some stress effects are implicated in more than one type of stress. For example, NaCl elicits both an ionic and an osmotic stress. The yeast responds physiologically in a similar way to an ionic and a nonionic solute such as sugar by initially shrinking, excluding the extracellular osmotica followed by intracellular accumulation of compatible solutes to restore cell volume and turgor. However, with NaCl additional stress is placed on the yeast by the intracellular accumulation of Na^+ that the cell must export in order to prevent general cellular damage (Wadskog and Adler 2003).

In nature yeasts might be exposed to various types of stress simultaneously or sequentially. *Saccharomyces cerevisiae* typically grows under fermentative conditions where high sugar and ethanol concentrations occur and the yeast needs to tolerate stress imposed by osmotic pressure, water stress and ethanol toxicity. During a wine fermentation the yeast is initially exposed to a sugar concentration often greater than 250 g/l that results in an osmotic stress response. As the sugar is fermented to ethanol, the osmotic stress is reduced but simultaneously ethanol increases, imposing water and ethanol stress on the yeast. In order to cope with the differing stresses, the yeast expresses a sequence of different stress response genes (Zuzuarregui and del Olmo 2004). Respirative yeasts, on the other hand, are exposed to reactive oxygen species as by-products of their cellular metabolism that impose an oxidative stress which can occur in combination with other stresses such as nutrient starvation in the stationary growth phase.

Yeasts that commonly proliferate in extreme environments would be expected to adapt more easily to environmental stress when compared with less tolerant yeasts. These yeasts have been studied poorly or not at all at the molecular level and while there are response mechanisms that appear to be conserved in all yeasts investigated to date, it appears that some response mechanisms might be specific to a yeast species and related to the particular habitat where the yeast is found. Investigation of the genomic makeup of the highly osmotolerant yeast *Zygosaccharomyces rouxii* has revealed that many of the genes involved in salt stress response in the less osmotolerant *S. cerevisiae* are also present in this yeast (Iwaki et al. 1998, 1999). However, the regulation of these genes might be different: in *Z. rouxii* induction of glycerol 3-phosphate dehydrogenase does not seem to respond to osmotic stress as observed in *S. cerevisiae* (van Zyl et al. 1991; Albertyn et al. 1994b; Iwaki et al. 2001). *Z. rouxii* also possesses a NaCl-induced glycerol uptake system that has not yet been reported to be present in *S. cerevisiae* (van Zyl et al. 1990).

In nature many types of stress would be expected to be imposed over an extended period, whereas in the laboratory because of limitations of available time and facili-

ties, most stress experiments are conducted over a much shorter period. *S. cerevisiae* has been found to adapt differently to short-term and long-term stress. For example, when hyperosmotic conditions are imposed on *S. cerevisiae,* growth ceases, the yeast rapidly loses water and plasmolysis occurs. This is followed by a recovery phase where compatible solutes are produced and accumulated until osmotic homeostasis is achieved to enable cell proliferation to recommence. The time required to recover is dependent upon the degree of osmotic stress imposed. The greater the stress imposed, the longer the time that the yeast requires to recover (Albertyn et al. 1994a).

9.3 General and Specific Stress Responses

An overview of the best studied examples of stress responses in *S. cerevisiae* is shown in Fig. 9.1.

9.3.1 General Stress Response

It has been discovered that yeast cells respond to a variety of stress conditions with a similar response at the transcriptional level and that this response is mediated

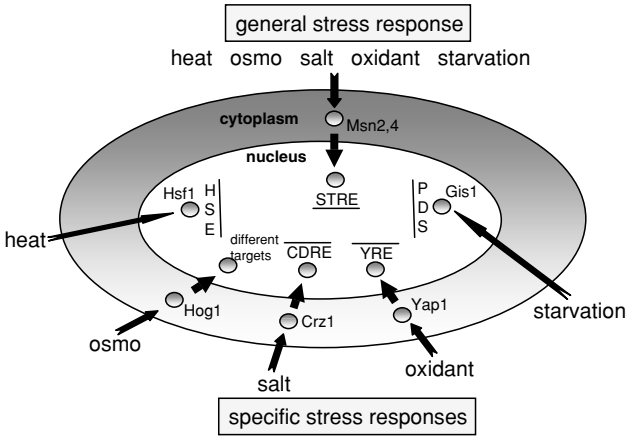

Fig. 9.1. Best-established stress response mechanisms in *Saccharomyces cerevisiae*. The general stress response is triggered by a variety of stressful conditions. It involves transfer of the Msn2 and Msn4 transcription factors into the nucleus, where they induce transcription by binding to the stress-response elements (*STRE*) in the promoters of target genes. In the specific stress responses heat-shock factor Hsf1 activates heat-shock-induced transcription through the heat-shock elements (*HSE*), whereas in the osmostress response the Hog1 protein migrates to the nucleus, where it interacts with several transcription factors to activate transcription of osmostress-induced genes. Salt stress specifically triggers entry of the Crz1 transcription factor into the nucleus, where it interacts with the calcineurin-dependent-response element (*CDRE*) in target gene promoters. Oxidative stress specifically causes accumulation of the Yap1 transcription factor in the nucleus, where it induces transcription of antioxidant genes through interaction with Yap1-response elements (*YRE*). Nutrient starvation activates the Gis1 transcription factor, which induces stationary-phase genes through interaction with the postdiauxic shift element (*PDS*)

by the widely distributed stress-response element (STRE) promoter (Marchler et al. 1993; Ruis and Schuller 1995). Recent work using genome-wide gene expression analysis has shown that about 9–14% of all genes in *S. cerevisiae* and *Schizosaccharomyces pombe* are involved in this general stress response (Gasch et al. 2000; Chen et al. 2003). Major targets of the general stress response in *S. cerevisiae* are the transcription factors Msn2 and Msn4, which bind to the STREs in the promoters of about 200 target genes, and are required for induction of a large number of stress-induced genes (Martinez-Pastor et al. 1996). Other genes depend on other transcription factors and many genes are also repressed as part of the general stress response. Genes induced are involved in carbohydrate metabolism, metabolite transport, fatty acid metabolism, maintenance of the cellular redox potential, detoxification of reactive oxygen species, autophagy, protein folding and degradation, cell wall modification, DNA-damage repair, secretion, vacuolar and mitochondrial functions, intracellular signalling, and a relatively large number of genes with unknown function. Genes repressed are mainly involved in protein synthesis (e.g. ribosomal protein genes) and other growth-related functions (Gasch et al. 2000). The transcriptional response to stress conditions is often transient with the largest change occurring rapidly after the stress (Rep et al. 1999). This allows rapid adaptation of the cells to the new environmental conditions. Initially it was thought that the general stress response might be triggered by a common effect of the different stress treatments by which it is activated, such as protein denaturation, change in intracellular pH, change in cyclic AMP level, etc. However, it appears that at least part of the integration of the response to different types of stress conditions might also occur at the level of the targets themselves, e.g. through different specific STREs occurring in the promoter of the same target gene. A typical consequence of the general stress response is the occurrence of cross-protection: submission of the cells to one stress condition renders them not only more resistant to the same stress condition but also to other stress conditions (Lewis et al. 1995).

Another pathway found in *S. cerevisiae* that is activated by different stress conditions, such as heat shock, low osmolarity, cell wall perturbing agents, but also internal signals generated in morphogenetic processes, is the cell integrity pathway. It appears to adapt the cell wall assembly to external stress conditions as well as to growth and developmental processes that require cell wall remodelling (Gustin et al. 1998). The Wsc1 and Mid2 plasma membrane proteins are considered to be sensors for cell wall strength. They control activity of protein kinase C, Pkc1, which in turn controls the expression of cell wall biosynthesis genes through an MAP kinase cascade (Levin et al. 1994; Heinisch et al. 1999).

In *S. pombe* the Sty1 MAPK pathway responds to a variety of stress conditions and also plays a role in cell cycle control and developmental switches (Degols et al. 1996). Although Sty1 is the homologue of Hog1 in *S. cerevisiae*, the latter appears to be more specifically involved in the response to osmostress (see further).

9.3.2 Response to Heat Stress

Apart from the general stress response (Wieser et al. 1991) heat stress is known to induce a specific response which is commonly known as the heat shock response and apparently occurs in all organisms (Lindquist 1986). In eukaryotic cells the heat

shock response is mediated by the heat shock transcription factor, which in yeast is encoded by the *HSF1* gene (Sorger 1991). Upon activation by heat shock, the Hsf1 transcription factor binds to heat-shock elements (HSE) in the promoters of target genes (Morimoto 1993). Many of the Hsf1 target genes are chaperones which often display a basal expression level that is also important for normal cellular functioning by stabilizing and refolding protein-folding intermediates or facilitating protein degradation (Morimoto et al. 1997). This explains why *HSF1* itself is also an essential gene under normal growth conditions. Interestingly, several heat shock genes contain both HSE and STRE in their promoter and these are used redundantly upon heat shock induction (Treger et al. 1998).

9.3.3 Response to Osmostress

The main pathway triggered by osmostress in *S. cerevisiae* is the HOG or 'high-osmolarity-glycerol' response pathway (Hohmann 2002). It causes rapid accumulation of the compatible solute glycerol and this is brought about by several distinct mechanisms. First, the *GPD1* gene encoding the main rate-limiting enzyme of glycerol biosynthesis, glycerol-3-phosphate dehydrogenase, is rapidly induced (Albertyn et al. 1994b). Second, the flux through glycolysis is stimulated at the level of phosphofructokinase which is apparently required for 'overflow' of glycolysis into elevated glycerol production (Dihazi et al. 2004). Third, the accumulation and release of glycerol from the yeast cells is favoured by the rapid closure and opening of the Fps1 glycerol channel in the plasma membrane in response to hyper- and hypoosmostress (Luyten et al. 1995). Osmostress probably affects the Fps1 channel directly. The stimulation of glycerol biosynthesis is triggered by a complex signalling pathway, starting with the putative osmosensors Sln1 and Sho1 in the plasma membrane which each stimulate a pathway leading to activation of the MAP kinase Pbs2, which in turn phosphorylates the MAP kinase Hog1. This leads to its accumulation in the nucleus and subsequent activation of several transcription factors, such as Sko1 and Hot1, but also the Msn2 and Msn4 factors (Hohmann 2002). The HOG signalling pathway also affects other targets besides glycerol production, such as cytoskeletal organization and plasma membrane composition.

Several components of the glycerol synthesis and HOG pathways have been identified in other yeast species. The genomes of both *S. pombe* and *Z. rouxii* contain a *GPD1* homologue but the expression of the gene in *Z. rouxii* in response to osmotic stress differs from that in *S. cerevisiae* and *S. pombe* (Ohmiya et al. 1995; Iwaki et al. 2001). A number of *FPS1* homologues have been found in other yeasts but surprisingly when the homologue was deleted in *S. pombe,* the yeast still released glycerol under hypoosmostress, suggesting that other mechanisms might regulate glycerol in this yeast (Kayingo et al. 2004). *HOG1* homologues are found in *Candida albicans* and *Z. rouxii* and their deletion also results in reduced glycerol production and osmosensitivity (San Jose et al. 1996; Alonso-Monge et al. 1999; Iwaki et al. 1999).

9.3.4 Response to Salt Stress

Addition of high salt concentrations not only evokes osmotic stress but also ionic stress. The plasma membrane transport systems such as the highly abundant

H^+-ATPases, sodium transporters and Na^+/H^+ antiporters of *S. cerevisiae* are prominent cell components involved in excluding NaCl from the cell, thereby maintaining a high intracellular K^+-to-Na^+ ratio and ion homeostasis (Serrano et al. 1986; Garciadeblas et al. 1993; Banuelos et al. 1998). Expression of Ena1, the major plasma membrane Na^+ efflux pump in yeast cells, is controlled at the transcriptional level by a complex network of pathways, including the HOG, PKA and Ca^{2+}/calcineurin pathways (Serrano and Rodriguez-Navarro 2001). Na^+ stress stimulates through the Ca^{2+}/calcineurin pathway entry of the Crz1 transcription factor into the nucleus, where it induces *ENA1* expression through the calcineurin-dependent-response element (CDRE) in the promoter (Mendizabal et al. 2001). The role of Crz1 in NaCl tolerance is supported by the failure of the *crz1Δ* mutant to induce *ENA1* expression and by their hypersensitivity to NaCl stress (Mendizabal et al. 1998). Accumulation of Na^+ in the vacuole is a second protection mechanism used to maintain a low cytosolic Na^+ concentration upon NaCl stress (Nass et al. 1997). On the other hand, comparison of transcriptional responses in *S. cerevisiae* with isoosmotic sorbitol and NaCl have shown similar expression patterns (Rep et al. 2000; Causton et al. 2001). Only ten additional genes were induced significantly more strongly by 0.7 M NaCl than an isoosmotic concentration of sorbitol (Rep et al. 2000).

9.3.5 Other Forms of Water Stress

Besides osmotic water stress also nonosmotic forms of water stress have been described (Hallsworth 1998). A net loss of water from the cell due to high levels of extracellular solutes unable to freely penetrate the cell membrane that reduce cell turgor has been described as an osmotic form of water stress (Hallsworth 1998). On the other hand, chaotropic compounds such as ethanol that diffuse through the plasma membrane impose a nonosmotic water stress. Ethanol reduces the intracellular water activity by decreasing the strength of hydrogen bonding and perturbing the structure and function of hydrated macromolecules, including nucleic acids, proteins and lipids. As is found with osmotic stress, compatible solutes are able to protect cell metabolism against nonosmotic water stress (Hallsworth 1998). Since ethanol is formed in high concentrations in natural fermentations of *S. cerevisiae* it is of paramount importance for the ecology of this yeast species. Apparently, the build-up of the high ethanol concentration combined with the high intrinsic ethanol tolerance of *S. cerevisiae* cells is the major factor that inhibits the growth of competing microorganisms and makes *S. cerevisiae* the dominating microorganism near the end of natural fermentations of sugar-rich media (Bauer and Pretorius 2000).

9.3.6 Response to Oxidative Stress

A cellular imbalance between the level of oxidants and the capacity of antioxidants and repair systems will lead to oxidative stress. Accumulation of reactive oxygen species, including different oxidation states of dioxygen (O_2) and singlet oxygen, superoxide anion (O_2^-), hydrogen peroxide (H_2O_2) and the highly reactive

hydroxyl radical (OH^*) at toxic levels have been implicated in oxidative stress (Halliwell and Gutteridge 1999). These reactive oxygen species are produced during respiration when O_2 is incompletely reduced and during fatty acid metabolism in the peroxisome (Toledano et al. 2003). They attack molecules containing aromatic rings, such as purine or pyrimidine bases, lipids, metal-containing proteins or amino acids such as cysteine and methionine. The concentration required to elicit an oxidative stress varies. Peroxides such as lipid hydroperoxides and H_2O_2 in the respective toxic concentrations of 0.05 and 5 mM have been used to investigate the oxidative stress response in *S. cerevisiae*, whereas menadione is toxic in the 100 μM range to *S. cerevisiae* (Toledano et al. 2003). H_2O_2 has been used extensively to generate oxidative stress in yeasts. The molecular response mechanisms to these various oxidants have a number of common features in *S. cerevisiae*, whereas other components of the response mechanism differ between the oxidants, suggesting the presence of both common and oxidant-specific defence mechanisms (Moye-Rowley 2003). Oxidative stress causes massive induction of the genes encoding the defence systems, as well as additional genes encoding heat shock proteins, trehalose biosynthesis enzymes and enzymes of the pentose phosphate pathway which are important for the generation of reducing power (Gasch et al. 2000). The major transcription factor in yeast specifically activated by oxidants is Yap1. Oxidative stress causes Yap1 to accumulate in the nucleus and this is due to inhibition of its rapid nuclear export rather than to stimulation of its import (Kuge et al. 1997). Yap1 binds to its recognition element, YRE, present in the promoters of the antioxidant biosynthesis genes (Kuge and Jones 1994; Wu and Moye-Rowley 1994). Although Yap1 is thought to be a redox sensor itself, it is still not known whether peroxides directly oxidize Yap1. Moreover, different oxidants appear to affect Yap1 in different ways (Kuge et al. 2001). Yap1 acts in association with Skn7 (Lee et al. 1999). However, the action of Skn7 is not specific to the oxidative stress response. In addition to Yap1, Skn7 also associates with other transcription factors that regulate stress and metabolic responses (Toledano et al. 2003).

9.4 Stress-Protection Mechanisms

In yeast, stress-induced cellular injury is to a certain extent specific for one type of stress, but can also be common for different types of stress. Different stress conditions seem to cause injury through common mechanisms, which renders it plausible that the mechanisms of protection and repair are likewise partially overlapping (Davidson et al. 1996; Beck et al. 2000; Mager et al. 2000; Pahlman et al. 2001; Rep et al. 2001; Tanghe et al. 2003). The major yeast stress-protection mechanisms recognized so far, i.e. trehalose accumulation, synthesis of molecular chaperones, synthesis of antioxidant proteins, accumulation of compatible solutes, synthesis of hydrophylins and adaptation of plasma membrane composition, have indeed been shown to protect the yeast cell against various stress types. On the other hand, expression of aquaporins has been identified as a mechanism specifically protecting against freeze stress and not against other stress conditions (Tanghe et al. 2003).

9.4.1 Trehalose Accumulation

The nonreducing disaccharide trehalose (α-D-glucopyranosyl α-D-glucopyranoside) is widespread in nature. For a long time it had been assigned a role only as a storage compound, but more recently its unique stress-protection properties have been revealed (reviewed in François and Parrou 2001; Elbein et al. 2003). In baker's yeast, for instance, improved stress tolerance is often correlated with higher trehalose levels as a function of the growth condition (Hottiger et al. 1987, 1994; de Virgilio et al. 1994; van Dijck et al. 1995). Further evidence for a stress-protective role of trehalose has been provided with yeast strains genetically engineered in trehalose metabolism, revealing a clear link between trehalose levels and tolerance to different stress types, including freeze, heat, dehydration, ethanol, osmotic and oxidative stress (Attfield et al. 1992; Eleutherio et al. 1993; Kim et al. 1996; Hounsa et al. 1998; Shima et al. 1999; Fillinger et al. 2001; Alvarez-Peral et al. 2002). This observation has also been extended to other yeast species, such as *S. pombe* (Ribeiro et al. 1997), *C. albicans* (Arguelles 1997), *Z. rouxii* (Kwon et al. 2003) and *Hansenula polymorpha* (Reinders et al. 1999).

How trehalose provides protection to cells is not entirely clear. Both in vitro and in vivo evidence has been obtained for a dual mechanism: preservation of the intracellular water structure and stabilization of membranes and proteins by replacing water (Sano et al. 1999). In line with the stress-protective effect of both endogenous (Hirasawa et al. 2001) as well as exogenous trehalose (Diniz-Mendez et al. 1999), it has been proposed that the protection exerted by trehalose requires its presence at both sides of the plasma membrane (de Araujo 1996).

9.4.2 Synthesis of Molecular Chaperones

Denaturation of proteins is a major injury factor following stress and, not surprisingly, the action of molecular chaperones is a major stress tolerance mechanism in yeast cells. Molecular chaperone proteins stabilize macromolecules to prevent them from aggregating. They recognize, selectively bind and reassemble proteins with an aberrant structure (Lindquist and Craig 1988; Buchner 1996). They might help to maintain a low degree of protein denaturation and reassemble damaged proteins during and after the imposition of the stress. Historically, molecular chaperones were discovered to be heat-shock-induced proteins but they are also involved in resistance to various other types of stress and also play an important role in many basic cellular functions where protein folding is involved.

There is increasing evidence that trehalose and molecular chaperones act synergistically as stress protectants (reviewed in François and Parrou 2001). During heat stress, trehalose has been shown to suppress the aggregation of denatured proteins in yeast, maintaining them in a partially folded state from which they can be activated by molecular chaperones (Elliott et al. 1996; Singer and Lindquist 1998a). However, the refolding activity of molecular chaperones itself is inhibited by trehalose. Hence, the rapid mobilization of trehalose upon removal of the stress condition is essential for the concerted action of trehalose and molecular chaperones in preventing cellular stress-induced injury (Singer and Lindquist 1998b).

Exposure of yeast cells to a mild dose of a particular type of stress results in the acquisition of resistance against a subsequent treatment with the same or another type of stress (Lewis et al. 1995). Accumulation of trehalose and synthesis of molecular chaperones are most likely to be the main players in these so-called acquired stress resistance and cross-protection phenomena (Soto et al. 1999).

The importance of trehalose and molecular chaperones as general stress protectants is also illustrated by the fact that the general stress resistance of yeast and other fungal cells strongly depends on the growth conditions. In yeast and many other fungi, slower growth is generally correlated with higher general stress resistance (Thevelein 1996) as well as high trehalose and molecular chaperone levels.

The concerted action of trehalose and molecular chaperones can nevertheless not account for all the stress resistance observed in yeast. There are studies reporting inconsistencies between stress tolerance and levels of trehalose and molecular chaperones, which apparently indicate the existence of other factors that are important or required for maintenance of viability under these conditions (van Dijck et al. 1995). Besides differences in trehalose and molecular chaperone levels, cells present in different growth phases or cultured under different conditions are likely to differ also in other properties which might contribute to stress tolerance. The importance of such other factors has only recently been revealed but their precise contribution is not clear yet (Versele et al. 2004). Evidence is forthcoming that the accumulation of antioxidant proteins, compatible solutes and hydrophylins, as well as the adaptation of plasma membrane composition together may at least account partly for the tolerance of yeast cells against different stress conditions.

9.4.3 Antioxidant Proteins and Other Molecules

The main antioxidant defences are a suite of metal-containing antioxidants: superoxide dismutases and catalases, and the thiol dependent antioxidants thioredoxin and glutathione. The breakdown of H_2O_2 to O_2 is catalysed by two catalase enzymes located in the cytosol and peroxisome in *S. cerevisiae*. Of the two superoxide dismutases found in yeast, the cytosolically located enzyme removes superoxide anions from the cytoplasm, whereas mitochondria are protected from superoxides generated during respiration by a mitochondrial-located enzyme (Jamieson 1998). Glutathione, α-tripeptide γ-L-glutamyl-L-cystinylglycine, is probably the most abundant redox-scavenging molecule in yeast. This molecule acts as a radical scavenger with the redox-active sulphydryl group reacting with oxidants to produce reduced glutathione (Jamieson 1998). Genes involved in glutathione synthesis have been identified in *S. cerevisiae* and mutants are hypersensitive to superoxide generators. Thioredoxin is a small sulphydryl-rich protein which acts as a reductant for thioredoxin peroxidase and for ribonucleotide reductase. The precise physiological function of thioredoxin is uncertain as deletion of the genes encoding thioredoxin is not lethal in *S. cerevisiae*. A number of other proteins, such as pentose phosphate pathway enzymes, metallothioneins and peroxidases, are also apparently involved in the protection of yeasts against oxidants (Juhnke et al. 1996; Jamieson 1998).

9.4.4 Compatible Solutes

Another damaging factor associated with many types of stress is osmotic disturbance. To be able to respond to changes in the osmotic pressure of their environment, yeast cells have so-called osmolytes, osmoprotectants or compatible solutes at their disposal (Kempf and Bremer 1998). In response to high osmolarity stress, they are able to accumulate those solutes through uptake and synthesis, whereas upon return to low-osmolarity conditions they can rapidly release them. Their accumulation is also correlated with higher tolerance to various other stresses, including heat stress, desiccation stress and freeze stress (Welsh 2000). The nature of these solutes in microorganisms is diverse, ranging from amino acid derived osmoprotectants such as proline and glycine betaine to sugar-related osmoprotectants such as trehalose, fructans and polyols. In *S. cerevisiae* and other yeasts, glycerol has been demonstrated to serve as the major compatible solute although other polyols such as arabitol and mannitol may also act as compatible solutes (Brown and Simpson 1972; Albertyn et al. 1994b; Tamás et al. 1999; Hohmann and Nielsen 2000). Notwithstanding its minor role as an osmolyte in yeast cells, proline at high levels has been reported to protect mature yeast ascospores against desiccation (Ho and Miller 1978) and intracellular accumulation of proline has been shown to improve freeze tolerance (Morita et al. 2003).

9.4.5 Hydrophylins

Proteins that meet the hydrophylin criteria – a high percentage of glycines and a high hydrophylicity – have mainly been found in plants but also in fungi (Garay-Arroyo et al. 2000). They are suggested to possess both water- and protein-binding regions, enabling them to protect enzymes from water loss, as has been demonstrated for some enzymes upon dehydration and freezing in vitro (Honjoh et al. 2000; Covarrubias et al. 2001). The so-called late embryonic abundant (LEA) proteins also belong to the family of hydrophylins. In baker's yeast, the LEA-like protein Hsp12 (Mtwisha et al. 1998) has been demonstrated to increase dessication-, ethanol- and barotolerance (Sales et al. 2000; Motshwene et al. 2004).

9.4.6 Plasma Membrane Composition

As the plasma membrane of unicellular organisms is in close contact with the surrounding medium, it is likely that its characteristics will influence the tolerance of the cells to all kinds of environmental challenges. A correlation has indeed been found between membrane composition and tolerance to heat, oxidation, ethanol and salt in several instances (Steels et al. 1994; Chi and Arneborg 1999; Swan and Watson 1999; Allakhverdiev et al. 2001; Sakamoto and Murata 2002). In addition, different types of stress have been shown to influence membrane composition (Low and Parks 1987; Singh et al. 1990; Sajbidor and Grego 1992). Fluidity and permeability are the most studied membrane characteristics. Freeze resistance, for instance, has been positively correlated with membrane fluidity (Calcott and Rose 1982) and membrane water permeability (Lewis et al. 1994; Tanghe et al. 2002). The precise

relationship between these membrane characteristics and stress tolerance is, however, not always clear (Swan and Watson 1997).

9.4.7 Aquaporins

Recently, a link was found between freeze tolerance and expression of the aquaporin encoding genes *AQY1* and *AQY2* (Tanghe et al. 2002). This observation supports a role for plasma membrane water transport activity in determination of freeze tolerance in yeast. It is proposed that rapid osmotically driven efflux of water during the freezing process might reduce intracellular ice crystal formation and resulting cell damage. This is in accordance with the observation that aquaporin-mediated improvement of freeze tolerance is limited to fast freezing conditions (Tanghe et al. 2004). Changes in aquaporin expression levels do not seem to alter other cell characteristics, including tolerance to other stress types (Tanghe et al. 2002).

9.5 Growth Conditions, Stress Tolerance and Activity of the Protein Kinase A Pathway

In *S. cerevisiae* there is a striking correlation between the growth conditions, the stress tolerance of the cells and the apparent activity of the protein kinase A (PKA) pathway. Although a relationship between stress tolerance and growth conditions has also been noted in other yeast species, very little is known about the underlying mechanisms in these species. When *S. cerevisiae* cells grow rapidly on fermentable sugars, like glucose and fructose, they display low stress tolerance. When they grow slowly on nonfermentable carbon sources or when they are in a stationary phase they display high stress tolerance (Schenberg-Frascino and Moustacchi 1972; Plesset et al. 1987). The connection between stress tolerance and the PKA pathway has been revealed by mutations either reducing or enhancing activity of the pathway. For instance, mutations in adenylate cyclase (Cyr1/Cdc35), in its activators Ras1,2 or Cdc25, or mutations in the catalytic subunits of PKA will reduce activity of the pathway and enhance stress resistance. On the other hand, mutations in the Ras inhibitors Ira1 and Ira2, or in the regulatory subunit Bcy1 of PKA, will enhance activity of the pathway and reduce stress resistance (Sass et al. 1986; Toda et al. 1987a, b; Cameron et al. 1988; Park et al. 1997). Extensive characterization of these mutants has revealed many other targets of the PKA pathway: trehalose and glycogen content, starvation survival, cell wall strength, sporulation and pseudohyphal growth capacity, etc. (Broach and Deschenes 1990; Chi and Arneborg 1999; Thevelein and de Winde 1999). The stress tolerance level and the status of the other targets of the PKA pathway indicate that in cells growing on rapidly fermented sugars, the activity of the pathway is apparently high, whereas in respiratively growing cells or in the stationary phase the activity of the pathway must be low. Downregulation of stress tolerance by the PKA pathway occurs at the level of the Msn2 and Msn4 transcription factors, which are excluded from the nucleus when PKA activity is high (Görner et al. 1998). A second system downregulated by the PKA pathway consists of the postdiauxic shift (PDS) element, which closely resembles the Msn2/Msn4-controlled STRE (Boorstein and Craig 1990). Downregulation

of the PDS element by PKA occurs through inhibition of the Rim15 protein kinase which is required for the activity of Gis1, the transcription factor that induces PDS-controlled genes (Reinders et al. 1998; Pedruzzi et al. 2000).

The presence or absence of a fermentable sugar in the growth medium is not the sole determinant for the level of stress tolerance in yeast cells. When yeast cells are starved of an essential nutrient, like nitrogen, phosphate or sulphate, in the presence of a fermentable sugar, they arrest growth and become highly stress tolerant. Hence, it is the combination of a fermentable sugar and active growth that in some way keeps stress tolerance low. The pathway involved has been called the 'fermentable-growth-medium-induced pathway' (Thevelein 1994). As a result, a rapid drop in stress resistance cannot only be induced by addition of a fermentable sugar like glucose to respiring cells (van Dijck et al. 1995) but also by addition of a nitrogen source or phosphate to cells starved in the presence of glucose for nitrogen (Donaton et al. 2003) or phosphate (Giots et al. 2003), respectively. Mutants and multicopy suppressor genes have been isolated that were more resistant to fermentation-induced loss of stress resistance ('fil') and they were identified as components of the cAMP–PKA pathway (Kraakman et al. 1999; Versele et al. 1999; van Dijck et al. 2000).

What is the reason for the correlation between stress tolerance and the growth conditions? It has been argued that the higher stress tolerance (which is due to the expression of stress-protection-related genes) on respirative carbon sources indicates that the cells are more 'stressed' on these carbon sources. If the amount of stress that cells experience can be deduced from the number of stress-protection mechanisms that are induced then this conclusion seems to be warranted. However, an alternative conclusion is that the cells induce stress-protection mechanisms because they expect to experience more stress; hence, as a preparation to survive stress. Resting stages, such as stationary-phase cells or ascospores in yeast, and seeds, cysts and other survival forms in other organisms are highly stress resistant clearly as a preparation for stressful conditions rather than as a reaction to the stress that is experienced. Hence, the higher stress resistance in yeast cells growing on nonfermentative carbon sources (in nature this will generally be ethanol) might be a preparation for the stress that the cells will experience due to the accumulation of the ethanol or as a preparation for the subsequent stationary phase which inevitably follows exhaustion of the ethanol.

Another point that needs attention is the general inverse relationship that exists in nature between metabolic activity and stress resistance. The more actively cells are metabolizing and multiplying the less stress resistant they are. Several explanations can be proposed for this observation. For yeasts cultivated in conditions that allow rapid growth, investment in maximal proliferation of the cells might be much more useful for survival in the long term than investment in higher stress resistance for the individual cells. Stress-protection mechanisms might also be incompatible with metabolic activity. Evidence in this respect has been reported for trehalose, which in high levels confers stress protection but reduces the activity of molecular chaperone proteins (Singer and Lindquist 1998a). Hence, folding activity of proteins, catalytic activity of enzymes and actually any protein activity might be hampered by stress-protection mechanisms. This would leave a yeast with a limited (or obligate?) choice between rapid proliferation and preparation for stress survival.

9.6 Stress and the Distribution of Yeasts in Nature

With respect to stress under natural conditions, it is important to emphasize that research on the molecular basis of stress responses and tolerance in the laboratory is generally conducted on uniform cell suspensions of yeast cells complemented with plate assays of growth. In nature yeasts usually grow in mixed populations and sometimes in special communities, such as biofilms. Cells in such mixed or specialized communities might respond quite differently to stress conditions. This is shown, for instance, by the much greater resistance to antifungals of *C. albicans* cells growing in biofilms (Baillie and Douglas 1998). The type of solid substrate on which the cells are growing and interactions with other microorganisms might also influence stress tolerance and stress response. Very little is known in this respect.

The ability of yeasts to tolerate stress and to grow in extreme environments is often species-specific (see Chaps. 15, 16). Certain yeast species like many other microorganisms have developed mechanisms that enable them to grow in extreme environments that exclude most other species. Well-known examples include the highly osmotolerant yeasts *Z. rouxii* and *Debaryomyces hansenii* (Brown 1978; Blomberg and Adler 1992). Pathogenic yeasts, such as *C. albicans*, can proliferate in the stressful environment of the host because part of its virulence factors constitute stress-protection mechanisms (Calderone and Fonzi 2001). Undoubtedly, the capacity to proliferate under specific stress conditions has a major impact on the distribution of the yeast in nature. Yeasts also have the ability to survive beyond the limits of growth in extreme conditions, such as low and high temperature, dehydration, extreme pH, excessive ethanol and limited nutrients. The survival mechanisms to these extreme conditions can be expected to be related in many instances to the adaptation mechanisms that yeasts use to grow under stressful conditions. The capacity of the yeast to survive under extreme conditions will also impact on its distribution in nature but this will be more difficult to reveal compared with that of yeast species able to multiply under extreme conditions.

9.7 Conclusions and Perspectives

We have shown in this review that the responses of yeasts to stress involve multiple components of cell metabolism. Most studies have focussed on the specific interaction of a certain aspect of metabolism and a stress condition. Recent investigations based on a global approach to the study of gene expression in some yeasts have shown that yeasts possess general stress responses as well specific responses to each stress type. These observations imply that the yeast cell is programmed to withstand the myriad of stresses that the yeast can expect during its lifetime. Some yeast species withstand the extremes of stress more successfully than others. However, in many instances we do not understand the basis for the greater stress resistance of one species compared with another. Furthermore, yeasts in nature seldom occur in pure culture but usually exist in complex communities consisting of numerous species together with other microorganisms. How these communities respond to stress has been poorly studied and the interactions between yeasts and their environment represent a future challenge for the yeast biologist to investigate. The tools to understand these

complex questions are now becoming available. With the recent explosion of yeast genomic sequences and improvements in computing power, the stage is now set not only to investigate specific changes to stress but rather to take an integrative approach that studies the interplay of genes, proteins, molecules and organelles with the environment, thereby obtaining a global picture of how the yeast operates under stress.

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Chapter 10

Antagonistic Interactions Among Yeasts

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10.1 Introduction

In every biocenosis, competition for nutrients and space is one of the major factors that determines which organisms succeed and become established. The reasons for such ecological success vary. In many cases, the microbes often alter the environment with their metabolic products, securing their dominance in the habitat as they create conditions unfavourable for the survival of other microorganisms. Such interaction is known as antagonism. Some of these antimicrobial compounds are nonspecific. For example, the antibacterial activity of yeasts is commonly caused by pH changes in the medium as a result of organic acid production or by producing high concentrations of ethanol. This chapter will focus on much more specific interactions stemming from antifungal agent secretion by yeasts.

By now two classes of such extracellular agents of yeasts are known: (glyco)proteins and glycolipids (Fig. 10.1). The proteinaceous compounds having fungicidal or fungistatic action are termed mycocins (zymocins, killer toxins) and they have been investigated for some 40 years, whereas the fungicidal activity of their extracellular glycolipids has only recently come to light.

10.2 Mycocinogeny

The synthesis of proteinaceous antimicrobial compounds (killer phenomenon) is not unique for yeasts; they are produced by various species ranging from bacteria to mammals (Nissen-Meyer and Nes 1997). As for microorganisms, secretion of proteins with a toxicity specific for related organisms, which is associated with specific immunity, is known in smut fungi (Koltin 1988), paramecia (Quackenbush 1988), slime molds (Mizutani et al. 1990) and bacteria (James et al. 1991). The bacterial protein antibiotics are termed bacteriocins and, to emphasize the general nature of such antagonistic interactions, it is preferable to call yeast toxins mycocins and killers mycocinogenic strains. Mycocins are antifungal proteins whose activity is directed against organisms which are taxonomically related to mycocin producers. The latter themselves are immune to their own mycocin but not to others mycocins.

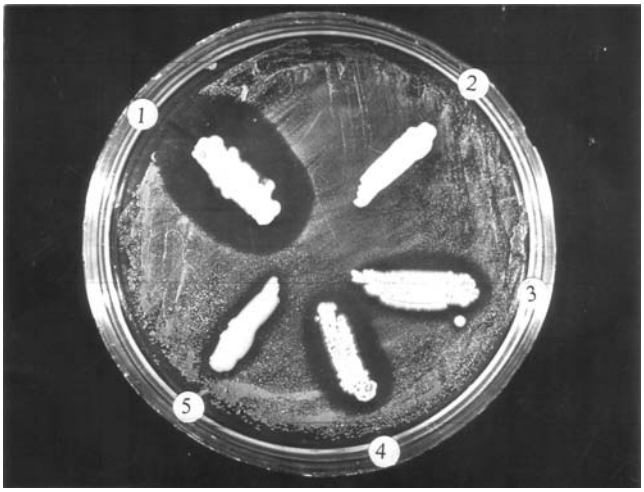

Fig. 10.1. Mycocin and cellobiolipid secretion by *Filobasidium capsuligenum* VKM Y-1439 (*5*) and *Cryptococcus humicola* 9-6, VKM Y-1613, 2238 (*1*, *3*, *4*) strains. They were streaked onto the surface of the medium that had been seeded with a lawn of a test culture that is insensitive to *Cystofilobasidium bisporidii* VKM Y-2700 (*2*) mycocin

In addition, there are some (neutral) strains which neither secrete mycocin nor are sensitive to it. Mycocins do not act against bacteria or protozoa, and no pharmacological activity has been detected in tests with animal organs (Ohta et al. 1984; Pfeiffer et al. 1988). A few reports of the antibiotic action of yeast strains against a wide variety of prokaryotic and eukaryotic organisms have come from an unwarranted interpretation of any observed growth inhibition as mycocinogeny (Polonelli and Morace 1986). No attempts were made to characterize the toxic substances cited in these reports and the growth inhibition was most likely to be due to metabolic products other than mycocins. With respect to antifungal activity, it is necessary to distinguish the mycocinogeny from other inhibition effects, for example, the arresting of growth from mating pheromones. The broad anti-yeast activity of *Metschnikowia pulcherrima* is associated with excretion of the iron-binding agent pulcherriminic acid (Vustin et al. 1990; Nguyen and Panon 1998).

10.2.1 Assay for Mycocinogenic Activity

Both the level and the expression of mycocinogenic activity depend on a number of variables. One of the most important conditions for its detection is the pH of the test medium. Killer activity is expressed under acidic conditions, usually between pH 3 and 6. As a rule, mycocins are most active at pH 4-5 (Woods and Bevan 1968; Young and Yagiu 1978; Middelbeek et al. 1979; Tolstorukov et al. 1989). Because of their proteinaceous nature, mycocins are inactivated at high temperatures, and in most cases 15–20°C is optimal for incubation when assaying for mycocinogenic activity. Adding glycerol (5–15%) to the medium produces broader inhibition zones around

Kluyveromyces and *Pichia* mycocinogenic strains and increases the sensitivity of the bioassay significantly (Lehmann et al. 1987b; Golubev and Blagodatskaya 1993). The antifungal action of mycocins produced by halotolerant yeasts (*Candida, Debaryomyces* and *Pichia* spp.) is evoked and enhanced in the presence of increasing (4–12%) NaCl concentrations (Kagiyama et al. 1988; Suzuki et al. 1989; Gunge et al. 1993; Llorente et al. 1997). Mycocins are stabler in a solid medium than in a liquid medium, and agitation can cause their inactivation (Woods and Bevan 1968; Wilson and Whittaker 1989). The concentration of sensitive cells influences the sensitivity of the bioassay; when the lawn of the target strain is too dense and the inoculum of the mycocinogenic strain is small, the inhibition zone can be narrow and rapidly overgrown or not developed at all. In addition, the composition of the medium and buffer solution may affect to the sensitivity of the assay (Panchal et al. 1985). In most cases, glucose–yeast extract–peptone agar with sodium citrate–phosphate buffer are used. The assay conditions are particularly important for detecting mycocinogenic strains with low activity and those organisms that are only slightly sensitive.

However, using optimal conditions does not guarantee successful screening, because the principal trait of mycocins is the specificity of their toxicity. Hence, the choice of appropriate sensitive strains is crucial for detecting mycocinogenic strains. Their incidence was found to be much higher when screening for mycocinogenic activity with target cultures of the same species (or one closely related taxonomically) as that being tested (Thornton 1986; Heard and Fleet 1987; Golubev and coworkers 1990, 1993).

After the discovery of mycocinogeny in *Saccharomyces cerevisiae* (Makower and Bevan 1963), it soon became evident that the production of mycocins is a general phenomenon for most, if not all, yeasts. At present, mycocinogenic strains have been found in over 100 species from more than 20 genera among both ascomycetous and basidiomycetous yeasts (Table 10.1). Several types of mycocinogenic strains were identified in some species (*Cryptococcus laurentii, Pichia membranifaciens*, *Rhodotorula mucilaginosa*, *Saccharomyces cerevisiae*).

10.2.2 Characteristics of Mycocins

All mycocins are either proteins or glycoproteins that often consisted of two or three subunits. Most yeasts secrete mycocins with a molecular mass of about 10–30 kDa, although those of *Kluyveromyces lactis*, *P. acaciae*, *P. anomala* and *P. inositovora* are much higher, about 100 kDa or greater (Sawant et al. 1989; Stark et al. 1990; McCracken et al. 1994; Klassen and Meinhardt 2003). The best known and most fully studied K1 mycocin (20 kDa) of *Saccharomyces cerevisiae* is secreted as a molecule consisting of α and β disulfide-bonded unglycosylated polypeptides with a relatively high content of hydrophobic and charged amino acids (Zhu et al. 1987). Its precursor has been synthesized in cells as a large single-strand polypeptide that has a δ–α–γ–β domain organization. The δ domain is a leader sequence that mediates folding and secretion; the α and β domains are separated by an interstitial glycosylated γ region which is required for maturation (Lolle and Bussey 1986). The β subunit is a lectin-like domain and is essential for recognition and binding to whole

Table 10.1 Yeast species for which mycocinogenic activity has been reported

Yeast species	References
Bullera alba	Golubev et al. (1997a, b)
B. hannae	Golubev et al. (1996)
B. sinensis	Golubev et al. (1997a)
B. unica	Golubev and Nakase (1998)
Candida albicans	Rogers and Bevan (1978)
C. berthetii	Buzzini and Martini (2000b)
C. cacaoi	Aguiar and Lucas (2000)
C. diversa	Abranches et al. (2000)
C. freyschussii	Buzzini and Martini (2000a)
C. glabrata	Sriprakash and Batum (1984)
C. homilentoma	Carreiro et al. (2002)
C. maltosa	Polonelli et al. (1987)
C. naeodendra	Suzuki et al. (1989)
C. nodaensis	Aguiar and Lucas (2000)
C. oleophila	Buzzini and Martini (2000b)
C. parapsilosis	Zekhnov et al. (1989)
C. silvae	Buzzini and Martini (2000b)
C. sonorensis	Starmer et al. (1987)
C. species	Yokomori et al. (1988)
C. stellata	Abranches et al. (2000)
C. versatilis	Vaughan-Martini et al. (1988)
Cryptococcus aerius	Carreiro et al. (2002)
C. albidus	Starmer et al. (1987)
C. aquaticus	Pfeiffer et al. (2004)
C. flavus	Buzzini and Martini (2000b)
C. laurentii	Golubev and Kuznetsova (1989)
C. luteolus	Buzzini and Martini (2000b)
C. nemorosus	Golubev et al. (2003a)
C. perniciosus	Golubev et al. (2003a)
C. podzolicus	Golubev (1991a)
Curvibasidium pallidicorallinum	Golubev (1992a)
Cystofilobasidium bisporidii	Kulakovskaya et al. (1996)
Cyst. infirmominiatum	Golubev et al. (2003b)
Cyst. species	Gacser et al. (2001)
Debaryomyces carsonii	Polonelli et al. (1987)
D. castellii	Vustin et al. (1993)
D. etchelsii	Buzzini and Martini (2000b)
D. hansenii (*C. famata*)	Suzuki et al. (1989); Aguiar and Lucas (2000)
D. occidentalis	Chen et al. (2000)
D. polymorphus	Vaughan-Martini et al. (1988)
D. vanrijiae	Vustin et al. (1993)
Fellomyces penicillatus	Aguiar and Lucas (2000)
Filobasidium capsuligenum	Golubev and Kuznetsova (1991)
Hanseniaspora uvarum (*Kloeckera apiculata*)	Rosini and Cantini (1987); Schmitt et al. (1997)
H. vineae (*K. africana*)	Abranches et al. (2000)
H. valbyensis (*K. japonica*)	Starmer et al. (1987)
Issatchenkia orientalis (*C. krusei*)	Lehmann et al. (1987a)
I. scutulata	Buzzini and Martini (2000b)
I. terricola	Abranches et al. (2000)

Table 10.1 Yeast species for which mycocinogenic activity has been reported—*cont'd*

Yeast species	References
Kloeckera lindneri	Abranches et al. (2000)
Kluyveromyces aestuarii	Vaughan-Martini and Rosini (1989)
K. dobzhanskii	Vaughan-Martini and Rosini (1989)
K. lactis (*C. sphaerica*)	Vaughan-Martini et al. (1988); Stark et al. (1990)
K. loddereae	Vaughan-Martini and Rosini (1989)
K. marxianus (*C. pseudotropicalis*)	Lehmann et al. (1987b); Polonelli et al. (1987)
K. phaffii	Comitini et al. (2004)
K. polysporus	Kono and Himeno (1997)
K. thermotolerns (*C. dattila*)	Choi et al. (1990)
K. waltii	Kono and Himeno (1997)
K. wickerhamii	Vaughan-Martini and Rosini (1989)
K. wikenii	Rosini and Cantini (1987)
Pichia acaciae	Bolen et al. (1994)
P. americana	Buzzini and Martini (2000b)
P. amethionina	Starmer et al. (1987)
P. anomala	Sawant et al. (1989)
P. antillensis	Starmer et al. (1987)
P. bimundalis	Polonelli et al. (1987)
P. burtonii	Buzzini and Martini (2000b)
P. cactophila	Starmer et al. (1987)
P. canadensis	Lehmann et al. (1987a)
P. ciferrii	Nomoto et al. (1984)
P. fabianii	Polonelli et al. (1987)
P. farinosa	Price et al. (1999)
P. guilliermondii (*C. guilliermondii*)	Zekhnov et al. (1989)
P. haplophila	Aguiar and Lucas (2000)
P. holstii	Polonelli et al. (1987)
P. inositovora	Klassen and Meinhardt (2003)
P. jadinii	Vaughan-Martini et al. (1988)
P. kluyveri	Zorg et al. (1988)
P. membranifaciens (*C. valida*)	Golubev and Blagodatskaya (1993)
P. mexicana	Starmer et al. (1987)
P. minuta	Polonelli et al. (1987)
P. ohmeri	Zekhnov et al. (1989)
P. opuntiae	Starmer et al. (1987)
P. petersonii	Nomoto et al. (1984)
P. pini	Zekhnov et al. (1989)
P. punctispora	Golubev and Blagodatskaya (1994)
P. quercuum	Zekhnov et al. (1989)
P. spartinae	Polonelli et al. (1987)
P. stipitis	Laplace et al. (1992)
P. subpelliculosa	Young and Yagiu (1978)
P. thermotolerans	Ganter and Starmer (1992)
Pseudozyma antarctica	Buzzini and Martini (2000b)
Rhodotorula dairenensis	Golubev (1989)
R. glutinis	Aguiar and Lucas (2000)
R. graminis	Buzzini and Martini (2000b)
R. lactosa	Buzzini and Martini (2000b)
R. lignophila	Buzzini and Martini (2000b)
R. mucilaginosa	Golubev and Churkina (1997)

Continues

Table 10.1 Yeast species for which mycocinogenic activity has been reported—*cont'd*

Yeast species	References
R. pallida	Golubev (1991b)
Saccharomyces cerevisiae	Wickner (1996)
S. exiguus (*C. holmii*)	Nagornaya et al. (1989); Vital et al. (2002)
S. paradoxus	Naumov (1985)
S. unisporus	Nagornaya et al. (1989)
Schizosaccharomyces pombe	Bonilla-Salinas et al. (1995)
Sporidiobolus pararoseus	Janderova et al. (1995)
S. salmonicolor	Golubev and Tsiomenko (1985)
Tilletiopsis albescens	Golubev (1998b)
T. flava	Golubev and Churkina (2001)
Torulaspora delbrueckii	Bonilla-Salinas et al. (1995)
Trichosporon asteroides	Buzzini and Martini (2000b)
T. capitatum	Morace et al. (1983/1984)
T. jirovecii	Carreiro et al. (2002)
T. pullulans	Golubev et al. (2002)
Williopsis pratensis	Vustin et al. (1991)
W. saturnus	Kimura et al. (1995); Komiyama et al. (1995)
W. saturnus var. *mrakii*	Kimura et al. (1999)
W. saturnus var. *subsufficiens*	Shemyakina et al. (1991)
Zygosaccharomyces bailii	Weiler and Schmitt (2003)
Z. fermentati	Buzzini and Martini (2000b)
Z. florentinus	Aguiar and Lucas (2000)
Z. microellipsoides	Buzzini and Martini (2000b)
Zygowilliopsis californica	Vustin et al. (1988); Theisen et al. (2000)

cells, while the α subunit acts at the cytoplasmic membrane to produce a collapse of a transmembrane proton gradient (Sturley et al. 1986; Douglas et al. 1988; Zhu and Bussey 1991). Unlike most reported mycocins, some produced by *Pichia* and *Williopsis* species show a higher thermostability and function over a wide pH range (Ashida et al. 1983; Ohta et al. 1984; Vustin et al. 1989). This stability appears to be due to the large number of disulfide bonds in these cysteine-rich molecules.

10.2.3 Modes of Action

After exposure to most mycocins, growing sensitive cells exhibit a reduction in intracellular pH and leakage of potassium ions, ATP and other cellular intermediates. Amino acid transport and proton pumping into the culture medium are also inhibited (Skipper and Bussey 1977; Middelbeek and coworkers 1980a–c; Ashida et al. 1983). All of these effects are indicative of an increase in proton permeability in mycocin-sensitive cells (de la Pena et al. 1981). Apparently, mycocin either inhibits a component of the proton pump or becomes incorporated into the cytoplasmic membrane and creates ion-permeable channels (Kagan 1983; Martinac et al. 1990; Santos and Marquina 2004). The K1 mycocin was shown to induce aberrant activity of ion channels, which leads to dysregulated potassium homeostasis (Ahmed et al. 1999).

The overall effect of pore formation disrupts the cell electrochemical potential across the plasma membrane and results in cell death (Fig. 10.2). Many, but not all mycocins, have a membrane-damaging activity. The *Kluyveromyces lactis* toxin, for example, does not elicit leakage of potassium ions and ATP but causes an arrest in the G1 phase of the cell cycle (Butler et al. 1991); while the mycocin of *Williopsis saturnus* var. *mrakii* inhibits β-1,3-glucan synthesis (Yamamoto et al. 1986) and the KT28 mycocin of *Saccharomyces cerevisiae* inhibits DNA synthesis (Schmitt et al. 1989).

Regardless of the mode of action of mycocins, it occurs in two phases. The first stage of cell interaction is a rapid and energy-independent binding to the cell wall surface. The second stage, the time-lag and energy-dependent process, involves mycocin translocation to the cytoplasmic membrane and interaction with a membrane receptor (Al-Aidroos and Bussey 1978; Bussey et al. 1979). Binding is pH-dependent and may be responsible for the pH range of mycocin activity on yeast cells. Binding sites may be particular cell wall receptors, which have other functions, such as the uptake of nutrients. Until now, the primary functions of these receptors have not been elucidated for any yeasts.

Comparison of the mycocin killing spectra on whole cells and on spheroplasts showed that it is the cell wall which determines the specificity of sensitivity to mycocins. *Saccharomyces cerevisiae* mycocin K1 can kill cells of the same species and those of *Candida glabrata*, but has a wide spheroplast-killing action and can

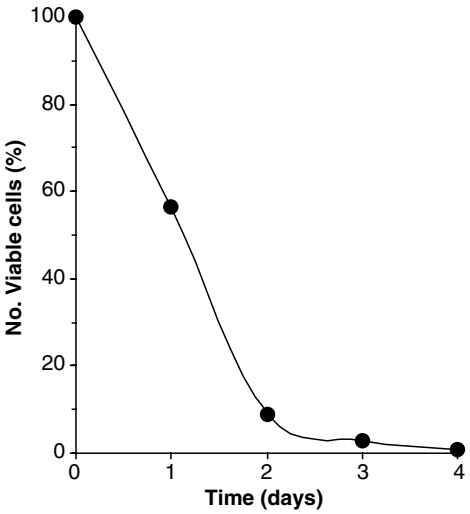

Fig. 10.2. Killing of *Mrakia nivalis* VKM Y-1443 cells incubated with *Cryptococcus aquaticus* VKM Y-2428 mycocin-containing culture filtrate (pH 4.5, 18°C). Survival is expressed as the percentage of cells able to produce colonies, where 100% is the number of colonies counted immediately before addition of the mycocin-containing filtrate

destroy the spheroplasts of *C. albicans*, *C. utilis*, *Kluyveromyces lactis* and *Debaryomyces occidentalis*. However, the whole cells of these species are insensitive to this mycocin (Zhu and Bussey 1989). Mutations that change the molecular structure of cell wall glucans and mannans alter their capacity to act as binding sites of mycocins and induce resistance to them (Schmitt and Radler 1988; Nakajima et al. 1989). As yeast and hyphal forms of the same organisms vary in the composition of their walls they can differ in sensitivity to mycocins (Golubev and Boekhout 1992). Any of the principal cell wall components can be involved in their binding. For mycocins produced by *Hanseniaspora uvarum*, *P. anomala*, *P. membranifaciens*, *S. cerevisiae* (K1 and K2 types) and *Williopsis saturnus,* the binding components have been identified as β-1,6-glucans; those for mycocins of *Debaryomyces occidentalis*, *S. cerevisiae* (K28 type) and *Zygosaccharomyces bailii* are mannans, whereas chitin may represent the binding site for mycocins of *Kluyveromyces lactis* and *P. acaciae* (Hutchins and Bussey 1983; Schmitt and Radler 1988; Sawant and Ahearn 1990; Radler et al. 1993; Takita and Castilho-Valavicius 1993; Kasahara et al. 1994; McCracken et al. 1994; Schmitt et al. 1997; Santos et al. 2000).

10.2.4 Genetic Basis for Mycocinogeny

Mycocin determinants can be either cytoplasmically or chromosomally inherited. In most cases mycocin production is presumed to be determined by nuclear genes but these have clearly been identified in a few species only (Sriprakash and Batum 1984; Goto et al. 1990; Kimura et al. 1993; Suzuki and Nikkuni 1994).

Much more attention is given to the extrachromosomal genetic elements, double-stranded RNA (dsRNA) viruses and linear double-stranded DNA (dsDNA) plasmids which are responsible for a mycocinogenic phenotype. The virally encoded mycocinogeny has been most intensively studied in *S. cerevisiae* (Wickner 1996), but has also been described for *Hanseniaspora uvarum* (Zorg et al. 1988) and *Zygosaccharomyces bailii* (Schmitt and Neuhausen 1994). In addition, the mycocin-coding dsRNA viruses have been identified in the basidiomycetous yeasts, *Cystofilobasidium bisporidii* (Karamysheva et al. 1991), *C. infirmominiatum* (Golubev et al. 2003b), *Sporidiobolus salmonicolor* (Kitaite and Čitavičus 1988) and *Trichosporon pullulans* (Golubev et al. 2002). Currently, these isometric dsRNA viruses with undivided genomes are classified in the genus *Totivirus* in the family *Totiviridae* (Ghabrial 1994). In contrast to most plant and animal RNA viruses, yeast viruses are noninfectious and are transmitted by vegetative cell division or through sexual fusion. The mycocins are encoded by different satellite dsRNA viruses (denoted M) which are dependent on another group of helper dsRNA viruses (denoted L). These encode capsid and RNA polymerase for replicating both virus types. Curing of viruses from mycocinogenic strains results in loss of activity and immunity to their own mycocins.

Mycocinogenic strains of *Kluyveromyces lactis* (Schaffrath and Breunig 2000), *P. acaciae* (Bolen et al. 1994) and *P. inositovora* (Ligon et al. 1989) contain two types of linear dsDNA plasmids differing in size. The loss of these plasmids also gives rise to cultures which are not only incapable of producing mycocins, but are also susceptible to them.

10.2.5 Taxonomic Implications of Sensitivity to Mycocins

By virtue of the fact that cell walls of yeast taxa vary in structure and chemical composition (Kreger-van Rij and Veenhuis 1971; Weijman and Golubev 1987; Fleet 1991), and as different wall components are involved in mycocin binding, it is reasonable to infer that mycocin activity against whole cells may be restricted to taxonomically related organisms, so that mycocin sensitivity patterns are of taxonomic importance (Golubev and Boekhout 1995). The differences between yeasts in this respect are associated with a whole range of taxonomic and phylogenetic markers, such as septal pore ultrastructure, monosaccharide composition of extracellular polysaccharides, and sequence similarity of small and large subunit ribosomal RNAs (Golubev 1998a). However, it must be emphasized that there are no definite taxonomic levels criteria for the action of mycocins, as their host ranges vary considerably. Although the mycocins are active against organisms phylogenetically and taxonomically related to the mycocinogenic strains, the degree of relatedness may vary from strains of the same species to species of related genera or even higher taxa. Apparently, the diversity of cell-wall receptors involved in binding of mycocins can be both unique and common for certain taxa and may provide the basis of differences in the ranges of mycocin action. In practice, this feature dictates that the use of each mycocin as a taxonomic tool must be preceded by a careful study of its killing pattern. The differences in host ranges may serve to resolve different levels of taxonomic organization. Broad-spectrum mycocins are apparently of most interest for overall phylogenetic evaluations, whereas narrow-spectrum ones may be used for clarification of the taxonomy of closely related organisms.

As a rule, ascomycetous yeasts are insensitive to mycocins produced by basidiomycetous yeasts and conversely. The exceptions are the mycocins of *Williopsis pratensis*, *Bullera alba* and *Curvibasidium pallidicorallinum*. That of *W. pratensis* is active not only against ascomycetous yeasts but also against some sporidiobolaceous species (Vustin et al. 1991), whereas that of *B. alba* is active against some ascomycetous yeasts (mainly the members of the Lipomycetaceae) in addition to basidiomycetous species (Golubev et al. 1997a). The mycocin produced by *C. pallidicorallinum* (previously identified as *Rhodotorula fujisanensis*) mainly acts against sporidiobolaceous yeasts and also shows a weak activity against some tremellaceous yeasts (Golubev 1992a). In most cases, all strains of the same species and closely related species of the same genus have identical responses to specific mycocins. However, some taxa are heterogeneous not only within a genus but also within a species. The major reason for such variability is the heterogeneity of many yeast taxa. Most teleomorphic taxa are homogenous, unlike the anamorphic taxa in which heterogeneity in sensitivity patterns is much more widespread. The additional reasons are the immunity of mycocinogenic strains. The responses to mycocins that are free from the effects of immunity are of taxonomic interest. There is the problem associated with distinction of immunity from resistance to mycocins. Resistance and immunity are similar phenotypically, and plate assays do not allow one to distinguish between these two types of insensitivity which differ both in their genetic mechanisms and in their taxonomic significance. Resistant yeasts lack the specific receptors necessary for the adsorption, and hence action, of mycocin.

Mycocinogenic strains, like sensitive ones, still possess these receptors, but the mycocins contain a component which gives immunity (specific for only one mycocin type) to mycocinogenic cultures. Immunity appears to be conferred at the cytoplasmic membrane level by a component which may act as a competitive inhibitor of mycocin by saturating membrane receptors (Boone et al. 1986; Hanes et al. 1986; Douglass et al. 1988). The so-called neutral strains contain genetic determinants for mycocin synthesis, either the mycocin is produced in an inactive form or it is not secreted. Consequently, such strains retain their immunity (Bussey et al. 1982; Wingfield et al. 1990). Thus, the insensitivity caused by immunity is a clone-related property, which coupled with possible cross-immunity between immunologically similar mycocins, may interfere with the resistance shown at the cell-wall level and conferred by the nuclear genotype. All these reasons taken together give a complicated picture of mycocin-sensitive relationships. Consequently many authors consider the sensitivity patterns to be strain-related and propose using them for fingerprinting in order to biotype the strains of a species (Morace et al. 1983/1984; Lehmann et al. 1987a; Vaughan-Martini et al. 1988; Vaughan-Martini and Rosini 1989).

Current molecular studies have shown that many classical features used to define taxa, such as the formation of pseudomycelium, the presence and morphology of spores, fermentation and assimilation of sugars and nitrate, have limited value. In this situation, sensitivity testing by the use of a set of mycocinogenic strains with known and different host ranges could be a very useful taxonomic tool. Although immunity to mycocins and the possible occurrence of resistant mutants limits the value of mycocin sensitivity patterns for identification, these difficulties can be overcome by using a panel of mycocinogenic strains with known and different host ranges. Mycocinotyping may then become an additional taxonomic tool with which to examine yeast classification.

10.3 Extracellular Glycolipids

While mycocinogeny was described at the beginning of 1960s, the antibiotic effect of extracellular glycolipids was only discovered 30 years later (Golubev 1992b), although these yeast compounds had long been known (Spencer et al. 1979). They were commonly considered as emulsifying agents associated with the growth of microorganisms on water-insoluble substrates. Thus far, the antibiotic activity of extracellular glycolipids has been recorded in only a few species: *Cryptococcus humicola* (Puchkov et al. 2002), *Pseudozyma flocculosa* (Cheng et al. 2003), *P. fusiformata* (Golubev et al. 2001) and *Sympodiomycopsis paphiopedili* (Golubev et al. 2004). The first species belongs to the tremellaceous yeasts, while the others are phylogenetically distributed among the Ustilaginomycetes. In this connection it should be mentioned that such compounds have long been known in smut fungi (Lang and Wagner 1987).

10.3.1 Characteristics of Glycolipids

Almost all known antifungal glycolipids contain cellobiose (4-*O*-*β*-D-glucopyranosyl-D-glucose) glycosidically linked to saturated fatty hydroxy acids (C16, C18).

So, *Ustilago maydis* produces a mixture of cellobiolipids in which cellobiose is esterified with 15,16-dihydroxyhexadecanoic or 2,15,16-trihydroxyhexadecanoic acid termed "ustilic acids". Similar compounds were also shown to be secreted by several yeasts. In *Cr. humicola* they are composed of a highly acetylated cellobiose linked to 2,16-dihydroxyhexadecanoic acid. The acyl chain forming aglycon can be replaced by 2,18-dihydroxyoctadecanoic, 2,16,18-trihydroxyoctadecanoic or 2,17,18-trihydroxyoctadecanoic acid (Puchkov et al. 2002). The structure of *P. flocculosa* glycolipid was 2-(2′,4′-diacetoxy-5′-carboxy-pentanoyl)octadecyl cellobioside (Cheng et al. 2003). The fatty acid moiety of *S. paphiopedili* cellobiolipid (Fig. 10.3) was 2,15,16-trihydroxyhexadecanoic acid (Kulakovskaya et al. 2004). The glycolipids secreted by *P. fusiformata* were only incompletely characterized but they are also cellobiose lipids (Golubev et al. 2001).

As with mycocins, antifungal activity of extracellular glycolipids occurs under acidic conditions, but in contrast to them, glycolipids have a much broader range of action. Their killing patterns are not taxonomically specific, but also include many of both ascomycetous and basidiomycetous yeasts, as well as mycelial fungi (Fig. 10.4, Table 10.2). Increased sensitivity of related organisms can only be noted (Golubev and Shabalin 1994; Golubev et al. 2001, 2004; Kulakovskaya et al. 2003).

10.3.2 Mode of Action

Cellobiolipids have a fungicidal action which involves a gross increase of the nonspecific permeability of the cytoplasmic membrane. Sensitive cells treated with cellobiolipids showed a leakage of intracellular compounds, including electrolytes

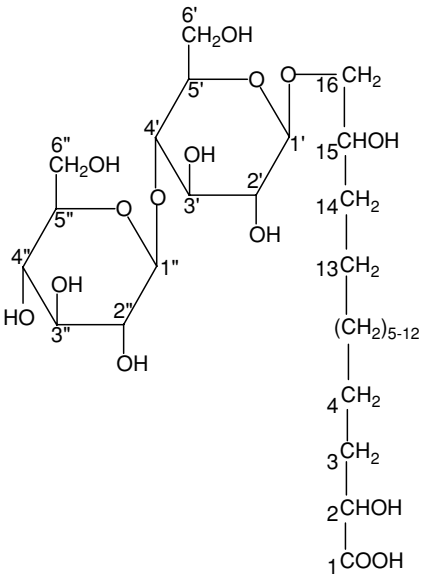

Fig. 10.3. Extracellular cellobiose lipid of *Sympodiomycopsis paphiopedili* VKM Y-2817

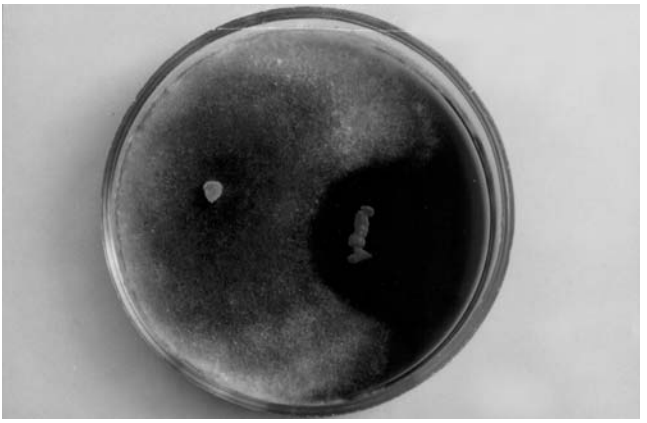

Fig. 10.4. Inhibitory activity of *Sympodiomycopsis paphiopedili* (streak) against *Sclerotinia sclerotiorum* (lawn). Glucose–peptone agar (pH 4.0, 20°C)

and ATP (Puchkov et al. 2001; Kulakovskaya et al. 2003). Probably, cellobiolipids can intercalate into the lipid matrix. This intercalation leads to permeability changes which disturb the membrane order and integrity, resulting in cytoplasmic disorder, cell disintegration and eventually in cell death.

Table 10.2 The genera of fungi (251 species tested) sensitive to the cellobiolipid of *Sympodiomycopsis paphiopedili*

Agaricostilbum	*Gymnosporangium*	*Schizosaccharomyces*
Arthroascus	*Holtermannia*	*Sclerotinia*
Arxula	*Issatchenkia*	*Septobasidium*
Bensingtonia	*Itersonilia*	*Sorosporium*
Bullera	*Kluyveromyces*	*Sphacelotheca*
Bulleromyces	*Kockovaella*	*Sporidiobolus*
Candida	*Kurtzmanomyces*	*Sporisorium*
Citeromyces	*Leucosporidium*	*Sporobolomyces*
Clavispora	*Lipomyces*	*Stephanoascus*
Cryptococcus	*Lodderomyces*	*Sterigmatomyces*
Cystofilobasidium	*Malassezia*	*Taphrina*
Debaryomyces	*Mastigobasidium*	*Tilletia*
Dekkera	*Mastigomyces*	*Tilletiaria*
Diaporthe	*Metschnikowia*	*Tilletiopsis*
Dioszegia	*Microbotryum*	*Torulaspora*
Dipodascus	*Mrakia*	*Trichosporon*
Endomyces	*Myxozyma*	*Trimorphomyces*
Endophyllum	*Nadsonia*	*Tsuchiyaea*
Erythrobasidium	*Pichia*	*Udeniomyces*
Exobasidium	*Protomyces*	*Ustilago*
Farysia	*Pseudozyma*	*Wickerhamia*
Fellomyces	*Puccinia*	*Williopsis*
Filobasidiella	*Rhodosporidium*	*Xanthophyllomyces*
Filobasidium	*Rhodotorula*	*Zygosaccharomyces*
Guilliermondella	*Saturnispora*	*Zygozyma*

Obviously, a difference in the cytoplasmic membrane lipid composition (specifically, sterol content, Avis and Bélanger 2002) among fungi is the major factor in determining their level of sensitivity to cellobiolipids. A difference in the cell envelope composition and surface charge may possibly also cause some variations in the sensitivity of fungi to these membrane-damaging compounds.

10.3.3 Genetic Basis

The synthesis of extracellular glycolipids may be controlled by chromosomal genes, since neither dsRNA viruses nor DNA plasmids were detected in any species secreting antifungal cellobiolipids (Golubev and Shabalin 1994). This is consistent with the observation that such a phenotype is cureless (Golubev and coworkes 2001, 2004). Moreover, multiple genes are probably involved in glycolipid production in *P. flocculosa* (Cheng et al. 2003).

10.4 The Ecological Role of Antagonistic Yeasts

Negative antagonistic interactions between microbes are more frequent than positive ones; and, indeed, production of antibiotic substances has long been established as a commonplace phenomenon among many groups of prokaryotic and eukaryotic microorganisms. Although yeasts are consistent and normal members of the microbial biota associated with plants, animals, soils and waters in all geographic areas, until recently they have been considered mainly as suppliers of growth-promoting substances (vitamins, sterols) for other members of biocenoses. The discovery of antagonistic activity in yeasts has important implications for a proper understanding of their role in natural communities. At the present time, only mycocinogeny can be considered from this viewpoint as secretion of fungicidal glycolipids by yeasts has only been revealed quite recently and the impact of this phenomenon on natural fungal communities remains to be studied.

Mycocin-producing strains of yeasts may be isolated from various sources, but they occur much more frequently in habitats where yeast populations reach relatively high densities, so that competition is more intense. Table 10.1 shows that most of the species listed are associated with plants and food; while mycocinogenic cultures have not been found among specific soil species (*Cryptococcus terreus*, *Lipomyces* spp., *Schizoblastosporion starkeyi-henricii*) that have been isolated exclusively from soils which usually have low yeast concentrations (Golubev, unpublished data). During the 4-year study of yeast communities from the phyllosphere and soil of the Prioksko-terrasny biosphere reserve (Russia), mycocinogenic strains were found to comprise one quarter of all isolates from steppe plants that had total yeast counts in the range 10^5–10^6 CFU/g, whereas no mycocinogenic yeasts were isolated from soils where the total numbers of yeasts were about 10^4 CFU/g (Golubev and Golubeva 2004).

Mycocinogeny has a marked impact on competition between related yeasts for their favoured ecological niche. This is because sensitivity to mycocins is specific, and only the yeast cells containing receptors for the mycocins are sensitive to them. The competitive advantage of mycocin production has been exemplified by

demonstrating the predominance of mycocin-producing *Trichosporon pullulans* isolates both in the spring exudates from birch and under laboratory-simulated conditions (Golubev et al. 2002). During the initial phase of the exudation, when the density of yeast populations is low (10^2 CFU/ml), the incidence of *T. pullulans* mycocinogenic strains was 3–4%; but towards the end of the exudation period the density increases to as much as 10^7 CFU/ml (Golubev et al. 1977). These represented 20–40% of all *T. pullulans* isolates. Mycocinogenic cultures are able to exclude sensitive ones from industrial fermentations (Vondrejs 1987).

Mycocinogenic yeasts isolated from particular habitats were found to have greater killing activity against yeasts of other habitats than against those in their own habitat. The activity (number killed per number tested) of *P. kluyveri* against yeasts from a fruit habitat was 12%, while the activity against yeasts from other habitats was 64% (Starmer et al. 1987). Further, only 9% of the yeasts originating from fruit were sensitive to *P. kluyveri* mycocinogenic strains, whereas 42% of the strains from habitats other than fruit were sensitive to the *P. kluyveri* strains tested (Starmer et al. 1992). Analogously, almost all soil isolates were sensitive to mycocins produced by yeasts originating from the phyllosphere but there were many resistant strains among isolates from plants, indicative of a selection within communities to the mycocins present (Golubev and Golubeva 2004). Clearly, the production of mycocins has an important function for mycocinogenic cells by defending an ecological niche against invading cells which have the same nutritional requirements and may occupy the same sites. In other words, mycocinogeny has a role in maintaining community composition by excluding "foreign" competitor yeasts from particular habitats.

The energy and cellular machinery used to produce mycocins cannot also be used for reproduction. Hence, mycocin synthesis may reduce the growth rate and competitiveness of mycocin producers (Pintar and Starmer 2003). Probably, the cost of mycocin production, together with spatial and temporal heterogeneity of habitats, makes possible the coexistence of mycocinogenic and sensitive populations, as is observed in natural communities.

10.5 Applications of Antagonistic Yeasts

Since its initial discovery, mycocinogeny has come to the attention of specialists in genetics, virology, biochemistry and molecular biology, who have used it as an excellent model to study host–virus interactions in eukaryotic cells (Wickner 1996), the mechanisms of regulation in eukaryotic protein processing (Riffer et al. 2002) and mycocin-based cloning vectors which are highly efficient for the effective secretion of heterologous proteins (Heintel et al. 2001). Antagonistic yeasts have also attracted attention not only from those doing applied research, but from those doing fundamental research as well.

10.5.1 Food and Fermentation Industries

Many commercial yeast strains used in the production of wine, beer and bread have been found to be sensitive to mycocins, and hence wild mycocinogenic yeasts can

cause protracted or block fermentations and negatively affect the quality of products. To protect industrial fermentations against contaminating yeasts, many attempts have been made to use suitably constructed strains with mycocinogenic activity as starter cultures (van Vuuren and Jacobs 1992; Javadekar et al. 1995; Schmitt and Schernikau 1997). In addition, mycocinogenic yeasts have been used for biotyping patented industrial strains (Buzzini and Martini 2000a; Buzzini et al. 2001).

10.5.2 Medicine

Considerable effort has also been devoted to biotyping medically important yeasts. Mycocin sensitivity patterns have been used as epidemiological markers for the intraspecific discrimination of pathogenic strains (Caprilli et al. 1985; Polonelli et al. 1985; Boekhout and Scorzetti 1997; Golubev et al. 2000). The use of mycocins as novel agents has also been proposed for treating fungal infections (Polonelli et al. 1986; Seguy et al. 1998). This use may be effective for treating superficial lesions, but mycocins cannot be used orally or intravenously, as they are protease-sensitive, antigenic, inactive at 37°C and active within a narrow pH range only. However, it was possible to obtain anti-idiotypic antibodies which apparently share the active site of the *P. anomala* mycocin and have anti-*Candida albicans* activity (Polonelli et al. 1997). Viral dsRNAs from yeasts were shown to have interferon-inducing activity (Nosik et al. 1984).

10.5.3 Agriculture

Mycocinogenic yeasts are commonly considered as promising natural biocontrol agents of plant-pathogenic fungi and in feed and food preservation from yeast spoilage (Petersson and Schnurer 1995; Walker et al. 1995; Kitamoto et al. 1999; Lowes et al. 2000). However, rare mycocins (for example, HMK mycocin of *Williopsis mrakii*) that have the broad spectra of activity, pH and temperature stability may be suitable for this purpose. Nearly all mycocins have narrow spectra of activity and are unstable. For this purpose, glycolipid-secreting yeasts are more promising as their thermostable cellobiolipids have much broader spectra of antifungal activity (Golubev et al. 1997b; Avis and Bélanger 2002).

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Chapter 11

Yeasts in Soil

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11.1 Introduction

Most reviews on the microbial ecology of soil barely mention the existence of soil yeasts, while the majority of ecological studies conducted on these yeasts were merely surveys utilizing classic microbiological techniques, indicating the presence of yeasts culturable on the isolation media used by the researcher. Thus, not all yeasts occurring in a particular soil sample may have been observed. Also, relatively little is known about the interactions of soil yeasts in situ, since the majority of studies in this regard were conducted in vitro or with soil microcosms under controlled conditions in the absence of a plethora of factors that may impact on soil yeast metabolism, when these organisms are growing in their natural habitat.

Nevertheless, ecological studies of culturable soil yeast populations provide some insight into the distribution of yeasts in soils and the role of soil yeast communities. In addition, in vitro studies on cardinal growth temperatures, nutrient assimilation and antibiotic resistance may provide us with a glimpse of the intrinsic abilities of particular yeasts (Lachance and Starmer 1998). Although not always a true indication of the ecological niche, the latter may provide insight into the fundamental niche of a yeast species. Similarly, binary interactions of yeasts with other microbes studied in vitro may help us to understand interactions of soil yeasts in their natural habitat (Fracchia et al. 2003). However, it must always be borne in mind that soil yeasts, in their natural habitat, may often intimately co-exist with a wide diversity of algae, moulds, prokaryotes, protists, macroscopic and microscopic fauna, as well as plant roots.

Growth and survival of a particular yeast species in soil may therefore not solely depend on the intrinsic abilities of the yeast, but is the cumulative result of a number of interactions within the soil microbial community. With the previous considerations as a background the purpose of this study was to review our current knowledge on soil yeast ecology and to provide a base for further investigations into this largely unexplored field of study.

11.2 Soil as a Habitat

A review on the ecology of soil yeasts would be incomplete without a brief discussion on their habitat, in which these fungi co-exist with other organisms. Soils consist of mineral and organic particles forming heterogeneous aggregates of various sizes, which contain a complex network of pores (Gray and Williams 1979; Young and Crawford 2004). The latter may be filled with atmospheric gasses, water vapour or aqueous solutions of various salts. Characteristic of soil as a habitat are the remarkable spatial and temporal heterogeneities regarding physico-chemical properties including available nutrients, pore size, temperature and water availability. This results in similar heterogeneities in microbial numbers and diversity, as well as biological processes brought about by soil microbes playing a pivotal role in ecosystem function.

Within the ecosystem, organic carbon acting as nutrient source for soil-borne microbial decomposers ultimately originates from plants (Wardle et al. 2004). The activity of these decomposers in turn indirectly regulates plant growth and community composition by determining the supply of available soil nutrients to plants. Plants may also provide organic carbon directly to organisms in the rhizosphere, such as root herbivores, pathogens and symbiotic mutualists like mycorrhizal fungi. These root-associated organisms and their consumers influence plants directly. In addition, they also influence the flow of energy and nutrients between plants and the decomposers. Considering these interactions of plants with soil microbes, the findings that vegetation type, or even individual plants, may influence the composition of soil microbial communities (Saetre and Bååth 2000; Grayston et al. 2004) come as no surprise.

It must be remembered, however, that despite the carbon inputs originating from plants and deposited in soil as dead organic matter, the amounts of available nutrients in soils are limited (Gray and Williams 1971; Poindexter 1981; Williams 1985). Water extracts from soils may contain less than 2 and 5 $\mu g\ ml^{-1}$ amino acids and carbohydrates respectively (Ko and Lockwood 1976). It is therefore understandable that soils are generally viewed as being in a state of oligotrophy (Williams 1985).

Soil yeasts are known to be able to grow under oligotrophic conditions (Kimura et al. 1998), but are also isolated from relatively nutrient rich habitats such as the rhizosphere (Kvasnikov et al. 1975) and organic debris like dung and decaying toadstools (Fell and Statzell-Tallman 1998). However, considering the previously mentioned heterogeneity of soil as a habitat, it can be expected that yeast numbers and species would be unevenly distributed in soil and would show temporal variation, depending on ever-changing environmental conditions.

11.3 Yeast Distribution in Soil

It is commonly known that yeasts occur in a wide range of soil types from a vast diversity of geographical areas ranging from the artic zones to the tropics (Carmo-Sousa 1969; Phaff and Starmer 1987; Spencer and Spencer 1997; Lachance and Starmer 1998). Many different ascomycetous and basidiomycetous yeasts were found in soil. In most cases, however, yeast numbers and species composition were found to be distributed quite unevenly and the numbers of these fungi are low com-

pared with that of prokaryotes and moulds. The number of yeasts that mostly occur in the top 10 cm of soil as a result of their ability to grow aerobically on a wide diversity of carbon compounds may range from less than 10 to 10^6 culturable cells per gram of soil. It was found that nutrient-rich moist soil may support a wider diversity of yeast species than nutrient-poor arid soils (Spencer and Spencer 1997). About 25–50% of the yeasts in nutrient-rich moist soils were found to be able to ferment carbohydrates. More yeasts are usually found in the soil beneath plants bearing fruits rich in carbohydrates, since the latter may act as a nutrient-rich yeast inoculum when the spoiled fruit is deposited in the soil (Phaff et al. 1966). Soil further away from a plant usually contains fewer yeast species associated with that particular plant. This phenomenon was already demonstrated in the 1950s for oak and pine trees and the associated genera *Saccharomyces* and *Schizosaccharomyces* (Carmo-Sousa 1969). More recently it was demonstrated that *Saccharomyces cerevisiae* and *Saccharomyces paradoxus* can be isolated from oak-associated soils using enrichment culturing medium containing 7.6% (v/v) ethanol (Sniegowski et al. 2002).

Other yeast species, belonging to the genera *Cryptococcus*, *Debaryomyces*, *Lipomyces* and *Schizoblastosporion* were repeatedly isolated from various soils, indicating that the ecological niches of these fungi occur in soil (Phaff and Starmer 1987). The ability of these autochthonous soil yeasts to survive in this habitat was ascribed to a number of traits. For example, most of these yeasts possess a wide spectrum of metabolic activities enabling them to assimilate the hydrolytic products of plant materials, generated by moulds and prokaryotes. *Lipomyces* and *Williopsis saturnus* are able to produce resistant spores. Some like *Lipomyces*, *Cryptococcus* and *Rhodotorula* produce exopolymeric capsules and it was suggested that this would enable them to survive better in habitats that are poor in available nutrients. It was found that semi-arid soils, low in nutrients and moisture, were mostly populated by cryptococci and related basidiomycetous yeasts (Spencer and Spencer 1997). Interestingly, it was found that some soil cryptococci are able to produce extracellular polymeric substances (EPS) and form biofilms when cultivated in flow cells under oligotrophic conditions (Joubert et al. 2003). The formation of these biofilms is a known mechanism whereby microbes are able to sequester and concentrate nutrients while growing in low-nutrient environments (Decho 1990). Figure 11.1 illustrates the ability of a common soil inhabitant, *Cryptococcus laurentii*, to grow under oligotrophic conditions as a biofilm on soil particles such as sand grains.

11.4 The Role of Yeast Communities in Soil

11.4.1 Dissipation and Transformation of Energy Through the Ecosystem

Within a functional soil ecosystem, dissipation and transformation of energy continually occurs away from the primary producers, i.e. plants, into organisms farther along the food chain, thus supporting a wide diversity of heterotrophs, including microbes and macroscopic fauna (Coleman and Crossley 1996). The primary agents of decomposition, able to degrade compounds directly or indirectly derived from plants, were found to be bacteria and fungi. However, the yeasts are also part of the fungal domain and the vast majority of yeasts discovered so far are saprotrophs

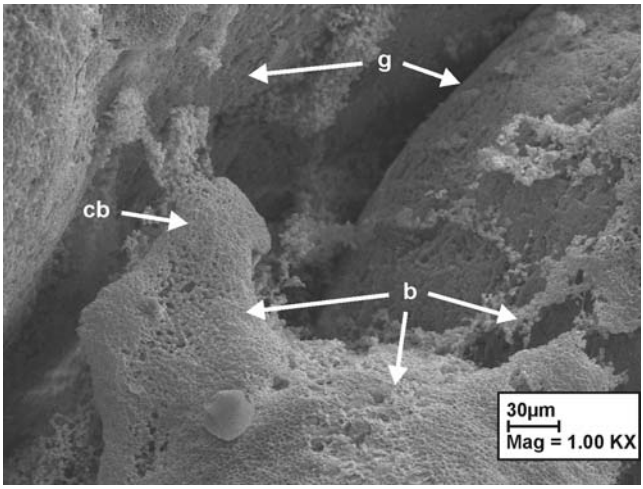

Fig. 11.1. Scanning electron micrograph illustrating biofilm formation (*b*) by *Cryptococcus laurentii* under oligotrophic conditions on sand grains (*g*), as well as a connective bridge (*cb*) formed between the sand grains as a result of excessive extracellular polymeric substances (*EPS*) production by the yeast. Sand grains with attached yeast cells were mounted onto stubs, sputter-coated with gold, and viewed unfixed and fully hydrated with a LEO 1430 VP scanning electron microscope operated at 7 kV. (Photograph; L. Joubert, Department of Microbiology, University of Stellenbosch)

contributing to mineralization processes in the environment by utilizing a wide range of organic carbon compounds. Some of these yeasts have the ability to ferment carbohydrates, but many are able to respire both carbohydrates and non-fermentable organic compounds (Kurtzman and Fell 1998a). The occurrence of autochthonous soil yeasts (Lachance and Starmer 1998) indicates that yeasts do play a role in the decomposition and dissipation of energy within the soil ecosystem as mentioned before for bacteria and fungi in general.

Most of the yeast species frequently encountered in soil (Table 11.1) are able to aerobically utilize L-arabinose, D-xylose and cellobiose. These carbohydrates are known to be products of the enzymatic hydrolysis of lignocellulosic plant materials by bacteria and moulds (Bisaria and Ghose 1981; Tomme et al. 1995). Some of the frequently encountered yeasts in soil were also found to assimilate intermediates of lignin degradation i.e. ferulic acid, 4-hydroxybenzoic acid and vanillic acid (Middelhoven 1993; Sampaio 1990). However, despite their ability to utilize the degradation products of woody material, it was suggested that soil yeasts do not play a major role in the decomposition of organic matter, because of their relatively low numbers compared with those of moulds and prokaryotes occurring in the same habitat (Phaff and Starmer 1987). Nevertheless, in some habitats, such as in the arctic zones, yeasts may be the dominant culturable soil microbes (Wynn-Williams 1982). This will enable them to make a significant contribution to decomposition of soil organic matter.

Table 11.1 Selected characteristics of the most abundant soil yeast species found during ecological surveys

Species according to Kurtzman and Fell (1998a)	Original identification during survey	Reference	Veg[a]	F[b]	Carbohydrate assimilation[c]			Aromatic compound assimilation[d]		
					xyl	ara	cel	pHA	VA	FA
Cryptococcus albidus	*Cryptococcus albidus/ diffluens/ terricolus*	Di Menna (1965); Bab'eva and Azieva (1980); Moawad et al. (1986); Polyakova et al. (2001)	F; G; T; V	–	+	+	+	+	+	+
Cryptococcus curvatus	*Candida curvata*	Di Menna (1965); Moawad et al. (1986)	F; G; V	–	+	v	+	+	–	–
Cryptococcus gastricus	*Cryptococcus gastricus*	Bab'eva and Azieva (1980)	T	–	+	+	+	+	–	–
Cryptococcus gilvescens	*Cryptococcus gilvescens*	Polyakova et al. (2001)	T	–	–	+	+	ND	ND	ND
Cryptococcus humicolus	*Candida humicola*	Di Menna (1965)	G; V	–	+	+	+	+	–	–
Cryptococcus laurentii	*Cryptococcus laurentii*	Bab'eva and Azieva (1980); Sláviková and Vadkertiová (2000)	F; T	–	+	+	+	+	–	–
Cryptococcus podzolicus	*Candida podzolica*	Bab'eva and Reshetova (1975)	V	–	+	+	+	–	–	–
Cryptococcus terreus	*Cryptococcus terreus*	Di Menna (1965)	G; V	–	+	+	+	+	+	v
Filobasidium uniguttulatum	*Cryptococcus uniguttulatus*	Bab'eva and Azieva (1980)	T	–	+	+	–	–	–	–
Cystofilobasidium capitatum	*Cystofilobasidium capitatum*	Sláviková and Vadkertiová (2000)	F	–	+	v	+	–	–	–
Leucosporidium scottii	*Leucosporidium scottii*	Sláviková and Vadkertiová (2000)	F	–	+	v	+	+	+	v

Continues

Table 11.1 Selected characteristics of the most abundant soil yeast species found during ecological surveys—*cont'd*

Species according to Kurtzman and Fell (1998a)	Original identification during survey	Reference	Veg[a]	F[b]	Carbohydrate assimilation[c]			Aromatic compound assimilation[d]		
Mrakia frigida	*Candida curiosa/gelida*	Bab'eva and Azieva (1980)	T	+	+	+	+	–	–	–
Rhodotorula aurantiaca	*Rhodotorula aurantiaca*	Sláviková and Vadkertiová (2000)	F	–	+	v	v	+	–	–
Rhodotorula glutinis	*Rhodotorula glutinis*	Moawad et al. (1986); Sláviková and Vadkertiová (2000)	F; V	–	v	v	v	+	+	v
Rhodotorula mucilaginosa	*Rhodotorula mucilaginosa*	Polyakova et al. (2001)	T	–	+	v	v	+	+	+
Schizoblastosporion starkeyi-henricii	*Schizoblastosporion starkeyi-henricii*	Di Menna (1965)	G	–	–	–	–	+	–	–
Sporobolomyces roseus	*Sporobolomyces roseus*	Polyakova et al. (2001)	T	–	v	v	v	+	+	+
Trichosporon cutaneum	*Trichosporon cutaneum*	Di Menna (1965); Sláviková and Vadkertiová (2000)	G; F; V	–	+	+	+	+	–	–

[a]General type of vegetation covering the soil that was sampled; *F* forest, *G* grass, *T* tundra, *V* various
[b]Ability of species to ferment carbohydrates according to Kurtzman and Fell (1998b); + able to ferment at least glucose, – unable to ferment carbohydrates
[c]Ability of species to aerobically assimilate carbohydrates according to Kurtzman and Fell (1998b); *xyl* D-xylose, *ara* L-arabinose, *cel* cellobiose, + positive, – negative, *v* variable
[d]Ability of species to aerobically assimilate aromatic compounds according to Middelhoven (1993) and Sampaio (1999); *pHA* *p*-hydroxybenzoic acid, *VA* vanillic acid, *FA* ferulic acid, + positive, – negative, *v* variable, *ND* not determined.

As part of the soil microbial biomass, yeasts may serve as a food source for bacteria and microbivorous eukaryotes. Invertebrate fauna such as micro-arthropods and nematodes, as well as protista are major soil predators (Bardgett and Griffiths 1997) and are known to graze upon soil yeast populations, thereby ensuring the continual dissipation and transformation of energy through the soil ecosystem. Both ascomycetous and basidiomycetous yeasts were digested in the gut of the soil millipede *Pachyiulus flavipes* (Byzov et al. 1998), while nematodes, belonging to the genera *Alaimus*, *Panagrellus* and *Rhabditis* were found to graze upon yeasts e.g. *S. cerevisiae* (Hechler 1970; Yeates 1971). Another soil nematode, *Caenorhabditis elegans*, was found to graze upon *C. laurentii* and *C. kuetzingii* (Nicholas 1984; Mylonakis et al. 2002). Under laboratory conditions, *C. elegans* was able to survive on both these yeast strains as the sole food source, maintaining brood sizes similar to when it is cultivated on *Escherichia coli*, the usual laboratory food source for this nematode.

Protista, of which the activity is limited to the water film within soil pores, may contribute up to 30% of the total net nitrogen mineralization that takes place in soil (Bardgett and Griffiths 1997). Although it is known that mycophagous protista do exist, very little is known about their interactions with yeasts. However, the fact that the majority of yeasts are found in the top cm of soil and that protists are more numerous in this part of the soil suggests that interactions between these two groups are inevitable within the natural soil environment. *Acanthamoeba* is a protist occurring in soil (Sawyer 1989) and it was found to feed on yeasts (Allen and Davidowicz 1990). Benting et al. (1979) found that when *Acanthamoeba polyphaga* was incubated with some strains of *Cryptococcus neoformans*, the amoeba was able to phagocytize and kill up to 99% of the yeast cells of within 9 days.

Yeasts were also found to be the nutrient source of bacteria, a number of Gram-positive bacteria isolated from soil were found to cause lysis of *S. cerevisiae* (Goto-Yamamoto et al.1993). It was also demonstrated that a group of Gram-negative bacteria commonly found in soil, the *Myxobacteria* (Reichenbach 1999), are able cause lysis of a wide diversity of yeasts including *Cryptococcus albidus*, *Filobasidium capsuligenum* (syn. *Candida japonica*), *Geotrichum candidum*, *Rhodosporidium toruloides, Rhodotorula glutinis* and *S. cerevisiae* (Yamanaka et al. 1993).

11.4.2 Dissolution of Rock and Release of Nutrients

It has also become apparent that some yeasts, because of their oligotrophic capabilities, may not only occur within soil, but are able to grow on rocks above ground (Sterflinger and Prillinger 2001; Burford et al. 2003). Such epilithic and endolithic fungi comprise a significant proportion of the microbial community in a wide range of rocks, including granite, gypsum, limestone, marble, sandstone and siliceous rock types such as silica, silicates and aluminosilicates. The yeasts and other fungi occurring in these rocks may contribute to weathering thereof, soil formation, as well as to the supply of soluble nutrients to the soil microbial community (Gadd and Sayer 2000; Burford et al. 2003). Dissolution of rock as a result of fungal metabolic activity may therefore result in phosphorous, sulphur and trace elements becoming bio-available. It was found that this dissolution is the result of the reactions of

metabolic end-products such as H^+ and organic acids, as well as siderophores. Solubilization of insoluble metal compounds in ores may therefore be the result of protonation of the anion of the metal compound, decreasing its availability to the cation. Sources of protons are the proton-translocating ATPase of the fungal plasma membrane and organic acids produced by these fungi. Soluble metal complexes may also be formed between organic acid anions and metal cations, depending on the relative concentrations of these ions, pH and the stability constants of the various complexes. Another mechanism whereby metals, specifically iron(III), are brought into solution and made bio-available is by reacting with low molecular mass (500–1,000 Da) iron-chelating compounds called siderophores. These ferric-specific ligands were found to be produced by a wide diversity of microbes (Neilands 1981), including basidiomycetous yeasts, such as *Leucosporidium scottii*, *Rhodotorula glutinis, R. mucilaginosa* and *Sporobolomyces roseus* (Atkin et al. 1970) which are commonly found in soil (Table 11.1).

Interestingly, both ascomycetous and basidiomycetous genera such as *Candida*, *Lipomyces, Rhodotorula* and *Trichoderma* were isolated from rock substrates (Sterflinger and Prillinger 2001; Burford et al. 2003), with euascomycetes taxa found in an even wider diversity of rock types. The latter taxa, which include meristematic fungi and the so-called black yeasts, may grow yeast-like in culture and represent the genera *Aureobasidium*, *Exophiala*, *Hormonema*, *Hortaea*, *Lecythophora*, *Phaeotheca*, *Rhinocladia* and *Sarcinomyces*. The melanized cell walls of these fungi, chlamydospore formation, as well as their clump- or yeast-like morphology with optimal surface-to-volume ratio, were found to make them well adapted for epilithic stress conditions, such as high levels of UV irradiation, large variations in temperature and available water, as well as low nutrient conditions (see Chap. 20).

11.4.3 Soil Aggregate Formation

Another way in which yeast populations may interact with their physico-chemical environment, and thus impact on biological processes in soil, is by producing EPS. Production of these extracellular compounds enables soil yeasts from the genera *Cryptococcus* and *Lipomyces* to play a role in the formation of soil aggregates, thereby impacting on soil structure (Bab'eva and Moawad 1973; Vishniac 1995). Figure 11.1 illustrates a connective bridge formed between two sand particles, as a result of cryptococcal EPS production, thereby contributing to aggregate formation. A high degree of aggregate stability and favourable soil structure was found to be associated with a decrease in erodibility, enhanced porosity and water-holding capacity, as well as improved soil fertility (Bronick and Lal 2004).

11.5. Ecological Interactions

11.5.1 Interactions with the Physico-Chemical Environment

Using sterile sandy loam soil as a growth medium it was demonstrated that the growth rate of the common soil yeast *C. albidus,* over the first 24 h after inoculation, was higher in the presence of 3–9% than in the presence of 1 or 2% added water

(Vishniac 1995). After monitoring yeast populations in different forest soils, seasonal changes in population sizes were partly ascribed to changes in soil moisture content (Sláviková and Vadkertiová 2000). Other factors are also known to impact on soil yeast populations. After sampling different soil types, it was found that a positive correlation (at 1% level) exists between soil yeast population size and both organic carbon (r=0.884) and organic nitrogen content (r=0.829) of the soil (Moawad et al. 1986). While no significant correlation was observed between the number of soil yeasts, soil $CaCO_3$ content, pH or soil texture. However, the study did not take into account the seasonal changes in the physico-chemical composition of the soils.

An investigation of the post-fire effects of vegetation covering different soils during a hot dry summer on the sizes of culturable yeast populations in the top 100 cm of soil revealed that yeast populations started to recover during the onset of the cooler, wetter weather of autumn (Cilliers et al. 2004). During this period the availability of macronutrients (P, K, Na, NO_3) was an important determinant of soil yeast population sizes. After further recovery during winter and spring, the most important determinants of soil yeast population sizes, however, were Mg, Na and soil texture.

Soil yeast populations, on the other hand, were also found to impact on the chemical composition of soils. Results indicated that nitrification of added ammonium, the hydrolysis of urea and the subsequent nitrification of released ammonium, all of which were stimulated after sugar beet emendation, may have largely been due to increased soil yeast numbers (Wainwright and Falih 1996). During these experiments representatives of *G. candidum* and *Williopsis californica* were found to increase in number in the emended soil.

Most of the previously mentioned studies on yeast interactions with their physico-chemical environment highlighted interactions of yeast populations in soil, rather than the interactions of individual species or strains in this habitat. The majority of studies conducted on the interactions of individual yeast strains with their physico-chemical environment were done in vitro mostly either to elucidate the physiology of the yeast cell while growing in monoculture (Rose and Harrison 1987) or to classify pure yeast cultures during taxonomic investigations (Kurtzman and Fell 1998a). These studies provided insight into the intrinsic abilities of particular species and were essential in the rapid development of yeast biotechnology. In addition, the studies highlighted the diversity of yeast species occurring in nature. During the course of these studies, however, in vitro investigations into cardinal growth temperatures, nutrient assimilation and antibiotic resistance may have provided only limited insight into the ecological niche of certain yeast species (Lachance and Starmer 1998). For example, while the ability of *Cryptococcus podzolicus* to assimilate carbohydrate products of the enzymatic hydrolyses of lignocellulosic plant materials (Table 11.1) may explain its presence in soil containing decaying plant material, the inability of this species to utilize monomeric aromatic acids associated with podzolic soils (Lunström et al. 2000) as sole carbon sources (Sampaio 1999) does not explain its presence in these soils (Bab'eva and Rheshetova 1975). Similar anomalies were uncovered regarding the ability of soil yeasts in their natural habitat to respond to perturbations with heavy metals. During enumeration of yeasts in virgin soil with a relatively low natural Cu content (approximately 2 mg kg^{-1} Cu), plate counts were dominated by hymenomycetous strains able to tolerate

Cu levels of up to 500 mg l^{-1} Cu in a liquid medium (Cornelissen et al. 2003). When 1,000 mg kg^{-1} Cu was added to the soil, a shift in the soil yeast community occurred, resulting in urediniomycetous strains, with similar high Cu tolerance levels, dominating the enumeration plates. The survival and growth of a particular yeast strain in soil therefore seems not to depend solely on the intrinsic abilities of the particular strain to maintain itself within the chemical environment. Other factors, such as interactions with members of the soil microbial community, may also be determinative factors in shaping the species composition of soil yeast populations.

11.5.2 Interactions with Soil Organisms

A list of the possible microbe–microbe interactions that terrestrial yeasts may participate in was provided by Lachance and Starmer (1998). These included amensalism, commensalism, competition, mutualism or symbiosis, neutralism, parasitism, predation and synergism. Taking the wide diversity of organisms into account that intimately coexist with soil yeasts in their natural habitat, it is conceivable that at a particular time a yeast in its ecological niche may participate in more than one of these microbe–microbe interactions. In addition, these yeasts are known to be grazed upon by soil fauna and protists. Therefore, to study a single interaction between two soil organisms in their natural habitat would seem to be a daunting task. Nevertheless, evidence for the intrinsic ability of some soil yeasts to participate in a number of these interactions was uncovered in different studies.

5.2.1 Amensalism

It was found that a yeast commonly found in soil, *C. laurentii*, is able to inhibit growth of filamentous fungal post-harvest pathogens on damaged fruit (Roberts 1990; Chand-Goyal and Spotts 1997). Environmental conditions, such as temperature (Roberts 1990) and available nutrients in agar plates (own unpublished results), were found to impact on the antagonistic effect of *C. laurentii* on filamentous fungi. No in situ experiments on yeast antagonism, however, have been conducted in bulk soil in the absence of plant roots. Also, the exact mode of action of this antagonistic effect of yeasts is still unclear, although it has been suggested that amensalism (chemical interference) such as killer activity or the production of enzymes, e.g. glucanases, may play a role (Roberts 1990; Fredlund et al. 2002; Masih and Paul 2002). In addition, the antagonistic effect has been ascribed to nutrient competition.

5.2.2 Competition

Considering the oligotrophic state of most soils (Williams 1985) the environmental factors most likely to be in short supply, and hence the objects of competition, are carbon and nitrogen sources (Lockwood 1992). Competition for ferric iron was also found to occur quite commonly in soil. It is known that such competition for nutrients impacts on the species composition of soil microbial communities. For example, it was demonstrated that yeasts such as *Candida tropicalis* emerge as dominant *n*-alkane-utilizing microbes when soil microcosms containing inocula from petro-

leum contaminated soil were treated with a mixture of n-alkanes (Schmitz et al. 2000). Competition experiments in soil microcosms with *n*-alkane-utilizing microbes from different culture collections confirmed that yeasts overgrow bacteria in sandy soil. This was partly ascribed to the acidification of the soil as a result of yeast metabolic activity, because when bentonite, a clay mineral with high ion-exchange capacity, was added to the soil, yeasts and bacteria belonging to the genus Pseudomonas coexisted in similar numbers. It was also found that *n*-alkane-utilizing yeast strains from culture collections, representing different yeast species, showed different levels of competitiveness in these *n*-alkane-treated microcosms. Strains of *Arxula adeninivorans*, *Candida maltosa* and *Yarrowia lipolytica* overgrew strains of *Candida shehatae*, *C. tropicalis* and *Pichia stipitis*. However, *C. maltosa* and *Y. lipolytica* were able to coexist in equal number within these soil microcosms.

5.2.3 Predation

While studying binary interactions of predatory yeasts with their prey on agar plates, it was found that some *Saccharomycopsis* strains are able to prey on a range of ascomycetous and basidiomycetous yeasts by forming infection pegs to penetrate and kill these organisms (Kreger-van Rij and Veenhuis 1973; Lachance and Pang 1997; Lachance et al. 2000). It can therefore be assumed that such interactions will also occur in soil under the appropriate environmental conditions, especially since two of the *Saccharomycopsis* species associated with predation, i.e. *S. fermentans* and *S. javanensis*, have been isolated from soil (Kurtzman and Smith 1998).

5.2.4 Synergism and Antagonism Regarding Nematodes

Recently a synergistic relationship was discovered involving ethanol production by *S. cerevisiae* resulting in enhanced growth of *Acinetobacter*, a bacterium which may occur in the same ecological niche as the yeast (Smith et al. 2004). The ethanol was found to also enhance pathogenicity of the bacterium towards the soil-borne predator *C. elegans*. It was suggested that by producing ethanol the yeast may therefore be able to indirectly reduce the numbers of its predator.

When preyed upon some yeast species may also exert a lethal effect directly on their predator. For example, when *C. neoformans*, a yeast periodically isolated from soil, was used as a food source for the soil nematode *C. elegans,* it caused distention of the nematode intestine when ingested (Mylonakis et al. 2002). The lethal effect was ascribed to the yeast capsule, as well as to a series of genes associated with mammalian virulence. However, killing of the nematode by an acapsular strain of *C. neoformans* was also observed. It was speculated that the nematode was killed either by toxins produced by the yeast, or by toxic components released during degradation of the cell wall within the nematode.

11.6 Yeasts Associated with the Rhizosphere

The rhizosphere is the narrow zone of soil extending for a few millimetres from the plant root surface out into the surrounding soil (Huang and Germida 2002). It may

also be considered as an interface through which energy is channelled away from the plant to the soil biota of the belowground ecosystem, because up to 18% of the carbon assimilated during photosynthesis is released from the roots. This may result in larger microbial populations in the rhizosphere than in the bulk soil away from the roots. As a result of root metabolic activity the chemical characteristics of the rhizosphere, such as pH, redox potential, as well as concentration and composition of organic compounds, may differ from those of the bulk soil. It was also proposed that, depending on soil conditions, the rhizosphere may select for specific microbial populations. This phenomenon was demonstrated when 18S ribosomal DNA (rDNA) fragments, that were amplified from soil and maize rhizosphere DNA, were cloned and sequenced (Gomes et al. 2003). On the basis of the sequence similarity of these rDNA fragments with known 18S rDNA sequences in a database, it was concluded that the rhizospheres of young maize plants seemed to select for *Ascomycetes* belonging to the order *Pleosporales*, while different members of the *Ascomycetes*, as well as basidiomycetous yeasts were detected in the rhizospheres of senescent maize plants. A group of filamentous fungi commonly associated with the rhizosphere and known to form mutualistic symbioses with plants is mycorrhizal fungi (Huang and Germida 2002). These fungi may facilitate uptake of up to 80% of the phosphorous and 25% of the nitrogen requirements of the host plant (Marschner and Dell 1994). In turn the plant provides photosynthate for the fungus. This nutrient exchange takes place via specialized fungal structures intimately associated with host root cells. However, a wide diversity of other microbes also occur in the rhizosphere; some are known to fix N_2, some are known to degrade complex organic compounds, thus participating in soil mineralization processes, while the ecological role of others has not been fully established yet.

A variety of both ascomycetous and basidiomycetous yeasts were isolated from the rhizosphere (Table 11.2). If the redox potential in the rhizosphere should decrease as a result of water logging (Huang and Germida 2002) some of these yeasts will still able to grow as result of a fermentative metabolism. It was found that, similar to the microbial populations in general, larger yeast populations normally exist in the rhizosphere than in the bulk soil away from the roots (Moawad et al. 1986). Root exudates containing a variety of potential yeast carbon and nitrogen sources (Table 11.3) may contribute to the growth and maintenance of yeast populations on or near the roots. As the chemical profiles of these exudates differ with plant species, genotypes and growth conditions (Fan et al. 2001) it may be assumed that these differences impact on the species composition of the yeast community in the rhizosphere. Figure 11.2 illustrates a yeast microcolony on the rhizoplane of a sorghum seedling growing in washed sand containing no carbon sources other than those provided by the plant. Figure 11.3 illustrates the relative abundance of yeasts in the rhizosphere and in the bulk soil away from the roots.

Although the biological interactions of the rhizosphere yeasts are largely unknown, it can be assumed that similar interactions would occur in the rhizosphere as discussed before. It was found that inoculation of legumes with viable *S. cerevisiae* increased nodulation and arbuscular mycorrhizal (AM) fungal colonization (Singh et al. 1991). Later, hyphal growth of the AM fungus *Glomus intraradices* colonizing cucumber roots was found to be enhanced by the presence of baker's dry yeast

Table 11.2 Some yeasts species that were found to occur in the rhizosphere during a number of surveys

Species according to Kurtzman and Fell (1998a)	Original identification during survey	Reference	F[a]
Bullera species	*Bullera* species	De Azeredo et al. (1998)	–
Candida azyma	*Candida azyma*	De Azeredo et al. (1998)	–
Candida krusei	*Candida krusei*	Kvasnikov et al. (1975)	+
Candida maltosa	*Candida maltosa*	De Azeredo et al. (1998)	+
Cryptococcus albidus	*Cryptococcus albidus/diffluens*	Kvasnikov et al. (1975); Moawad et al. (1986); de Azeredo et al. (1998)	–
Cryptococcus curvatus	*Candida curvata*	Moawad et al. (1986)	–
Cryptococcus humicolus	*Candida humicola*	Moawad et al. (1986)	–
Cryptococcus laurentii	*Cryptococcus laurentii*	Kvasnikov et al. (1975); de Azeredo et al. (1998)	–
Debaryomyces hansenii	*Debaryomyces hansenii/kloeckeri*; *Torulopsis famata*	Moawad et al. (1986); de Azeredo et al. (1998)	v
Debaryomyces polymorphus	*Debaryomyces phaffii/cantarelli*	Kvasnikov et al. (1975)	+
Debaryomyces vanrijiae	*Debaryomyces vanriji*	Kvasnikov et al. (1975)	v
Fellomyces species	*Fellomyces* species	De Azeredo et al. (1998)	–
Hanseniaspora uvarum	*Hanseniaspora apiculata*	Kvasnikov et al. (1975)	+
Leucosporidium scottii	*Leucosporidium scottii*	De Azeredo et al. (1998)	–
Metschnikowia pulcherrima	*Metschnikowia pulcherrima*	Kvasnikov et al. (1975)	+
Pichia guilliermondii	*Pichia guilliermondii*	De Azeredo et al. (1998)	+
Rhodotorula glutinis	*Rhodotorula glutinis*	Moawad et al. (1986); de Azeredo et al. (1998)	–
Rhodotorula minuta	*Rhodotorula minuta*	De Azeredo et al. (1998)	–
Rhodotorula mucilaginosa	*Rhodotorula mucilaginosa*	De Azeredo et al. (1998)	–
Torulaspora delbreuckii	*Torulaspora delbreuckii*	De Azeredo et al. (1998)	+
Tremella mesenterica	*Tremella mesenterica*	De Azeredo et al. (1998)	–
Trichosporon cutaneum	*Trichosporon cutaneum*	Kvasnikov et al. (1975)	–
Saccharomyces cerevisiae	*Saccharomyces cerevisiae*	De Azeredo et al. (1998)	+
Williopsis californica	*Hansenula californica*	Kvasnikov et al. (1975)	+
Williopsis saturnus	*Hansenula saturnus*	Kvasnikov et al. (1975)	+

[a]Ability of species to ferment carbohydrates according to Kurtzman and Fell (1998b); + positive, – negative, *v* variable

Table 11.3 Some components of root exudates that were found during different surveys (Campbell et al. 1997; Fan et al. 2001; Huang and Germida 2002)

Amino acids	Carboxylic acids	Carbohydrates	Phenolic acids	Mucilage	From sloughed-off cells
Alanine γ-Aminobutyric acid Asparagine Aspartic acid Glutamic acid Glutamine Glycine Isoleucine Leucine Phenylalanine Proline Serine Threonine Valine	Acetic acid *trans*-Aconitic acid Butyric acid Citric acid 2-Deoxymugineic acid Formic acid Fumaric acid Lactic acid Malic acid Malonic acid Oxalic acid Propionic acid Pyruvic acid Succinic acid Tartaric acid	Arabinose D-Fructose D-Galactose D-Glucose Maltose D-Raffinose Ribose L-Rhamnose Sucrose Trehalose Xylose	Caffeic acid Chlorogenic acid Coumaric acid Ferulic acid Hydroxyl benzoic acid Protocatechuic acid	Polygalacturonic acids	Plant cell lysates

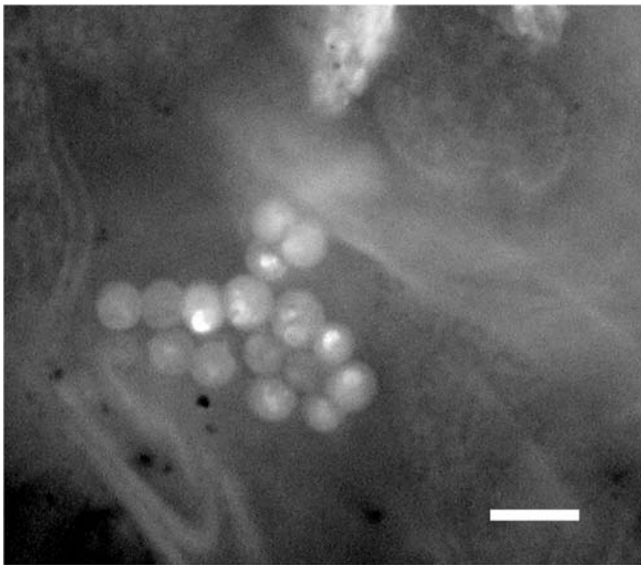

Fig. 11.2. Epifluorescence micrograph of yeast microcolony on the rhizoplane of a 2-week-old sorghum seedling growing in a medium of washed sand with no additional carbon source. Yeasts were visualized after staining with the fluorescent probes FUN 1 and Calcofluor White M2R (both from Molecular Probes). FUN 1 is a yeast-viability stain, marking intravacuolar structures of metabolically active cells. Calcofluor is a UV-excitable dye which labels yeast cell walls. The *bar* represents 10 μm. (Photograph; L. Joubert, Department of Microbiology, University of Stellenbosch)

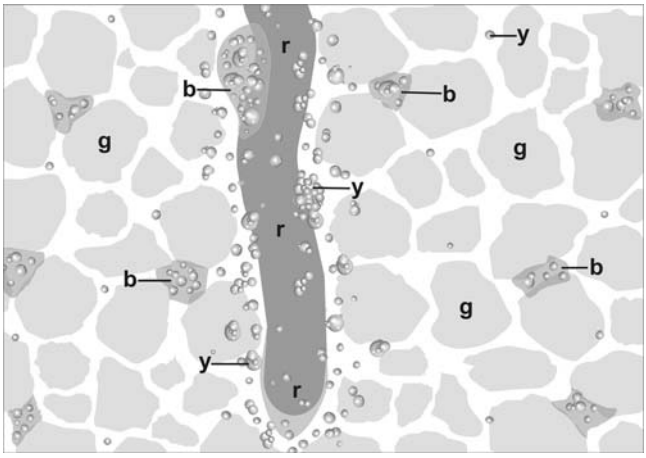

Fig. 11.3. A simplified illustration demonstrating only the distribution of soil yeasts (y) in relation to a plant root (*r*), omitting the remainder of the soil microbial community. More yeasts are found in the rhizosphere close to the root than further away from the plant in the bulk soil, where nutrients are less readily available. Yeasts in the latter nutrient-poor region are usually able to form biofilms (*b*) enabling them to sequester nutrients. These EPS-producing yeasts play a role in soil aggregate formation by binding soil particles (*g*) together

(Ravnskov et al. 1999). Uptake of phosphorous by the AM fungus, however, was unaffected by the yeast. When the ascomycetous yeast *Y. lipolytica* and *Glomus deserticola*, both entrapped in alginate gel, were used as an inoculum for tomato plants growing in soil microcosms enriched with rock phosphate, the yeast cells stimulated the level of mycorrhizal-root colonization (Vassilev et al.2001).

The basidiomycetous soil yeasts were also found to enhance AM fungal growth (Fracchia et al. 2003). *R. mucilaginosa* stimulated AM hyphal lengthening during in vitro spore germination of *Glomus mosseae* and *Gigaspora rosea*. This stimulatory effect on AM hyphal lengthening was also observed when exudates of the yeast produced in liquid culture were added to the spores. In addition, in glasshouse experiments it was found that these exudates increased AM colonization of soybean by *G. mosseae* and red clover by *G. rosea*. These results on the stimulatory effects of yeast exudates on AM fungi were later confirmed when the effects of *C. laurentii, R. mucilaginosa* and *S. kunashirensis* were studied on *G. mosseae* in similar in vitro and glasshouse trials (Sampedro et al. 2004). It was found that the presence of the yeasts, or their soluble and volatile exudates, stimulated the percentage spore germination and hyphal growth of the AM fungus. The percentage root lengths colonized by this AM fungus and plant dry matter of soybean were increased only when the soil yeasts were inoculated prior to the AM fungus.

Recently it was found that basidiomycetous yeasts, such as *Cryptococcus* and *Dioszegia* species, occur on AM fungal spores in soil, as well as on roots colonized with these fungi (Renker et al. 2004). The type of interaction that exists between these yeasts and mycorrhizal fungi is still unknown. Other yeasts isolated from the rhizosphere, i.e. *Candida valida*, *R. glutinis* and *Trichosporon asahii*, were found to be antagonistic towards growth of the fungal root pathogen *Rhizoctonia solani* (El-Tarabily 2004). The antagonistic effect of *C. valida* was ascribed to β-1,3-glucanase activity, of *R. glutinis* to the production of inhibitory volatiles and of *T. asahii* to the production of diffusible antifungal metabolites. It was also found that these yeasts were able to colonize sugar beet roots and protect the seedlings and mature plants from *R. solani* diseases in glasshouse trails. Interestingly, the three yeasts exerted a synergistic effect on disease suppression and promoted plant growth. The latter was ascribed to the production of indole-3-acetic acid and gibberellic acid by the yeast isolates used in the study. The study indicated that yeasts have the potential to be used as biological fertilizers.

11.7 Conclusion

Studies on yeast interactions were conducted on only a minute fraction of the vast diversity of soil yeasts. It can therefore be anticipated that when more yeast species are discovered and studied, more types of interactions will be uncovered and the role of yeasts in the soil ecosystem will be further elucidated. A challenge however, will be to determine the relative contribution of soil yeasts to ecological processes known to involve a wide range of soil organisms. Also, the fate of individual strains or species within a natural soil ecosystem needs to be studied. New technological advances in the field of rDNA sequence analyses of the total soil community DNA (Hunt et al. 2004), as well as fluorescence in situ hybridization (Spear et al. 1999),

make future studies of soil yeasts in their natural habitats an exciting possibility. Considering the enormous potential effect of soil yeasts on, for example crop performance, further investigation into this yet largely unexplored realm is expedient.

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Yeast Biodiversity in Freshwater, Marine and Deep-Sea Environments

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12.1 Introduction

Many investigators have reported a variety of yeasts in substrates such as water, sediment, plants, animals and other organic matter in aquatic environments, including rivers (Spencer et al. 1970, 1974; Simard and Blackwood 1971a, b; Kvasnikov et al. 1982; Sláviková and Vadkertiová 1997; Libkind et al. 2003), lakes and ponds (van Uden and Ahearn 1963; Spencer et al. 1974; Kwasniewska 1988; Sláviková et al. 1992; Sláviková and Vadkertiová 1995; Boguslawska-Was and Dabrowski 2001; Lahav et al. 2002; Libkind et al. 2004), estuaries, coasts, and mangrove areas (Goto et al. 1974a; Hagler and Mendonça-Hagler 1979; Hagler and Mendonça-Hagler 1981; Boiron and Agis 1982; Hagler et al. 1982, 1993; de Araujo et al. 1995; Soares et al. 1997; Gadanho and Sampaio 2004), oceans and the deep sea (Goto et al. 1974b; Yamasato et al. 1974; Fell 1976; Kohlmeyer and Kohlmeyer 1979; Hernandez-Saavedra et al. 1992; Nagahama et al. 1999, 2001b, 2003b; Gadanho et al. 2003). The accomplishments before 1987 have been expatiated upon in some reviews (Kriss 1963; Kriss et al. 1967; Morris 1968, 1975; van Uden and Fell 1968; Fell 1976; Hagler and Hagler 1978; Kohlmeyer and Kohlmeyer 1979; Sieburth 1979; Hagler and Ahearn 1987). In this paper, I will try to summarize the advances in the ecological and taxonomical study of aquatic yeasts during the last 2 decades, and will place in that context our investigation of yeasts taken from deep-sea floors in the Pacific Ocean.

12.2 Sampling and Isolation

The sampling methods for yeast isolation do not differ fundamentally from those used for bacteria. However, because the frequency of yeasts in natural aquatic environments is lower than that of bacteria, especially in places with low nutrient conditions, a higher volume of samples is required. Sterile bottles, boxes or containers have been used in various samplings from such accessible sites as the surface or

shallow regions of marine and freshwater environments. Whereas this sampling method is relatively easy to implement, sampling from offshore and deep-sea regions involves higher expenses and more complicated equipment. For water sampling, some known samplers are the Nansen bottle, the Niskin sampler and the van Dorn sampler. In order to avoid contamination, sterile samplers, such as the Niskin biosampler (or some modified form of it), can be used, as formerly discussed by Fell (1976). The J-Z sampler (or some modified form of it) has also been used frequently in bacterial studies. Yeasts in aquatic sediments have been studied less (Hagler and Ahearn 1987). The sampling of deep-water sediments has normally been performed with core samplers, though there is the risk of contamination. We have developed some samplers for the aseptic sampling of deep-sea sediments. A primitive version of this sterile sediment sampler is shown in Fig. 12.1 (Ikemoto and Kyo 1993); it can be incorporated into a manned or an unmanned submersible and can be handled by the manipulators (Fig. 12.1). However, the amount of sediment obtained through this method (maximum 50 ml) is too small to allow the isolation of yeasts, because this sampler was originally designed for bacterial sampling. Later, a second version was produced which could sample 10 times as large a volume as the primitive version; however, it was too heavy and bulky. The more advanced systems were capable of sampling water (Jannasch and Wirsen 1977; Jannasch et al. 1982) or sediment (Kyo et al. 1991) while retaining the hydrostatic pressure and low temperature characteristic of deep-sea environments, but their sampling capacity was very low.

The isolation procedure varies depending on yeast density, the volume and shape of the source, and the source itself (water, sediment, plant or animal material). Yeast cells in water samples were mostly filtrated through membranes and then used for isolation, because of the low number of yeast cells, especially from open and deep-sea waters. The solid sources, such as plants, animals and sediments from polluted

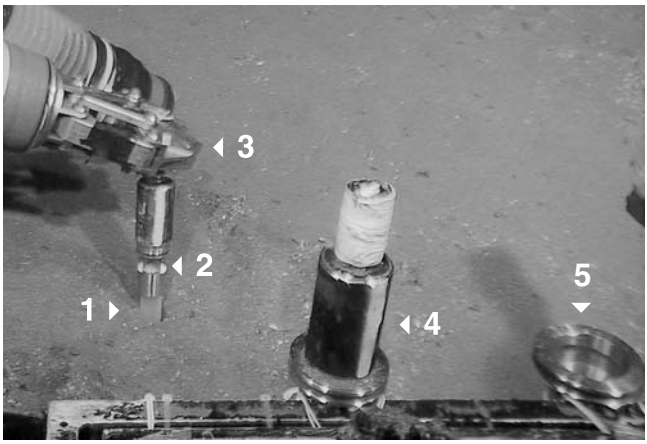

Fig. 12.1. Sterile sediment samplers. Each *numbered arrow* indicates the following: *1* a 50-ml polypropylene tube for retrieving sediment; *2* the entry point for sediment; *3* a manipulator of the submersible; *4* a sediment sampler in a sealed outer bracket; *5* an outer bracket

environments, which usually carry denser yeast populations, can be applied to the agar plate media directly or after being broken into pieces. These samples may be washed with water, and then the suspended cells in the water can be collected by filtration. Instead of filtration, organic contents, including yeast cells, have also been collected from water by using a precipitant (Sláviková and Vadkertiová 1995, 1997). When the sampling volume is too low owing to the capacity of the sampler, the sample can be enriched, but it should be noted that enrichment often changes the proportion of the yeast population and occasionally leads to a disappearance of the minority species or the slower-growing species. On the other hand, some enrichment media used for isolating specific yeast species from marine environments have been reported (Yanagida et al. 2002).

Because our ecological knowledge of the whole range of yeasts is very limited and many niches in aquatic environments have remained unexplored, it may be worth considering to attempt various new isolation media and conditions. The selection of media for isolating yeast strains from aquatic environments is important. Nevertheless, the media currently used are not so different from those used for terrestrial yeasts, even though aquatic environments generally contain lower organic nutrient concentrations than terrestrial environments. Most investigators prefer organic media mainly consisting of malt extract, yeast extract, peptone and glucose (Hagler and Ahearn 1987). Gadanho and colleagues (Gadanho et al. 2003; Gadanho and Sampaio 2004) employed media with MYP (0.7% malt extract, 0.05% yeast extract, 0.25% soytone) agar, broth, and no added glucose (Bandoni 1972). We also tried different media, but now we usually use five kinds of agar: yeast extract–malt extract (YM) agar, tenfold diluted YM agar, potato–dextrose agar, corn-meal agar and marine agar (all Difco), with 0.01% chloramphenicol. A new species of the genus *Cryptococcus* (Nagahama et al. 2003b) and a possibly an undescribed species of the genus *Dipodascus* (unpublished data) have been obtained as a result of cultivation in media unusual for yeast isolation, such as corn-meal and marine agar 2216. Seawater or sodium chloride was usually added, although this is not essential for the reproduction of most yeast species. But a marine yeast species which requires seawater for reproduction has been reported (Tong and Miao 1999). The temperature and pH of the media are often adjusted to prevent the growth of bacteria and filamentous fungi as competitors against the yeasts, as reviewed in many articles (Morris 1968, 1975; Fell 1976; Hagler and Hagler 1978; Hagler and Ahearn 1987). Recent investigators prefer to add chloramphenicol as an antibiotic, in concentrations sufficient to suppress the coexistent bacterial population (Sláviková and Vadkertiová 1995; Nagahama et al. 2001b, 2003b; Gadanho et al. 2003; Gadanho and Sampaio 2004); another method is acidification of the medium (Hagler et al. 1993; de Araujo et al. 1995; Soares et al. 1997; Libkind et al. 2003).

12.3 Identification and DNA Detection

Yeasts occurring in marine and other aquatic environments in high concentrations often comprised basidiomycetous yeasts. This made identification troublesome, owing to the unstable reactions in the assimilation tests and the lack of morphological information on the sexual reproduction of many basidiomycetes. But such

problems have been overcome with the progress of molecular taxonomy in the last 2 decades. The efficiency of using nucleotide sequences to identify marine yeasts was demonstrated in early reports (Fell and Kurtzman 1990; Fell et al. 1992). The extensive application of nucleotide sequences from the D1/D2 region of the large subunit of the ribosomal RNA gene (26S rDNA) and from the internal transcribed spacer (ITS) regions between the 18S rDNA, 5.8S rDNA and 26S rDNA domains vastly facilitated the identification of yeasts, which led to the discovery of novel yeast species (Kurtzman and Robnett 1998; Fell et al. 2000; Scorzetti et al. 2002). Combined analyses with other molecules may further improve the phylogenetic reliability of identifications within a taxonomic group if the issue is unresolved based on the D1/D2 or/and ITS sequences (Kurtzman and Robnett 2003). Molecular taxonomic identification has the advantage of giving scalable genetic distances, as opposed to conventional identification based on morphological and physiological characteristics. Conserved gene sequences such as 18S rDNA, 26S rDNA, translation elongation factor 1-α (EF-1α), or RNA polymerase II (RPB2) are useful for the inference of higher relationships, whereas the rapidly substituted molecules, such as ITS or intergenic spacers (IGS) between 26S rDNA and 18S rDNA, can be used to estimate the relationships between closely related species or the intraspecific variation between strains with different geographic origins. This method can successfully discriminate aquatic species or strains from terrestrials, the two never having been distinguishable phenotypically.

In contrast, small- or single-molecule sequence data are prone to give wrong or ambiguous results. In this, morphological, physiological, biochemical and molecular fingerprinting analyses, which are often called polyphasic taxonomic approaches (Vandamme et al. 1996), can lend added support. In such analyses, fingerprinting techniques such as those based on restriction fragment length polymorphisms (RFLPs) (Mantynen et al. 1999; Montes et al. 1999; Villa-Carvajal et al. 2004) and microsatellite-primed PCR (Gadanho et al. 2003; Libkind et al. 2003) are often performed prior to sequencing, with the aim of avoiding sequencing redundancy and minimizing the expenditure of reagents. These procedures should be considered if it is observed that identical strains tend to appear repeatedly in numerous isolates.

The traditional isolation approaches mentioned before depended on cultivation in the laboratory, and it was often not possible to grow and isolate the organisms in proportions reflecting their natural communities. On the other hand, a complete DNA-sequence database for yeasts enables the investigation of the microbial community and the identification of component species by means of direct DNA extraction from environmental samples. This cultivation-independent analysis has successfully detected uncultured prokaryotic microorganisms (Ward et al. 1990) and fungi in the soil and the rhizosphere (Crowe 2002; Anderson and Cairney 2004). In contrast to the microorganisms mentioned before, the direct analysis of yeasts from sources in marine and other aquatic environments is just beginning (Gadanho and Sampaio 2004), probably because uncultivable yeasts were not expected to be found in these environments. In the study of Gadanho and Sampaio, fingerprinting was performed by means of temperature-gradient gel electrophoresis and clone sequencing of the fungal PCR amplicon of 26S rRNA, from the DNA samples extracted directly or via enrichment from the estuarine water in Portugal. These approaches

may assist the discovery of new marine or other aquatic yeasts as well as their ecological niches.

Some new inexpensive techniques for examining the specific abundance of yeast species have been reported. In order to detect rapidly and specifically such clinically important species as *Candidas*, quantitative PCR has been utilized (Brinkman et al. 2003; Filion et al. 2003). A hybridization macroarray assay has been developed for the rapid identification of yeasts occurring frequently in marine environments (Kiesling et al. 2002).

12.4 Yeast Distribution and Biodiversity in Aquatic Environments

12.4.1 Cell Density

Water is common as a source to isolate yeasts from either freshwater or marine environments (Kohlmeyer and Kohlmeyer 1979). This may indicate that the main interest of many investigators has been water pollution. Yeast populations in fresh water are denser than in marine water (Hagler and Ahearn 1987), but the density varies with the substrate and the pollution level. Yeast densities are known to be below 10 cells/l in open ocean water and below 100 cells/l in unpolluted lakes and coasts. Lower yeast counts have been observed in an inaccessible lake in Patagonia, Argentina (Libkind et al. 2003). The exceptional increase of the cell counts in oceanic water is often associated with increased concentrations of invertebrates or plankton blooms, and may exceed 3×10^3/l (Fell 1976). The total yeast count usually increases with increasing pollution, and occasionally exceeds 2×10^8 cells/l (in sewage). A great variation in the number of yeast cells with depth was observed in the Gulf Stream (Fell and van Uden 1963). The number of yeasts in seawater above the Álvares Cabral Trench averaged about 5.4 cells/l and varied with depth (Gadanho et al. 2003).

In sediments, cell counts above 100/g have been reported in polluted areas (Hagler and Ahearn 1987). The yeast-cell frequency in sediment depends on the type of sediment (Fell et al. 1960). In our research of deep-sea floors, no yeast colony has been discovered so far in sandy sediments, except in hydrothermal vent areas. Hagler and colleagues have extensively surveyed the various substrates in the polluted estuaries of Sepetiba Bay, Rio de Janeiro, Brazil. The yeast population at the beach was 3.7×10^3 most probable number (MPN) cells/l in water, whereas the density in mud sediments was about 20 times higher, and that in shrimp intestines was more than 400 times higher than that in water, at the same site (Pagnocca et al. 1989). The average MPN count in invertebrates was about 37 times higher than that in water (de Araujo et al. 1995). In intertidal estuary sediments, the MPN counts were about 20–30 times higher than in the water (Soares et al. 1997). In contrast, some researchers reported that there were fewer yeast cells in invertebrates than in the surrounding sediments (Volz et al. 1974; Hagler and Ahearn 1987). It is possible that the obligatory localization of yeast vegetation in/on invertebrates depends on a certain association between the species and the environmental situation. Yeast counts in the water of mangrove bromeliads were from 10^4 to 10^5 cells/l (Hagler et al. 1993).

12.4.2 Fresh Water

Perfectly natural and unpolluted aquatic sources are almost impossible to find on Earth, and sampling while avoiding as far as possible the effects of human activity is difficult and usually expensive. In the past 2 decades, yeast communities in freshwater environments have been studied mostly in association with polluted water. Studies on freshwater yeasts have been focused mostly on their application as organic pollution indicators. Freshwater rivers and lakes in Europe, recreational lakes (Sláviková et al. 1992), fish ponds (Sláviková and Vadkertiová 1995), rivers (Sláviková and Vadkertiová 1997) and lagoons (Boguslawska-Was and Dabrowski 2001) were found to host mainly the genera *Candida*, *Cryptococcus*, *Pichia* (*Hansenula*) and *Rhodotorula*. Some species in these genera are regarded as bioindicators of the level of pollution (Dynowska 1997). Common species are *Aureobasidium pullulans*, *Cryptococcus albidus*, *Cr. laurentii*, *Debaryomyces hansenii* (*C. famata*) and *Rhodotorula mucilaginosa* (*Rh. rubra*) (Table 12.1). The black yeast

Table 12.1 Twenty species frequently isolated in four studies at European freshwater sites

Phylum	Species	Frequency (%)			
		Origins[a]			
Ascomycota	*Aureobasidium pullulans*	17.5	27.8	8.0	1.1
	Saccharomyces cerevisiae	–	2.0	17.3	7.8
	Pichia anomala	14.7	6.3	1.7	–
	Issatchenkia orientalis/ Candida krusei	11.2	2.2	6.6	–
	Debaryomyces hansenii/ Candida famata	0.6	0.9	1.4	11.5
	Pichia fermentans/Candida lambica	7.5	0.7	4.5	–
	Pichia guilliermondii/Candida guilliermondii	11.2	1.3	–	–
	Pichia burtonii	4.2	3.4	1.4	–
	Candida maltosa	–	–	8.0	–
	Geotrichum capitatum	–	–	6.9	–
	Candida sake	–	2.3	–	4.1
	Galactomyces geotrichum/ Geotrichum candidum	3.3	2.5	–	–
Basidiomycota	*Rhodotorula glutinis*	8.8	–	7.3	36.8
	Sporobolomyces roseus	–	22.9	–	–
	Cryptococcus albidus	0.6	3.6	5.2	6.8
	Cryptococcus laurentii	0.4	4.5	3.8	4.9
	Rhodotorula mucilaginosa/ Rhodotorula rubra	4.8	1.1	1.4	6.2
	Trichosporon cutaneum	–	5.2	3.5	4.0
	Cystofilobasidium capitatum	–	–	7.3	–
	Rhodotorula minuta	0.7	0.5	5.2	–

[a]*A* Sláviková et al. (1992), *B* Sláviková and Vadkertiová (1995), *C* Sláviková and Vadkertiová (1997), *D* Boguslawska-Was and Dabrowski (2001)

A. pullulans is an opportunistic pathogen (Koppang et al. 1991; Girardi et al. 1993; Redondo-Bellon et al. 1997), and accounts for the highest population in the total yeast population of recreational lakes (56–76%) and fishponds (38–48%) during autumn. It has only been found in water (Boguslawska-Was and Dabrowski 2001) and is considered to enter aquatic zones with plants, flowers, fruits, etc. A significant incidence of the black yeasts in lake water has been reported globally, including lake St. Clair, Canada (Kwasniewska 1988), and Lagoa Olhos d'Água, a lake on the karstic plateau of Lagoa Santa, Brazil (Rosa et al. 1995). *Cr. albidus*, *Cr. laurentii*, *D. hansenii* and *Rh. mucilaginosa* were found to be small, stable components at the European sites. The majority of ascomycetous yeasts, such as *Candida* spp. and *Pichia* spp., tend to be prevalent in summer. These are considered to be of human origin in recreational lakes (Slávikóvá et al. 1992), and may be related to the increase in phytoplanktons during summer in eutrophicated waters. The species relatively specific to studies in these European areas were *Bullera alba*, *Galactomyces geotrichum* (*Geotrichum candidum*), *Pichia burtonii* and *Lachancea* (*Saccharomyces*) *kluyveri*, although their proportions were low.

The increased numbers of red yeasts in autumn, including the ballistospore-forming *Sporobolomyces* spp. (Slávikóvá and Vadkertiová 1995, 1997), may be affected by the microbial effluents from the phyllosphere carried by the fallen leaves of terrestrial plants. *Rh. minuta* and *Rh. mucilaginosa* were found in relatively low proportions in almost all of the studies, whereas *Rh. glutinis* and *Sporobolomyces roseus* unexpectedly increased in their specific area (Table 12.1) and season (Slávikóvá and Vadkertiová 1995). It has been reported that red yeasts are found in higher proportions in the total yeast population of clean water, as compared with polluted water (Hagler and Ahearn 1987), but the response to pollution may depend on the red yeast species involved.

Rh. mucilaginosa was one of two dominant species found in high-salinity conditions in chemical wastewater evaporation ponds (Lahav et al. 2002). This species was prevalent also in many of the oligotrophic lakes in northern Patagonia, Argentina (Libkind et al. 2003), but it was not found in ultra-oligotrophic lakes with little exposure to human activity, and its niches were mainly occupied by some ballistosporogenous species comprising the undescribed yeasts *Sporidiobolus longiusculus* and *Sporobolomyces patagonicus* (Libkind et al. 2005). In the same series of oligotrophic lakes, members of the *Occultifur* lineage (Fig. 12.2), *Rh. minuta*, *Rh. slooffiae* and *Rh. pinicola*, along with ballistosporogenous yeasts such as *Sporidiobolus salmonicolor*, *S. ruberrimus* and *S. roseus*, have also been isolated.

Red yeasts have also been isolated from the deep groundwater (Ekendahl et al. 2003) and identified as *Rh. minuta* on the basis of 18S rDNA phylogeny and the physiological properties. In our study, these phylogenetic positions were reconsidered, and the strains J1, J2 and J3 were subsequently revealed to be different from *Rh. minuta* (Fig. 12.2). Strains J1 and J3 were found to be related to *Rh. slooffiae*, whereas the classification of strain J2 was unclear owing to low bootstrap reliability. Nevertheless, all three strains were at a remarkable distance from their closest relatives, and seemed to be new species. Unlike the type strain of *Rh. slooffiae*, strains J1 and J3, which cannot grow above 23°C, were found to be psychrophilic. Strain J2 had a unique physiology in that the optimum growth temperature was 4°C, although the maximum was 30°C.

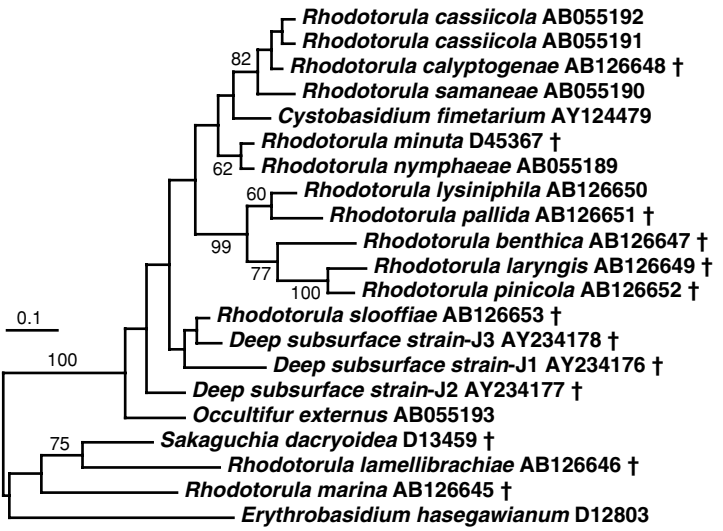

Fig. 12.2. The phylogenetic relationship within the *Occultifur* lineage and the positions of the red yeasts isolated from deep groundwater, based on the 18S ribosomal DNA (*rDNA*) sequences. The maximum likelihood tree was calculated by using the Bayesian phylogenetic inference on the program MrBayes (Huelsenbeck and Ronquist 2001; Ronquist and Huelsenbeck 2003) with the GTR+I+G model. The *numbers* on the branches are estimates of a posteriori probabilities. A *dagger* indicates species that have been isolated from aquatic environments

12.4.3 Estuaries and Mangrove Areas

Yeast communities of polluted estuary and mangrove ecosystems in subtropical marine environments were found to exhibit extreme diversity. The species diversity of basidiomycetous yeasts in these communities was less than or not notably different from that in other aquatic regions, but the ascomycetous yeasts comprised a remarkably greater number of species in these ecosystems than in other aquatic environments (Hagler and Mendonça-Hagler 1981; Hagler et al. 1982, 1993; Pagnocca et al. 1989; de Araujo et al. 1995; Soares et al. 1997). But highly dominant species tended to be absent, because many of the ascomycetous yeast species were represented by only one or a few isolates. Many of these species were considered to be allochthonous (de Araujo et al. 1995). Basidiomycetous yeasts were generally less frequent than ascomycetous yeasts in the polluted aquatic environments (Hagler and Ahearn 1987). The average proportion of basidiomycetous yeasts in the total yeast population in some studies conducted at Sepetiba Bay was 12.6% (Pagnocca et al. 1989; Hagler et al. 1993; de Araujo et al. 1995; Soares et al. 1997). The higher proportion of basidiomycetes in water (12.0%) as compared with sediment (3.8%) or invertebrates (3.5%) at same sites (Pagnocca et al. 1989) could be due to the fact that basidiomycetous yeasts are mostly oxidative and frequently occur on the water surface. *C. intermedia*, *D. hansenii*, *Issatchenkia occidentalis* (*C. sorbosa*), *P. guillier-*

mondii and *P. membranifaciens* (*C. valida*) were the ubiquitous ascomycetous species in the yeast studies conducted at Sepetiba Bay. The comprehension of a widely diverse ecology comprising numerous species, such as the yeast community in the subtropical mangrove ecosystem, may be impeded by the uncertain identities of the species owing to the ambiguous taxonomic results of phenotypic characterization.

Kluyveromyces aestuarii was found to be associated with detrital feeding invertebrates and sediments within specific mangrove areas (de Araujo et al. 1995; Soares et al. 1997). Previously, this species was presumed to be an obligate marine species (Kohlmeyer and Kohlmeyer 1979; Lachance 1998), and the other strains were isolated from shallow sediments of South Florida and the Bahamas and from the mangrove brackish area of the Everglades in Florida and the seawater from Torres Strait, Australia. *K. aestuarii* is phylogenetically placed in the *K. marxianus* lineage, genus *Kluyveromyces* nom. cons. (Kurtzman 2003) or the genus "*Zygofabospora*" (Naumov and Naumova 2002), and is ecologically similar to some members of the lineages *K. lactis* and *K. nonfermentans*. The "aquatic" strains of *K. lactis* (Naumova et al. 2004), which were isolated from rhizosphere sediments in the marine marshlands (Meyer et al. 1971), were suggested to be evidence of the possible evolution of the mangrove-inhabiting species *K. aestuarii* from the marshland-inhabiting species *K. lactis* (de Araujo et al. 1995; Soares et al. 1997). But the ITS phylogeny of the genus *Kluyveromyces* (Fig. 12.3) did not support the theory of direct evolution from *K. lactis* to *K. aestuarii*, whereas *K. aestuarii* obviously shared a common ancestor with *K. nonfermentans*, which was isolated from sediments and invertebrates in deep-sea environments. *K. nonfermentans* may have lost its fermentative ability as a consequence of adapting to oligotrophic oceanic environments.

Plant-associated yeasts on bromeliads in mangrove areas are distinct from those typical of polluted areas, and comprise a larger number of species and isolates with basidiomycetous affinities (Hagler et al. 1993). Some human-associated species, such as *C. parapsilosis* and *C. tropicalis*, and the prevalent species in polluted water, such as *Hanseniaspora uvarum* (*Kloeckera apiculata*), *I. orientalis* (*C. krusei*) and *P. anomala*, were absent, although they were common in other sources from the mangrove ecosystem.

Yeasts are prevalent in salt marshes or mangrove ecosystems where the yeasts play an important role in the detrital food web (Meyers et al. 1975), and they might be a food source for some marine invertebrates and zooplanktons. The fungus-growing behavior of the marine snail on the marine marsh grass was recently reported (Silliman and Newell 2003). In contrast, the infection of the freshwater prawns *Macrobrachium rosenbergii* by *C. sake* (Lu et al. 1998) and *Metschnikowia bicuspidata* (Chen et al. 2003) is known. In *Spartina* grass, in addition to *K. lactis*, *P. spartinae* has been previously found as the prevalent species in the outer- or intra-culm (fistulous stalk) cells and tissues, but PCR-based analysis of an ascomycetous community colonizing decaying *Spartina alterniflora* blades in the salt marsh was not able detect these two yeasts or any other yeast clones (Buchan et al. 2002). Further extensive research could reveal the ecological roles of these yeasts and their interaction with the other organisms of the salt marsh.

The yeast community from the estuary in Portugal, investigated with the help of fungal DNA clones, suggested the presence of some species known to be prevalent

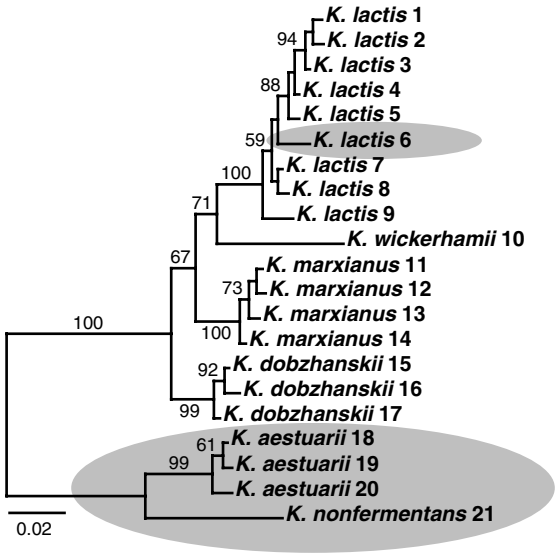

Fig. 12.3. The phylogenetic relationship of the genus *Kluyveromyces*, as inferred from internal transcribed spacer and the 5.8S rDNA sequences. The tree was drawn as for Fig. 12.2. The *numbers* on the branches are estimates of a posteriori probabilities. The marine species are in the *gray oval*. The accession numbers for the sequences (see the number after the name of the species) are as follows: *1* IFO 1090, AB011515; *2* JCM 6846, AB011516; *3* CECT 1121, AJ401702; CECT11401, AJ401718; CECT10366, AJ401708; CECT10364, AJ401707; CECT10361, AJ401706; CECT11395, AJ401715; CECT1961, AJ401704; CECT11401, AJ401718; *4* CECT 1122, AJ401703; CECT10669 AJ401710; CECT11380, AJ401713; CECT11394, AJ401714; CECT11396, AJ401716; *5* JCM 9563, AB011517; *6* CBS 6170, AY338967; CBS6171, AY628330; *7* UWO79-169, AY628331; CECT10390, AJ401709; CECT11337, AJ401711; UCD61-200, AY623808; CECT11340, AJ401712; UWO79-169, AY628331; UWO80-45, AY623809; *8* UWO79-127, AY626022; UWO80-12, AY626023; *9* UCD 69-8, AY338968; DV30, AY338969; *10* IFO 1675, AB011521; *11* IFO 10005, AB011518; JCM 1630, AB011519; CECT1123, AJ401692; CECT1031, AJ401694; CECT1058, AJ401698; CECT1066, AJ401699; *12* JCM 2188, AB011520; *13* CECT 1048, AJ401700; *14* CECT 1442, AJ401693; CECT1035, AJ401695; CECT1036, AJ401696; CECT1058, AJ401697; CECT1138, AJ401701; *15* IFO 10603, AB011514; NRRL Y-1974, AY046215; CECT 1952, AJ401719; *16* CBS 210, AJ229068; *17* CECT 10177, AJ401720; CECT 10180, AJ401721; CECT 10187, AJ401722; *18* NRRL YB-4510, AY046210; *19* NRRL Y-4510, U09324; *20* IFO 10597, AB011513; *21* JCM 10227, AB011507; JCM 10230, AB011508; JCM 10231, AB011509; JCM 10234, AB011510; JCM 10236, AB011512; JCM 10232, AB012264

in polluted aquatic environments, and six yeast species were regarded as putative undescribed species (Gadanho and Sampaio 2004). From among these yeast species, all five ascomycetous yeasts were relatively closely related to species which have been reported to occur in aquatic environments, e.g., *C. inconspicua*, *C. intermedia*, *D. hansenii* and *P. guilliermondii*, whereas the only basidiomycetous yeast, which, interestingly, was considered to be uncultured, was found to be related to *Cr. longus*, which had previously not been reported to be aquatic.

12.4.4 Offshore and Deep-Sea Environments

There have been a few yeast studies conducted in oceanic regions in the last 2 decades. This is probably because there has been little expectation of obtaining new findings in this area, and, in addition, the expenses for offshore and oceanic sampling are considerable. In an extensive study of yeasts in oceanic regions (Fell 1976), the yeast communities appeared to be constituted of the ubiquitous species and species restricted geographically, hydrographically and biologically. Among the ascomycetous yeasts, the halotolerant species *D. hansenii* was a typical ubiquitous species in oceanic regions as well as in other aquatic environments. Among the basidiomycetous yeasts, some species of *Cryptococcus*, *Rhodotorula*, *Sporobolomyces* and their teleomorphs were found to be widespread across various oceanic regions. Generally, basidiomycetous yeasts often account for the overwhelming majority of the total yeast population in oligotrophic oceanic water. *Candida* spp. also occur, but at lower frequencies than in the inshore or polluted freshwater regions, and the occurring species are also different in these habitats. Some of the *Candida* species appeared only in the oceanic regions around Antarctica, and, along with *Leucosporidium* spp. and *Sympodiomyces parvus*, they are known to be psychrophilic. They are probably autochthonous marine species (Lachance and Starmer 1998). *Metschnikowia* spp. are known to be associated with seawater, fresh water, invertebrates, fish and algae. The phylogenetic relationship (Fig. 12.4) shows that *M. australis*, *M. bicuspidata* var. *bicuspidata*, *M. bicuspidata* var. *chathamia*, *M. krissii* and *M. zobellii*, all of which are prevalent in marine environments, are monophyletic, whereas the occasionally aquatic species *M. reukaufii* and *M. pulcherrima* are distant, as previously reported (Mendonça-Hagler et al. 1993). The latter two are usually found to associate with terrestrials such as flowers, fruits and insects, like almost all of the other *Metschnikowia* species. The monophyly of the marine species suggests that their divergence has evolved in the course of association with marine environments.

The ubiquitous species in various marine habitats are usually regarded as being allochthonous. Especially because many basidiomycetous yeasts are often found to associate with the phyllosphere of terrestrial plants, the marine occurrences were considered to be run-offs from the phylloplane (Hagler and Ahearn 1987; Lachance and Starmer 1998). The yeasts of the ballistosporogenous genera *Sporobolomyces* and *Bullera* and their teleomorphs are typical inhabitants of the phylloplane. During a survey of yeasts in the Pacific Ocean off Mexico, yeasts of the genus *Sporobolomyces* (and *Bullera*) were the most commonly encountered (Hernandez-Saavedra et al. 1992). Interestingly, the frequencies increased with increasing distance from the coastline and increasing depth. The yeasts of these genera were also found in benthic invertebrates collected from deep-sea floors in the Pacific Ocean off Japan (Nagahama et al. 2001b). These facts may indicate that ballistosporogenous yeasts are not effluents from terrestrial plant foliages but are indigenous to the sea.

In a study of yeasts in the seawater of the Atlantic Ocean off Faro in the south of Portugal (Gadanho et al. 2003), however, few ballistosporogenous yeasts were found, and mainly basidiomycetous yeasts were isolated. *Rhodosporidium babjevae*

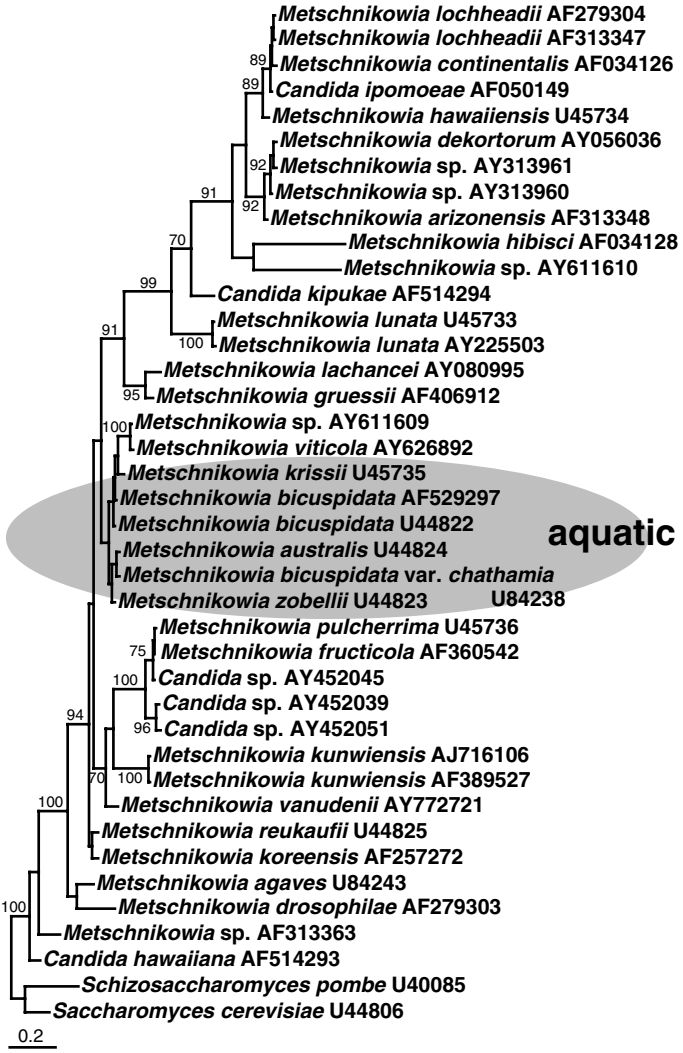

Fig. 12.4. A 26S rDNA D1/D2-based tree for the genus *Metschnikowia* and its relatives. The tree was drawn as for Fig. 12.2. The *numbers* on the branches are estimates of a posteriori probabilities. The species associated with aquatic environments are in the *gray oval*

and *R. diobovatum*, the two possible species previously identified as *Rh. glutinis*, and *Sakaguchia dacryoides* and *Pseudozyma aphidis* were frequent among the basidiomycetous yeasts. Notably, an ustilaginomycetous yeast, *P. aphidis*, was widely distributed in the area studied, but its ecological behavior in the sea is still unknown. As in the case of the *Rh. glutinis* species complex, a species previously identified as *Cr. laurentii* also occurred, and was found to be involved with undescribed species.

From quantitative surveys of microbiota in deep-sea sediment, abundance of yeast-like cells has been reported (Burnett 1981; Alongi 1987, 1992). During a survey of the yeast distribution in deep-sea environments around the Pacific Ocean, a number of yeasts have been isolated (Nagahama et al. 1999, 2001a, b, 2003a, b). Our most frequently visited site is around a cold seep at a depth of about 880–1,200 m near Hatsushima Island, Sagami Bay. At this site, giant white clams (*Calyptogena* spp.) and tubeworms (*Lamellibrachia* spp.), which inhabit the area, were collected. Suruga Bay (380–2,500 m), the Japan Trench (4,500–7,500 m) and Iheya Ridge (990–1,400 m) have been investigated a few times. The other sites (Kagoshima Bay, 220–260 m; the Mariana Trench, about 11,000 m; the Palau–Yap Trench, 3,700–6,500 m; the Manus Basin, 1,600–1,900 m) have only been visited once (Fig. 12.5). Iheya Ridge and the Manus Basin have biologically fertile spots owing to the hydrothermal vent ecosystem, and tubeworms (*Lamellibrachia* spp.) were collected at the former site.

R. sphaerocarpum, *Williopsis saturnus* and *C. pseudolambica* were found to be frequent species in a total account of all of the sites (Table 12.2), but their distributions were limited mostly to the sediments of Suruga Bay and Kagoshima Bay. *D. hansenii* occurred only in the sediments of Sagami Bay and Suruga Bay, although it had been known to be the commonest ascomycete in marine waters (Hagler and Ahearn 1987). Almost all ascomycetous yeasts were isolated only from sediments. The only exception was *K. nonfermentans*, which was common to both sediments and benthic invertebrates, whereas its distribution was limited to Sagami Bay and Suruga Bay. In contrast, *R. diobovatum* and *Rh. mucilaginosa* were widely prevalent in the various locations and sources.

The frequency of occurrence of each corresponding phylogenetic taxon is obviously different according to the source and geographical origin (Fig. 12.6). The ascomycetous yeasts constituted the majority of the total yeast population in the sediments of Sagami Bay, Suruga Bay and Kagoshima Bay, sites which are relatively inshore (5–20 km) near urban and industrial areas and where the deep-sea floors are affected by the human activities. But *P. anomala* and *H. uvarum*, which are known as the pollution-associated species, did not appear. Species in the *Erythrobasidium* clade have been isolated mostly from the benthic invertebrates, and the exceptional isolates from the sediments of the Manus Basin are considered to give clues about the hydrothermal ecosystems. Many of these species belong to the *Occultifur* lineage (Fig. 12.2), and some were found to be undescribed species (Nagahama et al. 2001a, 2003a). The association with animals is probably favorable for yeasts, owing to the abundance of nutrients (Hagler and Ahearn 1987). The reasons why species associated with animals are limited are unknown so far. Hymenomycetous species, mostly assigned to the genus *Cryptococcus*, were localized in the Japan Trench, Sagami Bay and Suruga Bay, and did not appear farther southwest. Species of Sporidiobolales were present at all of the sites.

However, there is poor evidence for physiological adaptation. Yeasts with an optimum growth under elevated hydrostatic pressure condition have not been reported, whereas many yeast species from both terrestrial and marine origins are moderately pressure-tolerant (ZoBell and Johnson 1949; Yamasato et al. 1974). Especially, the carotenogenic basidiomycetous yeasts such as *Rhodotorula* and *Rhodosporidium* were reported as psychro- and pressure-tolerant (Davenport 1980). A *Rhodotorula* species

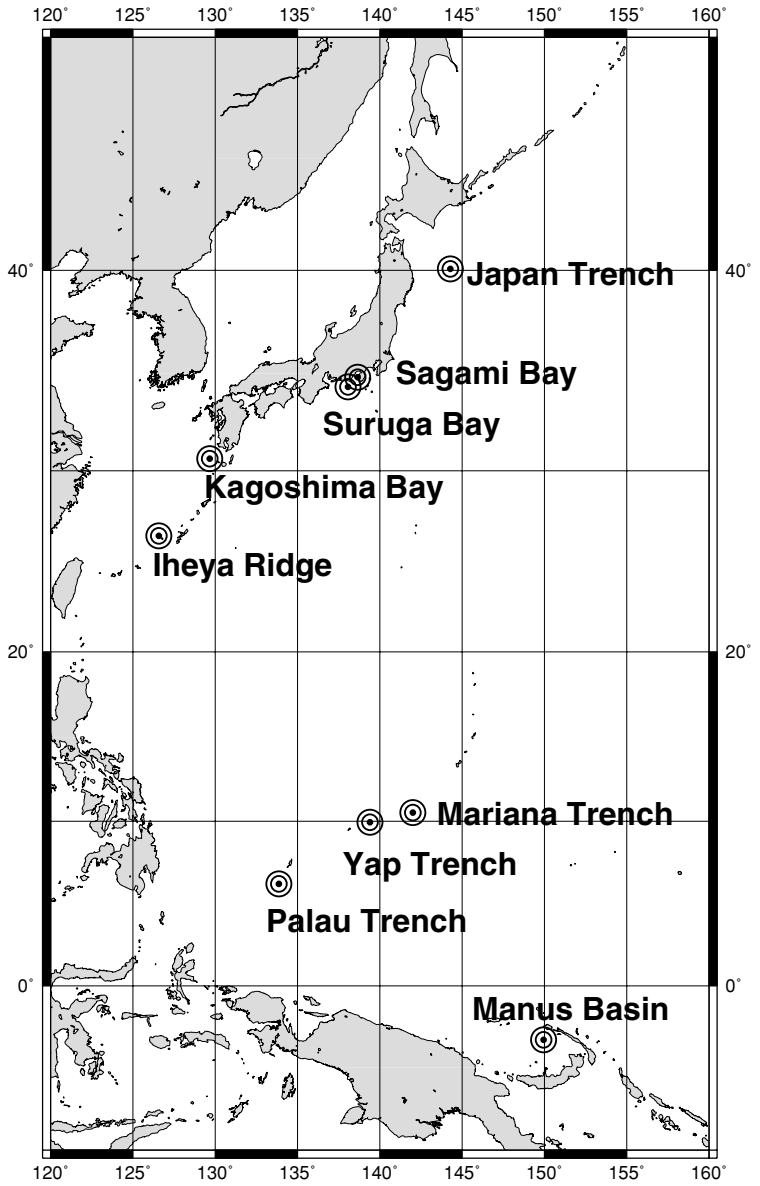

Fig. 12.5. Sampling sites in the Pacific Ocean

showed that growth at 20 MPa (equivalent to 2,000-m depth) was not significantly different as compared with that at 0.1 MPa and growth at 40 MPa was reduced to 20–30% (Lorenz and Molitoris 1997). But it should be noted that the growths were observed at a remarkably high temperature (27°C). In our study, the yeasts isolated from seafloors deeper than 4,000 m did not grow well under hydrostatic pressures

Table 12.2 The yeast species and the number of isolates from sediment and invertebrates collected from deep-sea floors around the northwest Pacific Ocean

Species[a]	Sediment	Invertebrates	Total ($n \geq 2$)
Rhodosporidium sphaerocarpum	33	–	33
Williopsis saturnus var. *saturnus*	30	–	30
Candida pseudolambica	27	–	27
Debaryomyces hansenii var. *hansenii*	25	–	25
Rhodosporidium diobovatum	16	9	25
Kluyveromyces nonfermentans	15	4	19
Rhodotorula benthica	10	7	17
Rhodotorula mucilaginosa	9	2	11
Candida vartiovaarae	6	–	6
Cryptococcus gastricus	6	–	6
Debaryomyces hansenii var. *fabryii*	5	–	5
Kondoa aeria	4	1	5
Rhodotorula lamellibrachiae like	1	3	4
Rhodotorula minuta	–	4	4
Candida melibiosica	3	–	3
Williopsis californica	3	–	3
Aureobasidium pullulans	3	–	3
Cryptococcus albidus	3	–	3
Cryptococcus laurentii like	2	1	3
Rhodosporidium toruloides like	3	–	3
Sporobolomyces salmonicolor	–	3	3
Candida boidinii	2	–	2
Candida sake	2	–	2
Pichia pinus like	2	–	2
Sarcinomyces petricola	2	–	2
Cryptococcus victoriae	2	–	2
Trichosporon pullulans	2	–	2
Rhodotorula dairenensis	2	–	2
Rhodotorula dairenensis like	2	–	2
Sporidiobolus pararoseus	1	1	2
Rhodotorula calyptogenae	–	2	2

[a]"Like" represents a similar but not identical species with greater than 1% differences in the comparisons of D1//D2 26S ribosomal DNA or internal transcribed spacer sequences.

corresponding to the sources at which they were collected (2–4°C, more than 40 MPa). But this result may also be due to the specifications of our compressed incubation system, which allows sharp pressure changes and insufficient oxygen supply. Psychrophilic strains have not been found so far in the deep sea, but many isolates were psychrotolerant and grew well at less than 4°C (unpublished data).

12.5 The Impact of Molecular Taxonomic Approaches on the Ecological Studies of Yeasts in Marine and Other Aquatic Environments

It is still difficult to give a convincing answer to an old question about marine yeasts: "Are there indigenous marine yeasts?" (Kohlmeyer and Kohlmeyer 1979). Even if

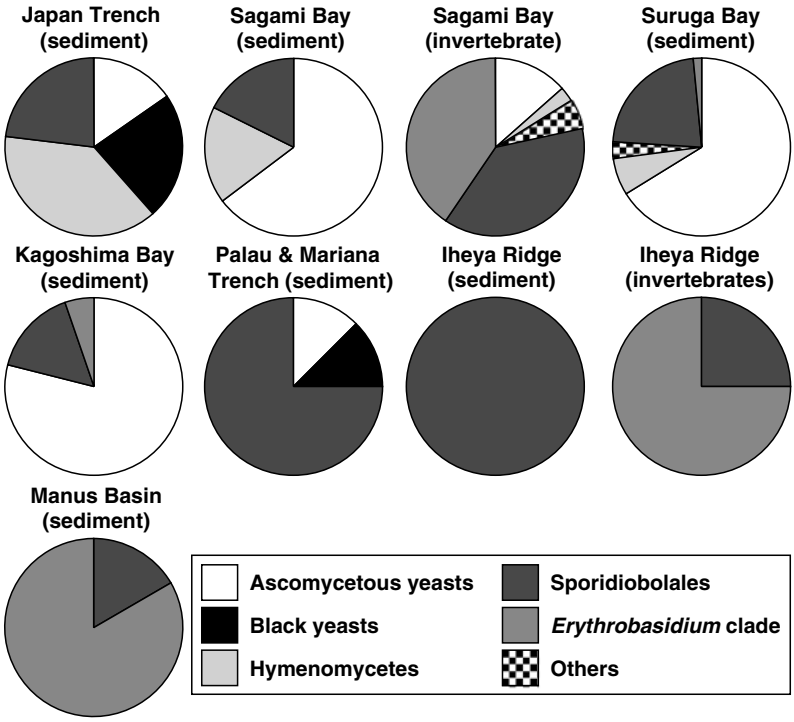

Fig. 12.6. The frequency of occurrence of each phylogenetic taxon by geographical origin and source

there is evidence that there are, there is another question: "Which are indigenous?" In general, the physiological properties of yeasts do not give clues as to whether they are marine species or terrestrial species. Although some marine species may be resistant to higher NaCl concentrations than related terrestrial species, almost all yeast species can grow well in media with NaCl concentrations exceeding those normally present in the sea (Kohlmeyer and Kohlmeyer 1979). Few yeast species with a physiological dependence on NaCl or other seawater components have been reported. Yeast concentrations in marine environments are known to decrease with increasing depth and increasing distance from land. But this tendency could be explained in two ways: one is the availability of nutrients coming from land, and the other is frequent encounters with terrestrial yeasts (Sieburth 1979). Thus, physiological characterization cannot discriminate between marine and terrestrial yeasts.

Many of the yeast species found in aquatic environments are considered to be allochthonous (Lachance and Starmer 1998). The number of yeast species prevalent in marine environments is limited, but each species of marine yeast is very similar to its terrestrial counterpart. Only a few of the isolated species have been found to be restricted to marine environments. These physiological and taxonomical observations could imply that most of the marine species are terrestrial contaminants.

Yeasts are known to be the dominant fungi in the open ocean, though the bacterial population is hundreds of times denser than the yeast population, according to counts of colony-forming units (Sieburth 1979). Because most natural seawater contains amounts of organic carbon nutrients insufficient for yeast-cell reproduction, many marine and other aquatic environments may be used by the yeasts as transit areas only. The candidates for autochthonous marine yeasts are not thought to be ubiquitous; their occurrence is thought to be restricted to specific geographical latitudes or to associations with certain macroorganisms, as in the case of the psychrophilic species from the circumpolar area or *Metschnikowia* spp.

Many of the yeast species that have a widespread distribution across aquatic environments often pose identification problems, owing to their ambiguous phenotypic characteristics. However, this problem has been overcome through the development of phylogenetic molecular identification. The aquatic strains that had been considered phenotypically identical to or indistinguishable from terrestrial strains can now be segregated genetically. Evidence of such imperceptible differentiation is offered by rapidly substituted molecules such as ITS or IGS. Thus, the aquatic strains of the species *K. lactis* were revealed to be differentiated as the variety level, by means of RFLP fingerprinting of the PCR replicons in IGS and the phylogenetic relationships based on ITS sequences (Naumova et al. 2004).

Red yeasts may also provide clues as to the differentiation of aquatic yeasts from terrestrials. These basidiomycetous yeasts are characterized as having the ability to produce carotenoids or carotenoid-like compounds, and are found in higher proportions in the total yeast population of clean freshwater than of open-sea water. The species *Rh. glutinis*, *Rh. minuta* and *Rh. mucilaginosa* are the predominant red yeasts in aquatic environments, although each has been considered to be multitypical. Notably, many strains previously identified as *Rh. glutinis* may genetically differ from the type strain of this species, and may be anamorphs of species in the genus *Rhodosporidium* or may belong to a novel species. The marine isolates related to the three *Rhodotorula* species have been found as undescribed species (Gadanho and Sampaio 2002; Nagahama et al. 2003a). The Sporidiobolales, which include *Rh. glutinis* and *Rh mucilaginosa*, and the *Occultifur* lineage, which includes *Rh. minuta* (Fig. 12.2), comprise many species which often are associated with aquatic sources.

Cr. laurentii is one of the commonest basidiomycetous yeasts in aquatic environments, but some aquatic strains previously identified as belonging to this species are possibly different from this type strain, as in the case of the *Rhodotorula* species mentioned before. Some new combinations have been recently reported in the *Cr. laurentii* species complex. *Cr. peneaus* is one of these, and has been isolated from the surface of shrimp. More new aquatic species may be recognized on the basis of further taxonomic studies of this complex. The widespread yeasts occurring across various ecological niches often form varieties or species complexes, and the identification of these yeasts based on traditional taxonomical approaches is difficult. A reexamination of aquatic strains previously classified into certain species is worth considering.

In contrast, *C. austromarina*, known as a psychrophilic marine species inhabiting the Antarctic Ocean, was regarded as a synonym of a mesophilic species, *C. sake*, because of the identical D1/D2 26S rDNA sequence. Because *C. sake* is widely

distributed in oceanic and other aquatic regions, *C. austromarina* is considered to be a variety indigenous to the Antarctic region. This conspecificity suggests the possibility that the psychro-adaptation occurred over a short period of time. Their genetic differentiation could be determined through further comparisons of rapidly substituted genes.

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Phylloplane Yeasts

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Abbreviations

BC	Ballistoconidiogenic
CFU	Colony forming units
DGGE	Denaturing gradient gel electrophoresis
EPS	Extracellular polysaccharide
FISH	Fluorescence in situ hybridization
rDNA	Ribosomal DNA
SEM	Scanning electron microscopy
SFM	Spore-fall method

13.1 The Phylloplane as a Microbial Habitat

Plant surfaces have been recognized as an important habitat for microorganisms for over a century. Different zones along the plant axis provide a multitude of topographical features, sources of nutrients and water, and a range of microclimatic conditions for correspondingly diverse communities of microbes, which in turn establish varied relationships with their hosts (Andrews and Harris 2000). These associations range from relatively inconsequential or transient (unspecific or ephemeral epiphytic saprophytes) to substantial or permanent (epiphytic residents, endophytes or pathogens). Phytopathogens have long been identified and studied owing to the economic impact of the diseases they cause on agricultural crops (e.g. Leben 1965; Morris 2001) but for many years much less was known about the identity or properties of the numerous saprophytic microbes that inhabit plant surfaces. However, the last few decades have witnessed a renewed interest in microbial epiphytes that apparently play important roles in nutrient cycling or in modulating population sizes of deleterious microbes, and some are being exploited as biological control agents for disease or frost control (Windels and Lindow 1985; Fokkema 1991; Andrews 1992; Lindow and Leveau 2002). Two main physical environments (or vertical stratification zones) for microbial colonization are distinguished: the aboveground surfaces, often referred to as the phyllosphere or the phylloplane, and the belowground

surfaces, termed the rhizosphere or rhizoplane (Diem 1973; Fokkema and Schippers 1986; Andrews and Harris 2000). In this chapter we will focus exclusively on the aerial surfaces of plants and in particular on the leaves. We have chosen the term phylloplane to refer to leaf surfaces (Last and Price 1969), although other authors advocate the use of the term phyllosphere (Fokkema 1991; Morris 2001; Lindow and Brandl 2003) originally proposed by Last (1955) and Ruinen (1956).[1] Our rationale was that the latter also comprises the internal leaf tissue that may be colonized by endophytes, which are not the object of this review.[2]

Plant leaves constitute one of the largest terrestrial microbial habitats and the total surface area available for colonization has been estimated to be about $2–6\times10^8$ km^2 (Morris 2001; Lindow and Brandl 2003). Leaf surfaces have been studied as habitats for microbial epiphytes since the 1950s and phyllosphere microbial ecology has been the theme of a regular series of scientific meetings since the early 1970s. The proceedings of those meetings summarize the progress made in understanding the nature of leaf habitats, their microbial colonists, and the interactions among microbes and between microbes and plants (Morris 2001, and references therein). The epiphytic (non-phytopathogenic) microbial communities of leaves are very diverse and their best-studied components have been bacteria and fungi, including yeasts (Andrews and Harris 2000; Hirano and Upper 2000; Morris 2001; Lindow and Brandl 2003). Bacteria are generally the earliest colonists of leaves and may be the most numerous, often being found in densities up to 10^7 cells/cm^2 (approximately 10^8 cells/g) of leaf under favourable environmental conditions, such as when high relative humidity or free water is present (Hirano and Upper 2000; Morris 2001; Lindow and Brandl 2003). Filamentous fungi are often considered transient inhabitants of leaf surfaces, being present predominantly as spores, whereas rapidly sporulating hyphomycetes, dimorphic species such as *Aureobasidium pullulans*, and yeasts appear to colonize this habitat more actively (Pennycook and Newhook 1981; Fokkema and Schippers 1986; Andrews and Harris 2000; Lindow and Brandl 2003). Some epiphytic mycelial fungi appear to be particularly active in leaf litter decomposition following leaf fall, at least in its initial stages (Mishra and Dickinson 1981; Osono 2002). However, extensive hyphal growth on healthy, intact, non-senescent leaves is relatively rare (except of course for biotrophic plant pathogens).

From a microbe's perspective the phylloplane is a continuously fluctuating physical environment both spatially and temporally, and may thus be considered an extreme environment (Hirano and Upper 2000; Morris 2001). Exposed to the atmosphere and the sun, leaf surfaces and, hence, their resident microbes are subjected to changes in aspects of microclimate such as temperature, relative humidity, wind speed, radiation, and others that occur on different time scales, from a few minutes to months. Water availability, for instance, is one of the most dynamic parameters of the phylloplane environment: condensation (dew, fog) and impaction of water

[1] It should be noted that in some texts the term phylloplane is used to refer to any aerial plant surface, including bark or fruits (Andrews and Harris 2000).

[2] Yeast endophytes in leaves have been reported by, for example, Larran et al. (2001) and Pirttilä et al. (2003).

droplets (rainfall) on leaf surfaces alternates with evaporation and run-off of water in cycles that are generally diurnal. The property that makes the phylloplane a harsh environment is not the extremes to which it is exposed, but the frequent, repeated, rapid shifts in those very different conditions, any one of which may be considered stressful to at least some of the prospective microbial colonists.

Cuticle composition and topographic features (stomata, trichomes, veins, etc.) are also highly variable both within a leaf and among different plant species (Baker 1971; Hallam and Juniper 1971) and may influence the composition and distribution of phylloplane communities (Kinkel 1997). Besides topography and microclimatic conditions, the nature and the size of epiphytic microbial populations are also shaped by the availability of nutrients and in this respect the phylloplane is generally considered oligotrophic (Andrews and Harris 2000). There are multiple possible origins of nutrients on the phylloplane. These can be endogenous and include diffusion of compounds out of the leaf tissue from hydathodes, trichomes or fissures in the cuticle accompanying weathering or injury, or exogenous, such as nutrients in guttation fluids or compounds contained in debris, pollen and honeydew, or originating in other organisms (microbes or insects). Molecules leached from plant leaves include a variety of organic and inorganic compounds, such as sugars, organic acids, amino acids, methanol and various salts (Blakeman 1971; Tukey 1971; Morris 2001). The abundance of such nutrients varies with plant species, leaf age and growing conditions. Exogenous nutrient sources, such as aphid honeydew and pollen, have been associated with dramatic increases in the microbial carrying capacities of some leaves (Diem 1974; Fokkema et al. 1983; Stadler and Müller 1996). However, in the common absence of such significant but ephemeral nutrient sources, plant leaves are still usually colonized by large microbial populations. Nutrients released to the leaf surface should thus be adequate to support microbial growth and depletion of sugars on the phylloplane by bacteria and yeasts has been demonstrated (Dik et al. 1991; Leveau and Lindow 2001). This suggests that epiphytic microorganisms normally compete for a limited amount of nutrients, which in turn determine the microbial carrying capacity of the leaf. Depending on the system studied, carbon compounds alone or both carbon and nitrogen compounds were shown to be limiting factors for bacterial and yeast populations on leaves (Bashi and Fokkema 1977; Mercier and Lindow 2000). Antimicrobial compounds may also leach to leaf surfaces and were found to affect the composition of phylloplane communities (Irvine et al. 1978; Fokkema 1991).

All the factors just discussed taken together account for the extraordinary plasticity and diversity of phylloplane microbial populations. Some of those aspects will be discussed in more detail in the following sections, with particular emphasis on yeasts.

13.2 Methods for Detection, Enumeration and Identification of Epiphytic Microorganisms

Several methods have been used for the detection, isolation and/or enumeration of microorganisms from the phylloplane, including yeasts (reviewed by Beech and Davenport 1971; Drahos 1991; Jacques and Morris 1995). Standard direct detection

methods, mainly in studies before the 1980s, were based on optical or scanning electron microscopy (SEM) of the leaf surface or corresponding leaf impressions (e.g. in nail varnish pellicles or acetate adhesive tape). These methods are generally quite laborious, there are difficulties in detecting small yeast cells or small cell densities against the background, and observations may be hindered by topographical features of the leaf surface. Some authors employed leaf clearing and staining procedures (Lindsey and Pugh 1976; McBride and Hayes 1977; Andrews and Kenerley 1978), which can alter the position of the epiphytic microorganisms on the leaf surface, therefore cancelling out some of the advantages of direct observation. Moreover, it is not usually possible to distinguish between different species with similar cell morphologies, or between viable, viable but non-culturable and dead cells. This is one possible explanation for the common finding that microscopy-based quantification of phylloplane microorganisms gives rise to higher counts than cultivation-based methods (Babjeva and Sadykov 1980; Mishra and Dickinson 1981). About one third of the 35 studies listed in Table 13.1 employed the direct observation of leaves and/or leaf impressions with optical microscopy, usually in complement to some other cultivation-based method. Five studies used SEM (Table 13.1). The main targets for the majority of these studies were filamentous fungi. In some cases yeasts were only detected using cultural methods, but not by direct microscopy (Hogg and Hudson 1966; Lindsey and Pugh 1976).

Enumeration of microbial populations associated with leaves relies almost exclusively on indirect methods, involving culture of the organisms on artificial agar media (Table 13.1). About two thirds of the studies in Table 13.1 employed methods based on the plating of leaf washings (or of leaf macerates) onto selected media for the isolation and enumeration of phylloplane microbial communities. These methods may underestimate the occurrence of non-spore-forming hyphal fungi and overestimate the spore-forming ones, but are generally considered appropriate for yeast quantification (Fokkema 1991). Leaves are usually cut into small pieces and submerged in a washing solution (e.g. water or Ringer solution, with or without low concentrations of a surfactant to aid dislodging of cells), which is then shaken more or less vigorously for a certain length of time (e.g. with the aid of a vortex, rotary shaker or sonicator). The resulting suspension is then serially diluted and aliquots are plated onto appropriate media. Several additives can be added to the culture medium, enabling the selective isolation of specific microorganisms (e.g. an antibacterial antibiotic such as chloramphenicol is normally used to prevent bacterial growth for isolation of fungi or yeasts). Isolation and quantification of yeasts from leaves is sometimes difficult owing to the growth of rapidly expanding moulds that overgrow yeast colonies. There is no single methodology or medium that inhibits one type of organism without affecting the other. For example, Azeredo et al. (1998) compared two agar media for yeast isolation from sugarcane leaf washings: yeast extract -malt extract and yeast nitrogen base with 1% glucose (both media supplemented with chloramphenicol to prevent bacterial growth). They claimed that the latter medium was superior owing to a lesser expansion of mould colonies. According to our own experience, however, yeast colonies develop poorly on this medium and are thus difficult to distinguish from one another. Some authors advocate the use of media supplemented with Rose Bengal (Pennycook and Newhook

Table 13.1 Studies of phylloplane microbial ecology that included or focused on yeasts

Plant(s)[a]	Geography, climate[b]	Dominant yeast genera or species (densities)	Methods[c]	Main subject of study and summary of conclusions	Reference
Lolium perenne (H); *Trifolium repens* (H); *Festuca arundinacea* (H)	New Zealand, C	*Cr. laurentii, Rh. glutinis, Rh. ingeniosa, Rh. marina, Rh. mucilaginosa, Rh. graminis, S. salmonicolor* (*Sp. odorus*); minor: *B. alba, Cr. albidus, Cr. diffluens, Cr. luteolus* (10^4–10^5 CFU/g)	A: PLW, B: P	Seasonal dynamics and species diversity (yeasts) on leaves of pasture plants; densities higher in summer and autumn and on senescent leaves; proportion of red yeasts lowest in winter (6%) and highest in late autumn (92%); soil yeasts rarely found on leaves; species composition independent of site	di Menna (1959, 1971)
Triticum aestivum (G)	England, C	*Sporobolomyces*	A: SFM	Seasonal dynamics (fungi); numbers of colonies increased with leaf age and were maximal when leaves died (while still attached)	Last (1955)
	Scotland, C	*Cr. albidus, Cr. laurentii, Cr. macerans, Rh. glutinis, Rh. ingeniosa, Sp. roseus, T. cutaneum*	A: PLW; PLM (w/washed leaves)	Seasonal dynamics and species diversity (fungi and yeasts); densities increased from early June to September	Flannigan and Campbell (1977)

Continues

Table 13.1 Studies of phylloplane microbial ecology that included or focused on yeasts—*cont'd*

Plant(s)[a]	Geography, climate[b]	Dominant yeast genera or species (densities)	Methods[c]	Main subject of study and summary of conclusions	Reference
Hordeum (G)	France, C	Pink yeasts (up to 10^4 CFU/cm^2)	A: PLW	Seasonal dynamics; densities increase from spring to autumn; bacteria always dominant; higher densities on plants in the centre of the cropping area than at the fringes	Diem (1967, cited in Last and Price 1969)
Beta vulgaris (D;H)	The Netherlands, C	*Cr. albidus*, *Cr. laurentii*, *Sp. roseus*	A: PLW	Seasonal dynamics (fungi and yeasts); densities increase from spring to summer but decrease in autumn; yeast numbers higher than those of other fungi	Kerling (1958, cited in Last and Price 1969)
	England, C	White and pink yeasts (10^6 CFU/g dry weight)	A: PLM, B: S	Short-term community dynamics (emphasis on bacteria); bacterial densities fluctuated: increased overnight and declined during daylight hours; yeast densities constant throughout the study	Thompson et al. (1995)

Pisum sativum (D;H)	England, C	Yeasts (up to 10^4 CFU/cm^2)	A: PLW, OMLI	Fungal dynamics with leaf age; yeast counts on plates lower than direct counts; yeast cells outnumbered fungal propagules; yeast-like cells increased sharply in senescent leaves	Dickinson (1967, cited in Last and Price 1969)
Typha latifolia (E;H)	England, C	*Sp. roseus*	A: PWL, SFM	Seasonal dynamics (fungi); *Sp. roseus* in mid-summer and on senescent leaves (only by SFM)	Pugh and Mulder (1971)
Oxalis acetosella (E;H)	Russia, D	*Cr. albidus*, *Cr. flavus*, *Cr. laurentii*, *Cy. capitatum*, *Le. scottii*, *Rh. fujisanensis*, *Rh. glutinis*, *Sp. roseus* (10^3–10^5 CFU/g)	A: PLM, B: P	Seasonal dynamics and species diversity (yeasts); densities and species richness higher in autumn and lower in spring; yeasts classified into 27 species (19 basidiomycetous species) belonging to 12 genera	Glushakova and Chernov (2004)
Various herbs/ bushes (tundra)	Russia, E	*Cr. laurentii*, *Mr. frigida*, *Le. scottii*, *Rh. glutinis* (10^5–10^6 CFU/g)	A: PLW, B: P	Species diversity (yeasts); yeasts dominate mycobiota and are present in high densities only on plants or litter (not in soil); some psycrophilic species	Chernov (1985)

Continues

Table 13.1 Studies of phylloplane microbial ecology that included or focused on yeasts—*cont'd*

Plant(s)[a]	Geography, climate[b]	Dominant yeast genera or species (densities)	Methods[c]	Main subject of study and summary of conclusions	Reference
Various plants (e.g. *Allium*, *Brassica*, *Malus*, *Tilia*, *Trifolium*)	Russia, D	*C. krusei*, *C. tropicalis*, *Cr. albidus* var. *albidus*, *Cr. albidus* var. *diffluens*, *Cr. laurentii*, *M. pulcherrima*, *Rh. glutinis* var. *glutinis*, *Rh. glutinis* var. *dairenensis*, *Rh. mucilaginosa*	A: PLW; LI, B: P	Yeast in rhizosphere and phyllosphere (agricultural plants vs. non-cultivated plants); isolation with and without pre-enrichment yielded 46 and 23 species, respectively; highest densities in August; basidiomycetous yeasts dominant; *Cryptococcus* and *Thichosporon* more abundant on agricultural plants	Kvasnikov et al. (1975)
Betula (D;T) and *Phleum* (G)	Russia, D	*Cr. albidus*, *Cr. laurentii*, *Cr. macerans*, *Rh. glutinis*, *Rh. rubra*, *Sp. pararoseus*, *T. pullulans* (minor: *Sp. roseus*, *Cy. capitatum*) (4–72×10^3 CFU/dm^2 – *Phleum*; 8–90×10^3 CFU/dm^2 – *Betula*)	A: PLW; LI; OML, B: P	Seasonal dynamics and species diversity (yeasts); direct counts 3–5 times higher than plate counts; 300 isolates (13 species and 7 genera); yeast densities higher in autumn	Babjeva and Sadykov (1980)

Fagus sylvatica D;T	England, C	*B. alba, Sp. roseus* and *Tilletiopsis minor*	A: PWL; SFM; OML	Seasonal dynamics (mainly filamentous fungi); yeasts detected right after unfolding and persisted thereafter (only by SFM)	Hogg and Hudson (1966)
Malus spp. (D;T)	New Zealand, C	*Cr. laurentii, Rh. ingeniosa* (buds and young leaves); *Sp roseus*, *Rh. rubra*, *Cr. laurentii*, *B. alba* and *U. pyricola* (expanding leaves) (10^4–10^5 CFU/cm^2)	A: PLM; SFM	Seasonal dynamics and species diversity (bacteria and fungi); densities increased throughout spring and early summer but decreased during autumn; densities increased with leaf age; comparison of buds, rosette leaves and shoot leaves	Pennycook and Newhook (1981)
	England, C	White (*Cr. laurentii*) and pink yeasts (*Sp. roseus* and *Rhodotorula*) (10^6 CFU/g)	A: PLW; PLM; SFM	Seasonal dynamics and species diversity (bacteria and fungi); effect of fungicides; bacteria and yeasts more numerous than that of moulds; densities increased from May to November on unsprayed trees	Hislop and Cox (1969)
	USA, C;D	*Sporobolomyces*, *Rhodotorula* and *Cryptococcus* (10^6 CFU/g)	A: PLW; LI; SFM; OML; SEM	Effect of pesticides on seasonal dynamics of non-target phylloplane microbiota; bacteria more severely affected than fungi; densities increased from May to September	Andrews and Kenerley (1978)

Continues

Table 13.1 Studies of phylloplane microbial ecology that included or focused on yeasts—*cont'd*

Plant(s)[a]	Geography, climate[b]	Dominant yeast genera or species (densities)	Methods[c]	Main subject of study and summary of conclusions	Reference
Malus spp.—*cont'd*	USA, C;D	*Sporobolomyces*, *Rhodotorula* and *Cryptococcus* (10^5 CFU/g – dormant buds)	A: PLW; SEM	Seasonal dynamics (bacteria and fungi) in buds and young leaves; after unfolding, densities decrease on expanding young leaves (yeasts lagged slightly)	Andrews and Kenerley (1980)
Acer pseudoplatanus (D;T)	England, C	*Sporobolomyces*	A: SFM; SEM	Seasonal dynamics (fungi); densities increase from May/June to August/September; *Sporobolomyces* present predominantly on upper leaf surface, mainly on veins (only by SFM)	Pugh and Buckley (1971)
Acer platanoides (D;T) and *Tilia platyphyllos* (D;T)	Scotland, C	Yeasts (*Acer platanoides*) (10^1–10^3 cells/cm^2 – optical microscopy; 10^1–10^2 CFU/cm^2 – leaf washings). White and pink yeasts (*Tilia platyphyllos*)	A: PLW; PWL; OMLI	Seasonal dynamics (fungi); effect of inhibitory substances from leaves (viz. gallic acid); yeast densities low throughout the season, with increase in September; increased fungal growth observed later in the season –	Irvine et al. (1978)

				coincident with decline in inhibitory activity of leaf washings	
Acer platanoides (D;T)	Scotland, C	Yeasts (*Sp. roseus* more abundant)	A: OMLI	Seasonal dynamics (fungi); low densities in early summer and maximum values during October and November	Breeze and Dix (1981)
Larix decidua (D;T)	Scotland, C	*Rh. glutinis*, *Sp. roseus*, *Cryptococcus* and *Torulopsis*(?) (10^4 CFU/g)	A: PLM; OMLI, B: P	Seasonal dynamics (bacteria and fungi); densities increase from June to November; leaf age is a more important factor for population development than weather or airborne inocula	McBride and Hayes (1977)
Ilex aquifolium (E;T)	England, C	Yeasts (10^4–10^5 cells/cm^2 – optical microscopy; 10–10^4 CFU/cm^2 – leaf washings)	A: PLW; PWL; OMLI; SEM	Seasonal dynamics (fungi); densities higher in autumn and early winter, and on older leaves; upper and lower leaf surfaces with similar yeast population size; litter appeared to provide a refuge for phylloplane yeasts during winter	Mishra and Dickinson (1981)

Continues

Table 13.1 Studies of phylloplane microbial ecology that included or focused on yeasts—*cont'd*

Plant(s)[a]	Geography, climate[b]	Dominant yeast genera or species (densities)	Methods[c]	Main subject of study and summary of conclusions	Reference
Various plants (spruce, alder and birch forests)	Russia, D	*Cr. albidus*, *Cr. laurentii*, *Le. scottii*, *Rh. glutinis*, *Rh. minuta*, *Rh. mucilaginosa*, *Sp. roseus* (up to 10^6 CFU/g)	A: PLW	Variation of species composition (yeasts) with substrate: plant leaves, leaf litter or soil; higher densities on plant surfaces; species composition differs with sample type (vertical stratification)	Maksimova and Chernov (2004)
Camellia sinensis (E;B) *Vicia faba* (D;B)	Sri Lanka, A England, C	*S. salmonicolor*, *Rh. mucilaginosa* and *Cryptococcus* sp. (*Camelia sinensis*); *Sporidiobolus* sp. and *B. alba* (*Vicia faba*)	A: PLW	Sensitivity of phylloplane yeasts to UV-B radiation; vulnerability to UV-B correlated with radiation doses of natural habitat; tolerance to UV-B may be related to pigmentation	Gunasekera et al. (1997)
Agrostis palustris (H;G)	USA, C	White (>95% – *Cryptococcus*) and pink yeasts (10^4–10^5 CFU/cm^2)	A: PLW	Seasonal dynamics (yeasts); effect of fungicides; densities higher in autumn; fungicide resistance widespread among phylloplane yeasts	Buck and Burpee (2002)
Various gramineous plants (steppe)	Russia, B;C	*Cr. diffluens*, *Cr. laurentii*, *Rh. glutinis*, *Sp. roseus* (*A. pullulans* dominant)	A: PLW, B: P	Variation of species composition (yeasts) with substrate: plant	Vinovarova and Babjeva (1987)

		(10^5–10^6 CFU/g)		surfaces, leaf litter or soil; higher densities on plant surfaces; species composition differs with sample type (vertical stratification)	
Hippophae rhamnoides (B)	Gibraltar, B;C	*Sporobolomyces*	A: PWL; SFM; OML; OMLI; SEM	Spatial distribution of fungi on leaves; higher densities associated with trichomes and midrib; yeasts isolated only by SFM, more frequent on upper leaf surfaces	Lindsey and Pugh (1976)
Various plants	Canary Islands, B;C	*D. hansenii*, *Cr. albidus*, *Cr. laurentii*, *Rh. glutinis*, *Rh. mucilaginosa*, *Pseudozyma* sp.	A: PLW, B: P	Species diversity and biochemical activities (yeasts); 44 isolates able to degrade and assimilate many high and lowmolecular weight plant constituents	Middelhoven (1997)
Various plants (subtropical deserts)	Southwest Asia, B	*Bullera* spp., *Cr. albidus, Cr. laurentii* (minor), *U. puniceus* (green leaves: $<10^3$ CFU/g; dead leaves: 10^5–10^6 CFU/g)	A: PLW, B: P	Species diversity (yeasts) on green leaves, dead leaves and soil; yeasts present only on plant surfaces	Chernov et al. (1997)

Continues

Table 13.1 Studies of phylloplane microbial ecology that included or focused on yeasts—*cont'd*

Plant(s)[a]	Geography, climate[b]	Dominant yeast genera or species (densities)	Methods[c]	Main subject of study and summary of conclusions	Reference
Acer monspessulanum (D;T), *Quercus faginea* (D;T), *Cistus albidus* (E;B), *Pistacia lentiscus* (E;B) and *Osyris quadripartita* (E;B)	Portugal, C	*Cr.* cf. *laurentii*, *E.* cf. *hasegawianum*, *L. inositophila*, *Rh. bacarum*, *Rh.* cf. *sloffiae, Sp. coprosmae*, *Sp.* cf. *gracilis*; putative spp. nov.: *Cryptococcus*, *Filobasidium*, *Rhodotorula* and *Sporobolomyces* (*Sp.* cf. *roseus*, *Sp. ruberrimus,* and *Tilletiopsis* spp. – SFM) (10^1–10^2 CFU/cm^2 – *Osyris*, *Pistacia*; 10^2–10^4 CFU/cm^2 – *Cistus*, *Acer*, *Quercus*)	A: PLW; SFM, B: P; MI	Seasonal dynamics and species diversity (yeasts); total counts and species richness varied according to plant species, season and sampling site; densities increased from spring to autumn, notably on deciduous trees; apparent specificity of some yeast species for *Cistus albidus* leaves (e.g. *Cryptococcus* sp. nov.)	Inácio et al. (2002); Inácio (2003)
Mangifera indica (E;T)	South Africa, C	*Cryptococcus* spp., *Sp. roseus* (minor: *Candida*, *Kluyveromyces*, *Rhodotorula*, *Torulopsis*, *Trichosporon*) (10^4–10^5 CFU/cm^2)	A: PLW; LI, B: P	Seasonal dynamics and species diversity (bacteria and fungi); densities higher in winter and spring; densities and species diversity increased with leaf age	de Jager et al. (2001)

Various plants	Indonesia, Suriname and Ivory Coast, A	*Cr. laurentii*, *Cr. luteolus*, *Rh. glutinis*, *Rh. graminis*, *Rh. mucilaginosa*, *Rh. rubra*, *S. ruineniae* (minor: *Candida*, *Rh. bogoriensis*, *Rh. diffluens*, *Rh. foliorum*, *Rh. javanica*, *Sp. roseus*)	A: PL; LI, B: P	Species diversity (yeasts); 65 strains representing 22 species (predominance of basidiomycetous yeasts)	Ruinen (1963)
Ananas comosus (E)	Brazil, A	*Cr. flavus*, *Cr. laurentii*, *Rh. minuta*, *Rh. rubra*, *Rhodotorula* sp. (10^3–10^4 CFU/cm^2)	A: PLW, B: P	Species diversity (yeasts); comparison of leaves, flowers, fruits and soil; yeast counts higher during flowering	Robbs et al. (1989)
Saccharum officinarum (G)	Brazil, A	*Cr. albidus*, *Cr. laurentii*, *Rh. mucilaginosa*, *Sp. roseus*, *Trichosporon* sp. (minor: *Bullera* sp., *D. hansenii*, *Di. hungarica*, *Rh. minuta*) (10^7 CFU/g dry weight)	A: PLW, B: P	Species diversity (yeasts); comparison of leaves, stems and rhizosphere; predominance of basidiomycetous yeasts on leaves	Azeredo et al. (1998)

[a]Plant type: *T* tree, *H* herb, *G* grass, *B* bush; *D* deciduous, *E* evergreen
[b]Köppen climate classification system: *A* tropical moist climates; *B* dry climates; *C* moist mid-latitude climates with mild winters; *D* moist mid-latitude climates with cold winters; *E* polar climates
[c]*A* detection and/or isolation methods: *OML* optical microscopy directly on leaves; *OMLI* optical microscopy on leaf impressions; *SEM* scanning electronic microscopy; *PLW* plating of leaf washings; *PWL* plating of washed leaves; *PL* plating of leaves; *PLM* plating of leaf macerates; *LI* leaf impressions onto solid media; *SFM* spore-fall method. *B* identification methods: *S* simplified; *P* phenotypic (morphology, physiology); *MI* molecular identification

1981; Inácio et al. 2002) or incubation at lower temperatures (e.g. 5˚C, Maksimova and Chernov 2004) to delay mould growth. The utilization of lower incubation temperatures may however select for psychrophilic yeast species (Beech and Davenport 1971). Rose Bengal and other dyes have the disadvantage of being taken up by some yeasts, thus inhibiting their growth or endowing their colonies with a pinkish tinge that can be mistaken for the carotenoid pigments of true "pink" yeasts. Instead of agar media some authors advocate the utilization of artificial substrates that mimic leaf nutritional conditions, such as a wax-based medium amended with nutrients (McCormack et al. 1994a). Counting and isolation of single colonies for subsequent characterization are usually done by selecting randomly from the plates or by picking colonies of each morphological type. Two major limitations of this procedure are that unrelated organisms may display similar colony morphologies and that fast-growing moulds may hamper the enumeration and isolation of other, more fastidious, organisms. Moreover, microorganisms from populations in numerical minority (less than 1% of the dominant species numbers) will most probably go undetected (Flannigan and Campbell 1977). All of the indirect methods mentioned previously are labour-intensive, time-consuming and have the inherent limitations of culture-dependent methods. It is often difficult to compare results from different studies owing to different sampling strategies, washing procedures, culture media and incubation conditions (Jacques and Morris 1995). For example, Azeredo et al. (1998) showed that although the two different washing methods used (sonication and agitation for 30 min on a rotary shaker) gave similar total yeast counts, they resulted in different proportions of the isolated yeast species. The second most used cultivation-based method for yeast isolation from the phylloplane was the spore-fall method (SFM), which is largely specific for ballistoconidiogenic (BC) yeasts (see also Sect. 13.4). Leaf samples are fixed under the lid of a Petri dish, suspended above the agar surface, and colonies develop from discharged spores that fall on the culture medium (Nakase and Takashima 1993). Only a few studies aimed mainly at epiphytic filamentous fungi used isolation methodologies based on the plating of leaves, with or without previous washing, or on leaf impressions onto agar media (Table 13.1).

In recent years several molecular methods have been used for studying the ecology of phylloplane microorganisms without the need of cultivation steps. The main target organisms have been bacteria, but in a few cases yeasts and dimorphic fungi were also included. For example, Yang et al. (2001) used denaturing gradient gel electrophoresis (DGGE) of DNA fragments amplified with universal primers for ribosomal DNA (rDNA) regions, followed by cloning and sequencing of the dominant amplicons, to study bacterial populations in the phyllosphere of *Citrus sinensis* (orange). Among several organisms detected, some DNA bands were ascribed to the ubiquitous epiphytic fungus *A. pullulans*. Bramwell et al. (1995) compared a range of DNA extraction protocols for microbial cells on leaf samples for this type of study. A different molecular technique, fluorescence in situ hybridization (FISH), has been used successfully in the direct detection and identification of microorganisms in their natural environments (e.g. Moter and Göbel 2000). The rationale underlying FISH is that, under appropriate hybridization conditions, the annealing between complementary sequences in the oligonucleotide fluorochrome-labelled probe and target molecules (usually rRNA sequences) can be detected by fluorescence microscopy (or flow

cytometry) in intact cells. Despite some methodological problems that arise when working with whole leaf samples or the respective washings (e.g. background fluorescence of chlorophyll of leaf tissues or topographical structures like trichomes), FISH has already been used for detecting and/or enumerating *A. pullulans* on apple leaves (Li et al. 1997; Spear et al. 1999; Andrews et al. 2002), *Taphrina deformans* on peach leaves (Tavares et al. 2004), and *Cryptococcus* sp. nov. and other yeasts on leaves of different Mediterranean plant species (Inácio 2003).

The motto 'no ecology without taxonomy' has often been applied to microbial ecology (Phaff and Starmer 1987; Fokkema 1991) and pertains to the crucial issue of accurate identifications of phylloplane microbes. Yeast identifications in the studies published until the late 1990s were based mainly on more or less extensive phenotypic characterizations (see Table 13.1) and in many cases can be merely considered tentative. However, the last decade has witnessed remarkable improvements in yeast (and fungal) systematics as a consequence of the so-called molecular revolution (Kurtzman and Fell 1998). In fact only the full implementation of molecular methods facilitates fast and unambiguous identifications, which are now possible owing mainly to the comprehensive rDNA sequence databases made available by the work of Kurtzman and Robnett (1998) for ascomycetous yeasts and of Fell et al. (2000) and Scorzetti et al. (2002) for basidiomycetous yeasts. The impact of these developments is especially significant in the ecology of epiphytic yeasts in view of the many changes that took place in the classification system of basidiomycetous yeasts in recent years (Fell et al. 2001; Sampaio 2004; see Sect. 3). Therefore, it cannot be ruled out that the apparent ubiquity of various epiphytic yeasts, irrespective of host plant and/or locality, stemming from many studies is a result of inaccurate identifications (see Sect. 13.4). In fact yeast species deemed to be ubiquitous phylloplane colonists have been shown to be taxonomically heterogeneous on the basis of molecular phylogenetic analyses only in the last 4 years: *Cr. albidus* (Fonseca et al. 2000; Sugita et al. 2001); *Cr. laurentii* (Sugita et al. 2000; Takashima et al. 2003); *Rhodotorula minuta* (Fell et al. 2000); *Sporobolomyces roseus* (Bai et al. 2002; Fell et al. 2002). A different picture of the composition of epiphytic yeast communities is indeed already emerging from the results of recent studies that used molecular identification methods (Inácio 2003; Prakitchaiwattana et al. 2004).

13.3 Plant Surfaces as Yeast Habitats

The occurrence of yeasts in association with plants has been acknowledged for many years (Lund 1954; Last and Price 1969; Phaff et al. 1978; Phaff and Starmer 1987). Their ability to utilize a plethora of organic compounds through respiratory processes greatly expands the range of ecological niches that they may colonize beyond sugar-rich substrates such as fermenting fruit juices. Many compounds, such as pentoses, polyols, organic acids, aromatic compounds and amino acids, which specific yeasts can assimilate, are either products of plant metabolism or products of other microorganisms from precursors in plants (Phaff et al. 1978). Examples of plant habitats that have been extensively investigated for their yeast inhabitants include the nectar of flowers (Golonka 2002; Herzberg 2004), tree exudates or slime fluxes (Phaff and Starmer 1987) and the necrotic tissues of cacti (Starmer et al.

1991). The yeast communities found therein and whose composition was specific for each type of habitat were generally dominated by ascomycetous species and insects were identified as the major vectors for the introduction and/or dispersal of those yeasts (Phaff and Starmer 1987; Babjeva and Chernov 1995). In contrast, communities found on plant surfaces such as leaves, flowers (excluding nectaries), immature or intact fruits and bark were dominated by basidiomycetous yeasts and the species composition of those communities was generally considered more uniform (Last and Price 1969; Phaff and Starmer 1987; Babjeva and Chernov 1995). The microenvironments that involve aerial organs of plants such as stems, fruits or flowers are analogous to those found on the phylloplane and it is thus not surprising to find parallels in the dynamics and composition of the respective yeast communities (Last and Price 1969; Buck et al. 1998). The distinctive nature of epiphytic yeasts received further support from comparisons with the communities found in the rhizosphere, ensuing from the early work of di Menna with pasture plants in New Zealand (reviewed in Carmo-Sousa 1969 and Last and Price 1969) and later confirmed by other workers (Kvasnikov et al. 1975; Fokkema and Schippers 1986; Maksimova and Chernov 2004). The results of those studies showed that although basidiomycetous yeasts were also dominant, the species found in soils near the roots of plants (e.g. *Cr. albidus*, *Cr. diffluens*, *Cr. humicola*, *Cr. curvatus*; di Menna 1959) did not coincide with those isolated from the aerial surfaces of the same plants (*Cr. laurentii*, *Rh. ingeniosa*, *Rh. graminis*, *Rh. mucilaginosa*, *Sp. roseus*; di Menna 1959). Another relevant observation by di Menna was that while the composition of the soil yeast communities varied with soil type but not with season, the phyllosphere populations changed with season but not with locality or plant (see Sect. 13.5). Similar conclusions ensued from the work carried out at the Department of Soil Biology of the State University of Moscow by Babjeva and co-workers since the 1980s (Babjeva and Chernov 1995). A reasonable corollary from these observations is that basidiomycetous yeasts seem to have distinct niches in the same geographic location despite displaying apparently similar phenotypes (strictly aerobic and generally oligotrophic). Another observation that points to the distinctive nature of epiphytic yeasts stemmed from studies of the communities found on the surfaces of fruits. Work by different authors revised in Last and Price (1969) and Phaff and Starmer (1987) suggested the frequent occurrence of important shifts in the yeasts populations present on intact fruits (generally dominated by basidiomycetous yeasts) when compared with those found on damaged or fermenting fruits (dominated by ascomycetous and/or fermenting yeasts). This trend was nicely demonstrated in a recent study of the yeast communities on the surface of grapes by Prakitchaiwattana et al. (2004), who found species of *Hanseniaspora* and *Metschnikowia* only on damaged, ripe grapes, whereas undamaged berries in different ripening stages were populated by basidiomycetous yeasts (besides *A. pullulans*). Some of these topics will be further discussed in the following sections.

Before proceeding we want to mention an apparently minor issue but one of relevance to the ensuing discussions on the nature and dynamics of phylloplane yeasts. It pertains to the euascomycete *A. pullulans*. This dimorphic fungus, sometimes inappropriately called yeast-like, is apparently ubiquitous on aerial plant surfaces in temperate or subtropical regions worldwide (Andrews et al. 1994) and has been

reported as the dominant fungal epiphyte of intact fruits (apple, Beech and Davenport 1970; pear, Chand-Goyal and Spotts 1996; grapes, Prakitchaiwattana et al. 2004) and leaves of trees such as *Acer platanoides* (Norway maple, Breeze and Dix 1981), *llex aquifolium* (holly, Mishra and Dickinson 1981), *Fagus sylvatica* (beech, Hogg and Hudson 1966), *Malus domestica* (apple, Andrews and Kenerley 1978) and *Mangifera indica* (mango, de Jager et al. 2001). In some studies *A. pullulans* was even considered a member of the yeast community (Breeze and Dix 1981; de Jager et al. 2001) but in this review we will focus mainly on the yeast taxa dealt with in Kurtzman and Fell (1998).

13.4 Diversity of Phylloplane Yeasts

Yeasts have been recognized as important phylloplane colonists since the classic publications by Last (1955), di Menna (1959) and Ruinen (1961). These and other early studies were reviewed in Last and Price (1969) and Phaff and Starmer (1987). We have summarized in Table 13.1 the results of those studies, as well as others not covered in the mentioned reviews or published more recently. The studies were grouped according to plant type and/or to the climatic characteristics of the sampling site. Whenever possible we have tried to update species names according to current notions on yeast classification (Kurtzman and Fell 1998; Sampaio 2004). Methods used for yeast isolation and identification are also indicated since they have a major influence on the results obtained (see Sect. 2).

The most obvious trend that emerges from the analysis of Table 13.1 is the clear dominance of basidiomycetous yeasts on leaves, a feature common to most aerial plant surfaces (see Sect. 3). Phylloplane communities usually comprise deeply pigmented species belonging to the genera *Rhodotorula* and *Sporobolomyces* (collectively referred to as 'pink yeasts' in many studies) and non-pigmented *Cryptococcus* species ('white yeasts') (Hislop and Cox 1969; McBride and Hayes 1977; Fokkema et al. 1979; McCormack et al. 1994b). In some cases, however, ascomycetous yeasts were reported as important leaf colonists (Phaff and Starmer 1987; Middelhoven 1997; Azeredo et al. 1998; de Jager et al. 2001). Attempts to draw inferences on distribution patterns of phylloplane yeast species from published data are hampered by the inherent limitations of the conventional methodologies used for strain identification in all but one of the studies listed in Table 13.1. In some cases identifications were admittedly tentative because the authors were essentially interested in unravelling the dynamics of the phylloplane populations (Hislop and Cox 1969; McBride and Hayes 1977; Irvine et al. 1978; Fokkema et al. 1979; de Jager et al. 2001). Some authors acknowledged the difficulties in achieving accurate identifications on the basis of phenotypic properties and noted the heterogeneity of certain yeast species (Hislop and Cox 1969; Flannigan and Campbell 1977; Azeredo et al. 1998). Comparison of the results of those studies is also constrained by the use of diverse isolation methods by different authors, an illustrative example being the use of the SFM specific for recovery of BC yeasts (Last 1955; Hogg and Hudson 1966; Lindsey and Pugh 1976; Pugh and Buckley 1971; Pugh and Mulder 1971). In fact, members of the genera *Bullera*, *Sporobolomyces* or *Tilletiopsis* are thought to be especially adapted to this kind of habitat owing to the production of forcibly ejected

conidia that ensure their efficient dispersal and are thus commonly isolated from leaves (Nakase 2000; Sampaio 2004). However, the SFM is not amenable to quantification (Pennycook and Newhook 1981; Fokkema 1991) and it is therefore not possible in those studies to estimate the relative size of BC yeast populations within the phylloplane communities. These yeasts will be dealt with separately at the end of this section.

Another general trend observed in studies that employed plating of leaf washings for yeast isolation is that populations are commonly dominated by relatively few abundant species (Table 13.1), a situation also observed for other microbial epiphytes (Andrews 1991). Nevertheless, minor species may account for a significant fraction of species richness on the phylloplane, especially in samples with large yeast counts, as demonstrated in some studies (Inácio et al. 2002; Maksimova and Chernov 2004) and many novel taxa were discovered among them (Nakase 2000; Inácio 2003). Amongst the dominant species, *Cr. laurentii*, *Cr. albidus* (less abundant than *Cr. laurentii* and not as widespread), *Rh. glutinis*, *Rh. minuta*, *Rh. mucilaginosa* and *Sp. roseus* appear to be prevalent regardless of plant type or geography (Table 13.1). However, as pointed out in Sect. 13.2, the majority of these species were recently found to be genetically heterogeneous and their apparent ubiquity thus needs revaluation. Whereas the presence of the previously mentioned species seems to be essentially independent of plant type and geography, their relative abundance varies not only with plant type but also with season (see also Sect. 13.5). For instance, non-pigmented *Cryptococcus* spp. (mainly *Cr. laurentii*) dominated phylloplane communities on pasture plants and other herbaceous plants (di Menna 1971; Buck and Burpee 2002; Glushakova and Chernov 2004) but pigmented yeasts (namely *Rh. glutinis* and/or *Sp. roseus*) were prevalent on barley and wheat (Diem 1967, cited in Last and Price 1969; Flannigan and Campbell 1977; Southwell et al. 1999) and possibly on conifers (McBride and Hayes 1977). Deeply pigmented *Rhodotorula* and *Sporobolomyces* spp. were also dominant on xerophytic shrubs when compared with other Mediterranean plant species sampled simultaneously (Inácio et al. 2002; Inácio 2003). Another trend that emerged from surveys on multiple plants was that yeast densities and species richness were consistently lower on leaves of xerophytic shrubs and coniferous trees than on those of hydrophytic or mesophytic herbs and deciduous trees (Inácio et al. 2002; Maksimova and Chernov 2004). On the other hand, some species appear to be restricted to specific biocoenoses or geographic regions: *Rh. ingeniosa* on pasture plants and apple leaves in New Zealand (di Menna 1971; Pennycook and Newhook 1981); *Rh. fujisanensis*, *Cystofilobasidium capitatum* and *Leucosporidium scottii* in subboreal forests in Russia, mainly on senescent leaves (Glushakova and Chernov 2004; Maksimova and Chernov 2004); *Rh. bacarum* as well as a few *Rhodotorula* and *Sporobolomyces* spp. belonging to the Erythrobasidium lineage (*sensu* Fell et al. 2000) on Mediterranean plants in Portugal (Inácio et al. 2002; Inácio 2003). Associations of particular yeast species to specific plants appear to be rare (Andrews 1991) but one such case was reported by Inácio and co-workers, who found consistently significant numbers of a putative novel *Cryptococcus* species with orange colonies on the leaves of the evergreen shrub *Cistus albidus* (grey-leaved cistus or white-leaf rock rose) but not on four other plant species sampled at the same sites for two consecutive years (Inácio et al. 2002; Inácio 2003).

Ascomycetous yeasts are usually rare on the phylloplane but the species *Debaryomyces hansenii* was found with high frequency on plants from the Canary Islands (Middelhoven 1997) and on sugarcane in Brazil (Azeredo et al. 1998) and was also reported to occur on leaves of forest plants in Russia (Babjeva et al. 1999; Glushakova and Chernov 2004; Maksimova and Chernov 2004). Yeasts related to the archiascomycete genus *Taphrina* and classified in *Lalaria* (Inácio et al. 2004) were found in high densities on Mediterranean plants in Portugal (Inácio et al. 2002; Inácio 2003). Putative *Lalaria* spp. were also reported on forest trees in Russia by Babjeva and co-workers (Babjeva and Reshetova 1998; Babjeva et al. 1999). This is an unexpected finding since members of the dimorphic phytopathogenic genus *Taphrina* are rarely isolated from plant material other than infected tissues of the host (Kramer 1987). Inácio et al. (2004) claim that their *Lalaria* isolates may represent genuine phylloplane inhabitants that have lost the parasitic stage altogether. The relatively rare occurrence of ascomycetous yeasts on the phylloplane is also evidenced by comparing the composition of epiphytic yeasts on fruits and leaves of the same plant species (Beech and Davenport 1970; Robbs et al. 1989). For example, on apple fruit skin (Beech and Davenport 1970; Bizeau et al. 1989) species of *Hanseniaspora* and *Metschnikowia* are commonly present together with the basidiomycetous species (and *Aureobasidium*) that are also found on the leaves, on which the former are absent (Pennycook and Newhook 1981). The unusual finding of species of *Torulaspora* and *Kluyveromyces* on elm trees in California was probably due to the sugary syrup secreted by its leaves (Phaff and Starmer 1987).

A different approach to the ecology of epiphytic yeasts has been undertaken by Babjeva and co-workers: instead of focusing on specific substrates (i.e. plant species) they have chosen to analyse the spatial structure and biogeography of yeast populations obtained from large numbers of samples in plant–soil systems from different geographic regions (Babjeva and Chernov 1995; Maksimova and Chernov 2004). They distinguished three yeast complexes occupying different substrates that correspond to a vertical stratification of each type of ecosystem studied (tundra, forest, steppe and desert): the epiphytous complex, i.e. the yeasts that occur on living, green aboveground plant parts (mainly the phylloplane); the litter complex, i.e. the yeasts that are present on senescent leaves and leaf litter; and the soil complex, i.e. the species inhabiting mineral soil horizons. The dominant species of the first complex, consisting mainly of *Cr. laurentii* and *Sp. roseus*, were regarded as non-geographic (similar species composition in different zones). However, species richness and minor species varied from tundra to desert ecosystems. Higher yeast densities and species richness were found in forest biotopes for which some differences in community composition were noted depending on the plant type (hydrophytic vs. xerophytic, conifer vs. broad leaf) or forest type (spruce, alder or birch) (Maksimova and Chernov 2004).

As mentioned previously, BC yeasts in general and the species *Sp. roseus* in particular are frequently associated with leaf surfaces and were already reported in the early phylloplane work by Last (1955), Kerling (1958, cited in Last and Price 1969) and di Menna (1959) (Table 13.1). Production of ballistoconidia provides a clearly efficient means for dispersal of those yeasts and appears to be stimulated by the nutrient-poor conditions found on leaf surfaces. In some studies the SFM was used

as the sole method for yeast isolation and therefore only that type of yeast was recovered from the phylloplane (Table 13.1). The majority of recognized species in the genera *Bensingtonia*, *Bullera* and *Sporobolomyces* were in fact isolated from phylloplane yeast surveys employing the SFM and were conducted mainly by researchers in Asia (Nakase 2000). However, it should be noted that BC yeasts may occur in very different habitats, such as seawater (Sampaio 2004), and that the SFM may yield non-BC yeasts (Inácio 2003; Inácio et al. 2004). The ubiquitous phylloplane species *Sp. roseus* was thought to have a worldwide distribution (Last and Price 1969) but may actually be more abundant in temperate regions (climate types C and D; Köppen's system). Babjeva and co-workers found *Sp. roseus* in high frequencies as a member of the epiphytous complex in tundra, forest and steppe ecosystems in Russia but not in desert biotopes (Babjeva and Chernov 1995). This species was dominant on steppe plants but in the different forest types studied it was never found on mosses despite being isolated from the phylloplane of all other herbaceous and ligneous plants (broad-leaved or conifers) sampled (Maksimova and Chernov 2004). *Sp. roseus* was also found on Mediterranean plants in Portugal but only using the SFM and was seldom detected on plates inoculated with leaf washings (Inácio et al. 2002; Inácio 2003). In an overview of the occurrence of BC yeasts in the Asia–Pacific region, Nakase (2000) claims that there is no correlation between the presence of each yeast species and the respective host plant but that there appear to be some geographic patterns. Once again *Sp. roseus* was apparently most frequently isolated in temperate and continental regions (climate types C and D) but not in the tropics (climate type A). According to the same author this pattern is shared by less frequent species such as *Udeniomyces pyricola*, *Sp. inositophilus*, *Sp. sasicola* and *Bensingtonia naganoensis*. Conversely, species that were isolated exclusively in the tropics include *Sporidiobolus ruineniae* and particular species in the genera *Kockovaella*, *Sporobolomyces* (namely those having hydrogenated CoQ10) and *Bullera*. Species such as *Bullera alba*, *B. crocea*, *B. variabilis*, *S. pararoseus* and *S. salmonicolor* were found in all the surveys reviewed by Nakase (2000) independent of climatic region.

13.5 Population Dynamics on the Phylloplane: Variation in Space and Time

One unifying feature of phylloplane microbial populations is their variability across a wide range of spatial and temporal scales (Kinkel 1997; Andrews and Harris 2000; Morris 2001). Some information is available about the patterns of epiphytic microbial community dynamics, at least for some of its components, but elucidation of the processes that generate those patterns is limited (Kinkel 1997). The dynamics of microbial populations on leaves are a function of four processes: immigration (I), emigration (E), growth (G) and death (D). The contribution of each process to the variability in phylloplane community sizes and composition is likely to differ markedly over time and for different organisms. Some studies dealing with changes in numbers of given species in the phyllosphere over time and with patterns of their localization on leaves focused mainly on bacteria (Kinkel 1991; Jacques et al. 1995; Hirano and Upper 2000) or fungi (Andrews et al. 1987, 2002; Kinkel et al. 1989) but

comparatively less is known about yeasts. However, some of the general trends observed may be extrapolated for different epiphytic microbes (Kinkel 1997).

The significance of the atmosphere as both a source (I) and sink (E) for phylloplane populations has been acknowledged by many authors (Andrews 1991; Kinkel 1997). However, despite a substantial body of literature on airborne propagules and their significance to dispersal of epiphytic microbes (yeasts: Last and Price 1969) there have been few attempts to correspond shifts in air inocula with specific changes in phylloplane populations and there is little quantitative information on the dynamics of those airborne cells in relation to immigration and emigration of microbes on individual leaves (Andrews et al. 1987). Emigration from leaves occurs as a function of active dispersal mechanisms generated by rain, water movement or wind. Immigration to leaves occurs by impaction of particles onto the leaf surface, gravity settling or sedimentation, or rain-splash dispersal to the leaf surface (Kinkel 1997). The uniformity in occurrence (but not necessarily population size; see later) of the dominant yeast species on leaves of different plants in the same geographic area (di Menna 1971; Babjeva and Sadykov 1980; Inácio et al. 2002; Maksimova and Chernov 2004) suggests that immigration is a quantitatively significant process in the build-up of phylloplane populations. Within-leaf processes of growth and death are a function of leaf age, season and plant species but are also influenced by the physical environment: growth of bacteria, filamentous fungi or yeasts on leaf surfaces is generally more significant when temperatures are moderate and moisture levels are high (fungi/yeasts: Bashi and Fokkema 1977; Inácio et al. 2002); death of microbes on plant surfaces is enhanced under conditions of intense UV radiation, high temperatures, low relative humidity and/or low availability of free moisture (Kinkel 1997). However, the presence of protected sites on leaf surfaces and the survival mechanisms of many microbial epiphytes allow individuals within the population to persist despite the prevalence of conditions non-conducive to growth or survival. In summary, differences in populations of microbial epiphytes among leaves of different plant species (and over time) may be initiated as a function of immigration and emigration, and the subsequent effects of differential growth and death are likely to act to further distinguish leaves as habitats (Kinkel 1997). Significant variations in relative sizes of populations of different yeast species on different plants in the same geographic area were demonstrated in the studies by Inácio et al. (2002) and Maksimova and Chernov (2004).

13.5.1 Patterns in Space

Microbial populations are unevenly distributed across individual leaf surfaces. Aggregated populations have been noted for bacteria (Morris et al. 1997), yeasts (Bashi and Fokkema 1976; Babjeva and Sadykov 1980) and filamentous fungi (Pugh and Buckley 1971; Mishra and Dickinson 1981). Aggregation of microbial cells is often correlated with specific structures on the leaf surface (leaf veins, trichomes, stomates, fungal hyphae, pollen grains) or may be a function of their existence within biofilms or within an extracellular matrix on the leaf surface (Kinkel 1997; Andrews and Harris 2000). The importance of discrete sites conducive to microbial growth and survival suggests that population densities are likely to be correspondingly

aggregated. Such patterns have been recently revealed in a study of the distribution of *A. pullulans* on the apple leaf surface using FISH (Andrews et al. 2002). The authors found that most of the leaf surface was not colonized and that there was a highly patchy occupancy of leaf space by *A. pullulans* often related to landmarks, especially the midvein. The reasons for this pattern in the distribution of microcolonies were not elucidated, but physiological and anatomical evidence suggests that veins may be zones of nutrient enrichment (Andrews et al. 2002). Veinal distribution patterns had already been shown for other phylloplane microbes (Kinkel 1997) and in particular for BC yeasts in studies that used the SFM (Last and Price 1969; Lindsey and Pugh 1976) and were confirmed by optical microscopy for other yeasts (Babjeva and Sadykov 1980) and by SEM for fungi in general (Pugh and Buckley 1971). Association of fungi with trichomes was revealed also using SEM on leaves of *Hyppophae rhamnoides* (sea buckthorn, Lindsey and Pugh 1976) and of apple (Andrews and Kenerley 1978). Inácio et al. (2002) suggested that the dense trichome cover on the leaves of the evergreen shrub *Cistus albidus* may have accounted for the high densities and species diversity of fungi on this plant when compared with those observed on other plants sampled concurrently. Andrews et al. (2002) also found that leaves were rarely seen with natural occurring wounds in early summer but that such damage (almost exclusively in the interveinal areas) increased over the course of a growing season. The authors claim that natural wounds accounted overall for an increasing fraction of the total *A. pullulans* population on leaves. If confirmed for other leaf–microbe systems, wounding, and the consequent increased leaching of nutrients, may contribute significantly to well-known but unexplained phenomena such as seasonal (see later) and leaf-to-leaf variations in microbial counts.

Epiphytic bacterial populations tend to be log-normally distributed even among identical leaves, as well as among small leaf segments (Kinkel 1997; Andrews and Harris 2000). Evidence based on colony forming units (CFU) counts on plates inoculated with washings from wheat leaves suggested that phylloplane yeasts and filamentous fungi were normally as opposed to log-normally distributed (Fokkema and Schippers 1986). However, more recent studies using improved plate count methods (Woody et al. 2003) or FISH (Inácio 2003) suggest that *A. pullulans* on apple leaves and *Cryptococcus* sp. nov. on *Cistus albidus* leaves, respectively, followed log-normal distributions.

Microbial populations tend to be higher on the lower (abaxial) than the upper (adaxial) leaf surface (Kinkel 1997), presumably owing to more favourable nutrient, microhabitat or microenvironmental settings, but this pattern possibly varies with plant type. Available data for yeasts are not consistent. Pugh and Buckley (1971) reported that *Sporobolomyces* evaluated with the SFM was present predominantly on adaxial surfaces and the same trend was found by Last and Deighton (1965, cited in Pugh and Buckley 1971) on elm leaves, by Lindsey and Pugh (1976) on leaves of *Hyppophae rhamnoides* and by Pennycook and Newhook (1981) on apple leaves. The opposite situation was found on leaves of chrysanthemum and rowan but no significant differences between the two surfaces were detected for barley (Last and Deighton 1965, cited in Pugh and Buckley 1971). Pennycook and Newhook (1981) claim that evidence for the distribution of *Sp. roseus* based on spore-fall data must be

treated with caution, since they found that spore discharge rates do not necessary correlate with population size (see Sect. 4). Moreover, using a maceration-dilution plating method they found that the yeast population on the abaxial surface was approximately twice as dense as that on the adaxial surface at all sampling dates (Pennycook and Newhook 1981). However, Andrews and Kenerley (1978) found no consistent differences between yeast counts on adaxial or abaxial apple leaf surfaces using a leaf-imprinting method. On the other hand, Mishra and Dickinson (1981) examined impression films taken from green *Ilex aquifolium* leaves of different ages and showed that the abaxial surfaces usually supported larger numbers of yeasts than adaxial surfaces, the differences between surfaces being most pronounced on younger leaves. Breeze and Dix (1981) observed the opposite situation for *A. pullulans* on the leaves of *Acer platanoides*, using the same technique. These apparent contradictory results could be due to different leaf topography of each surface (e.g. dense trichome cover of abaxial vs. adaxial surface of apple leaves; Andrews et al. 2002), which may have gone unnoticed and was not taken into account in most studies.

Variability in phylloplane population sizes among leaves is sometimes correlated with leaf position, especially height in the canopy, but varies with plant species (Kinkel 1997). For yeasts limited evidence suggests that the largest populations generally occurred on leaves from the basal portion of shoots (Mishra and Dickinson 1981) and on leaves closer to the ground (Andrews et al. 1980). Densities of phylloplane microbes are also determined by plant position in the field as shown in studies with barley (Diem 1967, cited in Last and Price 1969) and apple (Bakker et al. 2002).

13.5.2 Patterns in Time

The processes that govern the dynamics of epiphytic microbial populations depend on many intrinsic (e.g. abundance and composition of plant exudates) and extrinsic (e.g. temperature, humidity, solar radiation) factors, which in turn undergo significant seasonal and ontogenetic changes, thus causing pronounced temporal shifts in the species composition of phylloplane communities. Although short-term variations have been studied by some authors, they appear to be more significant for bacteria than fungi (including yeasts) (Thompson et al. 1995; Kinkel 1997). More importantly, the largest body of evidence comes from the numerous studies on seasonal dynamics of epiphytic populations and concerns long-term patterns (Kinkel 1997; Andrews and Harris 2000). The most consistent trends observed in those studies were (1) a general increase in population sizes over time (i.e. with leaf age and/or season) and (2) a seasonal succession in bacterial and fungal populations, featuring an early prominence of bacteria, followed by yeasts, and eventually filamentous fungi as leaves mature and senesce. These features also emerged from a significant number of detailed studies that focused on or included yeasts, listed in Table 13.1. Absolute values of yeast densities reported in different studies are difficult to compare owing to utilization of different methods of isolation and enumeration. However, there appear to be some significant differences in the maximum capacity of leaves depending on plant type and/or climate: values of 10^3–10^4 CFU/cm^2 (equivalent to approximately 10^4–10^5 CFU/g) are common on leaves of herbs or

deciduous trees in temperate climates but values as high as 10^6 CFU/cm^2 (approximately 10^7 CFU/g) were found in some cases (Table 13.1). Yeast population densities estimated from plate counts often exceeded those of filamentous fungi and this trend was corroborated by estimates from direct counts (Hogg and Hudson 1966; Dickinson 1967, cited in Last and Price 1969; McBride and Hayes 1977; Breeze and Dix 1981; Pennycook and Newhook 1981).

Studies that evaluated the dynamics of yeast populations and provided evidence for the increase of population sizes with leaf age focused on different plant types: a deciduous conifer (larch, McBride and Hayes 1977), a deciduous broad-leaved tree (apple, Pennycook and Newhook 1981) and two evergreen trees (holly, Mishra and Dickinson 1981; mango, de Jager et al. 2001). Other studies demonstrated similar trends but yeasts were evaluated solely by the SFM (Last 1955; Hogg and Hudson 1966; Pugh and Mulder 1971). The general increase in phylloplane populations was attributed to immigration processes, which should be more pronounced in the initial stages of leaf development, and to growth at the later stages mainly due to increased leaching of nutrients onto the leaf surface (see Sect. 5.1). Inoculation experiments on cereals provided circumstantial evidence that the limited nutrient availability on young leaves is a most probable cause for the relatively poor development of yeasts on non-senescing leaves (Bashi and Fokkema 1977). The fact that older leaves are more conducive to microbial growth has also been attributed to changes in surface properties, namely increasing wettability, i.e. lower hydrophobicity (Mishra and Dickinson 1981; Buck and Andrews 1999a), or to diminishing concentrations of antimicrobial compounds (Irvine et al. 1978). The interplay of the previously mentioned processes is evidenced in the thorough study of the mycobiota of apple buds and leaves by Pennycook and Newhook (1981): on newly unfurled rosette leaves the phylloplane yeasts were similar to those in unopened buds, but within 5 days some of the components began to disappear and new ones took their place; there was a sharp initial decline in population densities followed by a more gradual increase; the authors claim that the initial decrease was partly caused by the loss of those species of the bud microflora which were ill-adapted for survival on exposed leaf surfaces (D) and partly to dilution of the populations by the rapid increase in the surface area of each lamina during its expansion, the subsequent increase in population densities being due to multiplication of the surviving species (e.g. *A. pullulans*, *Cr. laurentii*, *Rh. ingeniosa*, *Rh. mucilaginosa*) of the bud microflora (G), and to deposition and establishment of new species (e.g. *Sp. roseus*) derived from the air spora (I). The presence of yeasts (and other microbes) inside leaf buds as possible sources of leaf inocula was confirmed by Andrews and Kenerley (1980). The prominence of yeasts on senescent leaves and on leaf litter in the initial stages of decomposition was demonstrated by Dickinson (1967, cited in Last and Price 1969), di Menna (1971), Mishra and Dickinson (1981) and Maksimova and Chernov (2004). The latter authors also noted important shifts in the yeast community composition on leaf litter of subboreal forests in Russia with the clear dominance of species that were minor components on green leaves, namely *Rh. fujisanensis*, *Cy. capitatum* and *Le. scottii*.

Several studies evaluated the seasonal dynamics of size and composition of phylloplane fungal and/or yeast communities on different plant types – pasture herbs,

di Menna (1959, 1971); wheat, Flannigan and Campbell (1977); wood sorrel, Glushakova and Chernov (2004); apple, Pennycook and Newhook (1981); larch, McBride and Hayes (1977); Mediterranean plants (Inácio et al. 2002) – while others focused mainly on population sizes (maple, Irvine et al. 1978; holly, Mishra and Dickinson 1981; mango, de Jager et al. 2001). A continuous increase in population size from spring to autumn/winter was the general rule but a significant decrease in autumn was observed on beet (Kerling 1958, cited in Last and Price 1969), apple (Hislop and Cox 1969; Pennycook and Newhook 1981) and wheat (Flannigan and Campbell 1977). A notable exception was the finding of higher densities of yeasts and fungi in winter and spring on mango leaves by de Jager et al. (2001), which the authors attributed to the winter flowering of this species that released large amounts of pollen (see Sect. 13.1). The increase in population size later in the growing season may be due to the factors mentioned before for leaf age but some authors also suggest the positive impact of the more conducive environmental factors that prevail in autumn, such as higher humidity and milder temperatures (Breeze and Dix 1981; Kinkel 1997). An almost universal trend in many of the studies mentioned before was that higher yeast densities were associated with larger numbers of species being recovered (species richness) (Inácio et al. 2002; Maksimova and Chernov 2004). However, important shifts in the composition of phylloplane yeast communities with season were reported in studies that evaluated the dynamics of species diversity in more detail: apple (Pennycook and Newhook 1981), wood sorrel (Glushakova and Chernov 2004), pasture plants (di Menna 1959, 1971). The most notable trend was the predominance of deeply pigmented *Rhodotorula* and/or *Sporobolomyces* spp. during the summer months that was attributed exclusively to environmental parameters such as prevailing temperatures, moisture levels and/or daily duration and intensity of sun exposure to which those yeasts are presumably better adapted. Conversely, *Cryptoccocus* spp., namely *Cr. laurentii*, dominated during the colder, more humid months (spring and late autumn/winter) when overall yeast populations were relatively sparse.

13.6 What are the Makings of a 'Phylloplane Yeast'?

Microbial phylloplane colonists (i.e. the residents as opposed to the transients) are presumably endowed with suitable phenotypes for survival and/or growth in their particular surface habitats. The adaptations shared by the diverse microbial colonists of a given region have been referred to as niche-specific traits. Some of these attributes include fast growth rates, the ability to compete for nutrients and to withstand periods of drought or intense light, varying nutrient levels, osmotic conditions and temperatures (Andrews and Harris 2000; Morris 2001; Lindow and Brandl 2003). Many fitness traits are intuitive and have been sustained experimentally but others are not, or are actually counterintuitive (Lindow 1991; Andrews and Buck 2002). Some of those traits have been elucidated for bacteria using modern molecular genetic tools and include production of extracellular polysaccharide (EPS), production of surfactants that modify surface properties such as wettability or production of compounds that stimulate release of nutrients (Lindow and Brandl 2003). Those methodological approaches have not yet been applied to fungi or yeasts

and available data on fitness traits in the latter organisms are mostly circumstantial. Is it possible then to propose a preliminary definition for 'phylloplane yeast'? In the absence of solid evidence we chose to offer some speculative thoughts that are intended merely as potentially useful hints for future studies.

Dominant phylloplane yeast colonists are mostly of basidiomycetous affinity. Phenotypic traits associated with these yeasts include a strictly respiratory metabolism, oligotrophic nutrition and production of ballistoconidia, and these characteristics seem most adequate for growth or dispersal on leaf surfaces (Last and Price 1969). However, basidiomycetous yeasts are also prevalent in quite different habitats, such as soil or aquatic environments (Sampaio 2004), suggesting that those traits are not unique to phylloplane colonists. Another interesting observation is that basidiomycetous phylloplane isolates are usually anamorphic yeast stages, whereas their teleomorphic filamentous counterparts are rarely found on leaves (Inácio 2003; Maksimova and Chernov 2004). An obvious if rather naive corollary is that unicellular growth forms are better suited for phylloplane colonization owing to more efficient nutrient uptake and dispersal, whereas a mycelial growth habit is better suited for invasion of leaf tissues. The latter situation occurs most likely during leaf decomposition and it concurs with the finding on leaf litter of filamentous yeast taxa (e.g. *Cy. capitatum*, *Trichosporon pullulans*) not present on green leaves (Maksimova and Chernov 2004).

A more tangible fitness trait, common to many phylloplane yeasts, is the production of EPS capsules (Bashi and Fokkema 1976; Babjeva and Sadykov 1980; Glushakova and Chernov 2004) mainly by members of *Cryptococcus* but also by some *Rhodotorula* spp. (Golubev 1991). EPS has already been shown to confer improved fitness to phylloplane bacteria (see earlier) and the importance of biofilms in bacterial colonization of surfaces is widely recognized (Andrews and Harris 2000). Experimental evidence for yeasts suggests that the role of capsules in providing increased fitness for survival and growth on natural substrates pertains to two environmental factors that are particularly significant on the phylloplane: water and nutrient stresses (Golubev 1991). On one hand capsules appear to act as cellular buffer systems preventing too rapid a loss of water and providing for efficient rehydration following periods of drought (Golubev 1991, and references therein). On the other hand capsular material was shown to bind both ionic and non-ionic nutrients providing for higher growth rates of encapsulated yeast cells versus non-capsulated (or hypocapsulated) variants on nutrient-poor media (Golubev 1991). Improved capacity for growth of encapsulated yeasts in oligotrophic conditions was also demonstrated by Kimura et al. (1998). Conversely, nutrient-poor media, particularly with low nitrogen content (high C-to-N ratio), which parallel the situation normally encountered on leaf surfaces, were shown to stimulate EPS production by yeasts (Golubev 1991). The presence of capsules confers yeast colonies with a mucous texture on solid media as was already noted by Ruinen (1963) in her pioneering studies of phylloplane yeasts. However, she attributed the observed mucilage to lipid production by the yeasts. Interestingly, lipid production was later confirmed in studies by Ruinen and other authors mainly by *Rhodotorula* spp. (e.g. *Rh. bogoriensis*, *Rh. graminis*) that do not produce considerable amounts of EPS (Hunter and Rose 1971). The significance of these compounds for leaf colonization has not been deter-

mined but they might well function as biosurfactants in a manner similar to that of equivalent compounds produced by phylloplane bacteria (see earlier). Bashi and Fokkema (1976) had already suggested that the mucilage secreted by *Sp. roseus* cells on barley leaves seemed to impregnate (but not dissolve) the wax layer of the leaf cuticle, thus changing surface properties (viz. wettability).

As previously mentioned oligotrophy appears to be a suitable trait for growth on the generally nutrient-poor leaf surfaces. Yeast species often found on the phylloplane (but also in soils) have been shown to be particularly fit to grow in media with extremely low concentrations of nutrients (Vishniac 1982; Kimura et al. 1998). This feature has been attributed to the exceptionally high affinity uptake systems for sugars and amino acids found in those yeasts (Kimura et al. 1998). A low nitrogen requirement by phylloplane yeasts was already noted by Ruinen (1963) and di Menna (1959) and although their findings have not been confirmed in later studies they clearly deserve to be further investigated. Ruinen's phylloplane isolates were actually obtained on nitrogen-deficient media which she used for isolation of nitrogen-fixing bacteria (Ruinen 1963). Oligotrophy is thought to provide an advantage in competition for nutrients on the phylloplane and oligotrophic microbes are likely to outcompete copiotrophic ones (Andrews and Harris 2000). This trait could explain preliminary results of co-inoculation of *A. pullulans* and *Tremella foliacea* (soil isolate) on an artificial wax substrate that was claimed to mimic the leaf surface (McCormack et al. 1994a).

Tolerance to UV radiation by means of efficient DNA repair systems and/or abundant production of pigments by both fungi and bacteria is thought to be a fitness trait for phylloplane colonists (Pugh and Buckley 1971; Lindow 1991; Moody et al. 1999; Morris 2001). Some solid evidence exists for bacteria (Lindow and Brandl 2003) but available data for yeasts are manifestly insufficient to prove a direct and major role of pigments in enhancing survival on the phylloplane (Pugh and Buckley 1971; Gunasekera et al. 1997). Nevertheless, it is noteworthy that a large majority of yeasts found on the phylloplane (notably deeply pigmented *Rhodotorula* and/or *Sporobolomyces* spp.) produce potentially photoprotective compounds, namely carotenoids, and that these yeasts occurred in higher frequencies in the summer months (see Sect. 13.5). A new class of UV-inducible compounds (mycosporines) was recently found in a number of basidiomycetous yeast species from freshwater lakes (Libkind et al. 2004), some of which are also found on the phylloplane (e.g. *Cr. laurentii*, *Rh. minuta*). It should be emphasized that mycosporines as well as some carotenoids (e.g. β-carotene) are not restricted to 'pink' yeasts but are also present in some of the non-pigmented (i.e. 'white') yeasts commonly found on the phylloplane (namely *Cryptococcus* spp.).

Production of hydrolytic enzymes (cutinases, pectinases, etc.) has been suggested to enhance the capacity of phylloplane colonists to actively extract nutrients from the plant tissue (Kinkel 1991; Morris 2001) but available evidence suggests that microbial epiphytes grow essentially at the expense of compounds that leach passively to the leaf surface (Mercier and Lindow 2000; Leveau and Lindow 2001). Conversely, production of hydrolytic enzymes is demonstrably essential for the efficient invasion of leaf tissues by many phytopathogenic bacteria and fungi (St. Leger et al. 1997; Hirano and Upper 2000). Nevertheless, some authors have

emphasized the biodegradative abilities of microbial epiphytes. In the case of yeasts, Ruinen (1963) found that the majority of phylloplane isolates produced extracellular lipases and she later claimed that those yeasts were able to degrade leaf cuticles (Ruinen 1966). However, later studies gave rise to conflicting results (Bashi and Fokkema 1976; McBride 1972, cited in Buck and Andrews 1999b). Middelhoven (1997) assayed many biochemical activities by the phylloplane yeast isolates he had obtained and found that the large majority were lipolytic and proteolytic and a considerable fraction of the basidiomycetous isolates were able to hydrolyse xylan and pectin (but not cellulose) and/or to assimilate phenolic compounds. Inácio (2003) also tested for different enzymatic activities (e.g. proteases, lipases, pectinases, xilanases) among a set of representative strains from a survey of yeasts on the phylloplane of Mediterranean plants. He detected significant extracellular hydrolytic abilities in *Cryptococcus* spp. belonging to the Filobasidiales (proteases, lipases, pectinases, cellulases and/or xilanases) and by some members of the Ustilaginomycetes (proteases and/or lipases); however, those were not the dominant species on the phylloplane of the sampled plants. Nevertheless, proteolytic, amylolytic and/or cellulotytic activities were detected in some strains of *Cryptococcus* spp. in the Tremellales and strong lipolytic activity was observed by some members of the Sporidiobolales, namely *Sp*. cf. *roseus* (in agreement with the results of Ruinen 1963), these yeasts being more prevalent on the same plants. It is not known if the observed activities are strain-specific and restricted to phylloplane isolates of each species or if they endow those yeasts with any competitive advantage on leaf surfaces.

Adhesion of cells to leaves is an intuitive pre-requisite for successful colonization of the phylloplane in analogy to what is known for other surface-inhabiting microbes, notably human and plant pathogens (Andrews and Harris 2000). However, available evidence for epiphytic bacteria and yeasts points to a more complex situation. Several publications by Buck and Andrews (reviewed in Andrews and Buck 2002) describe experiments aimed at unravelling the role and the mechanism of adhesion of the yeast *R. toruloides* to barley leaves. Their major findings were that although attachment of cells to the leaf could be demonstrated, especially by actively growing cells, this attachment was transient and the majority of cells could be easily removed by agitation. The authors concluded that adhesive ability does not appear to play a major role in leaf colonization and that instead efficient dispersal (i.e. emigration) and rapid recolonization (i.e. growth) may be more important attributes of a well-adapted phylloplane colonist (i.e. a resident). Transient adhesion should, however, be important to maintain a reservoir of cells that would expand quickly when nutrient availability and environmental conditions are conducive for growth allowing them to outcompete their less fit neighbours. However, rather surprisingly the yeast species chosen for this study is not a prevalent phylloplane colonist (the strain used most extensively was a soil isolate) and reports on the more or less ease of removal of yeasts, namely *Sp. roseus*, from leaf surfaces are contradictory (di Menna 1959; Preece and Dickinson 1971; Bashi and Fokkema 1976; Buck and Andrews 1999b).

Although the phenotypes cited here may be found in yeasts in a wide range of habitats (e.g. soil), many of the yeasts in these habitats are apparently incapable of growing and/or surviving on the phylloplane (see Sect. 13.3). Likewise, a large

number of the yeasts adapted to the phylloplane survive poorly in aquatic or soil ecosystems, for example. However, it seems clear that the key phenotypes essential for the fitness of phylloplane yeasts have not yet been fully elucidated.

13.7 Future Directions in Ecological Studies of Epiphytic Yeasts

In the previous sections we have tried to give a brief overview of the knowledge accumulated in recent years on the ecology of phylloplane microorganisms, with an obvious emphasis on yeasts. In spite of the considerable number of studies covering the topic many issues remain unresolved, including the defining characteristics of a phylloplane yeast (Sect. 13.6) and a more precise rendering of the diversity of epiphytic yeast populations on many different types of plants and climatic regions (Sect. 13.4). A better understanding of phyllosphere microbiology is important not only from a fundamental perspective, for leaf-inhabiting microbes are a fundamental component of terrestrial ecosystems and thus contribute to the nutrient cycling processes that occur therein, but also for their recognized potential as biocontrol agents or as sources of bioactive compounds of biotechnological interest (e.g. Fokkema 1991; Lindow and Leveau 2002). Here we provide some additional suggestions for future research efforts (see also Andrews 1991).

Firstly, sampling strategies and the methods used for isolation, enumeration and identification of phylloplane yeasts should be thoughtfully planned according to research goals. Aspects to be considered include the intensity of sampling (one plant vs. multiple plants, frequency of leaf collection, examination of other aerial organs for comparative purposes, etc.), the choice of protocols for removal of yeast cells from leaves and of adequate culture media and incubation conditions, etc. (see Sect. 13.2). A range of recently developed culture-independent methods have found application in microbial ecology (e.g. DGGE or temperature gradient gel electrophoresis, direct DNA extraction followed by cloning and sequencing) but only a few studies with phyllosphere microorganisms have made use of those methods (see Sect. 13.2); however, these methods have their own limitations and rather than replacing traditional isolation methods they should be used concurrently (Prakitchaiwattana et al. 2004). Sequence data available in public databases facilitate rapid and accurate identifications and it is imperative that such data be used in new surveys of phylloplane yeasts or that isolates from previous studies be re-identified using molecular approaches (see Sect. 13.4). Only then can phylloplane yeast communities be accurately appraised and their distribution patterns determined, which will in turn enable unravelling of possible associations with specific plants, biotopes and/or geography. Those patterns have already been disclosed for other yeast habitats such as tree exudates (Lachance et al. 1982), ephemeral flowers (Lachance et al. 2001) and cacti (Starmer et al. 2003). The resolution power of certain molecular typing methods provides the means to further discriminate distinct populations within a species and to test hypothesis about their turnover on leaves or the biogeography of ubiquitous phylloplane inhabitants such as *A. pullulans*.

The issue of population dynamics on the phylloplane remains vastly unexplored especially with respect to yeasts (see Sect. 13.5). Molecular genetics tools offer the possibility of examining the relative contributions of the processes of immigration,

growth and emigration to the development of microbial communities on leaf surfaces, but such studies have not yet been carried out with yeasts. The possible role of insects in the immigration and/or emigration processes of phylloplane yeasts has not been investigated, which is somewhat surprising given the well-known involvement of insect vectors in other yeast habitats (Starmer et al. 1991; Lachance et al. 2001). It is therefore desirable to assemble multidisciplinary teams for ecological surveys of phylloplane yeasts, which should involve the collaborative efforts of yeast biologists, entomologists and botanists. Knowledge of the topography, biochemistry and physiology of leaves is also important to understand the role of the leaf in shaping both the spatial and temporal patterns of its epiphytic microbial populations.

Another issue that deserves being looked into is the possible existence of yeast–plant interactions through molecular signalling mechanisms which might provide clues on why some yeast species are found on the phylloplane of certain plants and others are not. Although only a few yeast species were found to infect plants (Phaff and Starmer 1987) it is worth emphasizing in this respect the close phylogenetic relationship between some of the yeasts found on plant surfaces and dimorphic plant parasites (e.g. *Lalaria*/*Taphrina*; *Rh. bacarum*/*Microstroma* spp.; *Pseudozyma*/*Ustilago*). Likewise, interactions between the different members of the epiphytic communities have not been consistently explored and since bacteria constitute normally the early leaf colonists it would be most interesting to determine whether or not yeasts depend on those bacteria for their own colonization of leaf surfaces. Ruinen (1963) suggested that the common presence of nitrogen-fixing bacteria on the phylloplane might be beneficial for the ensuing colonization by yeasts. Some phylloplane yeasts have been found to produce antimicrobial compounds (McCormack et al. 1994b) but their ecological significance is not known. Moreover some yeasts with the ability to antagonize plant pathogenic fungi by producing lipids with fungicidal activity and that are being used as biocontrol agents may also be found on plant surfaces (Avis and Bélanger 2002).

Finally, it was not possible to identify with certainty which of the characteristics displayed by the yeasts commonly isolated from leaf surfaces define the required fitness traits for successful colonization of the phylloplane (see Sect. 13.6). For instance, while production of ballistoconidia is an obvious advantage as an efficient means of dispersal and is found in many yeasts inhabiting leaf surfaces, it is apparently not a pre-requisite for many other equally prominent phylloplane species (see Sect. 13.4). It would therefore be most interesting to uncover the selective pressures for the conservation of this phenotypic trait as well as its genetic determinants since it is well-known that BC yeasts tend to lose this capacity upon maintenance on nutrient-rich artificial culture media and are phylogenetically intertwined with non-BC taxa (Nakase 2000). Other frequent putative fitness traits of phylloplane yeasts are the production of EPS capsules and of photoprotective compounds. However, conclusive evidence of their relevance for yeast survival and/or growth on leaves is still lacking. The same applies to the adhesiveness of cells onto leaf surfaces which seemed like a good candidate to explain the observed differences between residents and transients in phylloplane colonization.

It seems clear that there are still many significant gaps in our understanding of the nature, dynamics and ecological role of phylloplane yeast populations. We are

maybe at the proverbial tip of the iceberg in accessing some of those topics and this appears to be the right time to make good use of the plethora of DNA-based methods and of molecular genetics tools already available for that purpose. In this context it seems fitting to quote Andrews (1991): "Renewed efforts at an experimental [i.e. hypothesis-testing] as opposed to a descriptive approach, integration of relevant advances from other disciplines, and recognition of the interplay among theory, experimentation, and observation, will be the essential ingredients for expanding our knowledge of phyllosphere ecology in the coming decades."

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Chapter 14

Yeast and Invertebrate Associations

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14.1 Introduction

The genesis of this review occurred at the 23rd International Special Symposium on Yeasts. The meeting was excellent, but, as I listened to the presentations, I was taken by the realization that most of presentations dealing with interactions between yeasts and their environments, especially presentations that could be thought of as applied science, focused on yeast–substrate interactions. This has been noted previously (do Carmo-Sousa 1969). Because the talks and posters presented interesting new data and insights, I did not think this a situation in need of correction but I did feel that yeast–animal interactions were a bit underrepresented. In several instances, yeast–animal interactions could have offered alternative explanations for the data or might have suggested answers to questions generated by the data. What caught my attention was that, in discussing their data, only one of the presenters made specific reference to animals as a possible alternative. Recalling this impression gave me a goal for this review. What I would like to accomplish is to present an argument that yeast–animal interactions are common, understudied, and a necessary part of understanding yeast ecology and evolution.

I will not review situations where yeasts are known to be animal parasites or pathogens. However, many interactions are not well understood and the nature of relationships is not fixed but is subject to evolutionary change. This sometimes made it difficult to set a proper boundary for the review and I chose to be inclusive rather than exclusive. It is organized according to the animals that are involved in the interactions. This decision was made because the field of study of yeast–animal interactions is organized, to the degree one can say that it is organized, in this fashion. The result is that ecological and evolutionary ideas may be revisited within separate sections. The alternative, organizing according to the evolutionary or ecological ideas presented, would mean revisiting animal groups instead of ecological concepts. More importantly, organizing the review by concept would give the impression that some overall scheme for animal–yeast interactions exists at this time. I believe that it would be misleading to imply conceptual unity beyond some broad generalizations. Animal–yeast interactions are varied, from mutualistic endosymbioses to simple

phoresy, from pairs of interacting animal and yeast species to interacting animal and yeast communities. So, if I am correct in my belief that much more remains to be discovered about yeast–animal interactions than we currently know, presenting the field as a coherent set of relationships might be premature.

Eliminating pathogenic and parasitic interactions has other ramifications. Vertebrate associations receive little attention here owing to a paucity of information about nonpathogenic interactions, although yeasts can be isolated from vertebrate guts (do Carmo-Sousa 1969; Abranches et al. 1998) and at least one yeast, *Cyniclomyces guttulatus* (Phaff and Miller 1998), is an endocytobiont. The scope of relationships reviewed here potentially involves fungivory, mutualism, commensalism, or amensalism (no interaction, simply co-occurrence). Other categories, such as competition or predation, are not likely. Symbiosis, another descriptor of interactions, does not seem to have a universally agreed upon definition. Some use it broadly for any relationship in which two organisms spend significant time in contact (parasites and pathogens may then be symbionts) and some authors restrict it to mutualistic or commensal interactions (the caveat about contact applying). Here, I will attempt to use the more restricted meaning when either observational or experimental evidence reasonably justifies the implication of a positive interaction.

Some broad generalizations are useful to state at the outset. When considered a subset of all fungus–animal interactions, there seems to be little that is unique to yeast–animal interactions. Most relationships are based on yeasts as a food for the animal and the animal as a vector for the yeasts. What is interesting is the widespread nature of the association. Table 14.1 is a reasonably complete and up-to-date list of yeasts associated with beetles found in wood and mushrooms. It is over 200-species long. For the vast majority of instances, we do not know if the association is happenstance or significant. I suspect that most are significant but do not involve obligate pairwise interactions between one species of yeast and one species of insect. If true, this would mean that most yeast–animal relationships must be studied in multispecies assemblages if the nature of the relationship is to be fully understood. The number of possible iterations becomes large, especially if variation in environmental factors, which may change the nature of yeast–animal interactions, are considered. It is a daunting task but there are also positive sides to the situation. Many necessary techniques have been perfected (obtaining axenic insects and pure cultures of yeasts, efficient means of differential yeast population counts, detection of spatial inhomogeneities with differential dye labeling, etc.) that might be applied to experiments on the dynamics of yeast–animal relationships. Both yeast and animal systematics are improving to the point that comparative phylogenetics may be used to generate a subset of testable hypotheses from a much larger set of possible hypotheses. Coalescence theory promises insights into basic ecological parameters (e.g., effective population size, dispersal patterns, and prevalence of recombination) and historical aspects of population biology (e.g., recent bottlenecks and founder events).

There have been several excellent reviews of the associations between yeast and animals, often as part of a more general review of yeast habitats (do Carmo-Sousa 1969; Phaff et al. 1978; Phaff and Starmer 1987). Here, I will focus, when possible, on recent additions to the field. Some terminological standards should be set at the

Table 14.1 Yeasts associated with beetles or beetle larval substrates (not including flowers)

Yeast	Scolytinae	Bark Beetle Frass and Galleries	Platypodinae	Ambrosia Beetles	Cerambycidae	Bostrichidae	Bupresidae	Tenebrionidae	Curculionidae	Scarabaeidae	From Decaying, Insect-infested Wood	Basidiocarps and Assoc. Beetles	Anobiidae	*Hypothenemus hampei*	Lucanidae	Erotylidae	Cucujoidae	Elatridae	Endomychidae	Passalidae	Source[a]
Candida diddensiae	1																				30
C. oregonensis	1						1														50
C. (Torulopsis) nitratophila	1						1														30, 32, 64
Cryptococcus albidus var. *diffluens*	1																				30
Cr. curvatus (*C. curvata*)	1																				32, 64
Cr. magnus	1																				30
Cr. skinneri	1																				32
Cuniculitrema polymorpha	1																				58
Debaryomyces hansenii	1																				30
Dipodascus aggregatus	1																				42
Myxozyma melibiosi (*T. melibiosum*)	1																				2
Pichia (*Hansenula*) *bimundalis*	1	1																			32, 43
P. (*H.*) *capsulata*	1						1														32
P. haplophila	1																				32
P. (*H.*) *holstii* (*C. silvicola*)	1	1			1																30, 32, 21
P. pini	1	1																			30, 32
P. (*E.*) *scolyti*	1	1																			32, 43
P. tolentana	1	1									1										30, 43
Rhodotorula aurantiaca (*crocea*)	1																				2
Rh. (*C.*) *hylophila*	1			1																	13, 30
Sporobolomyces (*Bullera*) *tsugae*	1																				2
Sp. singularis	1																				32

Continues

Table 14.1 Yeasts associated with beetles or beetle larval substrates (not including flowers)—*cont'd*

Yeast	Scolytinae	Bark Beetle Frass and Galleries	Platypodinae	Ambrosia Beetles	Cerambycidae	Bostrichidae	Bupresidae	Tenebrionidae	Curculionidae	Scarabaeidae	From Decaying, Insect-infested Wood	Basidiocarps and Assoc. Beetles	Anobiidae	*Hypothenemus hampei*	Lucanidae	Erotylidae	Cucujoidae	Elatridae	Endomychidae	Passalidae	Source[a]
Tsuchiyaea (*Sterigmatomyces*) *wingfieldii*	1																			33	
Ascoidea hylecoeti		1																			39
C. arabinofermentans		1																			47
C. ernobii		1											1								50, 21
C. homilentoma		1																			17
C. litsaeae		1																			60
C. melibiosica		1																			50
C. piceae		1																			57
C. ponderosae		1																			60
C. quercitrusa		1																			50
C. quercuum		1																			21
C. rugosa		1																			21
C. silvanorum		1		1																	13, 21
C. tenuis		1			1	1	1														2, 21
C. ulmi		1																			56
C. wickerhamii		1																			50
C. wyomingensis		1									1										57
Clavispora (*Candida*) *lusitaniae*		1																			21
Cr. humicolus		1																			21
Cr. laurentii		1																			21
D. vanrijiae var. *yarrowii*		1																			51
Issatchenkia scutulata var. *exigua*		1																			46
Ogataea (*P.*) *glucozyma*		1																			43
O. (*P.*) *henricii*		1																			43
O. (*P.*) *kodamae*		1																			32
O. polymorpha (*P. angusta, H. polymorpha*)		1																			43

Table 14.1 Yeasts associated with beetles or beetle larval substrates (not including flowers)—*cont'd*

Yeast	Scolytinae	Bark Beetle Frass and Galleries	Platypodinae	Ambrosia Beetles	Cerambycidae	Bostrichidae	Bupresidae	Tenebrionidae	Curculionidae	Scarabaeidae	From Decaying, Insect-infested Wood	Basidiocarps and Assoc. Beetles	Anobiidae	*Hypothenemus hampei*	Lucanidae	Erotylidae	Cucujoidae	Elatridae	Endomychidae	Passalidae	Source[a]
P. (*H.*) *americana*		1																			2, 32, 43
P. amylophila		1																			32
P. bovis		1																			21
P. canadensis (*Hansenula wingei*)		1																			43
P. euphorbiae		1																			20
P. (*H.*) *euphorbiaphila*		1																			18
P. hampshirensis		1																			31
P. heimii		1																			32
P. japonica		1																			31
P. media		1																			43
P. membranifaciens (*C. valida*)		1																			21
P. mexicana (*C. entomoea, C. terebra*)		1																			15, 21
P. meyerae		1																			19
P. mississippiensis		1																			3
P. nonfermentans		1																			21
P. pastoris		1																			43
P. philodendri		1																			21
P. ramenicola		1																			57
P. segobiensis		1			1	1															32, 43, 62
Stephanoascus smithiae		1																			54
Torulaspora delbruckii		1									1										44
Trichosporon cutaneum		1								1											2, 21
Williopsis californica		1																			45
Zygosaccharomyces bisporus		1																			21

Continues

Table 14.1 Yeasts associated with beetles or beetle larval substrates (not including flowers)—*cont'd*

Yeast	Scolytinae	Bark Beetle Frass and Galleries	Platypodinae	Ambrosia Beetles	Cerambycidae	Bostrichidae	Bupresidae	Tenebrionidae	Curculionidae	Scarabaeidae	From Decaying, Insect-infested Wood	Basidiocarps and Assoc. Beetles	Anobiidae	*Hypothenemus hampei*	Lucanidae	Erotylidae	Cucujoidae	Elatridae	Endomychidae	Passalidae	Source[a]
Z. rouxii		1																			21
Zygozyma oligophaga		1																			53
Ambrosiozyma (*P.*) *ambrosiae*				1																	9, 14
A. (*P.*) *cicatricosa*				1																	8, 14
A. (*E.*) *monospora*				1																	14
A. philentoma				1																	52
A. (*E.*) *platypodis*			1	1																	14
Ascoidea africana				1																	39
C. berthetii				1							1										32
C. (*T.*) *dendrica*				1	1																12
C. dendronema				1																	13
C. entomophila				1																	13
C. (*T.*) *insectalens*				1	1																12
C. insectorum				1																	15
C. maritima				1							1										25, 34, 50
C. mycetangii				1																	56
C. (*T.*) *nemodendra*				1																	12
C. (*T.*) *silvatica*				1																	12
C. trypodendroni				1																	48
Dipodascus ambrosiae				1																	42
Fellomyces (*Sterigmatomyces*) *polyborus*				1																	5, 48
O. (*P.*) (*H.*) *philodendri*				1		1															5
P. (*H.*) *dryadoides*				1																	7
P. (H.) sydowiorum (*C. nitrativorans*)				1	1	1															4, 15
P. acaciae				1	1	1	1														1
Rh. (*C.*) *philyla*				1																	12
Saccharomycopsis synnaedendra				1																	11

Table 14.1 Yeasts associated with beetles or beetle larval substrates (not including flowers)—*cont'd*

Yeast	Scolytinae	Bark Beetle Frass and Galleries	Platypodinae	Ambrosia Beetles	Cerambycidae	Bostrichidae	Bupresidae	Tenebrionidae	Curculionidae	Scarabaeidae	From Decaying, Insect-infested Wood	Basidiocarps and Assoc. Beetles	Anobiidae	*Hypothenemus hampei*	Lucanidae	Erotylidae	Cucujoidae	Elatridae	Endomychidae	Passalidae	Source[a]
Zygozyma smithiae				1																	36
C. ergastensis					1																32, 61
C. parapsilosis var. intermedia					1				1												50
C. rhagii					1	1	1														2
C. shehatae var. *inesctosa*					1		1														32,35,62
Schizosaccharomyces fibuligera					1	1															2, 32
?P. onychis (*P. xylopsoci*) – lost culture						1															10
C. (*T.*) *torresii*						1															32
C. fennica (*Trichosporon melibiosaceum*)						1															5
Cr. amyloentus (*C. amylolenta*)						1															15
P. (*H.*) *anomala*						1															32
P. burtonii (*Trichosporon behrendii*)						1								1							32, 63
P. (*H.*) *ciferrii*						1															32
P. stipitis					1					1					1					1	2
Bullera dendrophila							1														6
C. insectamans							1														15
C. naeodendra							1														16
C. silvicultrix							1														15, 32
D. vanrijiae var. *vanrijiae*							1														32
P. (*C.*) *guilliermondii*							1					1	1								32
T. behrendii							1														32
Blastobotrys elegans-like								1													32
C. ambrosiae-like								1				1				1					64
C. pyralidae-like								1				1									64
Malassezia restricta				1				1				1									64

Continues

Table 14.1 Yeasts associated with beetles or beetle larval substrates (not including flowers)—*cont'd*

Yeast	Scolytinae	Bark Beetle Frass and Galleries	Platypodinae	Ambrosia Beetles	Cerambycidae	Bostrichidae	Bupresidae	Tenebrionidae	Curculionidae	Scarabaeidae	From Decaying, Insect-infested Wood	Basidiocarps and Assoc. Beetles	Anobiidae	*Hypothenemus hampei*	Lucanidae	Erotylidae	Cucujoidae	Elatridae	Endomychidae	Passalidae	Source[a]
Stephanoasaus farinosus-like								1				1									64
C. athensensis									1			1				1	1				67
Candida sp.									1			1									64
T. scarabaeorum										1											65
Ascoidea corymbosa											1										39
A. rubescens											1										39
Blastobotrys capitulata											1										41
C. ambrosiae											1										59
C. ancudensis											1										28
C. antillancae											1										22
C. bertae											1										23
C. castrensis											1										22
C. chilensis											1										50
C. coipomoensis											1										29, 34
C. drimydis (same rDNA as *C. ancudensis*)											1										25
C. freyschussii											1										50
C. hungarica											1										61
C. ingens											1										34
C. laureliae											1										29
C. llanquihuensis											1										29
C. novakii											1										37
C. oleophila											1										26, 34
C. ontarioensis											1										48
C. parapsilosis											1										29, 34
C. peoriaensis											1										60
C. petrohuansis (same rDNA as *C. ancudensis*)											1										27
C. pini											1										50
C. pyralidae											1										59

Table 14.1 Yeasts associated with beetles or beetle larval substrates (not including flowers)—*cont'd*

Yeast	Scolytinae	Bark Beetle Frass and Galleries	Platypodinae	Ambrosia Beetles	Cerambycidae	Bostrichidae	Bupresidae	Tenebrionidae	Curculionidae	Scarabaeidae	From Decaying, Insect-infested Wood	Basidiocarps and Assoc. Beetles	Anobiidae	*Hypothenemus hampei*	Lucanidae	Erotylidae	Cucujoidae	Elatridae	Endomychidae	Passalidae	Source[a]
C. railenensis											1										23, 24, 34
C. santjacobensis											1										28
C. savonica											1										50
C. shehatae var. *lignosa*											1										50
C. shehatae var. *shehatae*											1										50
C. sophiae-reginae											1										26
C. tammaniensis											1										48
C. tepae											1										50
C. xylopsoci											1										59
Cephaloascus fragrans											1										40
Cr. huempii											1										25
D. carsonii											1										51
Leucosporidium scolyti											1										24, 34
Metschnikowia pulcherrima											1										27, 34
O. (*P.*) *dorogensis*											1										61
O. (*P.*) *methylivora*											1										43
O. (*P.*) *zsoltii*											1										61
P. castillae											1										43, 48
P. pilisensis											1										61
Rh. futronensis											1										24, 34
Rh. nothofagi											1										24, 34
C. anneliseae												1									66
C. atakaporum												1									66
C. bokatorum												1									66
C. bolitotheri												1									66
C. bribrorum												1									66
C. chickasaworum												1									66
C. choctaworum												1									66
C. elateridarum												1						1			67
C. emberorum												1									66

Continues

Table 14.1 Yeasts associated with beetles or beetle larval substrates (not including flowers)—*cont'd*

Yeast	Scolytinae	Bark Beetle Frass and Galleries	Platypodinae	Ambrosia Beetles	Cerambycidae	Bostrichidae	Bupresidae	Tenebrionidae	Curculionidae	Scarabaeidae	From Decaying, Insect-infested Wood	Basidiocarps and Assoc. Beetles	Anobiidae	*Hypothenemus hampei*	Lucanidae	Erotylidae	Cucujoidae	Elatridae	Endomychidae	Passalidae	Source[a]
C. fermentati												1		1							63
C. guaymorum												1									66
C. kruisii												1									50
C. kunorum												1									66
C. maxii												1									66
C. panamericana												1									34, 66
C. schatavii												1									50
C. smithsonii												1				1			1		67
C. taliae												1									66
C. terraborum												1									66
C. wounanorum												1									66
C. yuchorum												1									66
D polymorphus												1									51
Dipodascus armillariae												1									42
Dipodascus macrosporus (actually from a myxomycete)												1									42
Endomyces cortinarii (parasite of basidiocarp)												1									38
E. decipiens (parasite of basidiocarp)												1									38
E. polyporicola (parasite of basidiocarp)												1									38
E. scopularum (parasite of basidiocarp)												1									38
Kluyveromyces dobzhanskii												1									49
K. lactis var. drosophilarum												1									49
K. thermotolerans												1									49
K. waltii												1									49

Table 14.1 Yeasts associated with beetles or beetle larval substrates (not including flowers)—*cont'd*

Yeast	Scolytinae	Bark Beetle Frass and Galleries	Platypodinae	Ambrosia Beetles	Cerambycidae	Bostrichidae	Bupresidae	Tenebrionidae	Curculionidae	Scarabaeidae	From Decaying, Insect-infested Wood	Basidiocarps and Assoc. Beetles	Anobiidae	*Hypothenemus hampei*	Lucanidae	Erotylidae	Cucujoidae	Elatridae	Endomychidae	Passalidae	Source[a]
O. (*P.*) *minuta* var. *minuta*												1									43
Saccharomyces dairenensis												1									55
S. farinosus												1									54
C. karawaiewii													1								50
C. xestobii													1								50

[a] *1* van der Walt (1966), *2* do Carmo-Sousa (1969), *3* Pignal (1970), *4* Scott and van der Walt (1970a), *5* Scott and van der Walt (1970b), *6* van der Walt and Scott (1970), *7* Scott and van der Walt (1971a), *8* Scott and van der Walt (1971b), *9* van der Walt and Scott (1971a), *10* van der Walt and Scott (1971b), *11* van der Walt and Scott (1971c), *12* van der Walt et al. (1971a, *13* van der Walt et al. (1971b), *14* van der Walt (1972), *15* van der Walt et al. (1972), *16* van der Walt et al. (1973), *17* van der Walt and Nakase (1973), *18* van der Walt (1982a), *19* van der Walt (1982b), *20* van der Walt and Opperman (1983), *21* Bridges et al. (1984), *22* Ramierez and González (1984e), *23* Ramierez and González (1984a), *24* Ramierez and González (1984b), *25* Ramierez and González (1984c), *26* Ramierez and González (1984d), *27* Ramierez and González (1984f), *28* Ramierez and González (1984g), *29* Ramierez and González (1984h), *30* Leufvén and Nehls (1986), *31* Kurtzman (1987), *32* Phaff and Starmer (1987), *33* van der Walt et al. (1987), *34* Ramierez (1988), *35* Kurtzman (1990), *36* van der Walt et al. (1990), *37* Péter et al. (1997), *38* de Hoog (1998a), *39* de Hoog (1998b), *40* de Hoog and Kurtzman (1998), *41* de Hoog and Smith (1998), *42* de Hoog et al. (1998), *43* Kurtzman (1998a), *44* Kurtzman (1998b), *45* Kurtzman (1998c), *46* Kurtzman (1998d), *47* Kurtzman and Dien (1998), *48* Kurtzman and Robnett (1998b), *49* Lachance (1998), *50* Meyer et al. (1998), *51* Nakase et al. (1998), *52* Smith (1998a), *53* Smith (1998b), *54* Smith and de Hoog (1998), *55* Vaughn-Martini and Martini (1998), *56* Kurtzman (2000a), *57* Kurtzman (2000b), *58* Kirschner et al. (2001), *59* Kurtzman (2001a), *60* Kurtzman (2001b), *61* Péter et al. (2003), *62* Suh et al. (2003), *63* Vega et al. (2003), *64* Zhang et al. (2003), *65* Middelhoven et al. (2004), *66* Suh et al. (2004b), *67* Suh and Blackwell (2004c)

outset. The literature has some overlapping terms and terms with multiple meanings and I will try to use only one when multiples are available. A member of a symbiotic relationship can be either a symbiote or a symbiont and I will prefer symbiont. The term is more often applied to the microbe than the animal, which is commonly called the host. Mycetocytes are specialized animal cells that host endocytobionts (Jones et al. 1999 for the latter term). Many authors make no distinction based on the nature of the microbe but others use bacteriocyte when bacteria are resident and confine mycetocyte to situations when the resident is a fungus. I will use the term in the narrower sense. Association and interaction are not totally overlapping terms

in this review and the distinction is important. An association means that co-occurrence has been established. The use of "interaction" implies that some functional relationship has been established or that observational data strongly implies a functional relationship. Phoresy is the dispersal of small organisms, seeds, or spores by animals. Some authors use the term only for a commensal relationship and others use it no matter what sort of relationship exists between the carrier and the organism carried, which is how I will use it. Mycangia are external pockets on arthropods that hold microbes during dispersal by the animal. They may be almost anywhere on the host and range from simple depressions to more elaborate structures that may nourish the fungus or bacterium. "Mycetangia" looks confusingly similar to mycangia but refers to an organ or a cluster of cells within an organ that house an endocytobiont inside the body. Finally, several abbreviations occur more than once: LSU and SSU for large and small subunit plus ITS for internal transcribed spacer (of the ribosomal RNA gene); and YLS for a yeast-like symbiont.

14.2 Beetles

Most yeast–beetle interactions involve beetles that live on plants (although the interactions of yeasts and beetles associated with mushrooms discussed in the last part of this section are a significant exception). Beetles are closely associated with plants and Farrell (1998) has suggested that this association (especially with angiosperms) is a major cause of beetle diversity. Beetles use all parts of plants (see flower-associated beetles in Sect. 14.2.5) but a significant part of beetle diversity is due to the many beetles that use wood as a refuge, a nest, and a source of food. They feed on all parts of the woody stem: sap, phloem, cambial tissue, bark, wood, and the fungi that digest wood or attack the plant's living tissues (Lawrence 1989). The fossil record indicates they have done so for a very long time (Kirejtshuk 2003). Eating wood involves digestion of lignified cellulose and overcoming the toxins found there (Eriksson et al. 1990). (Farrell et al. 2001 point out that fallen logs are often protected by toxins, perhaps because mature trees are a combination of living and dead tissue and so have an interest in what eats dead wood.) Digestion and detoxification are often accomplished through symbioses between beetles and microbes that range from endocytobiosis to agricultural associations (Mueller et al. 1998; Farrell et al. 2001; Mueller and Gerardo 2002). Historically, most attention has been paid to xylophagic (or xylophagy-related) yeast–beetle interactions. It is my intention to discuss recent additions to this rich literature (see Buchner 1965; Phaff and Starmer 1987; Lawrence and Milner 1996 for previous reviews) and to cover some recent work on associations between floricolous yeasts and beetles and on yeasts and beetles from basidiocarps.

There are several feeding strategies used by beetles that feed on woody stems (Berryman 1989). Many Nitidulid beetles feed on fermenting plant sap and yeasts are among the microbes responsible for sap fermentation. Production of low molecular-weight volatile compounds by yeasts attracts nitidulid beetles (Nout and Bartelt 1998). Volatile production by bacteria growing on the same substrates as the yeasts did not attract the insect. Some nitidulids live in flowers and are associated with yeasts that feed on nectar and pollen. Many Scolytid beetles feed on the living tis-

sues beneath the bark. These bark beetles are often associated with euascomycete phytopathogens in the *Ophiostoma* and *Ceratocystis* clades as well as yeast and filamentous basidiomycetes. Infestation often leads to the rapid death of the plant as volatiles produced by attacking organisms attract more beetles to the attack. The relationship between the fungi and the beetles involves transport for the fungi and both facilitation of the attack (the fungi can block resin or latex channels from flooding beetle galleries) and nutrition for the beetles. Other Scolytid (as well as Platypodid) beetles, collectively termed ambrosia beetles, bore into the xylem of living trees where they feed on fungi that line their tunnels (galleries). However, most of the xylem eaten by beetles is eaten by beetles that bore into dead branches or trunks. Passalid beetles form multigeneration colonies in dead wood and are associated with yeasts able to digest xylose, a major component of lignocellulose. In addition, species from other beetle families also feed on xylem, including members of the Anobiid, Cerambycid, Buprestid, Bostrichid, and Scarabaeid beetle families. For many of these families, xylophagy is believed to be the ancestral feeding mode. Yeast or yeast-like endosymbionts have been isolated from the guts of many of them (Table 14.1).

In addition to the xylophagic beetles, fungi (including yeasts) are associated with other beetles and other beetle feeding modes. Recent efforts have identified yeasts associated with flower beetles and with beetles that feed on basidiocarps. Owing to the number of species that have been isolated from beetles, beetle frass, galleries, or rotting wood infested with beetles, they are summarized in Table 14.1, which does not include those found in association with flowers, which are summarized in Table 14.2.

14.2.1 Bark Beetles

The feeding strategies of Scolytid beetles have been summarized by Berryman (1989). There are three basic types: saprophagy (Scolytids that feed in rotting logs), phytophagy (bark beetles), and mycetophagy (ambrosia beetles). Berryman views saprophagy as the ancestral state. Saprophagous Scolytids feed on the fungi in the wood and use fermentation products to locate fallen wood. Perhaps because this group of beetles is not economically important, they remain understudied and specific associations among the fungi and beetles in this group have not been investigated. However, the use of fermentation products to locate suitable resources was probably an important preadaptation for both the phytophagous and mycetophagous life styles that followed. Both bark and ambrosia beetles use pheromones that are often wood components modified by fungal symbionts. Bark beetles use both aggregation (verbenol from pinene, Berryman 1989) and avoidance pheromones (verbenone from verbenol; Hunt and Borden 1990; Paine et al. 1997), so that adults are recruited to the tree during early stages of infestations and discouraged from joining the attack during later stages. Colonial living is characteristic of Scolytid beetles and Berryman believes that the use of fungal products to locate wood leads to colony formation, which he views as a preadaptation for both the phytophagous and mycetophagous life styles that followed. Bark beetles, the phytophagous group, are either colonial and virulent or solitary and far less virulent

Table 14.2 Recently described species associated with flowers and flower beetles

Clade First *Metschnikowia*	**Species**[a] *M. continentalis*	**Host plant** *Ipomoea*	**Insect** *Conotelus*	**Source**[b] D, P
	M. borealis	Convolvulaceae	*Conotelus*	D, P
	M. hibisci	*Ipomoea*, *Hibiscus*	*Conotelus*, *Aethina*	D
	M. lochheadii	various	*Conotelus*	K
	M. hawaiiensis	*Ipomoea*	*Conotelus*	A
	M. santaceciliae	*Ipomoea*	*Conotelus*	O
	C. ipomoeae	Convolvulaceae	*Conotelus*	B
	M. lochheadii-like	*M. tuberosa*	*Conotelus*	L
	M. hawaiiensis-like		Clerid beetles	L
Second *Metschnikowia*	*M. dekortorum*	Ipomoea	*Conotelus*	M
	M. arizonensis	*Opuntia*	*Carpophilus*	M
	C. kipukae	*Ipomoea*	*Conotelus*	O
Third *Metschnikowia*	*M. agaves*	*Agave tequilana* var. *azul*	No beetles	
	C. hawaiiana	*Ipomoea*	*Conotelus*	O
Other *Metschnikowia*	*M. reukaufii*	Convolvulaceae	No beetles	
	M. koreensis	*Ipomea*, *Lilium*		I
	C. tolerans	*Ipomoea*, *Hibiscus*	*Conotelus*, *Aethina*	F
	C. kunwiensis	*Ipomea*, *Helleborus*	Bees	N
	M. gruessii-like	Convolvulaceae	No beetles	L
	C. tolerans-like	Various	Beetles	L
Kodamaea	*K. anthophila*	*Ipomoea*, *Hibiscus*	*Conotelus*, *Aethina*	G
	K. kakaduensis	*Ipomoea*, *Hibiscus*	*Aethina*	F
	K. nitidulidarum	Cactus flowers	Nitidulid beetles	G
	C. restingae	Cactus flowers	Nitidulid and other beetles	G
Wickerhamiella	*W. occidentalis*	Convolvulaceae	Conotelus	*C*
	W. lipophila	*Ipomoea*	*Conotelus*	C, H
	W. australiensis	*Ipomoea*, *Hibiscus*	*Conotelus*, *Aethina*	C
	W. cacticola	Cactus flowers	Nitidulid and other beetles	C
	C. drosophilae	*Ipomoea*	*Conotelus*	C
	C. sorbophila-like	Various	*Conotelus*	L

Starmerella (associated with bees)	*S. bombicola*	Various	*Conotelus*	E
	C. tilneyi	Various	*Conotelus*	J
	C. powellii	Various	*Conotelus*	J
	C. powellii-like	Various	*Conotelus*	L
	C. floricola-like	*Ipomoea*	*Conotelus*	L
	C. geochares-like		*Aethina*	L
Other clades	*C. cleridarum*		Clerid beetles	J
	C. nanaspora-like	Various	Beetles	L
	Saccharomycopsis sp.	*Hibiscus*	Beetle	L
	Sporopachydermia sp.	Various	Beetles	L
	Fermentative yeast-like mold	*Ipomoea, Hibiscus*	*Conotelus, Aethina*	L
Various basidiomycetes				L

[a]*C.* represents *Candida*; other genera as in clade name

[b]*A* Lachance et al. (1990), *B* Lachance et al. (1998a), *C* Lachance et al. (1998c), *D* Lachance et al. (1998b), *E* Rosa and Lachance (1998), *F* Lachance et al. (1999), *G* Rosa et al. (1999a), *H* Lachance et al. (2000), *I* Hong et al. (2001), *J* Lachance et al. (2001a), *K* Lachance et al. (2001b), *L* Lachance et al. (2001d), *M* Lachance and Bowles (2002), *N* Hong et al. (2003), *O* Lachance et al. (2003), *P* Marinoni and Lachance (2004)

(Berryman 1989; Paine et al. 1997). The mechanism of tree death depends on the impact of the beetles, including their tendency to aggregate and to girdle the tree as they burrow tunnels, and the effect of the fungi, which attack the sapwood (many are blue-staining) and can range from deadly to mildly detrimental to the tree (Paine et al. 1997). The specificity of the relationship between bark beetle and fungi is low, with most beetle species associated with different pathogenic fungi at different times and places or on different host species. However, it is clear that the beetles exploit the fungi as food, as a means of reducing the toxicity of the tree, and as a means of overcoming the physical defenses of the tree, primarily by blocking resin or latex secretion that are primary defenses and by responding to the induced defenses (cell wall alterations, additional toxins, etc.) provoked by the attack on the tree (Paine et al. 1997; Farrell et al. 2001). However, the association may also involve cost to the beetles. In laboratory experiments, *Roptrocerus xylophagorum* and *Spathius pallidus*, Hymenopteran parasitoids, were attracted to odors from loblolly pine, *Pinus taeda* infected with strains of *Ophiostoma* spp. (Sullivan and Berisford 2004). Data from the field were less clear, as the fungus alone was not sufficient to explain long-range host location.

Fungi may benefit from the beetles' burrowing and from physiological changes their presence provokes in the tree's tissues but they clearly benefit from vectoring by the beetles. Many fungi have been isolated from the external surface of the beetles (Paine et al. 1997) but the clearest indication that the beetles vector fungi from their galleries is the presence of special cuticular structures, mycangia, that transport fungi, including yeasts (Whitney and Harris 1970). Mycangia are a diverse set of structures (including pits on various body parts such as the head and feeding appendages, grooves on the pronotum, and patches of setae that can hold spores and cells between the shafts) that are often secretory, producing waxes, fatty acids, and amino acids. The type of mycangia does not appear to be a diagnostic trait in Scolytid taxonomy (Berryman 1989). Mycangia are also found in the ambrosia beetles, in nonxylophagous Scolytids (Morales-Ramos et al. 2000), and even in other insect orders (Vasiliauskas and Stenlid 1999).

Bark-beetle-associated fungi include dimorphic *Euascomycetes* and *Hemiascomycetes*. The euascomycete species were once thought to belong to a single taxonomic group but recent DNA sequence analysis has not supported monophyly. The *Ophiostoma* and *Ceratocystis* clades are related perithecal euascomycete clades that contain plant pathogens and species associated with bark beetles (Spatafora and Blackwell 1994). They include numerous anamorphs, mostly in the genera *Ambrosiella*, *Leptographium* and *Pesotum*. Some have obligate associations with beetles and are transported in mycangia, while others have facultative associations and are vectored on the beetles mouthparts and body surfaces. Other teleomorphic genera (*Pyxidiophora*, *Kathistes*, and *Subbaromyces*), once included with *Ophiostoma* and *Ceratocystis*, are only distantly related (Blackwell and Jones 1997; Hausner et al. 2000). They have peritheca and are dispersed by beetles, but the former characteristic may be the result of convergent evolution due to their association with beetles.

It has long been unclear if many of the anamorphic and teleomorphic species in these clades deserve specific status or if the generic and specific designations repre-

sented monophyletic groupings. Recent molecular data have confirmed that there are ambiguities in the current classification. SSU ribosomal DNA (rDNA) sequence analysis has shown *Ambrosiella* to be polyphyletic, with some species related to *Ceratocystis* and others to *Ophiostoma*, which may be two separate clades (Cassar and Blackwell 1996; Rollins et al. 2001). Hausner et al. (2000) determined that neither *Leptographium* nor *Pesotum* were monophyletic. Problems can extend to specific designations, also. Using a multigene (rDNA, actin, β-tubulin, and transcription elongation factor 1α) and multistrain approach to the relatedness of a cluster of species physiologically and ecologically similar to *Ophiostoma clavigerum*, Lim et al. (2004) demonstrated that one (*L. terebrantis*) of the five species examined was paraphyletic in the combined tree and it was not clear if this would have been resolved with the addition of more loci to the analysis. Using a separate locus (rDNA ITS), ambiguous results were also obtained for a clade of teleomorph species related to *O. stenoceras* (de Beer et al. 2003). Although strains of *O. stenoceras* from Colombia, Kenya, Uruguay, and South Africa were included, there was no intraspecific variation linked to geographic origin and the sequences of both *O. albidum* and *O. ponderosae* were indistinguishable from those of *O. stenoceras*. The specific status was also ambiguous for the other two members of this clade, *O. narcissi* and *O. abietinum*. There was some sequence variability among *O. narcissi* strains and the species is paraphyletic as the *O. abiethinum* strains lie within the *O. narcissi* clade. It appears that, independent of the sequences studied, closely related species of *Ophiostoma* and its anamorphs need careful analysis to distinguish among species and physiological variants.

Yeasts are commonly isolated from bark beetles (Table 14.1). In an extensive sampling of *Dendroctonus ponderosae,* Six (2003) found yeasts in 80% of the mycangia (n=224) and was able to isolate them from the surface of 90% of the beetles (n=256) sampled in western USA. Unfortunately, the yeasts were only enumerated and not identified. Some *Ophiostoma* species isolated from bark beetles appear to be vectored both on the beetle's surface and in mycangia, while others appear not to survive well on the surface and show greater dependence on dispersal in mycangia (Six 2003). In a quantitative study of yeasts associated with a bark beetle, *Ips typographus*, Leufvén and Nehls (1986) found several species of yeasts on or in the beetle. Some species were present throughout the beetle's life cycle (Table 14.1), but *Pichia holstii* and *Candida diddensiae* outgrew the other species by several orders of magnitude as the galleries were excavated, eggs laid, and larvae reared. Gallery initiation corresponded to the lowest yeast population sizes, perhaps owing to the release of resin during this phase. Older galleries also did not support large yeast populations, perhaps owing to resource depletion. Because yeasts were isolated from whole-beetle homogenates, it was impossible to separate those on the surface from those in the gut and so it is not known if the beetles fed on yeasts. *C. diddensiae* (as well as two other less common species) is able to convert *cis*-verbenol (an important component of the beetle's aggregation pheromone) into verbenone (Leufvén and Nehls 1984). Bridges et al. (1984) reared wild-caught beetles in pine bolts brought into the laboratory. They sampled the bacteria, fungi, and yeasts from the larval chambers excavated by the wild-caught beetle's progeny. Fungi were absent from all substrates (frass and phloem in the vicinity of the egg niche, larval mines and chambers) at the

egg and young larvae stages. Whenever significant populations of fungi were present, all four substrates were dominated by yeasts [28 species in all, dominated by *Pichia pini*, *C. tenuis*, *P. capsulata*, and *Ogataea polymorpha* (*P. angusta*) – including three isolates from two basidiomycetous species]. Bacteria were present at all times, but at low numbers. Thus, yeasts may be of greater importance to larval development than isolations done from the portions of galleries occupied by the adults would suggest.

Yeasts recently isolated from beetles or beetle frass include a clade of anamorphic strains related to *C. tanzawaensis*. Kurtzman (2001b) isolated six new species, three from insect frass or tunnels (*C. ambrosiae*, *C. pyralidae*, and *C. xylopsoci*), one from soil (*C. canberraensis*, later isolated from beetle gut contents), and two from trees (*C. prunicola* and *C. caryicola*). The clade is only distantly related to a sexual genus (*Lodderomyces*). The collections were done from widely spaced sites (Illinois, USA; Natal, South Africa; and Canberra, Australia) and no beetles were examined, so it is not known if this represents a major clade of beetle symbionts but the data would indicate a speciose clade that has been undersampled in the past. Dimorphic basidiomycetes are not commonly isolated from bark beetles. Two species of *Fibulobasidium* were isolated near galleries and are probably associated with the beetles (Bandoni 1998). There is little doubt about a beetle association for *Atractocolax pulvinatus* as it was isolated from bark beetles (Kirschner et al. 1999). Kirschner et al. (2001) found a second, dimorphic basidiomycete, *Cuniculitrema polymorpha*, on bark beetles. It is very similar (physiological profile, LSU rDNA sequence, and DNA–DNA homology) to *Sterigmatosporidium polymorphum* and Kirschner et al. believe *Sterigmatosporidium* to be an anamorphic genus and *Cuniculitrema* its teleomorph. Little is known about the role these fungi might have in the bark-beetle system but it is suggested that *C. polymorpha* may be a mycoparasite owing to the presence of haustoria.

Stevens (1986) studied the biogeography of wood-boring beetles in the Scolytidae, Buprestidae, Cerambycidae, and Curculionidae in eastern North America. He found that the number of beetle species using dead wood depended on the size of the geographic range of the plant that supplied the wood. His data supported the hypothesis that plant species with greater ranges supported more beetle species because habitat diversity increased as the plant's range increased. However, Stevens was not able to suggest what sort of habitat diversity increased with plant range. His consideration of habitat diversity was confined to edaphic factors and did not stray into habitat differences related to the numbers or types of fungal species inhabiting the wood. Stevens mentions that beetle species that inhabit a plant's wood may be inexplicably missing from parts of the plant's range. Data on the distribution of the beetle's fungal associates may be pertinent in such cases.

There are cases of yeasts associated with Scolytid beetles that do not bore wood. An example is the coffee bean borer, *Hypothenemus hampei. P. burtonii* and *C. fermentati* were isolated from the internal organs of the beetle (Vega et al. 2003). Since caffeine is one of the better known insecticidal plant secondary compounds, Vega et al. (2003) investigated one of the yeast species for its ability to detoxify the beetle's diet. In this case, not only does *P. burtonii* not detoxify the beetle's diet, it suffers reduced spore production in the presence of the toxin at levels commiserate with those in coffee beans (*C. fermentati* was not tested). While this study failed to

identify any potential value of the yeasts to the beetle, it does underline the need to be cautious in inferring more to an association between yeasts and insects than has been experimentally demonstrated. Vega et al. suggest that the yeasts may be an important source of nutrition, but there is a stronger case for *Fusarium solani* as the beetle's fungus of choice, as the yeast provides ergosterol to the beetle (Morales-Ramos et al. 2000).

14.2.2 Ambrosia Beetles

There are over 3,400 species of ambrosia beetles. Ambrosial associations appear to have arisen at least 6 times within the Scolytid lineage (which includes the Platypodinae) (Farrell et al. 2001). There are several differences between bark and ambrosia beetles. Both adult and larval bark beetles tunnel but most ambrosia beetles have a form of parental care in which the adults tunnel, maintain the tunnel fungi, and feed the larvae, which may be solitary or aggregated in special chambers. Several clades of ambrosia beetles have developed sib-mating. The largest clade (1,300 species) has taken sib-mating to the extreme, abandoning outcrossing and developing flightless, dwarf males and haplodiploidy, perhaps as a means of controlling brood sex ratios (Jordal et al. 2002). Inbreeding may be a means of increasing the rate of establishment of successful colonies (Jordal et al. 2001). This suite of characters appears to be successful, as beetles from the haplodiploid clade dominate the wood-boring-beetle community in tropical lowlands (Jordal et al. 2000). Ambrosia beetles also respond to aggregation chemicals, but colonization by ambrosia beetles does not normally result in the death of the tree. These beetles form obligate mutualisms with vertically transmitted dimorphic fungi that are seen by entomologists as "domesticates" cultivated by diligent arthropod agriculturalists. Insect agriculturalists (including beetles, termites, and attine ants) can be important, even dominant, members of terrestrial herbivorous or macrodecomposer communities (Farrell et al. 2001; Mueller and Gerardo 2002). Some Lymexylid beetles have a life history that is similar to the ambrosia beetles. Adult *Melittomma* and *Elateroides* do not bore galleries but lay eggs coated with *Ascoidea* spp. on bark. The larvae bore tunnels that become lined with yeasts that are eaten by the larvae (Francke-Grosmann 1967).

In addition to the primary (ambrosial) fungus, yeasts (Table 14.1) and euascomycete fungi grow in ambrosial beetle galleries and can be isolated from mycangia of adults, although at lower numbers than ambrosial fungi. Batra (1966) demonstrated that these fungi could support the growth and development of ambrosia beetles. Most of the yeasts and fungi tested could not support all of the beetles tested and the proportion of successful larvae was lower for larvae reared on auxiliary fungi than for those reared on primary (ambrosia) fungi, so it appears that agricultural monocultures are potentially harmful to the beetles. The identification of an association between a yeast community and the beetles complicates the situation but raises many interesting questions about interactions between beetles, between the beetles and the fungi, and between beetle species.

Many of the fungi associated with ambrosia beetles are dimorphic and grow as hyphae in the beetle's galleries, including some of the *Hemiascomycetes*. However,

the literature is split on how they are vectored. Batra (1966) believed them to be vectored as yeast-like cells and was able to show that the transformation from hyphal to yeast forms occurred in the mycangia. Numerous subsequent authors have described the mycangia as specialized for fungal spore transport. Related to the transport issue is the means by which the beetles influence which species of fungi are present in their galleries. There is evidence that the adult beetles are responsible for eliminating airborne fungal contaminants from the system (Batra 1966). How this is accomplished is not known, although some beetles produce exocrine secretions with antiyeast activity (Gross et al. 1998). If the adults could potentially eliminate auxiliary yeasts from their galleries, then the continued presence of yeasts may indicate that they have a role to play in the ambrosia fungus–beetle system.

Several recent discoveries have enlarged the group of yeasts associated with ambrosia beetles. *C. trypodendroni*, an anamorph related to *C. insectorum* (on the basis of rDNA sequence data), has been isolated from *Trypodendron lineatum*, an ambrosia beetle (Kurtzman and Robnett 1998b). As it is in a poorly resolved clade, the relationship with *C. insectorum* is somewhat unclear, but the clade is rich with other ambrosia beetle and bark beetle yeasts, including another yeast described at the same time from insect frass in an insect tunnel in a dead oak, *C. tammaniensis*. Kurtzman and Robnett described a third species from frass in spruce trees, *C. ontarioensis*. This species partial LSU rDNA sequence was not very similar to any known yeast sequence (*C. entomophila* was most similar, but still 18% divergent). *C. mycetangii* and *C. ulmi* are also both beetle-associated, the former with an ambrosia beetle and the latter from insect frass (Kurtzman 2000a). It is not known if the newly described yeasts are functionally associated with beetles.

14.2.3 Anobiid Beetles

Anobiidae, a family in the Polyphaga, are related to powder-post (Lyctidae) and twig boring beetles (Bostrichidae). Xylophagy is thought to be the ancestral feeding habit of the family (Jones et al. 1999) although some species have become adapted to anthropogenic habitats. Two such species, *Lasioderma serricorne* (the cigarette beetle) and *Stegobium paniceum* (the drugstore beetle) have become pests. Both harbor fugal endocytobionts in the cecal cells between the midgut and the foregut. The YLS supply nutrients (vitamins and sterols, Pant and Fraenkel 1954; Pant et al. 1960; Buchner 1965; Nasir and Noda 2003) and detoxify various substances for their hosts (Dowd 1989, Shen and Dowd 1991). Unlike Homopteran YLS (see later), Anobiid YLS are culturable and have been the object of much systematic attention. They were first thought to be *Candida* (= *Torulopsis*) yeast but were later removed from the *Hemiascomycetes* and placed in the *Taphrinales* as *Symbiotaphrina kochii* and *S. buchneri* (van der Walt 1961; Gams and von Arx 1980). Using SSU rDNA, Noda and Kodama (1996) were able to confirm that the two were separate, related lineages (on the basis of sequence differences and the presence of five group I introns in the SSU locus of *S. buchneri*). However, their analysis removed these fungi from the *Taphrinales* and placed them in the paraphyletic *Discomycetes*. Sequence comparison of *ERG5* (a locus in the ergosterol synthesis pathway) from *Saccharomyces cerevisiae*, *Schizosaccharomyces pombe*, and *C. albicans* with the locus from beetle

and planthopper YLS clearly supports this conclusion (Noda and Koizumi 2003) although it does not help resolve their placement beyond that.

In addition to *S. buchneri* and *S. kochii*, there are other YLS found in Anobiid beetles and still classified as *Candida* species. *C. ernobii*, *C. karawaiewii*, and *C. xestobii* are all endocytobionts of Anobiid beetles (Jones et al. 1999). Other beetle families host YLS. Cerambycid beetles, only distantly related to Anobiids, are also xylophagous and harbor culturable YLS described as *Candida (C. rhagii*, *C. shehatae* var. *insectosa*, *C. ergatensis*, *C. parapsilosis* var. *intermedia*, *Rhodotorula glutinis,* and *C. tenuis)*. Except for *Rh. glutinis*, all of these are *Hemiascomycetes*, on the basis of both LSU and SSU rDNA sequences (Jones et al. 1999). For those species where analysis of their LSU sequences is possible (too few SSU sequences are available to allow a comparable analysis using SSU sequences), some Cerambycid YLS are siblings of teleomorphic species (*C. rhagii* and *P. heimii*, *C. xestobii*, and *P. guilliermondii*, Kurtzman and Robnett 1997). Some questions remain. LSU sequences indicate that *C. ernobii* and *C. karawaiewii* are conspecifics and related to *P. holstii* (Meyer et al. 1998). However, SSU sequence analysis indicates that they are not the same species (Jones et al. 1999). As Jones et al. (1999) point out, the origins of beetle YLS in both the *Euascomycetes* and the *Hemiascomycetes* are an interesting case of multiple, independent origins and convergent evolution occurring within (in the case of the Anobiids) a single host family. Further comparisons of YLS and their nonsymbiotic relatives might shed light on the adaptive characteristics (and preadaptations) for successful association with beetles.

14.2.4 Other Xylophagous Beetles

Beetles in the Passalidae are subsocial and live in multigeneration colonies in wood undergoing degradation by white-rot fungi. Larvae and adults feed on adult fecal pellets plastered onto tunnel walls by the adults. Coprophagy is a possible means of providing the microbes time and the proper conditions for further digestion of refractory food items such as lignocellulose. Suh et al. (2003) have isolated yeasts from the guts of two members of the family, *Odontotaenius disjunctus* and *Verres sternbergianus*, one common in temperate North America and the other from Central America. Two undescribed varieties were present and both were able to ferment xylose, possibly beneficial to both yeast and beetle. Xylose fermentation is characteristic of the clade that includes these yeasts and *P. segobiensis* and *P. stipitis*. It was not clear whether these lineages were separate species or varieties of *P. stipitis*. Both have sequences and physiologies that differ slightly from those of *P. stipitis*. One lineage had been identified previously as a *Euascomycete*, *Enteroramus dimorphus* (Lichtwardt et al. 1999), owing to its hyphal growth (all members of this clade are capable of hyphal growth).

Scarab beetles have also been found to harbor yeasts. The green June beetle, *Cotinis nitidia*, has no yeast in its gut during the larval or pupal stages but adults develop a diverse yeast flora that is vectored to new food items (Vishniac and Johnson 1990). The yeasts include *Aureobasidium pullulans*, *C. guilliermondii*, *C. krusei*, *C. sake*, *C. tropicalis*, *Cryptococcus albidus*, *Debaryomyces hansenii*, *Hanseniaspora uvarum*, *Rh. glutinis*, *Rh. rubra*, and *Trichosporon cutaneum*. The xylophagous scarabs, *Pachnoda*

ephippiata and the rose chafer *P. marginata*, have bacterial endosymbionts that contribute to their ability to digest plant fibers, but have not been sampled for yeasts (Cazemier et al. 1997; Egert et al. 2003; Lemke et al. 2003). The gut flora has been implicated in the beetle's ability to digest plant fibers (Cazemier et al. 1997). It should be noted that other insects (*Schistocerca gregaria*, the Cerambycid *Psacothea hilaris*, and the Phasmid, *Eurycanta calcarata*) are able to digest wood without the aid of microbes, so a microbial association is not necessary for this mode of feeding (Cazemier et al. 1997; Scrivener et al. 1997).

14.2.5 Flower Beetles

Flowers are visited by many insects, including beetles from several families. Recent efforts by Lachance and colleagues have isolated numerous species of yeasts either from floricolous beetles (mostly Nitidulids) or from the flowers with which they are associated. Rather than list individual instances, a table of recently described species is provided (Table 14.2).

The beetle–flower–yeast system is dominated by ascomycetous yeasts from the *Saccharomycetales*, which account for about 90% of the strains isolated from beetles (Lachance et al. 2001d). Many of the basidiomycetes and nonsaccharomycetales *Ascomycetes* from the system were either widely distributed (and, so, show little habitat specificity), dispersed through the air (*Aureobasidium*), or so rare that their occurrence was probably serendipitous. Lachance et al. (2001d) include many undescribed species, which have been included in Table 14.2. The table also includes species from flowers that are related to those from beetles with the exception of flower strains associated with bees, which are discussed in the section on Hymenoptera. There is a second set of yeasts from flowers (*Wickerhamiella australiensis*, *W. occidentalis*, *Metschnikowia santaceciliae*, *C. drosophilae*, *C. lipophila*, and *C. tolerans*) that have been isolated from *Drosophila* that lay eggs in the open flower, which they inoculate with yeast, and the larvae develop on the fermenting flower tissue (Lachance et al. 1998c, 1999, 2003). *M. santaceciliae* has been isolated from both beetles and flies, and so it included in Table 14.2. Cladograms based on partial LSU rDNA sequences show that flower-beetle-associated yeast cluster into several unrelated clades within the *Saccharomycetales* (Lachance et al. 2001d, or many of the references cited in Table 14.1) and Table 14.1 is organized by these clades. Each includes a single sporogenous genus, which gives the clade its name, and one or more asexual species (as defined by rDNA sequence divergence).

The *Metschnikowia* clade in Table 14.2 has been subdivided with information extracted from the several cladograms published for these species. The systematics of this clade is not completely understood. Hybridization experiments with auxotrophs showed that, like many sexual eukaryotes, some hybridization and horizontal gene transfer is possible between some species but that there is significant reproductive isolation among sexual *Metschnikowia* species (Marinoni and Lachance 2004). Some *Metschnikowia* species have several insertions in the region of the 26S rDNA used for phylogenetic studies (Hong et al. 2003). As the presence and sequences of the insertions can vary among species, it is not apparent whether or not to include this information in phylogenic analyses. Although the composition of subclades within

Metschnikowia are often well supported (over 70% bootstrap support), basal support for connecting the subclades is often weak. This impression is further strengthened by the cladogram (on the basis of the D1/D2 loop region of the LSU rDNA) presented by Suh et al. (2004a) as part of their description of *M. chrysoperlae* from lacewings. This species and two closely related anamorphs (*C. picachoensis* and *C. pimensis*) belong to a well-supported clade with *M. pulcherrima*, *M. fructicola*, and three undescribed species. However, much of the genus is part of a large polytomy consisting of well-supported subclades (including those in Table 14.1 and others). This ambiguity may indicate that the genus needs to be split or that species with transitional sequences have not yet been sampled. It is difficult to assess the degree of completeness of the known *Metschnikowia* sequences (indeed, this is difficult to know for any lineage). Lachance et al. (2001d) concluded that the biogeography of these yeasts depends on location and beetle, which are confounded (*Aethina* replaces *Conotelus* as the beetle species most often sampled in Australia and the South Pacific, except in Hawaii, where both are introduced). While their efforts have been extremely wide ranging (Table 14.1 includes samples from North America, Costa Rica, Brazil, Australia, Hawaii, Korea, and several South Pacific islands), there are many gaps (Europe, most of Asia, and Africa), which means that there may be systematic gaps in the current set of known species.

Sampling gaps or not, the conclusion that yeast species in the flower–beetle–yeast system tend to occur in restricted locales and that the distribution of the yeast is related to the distribution of both host plants and vectors is well supported. Within each clade, the teleomorphic species tends to have a wider distribution than the anamorphic species (Table 1 in Lachance et al. 2001d) but no species is truly ubiquitous throughout the system. This situation is similar to that in the cactus–*Drosophila*–yeast system. Both have several clades of related species. Some cactus–*Drosophila*–yeast system clades (such as the *Starmera* and *Phaffomyces* clades) conform to the plant–beetle–yeast model of geographic subdivision. However, some species (*P. cactophila* and *C. sonorensis*) seem to be ubiquitous, a situation without parallel in the flower–yeast–beetle system. This may be an historical artifact. Many of the species in the cactus–*Drosophila*–yeast system were described before sequencing became routine. Recent data from that system support the plant–beetle–yeast model. *Sporopachydermia cereana* was once listed among the ubiquitous members of the system but isolations were often reported as "*S. cereana* complex" owing to physiological variation. It is now known that this "complex" is composed of at least ten species, most with very restricted distributions (Lachance et al. 2001c). *C. sonorensis*, an exclusively asexual lineage, is genetically variable with a strong correlation between genetic variation and geography (Ganter et al. 2004). New collections from the Caribbean and South America indicate that *P. cactophila* may also have regional variants, some of which are heterothallic, in contrast to the widely distributed homothallic form (Ganter, Rosa, and Cardinali, unpublished data).

The occurrence of asexual lineages may be related to the means of yeast dispersal. Yeast species vectored by insects disperse to new habitat when their vectors do. Some asporogenous species may represent hybrid opportunists well adapted to very local situations as sometimes happens in plants (Grant and Grant 1971, 1980). Animal vectoring may produce founder events with interesting ramifications for

heterothallic species that occur as haploids. Lachance et al. (2001b) suggest that the Hawaiian species *C. kipukae* and *C. hawaiiana* may be stranded mating types of undiscovered *Metschnikowia* species not native to Hawaii. Only one of the mating types of *M. lochheadii* has been introduced into Hawaii, where it is among the most commonly isolated species from beetles and, more rarely, *Drosophila*. This species is hard to distinguish from some congenerics on the basis of physiology, but it is reproductively isolated (Lachance et al. 2001b). Hawaii has other *Metschnikowia* species, *M. reukaufii* and *M. hawaiiensis*. The mechanism of reproductive isolation from *M. reukaufii* has not been reported, but *M. lochheadii* is partially isolated from *M. hawaiiensis* (Lachance et al. 2001b). Only mating between the h+ mating type of *M. lochheadii* and h– of *M. hawaiiensis* produces sterile asci. Since the mating type of *M. lochheadii* found on Hawaii is h+ and it is ecologically isolated from *M. hawaiiensis*, hybridization is not expected in this instance, although other opportunities exist for hybridization between the invader and native *Metschnikowia* species (Lachance, personal communication). However, Lachance et al. (2001b) also point out that some asexual species (e.g. *C. ipomoeae*, a member of the *Metschnikowia* clade, and *C. azyma*, a member of the *Wickerhamiella* clade) are among the most frequently isolated yeasts from the flower–beetle–yeast system and have wide distributions that are not consistent with expectations for hybrid opportunists. Asexuality may be favored by more than one set of circumstances.

An unresolved question in this system is the degree to which beetles, yeasts, and flower are interdependent. The system is open in the sense that many animals visit the flowers, the beetles are not pollinators, and we do not know what the beetles eat. Whether or not the *Metschnikowia* clade is rightly subdivided, the apparent clustering of related lineages within this environment suggests that both yeast and beetle have specialized to some degree. That the presence of the beetle is valuable to the yeast has been demonstrated through experiments that exclude the beetles from flowers (Lachance et al. 2001d). With no beetles, no beetle-associated yeast colonizes the flowers. What is not yet demonstrated is the influence the yeasts have on the beetles. Nout and Bartelt (1998) found that *Carpophilus humeralis*, a member of a Nitidulid genus that includes some flower-associated species, was attracted to microbial fermentation of corn, its favorite substrate. The most attractive fermenters were all yeasts. Flower yeasts have been isolated from the frass of beetles with regularity owing to the method used to sample yeasts from beetles, so they at least pass through beetle guts. However, there is no direct evidence at this time on the nutritional value of yeasts to flower beetles.

14.2.6 Beetles Associated with Mushrooms

Our understanding of the yeast community associated with mushroom-associated beetles is undergoing a bit of a revolution. Suh, Blackwell and colleagues have embarked on a program to isolate yeasts from basidiocarps. As of their most recent publication, they have 650 isolates from beetles in 26 families. Many of the isolates represent undescribed species. One example is a relatively small clade including *C.* (*P.*) *guilliermondii* and *C. xestobii*, known from xylophagous beetles, *C. fermentati* a previously described species Suh and Blackwell (2004a) isolated from mush-

room beetles, and three new species: *C. smithsonii*, *C. athensensis*, and *C. elateridarum* (Suh and Blackwell 2004a). Although closely related, the yeast species seemed not to be associated with specific basidiomycete species, specific beetle hosts (six strains used to describe three new species were isolated from five beetle families), or specific places (*C. athensensis* was isolated in Panama and Georgia, USA). Recently they (Suh et al. 2004b) have described the largest clade from the collection: 164 isolates (30% of the total) in 17 species, one previously described (*C. ambrosiae*) and 16 new species, all anamorphic *Hemiascomycetes* related to *C. tanzawaensis* (*C. guaymorum*, *C. bokatorum*, *C. kunorum*, *C. terraborum*, *C. emberorum*, *C. wounanorum*, *C. yuchorum*, *C. chickasaworum*, *C. choctaworum*, *C. bolitotheri*, *C. atakaporum*, *C. panamericana*, *C. bribrorum*, *C. maxii*, *C. anneliseae*, and *C. taliae*). The collection locales varied from Vermont and the southeastern USA to Panama. The isolates in this clade were collected from the gut contents of members of 11 beetle families, although 85% came from Erotylidae and Tenebrionidae, with some of the new species possibly associated with particular beetle species. This clade has just been recently expanded from a single isolation from moss in Japan by the addition of six species from trees and beetle frass (see Sect. 2.1 and Kurtzman 2001b).

Considering that basidiocarps have been previously sampled for yeasts, Suh et al.'s work constitutes a remarkable demonstration that animal-associated yeasts are undersampled. The situation may be even more diverse than standard sampling methods will uncover. For some time, bacteriologists have amplified DNA regions useful for assessing biodiversity from environmental samples in search of lineages that do not grow under standard culture conditions. Comparisons between sequence diversity amplified from cultures and from environmental samples lead to the conclusion that only a small percentage of bacterial species are culturable (Torsvik et al. 1990; Kemp and Aller 2004). Environmental PCR has its pitfalls (Tanner et al. 1998; Qiu et al. 2001; Speksnijder et al. 2001) and is only beginning to be applied to yeast studies but has considerable potential (Lipson et al. 2002; Renker et al. 2004), especially in the area of yeast–animal interactions, where it is known that some YLS are not culturable. Using rDNA sequence amplification, Suh and Blackwell (2004b) report that as many as 150 new yeast species may be awaiting discovery in the guts of beetles. Zhang et al. (2003), sampling beetles collected from basidiocarps and rotting wood, found that there was less than expected overlap between the yeasts isolated from the beetles' guts and rDNA fragments amplified from their guts. Six sequences similar to known *Hemiascomycetes* sequences, five sequences from the Pezizomycotina, and two *Basidiomycetous* sequences were detected. Of these, only five were greater than 96% matches to known species and only one was a described yeast, *P. stipitis*. Only three of the six yeast sequences amplified from the gut matched sequences amplified from colonies isolated at the same time. Seventeen of the sequences from isolates did not occur in the sequences amplified directly from the gut. Considering that isolations are reasonable proof of the yeast's presence and the sensitivity of the PCR method, it is unexpected that so few of the isolated species' sequences were detected in the gut amplifications. Since both samples (isolation of viable colonies and direct amplification of gut contents) were small, it is difficult to know if the differences in the results are systematic and represent bias in the direct amplification method or are simply due to chance.

14.3 Termites

Most biologists associate termites with cellulose digestion mediated by the presence of protistans in the insect's gut. Study of the details of lignocellulose digestion and the distribution of microbes in the various sections of the highly differentiated termite gut have, for some termites, reduced the importance of the microbe–insect mutualism and raised questions about the role of nonprotistan microbes in both carbon (Wenzel et al. 2002; Ohkuma 2003) and nitrogen metabolism (Potrikus and Breznak 1977, 1980). Termites produce their own cellulose-degrading enzymes and the higher termites (75% of termite species) lack the protistan symbionts long thought to be a necessary component for digestion in termites (Slaytor 1992; Varma et al. 1994; Tholen et al. 1997; Tokuda et al. 2004). Some higher termites acquire cellulases from their fungal gardens (Martin and Martin 1978). Although prokaryotic and fungal symbionts have long been known from termites, the true complexity of the system is only now being dissected. Yeasts are a member of the system, and there role is still not understood. Fungi have been isolated and identified from termite mounds many times. Hendee (1935, cited in Prillinger et al. 1996) found dozens of genera of fungi associated with the lower termites and there has been considerable interest in the association between *Termitomyces*, a mycelial basidiomycete genus, and some higher termites in the Macrotermitinae (Aanen et al. 2002; Rouland-Lefevre and Bignell 2002; Ohkuma 2003). Fungus-cultivating termites can be the dominant macrofaunal decomposer in some tropical biomes (Abe et al. 2000), giving this association widespread ecological importance. A second association between termites and *Laboulbeniales* is known (Blackwell and Kimbrough 1976a, b; Rossi and Blackwell 1986; Blackwell 1994). The relationship is one of parasitism or, given the lack of impact on the host, perhaps a form of phoretic commensalism.

Yeasts have not received the attention of other termite-associated fungi. Boidin and associates collected *Trichosporon*, *Candida* (including *Torulopsis*), and *Saccharomyces* from African termites (cited in do Carmo-Sousa 1969). This promising start was not immediately followed up. In an extensive review of termite gut flora, Breznak (1982) did not discuss yeasts except to mention the utility of yeast extract in media used to cultivate some of the first protistan symbionts to be isolated. Subsequent reviews of termite microbes involved in lignocellulose digestion by Breznak and Brune (1994) and Varma et al. (1994) do not mention yeasts at all (although Varma et al. do use the term in their abstract). In 1996, Prillinger et al. isolated strains from six species of lower termite and a cockroach (as a sister group to the termites). They subjected the yeast isolates to an array of methodologies (randomly amplified polymorphism DNA, physiological profile, ultrastructure, cell wall constituents, ubiquinones, and 18s rDNA sequences) in order to develop useful taxonomic characters. Using this multifaceted approach, they isolated strains that could be grouped into 12 ascomycetous species from the lower termites (one basidiomycete isolate, probably from the genus *Trichosporon*, was collected). Three of the species were tentatively assigned to the *Lipomycetaceae*. Although the physiologies of the remaining ascomycetous species were similar to *Debaryomyces* species, the sequence data led Prillinger et al. (1996) to remove them from the *Hemiascomycetes* and place them in the *Ophiostoma – Sporothrix* clade within the *Ophiostomatales*

(see Blackwell and Jones 1997 for a discussion of the phylogeny of insect-associated *Ascomycetes*). It is notable that Prillinger et al. (1996) did not find a single isolate that they could identify as a member of a known species.

Prillinger et al. (1996) did not investigate the nature of the interaction between the yeast they isolated and their putative hosts except to conclude that the relationship was a stable symbiosis because they were able to isolate members of the same clades from a variety of termite species collected from widely dispersed locales. Using what appear to be some of the strains isolated by Prillinger et al. (1996), Schäfer et al. (1996) tested them and a larger set of bacterial isolates for their ability to produce a set of four enzymes (α-L-arabinofuranosidase, β-D-galactosidase, 1,4-β-xylanase, and β-D-xylosidase) that play a part in the digestion of hemicellulose. Twelve strains (from five of six termite species) had positive activity for at least one of the enzymes, although no strain had all four. From these results, Schäfer et al. concluded that yeast enzymatic activity may play a role in termite nutrition.

14.4 Ants

Although the association between fungi and ants is well established, evidence for a yeast–ant relationship remains more equivocal. Fungus-farming ants in the tribe Attini are associated with mycelial basidiomycetous fungi. The relationship has arisen independently at least three times from within the *Agaricales* (Chapela et al. 1994; Mueller et al. 1998; Mueller and Gerardo 2002). Fungi from this clade cultivated by members of the genus *Cyphomyrmex* grow as yeasts. It is not known if the switch to the yeast-like form is adaptive or is an outcome of chance and the particular details of this association. The yeast strains are not sexual and seem to be propagated by transfer from ant colony to daughter colony by the ants themselves (vertical transfer). Some horizontal transfer does occur (Green et al. 2002). While confirming that the yeasts cultivated by *Cyphomyrmex* species did form a single, well-supported clade within the larger ant-farmed clade, Mueller et al. (1998) found that ant colonies traded yeast cultivars, sometimes with ants from other species, and that members of this yeast clade could be collected from natural substrates outside of the nest. Indeed, the strain not isolated from an ant nest was sampled from a basidiocarp and did not grow as a yeast. This implies that the yeast habit is a consequence of the association with the ants and that adoption of the yeast growth form occurs when a free-living fungus is recruited by the ants.

Other yeasts have been observed in the gardens of attine ants that cultivate mycelial fungi (Craven et al. 1970), although the yeasts present were not identified. Several species of yeasts were isolated from *Atta sexdens rubropilosa* colonies that were maintained in a laboratory on *Eucalyptus albus* leaves collected from nature. *C. apis*, *C. colliculosa*, *C. famata*, *C. homilentoma*, *C. guilliermondii*, *C. robusta*, *C. sake*, *Cr. aerius*, *Cr. albidus*, *Cr. laurentii*, *Cr. haglerorum* (a new species), *Rh. aurantiaca*, *Rh. glutinis*, *Sympodiomyces attinorum* (another new species), *Sporobolomyces roseus*, *Tremella foliacea*, *T. jirovecii,* and several variant physiologies perhaps representing undescribed species were all present in the fungal garden but not on the leaves. *Hemiascomycetes* constituted 71% of the 84 isolates from the gardens, waste piles, surface of ants, or colony floors (Carreiro et al. 1997, 2004; Middelhoven et al.

2003). Quite different results were obtained from an attempt to isolate yeasts from gardens in the wild. A search of 14 colonies of *Acromyrmex octospinosus* (another Attine species) detected no *Hemiascomycetes* rDNA sequences among 41 fungal sequences amplified from workers (van Borm et al. 2002). The absence of yeasts from some gardens may not be due to chance. Fungi other than cultivated species have been isolated from nests in the wild and the laboratory, including both commensal and parasitic (*Escovopsis*) species (Currie et al. 1999). The ants have behaviors (weeding and grooming) and chemicals that may control contaminants. The chemical defenses include both those produced by the ants (metapleural and maxillary glands) and by cultivated *Actinomycetes* bacteria that produce toxins effective against the parasite (Currie 2001). However, the effectiveness of these defenses has not been tested, as the dynamics of the fungal populations are unknown.

Hemiascomycetes have been found in association with other ants. Zacchi and Vaughn-Martini (2002) isolated *Rh. mucilaginosa* and *P. guilliermondii* multiple times from the body fluid of *Iridomyrmex humilis* in Italy. *D. polymorphus* (*D. cantarellii*) and *D. vanrijiae* (*D. formicarius*) have been isolated from *Formica rufa* in eastern Europe (Golubev and Bab'eva 1972; Sláviková and Kocková-Kratochvilová 1980). In Texas, USA, Ba et al. (2000) found 13 yeast species in samples from the nest of the invasive red fire ant, *Solenopsis invicta*, and from the soil in the area of the nest but most yeast species were equitably distributed between brood chamber (the site of the nest samples) and surrounding soil. *C. parapsilosis* and *C. lipolytica* were strongly associated with the nest and these two species had previously been isolated from the ants themselves (Ba and Phillips 1996). There was some indication that *D. hansenii* was also associated with the ants and that *Cr. terreus*, *C. vini*, *Rh. minuta*, and *Rh. rubra* were excluded from the nests. The nature of the interaction (whether positive or negative) between any of the yeasts and the ant is not known.

A possible role for yeasts in ant nutrition has been suggested in a recent study of carpenter ants and yeasts. Carpenter ants, such as *Camponotus vicinus,* are wood-dwellers, although they are not xylophagous. Workers of *C. vicinus* ingest a liquid diet, which can include honeydew. The infrabuccal pocket, located just before the crop, filters large particles from the food stream but not microbes. Mankowski and Morrell (2004) isolated over 150 strains of yeasts from ant guts, their nest (including frass), and surrounding soil. Of the 18 taxa identified, six were found in the infrabuccal pocket. *D. polymorphus* comprised ten of the 17 isolates from the ant guts. No other species occurred more than twice. To see if the presence of the yeasts has any impact on the ant, Mankowski and Morrell (2004) fed workers (initially cleared of yeasts) on an artificial diet that had been previously shown to be adequate for workers of this species. Some of the workers were given access to *D. polymorphus*, while others were not. Those with yeasts in their diet were significantly heavier after 12 weeks. This, plus Ba and Phillips' (1996) observation that colonies with yeasts were more vigorous, suggests that yeasts can be important sources of nutrition for some ants.

14.5 Other Soil-Associated and Xylophagous Arthropods

Byzov and his associates have studied the interaction between yeasts and diplopods (*Pachyiulus flavipes*, *Glomeris connexa*, *Leptoiulus polonicus*, and *Megaphilium pro-*

jectum). Comparison of yeasts from the fore-, mid-, and hindguts with those from the surrounding soil exposed significant differences in both species composition and live yeast counts (Byzov et al. 1993). Yeasts in the litter that comprises the myriapod's food (numerous species were present and differed among the litter types sampled) were only rarely found in the midgut (Byzov et al. 1993). The hindgut and feces contained high densities of yeasts, mainly *D. hansenii*, *Torulaspora delbrueckii*, and *Zygowilliopsis californica*, but these were not found in the soil samples. Digestion of yeast species is selective. Midgut fluid is able to rapidly kill and enzymatically break the cell wall of most species commonly found in the soil. Those yeasts associated with the hindgut were either resistant to the effects of the midgut fluid or were killed and digested at a much slower rate (Byzov et al. 1998a, b). The common species in the hindgut were also able to use the insect's nitrogenous waste (uric acid) as their sole nitrogen source (Byzov et al. 1993). Byzov et al. (1998a) proposed that the soil microbes (fungi and bacteria) are the main foods of diplopods and that the hindgut flora (yeasts and bacteria) are mutualistic symbionts. The latter claim is open to some doubt in that they have only speculated on the benefits accruing to the animal. The hindgut flora clearly benefits as it grows on materials derived from the millipede, which also provides the habitat. Byzov et al. (1998a) argue that the flora provides benefits to the insect by becoming food when antiperistaltic movement carries cells from the hindgut into the midgut. The hindgut yeasts are typically found only in the posterior portions of the midgut and are not found in the foregut, which contains litter-associated yeasts, or the anterior portions of the midgut, which contains few yeasts. Those hindgut species that are susceptible to digestion may, in this fashion, contribute to the insect's nutrition but the contribution may only be a small portion of the insect's diet. The second benefit, detoxification of uric acid, was not measured in vivo and we do not know if the diplopods actually derive any benefit. The situation may be similar to that of cockroaches, which recycle nitrogenous waste through their bacterial microflora (Cochran 1985; Cruden and Markovetz 1987). Thus, while the symbiosis between yeasts and diplopods has been amply demonstrated, the prima fascie case for a mutualistic symbiosis, while reasonable, awaits experimental examination.

Cockroaches are soil-associated insects that receive attention here more for their potential as objects of study than for what is known about the yeasts associated with them. Although much is known about the rich bacterial community found in the guts of human-associated cockroaches (Cruden and Markovetz 1987), they have yielded only a single species of yeast, *Kluyveromyces blattae* (Henninger and Windisch 1976, from Prillinger et al. 1996), and the relationship between yeast and cockroach has not been explored. The outlook for yeast associations is brighter for the wood-eating cockroaches. Their gut microflora is similar to that of the lower (protistan-associated) termites and Prillinger et al. (1996) discovered a species of yeast from a wood-eating cockroach (*Cr. punctulatus*). While they could not assign a generic name to the three isolates they obtained, they did ascertain that the strains were similar to some yeasts isolated from termites and all three belonged to the same clade in the *Endomycetales*. Nothing is known about the interaction between the yeast and the cockroach but it remains a promising avenue for research. In addition to the cockroach-termite clade, xylophagy has been demonstrated in a Scarabaeidid

beetle, *Oryctes nasicornis* (Bayon 1980; Bayon and Mathelin 1980). Unfortunately, no attempt has been made to isolate the gut microbes so we do not know if yeasts are present in the system. One last note on xylophagy and yeast. Xylophagy is not the exclusive domain of insects. Nelson et al. (1999) report that a xylophagous catfish in the genus *Panaque* has a gut microflora capable of lysing cellulose and of sustaining itself on artificial media with cellulose as the sole carbon source. The identification work done on the microbes was minimal and no effort was made to isolate yeast. However, nonpathogenic yeasts are associated with fish guts (Andlid et al. 1995) and it would not be unexpected to find them present in *Panaque*.

In addition to termites and ants, there are several other soil-associated arthropod clades, including some Orthopera, Collembola, Thysanura, Diplopods, Diptera, Coleoptera, and Hymenoptera, that have known microbial associations. Yeasts have been identified only from a few of these groups, but that may simply result from a failure to look. The effort put into collecting yeasts from these sources has not been sufficient to eliminate yeasts from membership in any of these gut floras. *D. mycophilus* is a novel species isolated from an Oniscoid isopod (Thanh et al. 2002). Woodlice and pillbugs are common litter organisms long noted for consumption of rotting vegetation and may harbor more undescribed species.

14.6 Neuroptera

Lacewing larvae and adults are useful agents for biological control of aphids and other agricultural pests, including some mites and whiteflies. As such, their biology has received attention, first from agricultural entomologists and, more recently, yeast biologists. *Chrysoperla* (= *Chrysopa*, "golden pearls" for their golden eyes) is a large genus from which several biological control agents have come, including *C. rufilabris* and *C. carnea* (commonly used in the USA). Larvae can be purchased packaged in honeycombs of 500 cells, each with a single larva (they are cannibals). Investigation of *C. carnea* uncovered an unexpected interaction among aphids, yeasts, and lacewing adults. The feeding habits of *C. carnea* are unusual in that adults are not predaceous. Instead of eating the aphids, they feed on the honeydew extruded out of the anus of the aphid (Hagen and Tassan 1972). The honeydew is not a complete diet for the adults (it is deficient in several essential amino acids and lipids). Hagan and coworkers determined that endosymbiotic yeasts were able to make up any dietary deficiencies and observed that the standard laboratory food for rearing the adults consists of honey and *Saccharomyces cerevisiae* (Thompson 1999; Winterton 1999)

Hagan and Tassan (1972) found the yeasts in the crop and larger trachea of the adults but they could not find any in the predaceous larvae. Phaff and Starmer (1987) conclude that the adults must acquire yeasts through feeding or trophallaxis. The yeast population of the crop can grow until the adult deposits compact spheres of yeasts, which can be seen where the adults are ovipositing. Recently, there was a report that vertical transmission is possible and that the protocol for cleansing the adults of yeasts used by Hagan is not adequate (Gibson and Hunter 2003). Suh et al. (2004a) isolated yeasts from eggs, although it is not clear if the yeasts came from the surface or were internal. Although these observations establish the possibility of vertical transmission they would not exclude horizontal transmission as an important

dispersal process. Woolfolk and Inglis (2003) found no yeast present in the guts of newly eclosed *Chrysoperla* adults from larvae raised on laboratory food. The eggs were obtained from a mass-rearing facility. These data raise questions about vertical transmission but do not settle the question of transmission in nature. No work has been done to establish whether or not the appropriate yeasts are available from nonanimal sources in the adults' range (honeydew, plant surfaces, etc.) or if trophallaxis is common enough to ensure an adult has a complete diet. Seven of 24 adults sampled by Woolfolk and Inglis (2003) had no yeast, so adults can manage without the yeast for at least part of their adulthood. Clearly, more work needs to be done before we will know how yeast populations are established and maintained in this system and what effect they have on lacewings.

The distribution of yeasts in time and space was not random in Woolfolk and Inglis's study (2003). They sampled 24 *C. rufilabris* adults from two sites in Mississippi, USA, five times between October 2000 and June 2001. Fewer yeasts were collected in the cooler months, per capita colony-forming units were consistently lower at one site, and most yeasts were found in the diverticulum, with populations declining along the gut (the fore-, mid-, and hindguts were also sampled). Almost 90% of the isolates were assigned to *M. pulcherrima*. Isolates from other species were never recovered from the diverticulum and, in the other regions of the gut, almost always when no *M. pulcherrima* was present. These patterns hint at interesting ecological interactions among the yeast species but this awaits investigation.

The diversity of yeasts from lacewings follows a common trajectory: first a single species similar to, but not identical to, a published species is found and subsequent work redefines and adds to the initial discovery. Hagen and Tassan (1972) isolated a single species of yeast, which they felt was a *Torulopsis* (= *Candida*) species. Phaff and Starmer (1987) reported that they isolated only a single yeast from several *Chrysoperla* adults and that its physiological profile was roughly similar to *T. multisgemmis* (= *C. multigemmis*) but that its G+C value indicated that it was a new species. Woolfolk and Inglis (2003) originally divided their more than 750 isolates into five groups, two basidiomycetous and three ascomycetous, but analysis of rDNA sequences caused them to divide one basidiomycete group into two known species (*Cr. victoriae* and *Cr. luteolus*) and assign the other basidiomycete group to a new species, which they did not describe. Their sequence analysis suggested that the unknown species' sister taxon was *Bullera oryzae,* with which its physiological profile was consistent, but with which the new species shared only 63% sequence similarity. The three ascomycete groups' sequences were identical. Comparison with known sequences indicated that the ascomycete isolates from Mississippi formed a well-supported (bootstrap support) clade with *M. pulcherrima* as its sister group (also a well-supported clade). Owing to the physiological similarities between *M. pulcherrima* and the *Chrysoperla* isolates (and the presence of needlelike ascospores), they accepted them as members of the described species, although with reservations. Indeed, a very similar situation in their cladogram of basidiomycete isolates (Woolfolk and Inglis 2003, compare Figs. 5, 7) was used to suggest that the sister group to *B. oryzae* was a new species. I do not mean to suggest that Woolfolk and Inglis were being inconsistent but to remark on the difficulty of assigning isolates to species, especially when working with an undersampled group of yeasts.

The diversity of yeasts associated with lacewings has recently increased. Suh et al. (2004a) isolated 14 strains from *C. carnea* and *C. comanche* (two strains from eggs and the rest from adults). All were ascomycetes and the partial sequence of the LSU (from the D1/D2 loop region), SSU, and ITS region rDNA was determined for each strain. Three unique LSU sequences were found. The sequence differences corresponded to differences in physiological profile, so three new species related to *M. pulcherrima* were described: *M. chrysoperlae*, *C. picachoensis*, and *C. pimensis*. Comparison of the rDNA sequences from the strains isolated by Woolfolk and Inglis with those isolated by Suh placed the isolates identified as *M. pulcherrima* by Woolfolk and Inglis in *M. chrysoperlae* and matings of strains from both studies produced asci and needle-shaped ascospores. However, there are several physiological differences between the two groups, so there may be significant genetic differences below the specific level that are related to geography (Suh et al. collected in Arizona, USA). The two asexual species might never have been recognized without sequence data. Although there were significant differences among their rDNA sequences, there was very little difference in their physiologies. Sequence data place them in a separate clade that is the sister to several described and undescribed *Metschnikowia* species and which includes *M. chrysoperlae*. We can only speculate at this time on the origin of the asexual lineages and how they are maintained. It would be very interesting to know the age of the asexual lineages and if the asexuals are found with each other or with related *Metschnikowia* species.

14.7 Homoptera

A convincing case for mutualism between insect and yeast has been made for the interaction between Homoptera and their endosymbionts (in this case, intracellular YLS). All phloem-feeding Homoptera are believed to have mutualistic interactions with symbiotic microbes, most of which are bacteria (Wilkinson and Ishikawa 2001). YLS are found in planthoppers and some aphids. Some endosymbionts are found in special enlarged insect cells (bacteriocytes or mycetocytes for their respective symbionts). Aphid endosymbionts (various species of *Buchnera*) in bacteriocytes are referred to as primary symbionts. Secondary endosymbionts are sometimes found in aphid and planthopper fatbodies or hemolymph (but not intracellularly). Most aphid endocytobionts are bacteria, but a single clade of eusocial aphids from southeast Asia has no primary endosymbiont. Instead, they have YLS that are not intracellular (Fukatsu and Ishikawa 1992; Suh et al. 2001). They are found in the hemocoel and intracellular lacuna in the fat body, like secondary bacterial endosymbionts (Houk and Griffiths 1980; Lee and Hou 1987; Baumann et al. 1995; Douglas 1998). Because the association between aphids and bacteria is thought to predate the founding of the aphid clade (Munson et al. 1991), the loss of bacteria and acquisition of YLS is a derived state. It is not known if this happened more than once (Fukatsu and Ishikawa 1992). This is not the only time a loss of endosymbiotic bacteria is thought to have taken place. In those instances that have been examined, planthoppers that have abandoned phloem feeding in favor of piercing and eating individual plant cells have also lost their bacterial endosymbionts (Douglas 1998; Wilkinson and Ishikawa 2001).

Yeast-like endosymbionts of the rice planthoppers, *Nilaparvata lugens*, *Sogatella furcifera*, and *Laodelphax striatellus*, are obligate mutualists and are inherited vertically as a clump of cells within the egg called the symbiote ball (Lee and Hou 1987). Although the symbiont has not been cultured in vitro, YLS cells can be isolated by density-gradient centrifugation and protein and molecular work done on the extracted cells. This technique has allowed investigation of their taxonomic affiliations without isolation in pure culture. Noda and Kawahara (1995) determined that the symbiont genome sizes and electrokaryotypes were consistent with those of other ascomycetes. On the basis of the sequence of a portion of the 18s rDNA, Noda et al. (1995) concluded that the symbionts isolated from the rice planthoppers represented three different lineages (species determinations were impossible to make and the authors did not attempt to do so) that formed a single, well-supported clade. Analysis of a set of fungi chosen to represent all higher fungi convincingly placed the planthopper YLS clade in the *Euascomycetes*. A second sequence comparison with a set of species chosen to represent *Euascomycetes* placed all three symbiont sequences as a single clade within the *Pyrenomycetes*, in the order *Hypocreales* (*Sphaeriales*, reported by Noda et al. 1995, has been invalidated) with *Hypomyces chrysospermus* as the sister group. However, the distance between the three symbionts groups and *H. chrysospermus* was large enough to suggest that the sister group might actually be another *Euascomycetes* lineage. Using more strains and both LSU and SSU rDNA sequences, Suh et al. (2001) were able to confirm that the three planthopper YLS strains formed a well-supported clade within the *Clavicipitaceae*, one of three families in the *Hypocreales*, with *Cordycepioideus bisporus* as the most probable sister lineage. This represents an independently evolved adoption of a yeast-like morphology as the planthopper YLS lineage is not closely related the YLS from Anobiid beetles (Noda and Kodama 1996) nor any of the fungal lineages associated with beetles. A fourth sequence from an aphid YLS was also part of the clade, suggesting either a common YLS ancestor in the insect's common ancestor, horizontal transmission among planthoppers and aphids, or an unknown source of potential endosymbionts available to both groups (see later).

Phloem is rich in carbohydrates but amino acid poor. The role of the endosymbiont, whether bacteria or YLS, seems primarily to be to supply essential amino acids (Wilkinson and Ishikawa 2001). This has been extensively documented for some aphids and is reviewed by Baumann et al. (1995) and Douglas (1998). Wilkinson and Ishikawa (2001) were able to demonstrate similar effects of symbiont loss in an aphid (*Acyrthosiphon pisum*) and a planthopper (*N. lugens*). Both insects developed more slowly and were underweight without symbionts. However, the effect was less pronounced for the planthopper. This result is in agreement with the work of Lee and Hou (1987). *N. lugens* adults in which the number of symbiotic YLS had been reduced by heat treatment did not suffer increased mortality or shorter lives so the YLS seem not to have had a direct effect on adult survivorship in their study. However, heat-treated female planthoppers laid fewer eggs so it appears that the amino acids supplied by the YLS boost fertility. Of interest in the Wilkinson and Ishikawa (2001) study was the production of what they refer to as the "metabolic signature" of symbiont loss in both insects despite their phylogenetic distance from one another and the difference in their symbionts. *Acyrthosiphon*

pisum contains a bacterium (*Buchnera* sp.) and *N. lugens* a YLS. Asymbiontic adults of both insects had a decreased total protein percentage while at the same time an increased concentration of amino acids in their tissues when compared with conspecifics reared with symbionts. Both effects are seen as a direct outcome of the loss of essential amino acids from the insect's diets. Once again, the effect was not as large for the planthopper.

A third part of the "metabolic signature" in both insects without symbionts was the imbalance between essential and nonessential amino acids found in both Homopterans (Wilkinson and Ishikawa 2001). Asparagine and glutamine were predominant in the aphid, while alanine and glutamate were relatively abundant in the planthopper tissues. The use of the term "essential" is only conditionally true in this case. *N. lugens* nymphs with their normal symbionts were able to develop into adults no matter which amino acid was eliminated from their diets (Koyama 1985), which leads to the conclusion that the symbionts can supply whatever amino acids might be essential to the insect. The source of the nitrogen for amino acid synthesis is both the plant and the insect. *N. lugens* produces uric acid as its nitrogenous waste but does not excrete it. Instead, it appears to be metabolized by its YLS (Sasaki et al. 1996), thus recycling the waste and eliminating the need for excretion. On high-nitrogen diets, rice planthoppers can store waste in their tissues as uric acid. Under conditions of nitrogen stress, the uric acid is mobilized by uricase (urate oxidase) from their YLS (Hongoh and Ishikawa 1997; Hongoh et al. 2000). There may be a second source of nitrogen for the YLS. The pea aphid produces ammonia plus excess asparagine and glutamine as its nitrogenous recycling materials (Sasaki and Ishikawa 1995; Douglas 1998). Its bacterial symbionts metabolize all three. Wilkinson and Ishikawa's (2001) discovery of excess alanine and glutamate in asymbiontic planthoppers suggests that these amino acids are also part of its nitrogen recovery system, although Douglas (1998) feels that more work is needed before amino acid recycling is unequivocally established. Sasaki et al. (1996) offer an interesting explanation for the lack of uric acid production in the aphids. Recycling ammonia and amino acids requires fewer steps than the production of uric acid and, so, is more efficient. Planthoppers (and the cockroaches and termites with bacterial endosymbionts that recycle their uric acid) may not take advantage of the streamlining of the recycling process as aphids do because they all lay eggs, which may require nitrogen waste storage in the form of uric acid crystals. Aphids, with their viviparous development, may have no life history stage that needs to store waste and can, therefore, streamline the recycling by simply not producing uric acid. Cockroaches and some termites (under some conditions) also produce uric acid and do not excrete it but recycle it (Cochran 1985). The systems for recycling differ in the two groups but bacteria are involved in both (gut bacteria in the Reticulitermes; fat-body mycetocytes in the cockroaches).

The YLS of aphids is related to that of planthoppers (Fukatsu et al. 1994; Suh et al. 2001). The role of the YLS in aphids is not understood at this time. Aphids, generally, do not produce uric acid and analysis of the uricase gene sequences from aphids, planthoppers, and related, free-living fungi showed that, while present, some of the aphid genes had mutations that rendered them incapable of coding functional enzymes (Hongoh and Ishikawa 2000). Hongoh and Ishikawa (2000) tested one

species of YLS-associated aphid (which Fukatsu and Ishikawa 1992 identified as *Astegopteryx styraci*, Hongoh and Ishikawa 2000 named it *Tuberaphis styraci*, and Suh et al. (2001) subsequently called it *Hamiltonaphis styraci*) that lacked a functional uricase gene and determined that it produced no uric acid nor any endogenous uricase. However, some of the aphid sequences did indicate that a functional gene product was possible, although the product's function was not clear. Phylogenetic analysis of the sequences showed that the sequence of a functional aphid YLS uricase gene was closest to the sequence of the gene from free-living fungi, which were included as an outgroup. This puts the uricase genes in planthopper YLS in a derived position with respect to some aphid YLS genes. This supports the hypothesis that YLS were associated with aphids before planthoppers and implicates horizontal transfer as the means by which planthoppers acquired their endosymbionts.

Protein synthesis is not the only area of nutrition affected by the presence of YLS. Phytophagous insects depend on phytosterols for the production of their own sterol requirements. The exceptions to this may be insects with eukaryotic intracellular symbionts (bacteria do not seem to produce sterols – Douglas 1998). Noda et al. (1979) have made a case for YLS as the source of sterols for the rice planthoppers, *N. lugens, S. furcifera*, and *L. striatellus*. The phloem of rice contains β-sitosterol and a much lower concentration of campesterol. The sterols found in *L. striatellus* are primarily cholesterol and 24-methylenecholseterol with a small amount of β-sitosterol. Although the concentrations are not as expected, this situation might be explained as the outcome of the conversion of campesterol into cholesterol because 24-methylenecholesterol is an intermediary in the process. However, asymbiontic *L. striatellus* have negligible concentrations of 24-methylenecholesterol in their tissues and a reduced concentration of cholesterol, even though their plant diet is unchanged. The precursor of 24-methylenecholesterol is ergosta-5,7,24(28)-trienol. When planthoppers are maintained on artificial diets lacking sterols, the concentration of 24-methylenecholesterol in the insect increases, implicating YLS as the source of its precursor, since the insect cannot synthesize the precursor itself (Wetzel et al. 1992). This suggests that the source of cholesterol in the planthopper is its YLS. Noda et al. (1979) present other data suggesting that the YLS are the source of sterols in their host insects. Two other YLS-associated planthoppers on rice (*N. lugens* and *S. furcifera*) also have 24-methylenecholesterol in their tissues. A fourth rice planthopper, *Nephotettix cincticeps*, which is host to bacterial endosymbionts but not YLS, does not have detectable levels of 24-methylenecholesterol in its tissues, even though its natural plant diet is the same as the other three. It appears that the YLS, although the source of ergosta-5,7,24(28)-trienol, cannot metabolize it themselves (Noda and Koizumi 2003). It is an intermediate in the ergosterol synthetic pathway in yeast and is normally converted into ergosta-5,7,22,24(28)-tetraenol, the last precursor before ergosterol. However, planthopper YLS lack a functional gene for sterol C-22 desaturase, the enzyme responsible for the conversion.

The role of YLS in planthopper biology is not confined to nutrition. The fungi are not only passed on to offspring in the egg; they are a requisite part of normal planthopper development (Schwemmler 1974; Noda et al. 1979) in at least one

species, *N. lugens*. Embryos from which YLS had been eliminated or without YLS in their symbiote ball (because the eggs were laid by heat-treated females) failed to develop. They did not undergo blastokinesis or dorsal closure and the entire abdomen was missing (Lee and Hou 1987). The mechanism by which YLS affect planthopper development appears to be through direct supply of proteins needed for normal development. Eggs receive their YLS before yolk production and without YLS were deficient in more than one yolk protein (Lee and Hou 1987). Whether or not the YLS supply essential nutrients to the planthopper, the role of the YLS in the development of *N. lugens* makes the relationship with YLS obligatory.

Yeasts are associated with other members of the Homoptera but less is known about the nature of the association. Zacchi and Vaughn-Martini (2002) isolated seven yeast species from an olive tree scale insect, *Saissetia oleae*. Isolations were all from the internal organs, hemolymph, or the gut. A surprising number were basidiomycetous (*Bullera variabilis*, *S. roseus*, *Leucosporidium scottii*, and two undescribed species, one *Cryptococcus* and one *Rhodotorula*). They did not report serendipitous isolations but only those that occurred 10 times or more in their study. However, no species was present in all scale samples and no interactions between yeasts and insect were documented, although subsequent work determined that the number of yeasts in the body cavity fell as the insects developed (Zacchi and Vaughn-Martini 2003). Zacchi and Vaughn-Martini felt that the failure of the populations to grow lessened the likelihood that they were parasitic.

There are many unanswered and interesting questions pertaining to the evolution and ecology of YLS–insect associations. We do not know why different Homopteran lineages rely on different microbes. The answer to questions about the origins and effects of endosymbionts is likely to be found by studying variation in microbe, insect, and plant at the population level, but the few studies at this level have focused on bacterial endosymbionts. Baumann et al. (1995) and Douglas (1998) believe that YLS have replaced bacterial endosymbionts in some aphids, but no reason is suggested for how or why the replacement occurred. Ferrari et al. (2004) found that the presence of different secondary (facultative) bacterial endosymbionts in the pea aphid correlated both with differences in host plant utilization within that species and with resistance of the aphids to attack by different parasitoids, suggesting that the patterns of occurrence of facultative bacterial symbionts in Homopterans are not solely the outcome of chance. Chen and colleagues (Chen and Purcell 1997; Chen et al. 2000) demonstrated the possibility of horizontal transmission of endosymbiotic bacteria and that acquisition of facultative endosymbionts could profoundly affect both longevity and fecundity of their hosts. They believe that horizontal transmission has not been observed in the field owing to strong selection against it. Oliver et al. (2003) demonstrated that secondary bacterial endosymbionts were responsible for resistance to a Hymenopteran parasitoid. The facultative symbionts were fatal to the developing larvae of the parasitoid. We do not know if YLS can be horizontally transmitted under normal conditions (see later) or what the effects of transmission are.

Often these questions are studied through analysis of phylogenies, i.e., as history. Since the microbes are endocytobionts and (in the case of YLS) do not grow in pure culture, the switch from bacteria to YLS in the Cerataphidini has been viewed as a

single, historical event amenable to comparative study through phylogenetic analysis. If the microbes can only survive in an aphid and are vertically transmitted, then it is reasonable to assume there are no sources of the endocytobiont outside of its host. No mechanisms for horizontal transmission under such a scenario are known. However, recent data suggest that the assumption of no external sources might be wrong. Pea aphids without symbionts survived on bean plants but did so because their symbionts were replaced by a diverse set of microbes picked up from the environment, including all of the known YLS from planthoppers and aphids (Nakabachi et al. 2003). These remarkable results may mean that the relationship between the aphid and *Buchnera* sp., its primary symbiont, is not uniquely evolved, but that *Buchnera* is the competitive winner for the pea aphid mycetocyte niche. It also means that the presence of *Buchnera* or YLS may not be the result of an historical event but is the result of an ongoing dynamic process amenable to direct experimental study.

14.8 Bees and Wasps

Associations between yeasts and ants have already been discussed in the section on soil-associated arthropods. Here I will examine Hymenoptera other than ants. Bees are, of course, associated with flowers and it is in this context that most of the yeasts associated with bees have been isolated. A study of honeybee-associated yeasts in California isolated close to 400 strains from both pollen- and nectar-collecting bee guts (Phaff and Starmer 1987). Over 20 yeast species were isolated sufficiently often that Phaff and Starmer felt they could not be considered merely incidental. There were strong seasonal differences in the yeasts present in the guts. The number of isolations from pollen-collecting bees was negatively correlated with number in nectar-collecting bees, probably reflecting variation in flower types and abundance. Their interpretation was that the majority of yeasts were associated with the flowers on which the bees had been foraging and had no special relationship with the bees.

Yeasts associated with bees have been reported from numerous sources. *Debaryomyces robertsiae* (originally *Pichia*, van der Walt 1959) was collected from the pollen used to feed larvae of a bumblebee, *Xylocopa caffra,* in South Africa. Zacchi and Vaughn-Martini (2002) reported *C. bombicola*, *S. roseus*, and *P. guilliermondii* were commonly isolated from the body fluid of *Andrena* bees that feed on nectar and pollen. In an extensive survey of yeasts associated with seven *Bombus* species (*B. terrestris*, *B. lucorum*, *B. cryptarum*, *B. lapidarius*, *B. pascuorum*, *B pratorum*, and *B. hortorum*) and the flowers that they visit, Brysch-Hetzberg (2004) found that similar ascomycete species predominated in both flowers and bees. The ascomycetes were able to tolerate high sugar concentrations while the basidiomycetes were not. *M. reukaufii* and *M. gruessii* were the numerical dominants in both bee and flower, although they were not to be found in queens emerging from hibernation, leaving their means of overwintering still unknown. Brysch-Hetzberg suggests that two characteristics are most responsible for this domination: rapid growth on concentrated sugars and a pseudohyphal morphology (“airplane cell configurations”) that favors dispersal on insect setae. *C. kunwiensis* and *C. bombi*, less commonly isolated species, were present in the hibernating queens and may be more dependent on their

association with bees than the numerically dominant species in the system. Other osmotolerant species (*D. hansenii*, *D. maramus*, *M. pulcherrima*, *C. rancensis*, and *Zygosaccharomyces rouxii*), including a new species, *C. bombiphila* (Brysch-Herzberg and Lachance 2004), were also present in small numbers.

Although described by several authors over decades, many yeasts isolated from bees form a distinct clade within the *Ascomycotina* (Lachance et al. 2001d; Stratford et al. 2002; Teixeira et al. 2003). The only teleomorphs so far identified in this clade belong to the genus *Starmerella* (Rosa and Lachance 1998; Teixeira et al. 2003). Several anamorphic species related to *Starmerella* (on the basis of rDNA sequence similarity) have also been described from bees or wasps, including *C. apicola* from bees (Hajsig 1958); *C. bombi* from bumblebees (Montrocher 1967), *C. batistae* from solitary-nesting digger bees (Rosa et al. 1999b), *C. powellii* and *C. tilneyi* from bees (Lachance et al. 2001a), *C. bombicola* from bumblebee honey and alfalfa leaf-cutter bees (Spencer et al. 1970; Inglis et al. 1993; Rosa and Lachance 1998), *C. magnoliae* (Deak and Beuchat 1993; Gilliam 1997), *C. apis* (Barnett et al. 2000), and, most recently, *C. davenportii* from a dead bee found next to a tap on a sugar-syrup tank in a Dutch soft drink bottling plant (Stratford et al. 2002). This list is only suggestive of the diversity of bee-associated yeast within this clade. Lachance et al. (2001d) report *C. etchellsii*, *C. ipomoeae* and three undescribed taxa related to *C. etchellsii* from bees. *Starmerella bombicola* is usually isolated as a haploid and Rosa and Lachance (1998) suggest that many of the anamorphic species might be unmated teleomorphs.

Stratford et al. (2002) and Lachance et al. (2001d) believe that the relatedness of bee-associated yeast species is not a chance occurrence but indicative of a functional relationship between yeasts and bees. Lachance et al. (2001d) note that yeasts isolated from flower beetles and bees rarely overlap. However, the degree to which the yeasts and the bees are associated remains somewhat uncertain at this time. The phylogenetic evidence is somewhat mixed. Lachance et al. (2001d) collected (from bees) *C. glabrata*, *C. azyma*, and four undescribed taxa that do not belong to the "bee" clade. *C. bombicola* was initially described from honey in a bee nest (Spencer et al. 1970), but it mates with the type species of *Starmerella* (Rosa and Lachance 1998), which was collected from flowers (*Calystegia sepium*) and floricolous beetles (*Conotelus* sp.), not bees. Two other members of the clade, *C. powellii* and *C. tilneyi*, were described from both beetles and bees in Costa Rica. Most of the floricolous *Metschnikowia* species are associated with beetles or *Drosophila* (Lachance et al. 2001d) but *M. reukaufii* and *M. gruessii* were commonly collected from bumblebees (Brysch-Herzberg 2004). *C. kunwiensis*, a recently described anamorphic species associated with bumble bees in Germany (Hong et al. 2003), is also a member of the *Metschnikowia* clade. Although the phylogenetic evidence is suggestive of a functional association among bees and yeasts, experimental data on the interaction are needed.

Data on the functional relationships between yeasts and bees are scarce. One of the methods used to collect yeasts from bees, allowing the bees to deposit yeasts as they walk over the surface of agar plates, provides evidence that the bees vector the yeasts to a new habitat. Exclusion experiments from flowers associated with beetles indicate that insects are obligate vectors for flower yeasts (Lachance et al. 2001d).

However, the walking method does not elucidate where the yeasts are found in the bees, which might help clarify the utility of the yeasts to the bees. Some studies report yeasts from the surface of the insect (Hong et al. 2003), while others have found yeasts in the guts of workers but not larvae (Gilliam 1997; Teixeira et al. 2003). Honeybees can digest yeasts, which has long been an ingredient in artificial bee diets (Peng et al. 1984). Gilliam (1997) found a diversity of yeast species associated with pollen in the flower but noted that fewer species were found in the corbicular pollen (pollen carried in the pollen baskets on the bee's legs) of *Apis mellifera* and only a single species, *C. magnoliae,* in bee bread made from corbicular pollen. She concluded that the bees are using yeasts to process pollen before it is suitable as food. Teixeira et al. (2003) also found a single yeast associated with bee bread, although it was *Starmerella meliponinorum,* a relative of *C. magnoliae*. Rosa et al. (1999b) found a new member of the "bee" clade, *C. batistae*, and an undescribed yeast-like *Mucor* species in the pollen provisions and larvae from nests of two species of solitary bee. The suggestion is that the microbes are part of the nutrition of the larvae but more work needs to be done. Pupae had a lower incidence of yeasts than larvae and adults did not deposit any of the nest species on agar plates, although they did deposit other yeasts, which leaves open the question of how the nests were provisioned with microbes.

There has been little direct work on yeast in bee nutrition. Fermentation has been suspected as the means of transforming pollen into bee bread (Haydak 1958; Gilliam 1997) but it is not clear if yeasts or bacteria (or both) are responsible. The honey and bee bread yeast populations studied by Teixeira et al. (2003) were large enough to suggest that yeast growth had taken place in situ. Pain and Maugenet (1966, cited in Gilliam 1997) found that pollen fermented by *Lactobacillus* alone produced an unacceptable bee bread and suggested that yeasts are a necessary part of the process. At the present, yeasts are implicated in bee nutrition but have not been shown to play an important role.

There may be additional roles for yeasts in bee life histories. Some species of bees are aggressive invaders that displace native bees (Goulson 2003). Interactions between microbes, plants, and bees have not been specifically studied, so it is not known if floral microbes play a role in the invasion or if they are affected by the outcome of the invasion. Only scattered studies suggest that yeast might be involved. There are indications that flower yeasts can affect pollination in some plants (Eisikowitch et al. 1990a, b), suggesting that a change in the floral yeast community can have consequences for organisms that do not directly interact with the yeasts. Different species of bees are associated with different species of yeasts (Teixeira et al. 2003) and there may even be partitioning of the flower into distinct microbial communities (pollen versus nectar) and the bee's body as means of transport (body versus head and proboscis, Hong et al. 2003). Thus, a change might occur in the microbial community when one bee species replaces another. The implications for plant and animal ecology are important and deserve attention.

Yeasts may have negative as well as positive effects on bees. Stressed adult honeybees have increased yeast populations, although it is not known if the increase mitigates or worsens the situation (Gilliam 1973; Gilliam et al. 1974, 1977). Caged honeybees had more yeasts than wild adults. Streptomycin administered in their

diet also increased yeast populations in adult bees, suggesting that bacteria are responsible for keeping yeast population sizes low in adult honeybees. Yeasts were rarely isolated from healthy honeybee broods or queens (Gilliam and Prest 1977, 1987). It does not appear that all bees are stressed by the presence of yeasts. Teixeira et al. (2003) found yeasts in healthy adult *Tetragonisca angustula*, a stingless bee, and in their bee bread, bee glue, and garbage pellets.

Siricid wood wasps have symbiotic, species-specific associations with basidiomycetous fungi in the genus *Amylostereum* (Thomsen and Koch 1999; Vasiliauskas and Stenlid 1999; Slippers et al. 2002). The life history of these wasps is not unlike that of ambrosia beetles (including mycangial transport of the fungi) except that only larvae bore into wood. Presumably, their fungal symbionts have a relationship with the wasp that is similar to that of ambrosia fungi and beetles. Even wasps in the family without mycangia will only lay eggs in wood already infected with the fungus (Fukuda and Hijii 1997). Few yeasts have been isolated from wasps. In addition to *C. davenportii,* described earlier, *C. guilliermondii* has been isolated from fig wasps, *Blastophaga psenes* (summarized in Phaff and Starmer 1987). The wasp introduces the yeast and a bacterial species into figs, where their numbers increase but only to moderate levels (and, hence, do not spoil the fruit for human consumption). The presence of the yeasts did seem to increase the fig's attractiveness to various *Drosophila* species, which, in the process of ovipositing on the fig, brought a diverse yeast community. Eventually, beetles (*Carpophilus* sp.) were also attracted and brought another set of yeasts. The yeast community that developed was typical of that found in many fermenting fruits, including apiculate yeasts, *C. stellata*, and *C. krusei*.

14.9 Other Insects and Invertebrates

A new and interesting group of hemiascomycetous species has recently been described from the surface of nematodes in the genus *Panagrellus* living in beetle galleries, acid rots of grapes, and angiosperm slime fluxes. The isolates all belong to the genus *Ascobotryozyma* or its anamorph, *Botryozyma* (Kerrigan et al. 2004). All species described so far are oligotrophic and have determinate thallus growth. They are dimorphic, growing as yeast in pure culture on agar plates and as thalli on nematodes. The thallus begins with a branched basal cell that acts as a holdfast. A single cell buds from the basal cell's apex and multiple cells can bud from this second cell, with the first being another apical bud and others lateral to this. Three-celled vegetative propagules bud from above the first two apically budded cells. Conjugation can occur between any two cells from different thalli excluding the basal cell. Asci form as lateral buds and produce four lunate spores that are released from the ascus, which remains attached to the cell from which it buds. Strains propagated in the laboratory lose sexual reproduction, whether maintained on nematodes or not (Kerrigan et al. 2001).

Four species are known: *A. americana* on *Panagrellus dubius*, a nematode living in the galleries of the poplar borer, *Saperda calcarata*, in *Populus*; *A. cognata* from the same nematode in *Salix* and *Populus* galleries made by the bark beetles *Cryptorhynchus lapathi* and *S. calcarata*; *B. muscatilis* from *Panagrellus dubius* living

in *Populus* slime fluxes visited by beetles (*Carpophilus lugubris* and *Cryptarcha ampla*) and various Diptera; and *B. nematodophila* from *Panagrellus zymosiphilus* living in grapes and vectored by *Drosophila* (Kerrigan et al. 2001, 2004; Kerrigan and Rogers 2003). The first three are all from the northwestern USA and the last is from northern Italy. Superficially, the life history of *Ascobotryozyma* appears similar to that of the *Laboulbeniales* and its allies (Blackwell 1994). However, systematic data based on rDNA sequences places the *Ascobotryozyma* in the *Hemiascomycetes* (Kerrigan et al. 2004) and the *Laboulbeniales* outside (Blackwell and Jones 1997). This is an interesting case of convergent evolution (the *Trichomycetes* should also be mentioned here; Lichtwardt et al. 1999, 2001) to take advantage of animals as both a place to live and a means to reach fresh habitat.

Ascobotryozyma appears to be a commensal in that it attaches to the outside of the nematode and does not penetrate the cuticle. The nematodes can be successfully maintained with yeast as their only food, but they do not seem to eat the commensal. The nematodes can be covered with the fungi. Beyond observing that nematodes are able to feed and reproduce in culture with yeast attached, nothing is known of the impact the yeast has on the fitness of its host. This will remain a question until we know more about the yeasts, their hosts, and the plant system to which both belong. The role of the insect (beetle, *Drosophila*, or other) in this system may still be prominent. These nematodes require a vector, which may be beetles and *Drosophila* that visit slime fluxes and rotting acidic fruit or the beetles that drill galleries.

14.10 Drosophilids

Wherever *Drosophila* oviposit and feed, yeasts have been found if sampling has been done. Fly substrates include flowers, rotting fruits, mushrooms, soft rots, leaf litter, and tree bark, exudates, and fluxes. (See Sect. 2.5 for a list of some recently isolated *Drosophila*-associated yeasts from flowers.) Some unusual larval substrates have not been sampled for yeasts, such as spider egg cases (Heed 1968) or kleptoparasitic fly hosts (Sivinski et al. 1999). Even in some unsampled situations, there is reason to expect that yeasts are present. Three Drosophilidae, representing separate lineages, lay their eggs on land crabs. *Drosophila carcinophila* oviposits and feeds on the land crab, *Gecarcinus ruricola*, found on islands in the Caribbean basin. *Drosophila endobranchia* oviposits and feeds on *G. ruricola* and *G. lateralis* also in the Caribbean but does not co-occur with *D. carcinophila*. On Christmas Island (the one in the Indian Ocean), *Lissocephala powelli* oviposits on at least four of the resident species of land crab but pupates and feeds elsewhere. At first, it was felt that the flies were attracted to the crab's food, not the crabs themselves, but Carson (1967, 1974) observed that the *Drosophila* adults rarely left the crabs, even when he encouraged them to do so. In addition, when active on the crab, they were clustered near not only the mouth or feeding appendages as expected but also near the gill chambers and nephric grooves. These latter structures are filament-lined grooves in the carapace below the excretory pores. The hairs in the groove are "replete with an abundant, whitish, wet, caseous material which usually showed nematodes as well as mites" (Carson 1967). Both larvae and adult *D. carcinophila* feed on this substance, which might be predicted to

contain at least one species of yeast from its description. Carson felt that the material was microbial and that it might be there as a means of removing toxins from the crab's liquid waste, which exits the excretory pore, flows along the groove, and is (partially) reabsorbed at the end of the groove. Land crabs are adapted to dry conditions and wastewater recycling might be beneficial under such conditions. Larvae of the other fly species also fed in the gill chambers where microbial growth was also observed. It would be of interest to know if yeasts are present in all three cases and if they are also derived from separate lineages.

Except for a single exception (*Drosophila* endosymbiotic yeast, discussed later), yeasts and *Drosophila* have important but diffuse or indeterminate (with respect to the species involved) relationships. Indeed, all of the major *Drosophila* breeding habitats are also home to other animals, including beetles, other Diptera, nematodes, and mites. This does not lessen the importance of the relationship, as there is no evidence that animal-associated yeasts arrive at a new substrate without being vectored there (Gilbert 1980; Fogleman and Foster 1989; Lachance et al. 2001d) and most plant resources are poor-quality nutrition and can be improved for the fly by addition of yeasts (Sang 1978; Begon 1982).

The yeasts associated with *Drosophila* have been most recently reviewed by Begon (1982) and by Phaff and Starmer (1987) and I will briefly summarize their conclusions and concentrate on work done since or not covered in these reviews. Begon (1982) reviewed the qualities of yeasts as food and concluded that yeast species differ in composition and quality and that these differences depend on both the yeast and the environment in which the yeast grows. Laboratory experiments demonstrated that different *Drosophila* species and even genotypes within species grew and eclosed at different rates on different yeasts (Brito da Cunha et al. 1951, and works discussed therein). Begon further concluded that these differences might be the basis upon which the niches of regional *Drosophila* faunas were kept separate (Oakeshott et al. 1989; Morais et al. 1995b).

Supportive evidence for yeasts as a partitioned resource is habitat-dependent. The first samples of yeasts associated with *Drosophila* were yeasts associated with "domestic" species (*D. melanogaster* and *D. simulans*) attracted to fermenting vegetation in human dwellings. These carried in their crops a different set of yeasts from those found in the "wild" flies attracted to fermenting banana baits or captured in the wild. In addition, yeasts isolated from crops of adult wild flies often differed from yeasts found in the rotting vegetation where larvae fed and where it was at first presumed that the adults fed; thus, larval and adult yeast diets seemed to differ (Carson et al. 1956; Begon 1982; Phaff and Starmer 1987). Adult crop contents did not support a significant degree of dietary specialization and did not support resource partitioning. However, this conclusion was based on data from temperate woodland flies. As more habitats were sampled, partitioning gained greater support. Flies that feed on mushrooms, figs, or cactus rots had less variation between larval and adult yeast and each habitat was associated with different sets of yeasts (Phaff et al. 1978; Begon 1982; Starmer 1981b). This was also the case for fruit-breeding African *Drosophila* and Brazilian *Drosophila* (Begon 1982; Rosa et al. 1995), where adult diets corresponded to larval substrate yeast communities.

Pattern analysis of more recent species occurrence data has also supported a role for yeasts in the distribution of co-occurring flies. Fruit-breeding flies exploited a patchy yeast environment in different ways during the succession of yeasts and flies on rotting amapa (*Parahancornia amapa*) in a seasonally flooded (igapo) forest in Brazil, a condition which may have promoted coexistence of the *Drosophila* species (Morais et al. 1995a). Lachance et al. (1995) revisited the association between yeasts and *Drosophila* for temperate woodland flies. They included isolates from several *Drosophila* species in both the western and the eastern USA and based their comparisons on both species presence/absence data and on differences in combined yeast physiological profiles. They were able to distinguish among *Drosophila* species on the basis of either the yeast species isolated from them or the yeast's physiological profiles.

The inverse of the proposition in the preceeding paragraph, that yeast species differentiate their niches on the basis of their associations with *Drosophila* species, is harder to support. Cactophilic yeasts, in general, will grow on tissue from any cactus species (author's unpublished data) but analysis of their distribution supports the notion that cactus chemistry is an important distributional determinant (Starmer 1981a) and adult cactophilic *Drosophila* carry yeasts not found in their larval environment (Ganter et al. 1986). However, factors such as yeast–yeast interactions and yeast–insect interactions cannot be ruled out. Yeast species can have both positive and negative effects on the growth rates and maximal population sizes of co-occurring yeasts (Starmer and Fogleman 1986; Ganter and Starmer 1992). Yeast–yeast interactions are affected by the presence of fly larvae (Starmer and Fogleman 1986) and both may be affected by host tissue composition (Starmer and Aberdeen 1990; Lachance and Pang 1997). Separating these three factors is difficult and it may prove that the interactions among them are as important as any direct effect.

14.10.1 Cactophilic *Drosophila* and Yeast

There has been extensive investigation of the association between yeast and both larval and adult *Drosophila* that inhabit cacti. Cactus-breeding *Drosophila* lay eggs and feed in soft rots of cactus stems or closely associated habitats (most notably, *D. mettleri* breeds in soil soaked by outflow from persistent soft rots in giant columnar cacti such as saguaro, *Carnegia gigantea*). Each species oviposits in a small subset of host plants, often just one (Fellows and Heed 1972). The soft-rot pockets are also home to other insects plus a microbiota of bacteria, molds, and yeasts. Bacteria arrive first through aerial transport and are followed by insect-vectored yeasts about 2 days later (Fogleman and Foster 1989). Yeasts associated with this habitat are mostly specialists in this habitat and tend to be oligotrophic (Heed et al. 1976) but do not come from a single or even a small number of clades (Starmer et al. 2003). The cactus stem–*Drosophila*–yeast community is distinct from surrounding yeast communities associated with *Drosophila* (Ganter et al. 1986; Rosa et al. 1995) and there is almost no overlap among the yeasts found in cactus stems and those in fruits or flowers on the same plant, although insects are found in all three.

Since the first descriptions of cactophilic yeast, the number of species restricted to the cactus soft-rot habitat has steadily increased, often through recognition that cactus isolates had been misidentified owing to the small number of positive physiological tests and their resemblance to known species. The trend through time has been to recognize the cactus isolates as separate species. *P. cactophila,* the most commonly isolated species, was originally identified as *P. membranifaciens*. *P. heedii* and *P. deserticola* are specific to cactus stems. *Starmera* and *Phaffomyces*, small genera once classified as related groups of *Pichia* species, are cactus-specific. Some of what were once though to be regional variants of species are now known to be separate species. Originally, *Sporopachydermia cereana* (as its anamorph *Cr. cereanus*) was isolated from several cacti in the Sonoran Desert. As more collecting was done, variation in physiological profile and morphology led to awareness that more than one species was present. Isolates were referred to for a time as "*S. cereana* complex" members. This complex has yielded ten species up to now (Lachance et al. 2001c) and recent collections may increase the total (Lachance, unpublished data).

Some isolates once identified as cactophilic strains of species that also occur in other habitats are now known to be cactophilic specialists related to species in other habitats. *Candida ingens* from cactus is now known to be *Dipodascus starmeri*. *Hansenula polymorpha* from cactus has been recognized as a new species but it is not yet described (Naumov et al. 1997). *P. kluyveri*, which does occur in both fruit and cactus habitats, has varieties that occur only in cacti (Phaff et al. 1988). There are several unresolved situations. *C. sonorensis* shows surprising genetic variation and some lineages within this asexual species may have diverged enough to deserve specific status. *P. pseudocactophila* is a phenocopy of *P. cactophila* except that is restricted to columnar cacti in the Sonoran Desert. *P. norvegensis* is isolated from cacti in the new world with modest regularity but is rarely found anywhere else. The species was originally described as a rare human pathogen (as an anamorph) and the type strain is from a human vagina. It would not be surprising if the cactus strains were a separate species. Some cactophilic species are found only in particular hosts or are restricted geographically or both (Starmer et al. 1990). For species distributed widely on multiple hosts, the abundance may vary among host types. This variation means that the yeast community of each host differs in its proportional species composition (Starmer et al. 1990; Rosa et al. 1994) and these differences are relatively stable over both distance (Barker et al. 1987) and over time (Latham 1998).

14.10.2 Yeast as Food for *Drosophila*

Drosophila development is affected by the species of yeasts available as food for larvae (Starmer and Aberdeen 1990) and bicultures of yeasts are generally better food than monocultures (Starmer and Fogleman 1986; Starmer and Aberdeen 1990), although the biculture effect is not universal. Biculture effects (on development time or survivorship) were observed only when yeasts grew on their usual host tissue. The effect was clearest for host tissue that was poorest in nutrients and could be erased by supplementing the tissue with yeast extract, suggesting that the biculture effect might be due to nutrient complementation.

Both *Drosophila* larvae and adults prefer to feed on particular yeast species when offered a choice between pure cultures of species (Cooper 1960). Cooper worked

with temperate-forest flies and noted that larvae demonstrated stronger preferences than adults and that larval preferences did not always agree with those of adults. These observations are in accord with the lack of correspondence between yeast species isolated from the adults and those found in the larval breeding sites (discussed earlier). Larvae tended to prefer those species that best supported their development. Recent work has confirmed earlier observations that adult and larval cactus-breeding *Drosophila* have preferences for particular yeast species as food (Morais et al. 1994). Larvae of *D. mojavensis* preferred *P. cactophila* in laboratory tests and this yeast was commoner in their guts than in the cactus rots where they fed (Fogleman et al. 1981, 1982). *P. cactophila* is the most commonly isolated yeast from this habitat but preference and abundance do not always correlate. *C. sonorensis*, the second commonest species, was avoided by the larvae and was underrepresented in their guts (Fogleman et al. 1981).

Because the relationship between cactophilic *Drosophila* and yeasts is diffuse, interactions between any two species may change when other factors change. Data on the development of *D. mojavensis* will illustrate this. *Stenocereus* spp. tissue will not support *D. mojavensis* development although this is the fly's preferred larval habitat throughout most of its range (Starmer 1982b). The addition of yeasts will rectify the problem but the details involve the role of yeasts both as food and as an influence on the larval environment. Yeasts are far richer sources of organic nitrogen than cactus tissue (Starmer and Aberdeen 1990) and contain essential vitamins and lipids for the flies (Sang 1978). But other aspects of yeast activity are also important to the development of the fly. Most of the yeasts characteristic of *Stenocereus* rots are able to utilize uric acid or urea as a nitrogen source. Both waste products are toxic to larvae and the yeasts may improve the environment by lowering waste concentrations, although this has not been tested in the cactophilic community. Cacti differ in their stem chemistry and Starmer (1982b) was able to show that particular yeast species may be beneficial to a species of *Drosophila* under the right conditions. *Stenocereus thurberi* tissue (Organpipe, one of the preferred larval substrates of *D. mojavensis*) contains triterpene glycosides and unusual lipids that can slow both yeast and fly growth. The yeast community characteristic of this host includes yeasts that secrete extracellular lipases (*Dipodascus starmeri* and *P. mexicana*). *D. starmeri* is able to both resist the harmful effect of the lipids and utilize them as a sole carbon source. The lipolytic yeasts support *Drosophila* development both by detoxifying the tissue and by serving as food. Separating the two effects is difficult but it is clear that both effects are present and that rots with lipolytic yeast improve the quality of the habitat for *D. mojavensis* feeding on *S. thurberi*. In other parts of its range, *D. mojavensis* larvae feed on cacti with tissue that has more simple sugars and lacks most of the secondary chemicals present in *Stenocereus* spp. In these hosts, the relationship between lipolytic yeast and the fly is not as important.

Another of the problems in the study of yeasts as food in natural situations is the determination of the relative contribution of different yeast species to fly nutrition when multiple yeast species are present. Markow et al. (2000) have shown that stable isotope ratios ($\delta^{13}C$ and $\delta^{15}N$) can be used in combination to identify cactus species and that a change of diet (in this case, yeast substrate and not yeast species) is detectable in the fly over a period of 24 h. If yeast species are distinguishable with

this technique, it might be adapted to allow direct comparison of the contribution of different yeast species to both larval and adult nutrition.

Yeasts may play a significant indirect role in *Drosophila* nutrition through their production and assimilation of low molecular weight volatiles. Some *Drosophila* adults are able to assimilate small volatile molecules directly from the atmosphere (Starmer et al. 1977; van Herrewege and David 1978). A variety of volatiles, including methanol, acetic acid, and acetaldehyde, can produce the effect (Jefferson and Aguirre 1980; Parsons 1980, 1989). This may be especially important for species that have little access to a sugar-rich substrate, such as cactophilic flies (Starmer et al. 1977). Early studies concentrated on longevity experiments in which the adults were exposed to water vapor alone or water supplemented with low concentrations of volatiles (higher concentrations are toxic). These results raised questions about the mechanism by which longevity was increased and the relevance of the effect to the fitness of the flies, although Starmer et al. (1977) observed that the ovarioles were retained when *D. mojavensis* fed on atmospheric ethanol. Etges and Klassen (1989) confirmed that ethanol was a food resource for *D. mojavensis* and that the increase in life span is not due to physiological changes brought on by starvation. Ethanol increased longevity but also increased the metabolic rate and fecundity, effects inconsistent with starvation. Radiolabeling demonstrated that adults incorporate ethanol vapors into their tissues (Etges and Klassen 1989). Volatiles are also implicated in resource location and partitioning (Fogleman 1982; Oakeshott et al. 1982).

Volatile production varies with substrate and microflora, but cactophilic yeasts can produce acetaldehyde, acetic acid, propionic acid, methanol, ethanol, ethyl acetate, isobutyl alcohol, isobutyric acid, *n*-butyric acid, and isoamyl alcohol (Fogleman 1982; Barker et al. 1994). Other volatiles found in cactus rots include acetone and 1-propanol and 2-propanol, possibly owing to untested yeast's abilities or bacterial fermentation (Fogleman 1982; Starmer et al. 1986). Most of these compounds can also be metabolized by one or more cactophilic species. For this reason, interactions among volatiles, yeasts, and *Drosophila* are expected to be complex and have not, at this time, been adequately studied. Starmer et al. found that 2-propanol addition led to larger flies when the yeast present in the system could assimilate the volatile (in this case, *C. sonorensis* and *S. cereana*) but not when the yeast was unable to assimilate it. Interestingly, only *D. mojavensis* feeding on its preferred host, *Stenocereus gummosus*, benefited from 2-propanol. *D. arizonae* and *D. buzzatii* did not benefit but they both fed on *Opuntia* tissue in the experiment, which contains more simple sugar than does *S. gummosus* and this difference may have mitigated potential benefits from the presence of the volatile. Ganter et al. (1989) demonstrated that the longevity of *D. mojavensis* was increased by the presence of methanol and a yeast that could assimilate it, *C. sonorensis*, but there was no effect if other species of yeasts that could not assimilate the volatile were substituted. Yeast was inoculated onto agar supplemented with *S. gummosus* powder (no other nutrients were included) and the flies had access to the yeast during the experiment. Greater longevity was not observed for either *D. melanogaster* or *D. buzzatii*. The former is not a cactus breeder, while *D. buzzatii* is but it does not utilize *S. gummosus*.

Although the relationship between *Drosophila* and yeasts is diffuse, it is similar in some ways to fungal–insect interactions that are more specific. Although some vari-

ation existed, slime-flux breeders in a desert climate (where availability of alternative feeding sited may be limited, Begon 1982) carried yeasts similar to those found in their larval breeding sites (Ganter et al. 1986). For cactus breeding flies, the correlation was even closer (Ganter et al. 1986; Ganter 1988). In both cases, the host plant was of greater importance to the yeast found in the larval substrate than was locality (also see Barker et al. 1983, 1984, 1987). Cactophilic *Drosophila* vector a subset of yeasts present in the breeding site: those that are strong producers of volatiles, principally ethyl acetate (Ganter 1988). These are also the same species that cactus-breeding fly larvae preferred to eat (Fogleman 1982; Fogleman et al. 1982; Vacek et al. 1985). The larval preference may also be linked to production of ethyl acetate or other small volatile molecules by the yeasts (Fogleman 1982). *Drosophila* adults and larvae are found in younger cactus rots, where a yeast flora from other sources is less likely to be present (Starmer 1982a; Starmer et al. 1987; Ganter 1988). The result is that, when cactus-breeding adults arrive at a suitable rot, they deposit both eggs and the yeasts that will provision the rot for their offspring. Although not as species-specific as other instances, this too should be considered a case of insect agriculture (Mueller et al. 1998; Farrell et al. 2001; Mueller and Gerardo 2002).

There is yet another possible role of yeasts in the diet of *Drosophila* that goes beyond simply providing nutrition. Some *Drosophila* larvae and pupae develop more slowly and with greater mortality in the presence of sunlight, a situation which can be improved with the addition of yeasts to their diet (Bruins et al. 1991). The reason for the harmful effect of light is unknown, but may be linked to the production of oxygen radicals when light interacts with porphyrins. The yeasts may supply antioxidants that palliate the effect of the light.

14.10.3 Yeast and *Drosophila* Oviposition

Drosophila oviposition behavior depends on the microbiota found on their larval substrates but the exact form of the preference is linked to habitat and may vary with the successional stage of the rot. Most fruit-breeding *Drosophila* prefer to oviposit on yeast-colonized substrates over those where bacteria or molds predominate (Oakeshott et al. 1989). This is not true of *D. mojavensis,* which prefers to lay eggs on early-stage cactus rots. *D. mojavensis* was attracted to its favorite host tissue only after the addition of bacteria associated with the formation of the rot but was not attracted to mixtures of volatiles characteristic of later rot stages (Fogleman 1982). Not all cactophilic species show an oviposition preference for substrates without yeasts. Vacek et al. (1985) found that adult *D. buzzatii*, a cactus-breeder, preferred to feed and oviposit on the same species of yeast (*C. sonorensis*, *P. cactophila*, *P. barkeri*, *Phaffomyces opuntiae*, and *Clavispora opuntiae*) and, except for *Ph. opuntiae,* these were the most commonly isolated yeasts from their oviposition substrate (*O. stricta*). Bacteria from cactus soft rots were present and were one of the possible choices but were rejected. Oviposition preferences are variable among isolines of *D. buzzatii* and there is heritable genetic variation for the character (*P. cactophila* was once again the most preferred species) (Barker and Starmer 1999). Establishing the cause for such preferences is difficult as yeast–yeast interactions and preferences for yeast change with different combinations of yeast species (Barker 1992; Barker et al. 1994).

14.10.4 Yeast and *Drosophila* reproduction

Many studies of *Drosophila* life history have sought to understand the importance of trade-offs in the evolution of traits such as time to maturity, adult size, lifespan, fecundity, and egg size. An example of this is the potential trade-off in energy and resource allocation between metabolic (growth, maintenance, and storage) and reproductive functions. Yeasts in the diet of flies have been shown to directly affect egg production (by at least an order of magnitude) (Chippindale et al. 1993, 1997; Simmons and Bradley 1997) and to have a direct impact on the importance of such trade-offs for the fly (Leroi et al. 1994).

Male size is often an indicator of mating success for *Drosophil*a (Partridge et al. 1987), including *D. buzzatii* (Santos et al. 1988, 1992). However, the size of axenic *D. buzzatii* males had no effect on their reproductive success (Norry and Vilardi 1996). When the same strains of flies were allowed to feed on live yeast from their larval substrate (*O. vulgaris* soft rots), size-related sexual selection was observed. Males with wider faces were preferred if the face was contaminated with live yeast. Yeasts are transmitted between sexes during courtship (Starmer et al. 1988). The reason for the preference was not obvious so this effect cannot be labeled as a nuptial gift and there was no indication that the effect varied with yeast species. Some *Drosophila* males present their mates with a nuptial gift of a drop of fluid, rich in yeasts, regurgitated from the crop. Nuptial gifts are varied and their function may also be varied (Vahed 1998). Some gifts have potential interactions with associated microbes, such as the gift of stored nitrogen waste products by male Blattid cockroaches. Steele (1986b) found that *Drosophila* in the *obscura* species group produced a drop and, if the drop were of sufficient quality, the female would remain still while feeding long enough for the male to successfully mount her. Females kept on a low-quality diet that were fed a nuptial meal were more fecund than those that had no nuptial meal, so the meal may benefit both parents (Steele 1986a).

Drosophila mate choices can also be affected by the composition of cuticular hydrocarbons, which may function both in selection of a conspecific mate and in species recognition. In a study of both intraspecific and interspecific variation in hydrocarbon makeup, Stennett and Etges (1997) found that food type could significantly alter the composition of the cactus-breeding flies, *D. mojavensis* and *D. arizonae*. The relevance of this finding to this review is that they varied both the substrate and microbial components of the larval food (laboratory food with an uncontrolled microbial flora versus cactus tissue seeded with a subset of yeast species commonly isolated from it). As yeasts can supply insects with a significant portion of their lipid intake, it is not clear which component of the diets was responsible for their results. Yeasts may play an indirect role in mate choice and speciation by supplying the flies with a varied assortment of lipids in their diets.

14.10.5 Yeast Endosymbionts in *Drosophila*

One of the oldest known instances of endosymbiotic yeast involves *Drosophila* and *Coccidiascus legeri* (Chatton 1913; Lushbaugh et al. 1976). This is the only known *Drosophila* endosymbiont, which has been observed to produce ascospores but has

never been cultured. The means of transmission is not known, although, as it resides within gut epithelial cells and *Drosophila* larvae have been observed to feed on the carcasses of adults (Gregg et al. 1990), feeding is thought to play a role. The most basic question is about the nature of the relationship between yeast and fly. It has been found in temperate-woodland *Drosophila* (Ebbert et al. 2003) but it occurs in less than 4% of adults. This is consistent with a parasitic relationship. However, Ebbert et al. (2003) found that, in contrast to another member of the *Drosophila* gut flora (a Trypanosomatid flagellate), the yeast affects its host's life history in potentially beneficial ways. In laboratory experiments, infected fly larvae were more likely to eclose and did so an entire day earlier than uninfected individuals. The mechanism for this is not known but this does suggest a positive effect of the yeast on the fly. Most *Drosophila* biologists assume that flies that linger in the larval stage and expose themselves to predation and mischance are lowering their fitness and so, since no other habitat other than the gut epithelium is known for the yeast, the relationship appears mutualistic. At this time, it is not possible to reconcile the prevalence data with the experimental data.

14.11 Other Diptera

There are scattered reports of yeasts associated with Dipterans other than Drosophilids. Zacchi and Vaughn-Martini (2002) isolated *P. guilliermondii* from the guts of Heteromyzidae larvae that feed on truffles. *C. tropicalis* has been implicated as the causative agent in an outbreak of fermentation spoilage of ripening figs in Brazil. Gomes et al. (2003) believe that the fruit fly *Zaprionus indianus*, a member of the Drosophilidae and recently arrived in Brazil, is the sole vector of this yeast in this system. *Phorbia phrenione* is the principal pollinator of *Epichloë typhina*, a Clavicept endophytic parasite. The fly transfers conidia to a stroma of the opposite mating type before formation of perithecia (Bultman and White 1988). Perhaps haploid sexual yeasts rely on animal dispersal to bring mating types together as a part of their reproductive biology. Cranefly larvae (*Tipula abdominalis*) feed on leaf litter. Their hindgut is enlarged and home to microbes that may be important to the fly's nutrition (Breznak 1982).

14.12 Conclusions

The diversity of yeasts associated with animals may be severely underreported. It is hard to cite evidence that something has not happened, but there are hints that the previous is true from patterns existing in the current literature. Some habitats are understudied in general and when yeasts are reported from them, it is hard to tell how important they might be in that habitat. Yeasts from deep-sea tube worms and clams are an example of this (Nagahama et al. 2003). Collecting effort has not been uniform. The percentage of ambrosia and bark beetle yeasts known from South Africa is unexpectedly high (Table 14.1), perhaps because bark beetle yeast diversity is unusual in South Africa or the systematic search for these yeasts contributed by J.P. van der Walt and associates has not been duplicated in other regions. A similar observation may be made for the yeasts from decaying wood, which seem to reach a

diversity peak in Chile (Table 14.1, most described by C. Ramirez and A. Gonzalez), but this may also be a sampling artifact. Many of the yeasts identified from these two regions have not been collected elsewhere and one can only speculate that other species may fill these niches in other places. There are also more positive indications where habitats have only recently come under systematic scrutiny (the flower- and mushroom-beetle yeast communities) yet have proven to be very speciose and again the species have strong geographic associations. Although small, the nematode-associated *Ascobotryozyma* seems to be a potentially speciose clade, given that all recognized species have been sampled from a small geographic area and from only two species of Nematoda – itself one of the most speciose animal lineages known. One can argue (successfully) that part of the recent increase in yeast diversity is due to the application of molecular systematic techniques as predicted by Kurtzman (in Kurtzman 1994, Kurtzman and Robnett 1998a; and other sources). However, the techniques have thrown light on the diversity, not created it. What should interest us are the ecological and evolutionary processes that have created it. For the yeasts covered in this review, both insect associations and limited distributions seem to encourage diversification. These causative factors can be seen as interdependent because relying on animals as vectors may limit yeast dispersal range even as it increases dispersal success. That geography is important to the description of a yeast species has not always been accepted, but the previously given evidence is convincing that, for at least some habitats, it is essential.

Historically, much interest has been focused on substrate–yeast interactions rather than vector–yeast interactions. It was felt that different substrates would have the major effect on yeast evolution and that the vector's effect would not have much explanatory power. This is obviously not the case for mutualistic, endosymbiotic interactions, but these were seen as exceptional cases and not as central to a general understanding of yeast evolution or ecology. However, evidence is slowly building for the importance of animal–yeast interactions in yeast evolution. There are two important factors so far identified. First is the fundamental importance of dispersal. Animal-vectored yeasts seem to have populated the biosphere as many localized species, closely tied to geography and local conditions. This population structure is much closer to the population structure of the animals that vector them than to microbes with spores or dispersal morphs light enough and resistant enough to depend on air and dust to vector them. The model of wind-dispersed bacteria (Howard et al. 1985), where the genetic variation across a continent can be mirrored in the variation among strains from a single apple, or fungi (Gosselin et al. 1996), simply does not fit many yeasts.

The second factor is the importance of mutualisms that have diffuse, variable memberships rather than mutualisms between specific pairs of species. These mutualisms often involve communities of yeasts and animals. This makes them harder to detect and study but not less important in yeast evolution. An excellent example of the impact of diffuse mutualisms is the cactus–*Drosophila*–yeast system. While cacti are important in yeast evolution, they chiefly supply necrotic tissue to the system. Cactus rots are not initiated by the organisms that live in them (Fogleman and Foster 1989) and there is no evidence at this time that the cacti respond beyond limiting the amount of tissue available to the rot community. The opportunity for

coevolution seems limited. The *Drosophila*, while not the only vector in the system, are probably the most important vectors and are dependent on yeast for their nutrition. Thus, the probability of coevolution between fly and yeast seems more likely than cactus–yeast coevolution. However, at this time, we are much more likely to ask how variation in yeast genotype or phenotype relates to plant–yeast interactions than to animal–yeast interactions. Upon finding that there is variation among regional variants of a yeast species, are the differences more likely to be tied to adaptation to different hosts or to different vectors? Of course, the only answer is that both are vital to the yeast's success and both deserve study.

When is it reasonable to define an interaction as mutualistic? Yeasts can be animal parasites, commensals, or mutualists. The clearest cases for mutualism come from endocytobionts. That there is a cost to the host in terms of resource inherent in the presence of endocytobionts seems incontestable. Thus, the nature of the interaction depends on the benefit-to-cost ratio. Evolutionary costs and benefits must be measured in terms of fitness. What the drain of resources by the microbe costs the host in terms of fitness may be returned to the host in some benefit (production of essential molecules, detoxification, resistance to parasites) that equals or exceeds the cost. Unfortunately, costs are almost always measured in units (calories, millimoles of of nutrient, etc.) different from those used for measuring benefits so that they are not directly comparable. Fitness (or a component if fitness if both cost and benefit affect it) allows direct comparison but it is much more difficult to measure.

The advantage of considering animal–yeast interactions from an evolutionary perspective is that it opens up interesting lines of inquiry. When acquisition of an endosymbiont allows the host to exploit a new niche, there is a strong prima facie case that the relation is mutualistic. Phloem-feeding Homoptera cannot sustain themselves on plant sap without the presence of their endocytobionts. From an evolutionary perspective, questions arise about this relationship based on conflicts between hosts, between endosymbionts, and between host and symbiont. These conflicts complicate assessment of fitness for species involved in an endocytobiosis even when the likelihood of mutualism is high. There are asymmetries that need clarification if the system is to be understood beyond establishing that the presence of the microbe is necessary for exploitation of the niche. Does the host seek to limit the cost of and maximize the benefit from the symbiont cells, as though the host were the owner of the means of production and the endocytobiont only a worker in the factory? Do the symbiont cells cooperate with the host's limitations so that the host's, and therefore the microbe's, fitness is maximized or does conflict between symbiont varieties eliminate cooperation with the host beyond the minimum needed for host success? Once an endocytobiotic relationship is established, is the system open to invasion by other microbes and under what conditions does this lead to coexistence in the host versus replacement of one endocytobiont with another? (Jones et al. 1999; Suh et al. 2001) How are resources allocated in hosts with multiple endocytobionts, such as many Homoptera and Coleoptera? Is the host in charge or do the various endosymbionts compete? Is only one endocytobiont a mutualist while the rest exist as free-loading commensals, supplying only enough benefit to the host to cancel the cost of their presence? Horizontal transfer seems integral to the origins of the relationship between host and microbe and unculturable endocytobionts can still be present in an organism's environment

(Nakabachi et al. 2003) but there are few models for how and under what conditions horizontal transmission is replaced by vertical transmission. For established symbioses, it is necessary to know if horizontal transfer is possible and, if so, how likely must it be before it affects both host and endocytobiont? Considerations such as these make it difficult to assume that symbioses are mutualistic without experimental confirmation but the answers to the questions they pose are necessary if we are to fully understand animal–yeast interactions.

Note Added in Proof

Since completing the submitted draft of this chapter, several new insect-associated yeasts have been described. *Candida leandrae* was collected from a Drosophilid captured on the Hawaiian Islands (the LSU sequence places it in the *Kodamaea* clade near *C. restingae*) (Ruivo et al. 2004). *Metschnikowia hamakuensis, M. kamakouana,* and *M. mauinuiana* are all associated with nitidulid beetles on the same group of islands (Lachance et al. 2005). *Saturnispora hagleri* and *Geotrichum silvicola* were isolated from *Drosophila* captured in Brazil's Atlantic rainforest, although the latter was also isolated from silkworm caterpillars in India (Morais et al. 2005, Pimenta et al. 2005). The same forests also yielded *Candida riodocensis* and *Candida cellae,* two species isolated from bees and belonging to the Starmerella clade (Pimentel et al. 2005). *Komagataella phaffii* has been described from trees and sap fluxes (Kurtzman 2005) but its LSU sequence is identical to the *Pichia pastoris* strains collected from *Drosophila brooksae* by Ganter et al. (1986). Association with sap fluxes and the earlier misidentification argue for this being an insect-associated yeast. The variety of *Candida guilliermondii* isolated from fig wasps as been elevated to specific status as *C. carpophila* (Vaughan-Martini et al. 2005). Based on LSU sequence identity, this species also includes strains previously identified as *C. xestobii*.

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Chapter 15

Yeasts in Extreme Environments

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15.1 Introduction: a Definition of Niches Where Yeasts Are Able to Tolerate Harsh Surroundings

As stated by Rothschild and Mancinelli (2001) it is still fact that where there is liquid water on Earth, virtually no matter what the physical conditions, there is life. What we previously thought of as insurmountable physical and chemical barriers to life, we now see as yet another niche harbouring "extremophiles". An organism that thrives in an extreme environment is an extremophile; in more than one extreme it is a polyextremophile.

Extreme environments include different parameters (Rothschild and Mancinelli 2001):

-Physical extremes: temperature, radiation, pressure, gravity or vacuum

-Geochemical extremes: desiccation, salinity, pH, oxygen species or redox potential

It could be argued that extremophiles should include organisms thriving in biological extremes (for example, nutritional extremes, and extremes of population density, parasites and prey). Although all hyperthermophiles are members of the Archaea and Bacteria, eukaryotes are common among the psychrophiles, acidophiles, alkaliphiles, piezophiles, xerophiles and halophiles (which respectively thrive at low temperatures, low pH, high pH, and under extremes of pressure, desiccation and salinity) (Rothschild and Mancinelli 2001).

Although these characterizations seem straightforward, three philosophical issues need further exploration. First, what is "extreme"? Perhaps extreme is in the eyes of the beholder. It is clear that to a thermophile that dies at 21°C and a piezophile that finds atmospheric pressure extreme what determines an extremophily is based on definitions that are perhaps anthropocentric. Second, there are ecological aspects to determine a definition of extremo*tolerant* and extremo*phile* organisms. Besides, there is a very thin line between these two terms. Third, it has to be defined if an extremophile has to show its extreme physiology during all life stages, and under all conditions (Rothschild and Mancinelli 2001).

Life requires an input of energy, but must also be able to control energy flow. Redox chemistry is universal. As life is based on organic chemistry, such chemistry

must be allowed to operate. An extremophile must either live within these parameters, or guard against the outside world in order to maintain these conditions intracellularly (Rothschild and Mancinelli 2001).

Extremophilic microorganisms capable of developing in extreme environments have recently attracted considerable attention because of the challenge that their discovery has posed to our current notions of the limits of life and its possible origin, but, most importantly, because of their biotechnological potential (Boekhout and Phaff 2003; Gónzalez-Toril et al. 2003). In this paper we focus on yeasts that are able to survive in harsh surroundings and are therefore potentially useful in biotechnological applications.

15.1.1 Dry Environments

Water possesses many properties that seem to make it the essential solvent for life. It has high melting and boiling points with a wide temperature range over which it remains liquid, and a high dielectric constant important for its solvent action. Water expands near its freezing point, and it forms hydrogen bonds. No other compound possesses all of these traits. Thus, water limitation is an extreme environment (Rothschild and Mancinelli 2001). Water availability not only depends on the water content of an environment, that is, how moist or dry a solid microbial habitat may be, but is also a function of the concentration of solutes such as salts, sugars, or other substances that are dissolved in water. This is because dissolved substances have an affinity for water, which makes the water associated with solutes unavailable to organisms, so the salt or sugar solution can, in effect, be considered analogous to a dry environment (Madigan et al. 1997).

15.1.1.1 Low Water Activity

Water availability is generally expressed in physical terms such as water activity (a_w). a_w is defined as the ratio of the vapour pressure of the air in equilibrium with a substance or solution to the vapour pressure at the same temperature of pure water (Madigan et al. 1997). So, low a_w value defines an environment where water is in unavailable state for unadapted organisms. Such conditions can be found in freezing areas where water molecules form ice. Low a_w is also a characteristic feature for environments of high salinity [salt lake (a_w=0.75), salt fish, seawater, salami] and for environments of high concentrations of sugars [cereals, candy, dried fruit (a_w=0.7)] (Madigan et al. 1997). Limitation of water in the latter case is not meant in a way of a different state of water, as it is for ice, but the law of osmosis controls its availability.

15.1.1.1.1 Cold Environments

Much of the Earth's surface (over 80% of the total biosphere) experiences fairly low temperatures. The oceans, which make up over half of the Earth's surface, have an average temperature of 5°C, and the depths of the open oceans have constant temperatures of about 1–3°C. Vast land areas of the Arctic and Antarctic are permanently frozen or are unfrozen for only a few weeks in summer (Madigan et al. 1997;

Cavicchioli and Torsten 2000). These natural environments also include cold soils, in and on ice in polar or alpine regions, polar and alpine lakes, and sediments, caves, plants and cold-blooded animals (e.g., Antarctic fish). Artificial sources include many refrigeration appliances and much refrigeration equipment (Cavicchioli and Torsten 2000).

Cold environments have been successfully colonized by numerous organisms, in particular bacteria, yeasts, unicellular algae and fungi (Gerday et al. 2000). Temperature creates a series of challenges, from the structural devastation wrought by ice crystals at one extreme, to the denaturation of biomolecules at the other. At low temperatures with nucleation, water freezes. The resulting ice crystals can rip cell membranes, and solution chemistry stops in the absence of liquid water. Freezing of intracellular water is almost invariably lethal (Rothschild and Mancinelli 2001).

Active microbial growth in extremely cold environments is under the influence of ice formation and consequently of little biologically available liquid water. Thus, water activity (rather than extremely low temperatures) in habitats such as snow, sea ice and glacier ice is the dominant factor in the external chemistry that influences microbial activity. During freezing and binding of water in ice crystals, ions are expelled and the ion concentration in the remaining liquid water increases (Gunde-Cimerman et al. 2003).

The coldest, driest places on Earth are the dry valleys of Antarctica. The primary inhabitants for both hot and cold deserts are cyanobacteria, algae and fungi that live a few millimetres beneath the sandstone rock surface (Rothschild and Mancinelli 2001). Antarctica is a remote continent whose ice-free desert regions are subject to a unique combination of extreme environmental stresses (see Chap. 16). Paramount amongst these is the scarcity of water for the integrity of biomolecules and metabolic activity (biochemical processes) of living organisms. Precipitation is minimal, so transient summer melt water is frequently the sole source of moisture. Precipitation itself is in the form of snow whose ice crystals mainly sublime before they can melt under the influence of solar heating of the substratum. Little moisture enters the microhabitats of these desert ecosystems. There are frequently high concentrations of salts in the scarce melt water, resulting in surface evaporite habitats. These, by their osmotic effects in the microbial microhabitat, make the accessibility of water for metabolism energetically demanding. When desiccation becomes extreme, water replacement molecules, such as the sugar trehalose, are also essential to conserve the ultrastructure of fundamental biomolecules. These include membrane proteins associated with energetic sequences and photosynthetic systems, all of which require spatial organization. They also protect the tertiary structure of enzymes and the nucleic acids, DNA and RNA (Wynn-Williams and Edwards 2000).

In addition to desiccation stress and low-temperature constraints, the Antarctic also has the most extreme UVB flux on the Earth because of the ozone hole. UVB damages a variety of vital biomolecules. Not only does it cause mutation in DNA but it also disrupts proteins, including the lipoproteins of cell membranes and organelles. Experiments on the extraterrestrial environment of low Earth orbit are also valuable for augmenting environmental research in Antarctica, especially the effects of maximal ozone depletion with the biological consequences of its concomitant maximal UVB transmission (Wynn-Williams and Edwards 2000).

15.1.1.1.2 Hypersaline Environments

Organisms live within a range of salinities, from essentially distilled water to saturated salt solutions. Osmophily refers to the osmotic aspects of life at high salt concentrations, especially turgor pressure, cellular dehydration and desiccation. Halophily refers to the ionic requirements for life at high salt concentrations. Although these phenomena are physiologically distinct, they are environmentally linked. Thus, a halophile must cope with osmotic stress (Rothschild and Mancinelli 2001).

The definition of a hypersaline environment is one that possesses a salt concentration greater than that of seawater (3.5% w/v). For water-containing environments, the salt composition depends greatly on the historical development of the habitat, and the environments are normally described as thalassohaline (marine derived) or athalassohaline (the chemical composition is mainly determined by geological, geographical, and topographical parameters, for example, Great Salt Lake in Utah and the Dead Sea). In addition to lakes formed by evaporation in moderate climate conditions, hypersaline Antarctic lakes have been formed from the effects of frost and dryness in this environment. Antarctic and moderate-temperature soils may also contain salinities between 10 and 20% (w/v) (Cavicchioli and Torsten 2000).

15.1.1.1.3 Environments with High Concentrations of Sugars

A typical environment which has extremely high concentrations of sugars is honey. Microorganisms in honey may influence quality or safety. Owing to the natural properties of honey and control measures in the honey industry, honey is a product with minimal types and levels of microbes. Microbes of concern in post-harvest handling are those that are commonly found in honey (i.e., yeasts and spore-forming bacteria), those that indicate the sanitary or commercial quality of honey (i.e., coliforms and yeasts), and those that under certain conditions could cause human illness (Snowdon and Cliverb 1996).

15.1.1.2 Low Humidity

The relative humidity (RH) limit for functioning of the most resistant forms of life is near 70%. Data of the last few years show that the specificity of the role of water in living systems consists not only in its participation in forming the structures of biopolymers and membranes, but also in forming the environment for biochemical reactions. Water because of the complex character of its influence as stabilizing and as loosening factors determines the equilibrium of forces within such structures and its sensitivity to the regulation. The capability of functioning and reproducing as the necessary conditions for the existence of life is determined by requirements of regulation but not of durability or chemical resistance of structures, which have limited character. At low RH the interaction of water with the polar groups plays the basic role, but increase of the RH to 30% and more leads to a new effect – the competition of water for hydrogen bonds within the macromolecules of biopolymers and between the lipid molecules that form such bonds. This is the necessary condition for forming the native biological structures. This also determines the possibility of the enzyme reactions in the solid phase, the efficiency of photooxidation of cytochrome

in the photosynthesis chain and the lability of structures of the proteins and DNA. The next effect is determined by the appearance of the free water with high dielectric constant at a RH more than 60%, which makes weaker charge interactions and leads to lateral mobility within membranes. This water also causes the hydrophobic interaction, which stabilizes the structure of biopolymers and a set of other important processes for life. Any of these factors are necessary for functioning of the living systems that determine the previously mentioned limit of the RH for active life (Aksyonov 2002).

15.1.2 Acidic Environments

pH is defined as $-\log_{10}[H^+]$. Biological processes tend to occur towards the middle range of the pH spectrum, and intracellular and environmental pH often fall in this range (for example, the pH of sea water is approximately 8.2). However, in principle, pH can be high, such as in soda lakes or drying ponds, or as low as 0 ($[H^+]=1$ M) and below. Proteins denature at exceptionally low pH (Rothschild and Mancinelli 2001).

Environments with low pH includes volcanic soils and waters, acid mine drainages, acid soils, vinegar, gastric fluids and fruit juices. (Madigan et al. 1997). The majority of extremely acidic environments are associated with the mining of metals and coal (Cavicchioli and Torsten 2000). Acid-mine drainage water (e.g., effluent of a uranium ore mine) is an example of such an acidic environment and is an interesting model to obtain a better understanding of life in extreme acidic conditions and to elucidate the basis of the survival and role, if any, of acidotolerant yeasts. In these two cases tolerance to metals as well as several physiological parameters at acidic pH are important (de Silóniz et al. 2002b).

The microbial processes that produce the acidic environment are a result of dissimilatory oxidation of sulfide minerals, including iron, copper, lead and zinc sulfides. This process can be written as $Me^{2+}S^{2-}$ (insoluble metal complex)$\rightarrow Me^{2}+SO_4^{2-}$, where Me represents a cation metal. As a result of the extremely low pH in these environments, and due to the geochemistry of the mining sites, cationic metals (e.g., Fe^{2+}, Zn^{2+}, Cu^{2+} and Al^{2+}) and metalloid elements (e.g., arsenic) are solubilized; this process is referred to as microbial ore leaching (Cavicchioli and Torsten 2000).

The Tinto river (Huelva, southwestern Spain) is an environment where the pH remains low and rather constant year-round (mean 2.3), regardless of the temperature or the river flow. This is the consequence of the buffer effect produced by the presence of high concentrations of ferric iron along the river:

$$Fe^{3+}+H_2O \leftrightarrow Fe(OH)^{2+}+H^+$$
$$Fe(OH)^{2+}+H_2O \leftrightarrow Fe(OH)_2^{+}+H^+$$
$$Fe(OH)_2^{+}+H_2O \leftrightarrow Fe(OH)_3+H^+$$

When the river is diluted by tributaries or rain, ferric hydroxide is formed and protons are released, thus maintaining the acidic pH of the system. During the summer, when extremely high temperatures are reached, evaporation is increased, but the pH is maintained constant because the reverse reaction operates: ferric hydroxide precipitates dissolve, consuming protons (Gónzalez-Toril et al. 2003).

15.1.3 Environments with High Concentrations of Metals

Industrialization has long been accepted as a hallmark of civilization. However, the fact remains that industrial emanations have been adversely affecting the environment. Municipal water-treatment facilities in most developing countries, at present, are not equipped to remove traces of heavy metals; consequently every consumer is exposed to unknown quantities of pollutants in the water they consume. The main sources of heavy-metal pollution are mining, milling and surface finishing industries, which discharge a variety of toxic metals such as Cd, Cu, Ni, Co, Zn and Pb. They can be found regularly in municipal wastewaters and, in consequence, in sewage sludge and compost (de Silóniz et al. 2002a; Malik 2004).

15.1.4 Xenobiotic Environments

Xenobiotics are chemically synthesized compounds that have never existed naturally. Some of the most widely distributed xenobiotcs are the pesticides, which are common components of toxic wastes (Madigan et al. 1997). The most important and common pollutants among organochlorine pesticides are dichlorodiphenyltrichloroethane and its metabolites (DDTs), polychlorinated biphenyls (PCBs), hexachlorocyclohexane isomers (HCHs), chlordane-related compounds (CHLs), hexachlorobenzene (HCB), cyclodienes, dieldrin, etc. A great number of industries, such as textile, paper and pulp, printing, iron-steel, coke, petroleum, pesticide, paint, solvent, pharmaceutical and wood-preserving chemical, consume large volumes of water, and organic-based chemicals. These chemicals show a great difference in chemical composition, molecular weight, toxicity, etc. Effluents of these industries may also contain undesired quantities of these pollutants and need to be treated (Aksu 2004).

Another source of environmental pollution by xenobiotics is trinitrotoluene or TNT. Since the environmental conditions in explosive-polluted soils and waters are typically aerobic or microaerobic, the models of TNT transformation can reflect many real situations in such anthropogenic ecological niches where the microbial cells, growing on suitable accompanying substrates, can convert TNT into the early metabolites. The most important aspect of the environmental pollution with TNT and its metabolites is obviously the exposure of wildlife, food animals and humans to the toxic effects of these compounds. Presumably, the hydroxylamino derivatives of TNT, as the most toxic metabolites, may be responsible for the sharp reduction in both microbial numbers and population diversity in the petrochemical waste sludges exposed to TNT (Zaripov et al. 2002).

15.2 Yeasts in Extreme Habitats

It is difficult to determine a good definition of extremophilic yeast, so we shall limit our discussion of extremophiles to those yeasts that require or tolerate extreme environments for growth. Cavicchioli and Torsten (2000) also define an extremophile as an organism that is isolated from an extreme environment and often requires the extreme condition for growth. At the end of this definition they emphasize that,

again, "extreme" is anthropocentrically derived. While many organisms are able to tolerate extreme environments, true extremophiles exhibit optimal growth and function under extreme conditions.

15.2.1 Yeasts in Environments of Low Water Availability

15.2.1.1 Xerophilic Yeasts

Organisms that are able to grow in very dry environments (made dry by lack of water) are called xerophiles. This term is often mixed-up with osmophiles and halophiles, but the osmotic mechanism does not create the possibility to survive in very dry environments, because already at a RH of 50% the retention of water requires a pressure of about 1,000 atm. Investigations are trying to reveal the earlier unknown mechanism for retaining the free water. A study of more than 100 species and strains of yeasts, mosses and lichens by the NMR method has shown that the air-dry cells of species tolerant to water loss retain some of the mobile free water. In most cases the cells contain 1% or few percent of such water but some species of yeasts have been shown to contain about 30% of mobile water of the weight of air-dry biomasses. Such extreme ability is possessed by species of the genus *Cryptococcus* inhabiting high mountain deserts of the East Pamir, where sharp oscillations of temperature and humidity during a day are observed. The retention of water is evidently related to the ability of forming structures that are impermeable for rest water. The cells of *Cryptococcus* are known to have such a mechanism and therefore are able to survive subzero temperatures at night, keep the water collected through the night when the RH rises, and function during the day when temperatures become more suitable (but still extreme) for life. The polysaccharide capsule surrounding cells of *Cryptococcus* inhabiting high mountain deserts helps to keep the water intracellularly, while such a capsule is almost absent in similar species collected from Antarctica, where the diurnal oscillations of temperature do not have such a sharply marked character (Aksyonov 2002).

15.2.1.2 Psychrophilic Yeasts

Little is known about fungal diversity in extremely cold regions. In addition to low nutrient levels, low temperatures induce the formation of ice crystals and therefore also the creation of low water activity (a_w). These are the dominant factors in external chemistry that influence microbial biota in cold regions (Hirsch 1986; Gunde-Cimerman et al. 2003).

Organisms with low-temperature optima are called psychrophiles. A psychrophile can be defined as an organism with an optimal temperature for growth of 15°C, a maximum growth temperature below 20°C, and a minimal temperature for growth at 0°C or lower. Organisms that grow at 0°C but have optima of 20–40°C are called psychrotolerant (Madigan et al. 1997; Cavicchioli and Torsten 2000).

The lower temperature limit for growth of microorganisms is difficult to determine accurately. The problem is primarily a technical one since the amount of antifreeze substance required to be added to the media to prevent freezing is often

inhibitory to growth. Within this limitation, a low-temperature limit of between –5 and –7°C for growth of bacteria and yeasts has been reported (Larkin and Stokes 1968; Arthur and Watson 1976). Psychrophilic yeasts are of the genus *Candida*, and psychrotolerant members are mostly of the genera *Candida*, *Cryptococcus*, *Rhodotorula*, *Hanseniaspora* and *Saccharomyces* (Cavicchioli and Torsten 2000).

It has been reported that fungi can be isolated in concentrations from 6,000 to 7,000 CFU l^{-1} (where CFU is colony forming units) from melted sea ice and up to 13,000 CFU l^{-1} in melted glacier ice, while the majority of species represent yeasts. The dominant taxa are ascomycetous and basidiomycetous yeasts, melanized fungi, mainly represented by the genera *Cladosporium* and *Aureobasidium* plus different species of the genus *Penicillium*. The highest value of colony forming units per litre from melted ice was reported on media for moderate osmophiles – media with 20% glucose (a_w=0.941) and media with 5% NaCl (a_w=0.946). It shows an important connection between the environmental temperature and the a_w value (Gunde-Cimerman et al. 2003).

The most frequently isolated yeast species from Antarctic mosses were of the genus *Cryptococcus – Cr. albidus* and *Cr. laurentii* (Tosi et al. 2002). Several other species of the genus *Cryptococcus* were isolated from the Antarctic: *Cr. vishniacii*, *Cr. albidosimilis*, *Cr. antarcticus*, *Cr. friedmannii* and three new representatives of this genus, *Cr. adeliae* and *Cr. adeliensis*, xylanase producing basidiomycetous yeasts (Gomes et al. 2000; Petrescu et al. 2000; Scorzetti et al. 2000), and recently a novel psychrotolerant member of the hymenomycetous yeasts from Antarctica – *Cr. watticus* (Guffogg et al. 2004).

Mrakia frigida (formerly *Leucosporidium frigidum* and *Leucosporidium nivalis*) is classified as an obligate psychrophile since it is unable to grow at temperatures above 20°C (Arthur and Watson 1976; Barnett et al. 2000). *M. frigida*, which does not exist north of 62° S, has been subjected to much longer and more strenuous selective pressure than have other, more widely disseminated psychrophilic microorganisms, leading to adaptation of its metabolism to incessantly low temperatures (the temperature of Antarctic waters ranges from –2.2°C in shelf waters to 4°C in open waters, the average temperature being –1°C), and it can be considered as a representative source of cold enzymes (Turkiewicz et al. 2003). The growth of *M. frigida* at –0.5 and 17°C was essentially the same when either ethanol or glucose was used as the carbon source, except for a shorter lag phase with glucose as the substrate. The maximum cell yield was obtained at subzero temperatures, although the lag phase was considerably greater than at higher temperatures. Rapid growth occurred at 17°C; however, a slight increase in temperature to 18.5°C resulted in decreased growth. Strains of these yeasts have been reported to grow at –7°C (Larkin and Stokes 1968; Arthur and Watson 1976).

Barnett et al. (2000) reported 16 yeast species that are not able to grow at temperatures between 25 and 45°C: *Bullera huiaensis*, *B. hannae*, *Candida psychrophila*, *Cryptococcus aquaticus*, *Cr. consortionis*, *Cr. friedmanii*, *Cystofilobasidium capitatum*, *C. lari-marini*, *Fellomyces horovitziae*, *Leucosporidium antarcticum*, *M. frigida*, *Sporobolomyces coprosmicola*, *S. dracophylli*, *S. ruber*, *Tausonia pamirica* and *Udeniomyces puniceus*.

15.2.1.3 Halophilic Yeasts

Microbial life can be found over the whole range of salt concentrations from freshwater and marine biotopes to hypersaline environments with NaCl concentrations up to saturation. Organisms that require salt from 1–15% NaCl (Madigan et al. 1997) or above 0.2 M salt (Cavicchioli and Torsten 2000) for growth are called halophiles; those capable of growth in very salty environments [15–30% NaCl (Madigan et al. 1997) or 2.0–5.2 M (12–30%) NaCl with the optimum above 3.0 M (18%) NaCl (Cavicchioli and Torsten 2000)] are called extreme halophiles. Halophilic and halotolerant microorganisms are found in all three domains of life: Archaea, Bacteria, and Eucarya. Colonization of hypersaline environments such as salt lakes and salted food products by these microorganisms is often highly successful, and salt-loving and/or salt-tolerant microorganisms may reach high population densities in such ecosystems (Oren 1999).

A characteristic feature of the physiology of *Debaryomyces hansenii* is its resistance to NaCl. *D. hansenii*, a halophile yeast found in shallow sea waters and salty food products, grows optimally in 0.6 M of either NaCl or KCl, accumulating high concentrations of Na^+ or K^+ (Gonzalez-Hernandez et al. 2004). Since this yeast has been described as involved in spoilage, it is particularly important to understand the behaviour of the yeast when a high salt concentration is present together with other stress agents in the food environment. In general Na^+ is toxic to *Saccharomyces cerevisiae* but it improves the growth of *D. hansenii*. This is evident for growth at supraoptimal temperature. In contrast, K^+, a non-toxic cation, does not affect *S. cerevisiae*, but still improves growth of *D. hansenii*. The same pattern was found at high and low pH (Almagro et al. 2000). It has been reported that the presence of salt improves the performance of *D. hansenii* under normal conditions. From the results of work, Almagro et al. (2000) concluded that this improvement is more significant under stress conditions, contributing to protect the cells against those factors.

Gunde-Cimerman et al. (2000) presented data on fungal populations in a crystallization pond during the months of salt production, with salinity in the range 3–35% NaCl. The salterns as the natural ecological habitat of these yeasts represent an extreme and highly changeable environment (Kogej et al. 2004b). Among the black yeasts *Hortaea werneckii*, *Phaeotheca triangularis*, *Trimmatostroma salinum* and dimorphic fungus species *Aureobasidium pullulans* were detected with the highest frequency just before the peak of halite (NaCl) concentration. Since *H. werneckii*, *P triangularis* and *T. salinum* are not known outside saline environments, these results suggest that hypersaline water is their natural ecological niche (Gunde-Cimerman et al. 2000). *H. werneckii* is one of the most salt-tolerant species among all microorganisms and as such a highly appropriate model organism in which to study salt tolerance in eukaryotes (Petrovič et al. 2002).

Hypersaline water not only contains generally osmotolerant fungi, but also truly halophilic fungi. These taxa are all melanized and belong to a single order of the *Ascomycetes*, the *Dothideales*. They all have thick, melanized cell walls, slow, often meristematic growth and proliferation with endoconidiation. A similar morphology is observed with stone-inhabiting fungi, and this can thus be regarded as an

extremophilic ecotype. None of the known species of marine fungi were encountered; these fungi may therefore not be regarded as extremophilic, and belong to quite different orders of *Ascomycota*. Consequently the inhabitants of hypersaline waters are unlikely to have evolved from fungi living in seawater (Gunde-Cimerman et al. 2000).

The phylogenetic and physiological diversity among the halophiles suggest that halophily may have arisen more then once during evolution and is not a rarity. Because data from Mars missions suggest that Mars almost certainly had abundant liquid water on its surface at some time in the past it could have harboured some form of life. As Mars has lost its atmosphere it has not only become cold but also dry owing to water evaporation. As the water evaporated, the dissolved minerals became more concentrated and formed salty brine pockets. Therefore, if there was any life on Mars in water, there should have been some type of osmophile or halophile. Data regarding the survival of halophiles in permafrost, in evaporates and in freeze-thaw cycles suggest that these types of organisms may serve as a terrestrial analogue to the last vestiges of life on Mars (Mancinelli 2004).

15.2.1.4 Osmophilic Yeasts

Organisms able to live in environments high in sugar are called osmophiles (Madigan et al. 1997). Honey is a good example of such an environment; it is essentially water (average 17.2%) suspended in fructose (average 38.4%) and glucose (average 30.3%) (Snowdon and Cliverb 1996). These conditions make honey an appropriate environment for yeasts with osmophilic characteristic. Osmophilic yeasts use the pentosephosphate pathway. The regulation of the pentosephosphate pathway for osmophilic yeasts is not yet known (Burschäpers et al. 2002). They can grow under acidic conditions and are not inhibited by sucrose. Most samples of honey contain detectable levels of yeasts. Moreover, moulds and yeasts are the only microbes that have been reported to grow in honey. Certain bacteria will survive in honey but growth is unlikely. Although yeast counts in many honey samples are below 100 CFU g^{-1} (because of industry control efforts), yeasts can grow in honey to very high numbers (Snowdon and Cliverb 1996). An obligate osmophilic yeast that requires high sugar concentrations (10–20% glucose) for growth was identified in honey as *Saccharomyces bisporus* var. *mellis* (= *Zygosaccharomyces mellis*). Optimum growth for this strain was at 60% glucose (Munitis et al. 1976). There are many other osmophilic yeast genera that can be found in honey: *Debaryomyces*, *Hansenula*, *Lipomyces*, *Nematospora*, *Oosporidium*, *Pichia*, *Rhodotorula*, *Schizosaccharomyces*, *Schwanniomyces*, *Trichosporan*, *Candida*, and *Zygosaccharomyces* (Snowdon and Cliverb 1996).

Osmophilic or sugar-tolerant yeasts are a problem in the honey industry, because they can grow even at the limited level of water available in ripe honey. As a result, osmophilic yeasts readily ferment honey. Standard industry practices control yeast growth. However, honey has antimicrobial properties that discourage the growth or persistence of many microorganisms. Typically, honey can be expected to contain low numbers and a limited variety of microbes (Snowdon and Cliverb 1996).

The osmotolerant yeast *Pichia sorbitophila* was found to differ from other yeast species, not only from the conventional ones (*S. cerevisiae*, *S. pombe*), but also from

those widely known as osmotolerant (*D. hansenii*, *Z. rouxii*). *P. sorbitophila* was able to survive extremely high extracellular concentrations of salts (e.g., saturated solution of KCl) and other osmolytes (70% glucitol), although it is not classified as halophilic (or osmophilic). *P. sorbitophila* assimilated a broad range of carbon and nitrogen sources with extreme effectiveness. On solid media, *P. sorbitophila* created colonies of variable shapes and sizes in relation to media composition, number of colonies on the plate and cultivation conditions. The colonies were able to produce long-distance signals between each other that resulted in growth inhibition of the facing parts of both colonies, but were not inhibited by colonies of other yeast species growing on the same plate. Though sometimes *P. sorbitophila* has been indicated as a synonym of *P. farinosa*, comparative physiological studies together with PCR amplification of *P. farinosa* DNA fragments homologous to known *P. sorbitophila* genes provided a strong indication that this strain should be classified as a separate species (Maresova and Sychrova 2003).

James and Stratford (2003) have studied the osmophilic characteristics and the resistance to preservatives of *Zygosaccharomyces lentus*. Strains of *Z. lentus* grew over a wide range of temperature (4–25°C) and pH 2.2–7.0. Growth at 4°C was significant. All *Z. lentus* strains grew in 60% w/v sugar and, consequently, are osmotolerant. *Z. lentus* strains are resistant to food preservatives. It was confirmed that *Z. lentus* is an important food spoilage organism potentially capable of growth in a wide range of food products, particularly low-pH, high-sugar foods and drinks. It is likely to be more significant than *Z. bailii* in the spoilage of chilled products (Steels et al. 1999). The *Zygosaccharomyces* genus contains some of the most osmotolerant organisms known, and most are capable of growth at very low pH or in environments high in acids; *Z. bailii* is significantly resistant to weak acid preservatives and *Z. rouxii* and *Z. mellis* are known to be extremely osmotolerant. Foods particularly at risk are therefore acidic and contain relatively high levels of sugar. These include fruit juices, soft drinks, juice concentrates and sugar syrups, candied fruit, jams and preserves, honey, tomato sauce, mayonnaise and wines (James and Stratford 2003).

15.2.2 Thermotolerant/Thermophilic Yeasts

This category is not so extreme as are the others, because there are no yeasts adapted to such high, extreme temperatures like species of bacteria and archaea are. Moreover, there are no eukaryotes in environments with temperatures above about 60°C. This most likely involves the stability of organellar membranes, which must remain fairly porous to permit passage of large molecules like ATP and RNA. It is likely that porous membranes such as these would be more temperature-labile than the typical lipid bilayers of prokaryotes (or lipid monolayers of some hyperthermophiles). Thus, above 60°C, the organelles of eukaryotes cannot survive and the only life forms observed are prokaryotes (Madigan et al. 1997). Here again it must be said that this classification of extreme conditions is artificial and was constructed with the eye of the beholder, while temperatures above 40°C (Arthur and Watson 1976) represent a niche for thermotolerant/thermophilic yeasts.

It is clear that the definition of the terms psychrophilic and thermophilic as applied to microorganisms is dependent not only on whether the organism is a

prokaryote or a eukaryote but also on the species under consideration. With the yeasts, the upper temperature limit is close to 46°C, which is, by definition, the lower limit for thermophilic organisms (Arthur and Watson 1976; Madigan et al. 1997).

Yeast *Arxiozyma telluris* (= *Kazachstania telluris*) is classified as an obligate thermophile since it exhibits a narrow growth temperature range of between 28 and 45°C. *A. telluris* showed a maximum cell yield at 37°C when grown on ethanol. At 25°C growth was extremely slow, and at 20°C no growth was observed, thus indicating that this species possesses a more thermophilic character than thermotolerant yeast *Candida parapsilosis*. The upper temperature limit for growth of *A. telluris* was close to 45°C (Arthur and Watson 1976).

Yeast strain Y94T, which is capable of growth at high temperature, was isolated from soil in Korea. Characteristics of the strain include asexual reproduction by multilateral budding, the absence of extracellular starch-like compounds, a negative diazonium blue B colour reaction, and the absence of arthrospores, ballistoconidia and ascospores; the strain can therefore be placed in the genus *Candida*. A maximum growth temperature of 50–51°C, along with certain other physiological characteristics, and a unique 26S ribosomal DNA partial sequence separate this strain from other ascomycetous yeasts. This new species was described as *Candida thermophila* (Shin et al. 2001).

Barnett et al. (2000) reported five yeast species that are clearly (all the tests are marked with +) able to grow to grow at 25, 30, 35, 37, 40, 42 and 45°C: *Arxula adeninivorans*, *C. blankii*, *C. freyschussii*, *Pichia mississippiensis* and *Sporopachydermia lactativora*.

15.2.3 Acidotolerant/Acidophilic Yeasts

Each organism has a pH range within which growth is possible and usually has a well-defined pH optimum. Organisms that live at low pH are called acidophiles. Cavicchioli and Torsten (2000) define acidophiles as organisms that have a pH optimum for growth at or below pH 3. This definition excludes microorganisms that are tolerant to pH below 3, but that have pH optima closer to neutrality. It should be emphasized that despite the requirements of a particular organism for a specific pH for growth, the optimal growth pH represents the pH of the extracellular environment only; the intracellular pH must remain near neutrality in order to prevent destruction of acid-labile macromolecules in the cell. In extreme acidophiles the intracellular pH may vary by 1–1.5 pH units from neutrality (Madigan et al. 1997). Members of *Candida*, *Cryptococcus*, *Hanseniaspora*, *Metschnikowia*, *Rhodotorula, Sporobolomyces* and some others have acidophilic behaviour (BioloMIC 2005).

In general, growth rates decrease with pH. However, remarkable differences were obtained between species. Whereas *S. cerevisiae* was unable to grow at pH lower than 2.5, the isolates (*C. sorbophila* and *Rh. mucilaginosa*) from the effluent grew at pH 2. In addition to pH tolerance, a slight acidophilic behaviour was also observed, especially in *C. sorbophila*. This strain showed an optimum pH value of 2.5–3.0, and was able to grow at pH 2 and pH 4.5 at very similar rates (de Silóniz et al. 2002b). There was a report of another acidophilic yeast species of the genus *Candida*. The natural

habitat of *C. slooffiae* (= *Kazachstania slooffiae*) was reported to be the extremely acid environment of the stomach of domestic animals, in particular the pig and the horse, and it is not difficult to conceive that it had adapted to this extreme environment in such a way as to preclude the necessity for conventional mitochondrial metabolism (Arthur and Watson 1976). *Rh. mucilaginosa* showed the highest rate of growth at pH 3. Apparently, respiration was less affected than growth by acidic pH: even the control strain of *S. cerevisiae* was able to respire at pH 1, a pH value at which it was not able to grow (de Silóniz et al. 2002b).

It is very difficult to define the precise role and importance of each organism as part of a dynamic population in this habitat, especially with respect to non-acidophilic microorganisms. As an approach to this point, mixed cultures of yeast and chemolithotrophic bacteria were utilized, and the effect on ferrous oxidation carried out by the bacteria was studied. Finally, an evaluation was carried out to determine if the excreted metabolites could support the growth of yeasts *Rh. mucilaginosa* and *C. sorbophila*. When the yeasts were inoculated together with the bacteria in the same medium, an unexpected inhibitory and even lethal effect on yeast population was detected. Most probably, the inhibitory agent could be some metabolite excreted to the medium by bacteria, while nutritional competition, oxygen limitation and ferric ion were excluded. This antagonism would explain why yeasts have not usually been isolated from active leaching heaps, but only from the effluents, where the number of such bacteria is usually inferior (de Silóniz et al. 2002b).

The Tinto river is an extreme environment with a constant acidic pH (mean 2.3), a high concentration of heavy metals and a remarkable level of microbial diversity (bacteria, archaea, photosynthetic and heterotrophic protists, yeast and filamentous fungi). The extreme conditions found in the river are the direct consequence of the active metabolism of chemolithotrophic microorganisms thriving in the rich polymetallic sulfides present in high concentrations in the Iberian Pyritic Belt. Some of these microorganisms are important in several processes of biohydrometallurgical interest: acid mine drainage, biomining and bioremediation (Gónzalez-Toril et al. 2003).

15.2.4 Yeasts Resistant to High Concentrations of Metals

Chromium plays an important role in yeast metabolism, where its form and concentration in the microenvironment and the macroenvironment of the yeasts are the crucial factors (Batič and Raspor 1998; Paš et al. 1999, 2004). Batič et al. (1996) tested 35 yeast species for their tolerance to zinc(II) and chromium(III). Both *Yarrowia lipolytica* and *Arthroascus javanensis* were placed in the groups of the highest measured tolerance to both Zn(II) (above 16 mM) and Cr(III) (above 6 mM). Moreover, *Y. lipolytica* showed the highest tolerance to Cr(III) among 35 yeast species (49 yeast strains) tested, but with very high absolute deviation, while *A. javanensis* was the third in the list but had a more reliable result. Most of the strains of the species *S. cerevisiae* were placed in the group of the highest tolerance to Zn(II). Moreover, a *S. cerevisiae* strain was reported to be the most tolerant to Zn(II) among the all yeast species. However, *S. cerevisiae* is often used for metal bioremediation (Malik 2004) and a mathematical model was developed which shows the

behaviour (viability/mortality) of Cr(VI)-tolerant organisms, where *S. cerevisiae* represented this group (Raspor et al. 1999) and *Schizosaccharomyces pombe* (Belagyi et al. 1999; Czakó-Vér et al. 1999). High tolerance to Zn and Cr was also observed in the strains of *S. roseus*, *K. starkey*, *K. thermotolerans*, *Schizoblastosporion starkeyi-henricii* and some others.

Manganese toxicity is the second most important growth-limiting factor (after Al toxicity) in acid soil. The resistance of microorganisms to the toxic effects of manganese ions is an important factor for survival in acid soil. It was confirmed that the acid-tolerant yeast *Rh. glutinis* is also resistant to very high concentrations of aluminium and manganese ions (Nguyen et al. 2001). Another species of this genus, *Rh. mucilaginosa*, isolated from the effluent of the mineral heap, exhibited a higher tolerance than *S. cerevisiae*, except for zinc, for which this *S. cerevisiae* and *Rh. mucilaginosa* presented the same tolerance (de Silóniz et al. 2002b). On the other hand, a process and a system for removal of metals from groundwater or from soil by bioreducing or bioaccumulating the metals (Cr, Mo, Co, Zn, Ni, Ca, Sr, Hg and Cu) using metal-tolerant *S. cerevisiae* have been reported (Krauter and Krauter 2002). Another Cu-resistant strain (*S. cerevisiae* SN41) was found to successfully remove Cu from wine during fermentation (Brandolini et al. 2002).

The bioaccumulation of heavy metals (cadmium, nickel, cobalt and zinc) and the effects of these metals on the production of metallothionein and metallothionein-like proteins (MT) in *Y. lipolytica* were studied by electrochemical methods. The highest concentrations of heavy metals were found in the cell wall and membrane debris, while the lowest concentrations were detected in the cytoplasm. Cadmium and nickel showed the most significant effect on the production of MT. Sensitivity of the yeast to heavy metals can be classified as follows: the first class – zinc – exhibited a weak effect on living cells; the second class – nickel and cobalt – showed a medium effect on yeast cells; the third class – cadmium – is very toxic for cells. Therefore, the tolerance of the yeast to Zn, Co, Ni and Cd decreases as follows: Zn>Co~Ni>> Cd (Strouhal et al. 2003).

15.2.5 Xenobiotic Environments (Man-Made Xenoenvironments)

The organisms that are able to metabolize pesticides and herbicides are fairly diverse, including genera of both bacteria and fungi (Madigan et al. 1997). Yeasts such as *Saccharomyces* sp., *Candida* sp., and *Hansenula* sp. have been reported as capable of transforming a nitroaromatic hydrocarbon, TNT. Comparative toxicological assessment of TNT, isomeric monohydroxylaminodinitrotoluenes (HADNT), and hydride-Meisenheimer complex of TNT (H-TNT) chemical standards revealed that HADNTs are the most toxic compounds. The lowest mortality rates were observed with the supernatants of *Candida* sp. AN-L13, which converts TNT to H-TNT (Zaripov et al. 2002).

Unlike most of these microbes that are of terrestrial origin, the *Y. lipolytica* strain that Jain et al. (2004) have reported is a marine yeast transforming TNT. In addition to the transformation of TNT into products that can be utilized by other microbes, *Y. lipolytica* has the genetic make-up to degrade other environmental pollutants, such as triglycerides and aliphatic hydrocarbons.

Recek and Raspor (1999) reported that *Pichia ciferrii* could be used for reducing the chemical oxygen demand (COD) load of wastewater from ergot alkaloid production. This study is described more in detail in the "Bioremediation" section.

Although yeasts *K. marxianus* and *C. zeylanoides* have been explored in the context of aerobic biological processes for the removal of dyes from textile effluents (Aksu 2004), biochemical oxidation suffers from significant limitations since more dyestuffs found in the commercial market have been intentionally designed to be resistant to aerobic microbial degradation. Therefore, a wide variety of microorganisms including bacteria, fungi and yeasts are used for the biosorption of a broad range of dyes. Textile dyes vary greatly in their chemistries, and therefore their interactions with microorganisms depend on the chemical structure of a particular dye, the specific chemistry of the microbial biomass and characteristics of the dye solution or wastewater. Depending on the dye and the species of yeast used different binding capacities have been observed (Table 15.1).

15.2.6 Combined Extreme Environments

15.2.6.1 Low a_w – a Connection Between Cold and Hypersaline Environments

Halophilic and xerophilic fungi have been isolated from rocks and natural hypersaline environments. It can be speculated whether these species share some features with those isolated from ice. Whereas salinity creates both ionic and osmotic stress drought, low temperature per se cause osmotic stress. Freezing leads to cellular dehydration owing to reduced water absorption and conduction, whereas high salinity causes the same effects owing to osmotic imbalances. Compatible solutes are known to accumulate in response to certain physical stresses, such as desiccation and high salinity, but have so far been ignored with regard to protection against freezing temperatures. Since protection against dehydration damage is correlated with intracellular accumulation of compatible solutes, an increase in the amount of

Table 15.1 Data on the biosorption of dyes by various yeasts (Aksu 2004)

Yeast	Biosorption of dye
Candida spp.: *C. lipolytica*, *C. membranifaciens*, *C. guilliermondii*, *C. tropicalis*, *C. utilis*	Remazol Blue
Candida rugosa	Reactive Blue 19, Reactive Black 5, Sulfur Black 1
Cryptococcuss heveanensis	Reactive Blue 19, Reactive Black 5, Sulfur Black 1
Dekkera bruxellensis	Reactive Blue 19, Reactive Black 5, Sulfur Black 1
Kluyveromyces marxianus	Remazol Black B, Remazol Turquoise Blue, Remazol Red, Remazol Golden Yellow, Cibacron Orange, Remazol Blue
Kluyveromyces waltii	Reactive Blue 19, Reactive Black 5, Sulfur Black 1
Pichia carsonii	Reactive Blue 19, Reactive Black 5, Sulfur Black 1
Saccharomyces cerevisiae	Remazol Blue, Reactive Blue 19
Schizosaccharomyces pombe	Remazol Blue

non-freezable water may arise through the production of such solutes as low molecular weight sugar alcohols (polyols) or sugars (trehalose) (Gunde-Cimerman et al. 2003). Presumably low temperature and the presence of the salts are antagonistic in their effects on the synthesis of appropriate lipids for adaptation to each of these situations and this was clearly shown in the study of the effects of salts on *D. hansenii* and *S. cerevisiae* under stress conditions (Almagro et al. 2000).

Gunde-Cimerman et al. (2003) confirmed in their study that by using low-water-activity media for the isolations of halophilic/xerophilic fungi from hypersaline waters, higher CFU numbers than previously reported from ice samples can be expected to be obtained. This is also supported by the fact that the main groups of isolated fungi that were isolated in Kongsfjorden appear as well in solar salterns of the Mediterranean coast (Gunde-Cimerman et al. 2000).

A high proportion of melanin-producing microorganisms are known to be associated with environmentally stressed areas such as hot and cold deserts, alpine regions and the upper biosphere. In addition, melanins are known to confer protection to UV radiation, temperature extremes and desiccation, and they also provide an osmotic role (Kogej et al. 2004b). Some yeast species associated with extreme environments also produce pigments other than melanin. *Rhodotorula*, *Rhodosporidium*, *Cryptococcus*, *Cystofilobasidium*, *Sporidiobolus* and *Sporobolomyces* form red, orange and pink carotenoid pigments that do not diffuse into agar, while *Metschnikowia pulcherrima* forms diffusible pigment pullcherim (Bab'eva and Reshetova 1997; Raspor et al. 2000b).

Considering a low *a*w, made by a high concentration of salts or sugars, Barnett et al. (2000) reported 25 yeast species that are able to grow at 10 and 16% NaCl and 50 and 60% glucose (with fully positive results for these tests). The majority of the species belong to the genus *Candida* while the others are *A. adeninivorans*, *A. terrestris*, *D. melissophilus*, *D. nepalensis*, *D. robertsiae*, *D. udenii*, *P. acaciae*, *P. triangularis*, *Rh. acuta*, *Sterigmatomyces elviae* and *Sympodiomycopsis paphiopedili*.

15.2.6.2 Deep Igneous Rock Aquifers – High Salts, High Pressure, Anaerobic Environment

The diversity of prokaryotes in the groundwater deep below the surface of the Baltic Sea at the Äspö Hard Rock Laboratory (HRL) in southeast Sweden is well documented. In addition, there is some evidence that eukaryotes, too, are present in the deep groundwater at this site, although their origins are uncertain. It is interesting that all the eukaryotic strains isolated from Äspö groundwater between 201 and 444 m below sea level were fungi. Yeast strains isolated were most likely strains of the identified species *Rh. minuta* and of a new species of *Cryptococcus*. In addition, cultures related to *Rhodotorula* and *Cryptococcus*, which were also isolated in this study, were found in the deep-sea environment and even in sea-floor sediments. Scanning and transmission electron microscopy demonstrated that the strains possessed morphological characteristics typical for yeast, although they were relatively small, with an average length of 3 μm. Enumeration through direct counting and most probable number methods showed low numbers of fungi, between 0.01 and 1 cells ml^{-1}, at some sites. Studies revealed that the strains grew within a pH range of 4–10, between temperatures of 4 and 25–30°C, and

in NaCl concentrations from 0 to 70 g l^{-1}. These growth parameters suggest a degree of adaptation to the groundwater at Äspö HRL. Despite the fact that these eukaryotic microorganisms may be transient members of the deep biosphere microbial community, many of the observations of this study suggest that they are capable of growing in this extreme environment (Ekendahl et. al. 2003).

Red yeasts are commonest among yeasts isolated from the deep sea. Nagahama et al. (2001) isolated 99 yeast strains, including 40 red yeasts, from benthic animals and sediments collected from the deep-sea floor in various areas in the northwest Pacific Ocean. Comparing the yeast isolates from animals and sediments collected from shallow locations, they found the proportion of red yeasts differed considerably, comprising 81.5 and 10.6% of the isolates from animals and sediments, respectively. All of the red yeast isolates belonged to the genera *Rhodotorula* and *Sporobolomyces*. On the basis of morphological and physiological characteristics, the isolates were identified as *Rh. aurantiaca*, *Rh. glutinis*, *Rh. minuta* and *Rh. mucilaginosa* of the genus *Rhodotorula*, and *S. salmonicolor* and *S. shibatanus* of the genus *Sporobolomyces*. Only *Rh. glutinis* and *Rh. mucilaginosa* were isolated from sediments. All of the others were isolated from animal sources. Some strains assigned to known species on the basis of phenotypic features should be regarded as new species as suggested by the results of molecular analysis. Yeast isolates having ascomycetous affinities included members of the genera *Candida*, *Debaryomyces*, *Kluyveromyces*, *Saccharomyces* and *Williopsis*. Basidiomycetous yeasts were more frequently isolated from sediments from deeper regions (64.0%) than from sediments collected at depths of less than 2,000 m (there were no basidiomycetous yeasts other than *Rhodotorula*). This may be due to a difference in the amount of organic debris in the sediments or the difference in hydrostatic pressure at the sampling points. Red yeasts comprise a higher proportion of total yeasts in clean water than in polluted water. The other basidiomycetous yeasts isolated from sediments of deeper regions were members of the genus *Cryptococcus* (Nagahama et al. 2001).

The proportions of basidiomycetous yeasts and red yeasts among the total yeast isolates from benthic organisms were 88.5 and 80.8%, much larger than in the case of yeasts from sediments. The differences in the proportion of red yeasts recovered from the animals and the sediments collected from almost the same locations and depths suggest that the occurrence of red yeasts in the deep sea is strongly limited by the nutrient conditions of their habitats. Microbes associated with macroorganisms in deep-sea environments presumably are exposed to favourable conditions with a stable source of nutrients, whereas in deep-sea sediments there is less organic debris available to be utilized by yeasts than in sediments in shallow regions. The other basidiomycetous yeasts isolated from the clams were members of the genera *Cryptococcus* and *Pseudozyma* (Nagahama et al. 2001) (see also Chap. 12).

A large number of yeasts can utilize hydrocarbon compounds as sole sources of carbon and energy, for example, species of *Rhodotorula* and *Candida*. However, fungi must outcompete bacteria, a large obstacle, because prokaryotes are extremely successful in this environment. One may suspect that even though carbon sources should not be limiting, selective advantages such as secretion of digestive enzymes may prove essential for yeasts to survive in the deep biosphere. Perhaps the largest problem for subsurface yeasts is the absence of oxygen in the groundwater. If the

Äspö strains are aerobic, it may be that they produce spores under oxygen-limiting conditions, an occurrence which should be favoured under stressful environmental conditions. Therefore, oxygen limitation does not exclude the existence of yeasts in the deep subsurface but may curb their ability to grow (Ekendahl et al. 2003).

15.2.6.3 Association of Bacteria and Yeasts in Extreme Environments

Rikhvanov et al. (1999) studied the association of bacteria and yeasts in hot springs and they indicated that yeasts may exist in hot springs in association with bacteria and that this may influence the resistance of the yeasts to extreme temperatures. The thermophilic bacterium *Bacillus* sp. was isolated in association with the yeast *D. vanrijiae* from hot springs at 46°C. It was shown that the bacterium excreted thiamine into the culture broth, which not only promoted *D. vanrijiae* growth in mixed culture but also increased the maximal temperature for yeast growth.

15.2.7 A Competition for the Title

Some species of genera *Arxula*, *Candida*, *Cryptococcus* and *Rhodotorula* can be clearly marked as "extreme yeasts". These genera seem to be the most adapted yeast taxa to extreme environments. *A. adeniniovorans* is able to grow at high temperatures (45°C) and low a_w values (growth at 16% NaCl and at 60% glucose) (Barnett et al. 2000). Strains of the genus *Rhodotorula* have been reported from deep igneous rock aquifers – an environment of high salts, high pressure and anaerobiosis (Ekendahl et. al. 2003) – from sediments collected at depths of less than 2,000 m, where there were no basidiomycetous yeasts other than *Rhodotorula* (Nagahama et al. 2001), from honey (a_w=0.65) (Snowdon and Cliverb 1996) and a strain was reported to be resistant to high concentrations of metals (Nguyen et al. 2001; de Silóniz et al. 2002b). Moreover, red yeast isolated from the acidic water of Kusatsu hot spring could grow in an acidic medium of pH 1.5 and was identified as *Rh. glutinis*. The acid-tolerant yeast also showed strong resistance to both aluminium and manganese ions (Nguyen et al. 2001).

Such extreme features can also be found among the species of the the genus *Cryptococcus*. The most frequently isolated yeast species from Antarctic mosses were of the genus *Cryptococcus* (Scorzetti et al. 2000; Tosi et al. 2002; Guffogg et al. 2004): species of this genus inhabit high mountain deserts of the East Pamir – an environment of sharp oscillations of temperature and humidity during the day (Aksyonov 2002), they are capable of biosorption of dyes (Aksu 2004) and were isolated from deep-sea environment like species of the genus *Rhodotorula* mentioned before (Nagahama et al. 2001; Ekendahl et. al. 2003).

15.3 Mechanisms of Yeast Stress Responses and Tolerance to Harsh Conditions – Modes of Survival in Extreme Environments

It is critical for an organism to maintain function, and the easiest approach to achieve this is to keep the external environment out. If it is impossible to keep the environment out, evolutionary responses entail protective mechanisms, altering

physiology or enhancing repair capabilities. Research has focused so far on three key classes of biomolecules: nucleic acids, membrane lipids and proteins. For nucleic acids, function and structure are linked inextricably. DNA is especially vulnerable to high temperature, radiation, oxidative damage and desiccation. This can lead either to convergence or to multiple ways to solve the problem of living in a particular environment. Understanding the alternatives by using extremophiles on Earth as a sample should help us understand evolutionary processes on Earth, predict them elsewhere, and be useful in commercial exploitation of extremophiles (Rothschild and Mancinelli 2001) (See also Chap. 9).

15.3.1 Adaptation to Low Water Activity

15.3.1.1 Compatible Solutes

When an organism grows in a medium with a low water activity, it can obtain water from its environment only by increasing its internal solute concentration. An increase in internal solute concentration can be accomplished by either pumping inorganic ions into the cell from the environment or synthesizing or concentrating an organic solute. The solute used inside the cell for adjustment of cytoplasmic water activity must be non-inhibitory to biochemical processes within the cell; such compounds are called compatible solutes. The concentration of compatible solutes in the cell is a function of the level of external solutes, and in each organism the maximal amount of compatible solute(s) made or that can be accumulated is a genetically directed characteristic; this results in different organisms tolerating different ranges of water potential (Madigan et al. 1997). Compatible solutes detected in halophilic and halotolerant microorganisms include polyols such as glycerol and arabitol, sugars and sugar derivatives (sucrose, trehalose, glucosylglycerol), amino acids and derivatives, and quaternary amines such as glycine betaine. Compatible solutes are typically low molecular weight compounds, soluble at high concentrations in water, and either uncharged or zwitterionic at the physiological pH (Oren 1999).

The energetic cost of producing organic osmotic solutes is huge. An organism living in 4 M salt (by no means the highest salt concentration allowing the growth of many species that exclude salt from their cytoplasm) may contain close to 8 M organic solute in its cytoplasm to maintain osmotic equilibrium, with the exact value depending on the activity coefficients of the ions and the organic solutes involved. It is thus clear that the strategy of accumulating osmotic solutes is energetically very costly, the more so when larger organic molecules such as disaccharides (12 carbon atoms) are to provide osmotic balance. The use of smaller molecules such as glycerol (three carbons) or glycine betaine or ectoine (five and six carbons, respectively) is less energy consuming. The amount of energy needed for the production of the osmotic solutes may thus greatly exceed the energy requirement for the biosynthesis of proteins, nucleic acids, cell walls, etc. The total amount of glucose required for the formation of structural cell material may be thus 4.4 times as much as for a non-halophilic microorganism (Oren 1999).

It is obvious that of all known compatible solutes, glycerol is the simplest and energetically cheapest to produce. Its solubility in molar terms exceeds that of all

other solutes, since it is miscible with water at all ratios. Moreover, even at extremely high concentrations (4 M and more) it still supports excellent activity of intracellular enzymes. Most biological membranes are highly permeable to glycerol, and therefore a specially adapted membrane structure is needed to keep the glycerol produced within the cell. Glycerol seems to be restricted to the domain Eucarya, in particular to yeasts (Oren 1999).

Xerophilic yeasts produce mainly glycerol as a compatible solute (Madigan et al. 1997). In studies of a response to environmental changes in temperature (30–44°C) and osmotic pressure (resulting from concentrations of NaCl in the media ranging from 0 to 1 M NaCl) accumulation of trehalose and glycerol production in *S. cerevisiae* was observed (Carvalheiro et al. 1999). NaCl was found to be more important than temperature in glycerol production but the combination of the two factors still encloses a considerable synergistic effect. NaCl contributes with an effect fivefold that of temperature on both responses. Increase in trehalose concentrations can be regarded as part of thermotolerance and osmotolerance mechanisms. It was shown that both temperature and NaCl contributed in an interactive manner towards the accumulation of trehalose. The presence of both factors leads to a more effective synthesis of trehalose than each one per se. In fact, independent of the type of shock, the ratio between the parameters of produced glycerol and the parameters of the intracellular trehalose concentration for each stress type was similar (Carvalheiro et al. 1999).

The evolution of the denomination of these compounds went from osmotic effectors or osmolytes to compensatory compounds. These compounds osmotically compensate for the decrease in ion concentration. Their increase in concentration thus permits the maintenance of the ion concentration at a steady level, avoiding the disrupting effects of their increase in amount on cell macromolecular structures. In this regulation system, the organic solutes would "chemically compensate" for the disrupting effects of ions on macromolecular structures in the initial transient stress conditions. After acclimation, they would osmotically compensate for the osmotic gap left by maintenance of the ions at their normal level. In short, a major characteristic of the compensatory solutes is that they are preferentially excluded from the protein surface and its immediate hydration sphere. This exclusion appears to stabilize folded protein structures. It also promotes subunit assembly and salting-out of protein (Gilles 1997).

To sum up these studies, it is actually clear that the control of the level of the different compensatory solutes cannot be related to a single major mechanism. It appears that changes in the activity of different processes – catabolism, de novo synthesis, efflux, influx, equilibrium between macromolecular components and their constitutive organic solutes – can be implicated depending on the solute considered and on the conditions in which the cells are. Often also, control of the amount of a solute implicates more than one of these processes. A priori, control can be exerted in two different ways: directly, by one or several "modulating factors" acting on target systems – transporters, channels and/or enzymes (such an "allosteric" modulation would lead to a change in the kinetics characteristics of the target system), or indirectly, by regulation of expression of genes coding for the transporters, channels and/or enzymes implicated. In this case, there would be a change in the concentration

and/or in the kinetics characteristics of the target system. Also one system is not a priori exclusive of the other (Gilles 1997).

15.3.1.2 Cold Shock and Cold Acclimation

Despite the strong negative effect of low temperatures on biochemical reactions, these organisms breed, grow and move at rates similar to those achieved by closely related species living in temperate environments. They have therefore developed various adaptations in the form of finely tuned structural changes at the level of, for example, their membranes, constitutive proteins and enzymes, enabling them to compensate for the deleterious effects of low temperature (Gerday et al. 2000).

The yeast cell with its wealth of membrane organization, together with its rapid growth, reproduction, and relative genetic simplicity, is an attractive system in which to study the mechanism of thermal adaptation in eucaryotic microorganisms (Arthur and Watson 1976).

The factors controlling an organism's minimum growth temperatures are not clear. The cytoplasmic membrane must be in a fluid state for proper functioning. There are indications that the minimum temperature of an organism results from "freezing" of the cytoplasmic membrane so it no longer functions properly in nutrient transport. This explanation is supported by experiments in which the minimum temperature for an organism is altered to some extent by adjustments in membrane lipid composition. Studies on the composition of cytoplasmic membranes from psychrophiles have shown them to contain a higher content of unsaturated fatty acids, which help to maintain a semifluid state of the membrane at low temperatures (Madigan et al. 1997). Marked variations in both phospholipid composition and the degree of fatty acid unsaturation of cold- and warm-adapted yeasts indicate that the ability to alter these cellular components may constitute an adaptation mechanism to suit a particular environment. Considerable interspecific variations in phospholipid distributions were observed in the strains examined. *M. frigida* was chosen as representative of the psychrophilic strains and was found to contain a greater amount of phosphatidyl ethanolamine than did any of the other species. The formation of hydrogen bonds between the amino groups of phosphatidyl ethanolamine and the polar water molecules may serve to retard ice formation at subzero temperatures (Arthur and Watson 1976).

The extent of such ability may be an important controlling factor which determines the growth temperature limits of the microorganisms, the lower limit being that at which the membrane lipids solidify and the upper limit being that at which the membrane lipids melt. The membrane lipid composition of the different yeasts conforms well to this concept. There is a direct correlation between temperature adaptation and the degree of membrane lipid unsaturation: the lower the temperature, the greater the degree of fatty acid unsaturation. The high C18:2 (melting point, −5°C) and C18:3 (melting point, −11°C) content of the psychrophilic yeasts would permit the cell membranes to remain in a sufficiently fluid state to allow unimpaired functioning of metabolic processes even at subzero temperatures. On the basis of the melting points of the major fatty acids present, it could be proposed that the psychrophilic yeasts would be able to grow at temperatures as low at −10°C

(Arthur and Watson 1976). Also the concentration of dissolved oxygen may be particularly important in regulating the degree of lipid unsaturation at various temperatures since (1) the solubility of oxygen decreases with temperature, and (2) oxygen is obligatory in reactions involving the fatty acid desaturase enzymes of yeasts (Arthur and Watson 1976; Cavicchioli and Torsten 2000).

In studies of stress tolerance Deegenaars and Watson (1997) identified the presence of heat-shock proteins (HSPs) 90, 70 and 60 in protein extracts from *C. psychrophila*. These HSPs seem to be involved in adaptative reactions to thermal stress changes. However, the absence of strong induction of these HSPs upon a heat shock in this study argues against their role in the heat-shock-induced thermotolerance observed in the Antarctic psychrophilic yeast. On the other hand, the psychrophilic yeast showed the presence of a strongly heat shock inducible protein of about 110k Da. It may well be that this protein plays a role in thermotolerance in psychrophilic yeasts, similar to that of HSP104 in mesophilic species. In *S. cerevisiae*, temperature-shock-inducible protein 1 (TIP1) is a major cold-shock protein; it is targeted to the outside of the plasma membrane and appears to be heavily glycosylated with *O*-mannose, therefore invoking a role for TIP1 in membrane protection during low-temperature adaptation (Cavicchioli and Torsten 2000).

Psychrophiles produce enzymes that function optimally in the cold and that are often denaturated or otherwise inactivated at even very moderate temperatures (Madigan et al. 1997). Clearly, the strategy of adaptation is unique to each enzyme. Decreased levels of prolyl and arginyl residues and increased levels of glycyl residues could be involved (Gerday et al. 2000). The presence of a highly polar motif within the enzyme's N-terminal fragment can be considered as an indirect proof of the structural flexibility of LAP2 (the first reported extracellular subtilase of a psychrophilic yeast *Leucosporidium*), and this motif has not been detected in molecules of mesophilic representatives of the subfamily C of the subtilase clan (Turkiewicz et al. 2003). The stability of the cold-adapted enzyme can also be lowered by a general weakening of the intramolecular interactions, increased interactions with the solvent, weakening of the interdomain or intersubunit interactions and a decrease in the cation or anion interactions. All of these factors give rise to an increase in the overall or local flexibility of the molecular structure. A better accessibility of the catalytic cavity can also improve the accommodation of the substrate, giving rise to a higher specific activity at low temperatures (Gerday et al. 2000).

It was said that the membrane system and well-adapted proteins are crucial for surviving in very cold or very hot environments, but there was a report of a yeast species of the genus *Cryptococcus* which can survive in high mountain deserts of the East Pamir, where sharp oscillations of temperature and humidity during the day are observed (Aksyonov 2002). Therefore, the organism must act as a psychrophile and a thermophile in a period of 1 day. Adaptation through saturation and unsaturation of cell membrane phospholipids is therefore less likely, so other adaptation mechanisms must be involved. Characteristic for the genus *Cryptococcus* is a wide range of consumable carbon compounds (Fell and Statzell-Tallman 1998), formation of chlamidospores and very thick capsules, which protect against extreme environmental influences (Chernov 1997). These are the features that can assure survival in such harsh surroundings.

15.3.1.3 Resistance to High Salinity

Progressive salinization of irrigated land is one of the main problems for agriculture worldwide. This trend necessitates research into the development of genetically engineered crop plants with greater salt tolerance. An important step in this direction is to understand the mechanisms of salt tolerance in those eukaryotic organisms that are salt-tolerant by nature.

Two fundamentally different strategies exist within the microbial world that enable microorganisms to cope with the osmotic stress inherent to the presence of high salt concentrations.

1. Cells may maintain high intracellular salt concentrations, osmotically at least equivalent to the external concentrations (the "salt-in" strategy). All intracellular systems should then be adapted to the presence of high salt concentrations. This option is used by a few specialized groups of prokaryotes only.
2. (ii) Cells may maintain low salt concentrations within their cytoplasm (the "compatible-solute" strategy). The osmotic pressure of the medium is then balanced by organic compatible solutes. No special adaptation of the intracellular systems is required (Oren 1999).

Since biological membranes are permeable to water, cells cannot maintain a water activity of their cytoplasm higher than that of the surrounding brine, because this would lead to a rapid loss of water to the environment. Therefore, any microorganism living at high salt concentrations may be expected to keep its cytoplasm at least isoosmotic with the extracellular environment. Build-up of a turgor pressure requires a hyperosmotic cytoplasm. With the possible exception of the halophilic archaea of the order *Halobacteriales*, all halophilic microorganisms maintain a turgor pressure (Oren 1999).

Salt tolerance in eukaryotic microorganisms has been mainly studied in the yeast *S. cerevisiae*. This is probably not the best model organism since it is a rather salt-sensitive yeast which tolerates only moderate salt concentrations. To understand the mechanisms involved in salt tolerance, *D. hansenii* has been used as an eukaryotic model. Although Almagro et al. (2001) reported the existence of Na^+ efflux systems in *D. hansenii* – Ena proteins which seem to play an important role in maintaining balanced levels of intracellular cations, ensuring the ionic homeostasis of the cell – it has been shown that *D. hansenii* grows optimally in 0.6 M of either NaCl or KCl, accumulating high concentrations of Na^+ or K^+. In contrast to the statement that the "salt-in" strategy is restricted to a few specialized groups of prokaryotes only (Oren 1999), it can be concluded that Na^+ in *D. hansenii* is not excluded, but, instead, its metabolic systems must be resistant to high salt concentrations (Gonzalez-Hernandez et al. 2004). Moreover, it was shown that Na^+ even improves the performance of *D. hansenii* under different stress conditions (Almagro et al. 2000). Presumably that tolerance to salt can be conferred by gene transference; a proteomic approach to the study of salt stress responses in *D. hansenii* has been started and spots specifically regulated by the presence of high salt in the growth medium have been identified. It is planned to evaluate new unknown mechanisms together with the contribution of well-known processes involved in salt tolerance: production and

accumulation of compatible solutes, ion transport and enzyme sensitivity (Ramos 2004).

H. werneckii was isolated from solar salterns as one of the predominant species of a group of halophilic and halotolerant melanized yeast-like fungi. It can grow at salinities ranging from 0% to saturated solution of NaCl. As a response to high salinity, it activates a high osmolarity glycerol (HOG) signalling pathway that results in activation of glycerol-3-phosphate dehydrogenase gene expression and consequently in cytosolic glycerol accumulation (Petrovic et al. 2002; Lenassi et al. 2004). In a study of the cation quantities in the halophilic *H. werneckii* and halotolerant *A. pullulans,* Kogej et al. (2004a) showed that the sodium and potassium contents were relatively low in fully adapted cells of *H. werneckii* and *A. pullulans* in comparison to those for *D. hansenii*, which is known to accumulate high concentrations of Na^+ or K^+ in the cell (Gonzalez-Hernandez et al. 2004). These results show that accumulation of sodium and potassium ions does not contribute to osmoadaptation of *H. werneckii* or *A. pullulans*. The accumulation of glycerol seems to be more significant for their survival in a hypersaline environment. Kogej et al. (2004a) assume these yeasts have efficient ion pumps, which probably enable them to keep their cation contents at a low level even with high NaCl concentrations.

Petrovič et al. (2002) proposed a model by which the glycerol accumulation, the use of alternative energy production mechanisms, such as the glycerophosphate shuttle, the isovaleryl-CoA dehydrogenase/electron transferring flavoprotein: ubiquinone oxidoreductase system, or the Na^+ and/or the H^+ electrochemical potentials, and the post-transcriptional regulation enable *H. werneckii* to thrive at NaCl concentrations unparalleled by other eukaryotic species. *H. werneckii* is able to keep extracellular glycerol concentrations efficiently low and independent of salt concentration between 0 and 17% NaCl, but unchanged intracellular glycerol concentration between 10 and 25% NaCl points to the presence of (an)other compatible solute(s) in the cells of *H. werneckii* grown at salinities above 10% NaCl. At salinities higher than 17%, the extracellular concentration of glycerol started to increase. This effect correlates with the diminished growth of *H. werneckii* at salinities above 17% NaCl. As in *S. cerevisiae*, glycerol biosynthesis in *H. werneckii* is regulated mainly through transcriptional regulation of glycerol-3-phosphate dehydrogenase. Since glycerol presumably becomes one of the most abundant molecules produced by the cells, relatively less metabolic flux remains for the late stages of glycolysis and presumably also for the citric acid cycle. Increased expression of SOL5, a putative aconitase gene, at 17% NaCl and 25% NaCl, could also be related to this phenomenon. When the citric acid cycle becomes less important, a cell could gain enough ATP through alternative electron donors or it could make use of Na^+ and/or H^+ electrochemical potentials across the plasma membrane. Petrovič et al. (2002) describe that genes for SOL1, SOL2, SOL4p and SOL8 could be part of this scenario.

Additionally, Lanišnik Rižner et al. (2001) reported an increase of 17β-hydroxysteroid dehydrogenases (17β-HSD) activity in *H. werneckii* in the presence of salt. The enzyme activity at 17% NaCl was 13 times higher than that at 5% NaCl. They proposed that 17β-HSD could be a part of a detoxification mechanism in *S. pombe* and *C. albicans* and different types of 17β-HSDs might have evolved because of

different needs for detoxification in different environments. These enzymes are responsible for reversible interconversions of biologically active 17-hydroxy and inactive 17-keto steroids. Influencing their activity with altering NaCl concentrations is therefore an interesting feature for biotechnological applications in steroid production.

Another phenomenon, which contributes to better understanding of eukaryotic adaptations, enabling life in extremely saline environments has also been observed in research on lipids in halophilic microorganisms. Lipid saturation (increase of oleic acid and decrease of linoleic acid) was shown to be important for salt tolerance in *Z. rouxii* cells under a high-NaCl condition (Yoshikawa et al. 1995). Gostinčar et al. (2004) showed that the fatty acid composition of the *H. werneckii* cells is at least partially regulated through the transcription control of genes, encoding three important fatty-acid-modifying enzymes: elongase and two different types of desaturase. Expression of all three observed genes rose at downshift, while it decreased at increased salinity (upshift).

15.3.1.4 Osmotic Adaptation in Yeast

High glucose concentrations increase extracellular osmotic pressure, and as a consequence, osmotolerant yeasts accumulate glycerol as a compatible solute (Sahoo and Agarwal 2001). Yeast cells possess robust systems for osmotic adaptation. Central to the response to high osmolarity is the HOG pathway, one of the best-explored mitogen-activated protein (MAP) kinase pathways. This pathway controls via different transcription factors the expression of more than 150 genes. In addition, osmotic responses are also controlled by protein kinase A via a general stress response pathway and by presently unknown signalling systems. The HOG pathway partially controls expression of genes encoding enzymes in glycerol production. Upon hypoosmotic shock, yeast cells transiently stimulate another MAP kinase pathway, the so-called protein kinase C (PKC) pathway, which appears to orchestrate the assembly of the cell surface and the cell wall. In addition, yeast cells show signs of a regulated volume decrease by rapidly exporting glycerol through Fps1p. This unusual major intrinsic protein (MIP) channel is gated by osmotic changes and thereby plays a key role in controlling the intracellular osmolyte content. Yeast cells also possess two aquaporins, Aqy1p and Aqy2p. The production of both proteins is strictly regulated, suggesting that these water channels play very specific roles in yeast physiology (Hohmann 2002).

Osmotic hypersensitivity is manifested as cellular death at magnitudes of osmotic stress that can support growth. All strains of *S. cerevisiae* display the osmotic hypersensitivity phenomenon in qualitative terms, while the quantitative values differ. It was proposed that the growth rate does not dictate the level of osmotic hypersensitivity of *S. cerevisiae*. The phenomenon is physiological and not genetic because cells that do survive and form colonies under conditions of high stress will exhibit the same growth-related osmotic hypersensitivity if inoculated into fresh basal medium. The osmotic hypersensitivitiy phenotype is not related to any of the strictly growth linked functions in the cell, like DNA replication or biosynthesis. The phenomenon might be valid for many if not all species of yeast and is not restricted to specific

examples. Osmotic shock resistance seems not to be restricted to non-proliferating cells (Blomberg 1997).

15.3.2 Heat-Shock in Yeasts

The membrane lipid composition of the thermophilic yeasts is distinguished by the high percentage (30–40%) of saturated fatty acid, as compared with the mesophilic and psychrophilic yeasts. The latter contained approximately 90% unsaturated fatty acid, 55% of which was linolenic acid, $C_{\alpha\text{-}18:3}$. Changes in phospholipid composition in relation to temperature were also noted. The respiratory-deficient thermophile *C. slooffiae* was characterized by the absence of cardiolipin (sensitivity 0.1 μg of phosphorus) and cytochrome aa_3. The absence of conventional mitochondrial structures in this thermophilic microorganism is tentatively suggested, although low concentrations of cytochromes b, c and c_1 were detected by low-temperature spectroscopy. On the other hand, the respiratory competent thermophile, *T. bovina* (= *Kazachstania bovina*), was characterized by a high cardiolipin (25% of the total phospholipid) and cytochrome aa_3 content (1 nmol mg^{-1} of mitochondrial protein). Low-temperature spectra showed the presence of one b-type cytochrome in the thermophilic yeasts, two b-type cytochromes in the mesophilic yeasts and three b-type cytochromes in the psychrophilic yeasts. It was concluded that knowledge of the properties of the biological membrane is fundamental to understand the ability of a microorganism to grow and reproduce in different temperature environments (Arthur and Watson 1976).

On the other hand, Swan and Watson (1997) showed that there was no obvious relationship among membrane fatty acyl composition, membrane fluidity and stress tolerance in the yeast strains examined. They suggest that protein denaturation may be responsible for the observed effect of elevated temperature on membrane fluidity and viability – a decrease in membrane fluidity following thermal treatment, which coincided with a reduction in cell viability. They also suggest that the thermotolerant state of heat-shocked cells and cells entering the stationary phase may be associated with increased protein stability.

For walled cells such as yeast, a heat stress does not provoke any measurable variation of viable cell volume, while spheroplasts are rapidly affected by an increase in temperature of the medium that induced firstly a volume increase and secondly cell bursting. This constant volume over time could be explained by the protective role of the cell wall that mechanically maintains the plasma membrane. This could be explained by the sensitivity of the plasma membrane or of the cell cytoskeleton to heat. If the cytoplasmic membrane is maintained by a wall, as for yeast, the main envelope pressure resistance can then be assumed by the wall, which is still resistant to pressure in spite of the heat stress. For walled cells, the entry of water is prevented by the wall that mechanically balances the turgor pressure through the exertion of an overpressure on the plasma membrane (Gervais et al. 2003). Someone can speculate which composition of the cell wall structure enables the highest turgor pressure: the thicker β-glucan and additional α-glucan structure of ascomycetous yeasts or the thinner but lamellare β-glucan structure of basidiomycetous yeasts. A contribution to this knowledge was made by Nguyen et al. (2001), who studied

the physiological adaptation to influences of extreme environments in *Rh. glutinis* discussed in the following section.

15.3.3 Characteristics of Acidophilic Behaviour of Yeasts

Acidophiles keep their internal pH close to neutral. As a result, extreme acidophiles have a large chemical proton gradient across the membrane. Proton movement into the cell is minimized by an intracellular net positive charge and as a result the cells have a positive inside-membrane potential. This is caused by amino acid side chains of proteins and phosphorylated groups of nucleic acids and metabolic intermediates, acting as titratable groups. In fact, the low intracellular pH leads to protonation of titratable groups and produces a net intracellular positive charge (Cavicchioli and Torsten 2000).

The strength of an acid is defined by its dissociation constant (pK_a) – at this pH value the dissociated and undissociated forms of the acid are in equal amounts. The lower the pH, the greater the proportion of the acid in the undissociated form, which is membrane-permeable and therefore it can enter the cell. Once inside the cell, weak acids dissociate because of higher interior pH and become toxic, which ultimately inhibits cell growth owing to the acidification of the cell interior (Beales 2004). In yeast there are approximately 20 multidrug resistance (MDR) genes underlying tolerance to toxic compounds that are all involved in membrane transport. The yeast Mdr proteins are generally referred to as pleiotropic drug resistance (Pdr) proteins. They are composed of three major classes: the ATP-binding cassette (ABC) superfamily, such as Pdr12p, the major facilitator superfamily (MFS) and transcription factors, such as PDR1. Pdr12p is membrane-localized and its role is involved in weak organic acid resistance. HSP26 and HSP30 also seem to contribute to the establishment of a new level of cellular homeostasis in weak-acid adapted cells, latter by moderating the activity of the proton-pumping plasma membrane ATPase, which can consume about a half of the total cellular ATP produced. However, it is speculated that the net effect could be that cells (e.g., *Z. bailii*) effectively extrude the antimicrobial weak acid from the cytoplasm (Brul et al. 2003; Beales 2004).

In the study of de Silóniz et al. (2002b) important differences were found among the three strains in relation to changes in specific qO2 as a function of external pH. The respirometric results obtained over the pH range studied (respiratory quotient approximately 1.0), reflect an oxidative metabolism of glucose in *C. sorbophila* and *Rh. mucilaginosa*, as could be expected from the negative Crabtree character of these yeasts. On the other hand, a different effect of acid on the yeast cells could be observed when qO2 and growth rate results were considered together. Oxygen uptake by *Rh. mucilaginosa* was only slightly affected at low pH. Nevertheless, the bulk of energy obtained is probably needed to support the viability of cells between pH 2.5 and 1.0, and, in consequence, cells grow slowly or not at all. In contrast, at optimal pH (3.0–2.5), *C. sorbophila* was able to maintain low qO2 and slightly greater growth rates than at higher pH (4.5), and with very similar kinetics of glucose consumption.

Red yeast *Rh. glutinis* shows a high level of physiological adaptation to influences of extreme environments. Electron microscope observations (scanning electron

microscopy and transmission electron microscopy) showed that the cell envelope became wrinkled and thick as the pH values of the media became lower. The cell membrane grown at pH 1.5 was about 4 times as thick as that grown at pH 6.0. Furthermore, it was found that the densities of the cell membranes decreased in the acidic media. It was suggested that the change of the cell envelope plays an important role in the acid tolerance. Cellular proteins at pH 1.5 appeared to be different from those at pH 6.0 and the amounts of phospholipids and non-phospholipids increased and decreased under low pH conditions, respectively. The results suggested that the increase of phospholipid content prevented the penetration of excess protons. As the amounts of phosphatidylcholine and phosphatidylethanolamine increased slightly and the amount of the total phospholipids increased markedly at low pH compared with those at high pH, there is a possibility of the presence of phospholipids other than phosphatidylcholine and phosphatidylethanolamine. It was demonstrated that *Rh. glutinis* is capable of lowering the acidity of a low-pH medium and that it is also capable of neutralizing acidic media. As the neutralization occurred in the stationary phase, it could be thought that the neutralization depended on not only the alkaline materials derived from the cells but also the enzyme(s) capable of reducing metal ions (Nguyen et al. 2001).

15.3.4 Resistance to High Concentration of Metals

Yeast defence mechanisms to high concentrations of metal include bioaccumulation and the precipitation/chelation of the metal at the cell wall (biosorption), which prevents the metal from reaching sensitive intracellular components. Yeast tolerance to high metal concentrations is dependent on cell wall characteristics, which can regulate the number of cations that are able to reach the cell membrane and cytoplasm (Raspor et al. 2000a). The cell wall characteristics are determined by its structure and the distribution of homopolysaccharides (mannans and glucans), single saccharides and acid components (which can be good binding agents). Considering the growth of yeast it was observed that the hyphal type of growth of yeasts other than *Saccharomyces* has a better tolerance capacity to chromium in the environment than normal budding (Batič et al. 1996; Batič and Raspor 2000). Metal ion uptake in yeasts is known to involve an initial rapid biosorption of metal ions to negatively charged sites on the cell wall followed by a slower, energy-dependent entry into the cell (Raspor et al. 2003). Both the outer mannan–protein layer of the yeast cell wall as well as the inner glucan–chitin layer play important role in heavy-metal accumulation. A majority of intracellular metals become bound to polyphosphate granules localized in and near the vacuoles or may also get detoxified via binding to specific low molecular weight proteins, namely, metallothioneins and phytochelatins. Thus, it appears that in case of yeast and microalgae as well, most of the metals are accumulated intracellularly (Malik 2004).

Accumulation of cadmium and nickel in *Y. lipolytica* CCM 4510 was higher than that of cobalt and zinc, i.e., cobalt and zinc were less bonded on yeast biomass in comparison with cadmium or nickel. Moreover, cadmium and nickel were not detected on the surface of the cell wall, but mainly they interacted with the cell wall and membrane debris. The high level of cadmium and nickel in the cell wall and

membrane debris is probably due to the interaction of heavy metals with carboxylic groups, which are dominant functional groups in the cell wall. These carboxylic groups descended from peptides would be the potential sites for binding of heavy metals, while the binding process is not exactly described. The incorporation of heavy metals into individual cell compartments of *Y. lipolytica* decreases in the following order: (1) cell surface, Co>Zn » Cd, Ni; (2) cell wall and membrane debris, Cd, Ni» Zn>Co; and (3) cytoplasm of the yeast cell, Zn, Co>Cd» Ni (Strouhal et al. 2003). As an example, the distribution of accumulated chromium in yeast cells is shown in Fig. 15.1.

MT are an important class of eukaryotic stress-responsive proteins whose biosynthesis is induced by a variety of environmental and physiological stresses including metal sequestration and oxidative stress (de Silóniz et al. 2002a; Strouhal et al. 2003). The differences in MT levels in the yeast cell treated with heavy metals may arise owing to the variety of physiological-biochemical changes that take place during the growth process and the ageing of cells. Though the toxic metals (Cd, Ni) induce biosynthesis of MT more significantly than essential metals (Co, Zn), it was observed that when cytoplasmic concentrations of essential metals (Co, Zn) were high, the amount of MT was lower than that for toxic metals. Therefore it was supposed that the cell wall is the first protective barrier to prevent penetration of heavy metals into the cell (Strouhal et al. 2003).

De Silóniz et al. (2002a) compared the cell morphology of yeast *P. guilliermondii* between control cultures without copper and cultures with different concentrations of copper. They observed a shift to filamentous forms and no unicellular forms, and a change in colour to green in the copper cultures. A mixture of yeast-like and filamentous cells was always observed in the absence of copper, with the yeast cells

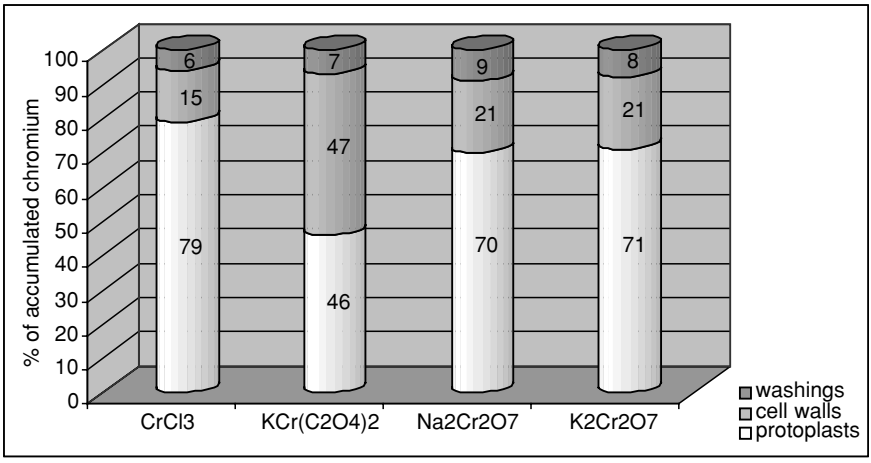

Fig. 15.1. Distribution of accumulated chromium in yeast cells, expressed as a percentage of Cr in cell walls, protoplasts and washings, after 12-h cultivation in the presence of 1 mM Cr(III) [as $CrCl_3$ or $KCr(C_2O_4)_2$] and 20 µM Cr(VI) (as $K_2Cr_2O_7$ or $Na_2Cr_2O_7$) (Paš et al. 2004)

being predominant. The addition of copper increased the proportion of filamentous forms, and this could be considered a primary response to copper toxicity. When the cells were adapted by being trained to grow at high concentrations of copper, only yeast-like forms developed. In this sense, a long-term response to copper toxicity could be responsible for the appearance here of yeast-like cells. Independent of the degree of stress, it seems that a process of bioaccumulation may occur in the presence of copper in both types of cells. These results indicate that unadapted cells growing in the presence of the metal must spend energy on the mechanisms involved in protecting the yeast cell from the toxic effect of copper. However, the mechanism of adaptation to copper in *P. guilliermondii* could be considered a system of all or nothing, i.e., once the mechanism of adaptation to high concentrations of copper is developed, the growth is stable and the concentration of copper has no influence on the metabolic parameters. The results of uptake indicate and confirm the microscopic observation that the resistance mechanism is a sequestration of copper, rather than a mechanism of avoidance. Consequently, it is possible that the metallothionein-mediated mechanism could be responsible for metal sequestration in *P. guilliermondii*. From the point of view of bioremediation, this mechanism contributes to the interest and feasibility of application of *P. guilliermondii* in such processes (de Silóniz et al. 2002a).

Generaly we relate mechanisms for metal toxicity to to cell stress response (Jamnik and Raspor 2003). There are few studies which consider this issue in more detail regarding genotoxicity (Plaper et al. 2002) and oxidative stress (Fujs et al. 2005).

15.3.5 Physiological Make-Up of Yeasts to Degrade Xenobiotic Compounds

Frequently, xenobiotic metabolism involves a combination of P450 and phase II enzymes. Yeast expression makes it possible to include at will some particular phase II activities, such as epoxide hydrolase activity (Pompon et al. 1997). A marine strain of *Y. lipolytica* has a set of enzymes for the bioconversions of nitroaromatic compounds. Very often, non-specific nitroreductases bring about these tranformations. The presence of glucose could preferentially modulate the choice of the reaction to the ring reduction and in the absence of glucose the nitro groups are preferentially reduced to amino derivates (Jain et al. 2004). Zaripov et al. (2002) reported that no strains analogous to *Candida* sp. AN-L13, which performs a practically unidirected reduction of the aromatic ring, were found among other strains in their collection. Such microorganisms are also unknown in the literature. Interestingly, the taxonomic affiliation and the type of metabolism (fermentative vs. respiratory) of yeasts correlated with the pathway of TNT degradation.

15.4 Biotechnological Potential of Yeasts from Extreme Habitats

Extremophiles have provided data that are basic to molecular biology, including information on protein folding. Enzymes from extremophiles have potential in multiple areas, either by using the enzymes themselves, or by using them as sources of

ideas to modify mesophile-derived enzymes. Human health may benefit from extremophiles indirectly through biotechnology and bioremediation. Antifreeze proteins show potential as cryoprotectants of frozen organs. It is now widely accepted that these microorganisms provide a valuable resource not only for exploitation in novel biotechnological processes but also as models for investigating how biomolecules are stabilized when subjected to extreme conditions (Herbert 1992; Rothschild and Mancinelli 2001). However, extremophilic yeasts can be used in many applications since yeasts are the most important and the most extensively used microorganisms in industry (Madigan et al. 1997) (see also Chap. 22).

15.4.1 Bioremediation

In modern society, an increasing number of hazardous organic compounds are being discharged into the environment. Most are degraded or detoxificated by physical, chemical and biological treatments before being released into the environment. Although the biological treatments are a removal process for some organic compounds, their products of biodegradation may also be hazardous. Moreover, some non-degradable compounds discharged into the environment along with the treated compounds can cause problems because they usually come back to human beings through several channels, such as bioaccumulation. Bioaccumulation is defined as the accumulation of pollutants by actively growing cells by metabolism-independent, temperature-independent and metabolism-dependent mechanism steps. Although bioaccumulation of dyes by yeasts was accomplished, there are significant practical limitations regarding the inhibition of cell growth at high dye concentrations and the requirement of metabolic energy externally provided. So there is a need to find alternative treatment methods that are effective in removing dyes and organic molecules that are not biodegradable from large volumes of effluents and are low in cost, such as biosorption. The term "Biosorption" is used to indicate a number of metabolism-independent processes (physical and chemical adsorption, electrostatic interaction, ion exchange, complexation, chelation and microprecipitation) taking place essentially in the cell wall rather than oxidation through anaerobic or aerobic metabolism (biodegradation). The main attractions of biosorption are high selectivity and efficiency, cost effectiveness and good removal performance. Biosorption is also becoming a promising alternative to replace or supplement the present removal processes of organic pollutants from wastewaters. Among these pollutants, dyes, phenolics and pesticides have recently been of great concern because of their extreme toxicity and/or persistency in the environment (Aksu 2004).

The use of dead microbial cells in biosorption is more advantageous for water treatment in that dead organisms are not affected by toxic wastes, they do not require a continuous supply of nutrients and they can be regenerated and reused for many cycles. Dead cells may be stored or used for extended periods at room temperature without putrefaction occurring. Their operation is easy and their regeneration is simple. Moreover, dead cells have been shown to accumulate pollutants to the same or to a greater extent than growing or resting cells. The mechanism of binding by inactivated biomass may depend on the chemical nature of the pollutant (species, size, ionic charge), the type of biomass, its preparation and its specific

surface properties and environmental conditions (pH, temperature, ionic strength, existence of competing organic or inorganic ligands in solution). As hydrophobic organic pollutants show a high tendency to accumulate onto microbial cells or sludge, the microbial biomass could be used as an adsorbent of biological origin for the removal of very low concentration hazardous organics from the wastewater (Aksu 2004).

Dönmez (2002) and Aksu and Dönmez (2003) reported the biosorption capacities and rates of nine yeast species (*S. cerevisiae*, *S. pombe*, *K. marxianus*, *Candida* sp., *C. tropicalis*, *C. lipolytica*, *C. utilis*, *C. guilliermendii* and *C. membranifaciens*) for Remazol Blue reactive dye from aqueous solutions. The yeasts studied were found to be more effective for concentrating Remazol Blue dye at different capacities according to the dye concentration. They explained the differences between yeast species for dye binding capacity in terms of the properties of the yeast (e.g., structure, functional groups, surface area and morphological differences depending on the yeast division, genera and species). They proposed that cell walls of yeasts contain polysaccharides as basic building blocks, which have ion-exchange properties, and also proteins and lipids and therefore offer a host of functional groups capable of binding dye molecules. These functional groups such as amino, carboxylic, sulfydryl, phosphate and thiol groups differ in their affinity and specificity for dye binding. Among the nine yeast species tested, *C. lipolytica* exhibited the highest dye uptake capacity (Aksu 2004).

Pharmaceutical wastewater remaining after ergot alkaloid production has a high COD load to sugar compounds, metabolic products of fungus *Claviceps* and organic solvents content (COD is a standard test that measures the amount of organic matter in wastewater that can be oxidized by a very strong chemical oxidant). The cost of discharging the wastewater into a wastewater treatment plant is forcing the pharmaceutical industry to pretreat it. With the aim to develop a more efficient system for wastewater treatment the isolation of a spontaneous population of microorganisms from a wastewater collecting tank was performed. Isolated strains of yeasts were identified as *P. ciferrii* and the results showed that the COD reduction in this case was significantly higher (75–80% in 60 h) in comparison with a previous selection among 60 yeast strains which showed that only three of them were able to grow in wastewater with a COD reduction efficiency of 60–64%. It was found that *P. ciferrii* could be used for reducing the COD load of wastewater from ergot alkaloid production (Recek and Raspor 1999; Recek et al. 1999, 2002).

Heavy-metal pollution represents an important environmental problem owing to the toxic effects of metals, and their accumulation throughout the food chain leads to serious ecological and health problems. Biotechnological approaches that are designed to cover such niches have, therefore, received a great deal of attention in recent years. At times, when pure biosorptive metal removal is not feasible, application of a judicious consortium of growing metal-resistant cells can ensure better removal through a combination of bioprecipitation, biosorption and continuous metabolic uptake of metals after physical adsorption. Such an approach may lead to simultaneous removal of toxic metals, organic loads and other inorganic impurities, as well as allow optimization through development of resistant species. However, the sensitivity of living cells to extremes of pH or high metal concentrations and the

need to furnish metabolic energy are some of the major constraints of employing growing cells for bioremediation (Malik 2004).

In order to overcome the problem, heavy-metal ions are commonly removed from wastewaters by chemical precipitation, ion-exchange or reverse osmosis processes. Such techniques can be very expensive and may have several disadvantages, therefore development of cost-effective alternatives, such as biosorption, has become an intensive area of exploitation over the past decade. Non-viable biomass has several advantages for metal biosorption from solution, especially as there is no requirement for maintenance and nutrition (de Silóniz et al. 2002a).

Some microorganisms are able to grow in extreme concentrations of different heavy metals in the environment. Yeasts have a great potential for removal and uptake of metals from the environment as well as for controlling environmental pollution (Batič et al. 1996).

Copper is one of the most abundant toxic heavy metals in municipal wastewaters and, in consequence, in sewage sludge and compost. The ability of a strain of the yeast *P. guilliermondii*, which was isolated from sewage sludge, to eliminate copper has been reported. It was found that raising the concentrations of copper affected both the morphology and the physiological parameters of the viable yeast, and it is thought that a process of bioaccumulation may be involved in its copper uptake. The growth rate of unadapted cells decreased with increasing concentrations of copper, mainly owing to a decrease in the biomass yield. The cells could be adapted by training them with increasing copper concentrations up to 317.7 mg l^{-1}. This adaptation was an all-or-nothing process: once the cells had adapted, the biomass yield, the metabolic flux and consequently the growth rate were constant and independent of the external copper concentration (de Silóniz et al. 2002a).

The acid-tolerant yeast *Rh. glutinis* showed strong resistance to both aluminium and manganese ions. It was found that *Rh. glutinis* R-l has the ability to adapt to an acidic environment by changing the components of its cell membrane and the shape of its cell envelope, and that the old cells can restore sterile environments such as acidic soil. It took only 5 days to reach neutral pH and the neutralization of the acidified culture could be repeated at least five times. An acidic medium (pH 3.0) containing these ions (100 mM) was shifted to neutral pH by long-term cultivation of the red yeast, suggesting the potential of using this yeast in the bioremediation of acidic soil containing these ions at a high level (Nguyen et al. 2001).

The microorganisms converting TNT quantitatively to the reactive metabolites can be useful for their immobilization through the detoxifying interaction with the soil components, such as humic compounds. Complex environmental contaminations such as explosives and petroleum hydrocarbons are not uncommon and in this regard the strain *Candida* sp. AN-L13, which was isolated earlier as one of the dominant microorganisms from oil-polluted peat bogs (Langepas, western Siberia, Russia), deserves special attention. In addition to its ability to perform the initial TNT conversion step, it is able to utilize crude oil and several individual aliphatic and aromatic hydrocarbons. This strain, as well as other microorganisms with comparable metabolic capabilities, is very interesting not only for academic research but it also possesses vast potential for bioremediation of areas with complex contaminations (Zaripov et al. 2002).

Another yeast was found to be useful for bioremediation purposes, like TNT bioconversion and oil degradation. The ability of a tropical marine strain of *Y. lipolytica* to transform TNT into products such as 2,4-DNT which in turn could be metabolized by other microbes has implications in the use of this yeast in the bioremediation of TNT-polluted marine environments (Jain et al. 2004). Oil contamination of soil and water is found frequently. Accidents are likely to occur in the form of pipeline leaks, train derailments, ship wreckages, storage tank ruptures, transport accidents, etc. Diesel oil, a distillate fraction of crude oil, is one of the major pollutants of soil and groundwater near petrol stations (Margesin and Schinner 1997). Among a number of biotechnological applications, *Y. lipolytica* is used in the bioremediation of soils contaminated by petroleum products (Strouhal et al. 2003). The effect of temperature on oil degradation by this psychrotrophic yeast in liquid culture and in soil was studied by Margesin and Schinner (1997). *Y. lipolytica* showed its capabilities for oil degradation, but the results demonstrated that bioaugmentation is not helpful in the case of oil pollution of alpine soil. The subsoil investigated harbours hydrocarbon-degrading indigenous soil microorganisms that are able to metabolize diesel oil at low temperature more effectively than the psychrotrophic oil-degrading microorganisms introduced. This yeast can be used for remediation of the environment contaminated by various pollutants, including heavy metals, and can grow in the presence of hydrocarbons. The *Y. lipolytica* cells can bind large amounts of heavy metals and produce specific protein metallothionein as a protective agent. This approach offers the possibility to apply the yeast in bioremediation of soil and water contaminated by petroleum products in connection with heavy metals (Strouhal et al. 2003).

15.4.2 "Cold enzymes"

Psychrophilic (cold-adapted) organisms and their products have potential applications in a broad range of industrial, agricultural and medical processes. Relative to this undisputed potential, psychrophiles and their products are under-utilized in biotechnology; however, recent advances, particularly with cold-active enzymes, have heralded rapid growth for this burgeoning field. Psychrophilic enzymes have two properties that have the most obvious biotechnological application: a high specific activity at low and moderate temperatures, and they are inactivated easily by a moderate increase in temperature (Margesin and Schinner 1994; Cavicchioli et al. 2002). These properties can be extremely useful in various applications; these enzymes are both innovative and invaluable. Using X-ray crystallography, these properties are beginning to become understood, and the rules governing their adaptation to cold appear to be relatively diverse. The application of these enzymes offers considerable potential to the biotechnology industry, for example, in the detergent and food industries, for the production of fine chemicals and in bioremediation processes. It is likely that the potential value of cold-adapted enzymes is greater, in view of the diverse capabilities of these enzymes in comparison with the annual market for thermostable enzymes (Gerday et al. 2000). There is a project to examine enzymes from Antarctica, some of which may have commercial potential. Enzymes arising from this work and some of the more obvious applications include α-amylase (used in breadmaking, textiles, brewing and detergents), cellulase (used in textiles and the pulp and paper industries, detergent additives), β-galactosidase (which

eliminates lactose from milk), lipase (used in detergents and flavourings), pectinases (in the fruit juice industry), proteases (used in detergents, meat tenderizing and baking processes), xylanase (baking processes) and enzymes in brewing and wine industries, cheese manufacturing and animal feed. The use of psychrophilic enzymes can be advantageous not only for their high specific activity, thereby reducing the amount of enzyme needed, but also for their easy inactivation. Cold-adapted enzymes offer economic benefits also through energy savings: they negate the requirement for expensive heating steps, function in cold environments and during the winter season provide increased reaction yields, accommodate a high level of stereospecificity, minimize undesirable chemical reactions that can occur at higher temperatures and exhibit thermal lability for rapidly and easily inactivating the enzyme when required (Gerday et al. 2000; Cavicchioli et al. 2002).

There is an industrial trend to treat foodstuffs under mild conditions in order to avoid spoilage and changes in taste and nutritional value at ambient temperatures. Therefore, cold-active enzymes are attractive for the processing of foods. Nakagawa et al. (2002) isolated a psychrophilic basidiomycetous yeast *C. capitatum* strain PPY-1, which could grow on pectin at 5°C. This is the first report of a psychrophilic yeast utilizing pectin as a sole carbon source. Moreover, it seems that the strain produced cold-active enzymes that degrade pectin, although the pectin-depolymerizing enzymes consisted of isozymes. On the basis of these facts, this strain and its pectin-degrading enzymes may be applicable to the food industry (Nakagawa et al. 2002).

Turkiewicz et al. (2003) first reported the extracellular serine proteinase of a *Leucosporidium* strain. The Antarctic enzyme is a newly found subtilase of the clan SB (family S8 of subtilisin and subtilases) and belongs to the group of so-called cold enzymes. This enzyme was termed LAP2 and is the first reported extracellular subtilase of a psychrophilic yeast. The proteinase LAP2 is specific towards synthetic substrates of chymotrypsin and subtilisin.

Ecologically, one of the important substrates is xylan, which is, after cellulose, the most abundant renewable polysaccharide on Earth (Scorzetti et al. 2000). Gomes et al. (2000) discovered another cold-enzyme.This was the first report on the production as well as on the properties of thermolabile xylanase produced by another Antarctic yeast *Cryptococcus adeliae* that exhibits optimal growth at low temperature. In the range from 0 to 20°C, the cold-adapted xylanase displays a lower activation energy and a higher catalytic efficiency. All these observations suggest a less compact, more flexible molecular structure (Petrescu et al. 2000). The cold-adapted xylanase can be used in dough fermentation, protoplast formation and in the wine and juice industry (Cavicchioli et al. 2002).

15.4.3 Glycerol Production

Maintaining high extracellular osmotic pressure is necessary for glycerol accumulation in either batch or continuous mode of operation. Multistage continuous culture techniques seem particularly suitable for possible enhancement of glycerol accumulation since the glucose concentration could be controlled at a relatively high level in the early bioreactors to induce enzyme formation and improve the activity of the enzyme for glycerol synthesis. Higher glycerol yield based on glucose consumed and higher productivity were also obtained with the increase of the initial glucose

concentration. The requirement for relatively high initial glucose concentration was due to the desirable high extracellular osmotic pressure to promote glycerol accumulation. The final glycerol concentration, glycerol yield and productivity could be improved by either raising the feed glucose concentration or employing sodium chloride as a regulator of osmotic pressure. However, the addition of NaCl to media is disadvantageous to downstream processing of product. Therefore, further studies involved higher feed glucose concentrations. A high extracelullar osmotic pressure could result in the efficient metabolic pathway of glycerol that inhibited other alternative metabolic pathways. The results for the effects of feed glucose concentration on continuous fermentation process showed that maintaining the high osmotic pressure in the early stages was necessary for improvements of glycerol yield, glycerol concentration and productivity. It was expected that productivity might be further improved if a multistage cascade bioreactor with cell recycling was used to avoid the disadvantages of low biomass concentration under high osmotic pressure and high dilution rates (Liu et al. 2002).

Trehalose and glycerol may act as protectors inducing a high tolerance to a range of stresses which appear in the production and industrial utilization of yeast. Trehalose is a technologically important parameter for baker's yeast performance as the accumulation of this compound strengthens the strain against stress conditions, namely increasing thermotolerance and storage stability (Carvalheiro et al. 1999). Glycerol production by osmophilic yeasts has a significant potential for large-scale production. The initial glucose concentration, the carbon-to-nitrogen ratio and the aeration rate significantly affected cell growth and glycerol production by osmophilic/halophilic yeast *C. magnoliae* I2B, while the variation of the medium pH in the range 3.5–5.0 had little influence on glycerol production. The availability of oxygen was suggested to be the most important factor controlling cell growth, glucose uptake and yield of glycerol (Sahoo and Agarwal 2001).

There are also some biotechnological applications that cannot be placed in one of the previously described categories, but which are very important in the fields of industry or ecology. Burschäpers et al. (2002) reported the production of sugar alcohols with the osmophilic yeast *Moniliella tomentosa* var. *pollinis* (= *Moniliella pollinis*). This process was conducted as a batch and fed-batch operation in a stirred tank. Kourkoutas et al. (2002) presented continuous wine fermentation using an alcohol-resistant psychrophilic *S. cerevisiae* AXAZ-1 immobilized on apple cuts at different temperatures. This technique was found to be suitable for continuous wine fermentation at temperatures between 5 and 15°C. The application of two halotolerant yeast species *C. versatilis* and *Z. rouxii* in soy-sauce production has been optimized. Immobilization of these two yeasts considerably decreases the total time required for the flavour development in soy-sauce processes. Especially, the application of immobilized salt-tolerant yeasts in a continuous bioreactor proved to be very effective by reducing the processes time by about 90%. This reduction seemed to be caused by the 10–100-fold higher concentration of yeast cells in the immobilized-cell process compared with the concentration in the conventional process. For immobilization of cells poly(ethylene oxide) rather than alginate gel seems to be suitable for use in soy-sauce processes (van der Sluis et al. 2001). Applications for various industrial processes and biotechnology using extremophilic yeasts are summed up in Table 15.2.

Table 15.2 Potential of some extremophilic yeasts for various industrial processes and biotechnology

Source	Industrial process	Advantages	Yeasts	Source
Psychrophiles				
Lipases, neutral proteases	Cheese maturation, dairy production	Stable at low temperatures	*Candida antarctica*	Rothschild and Mancinelli (2001); Cavicchioli et al. (2002)
Glucoamylase	Starch hydrolysis	Stable at low temperatures	*C. antarctica* CBS 6678, *Cryptococcus flavus*	Rothschild and Mancinelli (2001); Cavicchioli et al. (2002); Wanderley et al. (2004)
Proteases, amylases, lipases, cellulases	Degradation of polymers in detergents	Improved performance of detergent	*C. rugosa*, *Yarrowia lipolytica*	Ferrer et al. (2001); Fickers (2005)
Microorganisms	Bioremediation – reduction of oil spills	Works efficiently in cold waters	*Y. lipolytica*	Rothschild and Mancinelli (2001); Margesin and Schinner (1997)
Dehydrogenases	Biosensors, biotransformations		*C. tropicalis*	Takamizawa et al. (2000)
Polyunsaturated fatty acids	Pharmaceuticals, food additives, dietary supplements		*Saccharomyces kluyveri*	Oura and Kaiwara (2004)
Protease	Contact-lens cleaning solutions, meat tenderizing		*Pichia pastoris*	Cavicchioli and Torsten (2000); Sinha et al. (2005)
Subtilase			*Leucosporidium antarcticum*	Turkiewicz et al. (2003)
Ice nucleating proteins	Artificial snow, ice cream, other freezing applications in the food industry		*S. cerevisiae*	Cavicchioli and Torsten (2000); Hwang et al. (2001)
Antifreeze proteins and antifreeze glycoproteins	Binding to ice crystals and inhibiting ice crystal growth		*P. pastoris*	Li et al. (2001)
β-Galactosidase	Lactose hydrolysis in milk products		*Kluyveromyces lactis*	Cavicchioli and Torsten (2000); Becerra et al. (2004)

Continues

Table 15.2 Potential of some extremophilic yeasts for various industrial processes and biotechnology—*cont'd*

Source	Industrial process	Advantages	Yeasts	Source
Pectinase	Food industry	Treatment of foodstuffs under mild conditions in order to avoid spoilage and changes in taste and nutritional value at ambient temperatures	*Cystofilobasidium capitatum*, *Cyst. lari-marini*, *Cr. macerans*, *Cr. aquaticus*	Nakagawa et al. (2002); Birgisson et al. (2003)
Xylanase	Dough fermentation, protoplast formation and wine and juice industry	Lower activation energy and a higher catalytic efficiency	*Cr. adeliae*	Gomes (2000); Petrescu et al. (2000)
Microorganisms	Continuous wine fermentation		*S. cerevisiae*	Kourkoutas et al. (2002)
Halophiles				
Carotene	Food colouring	Inexpensive to produce	*Rhodotorula mucilaginosa*	Libkind et al. (2004)
Glucoamylase gene	Fermentation of starch		*Arxula adeninivorans*	Wartmann and Kunze (2000)
Glycerol, compatible solutes	Protein and cell protectants in a variety of industrial uses, pharmaceuticals	Inexpensive to produce	*C. magnoliae*	Rothschild and Mancinelli (2001); Sahoo and Agarwal (2001)
Membranes	Surfactants for pharmaceuticals		*C. bombicola*	Shepherd et al. (1995); Guilmanov et al. (2002)
Lipids	Liposomes for drug delivery and cosmetic packaging			Cavicchioli and Torsten (2000)
Microorganisms	Waste transformation and degradation		*C. utilis*	Cavicchioli and Torsten (2000); Zheng et al. (2005)
Microorganisms	Flavour development in soy-sauce processes		*C. versatilis*, *Zygosaccharomyces rouxii*	van der Sluis et al. (2001)

Eukaryotic homologues	Cancer detection, screening antitumor drugs			Cavicchioli and Torsten (2000)
Osmophiles				
Glycerol	Cosmetic, paint, tobacco, food, and pharmaceutical industries		*C. krusei*	Zhang et al. (2002)
Sugar alcohols	Production of sugar alcohols in stirred tank (batch and fed-batch operation)		*Moniliella tomentosa var. pollinis*	Burschäpers et al. (2002)
Genes phbA, phbB and phbC	Production of polyhydroxy-alkanoates		*A. adeninivorans*	Terentiev et al. (2004)
Acidophiles				
Microorganisms	Organic acids and flavour		*C. versatilis*, *Z. rouxii*	van der Sluis et al. (2001)
Alkaliphiles				
Cellulases, proteases, amylases, lipases	Degradation of polymers in detergents	Stable at high pH	Many	Rothschild and Mancinelli (2001)
Alkaline phosphatase	Molecular biology		*S. cerevisiae*	Cavicchioli and Torsten (2000)
Others				
Viable cells	Bioremediation of TNT polluted marine environments		*Y. lipolytica*	Jain et al. (2004)
			Candida sp. AN-L13	Zaripov et al. (2002)
Viable cells	Bioremediation of acidic soil containing aluminium and manganese ions at a high level		*Rh. glutinis*	Nguyen et al. (2001)
Viable cells	Copper elimination from wastewaters, sewage sludge and compost by bioaccumulation		*P. guilliermondii*	de Silóniz et al. (2002a)

Continues

Table 15.2 Potential of some extremophilic yeasts for various industrial processes and biotechnology—*cont'd*

Source	Industrial process	Advantages	Yeasts	Source
Viable cells	Metal bioremediation		*S. cerevisiae*	Stoll and Duncan (1997); Malik (2004)
Viable or dead cells	Biosorption of dyes from aqueous solutions		*S. cerevisiae*, *Schizosaccharomyces pombe*, *K. marxianus*, *Candida* sp., *C. tropicalis*, *C. lipolytica*, *C. utilis*, *C. guilliermondii*, *C. membranifaciens*	Dönmez (2002); Aksu and Dönmez (2003); Aksu (2004)
Viable cells	Aerobic biological processes for the removal of dyes from textile effluents		*K. marxianus*, *C. zeylanoides*	Aksu (2004)

15.5 Conclusion

Discussing extreme environments it is difficult to draw the line as to what is extreme in terms of organisms, since this conclusion is mainly based on human standards of extremes. Certainly, we put in the extreme group of microbes what we consider to be extreme according to the standards and knowledge from known data for physiological behaviour of organisms. We still do not use energy consumption as a regular indicator to measure extreme surroundings in specifically selected universal biomarkers, which we could apply in this process. We believe that in the future of "omics" we will develop suitable energy markers which will bring us a new toll for clustering organisms in terms of extremeness. This will be connected to stress response as well, and some traditional stress mechanisms will probably become universal markers for stress sensing in extreme environments. Among other issues we see cultivation and isolation of particular species from natural milieu as very important and should be carefully considered. In particular we should also focus on "viable but not cultivable" phenomena since organisms from this group deserve special attention Owing to their covered activity and consequently we will see development in this direction as well.

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Chapter 16

Yeast Biodiversity in the Antarctic

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16.1 Introduction

Yeast biodiversity in the Antarctic should be expected to be low. Biodiversity may be rich when an area provides varied habitats and/or when varied functions are assumable in a community but is richest when the organic energy supplies are large enough to support specializations in habitat utilization and community function. Primary productivity on the Antarctic continent is very low. It is inhibited by low temperatures and the resulting low availability of liquid water as well as by comparatively low insolation. Yeasts are saprophytes for which any other than a simple degradative role would be difficult in the Antarctic. Despite this, a variety of yeast species have been reported from Antarctic sources, and new species continue to be described. But it is impractical to apply biodiversity indices to Antarctic yeasts. Biodiversity indices require accurate identification of some systematic unit, typically species, and of the indigenicity of the populations identified. Both of these requisites are problematic in the Antarctic.

The problem of identification can be illustrated by the example of *Cryptococcus albidus*, a valid species with a readily available type culture to which other isolates can be compared. "*Cr. albidus*" isolates from continental Antarctica have been reported by di Menna (1960, 1966), Soneda (1961), Babyeva and Golubev (1969), Cameron et al. (1971), Artamonova and Krasnilnikov (1972), Atlas et al. (1978), Abysov et al. (1983), del Frate and Caretta (1990), and Baublis et al. (1991). Subsequent to these reports, Fonseca et al. (2000) used the currently popular method of sequencing the D1/D2 areas of ribosomal DNA (rDNA) to establish eight new species, elevate two former varieties to specific rank, and reinstate two synonyms in specific rank. In the apparent absence of current cultures, these Antarctic isolates cannot be assigned to any of the 13 resulting species, yet their accurate identification might well result in significant changes in any formal diversity index. While species described since the advent of such sequencing are stable for the nonce, many isolates have been ascribed to older species which have not been subjected to such broad examination as the *Cr. albidus* clade. Earlier attributions to such species as

Cr. laurentii (Sugita et al. 2000; Takashima et al. 2003) and *Rhodotorula minuta* (Fell et al. 2000), subsequently shown to lack homogeneity, cannot be taken at face value. Unidentifiable Antarctic isolates attributed to *Cr. albidus*, *Cr. laurentii* (di Menna 1960, 1966; Tubaki 1961; Babyeva and Golubev 1969; Atlas et al. 1978), and *Rh. minuta* (di Menna 1966) will not be further referred to; unreliable or tentative attributions to other species have been omitted (with one exception) from the tables. The practice of depositing voucher cultures in culture collections and the availability of sequencing at reasonable cost has already begun to obviate this problem. Sequencing of the D2 area alone suffices to distinguish most of the currently valid basidiomycetous yeast species – this is available commercially for submitted cultures so that the collecting laboratory need not be equipped to do more than the usual (now much expanded) phenotypic tests. The ecologists' need for accurate identification has made too much of the older literature useless.

Indigenicity is best indicated by the ability to reproduce in the habitat from which a microbe is isolated. Reproduction is fairly easy to observe in macrobiota, but somewhat more difficult in the case of microbiota. As applied to the Antarctic, this criterion is particularly important because microbes enter the region carried by wind, ocean currents, birds, humans, and other animals. Yeasts were reported as "a minor component" of airspora by Meyer (1962), have been isolated from a mummified penguin (Baublis et al. 1991), bird dung, and skua's nests (see later), and are common around field camps (see later). Such yeasts may survive for long periods under cold and dry conditions without being able to reproduce and might actually outnumber any natives in a given sample. Dehydrated baker's yeast cached at Cape Evans by the Scott Expedition contained both viable *Saccharomyces cerevisiae* and *Rh. pallida* after 50 years (Meyer et al. 1962).

Direct evidence of reproduction is possible. The insertion of sterile glass slides and pedoscopes into Antarctic soil has allowed essentially direct observation of microbial reproduction (Vishniac and Mainzer 1973; Uydess and Vishniac 1976) although no yeasts were seen. Wynn-Williams (1980, 1982) used laboratory manipulations of peat cores from Signy Island as microcosms to demonstrate effects on the seasonal reproduction of native yeasts there. Both of these methods rely on the presence of sufficiently large populations to make their way to the limited surfaces in the first method and to produce significant plate counts (i.e., from 30 to 300 cfu ml^{-1} of menstruum for spread plates) in the case of microcosms.

When such evidence is not available, less reliable indicators may suggest indigenicity without proving it – the possession of adaptations to life in a cold, arid, and energy-poor environment, such evidences of establishment as high population density with dominance in the community and enough genetic variation to imply a center of origin, persistence in the environment, or simply failure to occur elsewhere. Of course, failure to occur elsewhere may reflect only the failure of investigators' time and/or funds or the youth of a new species, while reports of extra-Antarctic occurrences may not need mean establishment in another venue, particularly when a single isolate is being reported. The number of isolations over the years may indicate dormant survival of individuals rather than establishment of a population. Soil culture has been a useful low-technology method of storing yeast isolates for many years; the author's personal collection has revived well after up to 11 years in storage.

Large populations are unusual in energy-poor habitats and in any case these yeasts have not been given the same intense investigation as, say, the origin of the human species. Adaptation is certainly expected of microbes growing in any habitat, but the absence of adaptation to extreme cold, aridity, and starvation need not indicate the tourist rather than the native.

Antarctica does provide less extreme habitats as well as those which caused it for a time to be considered a Martian analogue. The cold is moderated as one proceeds north (with major allowance for elevation and minor allowances for aspect, shading, and radiation received), yet the soil at Lake Vanda in the Ross Desert reached a maximum of 16.4°C and a 90-day austral summer mean of 3.9°C when the maximum at Marble Point on the continental margin was 13.9°C and the 90-day mean was 0.1°C. (To be sure, Lake Vanda experienced 30 freeze–thaw cycles rather than the 10–15 cycles of Marble Point and has frost-free surface soil for around 30 days rather than the 50 days of Marble Point.) The "Asgard Range" (presumably at a higher elevation than Lake Vanda in the Transantarctic Mountains) had no time when the soil surface was continuously above 0°C for several days (Campbell and Claridge 2000). Less extensive observations than the continuous monitoring of Campbell and Claridge have recorded higher temperatures as isolated effects of insolation, though none higher than the surface soil temperatures around active volcanoes where the soil and vegetation surface ranged from 41.5 to 43.4°C, with warm ground around fumaroles reaching 50°C (Bargagli et al. 1996). The highlands of the Ross Desert are nearly barren on the surface but the continental margin and the islands within the Antarctic Ocean are home to a flora of mosses, liverworts, algae, cyanobacteria, and lichens though phanerogams are found only on the Palmer Peninsula and islands of such latitude. (Detailed description of this flora is available from the Australian Antarctic Division. Search for "Antarctic flora" on the net.) The desert highlands are quite arid, but glacial melt streams are seasonally wet and drain into lakes which, like the streams, support cyanobacteria, algae, and the occasional moss. The Antarctic Ocean is productive enough to support large populations of krill.

Yeast biodiversity in the Antarctic has to be described under some disadvantage of taxonomic uncertainty and with recognition of habitat variation and the nature of possible adaptations.

16.2 Psychrophilic Yeasts in Antarctica

The most consistent characteristic of Antarctic habitats is relatively low average temperatures. It is accordingly not surprising that Babyeva and Golubev (1969) isolated more yeasts at 5°C than at "moderate... (20–30°C)" temperatures. Forty percent of their 63 isolates were designated "obligate psychrophiles", failing to grow above 20°C. A caveat – Artamonova and Krasilnikov (1972) remarked that for microbiota of all kingdoms "Antarctic psychrophiles adapt quickly to room temperature", a remark which leads one to suspect the original description. Indigenous yeasts are nonetheless expected to be psychrophiles, psychrophiles which may have to deal with frequent freeze–thaw cycles and with the possibility of temperature excursions above T_{max} as well as far below freezing.

Psychrophily, though literally "cold-loving", has been most usefully defined as failing to grow at 25°C. Maximum growth temperatures are usually available, while minimum growth temperatures (T_{min}) are often not determined – indeed, may be impossible to determine for psychrophilic yeasts because of the toxicity of the compounds required to prevent media from freezing (Larkin and Stokes 1968). The lowest temperatures at which currently recognized Antarctic psychrophiles are known to grow are –3°C for *Cr. vishniacii* (Vishniac and Hempfling 1979a) and below –7 C for *Mrakia frigida* (Fell et al.) (as *Candida frigida*, *C. gelida*, and *C. nivalis*) (Larkin and Stokes 1968).

Antarctic strains of psychrophilic yeasts of known T_{max} are listed by species in Table 16.1. This list contains a presently unreliable identification: that of *Rh. aurantiaca*. At the time the two strains listed were identified this species was heterogeneous (Inacio and Fonseca 2004). The isolate A19 of Sabri et al. (2000) is apparently available at the Mycotheque of the University of Louvain-la-Neuve, Belgium (registration number 40,267), but does not have a deposited rDNA sequence and differs in T_{max} from the better known mesophilic strains [T_{max}>25°C, V30°C,V35°C, <37°C (Barnett et al. 2000), >25°C, <30°C (Inacio and Fonseca 2004)]. These strains were included because, while their identification is presently uncertain, there is some prospect of confirming the identity at least one of them.

Four of the listed species – *C. psychrophila*, *Cr. vishniacii*, *Leucosporidium antarcticum*, and *M. frigida* – probably are obligate psychrophiles since they were first described (though not necessarily by the same epithets) over 35 years ago yet no mesophilic strains have surfaced. *L. antarcticum* has been isolated in Germany, from a mature willow catkin near Neubrandenburg, but the temperature of cultivation was not mentioned (Kockova-Kratochvilova et al. 1972). Mesophilic strains are known for *Cr. victoriae*, which was the dominant species in soil from Nome, Alaska (personal observation); apparently minor occurrences were recorded in Portuguese seawater (Gadanho et al. 2003), Thuringian roots (Renker et al. 2004), and Mississippian lacewing guts (Woolfolk and Inglis 2004). Some 27 % of isolates from a soil sample from Providenya (Russian Far East) were identified as *Cr. watticus* or a very close relative with T_{max} ranging from below 25°C up to 30°C or above (personal observation). *Cr. antarcticus* isolates with T_{max}>25°C represented significant proportions of yeasts in soils from Iceland (14% at one site, 20% at another) and 6% of isolates from Providenya (personal observation). *Dioszegia hungarica* Zsolt (Barnett et al. 2000 as *Cr. hungaricus*) and *L. scottii* have well-known mesophilic strains (Barnett et al. 2000). With some reservations and explanations required, mesophilic strains also exist for *C. sake* and possibly for *Cr. friedmannii*. *C*. (*Torulopsis*) *austromarina* was synonymized with *C. sake* on the basis of the identity of the D1/D2 regions of rDNA during the only detailed correlation of differences in these sequences with nuclear DNA (nDNA)/nDNA similarities (Kurtzman and Robnett 1998) but without a comparison of the nDNAs of these particular species. The case for synonymy is its probability; sequence identity can accompany a 46% nDNA reassociation, indicating lack of conspecificity, but all the other examples of sequence identity in Table 2 of Kurtzman and Robnett's paper show conspecificity of the tested strains. The reason for doubting this synonymy is the very wide range of T_{max} which would then appear in *C. sake*, the 15 degrees between 22° C

Table 16.1 Psychrophilic yeasts from Antarctic sites

Species	Substrate and site	T_{max}	Reference	Reported as
Candida psychrophila	Penguin dung, Cape Royds	>17°C, <20°C	Goto et al. (1969)	*Torulopsis*
Candida sake	Seawater, Antarctic Ocean	18–21°C	Fell and Hunter (1974)	*Torulopsis austromarina*
Cryptococcus antarcticus	Damp soil and dry drainage channel, University Valley	>15°C, <20° to <25°C	Vishniac and Kurtzman (1992)	
Cryptococcus friedmannii	Rock with cryptoendolithic lichen	>20°C, <25°C	Vishniac (1985)	
Cryptococcus nyarrowii	Snow petrel carnage, soil and lichen	22°C	Thomas-Hall and Watson (2002)	
Cryptococcus statzelliae	Soil, Lichen Valley, Vestfold Hills	22°C	Thomas-Hall et al. (2002)	
Cryptococcus victoriae	Moss, lichen, soil, Granite Harbour	<20°C	Montes et al. (1999)	
	Soil, Lichen Valley, Vestfold Hills	<25C	Thomas-Hall et al. (2002)	
Cryptococcus vishniacii	Soil, arid highlands, Ross Desert	>15°C, <23–25°C	Vishniac and Hempfling (1979a)	
Cryptococcus watticus	Soil,Vestfold Hills; stromatolite and shell, Watts Lake	>20°C, weak 25°C, <30°C	Guffogg et al. (2004)	
	Soil, Edmonson Point	>15 to >22°C, <23°C	Tosi et al. (2005)	
Dioszegia hungarica	Taylor Valley melt stream	< room temperature (~23°C max)	Personal observation	
Leucosporidium antarcticum	Seawater NE end of Palmer Peninsula	>12°C, weak 17°C, <19°C	Fell et al. (1969)	
	Taylor Valley melt stream	< room temperature (~23°C max)	Personal observation	
Leucosporidium scottii	Soil ± algae, Granite Harbour, Wright Valley, Ross Island areas	"~15°C"	di Menna (1960,1966)	*Candida scottii*
	Moss, penguin rookery, Haswell Island	<20°C	Babyeva and Golubev (1969)	*Candida scottii*
	Mosses, Cape Evans; lakeside algae, Lake Fryxell	>15°C, <20°C	Goto et al. (1969)	*Candida scottii*
Mrakia frigida	Soil ± photosynthesizers, "Ross Dependency"	"~20°C"	di Menna (1966)	*Candida frigida, gelida, nivalis*
	Antarctic snow	>20°C, <25°C	Sinclair and Stokes (1965)	*Candida* sp. P216

(Fell and Hunter 1974) and 37^0 C (Barnett et al. 2000), a range which exceeds the normal (Vidal-Leira et al. 1979; van Uden 1984). *Cr. friedmannii* is at the least closely related to *Cr. saitoi*, a species proposed (Fonseca et al. 2000) largely on the basis of differences from *Cr. friedmannii* in D1/D2 rDNA sequences of an extent not generally accepted as contraindicating conspecificity, even when internal transcribed spacer sequences (Scorzetti et al. 2002) are also considered. If *Cr. saitoi* does not have sufficiently low nDNA similarity to *Cr. friedmannii* to complete its establishment as a valid species, its T_{max} of "approximately 30°C" would disqualify *Cr. friedmannii* as obligately psychrophilic. *Cr. nyarrowii* and *Cr. statzelliae* have been too recently described for any definite decision about the obligate nature of their psychrophily.

The D1/D2 large subunit rDNA based phylogeny of the species listed in Table 16.1 suggests that psychrophily has arisen independently several times when mesophilic ancestors became adapted to Antarctic habitats. *C. psychrophila* and *C. sake* (in which psychrophily is confined to the former *T. austromarina*) are both in the *Debaryomyces*/*Lodderomyces* clade of ascomycetous yeasts, but in distant branches of this clade (Kurtzman and Robnett 1998). Of the basidiomycetous species, *Cr. antarcticus, Cr. friedmannii*, and *Cr. vishniacii* have all been placed in neighboring branches of the *Albidus* clade of *Filobasidiales*, *Hymenomycetes* (Fonseca et al. 2000). Each species is there most closely associated with mesophilic species. *Cr. nyarrowii* (Thomas-Hall and Watson 2002) and *Cr. watticu*s (Guffogg et al. 2004) were described as most closely related to each other and then to *Holtermannia corniformis* in the *Tremellales*, *Hymenomycetes* (T_{max}>25°C, <30°C). *Cr. statzelliae* appears in the otherwise borderline (T_{max}>20°C, V25°C, <30°C) *Dioszegia* clade of the *Tremellales* (Thomas-Hall et al. 2002) including *Dioszegia hungarica. Cr. victoriae* sits in yet another tremellalean lineage as near neighbor to *Cr. dimennae* and *Bullera globispora* (Thomas-Hall et al. 2002), both with T_{max}>25°C. The genus *Leucosporidium* contains one mesophilic species (Barnett et al. 2000); the psychrophilic species are separately embedded among mesophiles in the *Microbotryum* clade of the *Urediniomycetes* (Fell et al. 2000). *Mrakia* is a monospecific genus grouping most closely with the psychrophile *Cr. aquaticus* in the *Cystofilobasidiales*, *Hymenomycetes* (Fell et al. 2000), an order with varied T_{max}.

16.3 Effects of Temperature on Antarctic Psychrophiles

Life in a cold habitat requires a complex suite of adaptations; life in or on Antarctic soils is further complicated by inconstancy of habitat temperatures. Even on continental Antarctica, excursions in both directions occur. Soil surface temperature at a site in Taylor Valley (Ross Desert) reached 23°C under insolation during the austral summer (Cameron 1974) and 26°C at another desert site (Cameron 1971). Even greater temperatures need not result in damage to Antarctic psychrophiles. Van Uden et al. (1968) pointed out that the rate of thermal death for two strains of *M. frigida* (as *C. frigida* and *C. nivalis*) was dissociated from growth rates, unlike the death rates of eight mesophilic yeasts; the psychrophiles died in roughly the same temperature range as the mesophiles. More recently Deegenaars and Watson (1997) reported that "essentially 100%" of stationary-phase *C. psychrophila* survived a

60-min exposure to 35°C. Cameron noted that "for approximately three to four weeks during midsummer, many Antarctic soils are subjected to diurnal freeze–thaw cycles" (Cameron 1971). While the psychrophiles obviously survive these conditions, studies of freeze–thaw resistance have been largely vitiated by the unnatural conditions under which they were done, for example, that which concluded that a psychrophilic yeast (*M. frigida* as *L. stokesii*) was less resistant to freeze–thaw stress than a mesophile (*C. utilis*, anamorph of *Pichia jadinii*) even when the growth stage and the rate of cooling below −5°C were considered (Meyer et al. 1975). Such studies are more realistically conducted in microcosms, as when indigenous (unidentified) yeasts appeared in greater biomass after four and seven freeze–thaw cycles of Signy Island peat cores (Wynn-Williams 1982).

The role of unsaturated fatty acids in maintaining membrane function at varying low temperatures has long been recognized throughout the biological world. The unsaturation index calculated by Watson's formula [percentage monoenes + 2(percentage dienes) +3(percentage trienes)/100] (Watson et al. 1976) increased with decreasing temperature in *M. frigida* (as *L. frigidum*, *L. gelidum*, *L. nivalis*, in Watson et al. 1976) and in *C. psychrophila* (as *Torulopsis*) and the psychrophilic strain of *C. sake* (*T. austromarina*) (Watson 1980). Comparable data for *Cr. antarcticus*, *Cr. statzelliae*, *Cr. nyarrowii*, and *L. scottii* are shown in Table 16.2. Table 16.2 illustrates the dependence of unsaturation on temperature and the high degree of unsaturation of psychrophiles, but should not be used for precise comparisons, as the index is affected by medium and growth phase as well as temperature. Mesophilic yeasts do, however, tend to have similar index numbers when grown at suboptimal temperatures. *C. lipolytica* (anamorph of *Yarrowia lipolytica*), when grown at 10°C, had index numbers of 1.07 and 1.27 (calculated from Kates and Baxter 1962). *C. saitoana* (as *T. candida*), a mesophilic yeast grown under the same conditions as the *C. psychrophila* and *C. sake* psychrophiles and showing the same pattern of increasing unsaturation with decreasing growth temperature, produced a more similar unsaturation index at 15°C (1.42), though not the same distribution of fatty acids (Watson 1980). (The distribution of individual fatty acids tends to be species-specific.)

When *M. frigida* (as *L. stokesii*) attempted to grow at 23°C (a temperature barely above T_{max}), increased cell size, irregular wall growth, and the production of anucleate and aseptate buds ensued (Silver and Sinclair 1979). This was not lethal; downshift to 15°C restored normal morphology. The mechanisms underlying these defects were not entirely clear but failure of DNA synthesis at 23°C was considered to account for the anucleate condition of the buds, while the persistence of RNA synthesis (impaired at 25°C) and protein synthesis (unaffected at either 23 or 25°C) accounted for the increase in cell size (Silver et al. 1977). While inappropriately fluid membranes do leak in this species (studied as *C. nivalis*) at 25°C (Nash and Sinclair 1968), other systems begin to fail as well. Ninety percent of polypeptide synthesis activity in a cell-free ribosome preparation was lost after 5 min at 35°C and polypeptide synthesis had vanished entirely at 40°C, though only 60% of charged soluble RNA (sRNA) binding activity was lost at the latter temperature. Thirty minutes at 35°C abolished the activity of three essential aminoacyl–sRNA synthetases, and greatly diminished the activity of three more, though five escaped injury (Nash et al.

Table 16.2 Fatty acid unsaturation in Antarctic psychrophilic yeasts

Strain	Growth temperature (°C)	Unsaturation index	Reference
Candida psychrophila CBS 5956	0	1.78	Watson (1980)
	1	1.37	
Candida sake CBS 6179	0	1.87	
	15	1.44	
Cryptococcus antarcticus CBS 7687	14	1.33	Vishniac and Kurtzmann (1992)
Cryptococcus antarcticus CBS 7688	14	1.46	
Cryptococcus antarcticus CBS 7689	14	1.18	
Cryptococcus antarcticus CBS 7690	14	1.42	
Cryptococcus nyarrowii CBS 8804	6	1.39	Thomas-Hall (personal communication)
	15	1.34	
Cryptococcus nyarrowii CBS 8805	6	1.55	
	15	1.38	
Cryptococus statzelliae CBS 8925, 8926	15	~1.31	Thomas-Hall et al. (2002)
Leucosporidium scottii AL25	10	1.71	Kates and Baxter (1962)
Leucosporidium scottii 5AAP2		1.69	
Mrakia frigida CBS 5270	8	1.93	Watson (1980)
	19	1.32	
Mrakia frigida CBS 5272	8	1.5	
	18	1.41	
Mrakia frigida CBS 5266	8	1.82	
	18	0.91	

1969). Thirty minutes at 35°C also inactivated another thermolabile enzyme, pyruvate decarboxylase (Grant et al. 1968).

Increased cell size and leaking were also noted by Sabri et al. (2001) in *Rh. aurantiaca* A19 (a psychrophilic strain) grown at 18°C. They ascribed the leaking to a toxic accumulation of myristoyl-cofactor A (CoA), which was in turn ascribed to characteristics of a long chain acyl-CoA thioesterase in this strain. Substrate affinity and catalytic efficiency were optimal below 5°C and decreased dramatically with increases in temperature, corresponding to the buildup of myristoyl-CoA.

The question of the structural differences which made these enzymes inefficient or thermolabile and, indeed, why some enzymes (but not all) need to be so closely tailored to certain temperature ranges has not been examined in any of these Antarctic psychrophiles. It has been suggested that the catalytic efficiency required to maintain adequate metabolic rates at low temperatures requires methods of obtaining conformational flexibility which render the thermolabile enzymes unstable at higher temperatures – a trade-off (Feller and Gerday 1997). These authors adduced, among other examples, the xylanases of a pair of mesophilic cryptococci of the *Albidus* clade – *Cr. adeliensis* (as *Cryptococcus* TAE85), which has a cold-adapted enzyme, and *Cr. albidosimilis* (as *Cr. albidus*), which does not. (A note to clarify the identity of these yeasts: *Cr. adeliensis* CBS 8351, ATCC 201412, has also been referred to as *Cr. albidus* TAE85, and *Cr. adeliae* – a nomen nudum; ATCC 34633, referred to as *Cr. albidus* by Feller and Gerday, was identified as *Cr. albidosimilis* by Scorzetti et al. 2000.) The catalytic efficiency (K_{cat}/K_m) of the cold-adapted and thermolabile xylanase of *Cr. adeliensis* was nearly 4 times higher at 4°C (largely because of an increase in K_{cat}). Comparison of their molecular structures indicated that the cold-adapted enzyme had less compact hydrophobic packing, lost one salt bridge, and destabilized the macrodipoles of the helices (Petrescu et al. 2000). These are changes which are considered to increase both flexibility and thermolability (Feller and Gerday 1997).

Exponential phase cells do not have the thermotolerance of stationary-phase cells. Less than 0.1% of the *C. psychrophila* cells which survived a 60-min exposure to 35°C would have survived had they been in the exponential phase (Deegenaars and Watson 1997). Although unsaturated fatty acids are typically more abundant in stationary-phase cells, membrane fatty acid composition and membrane fluidity are unrelated to thermotolerance in *Saccharomyces cerevisiae* (Swan and Watson 1997) – this point has not been studied in psychrophiles. Thermotolerance can, however, be induced. Psychrophilic yeasts, like most living things, have a buffer for sudden and stressing raises in temperature – the heat shock proteins (hsps). *C. psychrophila* cells grown at 15°C acquire induced thermotolerance after mild heat shocks for 30 min at 25°C or 3 h at 20°C, with the latter and better pretreatment providing roughly 90% survival after 2 h at 35°C. Cells with induced thermotolerance contained two intrinsic hsps (hsps 60 and 90) and three induced hsps: hsp 70 and novel heat-shock-inducible proteins at 80 and 110 kDA (Deegenaars and Watson 1997). The 110-kDa protein was also inducible in *M. frigida* (as *M. stokesii*, *M. frigida*, and *M. gelida*) but not in *L. antarcticum*. Mesophilic strains of *L. scottii* and *L. fellii,* lacking hsp 60, also contained hsp 104, a protein lacking in the psychrophiles but considered important for thermotolerance in *Saccharomyces cerevisiae* (Deegenaars and Watson 1998).

16.4 Other Niche Determinants for Psychrophilic Yeasts

The variety of isolate sources listed in the second column of Table 16.1 implies the presence of more than one series of habitat types in Antarctica, suggesting that factors other than temperature may define the niches of Antarctic psychrophiles. Do isolate sources describe ecological niches or simply the coincidence of investigators and accidental yeast occurrences? The list of taxonomically approved isolations in Table 16.1 is short and would be little longer if T_{max} for each isolate were not required; it does not provide enough instances to directly derive niche definitions. The isolations of *C. psychrophila* and *Cr. friedmannii* (but see the taxonomic discussion above) certainly do not warrant even speculation. The factors suggested are highly correlated – water and organic energy availability form a geographically irregular cline in Antarctic soils, from the arid highlands of the Dry Valleys (Ross Desert) in the Transantarctic Mountains to the better watered and vegetated glacial melt streams and lakes and the continental margin and finally the embracing Antarctic Ocean. The distribution of significant populations more or less follows this cline.

The arid highlands of the Dry Valleys are the habitat of *Cr. vishniacii*. This species, though occurring at a population density of less than one microcolony per gram of soil and sometimes failing to occur in samples producing single mesophilic isolates, dominated overall in soil samples from the arid highlands (Vishniac and Hempfling 1979a, b; and, under a variety of synonyms, Klingler and Vishniac 1988a). Many soil samples from this area failed to yield any yeasts, a failure attributable to the high salt content typical of Antarctic soils (Klingler and Vishniac 1988b; Vishniac and Klingler 1988). This salt content had been modified, presumably by occasional snow melt, in the samples in which *Cr. vishniacii* was found. *Cr. vishniacii* is xerotolerant but not halotolerant or osmotolerant. None of the cryptococci have been reported to be halo- or osmotolerant, though a model cryptococcus, *Cr. albidus*, successfully competed with other soil microbiota only under conditions of low water potential (Vishniac 1995). The ability of *Cr. vishniacii* to survive solely on the meager deposits of substrates from the exfoliation of cryptoendolithic lichens (Friedmann and Weed 1987) and the ablation of organic matter from the Taylor Valley lakes (Lake Chad and Lake Hoare) (Parker et al. 1989) indicates that this species is also oligotrophic.

The melt streams of glaciers in the Dry Valleys provide both water and, in season, a varied population of photosynthesizing algae and cyanobacteria as energy sources. The soil adjacent to melt streams and lakesides also support a varied flora as well as a quite limited fauna. Di Menna (1966) collected soil samples between the glaciers of the "Ross Dependency." Since the majority of the samples contained visible algae, lichens, or mosses and many of those lacking visible producers were moist with melt water, it is not surprising that she recorded yeast populations far greater than those of the arid highlands. Six samples taken between Nimrod and Beardmore Glaciers, lacking visible or culturable producers, contained 12–40 cfu g^{-1} of soil; moist soil containing producers contained 10^4 (Koettlitz Glacier, Campbell-Mawson Glaciers) to more than 10^5 cfu g^{-1} (Mawson-Koettlitz Glaciers).

A dry melt stream in University Valley was the home of *Cr. antarcticus*. Although the population density of *Cr. antarcticus* was low, persistence over 2 years

and the isolation of four biotypes strongly suggest a center of population in this habitat. *Cr. antarcticus* is more halotolerant and less oligotrophic than *Cr. vishniacii* (Vishniac and Kurtzman 1992). An active melt stream in Taylor Valley produced yeasts with larger populations and greater diversity. The aqueous phase of a sample contained 70 cfu ml^{-1}, the solid phase 32.26 microcolonies per gram of which some 70% were identified as *Dioszegia hungarica,* the remainder comprised *L. antarcticum* (Vishniac and Klingler 1988, personal observation of density and *Leucosporidium* sp. identification) and two isolates tentatively identified (i.e., not confirmed by sequencing) as *M. frigida* (as *C. curiosa*) and *Rh. foliorum* (as *C. foliorum*). *L. antarcticum* appears to be an aquatic yeast, since it was originally described from a large population (46 isolates) collected at four oceanic sites off of Joinville Island, at the tip of the Palmer Penninsula, from ocean water at –0.19 to –1.70°C at the time of collection (Fell et al. 1969). *L. antarcticum* has also been reported from a peat bog in Russia (Golubev et al. 1981 – cited from Summerbell 1983) and from a willow catkin in northeast Mecklenburg (Germany) (Kockova-Kratochvilova et al. 1972). *Dioszegia hungarica*, named for its geographic origin (Zsolt 1957 – cited from Barnett et al. 2000), includes strains with T_{max}>25°C and is well known from substrates other than water – soil, cereals, and flowers at a number of sites outside of Antarctica.

The Vestfold Hills best represent yeast diversity on the continental margin. Davis Station provided three recently described species – *Cr. nyarrowii*, *Cr. statzelliae*, and *Cr. watticus*, species whose niche is difficult to ascertain, as well as novel isolates of *Cr. victoriae*. Their descriptions provide insufficient evidence for declaring this habitat a center of population, mentioning only two isolates of each species and being recent for species recognition in other venues. *Cr. watticus* does occur at another site on the continental margin (Tosi et al. 2005) and at sites in the Russian Far East (Yttygran Island), Alaska (Nome), and Iceland (personal observation). Another obligate psychrophile, *M. frigida*, appears widespread in the northern hemisphere, in soil from Greenland (di Menna 1966) and high-latitude Russian regions [Babyeva et al. 1976; Babyeva and Azieva 1980 (unavailable papers cited from Summerbell 1983); Polyakova et al. 200l], and in Nome and Yttygran Island (personal observation). The other obligately psychrophilic species have been unsuccessfully searched for, using appropriate methods, in Russian Far Eastern, Alaskan, and Icelandic soils by the author as well as by the observers cited. *Cr. watticus*, and *M. frigida* are therefore considered to form significant populations only in relatively rich habitats, as are the sometimes mesophilic *Cr. victoriae* and *L. scottii. Cr. victoriae* has been reported from three widely separated locations outside of the Antarctic and was the dominant yeast species in a well-watered and densely vegetated soil sample from Nome (Alaska) (personal observation). The wide distribution of *L. scottii* in boreal and temperate areas was both reviewed and investigated by Summerbell (1983).

The Antarctic Ocean is the home of the psychrophilic strain of *C. sake* (i.e., the former *T. austromarina*). It is well represented there. A total of 85 strains were isolated from two sectors of the Antarctic Ocean with temperatures of 2.06–3.59°C at the time of collection. As it has not been reported from elsewhere, it may be considered a marine yeast.

16.5 Mesophilic Yeasts in Antarctica

The much longer list of Antarctic mesophilic isolates is given in Table 16.3. This table includes some isolates which were described with T_{max}<25°C; the species to which they are attributed all have T_{max}>25°C according to Barnett et al. (2000). All of the listed species except the recently described *Cr. adeliensis* occur outside of Antarctica, even *Cr. albidosimilis,* which was isolated from sparrow plumage in the Czech republic (Scorzetti et al. 2000) and *Pseudozyma antarctica*, which was isolated from rice in Japan by Ito et al. (1974) as *Trichosporon oryzae*. Tubaki (1961), one of the earlier investigators of Antarctic yeasts, dismissed his mesophilic isolates as "not of the Antarctic". Table 16.3 does omit isolates from the food cache of the Scott Expedition as obviously "not of the Antarctic", but does include others which were quite possibly imported. The situation is ambiguous for several reasons: some mesophiles are psychrotolerant; the border between psychrophily and mesophily is artificial (T_{max} is a continuous variable); and the coastal areas include active volcanoes at which the soil temperature can reach 50°C (Bargagli et al. 1996). The coastal areas are heterogeneous but in general are relatively rich in plant and animal life; their soils are lower in salinity. The soils at Casey Station (on the coast of eastern Antarctica) have mean total organic carbon contents ranging from 5 to 45 mg g^{-1} in the surface layer (Beyer et al. 2004a), including compounds comparable to those found in other podzols (with the exception of lignin and its derivatives; mosses do not produce lignin) (Beyer et al. 2004b).

It may be possible for mesophiles to colonize the milder areas. The soils of volcanic areas have not been adequately explored. The sites from which the isolates of Table 16.3 were obtained are almost all in areas with the higher photosynthetic productivity that accompanies increased water availability. Investigators have naturally preferred to search readily available sites in the vicinity of the coastal bases and moister sites in the justified hope of obtaining greater results. Reports of mesophile presence become more meaningful as survival of yeast cells becomes less assured; zymophagous invertebrates also inhabit the less arid and better vegetated areas. Protozoa and nematodes are capable of outstripping yeast reproduction at 1.5°C. Protozoa studied in a peat core from Signy Island (held at 1.5°C) consumed 4.3 yeast cells per amoeba per day, nematodes 8.8 yeast cells per nematode per minute (Wynn-Williams 1983). Taking all of these factors, and proximity to human activity, into account, we can group these isolates in order of the probability that they are "of the Antarctic."

The most probable candidates for indigenicity are *Guehomyces pullulans* and *Cr. adeliensis. Guehomyces pullulans* [a comb. nov. for *Trichosporon pullulans* (Fell and Scorzetti 2004)], a common soil yeast, includes psychrophilic strains (Barnett et al. 2000). Such psychrophilic strains constituted 24% of yeasts in a soil sample taken near Vatnajokul (Iceland) (personal observation). An Arctic isolate (Grise Fjord, Northwest Territory, Canada) was highly psychrotolerant, capable of growth at a k (generations/time in hours) value of 0.020 at 0°C (Julseth and Inniss 1990b) (k=0.231 at the "optimal" temperature of 21°C; growth was inhibited at T_{max}=30°C with k=0.005). One wonders why di Menna (1966) found only a single isolate at Scott Base, one out of the 126 Antarctic soil samples she examined, but three in the

Table 16.3 Antarctic mesophilic yeasts

Species	Substrate and site	T_{max}	Reference	Reported as
Bulleromyces albus	Moss-bed soil, Schirmacher Oasis	>25°C, V30°C, <35°C[a]	Ray et al. (1989)	*Bullera*
Candida albicans	Soil adjacent to camp liquid waste barrel, Taylor Valley	>25°C, V30– 45°C[a]	Baublis et al. (1991)	
Candida parapsilosis	Ice tunnel, South Pole base	>37°C, V40–42°C, <45°C[a]	Jacobs et al. (1964)	
Candida saitoana	Soil containing photosynthesizers, "Ross Dependency"	>30°C, V35–42°C, <45°C[a]	di Menna (1966)	*Torulopsis candida*
	Moss, penguin rookery, Haswell Island	> "room temperature"	Babyeva and Golubev (1969)	
Candida sake	Algae and shore soil, Lake Bonney; penguin dung, Cape Royds	33°C	Goto et al. (1969)	*Candida australis*
Clavispora lusitaniae	Soil adjacent to camp liquid waste barrel, Taylor Valley	>42°C, V45°C[a]	Baublis et al. (1991)	*Candida lusitaniae*
Cryptococcus adeliensis	Decaying algae in ice, near Terre Adelie	>25°C, w30°C	Scorzetti et al. (2000)	
Cryptococcus albidus	Soil, Edmonson Point	>25°C, <30°C	Tosi et al. (2005)[b]	
Cryptococcus albidosimilis	Soil, Linnaeus Terrace (Mt. Oliver/Wright Valley)	>25°C, slow 30°C, <35°C	Vishniac and Kurtzman (1992)	
Cryptococcus diffluens	Soil, Wright Valley	> "room temperature"	di Menna (1960)	
	Moss, penguin rookery, Haswell Island and sites (± lichen) Molodozhnaya Station	> "room temperature"	Babyeva and Golubev (1969)	
Cryptococcus humicola	Water, Lake Vanda, lakeshore soil	>25°C, V30–37°C, <40°C[a]	Goto et al. (1969)	*Candida*
	Soil, Schirmacher Oasis	>25°C, <37°C	Ray et al. (1989)	*Candida*
Cryptococcus luteolus	Pool edge, Lake Vanda; soil ± photosynthesizers, "Ross Dependency"	>25°C, V30– 35°C, <37°C[a]	di Menna (1960, 1966)	
	Soil, The Strand Moraines, McMurdo Station		Atlas et al. (1978)	

Continues

Table 16.3 Antarctic mesophilic yeasts—*cont'd*

Species	Substrate and site	T_{max}	Reference	Reported as
Cryptococcus macerans	Soil + photosynthesizers, "Ross Dependency"	>25°C, <30°C[a]	di Menna (1966)	*Rhodotorula*
Debaryomyces hansenii	Soil, Showa Base	>30°C	Soneda (1961)	*Torulopsis famata*
	Soil, East Ongul Island and Antarctic continent	>25°C, V30–40°C, <42°C[a]	Tubaki (1961)	*T. famata*
	Soil + photosynthesizers, "Ross Dependency"		di Menna (1966)	*T. famata*, *Debaryomyces koeckeri*, *D. subglobosus*
	Moss, penguin rookery, Haswell Island	> "room temperature"	Babyeva and Golubev (1969)	*T. famata*
	Soil from skua's nest, Schirmacher Oasis	>25°C, V30–40°C, <42°C[a]	Ray et al. (1989)	
Guehomyces pullulans	Soil + photosynthesizers, Scott Base	>20°C, V25°C, <30°C[a]	di Menna (1966)	*Trichosporon*
Issatchenkia orientalis	Soil adjacent to camp liquid waste barrel, Taylor Valley	>40°C, V42,45°C[a]	Baublis et al. (1991)	*Candida krusei*
Pseudozyma antarctica	Sediment Lake Vanda	>30°C, <35°C	Goto et al. (1969)	*Sporobolomyces antarcticus*
Rhodosporidium sphaerocarpum	Antarctic Ocean near Palmer Peninsula and Archipelago	30°C	Newell and Fell (1970)	
Rhodosporidium toruloides	Water, Lake Vanda	>30°C, <35°C	Goto et al. (1969)	*Rh. glutinis* var. *refusa*
Rhodotorula aurantiaca	Moss	<20°C	Babyeva and Golubev (1969)	
	Accepted T_{max} range for *Rh. aurantiaca*	>25°C, V30,35°C, <37°C[a]	Barnett et al. (2000)	
Rhodotorula diffluens	Water and sediment, Lake Miers, Lake Vanda	~25°C	Goto et al. (1969)	*Candida*

Rhodotorula glutinis	Moss, penguin rookery, Haswell Island and lichen sites Molodozhnaya Station	>"room temperature"	Babyeva and Golubev (1969)	
Rhodotorula graminis	Soil, Wright Valley and soil with photosynthesizers, "Ross Dependency"	>20°C	di Menna (1960, 1966)	
	Soil, Asgard Range	>30°C, <35°C[a]	Atlas et al. (1978)	
Rhodotorula laryngis	S. Onofri isolate, soil, Edmonson Point	>25°C, <30°C	Tosi et al. (2005)	
Rhodotorula marina	Soils with algae between Campbell and Mawson glaciers	>30°C, <35°C[a]	di Menna (1966)	
Rhodotorula minuta	Water, Lake Miers and Lake Vanda	30°C	Goto et al. (1969) ‡	*Rh. texensis*
Rhodotorula mucilaginosa	Soil, "the continent" and West Ongul Island	>25°C, <30°C	Soneda (1961)	
	Soil + photosynthesizers, "Ross Dependency"	>30°C, V35–37°C, <40°C[a]	di Menna (1966)	
	Moss, penguin rookery, Haswell Island; sites (+ lichen) Molodozhnaya Station	> "room temperature"	Babyeva and Golubev (1969)	
	Water and soil, Lake Vanda, Lake Miers	33°C	Goto et al. (1969)	*Rh. rubra*
	Soil, Victoria Valley	>30°C, V35–37°C, <40°C[a]	Atlas et al. (1978)	
	Lake shore, lake sediment and under rock, Schirmacher Oasis	>25°C, <37°C	Ray et al. (1989)	
	Soil adjacent to camp liquid waste barrel, Taylor Valley	>30°C, V35–37°C, <40°C[a]	Baublis et al. (1991)	*Rh. rubra*
Rhodotorula pallida	Soil + photosynthesizers, "Ross Dependency"	> "room temperature"	di Menna (1966)	
Sporidiobolus johnsonii	Soil, Ross Desert	>25°C	Vishniac and Hempfling (1979b)	*Sporobolomyces holsaticus*
Sporidiobolus pararoseus	Soil and lichens, vicinity of Molodozhnaya Station	> "room temperature"	Babyeva and Golubev (1969)	*Sporobolomyces pararoseus*

Continues

Table 16.3 Antarctic mesophilic yeasts—*cont'd*

Species	Substrate and site	T_{max}	Reference	Reported as
Sporidiobolus salmonicolor	Soil, Wright Valley	>30°C, V35–37°C, <40°C[a]	di Menna (1960)	*Sporobolomyces odorus*
	Soil, Marble Point and Brown Peninsula	>30°C, V35–37°C, <40°C[a]	Atlas et al. (1978)	*Sporobolomyces*
Sporobolomyces roseus	Lakeshore sediment and lichen, vicinity of Molodozhnaya Station	> "room temperature"	Babyeva and Golubev (1969)	
	Soil, Ross Desert	>25°C	Vishniac and Hempfling (1979b)	
Stephanoascus ciferrii	Microbial mat, Lake Hoare	>35°C, V37–45°C[a]	Baublis et al. (1991)	*Candida ciferrii*
Torulaspora delbrueckii	Soil, Victoria Land	>30°C, V35–40°C, <42°C[a]	del Frate and Caretta (1990)	
Trichosporon cutaneum	Soil, Showa Base	>25°C, <30°C	Soneda (1961)	
	Soil, Showa Base	>30°C, V35°C, <37°C[a]	Tubaki (1961)	
	Soil adjacent to camp liquid waste barrel, Taylor Valley		Baublis et al. (1991)	*Trichosporon beigelii*
Trichosporon moniliiforme	Lake water, Lake Vanda	>30°C, <35°C	Goto et al. (1969)	*Tr. cutaneum* var. *antarcticum*

V response is variable (strain-dependent)
[a] T_{max} taken from Barnett et al. (2000)
[b] Other reports of these species are considered unreliable (see text).

eight Greenland soil samples she investigated. The T_{max} of di Menna's isolate was not reported. The Canadian strain produced 11 or 12 inducible hsps (depending on the method of shocking) (Berg et al. 1987; Julseth and Inniss 1990a); it was also the first yeast which evinced the induction of 10–26 cold-shock proteins (csps) (again depending upon the method of shocking) (Julseth and Inniss 1990b). These proteins act as buffers; they do not extend growth beyond the previously determined range of temperatures. The hsps significantly extended survival at 45°C, (but not 50°C); the csps were induced by suddenly switching cells from near optimal temperatures to 5°C, a temperature well within the normal growth range of this isolate. Yet buffers may be very useful when sudden changes in insolation occur.

Cr. adeliensis also grows (weakly) at 30°C. The single known isolate grew better than the Czech isolate of *Cr. albidosimilis* at 4°C, a situation that was reversed at 28°C (Scorzetti et al. 2000). In another contrast to *Cr. albidosimilis*, the xylanase of *Cr. adeliensis* was cold-adapted (vide supra). *Cr. adeliensis* was isolated from a relatively rich source of organic energy. These factors, taken together, make *Cr. adeliensis* a possible Antarctic native.

The least probable candidates for indigenicity are those associated with field camps, are fermentative (presumably copiotrophs), or have T_{max} well above 30°C. When considering human contamination it is important to remember that a "base" is not a field camp; a base is a geographic entitlement which contains at least the equivalent of a field camp but is not limited to its immediate vicinity. Isolates from a base need not therefore be suspect. Rookeries may also be centers of imported propagules but it is premature to declare every yeast isolated there an unsuccessful immigrant. Of the yeasts isolated from soil adjacent to the field camp liquid-waste barrel by Baublis et al. (1991) *C. albicans*, *Clavispora lusitaniae* (as *C. lusitaniae*), and *Issatchenkia orientalis* (as *C. krusei*) fail on all three criteria. *Trichosporon cutaneum* is a "rather rare species"(Gueho et al. 1992), a superficial pathogen of warm-blooded animals (Gueho et al. 1994) and so surely a result of human presence. *Saccharomyces cerevisiae* and *Stephanoascus ciferrii* (as *C. ciferrii*), isolated from "benthos box core strata" by the same group may not have been contaminated by human presence, but still fail on one or two counts. The work of Meyer et al. (1962) on the refuse of the Scott Expedition demonstrates that ascomycetous yeasts entering Antarctica by whatever means could have a lengthy survival. The *C. parapsilosis* in the South Pole ice tunnel (Jacobs et al. 1964) fails on the same criteria as *C. albicans*.

We are left with a still considerable list of species which may or may not have been able to colonize. Many of these species were reported after the appearance of only one or two isolates, lending an adventitious aspect to their occurrence in Antarctica. A single cell may easily divide during the manipulations required for isolation. Population densities strongly hinting at establishment were reported for *Rhodosporidium sphaerocarpum* and *Rh. mucilaginosa. Rhodosporidium sphaerocarpum* has a mesophilic T_{max}, but 37 isolates were obtained from the 15 associated sites in the Antarctic Ocean, from water temperatures of −1.68 to 1.33°C (Newell and Fell 1970). The seven reports of *Rh. mucilaginosa* from Antarctic areas did not always make it clear how many isolates came from each site (an annoying omission in several other reports as well) but Babyeva and Golubev (1969) isolated 15 strains

from a moss collected in the penguin rookery on Haswell Island where the only other species yielding more than one or two isolates was a psychrophilic strain of *Rh. aurantiaca* (three isolates). While T_{min} is not recorded for Babyeva and Golubev's Antarctic strain, the strains of *Rh. mucilaginosa* isolated by Goto et al. (1969) were declared to be capable of growth at 0°C. *Debaryomyces hansenii* turned up at five locations, but never in quantity; this species is ascosporogenous and distributed so widely that it would be remarkable if it did not turn up in Antarctica. Had isolates of the remaining species simply not met their consumers yet?

16.6 Conclusion

Any assessment of yeast biodiversity in the Antarctic must remain incomplete at this time. The Antarctic provides a variety of habitats, some of which have never been adequately examined: the warm volcanic soils; the streams whose photosynthetic flora are meticulously catalogued, but the yeasts barely so; the phanerogams of the Palmer Peninsula. Antarctica is open to any yeast that can survive the various means of transport, but the absence of experimental evidence of the inability of putatively allochthonous yeasts to colonize leaves the position of many isolates ambiguous. It does appear that yeast biodiversity correlates with the availability of water and organic sources of energy. The arid highlands of the Ross Desert provide a unique habitat which contains a single species adapted to this habitat and which is unknown elsewhere – *Cr. vishniacii*, the eponym of a scientist who met with a fatal accident there – while the streams and margins of the continent contain six or more species of psychrophiles, many of which have also been found in Arctic or near-Arctic regions with temperatures approaching (at least seasonally) the cold of the Antarctic margins and with more varied vegetation.

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Yeast Biodiversity in Tropical Forests of Asia

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17.1 Introduction

It is well known that the tropical region of Asia, especially southeastern Asian countries, has a long history of utilizing microorganisms for the production of foods and beverages. A number of unique fermented foods and beverages have been produced and consumed in this region. Quite naturally, the investigation of yeasts associated with these fermented products, including starters for fermentation, were started in the early stage of the history of microbiology of Asia by European and Japanese researchers. However, different from mushrooms and filamentous fungi, yeasts living in the natural environment such as forests were not studied until recent years. The recent studies from the viewpoint of biodiversity revealed the richness in the species diversity of yeasts in tropical forests though such studies have just started and further extensive studies are required. This chapter discusses yeasts found in forests of tropical and subtropical regions of Asia. The irreducible minimum of yeasts associated with fermented products is also discussed if it is necessary for comparing them with those associated with forests.

17.2 Yeasts in Tropical Forests of Asia

17.2.1 Yeasts Found in Forests of Thailand

Among tropical Asian countries, yeasts have been most extensively studied in Thailand. However, the study of yeasts living in the natural environment has just started. Yeasts are a group of microorganisms that require more intense study. Currently some 150 species have been recorded in Thailand, from fermented products and substrates in the natural environments.

Generally speaking, yeasts found in fermented foods and related substrates are not rich in diversity. Ascomycetous yeasts are dominant in these substrates and species are common to other countries. Saito et al. (1983) isolated 386 strains of yeasts from 54 samples of fermented foods and related substrates and identified

them as 21 species in 11 genera. *Saccharomyces cerevisiae* was the dominant species and occupied 30.6% of the isolates and was found in 39.4% of samples, followed by *Issatchenkia orientalis* (anamorph *Candida krusei*) (28.2%), *Hanseniaspora valbyensis* (9.8%), *C. tropicalis* (5.4%), *Pichia membranifaciens* (4.7%), *P. ohmeri* (4.4%) and *Saccharomycopsis fibuligera* (3.9%). The remaining 14 species occupied 1.8–0.3% of the isolates. In this study, all of the isolates belong to known species. However, yeasts were identified by keys devised by Barnett et al. (1979) in this study so that some misidentification might be inevitable.

Saccharomycopsis fibuligera is a unique species found in fermented foods in southeastern Asian countries. This species has strong amylolytic activity and shows alcoholic fermentation, and is often found in various kinds of starters in this region. Limtong et al. (2002) reported that this species is contained in most of the *loog-pang* solid starter for fermentation in Thailand.

Suzuki et al. (1987) studied 80 strains of yeasts that were isolated from fermented foods and related substrates in 1984 by several Thai researchers. They identified these yeasts as 17 species in nine genera. *I. orientalis* is the dominant species and occupied 42.5% of the isolates, followed by *Saccharomyces cerevisiae* (11.3%), *I. occidentalis* (anamorph *C. sorbosa*) (10.0%) and *C. tropicalis* (6.3%). In this study, a strain was found to represent a new species and described as *C. stellimalicola* (Suzuki et al. 1994). In these two studies on yeasts associated with fermented foods and related substrates, yeast species were common to those found in fermented foods in other countries, not only in southeastern Asian countries but also in European countries and in Japan. Further, more than 80% of the isolates belong to species that are commonly found in these two studies.

Jindamorakot (2000) studied yeasts associated with fermented foods and related substrates using high-salt-content media. She isolated 137 strains and identified 123 of them as 33 known species. *I. orientalis* is the dominant species and occupied 27.0% of the isolates, followed by *S. cerevisiae* (8.8%), *C. parapsilosis* (7.3%) and *C. glabrata* (5.8%). In this study, 57.6% of the yeasts belonged to species commonly found in studies by Saito et al. (1983) and/or by Suzuki et al. (1987), in spite of the use of high-salt-content media for isolation. Two strains from fermented soybean and dried salted squid were identified as *Citeromyces matritensis* in this study. However, further detailed studies revealed that the two strains represented a new species. They were described as *Citeromyces siamensis* (Nagatsuka et al. 2002). This is the second species of this genus. Jindamorakot assigned the remaining 12 strains to the genera *Debaryomyces*, *Saccharomyces* and *Candida* but she could not identify them as any known species of these genera. Probably, these strains represent hitherto undescribed species.

It is concluded that yeasts associated with fermented foods and related substrates in Thailand are not rich in biodiversity and that most of them belong to already described species that are common to other southeastern Asian countries, European countries and Japan.

In contrast to fermented foods and related substrates, yeasts found in the natural environment are rich in biodiversity and many undescribed species have been found so far as discussed later.

Basidiomycetous yeasts, especially ballistoconidium-forming yeasts, have been extensively studied in Thailand. In 1987 and 1990, isolation studies of this kind of

yeast were carried out as a joint study of Thailand Institute of Scientific and Technological Research (TISTR) and Japan Collection of Microorganisms (JCM).

In 1987, 42 samples of various plants were collected in the forests, fields and rice fields and from the roadsides in the western suburb of Bangkok and near Ayutthaya, and also in the urban areas of Bangkok, including plant leaves collected in several markets. Yeasts were isolated from these samples at 23 and 30°C by the ballistoconidium-fall method (Nakase and Takashima 1993). Sixty-three strains of ballistoconidiogenous yeasts were isolated from 20 samples (50%) examined. The frequency of isolation reached 81.2% when samples were collected in the suburbs of Bangkok and Ayutthaya but it was very low (15%) when samples were collected in the urban areas of Bangkok, including markets (Nakase et al. 2001).

Sixty-three isolates were identified as 16 species in the genera *Bullera*, *Kockovaella*, *Bensingtonia*, *Sporidiobolus*/*Sporobolomyces* and *Tilletiopsis*. Three strains produced non-ballistoconidiogenous stalked conidia and budding yeast cells in addition to ballistoconidia. This kind of conidiogenesis has not been found in hymenomycetous ballistoconidium-forming yeasts so far; therefore, a new genus *Kockovaella* was proposed for these strains. Two species, *K. imperatae* and *K. thailandica,* were included in the genus (Nakase et al. 1991). In addition to new species of *Kockovaella*, *Sporobolomyces nylandii* and *S. vermiculatus* were also described (Takashima and Nakase 2000). Three strains out of six of yeast-like fungi were assigned to *Tilletiopsis*. They were found to represent three new species, *T. derxii*, *T. oryzicola* and *T. penniseti* (Takashima and Nakase 2001a).

In 1990, 73 ballistoconidiogenous yeast strains were isolated from 33 plant materials (82.5%) out of 40 collected in forests, grasslands and rice fields along the southeastern seacoast from Bangkok to Pattaya. The isolation of yeasts was carried out at 25°C. These yeasts were identified as 13 species in the genera *Bullera*, *Kockovaella*, *Bensingtonia* and *Sporidiobolus*/*Sporobolomyces. Bensingtonia musae* (Takashima et al. 1995), *Bullera penniseticola*, *K. sacchari* (Takashima and Nakase 1998), *Sporobolomyces blumeae* and *S. poonsookiae* (Takashima and Nakase 2000) were described as new species. In this isolation study, *Tilletiopsis* strains were not isolated though they are commonly found in the samples.

A total of 136 strains isolated in 1987 and 1990 were identified as 21 species, 105 strains (77.2%) as nine known species and 31 strains (22.8%) as 12 undescribed species (Nakase et al. 2001). Eight of them were commonly isolated in 1987 and 1990, but the remaining 13 species were isolated in either year. In the isolation study of 1987, *Sporobolomyces shibatanus* (teleomorph *Sporidiobolus pararoseus*) was the dominant species and was found in 21.4% of plants examined, followed by *B. sinensis* (19.0%), *B. crocea* (16.7%) and *S. salmonicolor* (11.9%). In the isolation study in 1990, the most frequently isolated species was *B. sinensis* and was found in 52.5% of plant samples examined, followed by *Sporobolomyces shibatanus* (30.0%), *Sporidiobolus ruineniae* (17.5%) and *Sporobolomyces poonsookiae* (15.0%). *Sporobolomyces roseus*, the most frequently encountered ballistoconidium-forming species in the temperate zones, was not found in these studies.

In addition to the ballistoconidiogenous yeasts just mentioned yeast colonies excreting black pigment were found from several samples of 1987 and 1990. These colonies are assumed to be anamorphs of *Entyloma* (Boekhout, personal communication).

Among the ballistoconidium-forming yeasts isolated in 1987 and 1990, a strain had Q-9 as the major component of ubiquinone and was described as *Bensingtonia musae* (Takashima et al. 1995). The remaining yeasts had Q-10 and were assigned to the genera *Bullera*, *Kockovaella*, *Sporidiobolus*, *Sporobolomyces* and *Tilletiopsis* (Nakase et al. 2001). No yeast was found to have Q-10 (H_2) as the major ubiquinone.

In the studies carried out in 1987, two incubation temperatures, 23 and 30°C, were used for the isolation of yeasts. Twelve species were isolated at 23°C, eight were isolated at 30°C and four were isolated at both temperatures. Apparently, the use of 23°C is better than 30°C to recover a variety of yeast species. It is interesting that several undescribed species of ballistoconidiogenous yeasts found in Thailand have relatively low maximum growth temperatures below 30°C in spite of the high atmospheric temperatures during the isolation study (Nakase et al., unpublished). We examined the maximum growth temperatures of yeasts isolated in Thailand and Japan and found that there is no relationship between the maximum temperature of yeasts and the atmospheric temperature of the places where the yeasts were isolated, as discussed later.

Further isolation studies of ballistoconidium-forming yeasts were carried out in the project named "Asian Network on Microbial Research" supported by the Science and Technology Agency of the Japanese government. In 1996, Fungsin isolated 175 strains of yeasts from plants collected in a protected tropical rain forest in Sakaerat, Nakhon Ratchasima Province, northeastern Thailand, by the ballistoconidium-fall method (Fungsin 2003). After confirming the ballistoconidium-forming ability, he assigned 151 strains to the genera *Bullera* (51 strains), *Dioszegia* (two strains), *Kockovaella* (four strains), *Bensingtonia* (ten strains), *Rhodotorula* (six strains), *Sporidiobolus* (two strains), *Sporobolomyces* (57 strains) and *Tilletiopsis* (18 strains) as shown in Table 17.1. He identified 141 of them as 47 species and a variety, 14 known species, 33 undescribed species and an undescribed variety. Among the 141 strains identified, 106 (75.2%) belonged to undescribed species or an undescribed variety. The frequency of isolation of the undescribed taxa was more than 3 times that of those from plants in Bangkok and along the seacoast from Bangkok to Pattaya (Nakase et al. 2001). This result clearly suggests that ballistoconidium-forming yeasts associated with plants in protected forests of Thailand are rich in biodiversity and numerous unknown species live in these substrates.

Among the undescribed species just mentioned, six were described as new species in the genera *Bensingtonia*, *Kockovaella* and *Bullera*, i.e., *Bensingtonia thailandica* (Fungsin et al. 2001), *K. barringtoniae* (Fungin et al. 2002a), *B. arundinariae* (Fungsin et. al. 2002b), *B. siamensis*, *B. panici* (Fungsin et al. 2003a) and *B. sakaeratica* (Fungsin et al. 2003b). Fungsin named two more new species, *B. lagerstroemiae* and *B. nakhonratchasimensis*, but they have not yet been validly described (Fungsin 2003). The two undescribed *Bullera* species were located in the *Trichosporonales* clade in the phylogenetic trees based on the 18S ribosomal DNA (rDNA) and D1/D2 domain of 26S rDNA sequences though they did not produce arthroconidia, a characteristic feature of the *Trichosporon*. Further, he found that one strain (TY-213) belonged to *Sporobolomyces* by its morphology of ballistoconidia but is located in *Ustilaginomycetes* and not in *Urediniomycetes* in the phylogenetic tree based on 18S rDNA sequences. He presumed that this strain may represent a new genus of ustilaginomycetous yeasts.

Table 17.1 Ballistoconidium-forming yeasts found in Thailand (Fungsin 2003)

Species	No. of strains		Species	No. of strains	
Hymenomycetous yeasts			Urediniomycetous yeasts		
Bullera	51		*Bensingtonia*	10	
B. arundinariae		1	*B. thailandica*		10
B. boninensis		3	*Rhodotorula*	6	
B. coprosmaensis		8	*R. marina*		5
B. hannae		1	*R. bogoriensis*		1
B. lagerstroemiae		1	*Sporidiobolus*	2	
B. panici		1	*S. ruineniae*		2
B. sakaeratica		2	*Sporobolomyces*	57	
B. schimicola		1	*S. odoratus*		4
B. siamensis		3	*S. vermiculatus*		1
B. sinensis		4	*Sporobolomyces* spp., Q-10[b]		33
B. variabilis		3	*Sporobolomyces* spp., Q-10(H_2)[c]		19
B. nakhonratchasimensis		6	Ustilaginomycetous yeasts		
Bullera spp.[a]		17	*Sporobolomyces*	1	
Dioszegia	2		*Sporobolomyces sp.* TY-213		1
D. zsoltii var. *thailandica*		2	*Tilletiopsis*	18	
Kockovaella	4		*Tilletiopsis* spp.		18
K. barrintoniae		1			
K. sacchari		1			
K. thailandica		1			
Kockovaella sp.		1	Total		151

[a]Seven species are recognized.
[b]Twelve species are recognized.
[c]Two species are recognized.

In this isolation study, Fungsin (2003) reported that yeasts with Q-10 were recovered from all of the samples examined and occupied 80.9% of isolates, yeasts with Q-10(H_2) were found in 57.7% of plant samples and occupied 12.6% of the total isolates and yeasts with Q-9 were recovered from 26.9% of samples and occupied 6.6% of the isolates. This result is quite different from the studies in 1987 and 1990 on plants collected in urban and suburban areas of Bangkok and the southeastern seacoast from Bangkok to Pattaya where no yeasts with Q-10(H_2) and only one strain of yeast with Q-9 were isolated (Nakase et al. 2001). Yeasts with Q-10(H_2) are known to be rare in the temperate zones but rich in the tropical and subtropical regions (Nakase 2000). Fungsin's data suggested the richness of yeast diversity in protected forests of Thailand.

According to Fungsin (2003), ballistoconidium-forming strains of *Rhodotorula marina* and *R. bogoriensis* were isolated from a forest in Sakaerat. Apparently, ballistoconidium-forming ability has no phylogenetic value though their morphology has a certain taxonomic value (Nakase 2000). However, this property is very useful for the selective isolation of basidiomycetous yeasts from substrates in the natural environment. Generally speaking, basidiomycetous yeasts grow slower than ascomycetous yeasts and are often suppressed on isolation media by the latter yeasts.

In addition to the basidiomycetous yeasts mentioned before, Prillinger et al. (1997) found a new stalked conidium-forming yeast from a lichen in Thailand and described it as *Fellomyces thailandicus*.

The study of ascomycetous yeasts in the natural environment followed the study of basidiomycetous yeasts. Limtong and coworkers carried out extensive studies on the thermotolerant methylotrophic yeasts and found that these kinds of yeasts were widely distributed in the natural environment of Thailand. Three of them were described as new species of the genera *Pichia* and *Candida*, *P. siamensis*, *C. krabiensis* and *C. sithepensis* (Limtong et al. 2004).

Recently, Nakase and coworkers carried out extensive isolation studies of yeasts from substrates collected in the natural environment of Thailand using the direct streaking technique and enrichment methods using yeast extract–malt extract agar supplemented with 100 ppm chloramphenicol and 0.2% sodium propionate. They sequenced the D1/D2 domain of 26S rDNA of 283 yeast isolates from insect frass, mosses, flowers, wild mushrooms and several other substrates. One hundred and ninety-four strains belonged to ascomycetous yeasts and the remaining 89 belonged to basidiomycetous yeasts (unpublished). These yeasts were tentatively identified on the basis of the D1/D2 sequences by consulting a guideline of Kurtzman and Robnett (1998), namely, strains having a nucleotide difference of zero to one are conspecific, those having a nucleotide difference of two to three are conspecific or sister species of earlier described species and those having a nucleotide difference of four or more are different species. In the case of known yeasts, morphological and physiological properties were also compared.

On the basis of the above mentioned standard, 194 strains of ascomycetous yeasts were classified into 134 species. Ninety-six strains were identified as 41 known species (Table 17.2). In this study, yeast isolates having the same D1/D2 sequences as yeasts whose sequences were already registered in DNA data banks were dealt with as known species because they were already found in places other than Thailand. The 41 known species just mentioned include such seven species consisting of ten strains (Table 17.2). *C. tropicalis* (nine strains) is the most frequently isolated and is followed by *S. kluyveri* (eight strains), *S. cerevisiae* (five strains), *Metschnikowia koreensis* (five strains) and *Debaryomyces nepalensis* (four strains).

Eighty-four ascomycetous strains were found to represent 79 undescribed species and the remaining 14 strains showed two to three nucleotide differences from known species. The latter 14 strains are considered to represent 14 different species but have not yet been identified. Probably, some of them are conspecific with known species and some others represent undescribed species. Further molecular studies such as DNA–DNA reassociation experiment are required for the identification of these 14 species.

Eighty-nine strains (31.4%) included in the basidiomycetous yeasts were classified into 53 species, 17 known species (41 strains), 22 undescribed species (31 strains), and 14 not yet identified species (17 strains). The latter 14 species showed two to three nucleotide differences from known species. Probably, several of them belong to known species and some others represent undescribed species. As shown in Table 17.3, *Cryptococcus heveanensis* (eight strains) is the most frequently isolated and is followed by *Exobasidium vexans* (four strains), *Sporobolomyces* sp. (four strains) and *Trichosporon asahii* (four strains).

Table 17.2 Known species of ascomycetous yeasts isolated from substrates in the natural environment of Thailand

Species	No. of strains	Species	No. of strains
Ambrosiozyma monospora	2	*Hanseniaspora guilliermondii*	1
Aureobasidium pullulans	2	*H. opuntiae*	3
Blastobotrys capitulata	1	*H. vineae*	1
Candida diversa	3	*Hanseniaspora* sp. CBS 8772	1
C. fukuyamaensis	3	*Kloeckera lindneri*	1
C. gotoi	2	*Kluyveromyces lactis*	2
C. leandrae	1	*Kodamaea ohmeri*	3
C. natalensis	1	*Metschnikowia koreensis*	5
C. palmae	1	*Pichia nakazawae* var. *akitaensis*	2
C. parapsilosis	3	*P. stipitis*	2
C. rancensis	1	*P. sydowiorum*	2
C. sithepensis	1	*Pichia* sp. UWO(PS)99-305.1	1
C. tropicalis	9	*Saccharomyces cerevisiae*	5
Candida sp. NRRL Y-17456	4	*S. kluyveri*	8
Candida sp. UWO(PS)00-147.3	1	*S. unisporus*	2
Debaryomyces polymorphus var. *africanus*	1	*Stephanoascus smithiae*	3
		Torulaspora delbrueckii	1
D. nepalensis	4	*Torulaspora* sp. IFO 11061	1
D. polymorphus	3	*Williopsis saturnus* var. *mrakii*	1
D. vanrijiae var. *yarrowii*	3	*W. saturnus* var. *subsufficiens*	1
Debaryomyces sp. NRRL-7804	1	*Zygosaccharomyces* sp. IFO 11070	1
Geotrichum fragrans	2	Total 41 species	96

Species whose D1/D2 sequences are registered at DNA Data Banks are dealt with as known species (Nakase et al., unpublished).

Several undescribed species found in these isolation studies were described as new species, *C. easanensis*, *C. pattaniensis* and *C. nakhonratchasimensis* (Jindamorakot et al. 2004), but the majority of them are still under study.

Among 101 undescribed species found in this study, 93 species (92.1%) comprised one strain and the remaining eight species comprised two to five strains. Further, among 28 species of not yet identified species, 27 (96.4%) comprised one strain and the remaining one species comprised three and four strains. This is quite a contrast to known species where two or more strains up to nine were isolated for 32 species (55.2%) and 27 species (44.8%) comprised one strain (Tables 17.2, 17.3). This fact clearly indicates that species diversity of yeasts is extremely rich in forests of Thailand and a vast number of unknown yeasts live there. This is quite different from yeasts associated with fermented foods. Further extensive studies are required to clarify the yeasts living in forests of Thailand.

Table 17.3 Known species of basidiomycetous yeasts isolated from substrates in the natural environment of Thailand (Nakase et al., unpublished)

Species	No. of strains	Species	No. of strains
Bullera dendrophila	1	*Rhodotorula nothofagi*	1
Bullera sinensis	2	*Sporidiobolus ruineniae*	3
Cryptococcus heveanensis	8	*Sporobolomyces bannaensis*	1
Cryptococcus sp. CBS 8372	1	*S. odoratus*	3
C. humicola	2	*S. poonsookiae*	1
C. laurentii	3	*Sporobolomyces* sp. TY-241, etc.[a]	4
Exobasidium vexans	4	*Tilletiopsis* sp. TY-235[a]	1
Rhodosporidium paludigenum	1	*Trichosporon asahii*	4
Rhodosporidium toruloides	1		

Species whose D1/D2 sequences were registered at DNA Data Banks were dealt with as known species.
[a]These two species are the same as those reported by Fungsin (2003).

17.2.2 Yeasts Found in Forests of Indonesia

In Indonesia, like other southeastern Asian countries, yeast research started in the fairly early stage of microbial studies of traditional fermented products, foods, beverages and starters of fermentation. The study of yeasts living in forests and other natural environments started some 40 years ago. Deinema (1961) isolated yeasts for the study of lipid production. In this study, a new yeast species was found from the surface of leaves of the flowering shrub *Randia melleifera* (Rubiaceae) in Bogor and was described as *C. bogoriensis*. This *Candida* species showed basidiomycete affinity so it was transferred to the genus *Rhodotorula*, as *R. bogoriensis* by von Arx and Weijman (1979).

Ruinen (1963) isolated yeasts living on leaves of trees and shrubs collected in several tropical countries, Indonesia in Asia, Surinam in South America and Ivory Coast in Africa, and studied lipid production from these isolates. In Indonesia, leaf samples were collected from Bogor Botanical Garden, Bogor, Java Island. These Indonesian yeasts were identified as *Hansenula anomala* var. *sphaerica*, *H. anomala* var. *heteromorpha*, *Sporobolomyces salmonicolor*, *S. roseus*, *Cryptococcus luteolus*, *C. guilliermondii*, *C. bogoriensis*, *R. glutinis*, *R. graminis*, *R. rubra* and *Pullularia* (*Aureobasidium*) sp. In addition to these species, two new species, *C. javanica* and *C. foliorum*, and a new variety, *C. bogoriensis* var. *lipolytica*, were found from these isolates. Van Uden and Buckley (1970) did not accept the latter variety. It is now regarded as a synonym of *R. bogoriensis* (Fell and Statzell-Tallman 1998). Since the two new *Candida* species demonstrated basidiomycetous nature, they were transferred to the genus *Rhodotorula*, an urediniomycetous genus, as *R. javanica* and *R. foliorum* (Weijman et al. 1988; von Arx and Weijman 1979). These basidiomycetous yeasts isolated in Indonesia are rich in cellular lipids.

Among the yeasts just mentioned, Ruinen (1963) discussed the properties of six strains of *Sporobolomyces salmonicolor*. She stated that "the presence in these strains of a buckled mycelium and with chlamydospores would make the identification with the genus *Sporidiobolus* even more plausible." Holzschu et al. (1981) validated this species as *Sporidiobolus ruinenii,* and it is now listed in *The yeasts, a taxonomic study, 4th edn* as *Sporidiobolus ruineniae* (Statzell-Tallman and Fell 1998).

In 1996, Haryono et al. collected 40 plant leaves in the suburb of Yogyakarta, Java Island, and isolated more than 300 strains of yeasts by the ballistoconidium-fall method. They examined the ballistoconidium-forming ability of 70 strains of them isolated from Kaliurang area, 900–1,000 m above sea level, and found that 61 strains were ballistoconidiogenous. These strains were assigned to the genera *Bullera* (11 strains), *Bensingtonia* (14 strains) and *Sporobolomyces* (36 strains) (Haryono et al. 1998). Among the strains assigned to *Sporobolomyces*, eight strains possessed monosaturated ubiquinone, Q-10(H_2), as the major component of ubiquinones. They differed in the internal transcribed spacer region sequences from the known Q-10(H_2)-containing species, *Erythrobasidium hasegawianum* and *Sporobolomyces elongateus*, and were divided into two groups on the basis of their physiological characteristics (Haryono et al. 1999).

Tropical fruits are considered as good habitats for yeasts. Sjamsuridazal and Gandjar (1994) studied yeasts associated with overripe fruits collected in Jakarta and vicinities of Java Island and isolated 17 yeast strains. Fifteen strains were identified as *Kloeckera apiculata*, *Kluyveromyces marxianus*, *K. blattae, K. waltii*, *C. oleophila*, *C. tropicalis*, *P. burtonii*, *P. humboldtii*, *P. acaciae*, *P. etchelsii*, *P. pijperi*, *P. strassburgensis* and *Trichosporon cutaneum*. The respective species comprise one strain except for *K. marxianus* and *P. humboldtii*, for which four and two strains were isolated, respectively. The remaining two strains were dealt with as *Dekkera* sp. and *Pichia* sp. Further, Oetari et al. (1999) studied 25 strains of yeasts isolated from 21 samples of 17 banana cultivars. The yeasts isolated mostly belonged to *Pichia* followed by *Saccharomyces*. Nineteen strains of *Pichia* were identidied as *P. amethionina* (one strain), *P. chambardii* (one strain), *P. farinosa* (one strain), *P. kluyveri* (one strain) and *P. membranifaciens* (seven strains). Three *Saccharomyces* strains found in three banana cultivars were identified as *S. cerevisiae* (two strains) and *S. kluyveri* (one strain). The remaining three strains were identified as *Hanseniaspora uvarum, K. marxianus* var. *wikenii* and *Zygosaccharomyces rouxii*. In their studies, samples were collected from traditional markets around Jakarta, Depok and Bogor, Java Island, but not from orchards or forests. However, yeast flora is supposed to be similar to those of banana in the orchards or forests.

Naruki et al. (1999) isolated 109 strains of sugar-tolerant yeasts from several plants in the natural environment together with foods and fruits and their products and studied sugar alcohol production. On the basis of the morphological and physiological characteristics described in *The yeasts, a taxonomic study, 3rd edn* (van der Walt and Yarrow 1984), they predicted the taxonomic positions of 74 strains. The predicted species include, for example, *S. cerevisiae*, *S. kluyveri*, *S. capensis*, *S. steineri*, *D. polymorphus*, *D. hansenii*, *H. anomala* (*P. anomala*), *P. guilliermondii*, *P. ohmeri*, *Kluyveromyces marxianus*, *Torulaspora delbrueckii*, *C. haemulonii*, *C. magnoliae*, *C. catenulata*, *C. insectorum*, *C. famata*, *C. pelliculosa*, *C. sorboxylosa*, *Schwanniomyces*

occidentalis, *Schizosaccharomyces pombe* and *Zygosaccharomyces fermentati*. Several strains were identified at generic level. This is an excellent study from the viewpoint of applied microbiology. However, it is doubtful whether their identification is correct because they reported the isolation of *Malassezia* species. It is not easy to isolate yeasts of the genus *Malassezia* from the natural environment using ordinary isolation media for yeasts because they require fatty acids for growth (Ahearn and Simmons 1998). It is assumed that most of the species were correctly predicted but some might have been mispredicted.

Yeast biodiversity in the forests of Indonesia is assumed to be rich as well as in those of Thailand. However, further detailed isolation studies and the identification based on molecular methods are required to clarify the yeasts living in forests of Indonesia. Probably, many undescribed species will be found in there.

17.2.3 Yeasts in Tropical Forests of Vietnam

Luong et al. (1999) isolated 151 strains of yeasts from 20 samples of plants collected in a tropical rain forest of Cuc Phuong National Park, Ninh Binh Province, Vietnam, using the ballistoconidium-fall method. One hundred and twenty-one of them produced ballistoconidia. They carried out taxonomic studies of 85 strains and assigned them to the genera *Bullera* (39 strains), *Kockovaella* (five strains), *Sporobolomyces* (39 strains) and *Tilletiopsis* (two strains). Among strains assigned to *Sporobolomyces*, 34 have Q-10 and the remaining five have Q-10(H_2) as the major component of ubiquinones.

All five strains of *Kockovaella* belonged to hitherto undescribed species and were described as four new species, *K. calophylli* (one strain), *K. cucphuongensis* (two strains), *K. litseae* (one strain) and *K. vietnamensis* (one strain) (Luong et al. 2000). According to Luong (personal communication), two new species have been found among *Bullera* strains so far. It is assumed that the ballistoconidium-forming yeasts found in Cuc Phuong National Park included many undescribed species as easily suggested by the fact mentioned before that all of *Kockovaella* isolates represented new species.

17.3 Yeasts in the Subtropical Forests of Asia

17.3.1 Yeasts in Forests of Taiwan

The study of yeasts in Taiwan started about 70 years ago but the study is related to the alcoholic fermentation such as the isolation of *S. formosensis* (= *S. cerevisiae*), a powerful alcoholic fermenter from molasses, and yeasts living in the natural environment were not studied until recent years.

Lee et al. (1994) described a new species, *Arthroascus fermentans*, from soil in orchards. Later, this species was transferred to the genus *Saccharomycopsis* (Kurtzman and Robnett 1995).

In 1997, Nakase and coworkers isolated 154 strains of yeasts from plants collected in a protected subtropical rain forest in Fu-Shan Experimental Forest, Taiwan Forestry Research Institute, located in northeastern Taiwan, by an improved

ballistoconidium-fall method (Nakase and Takashima 1993). One hundred and forty-six of them produced ballistoconidia. These ballistoconidium-forming yeasts were assigned to the genera *Bullera* (90 strains), *Dioszegia* (one strain), *Kockovaella* (one strain) and *Sporobolomyces* (54 strains) though the identification is not yet complete (Table 17.4). Eight new species were described from these isolates, *B. taiwanensis*, *B. formosensis* (Nakase et al. 2002), *Sporobolomyces magnisporus* (Nakase et al. 2003), *B. begoniae*, *B. setariae* (Nakase et al. 2004a), *B. melastomae*, *B. formosana* (Nakase et al. 2004b) and *S. fushanensis* (Nakase et al. 2005). In addition to new species just mentioned, at least four new species have been recognized so far, three of *Sporobolomyces* and one of *Kockovaella. Sporobolomyces roseus*, the commonest ballistoconidium-forming species in the temperate zones, is a minor species in Taiwan (Table 17.4). The identification of this species needs to be confirmed because *S. roseus* is heterogeneous and consists of several different species (Fell et al. 2000; Bai et al. 2002b). Therefore, this species is tentatively dealt with as *S. roseus* complex (Table 17.4).

B. formosensis is located in *Trichosporonales* clade in the phylogenetic tree on the basis of the 18S rDNA sequences though this species does not produce arthroconidia. Probably, this is the second ballistoconidiogenous yeast in this clade because

Table 17.4 Ballistoconidium-forming yeasts found in a subtropical rain forest in Taiwan

Species	No. of strains	References
Bullera	90	
B. taiwanensis[a]	1	Nakase et al. (2002)
B. formosensis[a]	1	Nakase et al. (2002)
B. begoniae[a]	1	Nakase et al. (2004a)
B. setariae[a]	1	Nakase et al. (2004a)
B. melastomae[a]	1	Nakase et al. (2004b)
B. formosana[a]	1	Nakase et al. (2004b)
B. coprosmaensis	1	
B. oryzae	1	
B. sinensis	8	
B. variabilis complex	16	
B. sinensis complex	18	
Bullera spp.	40	
Dioszegia	1	
D. zsoltii	1	
Kockovaella	1	
Kockovaella sp.	1	
Sporobolomyces	54	
S. magnisporus[a]	2	Nakase et al. (2003)
S. fushanensis[a]	1	Nakase et al. (2005)
S. shibatanus complex	27	
S. roseus complex	3	
Sporobolomyces spp.	21	
Total	146	

[a]New species found in Taiwan (Nakase et al., unpublished)

Sporobolomyces albidus described by Ramirez Gomez (1957) is assumed to be the first ballistoconidium-forming yeast in this clade. For many years *S. albidus* had been dealt with as a synonym of *C. humicola* (= *Cryptococcus humicola*) because no one observed ballistoconidia after the description of this species. Takashima et al. (2001b) reported that this yeast is located in *Trichosporonales* in the phylogenetic tree on the basis of 18S rDNA sequences and gave it a new name, *Cryptococcus ramirezgomezianus*. Nakase et al. (2002) stated that *B. formosensis* produces bilaterally symmetrical ballistoconidia as reported for *Sporobolomyces albidus* (Ramirez Gomez 1957).

It is assumed that many unknown species of ascomycetous yeasts live in forests of Taiwan as well as ballistoconidium-forming yeasts. However, such reports have not been published so far.

17.3.2 Yeasts in Forests of Yunnan, China

In 1996, Bai and coworkers isolated 670 strains of ballistoconidium-forming yeasts from 43 semidried leaf samples collected in subtropical forests of Yunnan Province, China, by the ballistoconidium-fall method. They selected 109 strains and characterized them morphologically, physiologically and chemotaxonomically, and recognized 25 groups. Among these groups they carried out detailed studies on five groups including 16 strains that form symmetric ballistoconidia and contain xylose in the whole cell hydrolysates. One strain was identified as *B. mrakii*, seven strains as *Bulleromyces albus* and the remaining eight strains in three groups represented three undescribed species of the genus *Bullera* (Bai et al. 1999). The latter strains were described as *B. kunmingensis* (Bai et al. 2001a), *Dioszegia zsoltii* including two varieties, *D. zsoltii* var. *zsoltii* (four strains) and *D. zsoltii* var. *yunnanensis* (two strains) (Bai et al. 2002a) and *B. anomala* (Bai et al. 2003). They studied another strains isolated from plants in Yunnan Province and described additional three new species, *B. pseudovariabilis* (Bai et al. 2003), *Sporobolomyces phaffii* (Bai et al. 2002b) and *S. yunnanensis* (Bai et al. 2001c). *B. pseudovariabilis* is a second species of the *B. variabilis* cluster and *S. yunnanensis* is a Q-10(H_2)-containing species in the *Erythrobasidium* cluster. Three strains of *Sporobolomyces roseus* were also isolated in Yunnan Province (Bai et al. 2002b). As formerly stated, this species is very common in the temperate zones but is rare in the tropical regions.

17.3.3 Yeasts in Forests of the Ogasawara Islands in the Pacific Ocean

The Ogasawara (Bonin) Islands are isolated islands in the subtropical region of the Pacific Ocean about 1,000 km from Tokyo, Japan, and are known to have a unique flora of higher plants. In the autumn of 1994, we received various plant samples collected in these islands from T. Sato, and isolated yeasts by the ballistoconidium-fall method. The number of yeast colonies that appeared on the agar plates was less than 1% of the number from plants collected from other regions of Asia including the tropics, subtropics and the temperate zone. According to T. Sato, the plant samples were not stored in good condition and some of them were infected by molds. Furthermore, the islands are small and plants are greatly affected by the strong wind

from the sea that results in a high salt concentration on the surface of plants. In spite of the small number of colonies, we found that the species diversity of yeasts is very high in these islands. A total of 199 strains were isolated from 24 samples (66.7%) of 36 examined. One hundred and ninety-seven of them produced ballistoconidia. These strains were assigned to seven genera on the basis of the morphology of ballistoconidia, the presence of xylose in the cell hydrolysates and the ubiquinone types (Table 17.5).

Forty-six strains of the genus *Bullera* isolated from ten samples (27.8%) were identified as six species (Table 17.5). Three of them were described as new species, *B. boninensis*, *B. schimicola* and *B. waltii* (Sugita et al. 1999). *Bullera* sp. 1 and 2 seem to represent new species and *Bullera* sp. 3 is closely related to *B. variabilis*, a taxonomically heterogeneous species comprising several different species (Bai et al. 2001b).

Sixteen strains isolated from three samples (8.3%) represented three new species of *Kockovaella* and were described as *K. machilophila*, *K. phaffii* and *K. schimae* (Gibas et al. 1998). Two strains of *Bensingtonia*, which were isolated from the same sample, was found to be a new species and was named *B. sakaguchii* (Sugita et al. 1997).

One hundred and thirty-three strains were assigned to the genus *Sporobolomyces* in which 103 strains possessed Q-10 and the remaining 30 strains

Table 17.5 Basidiomycetous yeasts found on the plants of the Ogasawara Islands

Species	No. of strains	Frequency of isolation (%)[a]	Species	No. of strains	Frequency of isolation (%)[a]
Hymenomycetous yeasts			Urediniomycetous yeasts		
Bullera	46	27.8	*Bensingtonia*	2	2.7
B. boninensis	16	8.3	*B. sakaguchii*	2	2.7
B. schimicola	1	2.7	*Bannoa*, Q-10(H_2)	6	11.1
B. waltii	5	13.9	*B. hahajimensis*	6	11.1
Bullera sp. 1	1	2.7	*Sporobolomyces*, Q-10(H_2)	24	27.8
Bullera sp. 2	1	2.7	*S. ogasawarensis*	19	16.7
Bullera sp. 3	2	5.6	*S. bischofiae*	1	2.7
Kockovaella	16	8.3	*S. syzygii*	4	5.6
K. machilophila	1	2.7	*Sporobolomyces*, Q-10	103	52.8
K. phaffii	14	5.6	*Sporobolomyces* spp.	103	52.8
K. scimae	1	2.7			
Fellomyces	2	2.7	Subtotal	135	
F. ogasawarensis	1	2.7			
F. distylii	1	2.7			
Subtotal	64		Total	199	66.7

[a]Percentage of plant samples from which corresponding yeasts were isolated

were found to possess Q-10(H_2) as the major component of ubiquinones. The latter 30 strains were found in ten samples (27.8%) of plants examined. The Q-10(H_2)-containing strains constituted a cluster which was close to the cluster where *Erythrobasidium hasegawianum* and *Sporobolomyces elongatus*, known species of Q-10(H_2)-containing yeasts, are located. On the basis of a DNA–DNA hybridization experiment, these strains were found to represent four different species, including a species demonstrating a sexual stage. *Bannoa hahajimensis* was proposed for this teleomorphic species (Hamamoto et al. 2002). *Bannoa* is the second genus for Q-10(H_2)-containing basidiomycetous yeasts. Three new species, *Sporobolomyces ogasawarensis*, *S. syzygii* and *S. bischofiae*, were proposed for Q-10(H_2)-containing anamorphic species.

In addition to ballistoconidium-forming yeasts, two strains of non-ballistoconidiogenous, stalked conidium-forming yeasts were also isolated in the phyllosphere of the Ogasawara Islands. These yeasts represented two different new species and were named *Fellomyces distylii* and *F. ogasawarensis* (Hamamoto et al. 1998).

17.4 Growth Temperature of Yeasts from the Tropics and the Temperate Zone of Asia

It is quite natural to assume that yeasts living in the tropical region have higher growth temperatures than those living in the temperate zones. Probably, however, this assumption seems to be erroneous. In the isolation study of ballistoconidium-forming yeasts in 1987, as mentioned before, we found that 23°C is better than 30°C to recover a variety of yeast species though the atmospheric temperatures was 24–34°C during the period of the isolation studies.

We examined the maximum growth temperatures of 1,111 yeast strains, 889 ascomycetous yeasts and 222 basidiomycetous yeasts, isolated in Thailand and Japan as representatives of the tropics and the temperate zones, by incubating them at an interval of 1°C using water baths or metal block baths whose temperatures were accurately controlled. Regardless of their locality, the average value of the maximum growth temperatures of ascomycetous yeasts was between 36 and 37°C (Table 17.6); those of the basidiomycetous yeasts were between 30 and 31°C in yeasts isolated in Thailand and between 31 and 32°C in yeasts isolated in Japan. The isolation temperatures of these yeasts were 23, 25 and 30°C in Thailand and 17 or 25°C in Japan. In Japan, 14.2% of basidiomycetous yeasts have their maximum growth temperatures below 25°C. These psychorophilic yeasts were isolated by incubation at 17°C. Psychrophilic yeasts have not been isolated in Thailand so far but we cannot discuss the psychrophiles in Thailand at present because psychrophilic yeasts cannot grow at the temperatures used for the isolation of Thai yeasts. Further study is required in this respect using low isolation temperatures below 20°C and samples collected in mountainous areas of northern Thailand.

It is concluded that yeasts living in the tropics have similar growth temperatures to those in the temperate zones. The growth temperatures of yeasts depend on the species and ascomycetous yeasts have higher growth temperatures than basidiomycetous yeasts. However, the number of basidiomycetous yeasts sampled is not enough to compare the temperature relationships of yeasts in Thailand and Japan.

Table 17.6 Maximum growth temperatures of yeasts isolated in Thailand and Japan (Nakase et al., unpublished)

Maximum growth temperature (°C)	Ascomycetous yeasts				Basidiomycetous yeasts			
	Thailand		Japan		Thailand		Japan	
	No. of strains	Cumulative %	No. of strains	Cumulative %	No. of strains	Cumulative %	No. of strains	Cumulative %
22–23							1	0.6
23–24							20	12.4
24–25							2	14.2
25–26							1	14.8
26–27							7	19.1
27–28					5	8.2	12	26.6
28–29					10	24.6	11	33.4
29–30					13	45.9	11	40.2
30–31	1	0.5	18	2.6	4	52.5	6	43.9
31–32	4	2.5	23	6.0	7	62.3	13	52.0
32–33	8	6.4	42	12.1	7	73.8	14	60.7
33–34	12	12.3	46	18.8	5	82.0	10	66.9
34–35	17	20.7	97	32.9	2	85.3	16	76.8
35–36	40	40.4	72	43.4	6	95.1	11	83.6
36–37	28	54.2	84	55.6	1	96.7	5	86.7
37–38	19	63.6	34	60.6			17	97.3
38–39	31	78.9	86	73.1				
39–40	27	92.2	41	79.1			2	98.5
40–41	7	95.6	32	83.8				
41–42	8	99.5	41	89.8			1	99.1
42–43			39	95.5	1	98.3		
43–44	1	100	9	96.8	1	99.9	1	99.7
44–45			22	100				
Total	203		686		61		161	

Apparently, further studies are required to confirm the temperature relationship between yeasts of the two countries.

17.5 Conclusion

Since long ago, Asian people have utilized microorganisms for human beings and produced various kinds of fermented foods and beverages. Quite naturally, the study of yeasts associated with fermented products preceded the study of yeasts living in the natural environment. It has been reported that yeasts found in fermented products and related substrates such as raw materials of fermentation are poor in biodiversity and most yeast species associated with such products of tropical Asia are common to other countries in the temperate zones. In recent years, yeasts associated with plant leaves, especially ballistoconidium-forming yeasts, a group of basidiomycetous yeasts, have been extensively studied in tropical and subtropical Asia. More recently ascomycetous yeasts in Asian forests, especially those in Thailand, have been studied using molecular methods. In contrast to yeasts in fermented products, yeasts in the forests are rich in biodiversity and many undescribed species were found from various substrates of tropical Asian forests. However, only some of these unknown species have been described as new taxa so far.

Yeast species having Q-10(H_2) have long been considered to be rare among yeasts. Only two strains belonging to two species, *Erythrobasidium hasegawianum* and *Sporobolomyces elongatus*, were reported to have this ubiquinone homologue as the major component (Yamada et al. 1973; Nakase and Suzuki 1986). The former was isolated from an old culture of beer yeast in Philadelphia, USA. (Sugiyama and Hamamoto 1998) and the latter was from a leaf of *Callistemon viminalis* in Australia (Shivas and Rodrigues de Miranda 1983). The recent isolation studies of ballistoconidium-forming yeasts, however, proved that Q-10(H_2)-containing ballistoconidium-forming yeasts are widely distributed in the tropical and subtropical regions in Asia – Thailand (Fungsin 2003), Indonesia (Haryono et al. 1999), Vietnam (Luong et al. 1999) – and the Ogasawara Islands in the Pacific Ocean (Nakase 2000).

The diversity of molecular species of ubiquinones is a good example of rich yeast biodiversity of tropical forests of Asia. It is assumed that many new groups of yeasts will be found in tropical forests of Asia in the near future. It should be emphasized that studies of yeasts in tropical forests of Asia has just started and further extensive studies are required to clarify the whole profile of yeasts in forests of this region. This kind of study must be done for the progress of yeast systematics and for the utilization of useful functions of yeasts for the welfare of humans. A fruitful achievement is expected from this kind of yeast research.

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Chapter 18

Yeast Communities in Tropical Rain Forests in Brazil and other South American Ecosystems

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18.1 Introduction

Yeast ecology has a strong emphasis on the description of novel taxa of yeasts colonizing a substrate. Studies on the structure and functioning of yeast communities are rare and based on a multitude of methodological approaches and different strategies of collection used by different researchers. Among fungi, yeasts are immotile, predominantly unicellular organisms that are functionally heterotrophic colonizers of sugary substrates. Yeasts are known as specialized organisms with limited physiological characteristics (Lachance and Starmer 1986; Phaff and Starmer 1987). Ecological studies aim to understand colonization and distribution of yeasts in different ecosystems and substrates (Phaff 1990), and have shown that the majority of yeast species and groups have specialized habitats. It is possible to isolate yeasts typical of different natural substrates in the geographical areas where they occur (Phaff and Starmer 1987).

The commonest difficulty in studies of yeast ecology in natural ecosystems is to define a yeast community. Möbius (1877) made the first attempts to describe an ecological community when describing oyster banks in marine ecosystems, which he called biocenosis. According to Odum (1994) communities are defined as groups of different species living together in a geographic area, or else to say in space and time defined altogether. Brewer (1988) describes communities as systems composed of species populations bound by coactions. Guilds are defined as groups of species that share a resource in a community. Lachance and Starmer (1986) consider a yeast community as a multidimensional array, the elements of which are its component species or the characteristics of these species. Differing from bacterial communities,

yeast communities present physiological homogeneity since very few yeast species have highly specialized nutritional requirements or high degrees of tolerance to extreme physicochemical conditions. Such homogeneity normally precludes the description of yeast communities as complex networks of nutritional specialists (Lachance and Starmer 1986). In an attempt to solve the problem of characterization of yeast communities, Lachance and Starmer (1986) offer a strategy based on physiological profiles of yeast assemblages. Physiological profiles are vectors of the proportions of yeasts, in a particular community, which give a positive response on each of a series of physiological tests and are considered to be sometimes more informative, as multivariate descriptors, than vectors of yeast taxa frequencies. Lachance and Starmer (1986) calculated the mean responses of all described yeast species to 33 selected physiological tests and revealed that some physiological attributes are characterized by high responses while others are comparatively low. They estimated the probability that each yeast in a random collection will be positive for each attribute *Y*. According to them, if in an actual collection of yeasts, the number of isolates positive for the attribute *Y* departs substantially from the expected mean, then ecological specificity may be significant.

In this chapter, we will consider yeast communities as guilds of taxonomically defined groups of species having similar functional/physiological attributes in a geographically limited habitat. The environment of a yeast community may be viewed as a multidimensional geometric space following Hutchinson's (1958, 1959) model and that Brewer (1988) defines as a biotope – the specific set of environmental conditions under which an individual species or community exists. Sometimes restricted to conditions of the physical environment, its dimensions would include physical factors, as well as biotic factors such as predation, competition, or food. The habitat of a yeast species is generally well defined in most studies, specially when studying yeasts associated with trees and other plant substrates, but it is less clear when dealing with yeasts associated with animals, that would include mammals and insects, mostly. There are many reports of yeasts isolated from flowers, tree saps and fruits of angiosperms (Miller and Mrak 1953; Shehata et al. 1955; Carson et al. 1956; Phaff and Knapp 1956; de Camargo and Phaff 1957; da Cunha et al. 1957; Prada and Pagnocca 1997; Lachance et al. 2001, 2003) and visiting insects, mainly drosophilids (da Cunha et al. 1957; Morais et al. 1992a, 1995a, b, 1996; Morais and Rosa 2000), and a few have studied the yeasts associated with mushrooms (Ramirez Gomes 1957).

18.2 Specificity of Association Between Yeasts and Substrates

The distribution and specificity of yeast communities depend mainly on the composition of nutrients present in the substrate, the presence of inhibitory compounds and the vectors that utilize those substrates as feeding and breeding niches (Gilbert 1980; Phaff and Starmer1987; Ganter 1988; Starmer et al. 1988). Yeasts have a nutritional dependence on the substrate, and different substrates bring up different assemblages of yeasts. It is well accepted that yeast ecology is more than just a more or less random distribution of these organisms in substrates where sugars are present at detectable levels. Plants that serve as habitats for large yeast communities are

often sites of ecological interactions involving other organisms and the substrate itself (Phaff and Starmer 1987).

Starmer (1981) demonstrated that the physiological attributes of yeasts are useful indicators of habitat characteristics, but a few studies apply the other approach: to use the biochemical characteristics of habitats to understand the associated yeast communities. Extensive knowledge of yeast habitat characteristics, such as sugar and water content and composition of fruits, tree exudates, nectar and pollen, would be of help in defining habitat specificity. Nevertheless, those efforts were found solely in the works of Starmer (1981) when looking for explanations of the specificity of interactions between *Drosophila* and yeasts. One would argue that is difficult to find information on the chemical composition of most natural substrates in forests, and limitations could be due to this scarcity. There is certainly a need for an interdisciplinary approach to the study of the relations between yeasts and specific substrates, and to fill the missing information on substrate chemistry.

18.3 Vectors of Yeasts

Yeasts are immotile organisms, which need to be carried by vectors. This association makes yeasts dependent on vectors, mainly insects, and probably they benefit from yeasts. A great number of studies have isolated yeasts associated with insects, especially *Drosophila*. Gilbert (1980) and Starmer et al. (1988) showed that these flies are important vectors of yeasts, which are important food sources for adult and larval stages of the flies (Begon 1982). *Drosophila* species show specific relation to certain species or groups of species of yeasts, usually owing to substrate composition or to biogeographic distribution of insects (Starmer et al. 1990). Lachance et al. (2001) discuss the role of nitidulid beetles in the distribution of yeasts in different biogeographical areas. Nevertheless, most studies on forests in Brazil have focused only on drosophilids, and efforts must be made to search for other associations that may be as important as the association between *Drosophila* and yeasts.

18.4 Forests as Ecosystems for Yeast Communities

Forest ecosystems are an attractive site for the collection of yeasts, and various researchers have made efforts to study the yeast diversity in those forests. Today, forests occupy approximately one third of Earth's land area, account for over two thirds of the leaf area of land plants, and contain about 70% of the carbon present in living things of terrestrial and aquatic habitats. Tropical forests are characterized by the greatest diversity of species. They occur near the equator, within the area bounded by latitudes 23.5° N and 23.5° S. One of the major characteristics of tropical forests is their distinct seasonality: winter is absent, and only two seasons are present (rainy and dry). The length of daylight is 12 h and varies little. The temperature is on average 20–25° C and varies little throughout the year: the average temperatures of the three warmest and the three coldest months do not differ by more than 5°C. Precipitation is evenly distributed throughout the rainy period of the year, with annual rainfall exceeding 2,000 mm. The tree canopy in tropical forests is multilayered and continuous, allowing little light penetration and the flora is highly

diverse since one square kilometer may contain as many as 100 different tree species. The trees are 25–35-m tall, with buttressed trunks and shallow roots, mostly evergreen, with large, dark-green leaves. Plants such as orchids, bromeliads, vines (lianas), ferns, mosses, and palms are present in tropical forests. The fauna includes numerous birds, bats, other small mammals, and insects.

Theoretical ecology indicates that ecosystems such as forests are in fact a mosaic of patchy habitats for organisms – and one could even state that they represent mosaics of ecosystems for microorganisms. The vertical arrangement of forests, from soil, litter, tree stems, trunks and canopy, together with flowers and fruits in different stages of ripening represent different niches for colonization of yeasts. Because they have a sugary composition flowers and fruits are ephemeral substrates constantly visited by insects and other vectors of yeasts creating a diversity of abiotic and biotic interactions that would probably support a huge biodiversity of microorganisms.

Differing from temperate forests, in tropical forests different plant species present flowers and fruits in different periods of the year, a characteristic that would produce food and substrates for colonization of yeasts throughout the year.

Brazil is the largest country in South America. Approximately 2.3 million square kilometers – 27% of Brazil's total area of 8.5 million square kilometers – can be considered "frontier" forest. Brazil's frontier forests comprise 17% of the world's remaining frontiers, making it the third highest ranked country in terms of remaining frontier forest. Brazil has the third largest block of remaining frontier forest in the world and ranks first in plant biodiversity among frontier forest nations.

Brazil's geographic space presents a great diversity of climate types, physiognomy, soils, and vegetation. As early as the nineteenth century, C.F.P. Martius recognized five phytogeographic provinces (defined as geographic spaces containing endemism). Such endemisms reflect diverse environmental conditions that create geographical isolations and allowed the birth to a highly diverse biota. Ab'Saber (1977) classified those provinces as dominions based on morphoclimate and phytogeography. A dominion is a geographic space having subcontinental extension where morphoclimatic and phytogeographic characteristics prevail that are distinct from those in other areas. This means that in a particular dominion, different biomes can be found. For example in the Dominion Cerrado (savannas) we can find forest ecosystems representative of biomes other than savanna. In the dominion of Cerrado, savannas dominate, but fragments of dry (deciduous) forests, semideciduous forests, gallery forests and tropical rain forests are also found. The dominion Caatinga, dominated by dry vegetation, also presents different forest ecosystems, including dry and humid forests. This increases the impact of the rarity of studies on yeast from Brazil's forest ecosystems, since the studies until now have focused mainly on Atlantic rain forest and Amazon forest sites.

18.5 Yeast Studies in Atlantic Rain Forests

The Atlantic rain forest represents one of the areas of the planet with the greatest biodiversity (Guedes-Bruni and Lima 1997) and is considered the second most threatened tropical forest among 15 regions denominated as hot spots (areas with high biodiversity, elevated levels of endemism and great antropic pressure) (Myers

et al. 2000). Originally the Atlantic forest was the forest with the largest latitudinal extension of the planet, going from about 6 to 32° S. In those days it covered about 11% of the national territory. Today, however, the Atlantic forest covers only 4% of its original area (Aidar et al. 2001). The climate along its extension varies, ranging from superhumid in the south to tropically humid and semiarid in the northeast. The uneven relief of the coastal zone adds more variability to this ecosystem. In the valleys, the trees grow a lot, forming a dense canopy. At the coastlines this forest is less dense, owing to the frequent fall of trees. At the southern end the Atlantic forest gradually mixes with the forest of Araucarias. In the state of Rio de Janeiro, where it represented 97% of the original plant coverage, it is nowadays reduced to less than 10% as a result of the ever-increasing rate of reckless destruction (Guedes-Bruni and Lima 1996). The forest types mentioned before are different from the Amazonian Hylaea in many respects, especially in relation to the greater availability of light at the lower portions. This is due to the layered distribution of trees in often-steep mountains, which generates a discontinuous canopy, allowing the entrance of more light (Silva and Leitão Filho 1982; Shepherd 2002). The greater availability of light makes possible the existence of different plant species at different levels in the forest, greatly boasting its biodiversity and making it in one of the most complex ecosystems of the planet (Kricher 1999).

Studies of yeasts in the Brazilian Atlantic forest started in the late 1950s with the study of da Cunha et al. (1957), who streaked individual crops of 281 *Drosophila* captured in Serra da Cantareira Forest reserve and other forest sites in São Paulo on propionate–malt agar at 25°C. They isolated 417 strains, of which 391 were identified to species level. The other 186 isolates were probably new species that could not be identified at that time. Most *Drosophila* crops (69%) contained only one species of yeast, whereas 22.8% contained two species, 7.1% contained three species and 1.1% contained four species of yeasts. They found 43 different yeast species, with 79% isolated one to six times, 9.3% found 10–20 times, 9.3% isolated above 20–30 times, and 4.6% found around 40 times. *Kloeckera apiculata* was isolated 122 times, and it was considered to have a uniform distribution among *Drosophila* species. *Candida krusei* and *C. mycoderma* were consistently associated with the medio group of *Drosophila*, whereas *C. parapsilosis* var. *intermedia*, *C. brasilae* (undescribed yeast species that does not have a synonym in the literature), and *Pichia membranifaciens* were associated with *Drosophila bocainensis. P. fermentans* was clearly associated with *D. willistoni*. The study also concluded that food preferences were different among *Drosophila* flies, because *P. fermentans* comprised 20.6% of the yeasts isolated from crops of *D. willistoni*, and *P. membranifaciens* represented 19.6% of the yeasts from crops of *D. griseolineata* and 11.1% of *D. willistoni. C. krusei* was 24.2% of the total isolates from crops of *Drosophila* of the medio group and 14.3% of the yeasts from *D. willistoni* crops. *C. mycoderma* constituted 14.9% of the yeasts from crops of *D. bocainensis*, 7% of yeasts in crops of *Drosophila* of the medio group and 5% of yeasts in crops of *D. guaramuru*, and it was not isolated in other drosophilids. *C. brasilae* was frequent (12.3%) in crops of *D. bocainensis*, being rare or not existing in other drosophilids. *K. apiculata* was prevalent in all *Drosophila*, being dominant in crops of *D. paulistorum* (58.8%), *D. griseolineata* (43.5%), *D. guaramuru* (38.5%), *D. bocainensis* (32.4%), *D. willistoni* (22.2%) and medio group (16.7%).

In the same work, da Cunha et al. (1957) isolated 46 yeasts from ten species from fruits at the same sites where they collected *Drosophila. C. brasilae* and *K. apiculata* were prevalent species, and *C. guilliermondii*, *C. krusei*, *C. parapsilosis*, *Cryptococcus luteolus*, *P. fermentans*, *Rhodotorula mucilaginosa* and *Saccharomyces acidifaciens* (= *Zygosaccharomyces bailii*) were also isolated. Among those, only three were not isolated from flies.

Morais et al. (1992a) studied the distribution and diversity of yeast species associated with *Drosophila* in three forest fragments in Rio de Janeiro. They isolated 557 cultures from the surface of 228 insects and 305 from crops of 286 flies collected at two sites in the Tijuca National Forest reserve and at one site of the fruit orchard at the Universidade Federal do Rio de Janeiro. The flies were attracted by means of baits and set to walk on yeast extract–malt extract agar (YMA) plates with chloramphenicol (100 mg/l) for 15 min, after which they were stored for identification. The other 286 flies were identified and crops dissected from them were streaked on the surface of YMA. Seventy-two species of yeasts were obtained after 3 days at room temperature. Apiculate yeasts, especially of the species *K. apis*, *K. japonica* and *K. javanica* were the most frequent isolates from *Drosophila* vectors, and *Aureobasidium pullulans, C. apicola*, *C. citrea*, *Debaryomyces vanrijiae*, *Geotrichum* spp., *P. beckii* and *Williopsis californica* were also found. This work could establish a specific association between yeast and *Drosophila* species. For example, *Kluyveromyces delphensis* was associated with the *D. willistoni* group, from which all but one of 34 strains were isolated. *D. vanrijiae* var. *yarrowi* was the predominant yeast associated with the *D. tripunctata* group. *K. javanica* was frequently isolated from the *D. melanogaster* group. Together with *P. beckii*, *D. vanrijiae* var. *yarrowi* was isolated from external parts of flies, and rarely from crops. *Saccharomyces cerevisiae* was represented by only three isolates. Two of these isolates were later shown to represent a new species, and they were described as *S. cariocanus* by Naumov et al. (2000). A few strains of *Pichia* and *Williopsis* were isolated from disturbed areas, but *P. angusta*, *P. beckii*, *P. carsonii*, *P. kluyveri*, *W. californica* and *W. saturnus* were represented by multiple isolates at primary forest sites and were associated with *D. guarani*, *D. tripunctata* and *D. willistoni groups*. With exception of *C. citrea* and *C. colliculosa*, the species of the genus *Candida* were restricted to primary forest sites. Basidiomycetous yeasts and their anamorphs were represented by only a few isolates. More extensive collections of insects would probably enlarge the list of yeast species.

In the same work, taxonomic questions were set, since differences were noted in physiological profiles between the type strains and those isolated from *Drosophila*. Some biotypes differed from the standard descriptions of species by growing at higher temperatures than expected. Among the apiculate yeasts, 68 strains of *K. apis* grew at 40°C, and 117 strains of *K. javanica* and four strains of *K. africana* grew at 37°C (Morais et al. 1992b). Strains of *C. citrea* grew at 37°C and strains of *C. berthetti*, *C. guilliermondii*, *P. acaciae* and an unidentified *Candida* species positive for the Diazonium Blue B (DBB) test grew at 43°C. Six strains were named under the epithet "like" that meant they did not fit a standard description and correspond to potential new species.

Morais et al. (1995a) isolated yeasts from a specific group of *Drosophila* in forests – the *fasciola* subgroup of the cactiphilic *repleta* group in the Tijuca National Forest

Park and Ilha Grande State Park in Rio de Janeiro. They isolated 61 yeast species vectored by 202 flies allowed to walk 8-12 h on YMA, and 38 species from crops of 205 flies that were dissected and streaked on YMA plates. A *P. membranifaciens* like species, *C. colliculosa* and *K. apiculata* were the most frequent yeasts, summarizing 35% of the isolates. The frequency of yeasts in male and female flies did not differed except for *K. apiculata*, which was present solely in females that also presented a higher diversity of associated yeast species. Taxonomic difficulties were encountered, and many strains did not follow the description of the type strains of species. Twenty-eight strains of *Candida* spp., nine strains of *Pichia* spp., six strains of *Saturnispora* sp. and 13 strains of *Sporopachydermia* spp. were probably new species, together with four isolates of a species similar to *C. blankii*, one isolate of a species similar to *C. buffoni*, five of a species similar to *C. colliculosa*, one isolate similar to *C. stellata*, 47 isolates similar to *C. valida*, 133 isolates similar to *P. membranifaciens* and one similar to *P. silvicola*, indicating that the diversity of yeasts in Atlantic rain forests in Brazil is almost unknown. The sequence of the D1/D2 domains of the large subunit ribosomal DNA (rDNA) of a *Saturnispora* strain showed that this yeast is a sister species of *S. dispora*. It is described as *S. hagleri* (Morais et al. 2005).

Morais et al. (1996) isolated 223 strains of 40 species of yeasts from 103 flies of the *D. quadrum* group in forests of Rio de Janeiro. The authors isolated 139 strains of 33 species in external parts of 82 flies that were allowed to walk on YMA plates, and 36 strains of 18 species of yeasts from 21 crops streaked on the surface of YMA plates after dissection. The prevalent yeast species associated with *D. quadrum* in the forest floor were *D. melissophilus* and *D. vanrijiae*, together with *C. guilliermondii* and *K. apis*, that are usually associated with flowers. Black yeasts, *C. antarctica* and *Rh. rubra* are typical of plant surfaces and were also frequent in samples. *C. diversa*, *C. insectamans*, *C. quercitrusa*, *P. membranifaciens* and its anamorph *C. valida* were also isolated. Yeasts belonging to this community did not significantly differ from the standard description of the species. The feeding niche of *D. quadrum* was probably limited to the forest floor and vicinities which this fly species visited and fed on yeasts colonizing fruits in late states of decomposition and flowers.

Yeast occurrence in bromeliad tank waters was studied at an Atlantic rain forest site in Rio de Janeiro (Araújo et al. 1998). Samples from *Nidularium procedurum* and *Quesnelia arvensis* were collected, and many yeast isolates found in these microhabitats were phenotypically similar to *S. cerevisiae*, and including a probable new species belonging to the *Saccharomyces sensu lato* group. Other isolated species were *C. famata*/*D. hansenii*, *C. intermedia*, *C. tropicalis*, *Cr. albidus*, *D. vanrijiae*, *Rh. mucilaginosa* and *W. saturnus*.

Abranches et al. (1998) recorded a high yeast diversity associated with some wild small mammals in a patchwork of habitats including cultivated fields, small Atlantic forest fragments, pasture and grasslands. The yeasts were isolated from fecal pellets from the traps in which the animals were caught. Fecal pellets from the marsupials *Didelphis marsupialis* (opossum, Didelphidae) and *Philander frenata* (Didelphidae) and from the rodents *Akondon cursor*, *Oryzomys* sp. and *Nectomys squamipes* (Akodontinii) were studied. The most frequent isolates were *P. membranifaciens*, *Issatchenkia orientalis* and its anamorph *C. krusei*, and *D. hansenii*. From 57 yeast species isolated, 21 were probably new species or biotypes. Most of the yeast species

isolated were typical of fruits. The high diversity of yeasts found in this work probably reflected the diversity of food items that these animals ate. The authors suggested that the fecal pellets of these animals could serve as an additional tool for assessing the diversity of yeasts in a given habitat.

A survey of the yeasts found in association with native fruits of a tropical rain forest in Brazil was made by Prada and Pagnocca (1997) at the Ecological Station of Juréia-Itatins, São Paulo, Brazil. During this study, 202 strains were isolated from ripe and/or unripe fruits from 20 representative angiosperms. Most (74%) of the isolates had ascomycetic affinity with *Candida* and *Kloeckera* (including its teleomorph *Hanseniaspora*) being the predominant genera, followed by *Cryptococcus*, *Sporobolomyces*, *Pichia*, *Bullera* and *Hansenula* (*Pichia*). *Candida* was the most frequently found genus (51%) with 30 species, whereas *K. apiculata* was the predominant species isolated (12.7%). The following species were identified in decreasing order of frequency: *C. valida*, *C. magnoliae* like, *C. versatilis* like, *P. kluyveri*, *C. boidinii* and *C. colliculosa*. Together, they represented 34.1% of the total number of isolates identified at the species level (n=126). It is important to emphasize that many of the strains isolated differed from the standard description of known species suggesting that they could represent new biotypes or even new species. Unfortunately at that time the methodology of sequencing of rDNA was not available for the authors and they could not confirm this hypothesis.

Similar research was made from 1999 through 2002 at a site close to the same ecosystem (Serra do Mar State Park, Picinguaba area). This area of around 47,000 ha is part of Ubatuba city, São Paulo state, on the border with Rio de Janeiro state. Three hundred and eleven flowers from 13 plant species, 72 fruits of six plant species and 75 water deposits in tanks of two bromeliad species were sampled in order to isolate the yeasts associated with them. Three hundred and twenty-six strains of yeasts were obtained, with 75.8% of them having ascomycetic affinity (Ruivo 2005). *Candida* was the predominant genus, followed by *Metschnikowia*, *Hanseniaspora*, *Bullera* and *Cryptococcus*. Among the ascomycetous yeasts and their anamorphs, 37 species were identified. *Hanseniaspora uvarum* was the prevalent species in flowers and fruits. These data are still being analyzed but some light has already been put on the richness of the yeast communities of this environment. Eight isolates of *C. leandrae*, a new ascomycetous yeast, were found in decaying fruits of *Leandra reversa* (Melastomataceae) (Ruivo et al. 2004). Additionally, another five new ascomycetous yeast species were found. Two of them, *C. bromeliacearum* and *C. ubatubensis*, were isolated from the bromeliad tank of *Canistropsis seidelii* (Bromeliaceae) (Ruivo et al. 2005a) and three others, *C. heliconiae, C. picinguabensis* and *C. sanpauloensis*, were isolated from *Heliconia velloziana* (Heliconiaceae) (Ruivo et al. 2005b). Some other strains did not fit well the standard description and some biotypes initially considered as *Candida* sp. A, B, C, D, E, F, G, H, I and *Debaryomyces* sp. A, B, C, among others, are still under investigation and it is possible that new yeasts species will be found.

Pimenta (2001) isolated 54 yeast species from different substrates at an Atlantic rain forest site (Rio Doce State Park) in Minas Gerais state in Brazil. Yeasts were isolated from fruits, mushrooms, plant exudates and *Drosophila* flies. Twenty-two species differed from known species in their physiological characteristics, and they

probably represent new yeast species. A new species of *Geotrichum*, *G. silvicola*, was described. This new species was associated with *Drosophila* flies (Pimenta et al. 2005).

18.6 Yeasts from Amazon Forest Sites

The Amazon Forest occupies the northern area of Brazil, embracing about 47% of the national territory. It is the largest forest formation of the planet, conditioned by the humid equatorial climate. It has a great variety of flora and fauna, living between dense forests and open fields. Three types of forest are common: dense forests on firm soil (*terra-firme*), periodic forested floodland (*várzea*) forests, and seasonally flooded (*igapó*) forest. The Amazon region attracted attention in the early 1950s with works of A.C. Batista focusing on yeasts in fruits, but an ecosystem approach was not done because fruits were collected in the markets of Manaus (Batista et al. 1961).

Mok et al. (1982) captured a total of 2,886 bats in the Amazon Basin of Brazil and processed these samples for the isolation of fungi. From the livers, spleens and lungs of 155 bats (5.4%), 186 fungal isolates of the genera *Candida* (123 isolates), *Trichosporon* (26 isolates), *Torulopsis* (*Candida*) (25 isolates), *Kluyveromyces* (11 isolates) and *Geotrichum* (one isolate) were recovered. Seven known pathogenic species were present: *C. parapsilosis*, *C. guilliermondii*, *C. albicans*, *C. stellatoidea (C. albicans)*, *C. pseudotropicalis*, *Trichosporon beigelii* (= *T. ovoides*), and *Torulopsis glabrata* (= *C. glabrata*). In another work, Mok et al. (1984) studied the ecology of pathogenic yeasts in Amazonian soils. The authors collected 1,949 soil samples from diverse geographical and ecological settings of the Brazilian Amazon basin, and analyzed them for the presence of nonkeratinophilic fungi by the indirect mouse inoculation procedure and for the presence of keratinophilic fungi by the hair bait technique. Two hundred and forty-one yeast and yeast-like isolates were obtained from 12% of the soil samples. *Candida* was the prevalent genus representing 89% of isolated species. *C. glabrata*, *C. famata* (= *D. hansenii*), *T. candida* (= *D. hansenii*), *C. guilliermondii* and *T. dattila* (= *C. dattila*) were the most frequent species. Forty-six isolates could not be classified and were composed of 25 *Torulopsis* isolates, 13 *Candida*, and eight nonascosporic white yeasts.

Morais et al. (1995b) presented a study focusing on fruits of *Parahancornia amapa* (*amapa*, Apocynaceae), *Anacardium giganteum* (*cajuí*, Anacardiaceae), *Helycostis* sp. (Moraceae), *Platonia insignis* (*bacuri*, Guttiferae) and *Clusia grandiflora* (*cebola da mata*, Clusiaceae) in two upland (*terra-firme*) forest sites near Belem and on Marajo Island, in Para. Yeasts were isolated from fallen fruits with different days of falling and counts were generally between 5×10^5 and 1×10^6 cfu/g, reaching 2×10^7 and 8×10^6 cfu/g. The most frequent species in amapa fruits were *K. apiculata*, which represented 16% of total isolates, and it presented the highest populations. *C. amapae*, *C. krusei*, *C. sorbosa* like complex, *C. fructus*, *C. sorboxylosa* like complex and *P. kluyveri* var. *kluyveri* were also frequent. These species constituted 50% of all isolates from amapa fruits. The yeasts *C. amapae* and *C. krusei* can be considered as consistently associated with amapa fruits, since they showed a high frequency in amapa fruits, but were not isolated from other fruit substrates at the same forest

sites. The most frequent species in *A. giganteum* fruits were *C. guilliermondii*, *K. apiculata* and *P. membranifaciens* like complex. In *Helycostis* fruits, *K. apiculata*, *K. apis* and *C. sorboxylosa*-complex were the most frequently isolated yeasts. In *Platonia insignis*, *K. apiculata* and *C. sorboxylosa* complex were commonly isolated. In *C. grandiflora* fruits, black yeasts, *Cr. humicola* and *K. apiculata* were frequently isolated.

In this study, the succession of yeasts colonizing 99 fallen fruits from amapa trees was verified from the first to the fourteenth day. Yeast succession was represented by 19 species, among which *C. amapae* and *C. krusei* were isolated throughout the course of succession in fallen fruits. *C. sorbosa* like, *K. apis* and *P. membranifaciens* like dominated the fruits soon after they had fallen, *C. guilliermondii*, *C. sorboxylosa* like and *K. apiculata* appeared during early ripening (1–2 days), whereas *C. fructus*, *C. insectamans*, *C. norvegensis*, *P. kluyveri*, *P. membranifaciens* and *P. pijperi* appeared after the third to tenth day after the fruits had fallen. Of these, *P. kluyveri* and *C. fructus* secreted killer toxins, which enabled these yeasts to persist longer. After day 8, the counts were lower and the yeast species were represented by *C. diversa*, *C. karawaiewii* and *C. quercitrusa*.

In the same work, Morais et al. (1995b) isolated 215 isolates of 18 species of yeasts from surface of 45 flies of the *D. willistoni* group, 26 flies of *D. malerkotliana* and 11 *D. sturtevanti* flies associated with fallen amapa fruits. The yeasts most frequently vectored by flies included *K. apiculata*, *P. membranifaciens* like complex and *C. citrea*. Sixteen species were associated with the *D. willistoni* group, with which a high diversity of yeast species were associated, although *P. membranifaciens* was dominant. Flies of *D. malerkotliana* were associated with seven yeast species, dominated by *K. apiculata*, and *D. sturtevanti* was associated with five species of yeasts, *C. sorboxylosa* and *K. apiculata* being the most frequent ones. Among male flies of the *D. willistoni* group, *C. insectamans* was isolated only from *D. willistoni*, showing a close association with this fly species. *D. tropicalis* was frequently associated with *C. norvegensis*. The authors also isolated yeasts from crops of 55 flies of the *D. willistoni* group, 20 *D. malerkotliana* flies and six *D. sturtevanti* flies. The most frequent isolates in crops of flies were *K. apiculata*, *C. insectamans* and *P. membranifaciens* like. *C. citrea*, frequently isolated from fly surfaces, was not isolated from crops. Twelve yeast species were isolated from crops of flies of the *D. willistoni* group, *K. apiculata* being dominant, together with *C. insectamans*, which was isolated solely from this fly group. The *P. membranifaciens* like complex was frequently isolated from crops of male flies of *D. paulistorum*, and it was rarely isolated from crops of females of this fly or from other groups. *C. norvegensis* was isolated only from crops of *D. tropicalis* males, together with *C. fructus*, and varieties of *C. sorboxylosa* complex, indicating diet specificity in flies of the group. Five species were isolated from *D. malerkotliana* crops, and *K. apiculata* was the dominant yeast. *K. apiculata* and *P. pijperi* were the only species isolated from crops of *D. sturtevanti*.

This work resulted in the description of a new species, *C. amapae* (Morais et al. 1994). D1/D2 sequences of the 26S subunit of the rDNA showed that this species is closely related to *Saccharomycopsis crataegensis* (Kurtzman and Robnett 1998). Eleven species were assigned as species similar to *C. blankii*, *C. deformans (Yarrowia lipolytica)*, *C. diddensiae*, *C. quercitrusa*, *C rugopelliculosa*, *C. sorbosa*, *C. sorboxy-*

losa, *C. terebra* (= *P. mexicana*), *P. kluyveri*, *P. membranifaciens*, *P. muscicola* and *P. sargentensis*. They are probably new yeast species that wait description.

Vital et al. (2002) isolated and identified 240 yeasts from soils of the Maraca Ecological Station, located on Maracá Island, in the Amazon state of Roraima, in the Brazilian Amazon. These isolates were grouped into 16 genera and 66 species, with 82% of the strains having ascomycetous affinity. Some isolates had an intermediate level of partial D1/D2 sequence similarity with known species, and represented new undescribed species.

18.7 Other Ecosystems in South America

Gonzalez et al. (1989) in a study of yeasts during the delignification and fungal transformation of wood into cattle feed in continental and island locations of rain forest of southern Chile isolated 327 yeast strains from 33 samples of wood from *Eucryphia cordifolia* (Eucyphiaceae), *Nothofagus dombey* (Nothofagaceae), *N. oblique* (Nothofagaceae), *Laurelia sempervirens* (Atherospermataceae, Laurales), *L. phillipiana* (Atherospermataceae, Laurales), *Drimys winteri* (Winteraceae) and *Myrceugenia planipes (*Myrtaceae) at different stages of degradation. Most representative yeasts belonged to 37 species whose occurrence was not influenced by the type of wood. The most abundant yeasts were *Schizoblastosporion chiloense*, *C. castrensis* and *Apiotrichum futronensis* (= *Rh. futronensis*). Several new species were described among the strains isolated in this study (Ramirez and Gonzalez 1984a–j). Different stages of decay by *Ganoderma applanatum* were dominated by different yeast species: *C. parapsilosis* and *C. railenensis* were dominant at the initial stage; *S. chiloense*, *Rh. rubra* and *C. sophiae-reginae* at the intermediate stage; *Citeromyces matritensis* at the advanced stage; and *C. bertae*, *Cr. albidus* var. *diffluens*, which was present during the four stages, and *C sake* prevailed at the final stage. The relative abundance of basidiomycetous yeasts was low at the initial stage of decay, but significantly higher at more advanced stages (40–50% of colonies), particularly in samples of wood decayed by the mushroom *Armillariella limonea*, where they were represented by *A. humicolum* (= *Cr. humicola*), *A. futronensis* and *A. eucryphiae* (= *Leucosporidium scottii*).

Spencer et al. (1992) isolated 127 strains of yeasts from spoiled fruits, oranges, lemons and mandarins from a shop in Tucuman, Argentina, that included *C. guilliermondii*, *C. famata*, *C. stellata* and *P. kluyveri*. They also isolated probable new species, including ten strains belonging to the *P. membranifaciens* group, 13 unidentified strains of *Candida* spp., 20 strains of *Kloeckera* spp., 31 strains of *Pichia* spp. and two strains of *Cryptococcus* spp.

The distribution of yeasts was studied in different plant substrata of sandy plains along the southeastern Brazilian coast (Rosa et al. 1995). These formations are classified under the broad category of "restinga" vegetation. Restinga ecosystems are located at the interface between marine and continental (Atlantic rain forests) environments. Plant species living in these ecosystems are adapted to stressful environmental conditions such as high solar radiation, nutrient deficiency, drought, salt spray and high winds (Lacerda et al. 1984). Yeast communities were sampled from the cacti *Cereus pernambucencis*, *Selenicereus rizzini*, *Opuntia vulgaris* and *Opuntia*

sp., from flowers of *Ipomoea pes-caprae* and *I. litoralis* (Convolvulaceae) and from extrafloral nectaries of *Senna australis* and *S. bicapsularis* (Leguminoseae). Fifty-seven yeast species were isolated in this study, and nineteen were possible new species. Three distinct groups were noted among the different yeast communities studied. Yeast communities from necrotic tissues of cacti were grouped together; a second group was formed by yeasts from cactus flowers; and the last group was formed by yeast of the other non-cactus substrata. The species identified as *C. domercqii* like was described as a new species of *Wickerhamiella*, *W. cacticola* (Lachance et al. 1998). The *P. ohmeri* like strain represented a new species, and it was described as *Kodamaea nitidulidarum* (Rosa et al. 1999). A yeast identified as *Pichia* sp. C in this work was described later as *C. restingae*, a species belonging to the *Kodamaea* clade (Rosa et al. 1999). These three species were associated with cactus flowers.

Flowers of *I. litoralis* and *I. pes-caprae* were collected within 100 m of the ocean and were subjected to salt spray. This may have influenced the high occurrence of species capable of growing in media containing 10% NaCl in these yeast communities. Most yeast species from *Ipomoea* flowers and extrafloral nectaries produced pigments, extracellular polysaccharides and utilized a wide array of carbon sources. These characteristics could be important adaptive traits to help these yeasts colonize the microhabitats occurring in the restinga ecosystems (Rosa et al. 1995).

Santos et al. (1996) isolated yeasts from flowers and fruits from cashew (*A. occidentalis*), cajá (*Spondia lutea*) and umbu (*Spondia* sp.) in a semiarid region near Campina Grande and João Pessoa, state of Paraíba, Brazil. The most frequent yeasts in flowers of these three trees were basidiomycetous anamorphs and black yeasts. Green fruits of cajá and umbu had a high number of yeast species with prevalence of black yeasts, *Cr. laurentii*, *C. entomaea* like and *Candida* spp. Cajá ripe fruits had the highest yeast diversity, and *K. javanica*, *I. orientalis*, *C. entomaea* like and *I. terricola* as prevalent species.

Yeasts were isolated from exudates of algarrobo (*Prosopis* spp.) trees from nine sites in northwestern Argentina, three in the Chaco region, from pods of algarrobo and acacia, collected in the Quebrada de Cafayate (Spencer et al. 1996). This region is arid to semiarid. Among 379 yeast isolates from exudates of algarrobo most were identified as *Bullera variabilis*, *C. famata*, *Cr. albidus* and other *Cryptococcus* species, *D. hansenii*, *P. angusta* (*Ogataea polymorpha*), *P. ciferrii*, *P. farinosa* and *Torulaspora delbrueckii*. Other *Candida*, *Kluyveromyces* and *Pichia* species were also found. Most species were osmotolerant. The high sugar content of the exudates influenced the nature of the yeast species present. The pods of *Acacia* and Algarrobo contained *Cryptococcus* spp., *C. famata* and *C. ciferrii*. Half the species isolated from rotting cactus were *Cryptococcus* species. *P. membranifaciens* and *T. delbrueckii* were also isolated.

Trindade et al. (2002) isolated yeasts from four different tropical fruits: pitanga (*Eugenia uniflora*), mangaba (*Hancornia speciosa*), umbu (*Spondia tuberosa*) and acerola (*Malpighia glaba*). Fruits were collected in small farms in northeastern Brazil. The most frequent yeasts were *K. apis*, *Pseudozyma antarctica*, *Cr. laurentii* and several species of *Rhodotorula*. Frozen pulps of these fruits present *C. sorbosivorans*, *C. spandovensis*, *C. spandovensis* like, *P. membranifaciens* and *Schizosaccharomyces pombe* as

prevalent species. From 70 yeast species isolated in this study, 24 did not fit in any previously described species, and were probably new biotypes or new species. Trindade et al. (2004) showed that the species identified as *C. spandovensis* like was phylogenetically related to *C. spandovensis* and *C. sorbophila*, species belonging to the *Wickerhamiella* clade. This new species was described as *C. sergipensis*.

Currently, a study of yeasts associated with flowers of different plant species in the dominion of Cerrado is being done in middle northern Brazil. It included at this time 55 samples of tree exudates in *ipucas*, natural forest fragments in flooded plains that resulted in the isolation of 45 strains of yeasts which included a new methylotrophic yeast belonging to the genus *Ogataea*, *Ogataea falcaomoraisii* (Morais et al. 2004). The sampling effort included 86 flowers of *Tabebuia aurea* (Bignoniaceae), from which 125 yeast strains were isolated, including five new ascomycetous species that await description, and 106 flowers of *Ipomoea* spp., from which 187 strains were isolated that included yeasts such as *Metschnikowia continentalis*, *M. colocasiae*, a new species of *Metschnikowia, K. apiculata*, five new species of *Starmerella*, and *P. antarctica* (Morais and Rosa, unpublished).

18.8 Physiological Profiles of Yeast Communities in Tropical Rain Forests

Physiological characteristics and metabolic abilities of yeast communities show a pattern that accompanies the evolutionary history of the associated substrates and vectors (Starmer 1981; Lachance and Starmer 1982). Communities evolved from the colonization of foliar substrates and mushrooms, sites associated with yeasts presenting large physiological profiles, to colonization of flowers, fruits and specific substrates such as necrotic tissues, which are more specialized and evolutionarily more recent (Starmer 1981). According to Lachance and Starmer (1982), the physiological structure of yeast communities is a function of evolutionary divergence in tree taxa, as viewed by Dahlgreen (1980) and Hutchinson (1969). They showed a gradient from polytrophic communities associated with Betulaceae – trees deemed more "primitive" – at one end to the nutritionally selective yeasts linked to the Campanulaceae and the Myoporaceae – more "advanced" (Sporne 1980) – at the other end. It is probable that yeast communities are shaped in response to changes in nutrient composition of tree habitats, and that chemical breadth, rather than the absence of specific compounds, is the major determining factor.

A comparison was made by Morais et al. (1992a) of physiological profiles of yeasts associated with *Drosophila* from Atlantic rain forests and temperate forests (from Heed et al. 1976), which showed that most yeasts ferment glucose in both environments, but they differ in assimilation characteristics. Most yeast species associated with *Drosophila* in Atlantic rain forests were not unique but were similar to yeast communities associated with fruits and other fermentable substrates. The yeast biotypes assimilative abilities were mostly restricted to a few compounds that include glycerol, D-xylose, sucrose, cellobiose, mannitol and succinate. In contrast, yeasts from temperate American forests were largely assimilative of galactose, sucrose, maltose, trehalose, cellobiose, salicin, glycerol, mannitol and succinate. Those yeasts differed strongly from yeast profiles from other habitats such as cacti and associated *Drosophila* in

desert sites. The genera *Pichia* and *Hanseniaspora*/*Kloeckera* predominate among yeasts of flies from tropical forests and *Drosophila* in Brazil.

According to Morais et al. (1995b), the physiological abilities of the yeast community associated with amapa fruits were mostly restricted to the use of glycerol, cellobiose, D-lactate, salicin, and L-sorbose among 36 carbon compounds tested. The community is strongly fermentative (82% of isolates) and capable of growing at 37°C more than other tropical isolates. In Amazonian forests, a large variation of responses to temperature, including ability to grow at temperatures higher than that described for the species, was a general rule among yeasts, and Morais et al. (1995b) correlate this to the high daily variation in temperature (up to 6°C) in rainy forests of Brazil.

The different stages of deterioration of the amapa fruit should be regarded as a microhabitat mosaic in which different yeast species colonize different stages, in a successional pattern directed by the chemical composition of the substrate, and mainly by visitation of vectors attracted to it by yeasts. In the first stages the fruits were colonized by yeasts fermenting simple sugars and presenting restricted assimilation profiles, such as *C. sorbosa* like complex and *K. apis*, whereas later stages corresponded to colonization of yeasts with larger assimilative characteristics, such as *C. diversa*, *C. karawaiewii* and *C. quercitrusa*.

18.9 Ecological Interpretation of Yeast Diversity in Forests

Fruits, flowers and *Drosophila* flies provided a good estimate of yeast diversity in tropical ecosystems (Table 18.1). Yeast community structure and species diversity seems to reflect the degree of disturbance in tropical forests. Comparisons among habitats have shown that yeast diversity is higher in primary forest sites, probably as a result of more diversified food sources in primary than in disturbed areas. A higher diversity of food sources is also supported by the extensive physiological profiles of yeast communities associated with *Drosophila* species that prevails at primary sites (Morais et al. 1992a).

Morais et al. (1995b), studying yeasts associated with ripe Amazon fruits at two Amazon rain forest sites, detected a higher diversity at the more heterogeneous sites in protected areas with higher plant diversity than at homogenous sites of lower plant diversity. Yeast species composition and diversity could indicate habitat heterogeneity in tropical ecosystems, since environmental degradation and patch removal seem to extinguish populations and decrease diversity (Nee and May 1992).

18.10 Concluding Remarks

Many of the isolates in the studies of yeasts from forests in Brazil and other countries of South America varied from the descriptions of known species, confirming the need for further taxonomic studies of yeasts from tropical forests. We can conclude from the studies already made that South American forests are yet to be screened for yeasts. It was shown that yeast vectors such as drosophilids are an important sampling unit for further investigations, and will allow comparisons among yeasts communities from different ecosystems and biomes. There is a

Table 18.1 Comparison of yeast diversity associated with plants and insects in three different ecosystems in Brazil[a]

Yeast species	Sandy coastal plains of Rio de Janeiro		Atlantic rain forests, Rio de Janeiro			Amazonian rain forest	
	Flowers of *Ipomea* spp (*n*=91)[b]	Extrafloral nectaries of *Senna* spp (*n*=109)	*Drosophila fasciola* (*n*=399)	*Drosophila quadrum* (*n*=103)	*Drosophila* spp. (*n*=624)	*Parahancornia amapa* fruits (*n*=127)	*Drosophila* species (*n*=173)
Aureobasidium pullulans	40	38	19	8	21	6	
Candida amapae						29	
C. antarctica	55	25	4	1	2		
C. apicola	1		1	7	23		
C. apis				3	14		
C. berthetii					2		
C. bimundalis (= *Pichia bimundalis*)			1		1		
C. blankii like[c]			3	1		1	
C. boleticola			4		7		
C. citrea (= *P. nakasei*)			4		48	4	14
C. colliculosa			145	1	20		
C. colliculosa like			5				
C. datilla (*Lachancea thermotolerans*)			2				
C. deformans like						1	
C. dendrica			2			1	
C. diddensiae like						1	
C. diversa			7	5	1	18	8
C. fabianii (= *P. fabianii*)			1				
C. fructus			2	7	15	24	6
C. guilliermondii	2			16	2	16	
C. holmii (= *Kazachstania exigua*)						1	
C. humicola (Cr. humicola)					3		

Continues

Table 18.1 Comparison of yeast diversity associated with plants and insects in three different ecosystems in Brazil[a]—*cont'd*

Yeast species	Sandy coastal plains of Rio de Janeiro		Atlantic rain forests, Rio de Janeiro			Amazonian rain forest	
	Flowers of *Ipomea* spp (n=91)[b]	Extrafloral nectaries of *Senna* spp (n=109)	*Drosophila fasciola* (n=399)	*Drosophila quadrum* (n=103)	*Drosophila* spp. (n=624)	*Parahancornia amapa* fruits (n=127)	*Drosophila* species (n=173)
C. humicola like					1		
C. insectamans				1		10	30
C. intermedia				1			
C. karawaiewii			2			6	
C. karawaiewii like					1		
C. krissii like	2						
C. krusei			107			27	
C. lambica (= *P. fermentans*)			4	1	5		
C. lypolitica like						2	
C. magnoliae			1		2		
C. melinii (= *P. canadensis*)				2	11		
C. melinii like	1						
C. norvegensis			14		4	8	9
C. norvegensis like					3		
C. parapsilosis			3			8	
*C. pelli*culosa			3	3			
C. pulcherrima (= M. pulcherrima)					15		
C. quercitrusa				11	14		
C. reukafii like	12						
C. rugopelliculosa			1				
C. santamariae like	5						
C. sorbophila			1				
C. sorbosa			14				
C. sorbosa like	25	25			1	30	25

C. sorboxylosa like	17	17				75	17
C. sorboxylosa			40				
C. steatolytica (= Zygoascus hellenicus)	1						
C. stellata like			1				
C. tepae like					1		
C. terebra like							1
C. valida (P. membranifaciens)	1						
C. valida like			48				
Candida spp.	29	2	9		3		
Clavispora opuntiae			7				
Cryptococcus albidus				1			
Cr. albidus var. aerius		12					
Cr. albidus var. albidus		32					
Cr. flavus	11	4					
Cr. infirmominiatus (Cystofilobasidium infirmominiatum)					1		
Cr. humicolus				1		5	1
Cr. hungaricus		4	4	4			
Cr. laurentii	48	9					
Cr. luteolus		1		8			
Cr. macerans	1	15			2		
Cryptococcus sp.	1						
Debaryomyces melissophilus			2	17	7		
D. vanrijae var. yarrowii				28	25	10	
Geotrichum sp.			25	11	47	16	1
Hanseniaspora guilliermondii			16				
H. occidentalis			34		2	2	3
H. uvarum				2	8		
H. valbyensis					2		
H. vineae					1		
Issatchenckia occidentalis				2		1	
Kloeckera africana				1	10	3	
K. apiculata	2		84			128	150

Continues

Table 18.1 Comparison of yeast diversity associated with plants and insects in three different ecosystems in Brazil[a]—*cont'd*

Yeast species	Sandy coastal plains of Rio de Janeiro		Atlantic rain forests, Rio de Janeiro			Amazonian rain forest	
	Flowers of *Ipomea* spp (n=91)[b]	Extrafloral nectaries of *Senna* spp (n=109)	*Drosophila fasciola* (n=399)	*Drosophila quadrum* (n=103)	*Drosophila* spp. (n=624)	*Parahancornia amapa* fruits (n=127)	*Drosophila* species (n=173)
K. apis	29		75	18	145	42	2
K. corticis					3		
K. japonica				1	86	6	
K. javanica			23	1	117		
Kluyveromyces delphensis (= Nakaseomyces delphensis)					34		
K. marxianus var. drosophilarum			3	1	10		
K. marxianus var. marxianus					1		
K. thermotolerans (= L. thermotolerans)					2		
K. wickerhamii					5		
Nadsonia elongata like			5				
Pichia abadiae					1		
P. acaciae				9	1	1	2
P. angusta (O. polymorpha)					11		
P. anomala				1	1		
P. beckii				2	34		6
P. cactophila			13				
P. carsonii			2		5		
P. dryadoides					4		
P. etchelsii (= *D. etchelsii*)						7	
P. fermentans						7	1
P. holstii					4		
P. kluyveri			121	1	8	21	1

P. kluyveri like						35	18
P. lindneri (= *P. minuta*)				4			
P. membranifaciens like			133	10		53	73
P. muscicola like						1	
P. ohmeri (= *Kodamaea ohmeri*)	4						
P. pijperi						13	4
P. sargentensis like						3	
P. silvicola					2		
P. silvicola like			1				
P. toletana					1		
Pichia spp.			9				
Rhodotorula araucariae					1		
Rh. aurantiaca	17	1			1		
Rh. fujisanensis					1		
Rh. glutinis	48	38	2	4	1		
Rh. lactosa		1					
Rh. minuta	1	3			2		
Rh. rubra		5	1	17	2		
Saccharomyces cerevisiae					3		
Saccharomycodes ludwigii			1				
Saturnispora sp.			6				
Sporobolomyces roseus	24						
Sporopachydermia spp.			13				
Torulaspora delbrueckii			1	5	3		
T. globosa			5				
T. pretoriensis			7		3		
Tremella globispora	2						
Tremella spp.		5					
Williopsis californica					22		
W. saturnus					8		
Zygossaccharomyces rouxii			1				
Total yeast species	25	17	58	40	70	34	24

[a]Morais et al. (1992a, 1995a, b, 1996) and Rosa et al. (1995)
[b]Number of samples
[c]Probable new species similar in characteristics to the species indicated

significant amount of literature on Lepidoptera, Coleoptera and Hymenoptera, especially ants and bees that could guide a strategy for research of yeasts associated with insect guild and communities in different landscapes of the most threatened biomes. We need to make an effort to test methodologies applicable to interdisciplinary studies, such as field traps that could be employed to aseptic collections, collection procedures for yeast isolation less destructive to insects, and standard approaches to taxonomy, ecology and phylogeny of both groups.

Fruits and flowers are a very attractive sampling unit, since they are easy to collect and to handle in the laboratory, and many methodologies could be employed to isolate yeasts from those substrates. Also, phenological information on and the distribution of species in ecosystems and ecological characters are already known for many species of plants.

A map of known yeast communities in forests shows that most hot spots for biodiversity – also threatened ecosystems – are yet to be studied. A strategy focusing on biodiversity hot spots is important to direct efforts to understand yeast community structure and composition in forests, and that will result in magnification of yeast biodiversity in forests in South America.

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The Biogeographic Diversity of Cactophilic Yeasts

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19.1 Introduction

Two commonly used measures of biological diversity are the number of unique species (species richness) and the uncertainty of species identity (diversity measured by H'; Pielou 1975). These metrics are affected by the availability of suitable habitats and are ultimately a function of speciation, extinction, and immigration patterns (Ricklefs 1987; Zobel 1997; Hubbell 2001). For this last function, it is generally thought that the size of an organism is important in its capacity to disperse and that very small organisms (microorganisms) freely disperse over large areas, usually as passive propagules in currents of air or water. In this situation the species in a local area are expected to be the same as species over larger, even global, expanses (Godfray and Lawton 2001). This "bugs are everywhere" hypothesis is, however, without foundation for many small organisms that have formed obligate relationships with vectors that constrain their dispersal patterns. Among the fungi, ascomycetous yeasts are often constrained by their vectors (Phaff and Starmer 1987). Two examples are the yeasts associated with drosophilids that feed and breed in the necrotic tissues of cacti (Barker and Starmer 1982) and yeast communities in blossoms of Convolvulaceae (morning glory, bindweeds) and *Hibiscus* species. This latter group has been reviewed and studied in a biogeographic context by Lachance et al. (2001b). They concluded that geographic factors may not act directly to determine yeast distributions but rather act indirectly through their insect vectors (i.e., Coleoptera) that distribute the yeasts to their flower–host resources. These insects have a major influence on the biogeographic diversity of flower-specific yeasts. The extensive records and wide ranging sampling that characterize studies of the flower-inhabiting yeasts make them a useful system for studying yeast ecology and insect–yeast interaction. The system is comparable to the cactophilic yeasts (Starmer and Fogleman 1986; Starmer et al. 1991).

The cactophilic yeasts have been collected and studied for over 30 years and provide insights into the origin of new species (Starmer et al. 1980), host and vector ecology (Ganter et al. 1986), community stability (Latham 1998), yeast diversification

(Lachance et al. 2000, 2001b), origin of yeast communities (Starmer et al. 2003), and the relationship of phylogeny to yeast community organization (Anderson et al. 2004). Only four dominant yeasts are found world-wide in the cactophilic yeast community [*Pichia cactophila*, *Candida sonorensis*, *Sporopachyderma cereana* (and related sister species) and *Clavispora opuntiae*]. Several species are very common (*Myxozyma mucilagina*, *S. amethionina* clade species, *Dipodascus starmeri*, *Pichia deserticola* and *Pichia kluyveri* v. *eremophila*). All of these species are cactus-specific; they are rarely recovered from other habitats such as sympatric fruit rots (Starmer et al. 1987) or slime fluxes of trees (Ganter et al. 1986). These species have diverse origins. They do not belong to a single cactus-yeast clade but evolved from distinct ancestors found in separate clades that show affinity to fruit-rot and tree-flux habitats (Starmer et al. 2003). Overall 80 species have been detected in decaying columnar-cactus stems or *Opuntia* cladode rots. In this chapter, we shall analyze the pattern of diversity for these 80 species found in cacti and discuss possible reasons for those patterns.

The biogeography of cactophilic yeasts has been described, compared and discussed in terms of host and geographic determinants of species and community distributions (Starmer et al. 1990). That analysis compared four community types (host cactus categories) across five semiarid regions of the southern USA, Mexico, northern Venezuela, as well as Caribbean and Bahamian islands. The analysis showed that yeast communities from the same host cactus type were more similar to one another across the five regions as compared with yeast communities from different host cactus types within regions. It thus appeared that the host plant had a larger influence on the diversity and the composition of the yeast community than geographic separation. However, this conclusion for community similarity was not a generality that extended to all species, where some geographic factors may be important to the diversification and speciation of some cactus specific species complexes (i.e., *Sporopachyderma* spp., Lachance et al. 2001b; *Phaffomyces* spp. Starmer et al. 2001; and *Starmera* spp. Starmer et al. 1990). Since the last review of the biogeography of the yeasts associated with cacti (Starmer et al. 1990) new collections and new species have been added to the database, and records from other continents (primarily Australia, where *Opuntia* cacti were introduced) have been incorporated. The additional data for Australian localities add another level to the determinants of biodiversity. This situation is unique because not only were the cacti introduced but the cactus yeasts (from the Americas) were also introduced when biological control was attempted in efforts to remove *Opuntia* from large geographic areas of eastern Australia (Starmer et al. 1987). In addition, the local yeasts already in Australia are expected to be significantly different from those on other continents. This difference in indigenous microbiota is expected to add significantly to the species richness and diversity in the rotting cladodes of *Opuntia* in Austrialia.

19.2 Methods

19.2.1 Measuring Diversity

Even though diversity can be quantified by many different metrics (for example, 24 measures of β diversity, Koleff et al. 2003), a useful method is to partition the global

diversity, γ, into diversity between and within levels of a hierarchy. The simplest partition is $\gamma = \alpha + \beta$, where α is the average diversity for all of the samples and β is the between-sample diversity or the difference between γ and α (Lande 1996; Loreau 2000). When several levels exist in a hierarchy, e.g., host plants, local areas, and regions such that n=3, then $\gamma = \alpha + \beta_1 + \beta_2 + \beta_3$. In this example α is the average diversity of the yeasts in the host plants, β_1 is the diversity between host plants, β_2 is the diversity between localities, β_3 is the diversity between regions, and γ is the total diversity.

The additive partition can be used for species richness S (defined as the total number of unique species in the entire collection), the Simpson index $d=1-\Sigma p_i^2$ or the Shannon–Weiner index $H' = \Sigma p_i \ln(p_i)$, where p_i is the proportional representation of each species in the level under consideration. This approach has been reviewed by Veech et al. (2002) and is becoming widely used by ecologists to understand the relationship between scale and biological diversity (Godfray and Lawton 2001). For an extensive discussion of β diversity see Vellend (2001). Veech et al. (2002) suggest converting the diversity components in the hierarchy into percentages or proportions of the total (γ) for comparison of the relative contribution to the total species richness (S), Simpson's index (d), or the Shannon–Weiner index (H'). We have followed their suggestion and present statistics for S and H'.

19.2.2 Hosts

Cactus hosts were categorized according to the systematic groupings outlined by Gibson and Nobel (1986) and conform to the listing given in Starmer et al. (1990). The major divisions we use in our analysis are given in bold in Table 19.1. They were chosen for study because they represent a nested taxonomic hierarchy and have adequate sample sizes.

19.2.3 Geography

The database used in this study includes records for 188 distinct collection localities in eight regions (listed in the following with the number of localities for each, see Fig. 19.1 for a map of the New World regions):

1. AU (44): Australia (Queensland and New South Wales)
2. CA (24): Caribbean Islands [Greater Antilles: Cuba, Cayman Islands, Jamaica, Navassa, Hispainola (Haiti and Dominican Republic); Lesser Antilles: Montserrat, US & British Virgin Islands, and Islas Los Roques]
3. FB (7): USA(Florida), Bahama Islands (Great Inagua and Conception Island)
4. HA (2): USA (Hawaii)
5. SM (25): Mexico (Chiapas, Guerrero, Hidalgo, Jalisco, Michoacan, Oaxaca, and Puebla), Honduras
6. SD (71): USA (Arizona and California), Mexico (Sinaloa and Sonora)
7. TX (5): USA (Texas)
8. VZ (10): Venezuela (northern)

Table 19.1 Cactus hosts sampled according to systematic groups. Those groups given in *bold* were used as categories for analysis. The number of yeast species and the number of plants sampled are in *parentheses*. Only the number of plants sampled is given for each genus

Family	Subfamily	Tribe	Subtribe	Genus
Cactaceae (80, 2649)	**Opuntioideae** (67, 1651), North America: (42, 950), introduced: (46, 701)			*Opuntia* (1602), *Nopalea* (49)
	Cactoideae (49, 998)	Cacteae		*Ferocactus* (13)
		Cereeae		*Cereus* (36), *Melocactus* (18)
		Hylocereeae		*Acanthocereus* (7)
		Leptocereeae		*Neoabbottia* (12)
		Pachycereeae	**Pachycereinae** (36, 335)	*Lophocereus* (80), *Carnegiea* (54), *Backebergia* (1), *Cephalocereus* (28), *Neobuxbaumia* (1), *Pachycereus* (37), *Pilosocereus* (134)
			Stenocereinae (38, 572)	*Stenocereus* (533), *Myrtillocactus* (28), *Escontria* (16)

The hierarchy available for cactus yeasts is easily viewed as a nested geographic set, starting from the global or continental level, which is divided into distinct regions, that are further divided into subregions composed of a group of localities in which individual rot pockets of plants are sampled to yield the incidence of yeast species. Thus, each yeast species has a plant, locality, subregion, region, and continental designation such that diversity can be viewed at all levels. Specific definitions of the levels for this study are as follows.

2.3.1 Plants

Individual plants are discrete. Even though a single plant can have multiple rot pockets, our collections have generally been limited to one sample per plant. The number of yeast species present in a plant-rot pocket is the species richness (S). The overall database does not allow calculations of p_i within a plant because the cell numbers for each species were not accurately estimated in all collections. As a consequence the diversity index H' could be calculated only for localities, subregions, regions, and continents. However, we do include the categories "within-plant" and "between

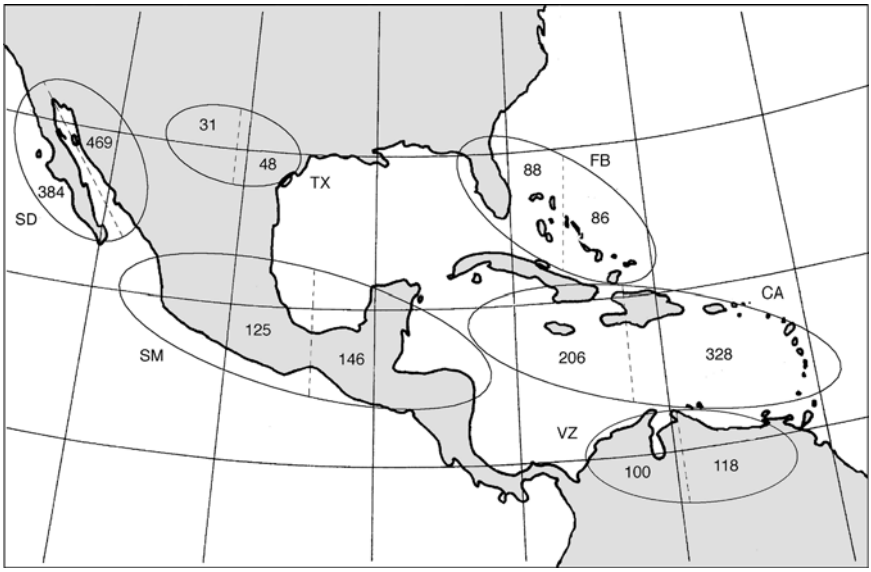

Fig. 19.1. Geographic areas where cactus necroses were collected. *Shaded oval areas* show the six distinct regions that were sampled (see Sect. 2.3 for the regional definitions). Each region is divided by a *dashed line* into subregions. The *numbers* within in each subregion are the number of plants sampled

plants" for studies of two *Stenocereus* species, *S. gummosus* (Pitaya agria) and *S. thurberi* (organpipe), for which detailed within-plant yeast-cell counts were made by selective-isolation methods (Starmer 1982; Fogleman and Starmer 1985). In order to eliminate noise from estimates of H', we excluded locations with small numbers of yeast isolates ($n<8$; e.g., a location with only one or a few plant samples).

2.3.2 Localities

Localities are somewhat subjective but were discrete. They range in size from a few square meters to several hectares. Separate localities were usually isolated in space by 10 km or more. Localities can be viewed as having more dispersal potential within than between.

2.3.3 Subregions

Subregional divisions of larger regions were mainly arbitrary but represented two more or less equal east–west contiguous partitions, except for Australia, where the division was north–south, and Hawaii, where no subregional division was warranted.

2.3.4 Regions

Regions were geographically distinct in the sense that dispersal between regions was severely limited by large expanses such as cactus-free terrain, bodies of water, or mountains.

2.3.5 Continents

Continental division was only possible for *Opuntia* hosts and is a "natural" versus "introduced" categorization of the *Opuntia* cacti. Those cacti in continental New World regions (North America, the Caribbean, the Bahamas, and northern Venezuela) were native, whereas those in Australia and Hawaii were introduced.

19.2.4 Yeasts

The localities were partitioned into eight regions and 15 subregions. Figure 19.1 shows the regional and subregional extent for North America. The number of plants sampled in each is shown for each subregion. The yeast taxa recovered from the entire collection and their frequency of isolation on a per plant basis are listed in Table 19.2 along with their isolation frequency in each of the eight regions.

19.3 Species Richness

Comparison of the proportion of diversity (S) explained as the scale of sampling increases shows a distinct trend for yeasts both from native columnar cacti and from *Opuntia* (Table 19.3, Fig. 19.2). The β diversity for species richness (S) between regions is over 50%. This differentiation at the highest level of division could have several causes that function to render the regions different from one another in their species composition: (1) the discontinuous geographic regions may severely limit the likelihood of dispersal from region to region, (2) allopatric speciation events result in differential community composition, (3) opportunistic local yeasts (non-cactus-specific) differ in the regions such that rare entry into the community causes the species richness to increase on pooling species from all regions, (4) cactus-specific species that were once widespread have become extinct in some regions but not all (i.e., relics), and (5) species from different regions are different but ecologically equivalent (Shmida and Wilson 1985).

19.3.1 Yeasts of Columnar Cacti

In order to evaluate the factors just listed we compared the species composition for the nature of species that were not shared among regions. Of the 49 species in the columnar community, 24 were unique (or essentially unique) to an individual region. In two instances the unique species were commonly recovered from cactus rots and also were closely related, i.e., *Phaffomyces antillensis* was found in the Caribbean region and the sister species *P. thermotolerans* in the Sonoran Desert; *Starmera caribaea* was exclusive to the Caribbean and *S. amethionina* in the Sonoran Desert.

Table 19.2 Number of each yeast species isolated from the entire collection and the eight geographic regions (see Sect. 2.3 for a description of each region). Yeast species listed as sp. "1", etc. are unknown or unidentified taxa

	Global	AU	CA	FB	HA	SM	SD	TX	VZ
Pichia cactophila	1,245	251	271	48	22	100	394	29	130
Candida sonorensis	862	314	106	54	19	35	220	54	60
Sporopachydermia cereana	592	45	108	29	2	54	282	26	46
Clavispora opuntiae	377	228	46	8	21	17	27	9	21
Myxozyma mucilagina	174	39	–	13	1	1	93	23	4
Starmera amethionina	166	27	–	–	1	–	125	13	–
Dipodascus starmeri	161	–	19	1	–	23	96	–	22
Pichia deserticola	140	–	47	–	–	15	70	8	–
Pichia kluyveri v. *eremophila*	119	–	–	–	–	10	76	33	–
Pichia mexicana	86	–	11	5	–	4	34	–	32
Pichia heedii	72	–	–	–	–	–	72	–	–
Pichia angusta	69	1	1	1	–	–	59	7	–
Cryptococcus albidus	67	30	3	–	1	5	21	2	5
Starmera caribaea	64	–	33	18	-	2	2	4	5
Candida boidinii	62	33	–	4	6	2	9	8	-
Pichia opuntiae	61	53	–	–	–	1	5	–	2
Pichia barkeri	57	41	8	4	–	4	–	–	–
Pichia kluyveri	48	15	12	8	3	3	6	–	1
Rhodotorula minuta	46	34	1	–	–	–	9	1	1
Pichia norvegensis	45	–	7	18	9	–	11	–	–
Cryptococcus laurentii	40	21	6	–	–	2	5	4	2
Pichia membranifaciens	39	4	–	1	–	2	1	–	31
Clavispora lusitaniae	32	–	–	–	–	29	3	–	–
Phaffomyces thermotolerans	26	–	–	–	–	–	26	–	–
Candida caseinolytica	26	–	–	–	–	1	24	1	–
Pichia pseudocactophila	25	–	–	–	–	5	20	–	-
Rhodotorula mucilaginosa	24	16	1	–	–	1	–	–	6
Debaryomyces hansenii	23	19	2	–	–	–	1	–	1
Kloeckera apiculata	22	11	3	–	3	–	–	5	–
Phaffomyces antillensis	20	–	20	–	–	–	–	–	–
Cryptococcus sp. "1"	15	–	–	–	–	–	–	–	15
Pichia guilliermondii	13	1	–	1	–	1	3	3	4
Kluyveromyces marxianus	13	–	–	–	–	–	12	1	–
Rhodotorula graminis	12	–	6	1	–	1	2	1	1
Williopsis californica	9	9	–	–	–	–	–	–	–
Rhodotorula glutinis	8	7	–	–	–	–	–	–	1
Cryptococcus macerans	8	8	–	–	–	–	–	–	–
Candida zeylanoides	8	7	1	–	–	–	–	–	–
Metschnikowia pulcherrima	7	7	–	–	–	–	–	–	–
Kloeckera apis	6	–	3	2	–	1	–	–	–
Cryptococcus luteolus	5	2	1	2	–	–	–	–	–
Candida sp. "2"	4	–	1	–	–	1	1	–	1
Candida vini	4	4	–	–	–	–	–	–	–
Candida orba	4	4	–	–	–	–	–	–	–
Trichosporon cutaneum	3	–	–	–	–	–	–	–	3
Rhodotorula fujisanensis	3	3	–	–	–	–	–	–	–
Pichia sp. "TM"	3	–	–	–	–	–	3	–	–
Candida viswanathii	3	3	–	–	–	–	–	–	–
Candida krissii	3	3	–	–	–	–	–	–	–
Yarrowia lipolytica	2	2	–	–	–	–	–	–	–
Rhodotorula aurantiaca	2	–	2	–	–	–	–	–	–

Continues

Table 19.2 Number of each yeast species isolated from the entire collection and the eight geographic regions (see Sect. 2.3 for a description of each region). Yeast species listed as sp. "1", etc. are unknown or unidentified taxa—*cont'd*

	Global	AU	CA	FB	HA	SM	SD	TX	VZ
Pichia onychis	2	–	1	–	–	–	1	–	–
Issatchenkia orientalis	2	–	–	1	–	–	1	–	–
Issatchenkia occidentalis	2	2	–	–	–	–	–	–	–
Cryptococcus skinneri	2	–	–	–	–	1	1	–	–
Pichia burtonii	1	1	–	–	–	–	–	–	–
Trichosporon sp. "2"	1	–	–	–	–	–	1	–	–
Trichosporon sp. "1"	1	1	–	–	–	–	–	–	–
Debaryomyces sp."1"	1	–	1	–	–	–	–	–	–
Rhodotorula sp. "2"	1	–	1	–	–	–	–	–	–
Rhodotorula sp. "1"	1	1	–	–	–	–	–	–	–
Rhodotorula marina	1	–	1	–	–	–	–	–	–
Rhodotorula auriculariae	1	1	–	–	–	–	–	–	–
Pichia strasburgensis	1	–	–	–	–	–	1	–	–
Pichia nakasei	1	1	–	–	–	–	–	–	–
Pichia glucozyma	1	1	–	–	–	–	–	–	–
Pichia farinosa	1	–	–	–	–	–	1	–	–
Kloeckera japonica	1	1	–	–	–	–	–	–	–
Dipodascus capitatus	1	1	–	–	–	–	–	–	–
Cryptococcus magnus	1	–	–	–	–	1	–	–	–
Cryptococcus infirmo-miniatus	1	1	–	–	–	–	–	–	–
Cryptococcus flavus	1	–	–	–	–	–	1	–	–
Candida sp. "3"	1	1	–	–	–	–	–	–	–
Candida sp. "1"	1	–	–	–	–	–	1	–	–
Candida vanderwaltii	1	1	–	–	–	–	–	–	–
Candida tropicalis	1	–	–	–	–	–	1	–	–
Candida tenuis "like"	1	1	–	–	–	–	–	–	–
Candida inconspicua	1	–	–	–	–	–	1	–	–
Candida diddensiae	1	1	–	–	–	–	–	–	–
Candida catenulata	1	–	1	–	–	–	–	–	–
Total	4,958	1,257	724	219	88	322	1,722	232	394

Both cases represent speciation events as sources of the overall species richness. Two other common, but unrelated cactus-specific yeasts, *Pichia heedii* and *Candida caseinolytica*, were region-specific (Sonoran Desert) and could represent old lineages that have only survived in the Sonoran Desert, i.e., relics. Alternately, they might be autochthonous members of other sympatric communities living in other plants but we have little evidence for this possibility (Ganter et al. 1986). *Pichia membranifaciens* was found frequently in cacti collected in Venezuela, occurring multiple times in different localities. This species is not cactus-specific and is found in a number of habitats, including fruit rots and tree fluxes. This may be a case where the species diversity is increased as a consequence of ecological equivalence. However, because the *P. membranifaciens* phenotype is convergent with a number of cactus-specific yeasts and because these strains are no longer available, it is not possible to verify their identity. They may in fact represent cryptic species similar to *P. membranifaciens*, as is known to be the case in recent studies of sap-flux yeasts in Costa Rica

Table 19.3 Species richness (*S*) and diversity (*H′*) for host categories in the geographic hierarchy. *Numbers* in *bold* are the percentage contribution of the total species richness or diversity. β diversity represents contributions of between-level components. α diversity is the within-sample diversity (see Sect. 2.1 for details on calculations)

	Global	Continental	Regional	Sub-Regional	Local	Plant	Within plant
Opuntioideae:*S*	67	44	22.13	16.60	5.78	1.94	–
β_c:β_r:β_{sr}:β_l:β_p:α	**34.3**	**32.6**	**8.2**	**16.2**	**5.7**	**2.9**	–
H′	2.68	2.505	2.189	2.08	1.669	–	–
β_c:β_r:β_{sr}:β_l:α	**6.5**	**11.8**	**4.1**	**15.3**	**62.3**	–	–
Cactaceae:S	–	56	26.17	19.83	7.93	1.95	–
β_r:β_{sr}:β_l: β_p:α	–	**53.3**	**11.3**	**21.3**	**10.7**	**3.5**	–
H′	–	2.642	2.299	2.193	1.705	–	–
β_r:β_{sr}:β_l: α	–	**13.0**	**4.0**	**18.5**	**64.5**	–	–
Opuntioideae:S	–	42	20.17	14.67	5.67	1.94	–
β_r:β_{sr}:β_l:β_p:α	–	**52.0**	**13.1**	**21.4**	**8.9**	**4.6**	–
H′	–	2.552	2.197	2.044	1.671	–	–
β_r: β_{sr}:β_l: α	–	**13.9**	**6.0**	**14.6**	**65.5**	–	–
Cactoideae:*S*	–	49	20.2	14.3	5.57	1.59	–
β_r:β_{sr}:β_l:β_p:α	–	**58.8**	**12.0**	**17.8**	**8.1**	**3.2**	–
H′	–	2.525	2.091	1.959	1.619	–	–
β_r:β_{sr}:β_l: α	–	**17.2**	**5.2**	**13.5**	**64.1**	–	–
Pachycereinae:*S*	–	36	13.6	9.89	3.81	1.34	–
β_r:β_{sr}:β_l:β_p:α	–	**62.2**	**10.3**	**16.9**	**6.9**	**3.7**	–
H′	–	2.567	2.005	1.820	1.501	–	–
β_r:β_{sr}:β_l: α	–	**21.9**	**7.2**	**12.4**	**58.5**	–	–
Stenocereinae:*S*	–	38	17.5	12.38	4.85	1.44	–
β_r:β_{sr}:β_l:β_p:α	–	**53.9**	**13.5**	**19.8**	**9.0**	**3.8**	–
H′	–	2.299	1.986	1.871	1.579	-	–
β_r:β_{sr}:β_l: α	–	**13.6**	**5.0**	**12.7**	**68.7**	-	–
Stenocereinae[a]							
H′	–	2.299	1.986	1.871	1.420	0.9145	0.7075
β_r:β_{sr}:β_l: β_p:β_w: α	–	**13.6**	**5.0**	**19.6**	**22.0**	**9.0**	**30.8**

[a]Local, plant, and within-plant categories are estimated from data of *Stenocereus gumosus* and *S. thurberi* collections in the Sonoran Desert that selectively isolated and recorded cell counts of yeast species within plants, while the other categories include *Stenocereus* species from all regions.

and *Clermontia* flowers in Hawaii (Lachance et al., unpublished). All other species (17) that were unique to one region were either single isolates (11) or only occurred in low numbers (6). These include basidiomycetes such as *Cryptococcus* and *Rhodotorula* species and ascomycetous species found commonly in fruit rots or tree fluxes (*Kloeckera* spp. and *Pichia* spp.). The overall assessment of the 24 unique yeasts is four are due to speciation, two are relics, one is uncertain and 17 are rare opportunistic non-cactus species.

19.3.2 Yeasts of *Opuntia* Cacti

A similar comparison of the 42 yeast species in the *Opuntia* yeast community showed that 11 were found in only one region. Eight were single isolates, of which two were normally specific to columnar cacti (i.e., *Pichia pseudocactophila* and

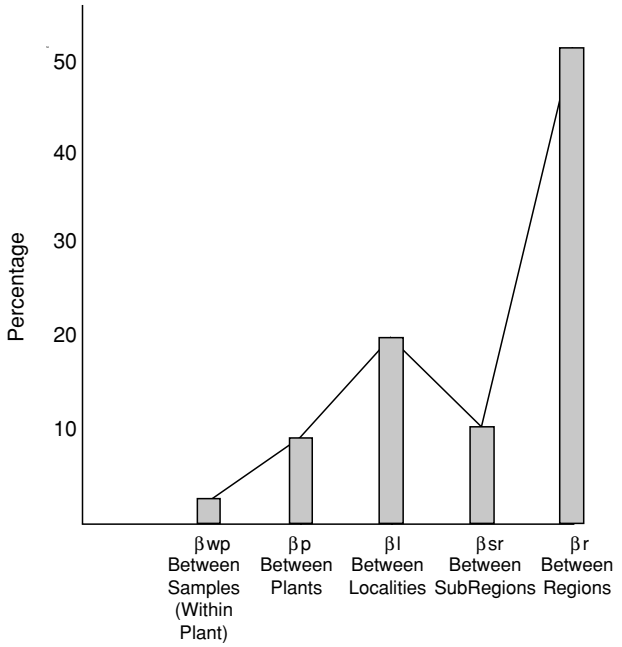

Fig. 19.2. Partition of species richness for yeasts in the Cactaceae (S=56) as a function of geographic level (Table 19.3)

P. thermotolerans) while the others were non-cactus yeasts usually associated with fruit or tree fluxes (*Kloeckera* spp., *P. membranifaciens*) or potentially airborne basidiomycetous species. These unique regional yeasts in *Opuntia* are mostly opportunistic and not products of speciation or extinction processes.

The global comparison of S for yeasts from *Opuntia* cladode rots shows a substantial increase in species richness when the Australian and Hawaiian yeast collections are included. Inspection of Table 19.2 shows that this increase is not due to replacement of one set of dominant cactus yeasts by another but is more likely a consequence of different rare indigenous yeasts in the local environments. Thirteen unique yeast species were recovered only once and only in Australia. A comparable number of single isolates were unique to the New World. Furthermore ten species found only in Australia had a low frequency of isolation (less than 10), whereas a number of common species in the New World have been found neither in Australia nor in Hawaii. These include seven species that were isolated between 161 and 45 times in the New World. Notable (Table 19.2) is that the top six cactophilic yeasts are found in Australia and other regions of the world (i.e., introduced and natural). It may be possible to trace the origin of the cactus-specific yeasts that found their way to Australia from the Americas during the campaign to eradicate the undesirable "prickly pear." However, because the introduction of yeasts and their vectors occurred a number of times (67 shipments of rotting plant material infected with

microorganisms and larval stages of insects) and from a number of different localities (59 from North America and eight from South America) in the New World (Dodd 1940; Starmer et al. 1988) multiple sources are likely.

19.3.3 Other Studies

Cactus rots have been investigated in Brazil by Rosa and colleagues (Morais et al. 1994; Rosa et al. 1995). Their species lists from columnar and *Opuntia* cacti contain the same dominant species we found in our surveys, with three to four additional unidentified or unknown *Candida* species collected in low numbers. It is likely that more extensive sampling of other localities in South America will increase the species richness, although the cactophilic yeast community clearly is very similar for both continents.

19.3.4 Biases Affecting Estimates of Species Richness

Among the factors that could bias our estimates of species richness are the methods that we use to detect yeasts in the individual rot pockets. Using general isolation media such as acidified yeast extract–malt extract agar or medium supplemented with chloramphenicol will necessarily cause one to overlook species present in low numbers, especially under conditions where the dominant yeasts are very numerous. We have shown this to be the case when we used selective isolation media to screen for rare yeasts and to obtain accurate counts of common yeasts (Starmer 1982; Fogleman and Starmer 1985; Latham 1998). However in those studies the yeasts that were revealed were still a subset of those found in the larger survey that only exposed those species with the greatest number of cells. Another factor that may prove important is the undetected diversification of taxa that we have called single species. For example, we expect the taxon *Pichia mexicana* to consist of a number of cryptic species. This was our initial finding when we identified the common dominant cactus yeast as *Pichia membranifaciens* in the mid 1970s (Heed et al. 1976; Starmer et al. 1976). Detailed study of host plant distribution, physiology, sexuality, GC content of the DNA, and DNA reassociations revealed several distinct species and complexes that were originally identified as *P. membranifaciens*. Among these were *Pichia cactophila*, *Pichia pseudocactophila*, *Pichia deserticola*, *Phaffomyces opuntiae, P. thermotolerans, Pichia eremophila,* and the *S. amethionina* complex, all of which have restricted physiologies that are convergent on a similar phenotype. In a like manner it is now recognized that *Sporopachydermia cereana* is a highly heterogeneous complex of species that show considerable geographic diversification (Lachance et al. 2001a).

19.4 Yeast Diversity (Shannon–Weiner)

The diversity index (H') has a very different pattern of change in the geographic hierarchy as compared with species richness (S). The most detailed comparison is available for yeasts found in the columnar cacti in the subtribe Stenocereinae (Table 19.3). In this case most of the variability occurs at three levels: within samples of

individual plants, between plants in a locality, and between localities in a region (Fig. 19.3). These three levels account for 72% of the diversity. Only a small amount of diversity remains for the other levels (i.e., within a plant or between samples, between subregions or between regions, Fig. 19.3). This result is explained by the following arguments.

Each sample of cactus tissue has about two to three species and if samples are taken repeatedly from the same rot they yield about the same number of cells of the same species, i.e. samples are homogeneous in the rot pocket and pooling them does not increase the diversity by much. However, each plant may represent a different inoculation history or may be at a different stage of the decay process. As a consequence there would be a large increase in diversity when plants from a single locality are pooled. Combining localities of a subregion also increases diversity but in this case the increase is likely due to changes in habitats and local species availability. This is reflected in the sharp increase in species richness (Fig. 19.2) seen for β diversity (between localities).

Comparison of the diversity index for categories where yeast cell numbers for each plant were not available gives no information for within-plant diversity. In these cases α diversity is for yeast species within localities. In all host categories the α diversity comprises most of the total diversity. The α partition accounts for

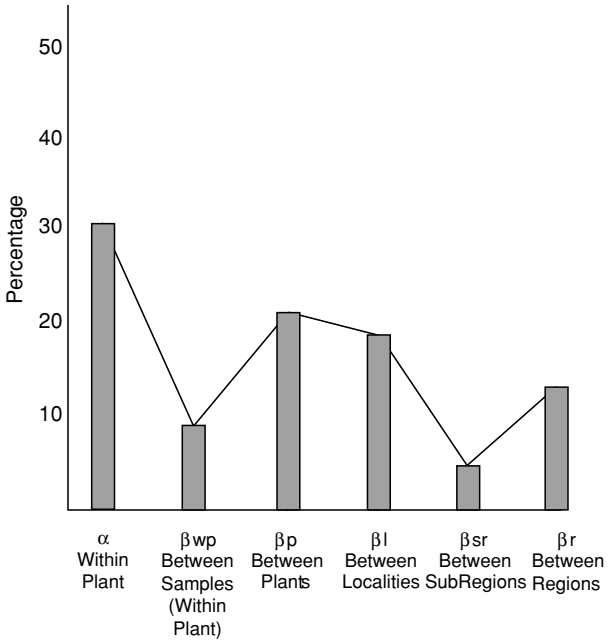

Fig. 19.3. Partition of species diversity for yeasts in the Stenocereinae (H'=2.299) as a function of geographic level (Table 19.3)

58.5–68.7% of the total diversity. This magnitude of increase in the metric likely reflects (as mentioned before) the inoculation history and the stage of the rotting process sampled in each locality. Likewise the increase (12.4–18.5%) for between-locality diversity is interpreted to be a function of the local species availability as a consequence of living in different habitats. There was little increase in diversity (4.0–7.2%) between subregions, whereas diversity between regions increase by 3 times as much (13.0–21.9%). The distribution of yeasts in the Stenocereinae would seem to argue in favor of the ubiquitous model of microbial diversity (Fenchel and Finlay 2004). However, it should be noted that these cacti are limited in distribution, such that the distinction between β and γ diversity would be expected to be less pronounced.

19.5 Conclusions

There is a striking difference when the geographic hierarchy is used to partition species richness (S) as contrasted to species diversity (H'). Most of the species richness is due to finding different relatively rare non-cactus-specific yeast species in different regions or continents. In this case the increase is most likely due to accidents, contaminants, or otherwise unusual circumstances and as such provides a "random" reason for an increase in biodiversity. Diversity as reflected in relative proportions of species and their uncertainty is influenced primarily by the number of cells (or abundance) of a small number of species (two or three) within a rotting sample (usually about 1 cm^3) of cactus tissue. Thus the α diversity, which almost always includes the core cactus-specific species, accounts for most of the information. This diversity metric has a biological meaning that reflects the salient factors mentioned in the "Introduction," i.e., habitat suitability, speciation, extinction, and immigration. Our previous work on habitat suitability and the insect (vector) yeast relationships has emphasized the likely mutualistic interactions among the core cactus yeasts and has shown experimentally that the cactus-yeast community is mutualistic with their vectors (Starmer et al. 1991). These relationships are not obligate but apparently strong enough to (1) maintain yeast communities that are stable over time and space (Latham 1998) and (2) restrict them to decaying cactus stem and cladode tissues (Ganter et al. 1986). These factors argue strongly that not all microorganisms are everywhere and that not all microorganisms freely and passively disperse to achieve world-wide distributions.

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Chapter 20

Black Yeasts and Meristematic Fungi: Ecology, Diversity and Identification

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20.1 Black Yeasts, Meristematic, Microcolonial and Dematiaceous Fungi – a Disambiguation of Terms

For the morphological classification of the fungi discussed in this chapter three terms are used in the literature with overlapping meaning: black yeasts, meristematic fungi and microcolonial fungi (MCF). As will be described later, because of pleomorphic behavior the clear separation of fungi into one or the other group is impossible and fungi can just be clustered into one of those form groups according to their predominating morphological characteristics.

"Black yeast" is a *terminus technicus* subscribing a group of fungi that is quite heterogeneous from the taxonomic and phylogenetic point of view but having in common melanized cell walls and the formation of daughter cells by yeast-like multilateral or polar budding (Fig. 20.1). The resulting daughter cells may be encapsuled in a matrix of extracellular polymeric substances (EPS). Most black yeasts additionally exhibit mycelial growth and generate conidia from simple phialides, from phialides with collarettes, from annelated phialides, on rhachides or on undifferentiated conidiogenous cells (de Hoog and Hermanides-Nijhof 1977). Conidia may be unseptated or otherwise have up to three transversal septa. Also the formation of arthroconidia from fragmenting hyphae can occur in some genera. Only very few species, for example, *Phaeococcomyces exophialae*, do not form any hyphal states.

The term "meristematic fungi" was introduced by de Hoog and Hermanides-Nijhof (1977) for fungi that form aggregates of thick-walled, melanized cells enlarging and reproducing by isodiametrical division (Fig. 20.2). Propagules are liberated by breaking apart of aggregates as in the genus *Sarcinomyces* (Fig. 20.3) or by endogenous conidiogenesis with subsequent disruption of the mother cell wall as in the genus *Phaeotheca*. Some meristematic fungi might form blastic conidia from fairly undifferentiated cells, for example, in *Capnobotryella,* or even yeast-like budding cells as in *Hortaea werneckii*. Thus, some meristematic fungi can also be

Fig. 20.1. Hyphae, budding cells and annelidic conidiogenesis of the black yeast *Exophiala* sp. (2-week-old culture on 2% malt-extract agar, MEA, 21°C)

classified morphologically as black yeast and vice versa. A close phylogenetic relationship of both forms was suggested by de Hoog and Hermanides-Nijhof (1977) and was confirmed by molecular methods (Sterflinger et al. 1999).

A third term commonly used in the literature refers to the *in situ* growth pattern of the meristematic fungi and some black yeasts: MCF. MCF were first described by Staley et al. (1982) and the term characterizes fungi growing on mineral substrates –

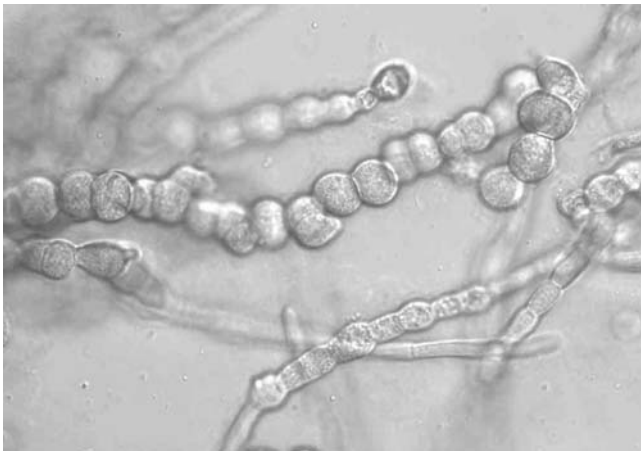

Fig. 20.2. Species of *Coniosporium* form conidial chains; the secondary formation of longitudinal and transverse septa results in meristematic aggregates (2-week-old culture on 2% MEA, 21°C)

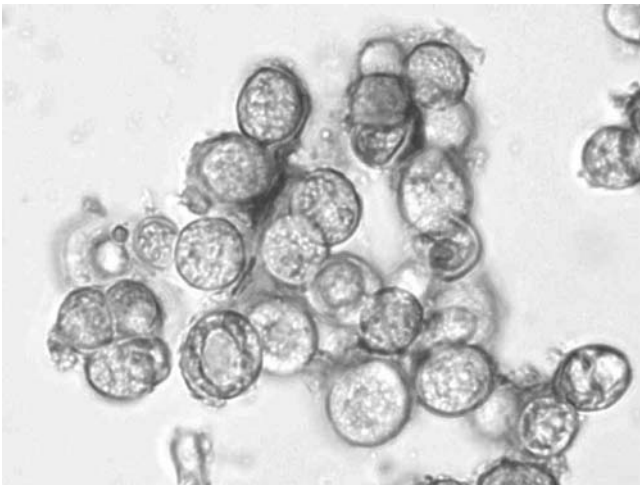

Fig. 20.3. Cell packages (*sarcina*) of *Sarcinomyces* sp. result from isodiametical division; solitary cells are released by breaking up of the common cell wall (2-week-old culture on 2% MEA, 21°C)

mostly rock but also glass or metal – and forming black cauliflower-like colonies consisting of densely aggregated thick-walled cells (Fig. 20.4). A single colony might be up to 1 mm in diameter and can be visible to the naked eye. Morphologically, colonies of MCF are absolutely indistinguishable from each other but after isolation onto suitable growth media they often differentiate into various morphologies, thus manifesting different genera and species (Sterflinger et al. 1999).

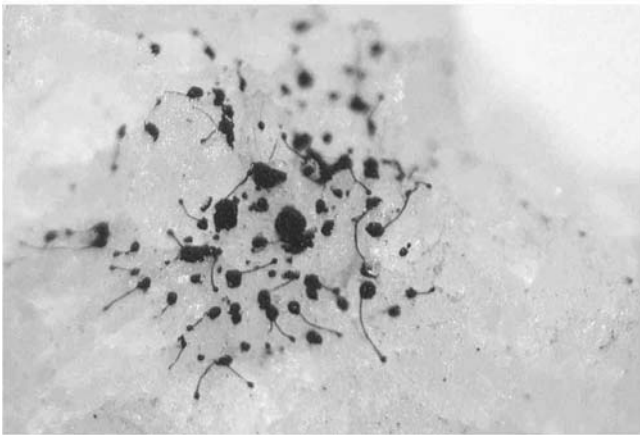

Fig. 20.4. Meristematic fungi form microcolonies with stolons and satellites on mineral surfaces; here on marble from Turkey

All black yeasts, meristematic fungi and MCF together with other darkly pigmented fungi are colloquially subsumed as so-called dematiaceous fungi. *Dematiaceae* stands for a highly artificial group the sole common feature of which is the pigmentation and thus the term should no longer be used as a taxonomic one. For practical reasons, however, it still has an significant impact for mycologists in routine diagnosis because the most important genera of melanized fungi are included in the keys "Dematiaceous Hyphomycetes" and "More Dematiaceous Hyphomycetes" (Ellis 1971, 1976).

20.2 Ecology of Black Yeasts and Meristematic Fungi

Black yeasts and MCF conquer extreme environments characterized by oligotrophic nutrient conditions, elevated temperatures, UV radiation, matrix and osmotic stress and combinations of these factors. The combined influence of these stress factors exerts a high selective pressure on the microbial community and as a consequence black yeasts and MCF are rarely found in complex microbial populations but solitary or in spatial association with comparably stress resistant organisms such as lichens and cyanobacteria in very special habitats.

Originally, darkly pigmented fungi with meristematic and/or yeast-like growth were described as inhabitants of living and dead plant material, including needles, leaves, bark, fruits and wood. While species of *Hormonema* and its teleomorph *Sydowia* are nearly exclusively found as opportunistic pathogens on plants – especially *Pinus* and *Prunus* – the closely related species *Aureobasidium pullulans* has a wide distribution on various materials, including plants, foodstuff, soil, textiles, metallic equipment, hypersaline water and rock (de Hoog et al. 1999). Also *Coniosporium* and *Trimmatostroma* are plant-associated, whereas single species of those genera are more restricted to discrete habitats: *Coniosporium perforans* does exclusively occur on rock (Sterflinger et al. 1999) and *Trimmatostroma salinum* was recorded only from hypersaline waters (Zalar et al. 1999). *Capnobotryella renispora* was detected on roof tiles and on *Sphagnum* (Hambleton et al. 2003), *Trimmatostroma abietis* from *Abies* and from sandstone (Butin et al. 1996), *H. werneckii* from rock, hypersaline water and as a causative agent of *Tinea nigra* on human skin. Additionally, lichenicolous fungi in the genera *Mycocalicium*, *Taeniolella*, *Trimmatostroma* (Ellis 1976) and *Intralichen* (Hawksworth and Cole 2002) form microcolonial colonies on and in lichen thalli.

Meristematic fungi are colonizers of bare rock surfaces in hot deserts and in semiarid climatic regions such as the Mediterranean (Staley et al. 1982; Gorbushina et al. 1993) but are also part of the cryptoendolithic community of Antarctica (Onofri and Friedman 1999). *Sarcinomyces petricola*, *Exophiala* species with close relationship to *Exophiala jeanselmei*, several *Coniosporium* species, strains of *Trimmatstroma* as well as *Phaeotheca* and *Phaeosclera* are very common rock inhabitants. Those fungi gained additional attention because they have a high destructive potential against historic buildings and works of art and are able to destroy even hard materials such as marble, sandstone and glass (Wollenzien et al. 1997; Sterflinger 2000). *H. werneckii* has its natural niche in saline to hypersaline water, in sea spray areas and is associated with salt efflorescence on walls. Together with

Trimmatostroma salinum, *Phaeotheca triangularis* and *Aureobasidium pullulans* it was isolated from hypersaline water of Slovenian salterns (Zalar et al. 1999).

Black yeasts and meristematic fungi are also found in human environments and as human pathogens or opportunists. Air conditioning systems with moisteners and humidifiers provide good living conditions for oligotrophic, stress-resistant fungi. The type strain of *P. triangularis* – a meristematic fungus with endogenous conidiogenesis – was isolated from an air conditioning system (de Hoog et al. 1997). Also strains of *Exophiala* and *Sarcinomyces* are frequently found in humidifiers as well as in bathing and sauna facilities (Matos et al. 2002). Especially black yeasts are of high clinical importance because they are causative agents of superficial and systemic mycosis. As mentioned before *H. werneckii* causes human *Tinea nigra* especially on the palms of the hands. Species of *Exophiala* are causative agents of chromoblastomycosis, phaeohyphomycosis and phaeohyphomycotic cysts and keratitis; they occasionally occur associated with pneumonia and sinusitis and they were isolated from subcutaneous infections (de Hoog et al. 2000). *E. dermatitidis* is of special importance because together with the closely related species *Cladophilalophora bantiana* and *Ramichloridium mackenziei* it causes lethal infections of the human brain (Horré and de Hoog 1999; Kantarcloglu and de Hoog 2004). *E. pisciphila* and *E. salmonis* infect fish, the latter causing the phenomenon of tumbling in salmon and trout owing to cerebral infections.

The habitats described seem to be quite different and widely apart at first sight but in fact rock and material surfaces, the phyllosphere and living mammalian tissue share some main ecological similarities, for example, raised temperature, osmotic stress, UV radiation and oxygenic action. Several properties are involved in stress tolerance of black yeasts and meristematic fungi:

20.2.1 Melanin, the All-Around Protective

One of the most important factors in stress resistance of black yeasts and meristematic fungi is the production of melanins and the incrustation of the cell walls with this high-molecular substances. In fungi, 3,4-dihydroxyphenylalanine (DOPA) and 1,8-dihydroxynaphtalene (DHN) melanin occur, while DHN seems to be predominant in meristematic fungi (Kogej et al. 2003). Melanins are not only responsible for the dark-green, brown and black color of the fungi but also for a number of properties helping the cells to survive environmental stress. Since in some habitats, for example, in Antarctica, UV radiation is the limiting factor for fungal diversity (Tosi et al. 2004), melanin together with some mycosporines (Volkmann et al. 2003) plays an important role as a UV-protection substance. However, as described by Langfelder et al. (2003), the relevance of fungal melanin is far beyond that. Wheeler and Bell (1986) described that melanins are not only UV-protective but also important for the penetration of host tissue in plants as well as in animal/human tissue. Melanin-deficient mutants are unable to generate appressorial turgor and thus lose their pathogenic potential. However, melanin alone does not define a plant or a human pathogenic fungus. Also fungi that are known for their capability to penetrate hard inorganic material mechanically are melanin-pigmented but otherwise nonpathogenic. Both DOPA as well as DHN melanin protect fungal cells against

osmotic stress, from reactive nitrogen species, from reactive oxygen, shelter them against phagocytosis in host tissue and inhibit tumor-necrosis factors (Langfelder et al. 2003). In human pathogenic black yeasts such as *E. dermatitidis* and *Cryptococcus neoformans* melanin is one of the most important virulence factors – albeit not the only one; in environmental and especially in rock-inhabiting fungi it is a major survival factor.

20.2.2 Morphological Adaptation

A general tendency in black yeasts and meristematic fungi seems to be that yeast-like stages are more associated with fungi inhabiting living tissue – mammalian as well as plant – and that meristematic/microcolonial growth is linked to bare material surfaces and to the highest demands towards stress resistance. Intermediate forms are also known: *E. dermatitidis* forms meristematic aggregates to survive low pH conditions in the digestive tract through which humans are invaded (Horré and de Hoog 1999). Generally, meristematic morphology is interpreted as a response to multiple stress factors supporting temperature tolerance and decreasing the rate and speed of desiccation by keeping the volume-to-surface ratio optimal (Wollenzien et al. 1997). Additionally, multilayered cell walls are developed as a response to raised temperature (Sterflinger and Krumbein 1995). As a consequence and in contrast to other fungi, only the spores or chlamydospores of which are stress-resistant, in colonies of meristematic fungi each vegetative cell is resistant and can additionally serve for propagation. In their natural habitat the development of spores or conidia was never observed for meristematic fungi; instead satellite colonies formed on stolons and colony fragments serve for propagation (Fig. 20.4). Moreover, the production of EPS in the form of loose slimes or dense capsules can be significant for survival under humid and hot conditions. *E. dermatitidis* forms slimy colonies and can grow up to 42°C, whereas the closely related species *Sarcinomyces phaemuriformis* and *E. mesophila* have their growth limit at 23 and 38°C, respectively (Matos et al. 2002). Since thermotolerance is a prerequisite for pathogenicity, a role of EPS in the virulence of *E. dermatitidis* was suggested by Yurlova and de Hoog (2002).

20.2.3 Desiccation Tolerance

As mentioned before modifications of the cell wall and the meristematic morphology are successful tasks in order to limit loss of intracellular water. However, in deserts and other extremely dry environments, for example, indoor environments, it is necessary to survive nearly complete dehydration in a dormant state. For this reason intracellular accumulation of the disaccharide trehalose is obligate in several rock-inhabiting fungi (Sterflinger 1998). Trehalose stabilizes the conformation of enzymes and lipid bilayers and plays a major role for so-called anhydrobiotic organisms capable of surviving complete dehydration. Taking together the oligotrophic situation in environments of black yeasts and meristematic fungi and the high carbon and energy demand caused by biosynthesis of trehalose, it can well be explained why the majority of those fungi have very low growth rates.

20.2.4 Temperature Tolerance

The growth of black yeasts and meristematic fungi from nature is limited to temperatures below 32°C, whereas phylogenetically closely related species that are causative agents of human chromoblastomycoses are able to grow at 37°C. The fact that none of the environmental species ever attracted attention as human pathogens indicates that the maximum growth temperature might play an important role as a virulence factor. Irrespective of that, conditions in the natural environment occasionally necessitate tolerance of temperatures significantly above the maximum growth temperatures. Sun-exposed surfaces reach temperatures up to 70°C and an additional, local temperature increase is caused by selective absorption of solar radiation by the black colonies themselves. For physiologically active and fully hydrated colonies the lethal temperature is between 35 and 75°C but dehydrated colonies withstand temperatures up to 120°C for at least 0.5 h and gain full growth activity after transfer to fresh medium (Sterflinger 1998). Thus, desiccation is a prerequisite to withstand temperature stress; similar results were reported for wood-inhabiting *Trimmatostroma* strains.

Cryophilic and cryotolerant meristematic fungi are described as important inhabitants of Antarctic rocks and thus conquer an ecological niche at the opposite end of the temperature scale. Especially in the *Friedmanniomyces* group strains can grow in temperature ranges from 0°C upwards. Amongst other features their cryotolerance is the reason why Antarctic fungi may be promising models to investigate exobiology under present Martian conditions (Onofri et al. 2004).

20.2.5 Osmotolerance

Although several species of black yeasts and MCF are osmotolerant, true halophilism seems to be rare and was merely found in *P. triangularis* (Zalar et al. 1999). In *H. werneckii*, however, halophilism is adaptive and the fungus can grow in up to 30% NaCl, which is almost the saturation point. Glycerol is the main compatible solute synthesized as a response to increased salinity (Sterflinger 1998). Because the spatial organization and density of melanin in the outer part of the cell wall is denser in cultures grown in increased salt concentration an osmoprotectant role of melanin was suggested by Kogej et al. (2001). Also alterations of the membrane properties seem to influence osmotolerance since the regulation of sterol biosynthesis in halophilic black yeasts was demonstrated to be different than in mesophiles (Petrovic et al. 1999).

20.2.6 Physiology

With respect to carbon and nitrogen both black yeasts and meristematic fungi have a fairly wide physiological spectrum; all strains known so far are restricted to an aerobic, oxidative metabolism; no fermentation of sugars was ever observed. The spectrum of sugars includes L and D forms of monosaccharides and disaccharides and sugar alcohols, a specialty of black yeasts is the usage of *meso*-erythritrol as a carbon source. Diagnostic differences can be the ability to assimilate sucrose, ribitol, rhamnose, sorbose and xylose. Nitrate, nitrite, ethylamine and complex proteins are used as nitrogen sources. Ethanol can be degraded by most species, whereas

the oxidation of methanol is rare. Several isolates from rock are able to degrade hydrocarbons as diesel oil, gasoline and kerosene; also the degradation of polycyclic aromatic hydrocarbons is observed in some rock inhabitants (Sterflinger, unpublished data). The wide spectrum of degradable carbon and nitrogen sources is an important prerequisite to conquer oligotrophic habitats where organic detritus and organic air pollutants that were carried by dust that has settled are the only nutrient sources available. In contrast to their mostly oligotrophic habitats the energy and nutrient demand for the synthesis of melanins, trehalose and polyols necessary for survival is comparatively high. Consequentially those fungi do not produce or excrete organic acids, biotechnological important secondary metabolites or any product of excessive metabolism.

20.3 Taxonomy and Phylogeny

As mentioned before the terms black yeast, meristematic fungus as well as dematiaceous fungus are applied for morphological groups and do not reflect natural phylogenetic relations. The identification of black yeasts and meristematic fungi is still a challenge for mycologists and hitherto we know that it is rather impossible to perform a reliable species identification based on morphology alone. Microscopic identification fails in species and sometimes even in genus determination because of two main facts:

1. Especially in meristematic fungi a lack of differentiation hampers the determination of their systematic position. The *in situ* morphology is nearly identical even in different genera and in most cases their black, cauliflower-like colonies do not allow differentiation (Fig. 20.2, 20.3, 20.4). In culture some genera switch to mycelial growth with meristematic conidiogenesis, such as *Coniosporium*, while others, such as *Phaeotheca*, remain meristematic even on different media. The conidiogenesis often takes place on undifferentiated hyphae without any discrete conidiogenous cell or conidiophore. Some genera, for example *Trimmatostroma*, form conidia that resemble hyphal fragments with secondary transformations such as thickening of cell walls and bending. Morphologically very similar meristematic fungi can be phylogenetically widely apart. On the basis of their morphological similarity, *S. phaeomuriformis* from human skin, *S. crustaceus* from conifer wood and *S. petricola* from rock were classified in a single genus, despite a large phylogenetic distance exceeding the ordinal level.
2. For black yeasts pleomorphic growth combined with variable morphologies of conidiogenesis leads to confusing taxonomic results. Black yeast genera such as *Exophiala* and its teleomorph *Capronia* are polymorphic with very fine differences in conidiogenesis that hamper the morphological identification even for routine adepts of the groups. A single species may exhibit morphologically widely different synanamorphs. Such a situation is known in the meristematic species *S. phaeomurifomis*, where some of the strains with molecular identity to the type strain were morphologically indistinguishable from the hyphal, annellidic species *E. dermatitidis* (Uijthof et al. 1994). In *Coniosporium apollinis* some strains exhibit a yeast-like growth pattern and other isolates display a meristematic growth pattern.

In order to overcome this confusion effort was put into the analysis of molecular data in order to be able to cluster morphologically divergent strains, to distinguish morphologically similar ones and to determine their phylogenetic positions. The first method introduced as routine was the restriction fragment length polymorphism (RFLP) analysis of the small ribosomal subunit (SSU). On the basis of the variations observed in SSU-RFLP patterns it was concluded that black yeasts and meristematic fungi are genotypically much more diverse than could be assumed from morphology or physiology (Uijthof and de Hoog 1995; Sterflinger and Gorbushina 1997).

Today, owing to emerging technical facilities, sequencing of DNA followed by homology search in the databanks of the European Bioinformatics Institute (http://www.ebi.ac.uk) and the National Center for Biotechnology Information (NCBI, http://www.ncbi.nlm.nih.gov) by alignment and phylogenetic tree construction is the general praxis for resolving the taxonomy and phylogeny of black yeasts. However, for the detection of intraspecific variability and populations, DNA fingerprint methods still are valuable tools. For example, in *E. dermatitidis* seven populations were determined on the basis of random amplified polymorphic DNA patterns (RAPD). *E. dermatitidis* is the main neurotropic agent in east Asia and in Europe and it causes cystic fibrosis in the lung. Fingerprints showed that the pathogenic strains cluster in two main populations (Uijthof et al. 1994). Also within *E. jeanselmei* different populations were detected by RAPD which refer to different potentials of pathogenicity (Nucci et al. 2002). Intraspecific variability with three populations in *Aureobasidium pullulans* was analyzed using RFLP (Yurlova et al. 1995).

For the analysis of their position in fungal divisions DNA sequencing followed by alignment and phylogenetic analysis is necessary because teleomorphs are still nearly unknown for black yeasts and meristematic fungi. An exception is *Exophiala*, for which *Capronia* is known to be the teleomorph. Phylogenetic trees based on SSU sequencing showed that the black yeasts are phylogenetically far away from the class of Hemiascomycetes comprising the classical yeast genera *Saccharomyces*, *Candida* and *Pichia* (Haase et al. 1995). This result is confirmed by chemotaxonomical data since the cell walls of black yeasts and meristematic fungi contain chitin rather than mannose or glucose. With few exceptions both black yeasts and meristematic fungi are Euascomycetes (Fig. 20.5). In the Basidiomycetes *Trichosporonoides nigrescens*, *T. oedocephalis*, *Moniliella suaveolens* and the asexual state of *Ustilago maydis* are melanized and yeast-like; *Trichosporon asteroides* is the only fungus within the *Basidiomycetes* with meristematic conidiogenesis (de Hoog et al. 2000). Within the *Euascomycetes*, however, the melanized meristematic fungi are phylogenetically quite diverse. SSU-based phylogenetic trees by Spatafora et al. (1995) and Sterflinger et al. (1999) showed close relationships to the following three orders of *Ascomycetes* (Fig.20.5):

1. Chaetothyriales. Molecular data proved that the black yeasts within the Herpotrichiellaceae are an evolutionary hot spot with many genotypically divergent species in the genera *Exophiala*, *Phialophora* and the herpotrichiellaceous teleomorphs in *Capronia* (de Hoog et al. 2003). *Exophiala* is the main genus of the black yeasts; its synanamorph *Phaeococcomyces* does not form mycelia at all but remains completely yeast-like, *Phialophora* forms collarettes and *Rhinocladiella* is determined

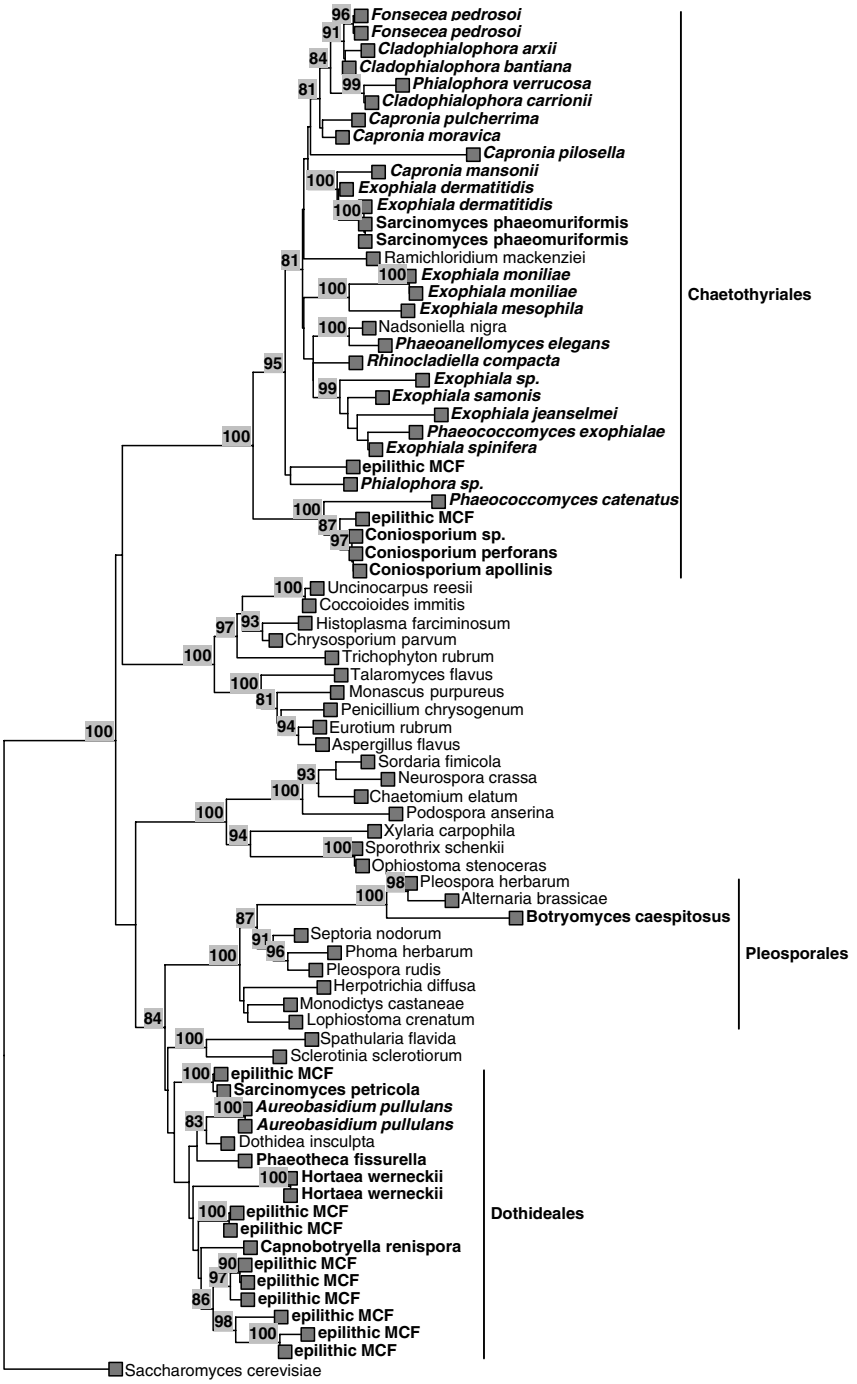

Fig. 20.5. Phylogenetic tree based on 1,700 positions of small subunit ribosomal DNA. Fungi with predominating black-yeast morphology are printed in *bold italics*; fungi with predominating merstematic growth are printed in *bold*. Bootstrap values were generated from 500 trees using the Felsenstein method

by sympodial conidiophores. In spite of this morphological classification, differences between species albeit constant are often difficult to perceive even for the mycologists specialized on this groups. Neurotropic species of *Cladophialophora*, *Ramichloridium* and *Exophiala* are distributed over the entire Herpotrichiellaceae. *S. phaeomuriformis* is known to be a meristematic member of the Herpotrichiellaceae (Haase et al. 1995), closely related to *E. dermatitidis* (Uijthof 1996) Also the agents of chromoblastomycosis, having meristematic tissue forms, are members of the family on the basis of molecular phylogeny (Haase et al. 1999).

2. In the order Dothideales several members are found which are meristematic during their whole life cycle (*Phaeotheca*, *Phaeosclera*, *Hyphospora*) or are able to produce meristematic synanamorphs (*Aureobasidium*, *Hortaea*). A further species found here is *Capnobotryella renispora*. 5.8S ribosomal DNA data suggest that several lichenicolous and lichen-associated meristematic fungi such as *Mycocalicium victoriae* are also members of the Dothideales (Sert and Sterflinger, unpubl. data). The SSU phylogenetic tree including new strains from Mediterranean habitats indicates that there are many species and genera with meristematic morphology that were hitherto unknown and will have to be described in the future (Fig. 20.5).
3. In the Pleosporales hitherto only one meristematic species was found. *Botryomyces caespitosus* is morphologically very similar to *S. phaeomuriformis* but can be distinguished by having pink colonies when young. Internal transcribed spacer (ITS) sequences suggest a close relationship of *Botryomyces caespitosus* to *Alternaria* (de Hoog et al. 2000).

Haase et al. (1995) suggested the 1,340–1,389 position in the SSU as a promising region for species specificity. However, the SSU turned out to be too conserved to resolve the enormous interspecies and intraspecies variability existing in black yeast species. For the species determination the ITS region 1 (ITS 1) evolved as suitable tool because this region is nearly identical within a single species of black yeasts (de Hoog et al. 2003).

Many of the meristematic fungi cannot be attributed to any of the known and sequenced orders of the Ascomycetes. Only few species of *Trimmatostroma* have hitherto been sequenced. *Trimmatostroma microsporum* has its anamorph in *Teratosphaeria* and an affiliation to Dothideales or Pleosporales has been discussed (Taylor and Crous 2000). *Taeniolella*, *Bispora*, *Lichenothelia*, *Scolecobasidium* and *Intralichen* have not been investigated at all in view of their genotypic consistency and phylogenetic position. The results obtained so far suggest that at least *Trimmatostroma* and *Taeniolella* are heterogeneous (Sert and Sterflinger, unpublished) and there is indication that these genera must also be regarded as mere form-taxa with phylogenetically separate species. The genus *Lichenothelia* for microcolonial nonlichenized rock inhabitants was introduced by Hawksworth (1981) and several species were described by Henssen (1987). However, for the whole genus only descriptions of the in situ morphology exist. It was later described by other authors that a wide diversity of fungal species can be camouflaged in situ by this microcolonial growth. Thus, the genus *Lichenothelia* must possibly be seen as an ecotaxonomic description of a habitat-specific growth pattern.

The overall phylogeny of black yeasts and meristematic fungi is a complex task and demands a polyphasic approach. While the sequencing of the SSU is suitable for placement into the right classes, ITS 1 is a taxonomic tool for species identification. The 5.8S and part of the ITS 2 region can close a gap between the species level (ITS 1) and higher taxonomic levels although the 5.8S gene sometimes indicates a higher heterogeneity than the ITS 1 region (de Hoog et al. 1999). Thus, the careful observation of morphology and life cycles as well as physiology is still an important tool for anamorph classification and for the understanding of generic placement. As described before assimilation patterns of black yeasts are broad and thus a physiological key can only be applied to very restricted groups, for example, for *Capronia* (Untereiner et al. 1999). New insights into phylogeny could possibly be derived from intergenic spacer regions, from the β-tubulin gene or from the translation elongation factor 1 that is used for identification of several hyphomycete genera. However, these genes have not yet been analyzed with respect to their suitability for taxonomy of black yeasts and meristematic fungi.

20.5 Working with Black Yeasts and Meristematic Fungi

Meristematic fungi and some black yeasts are characterized by slow growth rates. In nature the formation of new microcolonies may take several months, the single colony never exceeding more than 1 mm in diameter. In culture the maximum growth velocity of a single colony is 5 mm/week albeit some black yeasts may have faster growth rates in liquid cultures. Thus, on agar plates meristematic fungi are easily overgrown by fast-growing hyphomycetes. For this reasons the following points should be taken into account for the isolation and cultivation of meristematic fungi:

1. The isolation itself should be as selective as possible. Single colonies are best picked from the substratum using fine but hard needles – canulas are suitable tools – and transferred onto agar plates.
2. Meristematic fungi grow well on malt-extract agar but in order to limit growth of contaminants growth-inhibiting media should be used additionally. Dichlorane–rose bengal medium is suitable for this.
3. Isolation of those fungi needs patience and the incubation time may be up to 4 weeks until colonies become visible to the naked eye.

As described before several black yeasts and their close relatives are human pathogens. Some black yeasts belong to biological safety level 2 or even 3 (de Hoog et al. 2000) and thus while working with black yeasts all precautions necessary for these safety levels have to be followed carefully. This is of special importance if new unknown species are isolated from the environment or from clinical specimens.

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Chapter 21

Yeasts as Indicators of Environmental Quality

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21.1 Introduction

Monitoring environmental quality is a growing concern as the increased human population taxes the Earth's resources to the limit and we need to better manage resources to allow sustainable development. Although invisible to us, microorganisms are largely responsible for the environmental conditions allowing our survival. Their populations respond quickly to environmental changes and some of them can be used to measure the quality of the environment relative to our needs. Yeasts are part of the invisible biodiversity that surrounds and even inhabits us (Kurtzman and Fell 1998; Lachance and Starmer 1998). Considering their wide distribution, ease of cultivation, well-developed taxonomy, and the amount they have been studied in the environment, it is surprising how little use has been made of them as indicators of environmental quality. Different yeast communities are associated with different habitats and population shifts of their component species should reflect conditions within the habitat. For this application they do not need to be pathogenic or otherwise disagreeable organisms themselves, but should have typical population levels in a habitat and change this level in response to the change of an important parameter of environmental quality. The presence of a species in a habitat other than its own can show the influence of one habitat on another.

Yeasts are largely unicellular immotile higher fungi including some that have important associations with humans. Whereas the filamentous fungi are more adapted to solid substrates, the mostly smaller and unicellular yeasts are more adapted to fluid substrates (Kornillowicz 1994). Their small size helps keep them suspended in their habitat and they are found associated with many industrial fermentations especially for production of alcoholic beverages. Much of the waste of the over six billion humans is discharged into the water, atmosphere and soil. So we should expect a response of yeast populations to nutrient enrichment in these natural substrates. Some yeast species are closely associated to man as an organism or his technological applications especially in the food industry. An obvious positive aspect of this association for humans is the domesticated species *Saccharomyces cerevisiae* used

extensively in industry especially in production of the bread and fermented beverages that are part of our everyday lives. But, there are also a few dozen species known to be opportunistic pathogens, most notably *Candida albicans* and *Cryptococcus neoformans* (Ahearn 1998).

About 1,000 different yeast species have been described, but it is typical that about half or more of the yeast species found in the communities of previously unstudied habitats do not fit any described species (Araujo et al. 1995; Hagler et al. 1997). This suggests that the total number of yeast species is much higher than those already known to exist. Most yeast species have been isolated only a few times and little is known about their function or distribution in nature. Many of these may be associated with specific microhabitats or regions that have received little study by zymologists and could have potential as indicators. Other species are ubiquitous in nature and in their associations with humans, making them more obvious targets for use as indicator organisms since a method based on them can be applied in diverse habitats. When a yeast species is consistently associated with a particular microhabitat, such as faeces of warm-blooded animals, it can indicate an influence of that source material in other segments of our lives. Also some yeast species may be typical of habitats in the pristine state and have decreased populations when that habitat is perturbed. These situations, combined with the ease of cultivation of most yeasts, make them good targets for application as indicator organisms. They can be detected and enumerated using selective culture media and also various molecular genetic methods that have been applied in studies of yeasts. Many of these same indicator organisms, and the methods used to detect and enumerate them, can also be applied to quality control of products such as foods. However, here we will look at them as indicators of the quality of the water, soil and air of our human scale macroenvironments.

21.2 Methods for Yeasts as Environmental Quality Indicators

The methods for applications of yeasts as indicators of environmental quality are specific applications of those available for yeast ecology and quality control of products. These subjects are covered in more detail in other chapters of this book.

21.2.1 Collection of Water, Air, and Soil from Natural Habitats

Aseptic methods should be used for collection of samples to avoid contamination with yeasts not actually present in the material under study. Because of their small size, the immediate environments of microbes are measured in millimeter dimensions or smaller, so minor differences in sampling strategy can have a dramatic influence on results. Careful selection of each specific sample site is essential so that the material collected will be representative of the study area. Samples taken near a point source of pollution are important, but should not be assumed to be typical of the general conditions of a habitat and could give the impression of the problem being more serious than it actually is. In pollution studies it is important to include uncontaminated sites as controls to determine background levels of the indicator organisms. Water samples can be taken from the surface by simply using sterile wide-

mouth screw-capped bottles or tubes of appropriate volume and taking care to avoid contamination (American Public Health Association 1998). Where water is relatively shallow and well mixed by currents or tidal action, surface samples are appropriate. However, surface samples may not be typical of a body of water especially under static conditions. Subsurface devices for sterile sampling are available, including the Zobell and Niskin samplers for deep water or simple mechanisms to open and close a sterile bottle at a few meters depth (Hagler and Ahearn 1987). Soil or intertidal beach sediment can be removed from different depths with a disinfected shovel or similar tool or taken as an intact core to preserve soil structure (Hagler et al. 1982). Air sampling is best done with an appropriate sampling device, like the Anderson sampler, and selecting the stages or sample fraction expected to have particles the size of fungal spores. It is possible to use particles settled out of the air by gravity directly on solid media or collected as dust, but such sampling will not be complete. Indirect measures of air quality can be done by sampling plant leaves although this is influenced by many factors, such as the type of plant and age of the leaves (Dowding 1987).

21.2.2 Preisolation Treatments

Environmental samples should be processed as soon as possible after collection, and maintaining them at low, but not freezing, temperatures can help preserve the yeast populations in them. Treatment of samples to more effectively extract, disperse and concentrate yeast cells is often ignored, but this is an important part of effective isolation and enumeration procedures (Martini et al. 1980). If counts are to be made by cultivation methods, cells associated with each other in colonies or on the substrate should be dispersed. The yeast cells should be separated or diluted from substances that can interfere with the detection of the target species of the assay. Sometimes the growth-inhibiting substances cannot be easily removed and diluting the sample may reduce their levels enough to allow isolation of yeasts. Sterile distilled water or protective solutions like 0.1% peptone or 0.85% NaCl can be used for this. Yeasts in aquatic samples may not be evenly distributed because of association with particles or because they are present as colonies. Also toxic compounds such as from algae or pollutants may be present in the samples and their removal should be considered in these procedures. Sedimentation of heavier particles, prefiltration that allows yeasts to pass but not larger fungi, or differential centrifugation has been used for this (Beech and Davenport 1971; Simard and Blackwood 1971a). Vigorous shaking is recommended as described in the APHA standard methods for analysis of water and wastewater by leaving a head space in the sample container and inverting vigorously 25 times (American Public Health Association 1975). Blending or sonification under aseptic conditions can be applied since yeasts have strong cell walls and are not easily disrupted (Martini et al. 1980; Santos et al. 1996). Sonification can also reduce the number of more delicate bacteria in the sample to facilitate yeast counts (Hassen et al. 2001). Aquatic sediment and soil samples are typically suspended in 10 vol of sterile water or an extraction solution that can contain substances like Tween detergents or peptone to facilitate dispersal and survival of the extracted yeasts. Vortexing or vigorous shaking by hand can be effective to extract

cells when sand particles are present to facilitate dispersal. In air sampling, prolonged exposure to strong air currents can cause drying of the medium surface of agar plates and can interfere with growth of samples. When the populations are too low to be detected or counted in the 0.1-ml volumes typically applied on spread plates, larger volumes can be concentrated by centrifugation (Slavikova et al. 1992) or on membrane filters (black filters with 0.8-μm pores are recommended) and these may be placed directly on culture media (American Public Health Association 1998). Preinoculation treatment can have a profound effect on the results of detection and enumeration of yeasts by cultivation methods.

21.2.3 Selective Culture Media for Monitoring Yeasts

All culture media are selective and their compositions will determine which of the yeasts extracted from the sample will be recovered. Selective and differential media may be employed for more specific isolation and presumptive identification of target organisms. Combinations of nutrients and conditions allowing the target organisms to grow are combined with substances and conditions inhibiting growth of nontarget organisms. Since bacteria are typically more numerous than yeasts in samples, their growth must be controlled if yeasts are to be cultivated successfully for study. This can be done by acidification of the medium, for example, to about 3.7–4.5 with HCl, and the use of antibiotics such as chloramphenicol at 200–400 mg/l (Buck 1975; Phaff et al. 1978). No single cultivation method should be expected to allow all the different yeast species in a sample to develop and incubation conditions like temperature can also select for different species (Buck 1975; Hagler et al. 1986). Buck et al. (1977) observed that some yeasts were isolated by enrichment technique that did not appear when plating methods were applied to the same samples. They also noted that total yeast counts, even at the restrictive temperature of 37°C to select for human-associated yeasts, may not provide useful information for quality control purposes and that application of certain species for monitoring requires development of selective media. After the yeasts have grown on solid media, distinctive colony morphology or indicator dyes can assist in presumptive identification of colonies that may be the targets of the assay. Examples are MCa medium to enumerate *C. albicans* (Buck and Bubucis 1978) and Chromagar *Candida* medium for isolation and presumptive identification of clinical yeasts (Odds 1993; Odds and Bernaerts 1994; Odds and Davidson 2000). Although these differential media with indicator dyes were intended for presumptive identification of a few pathogenic species in clinical samples, they can also help distinguish between different species appearing on isolation plates from other habitats and help increase the amount of diversity recovered in environmental studies. The development of methods to detect and count potential indicator groups is a key to further application of yeasts as environmental quality indicators.

21.2.4 Yeast Detection and Enumeration Methods

Agar plating methods can be in the form of streak plates to detect the presence of an indicator organism, or spread plates and pour plates to allow colony counts

(colony-forming units, CFU), but the results from these methods are mostly limited to the prevalent species making up a minimum of about 1% each the colonies obtained from samples. The 0.1-ml inoculum that can be applied for spread plates or the 1 ml inoculum for pour plates is a limiting factor for the sensitivity of plating methods. However, membrane filters or centrifugation can be used to concentrate yeasts from larger volumes, but keeping in mind there is a practical limit to the number of colonies that can be counted on a single plate or filter. At least some oligotrophic yeasts can be detected by cultivation on relatively low simple sugar content media like corn meal agar or yeast nitrogen base (YNB) with little or no added carbon source (Phaff et al. 1978). Most known yeasts grow at relatively high nutrient levels with glucose and reduced nitrogen sources but carbon source concentrations above about 2% produce large colonies and further limit the number of them that can fit on a single Petri dish. Larger sample volumes can be used in enrichment broth cultures using selective media to increase the sensitivity of detection if target species and populations can be estimated in them (Cooke et al. 1960; Buck et al. 1977; Hagler et al. 1986). This can be done by noting the presence or absence of the indicator organism in a single known volume (P/A test) or a series of measured volumes of inocula (typically done by decimal dilutions in an extinction dilution method) to yield an indicated number (IN) for approximation of the order of magnitude of the indicator population. When the IN is applied with multiple tubes, typically three or five at each sample volume (or dilution) tested, it can be used to determine the most probable number (MPN) for a more precise estimate of the population (American Public Health Association 1975). A method recommended for wastewaters by the American Public Health Association (1975, 1998) allows isolation and estimates of the yeast population in the presence of large populations of filamentous fungi. This approach is worthy of consideration since overgrowth by molds is a limiting factor in studies of yeasts in many habitats. In this enrichment method 15-ml samples are shaken with 135 ml of sterile water in a 250-ml Erlenmeyer flask at 120–150 rpm for 30 min. Two 250-ml Erlenmeyer flasks containing YNB with 1 and 20% glucose are each inoculated with 1 ml of sample and these are incubated at room temperature on a rotatory shaker at 120–150 oscillations per minute for at least 64 h. The flasks are then allowed to settle for 4–5 h and yeast cells will settle to the bottom leaving bacteria and filamentous fungi in suspension. A loopful of sediment is then removed from a tilted flask and streaked out on yeast extract–malt extract (YM) agar using three plates per flask. After 2–3 days' incubation reasonably isolated colonies that are morphologically different are picked from the plates to obtain pure cultures. The reciprocal of the highest positive dilution is the IN of the yeast population. These enrichment methods provide a low precision of counts but can be very sensitive since a single cell can be detected and the method can be made highly selective for a group of indicator organisms when the medium and cultivation scheme is well designed.

21.2.5 Cultivation-Independent Methods

Cultivation-independent methods have been applied using fungal specific primers in some habitats in which yeasts are among the prevalent fungal species. Cultivation-independent methods depend on efficient extraction of DNA without inclusion of

PCR-inhibiting substances. These methods combine PCR amplification of the DNA extracted and purified from samples with separation of the DNA from different species by methods like denaturing gradient gel electophoresis or in thermal gradient gel electrophoresis polyacrylamide gels (Gomes et al. 2003; Gadanho and Sampaio 2004; Prakitchaiwattana et al. 2004). They do offer an advantage in distinguishing between different prevalent species that produce colonies similar in morphology when cultivated and also in detecting species not growing on commonly used cultivation media, but are limited in sensitivity to prevalent species as is cultivation on solid media. Real-time PCR with specific primers for the indicator species would seem a very attractive approach for cultivation-independent monitoring of indicator organisms (Bleve et al. 2003; Brinkman et al. 2003). These molecular methods can be applied after enrichment to detect some lower population level species (Gadanho and Sampaio 2004). DNA hybridization methods such as microarrays using appropriate probes also have potential applications (Kim et al. 2004).

21.3 Applications of Yeasts as Environmental Quality Indicators

21.3.1 Yeast Counts as Quality Indicators in Aquatic Habitats

Populations of a few yeasts or less per liter are typical of clean water in oceans and pristine fresh waters. This contrasts with counts that can reach thousands per liter in eutrophic waters (Hagler and Ahearn 1987). Low populations of oligotrophs may exist as autochthones in low-nutrient pristine habitats, but they are expected to be at low population levels and not necessarily adapted to growth on the rich culture media normally used to isolate yeasts. Oceanic waters are a good example with typical yeast populations of a few cells per liter at best (Fell 1974).

Yeasts have been applied as indicators of sewage contamination and recreational water quality as a complement for the coliform and faecal *Streptococcus* counts used as indicators of recent fecal pollution. Some yeasts are part of the normal fecal flora of many animals, including humans (Ahearn 1998). Although yeasts present in feces are expected to be associated with the types of foods consumed, some opportunistic human pathogens, including *C. albicans*, are associated with warm-blooded animals and can be washed off the body during bathing in addition to being included in feces. Some of these are more resistant to chlorine treatment than most microbes, making them valuable as indicators for swimming pools or other chlorinated bathing waters (Engelbrecht et al. 1974). Methods for yeasts have been included for decades in the APHA standard methods (American Public Health Association 1975, 1998) and were based on the work of Cooke et al. (1960) and Cooke (1965). APHA recommends that in addition to noting yeasts appearing in pour plates with media for cultivation of fungi the enrichment procedure described before be used.

The yeasts associated with the feces of warm-blooded animals include various fermentative species and there is a higher proportion of fermentative yeasts in polluted than in clean water (Ahearn et al. 1968; Cooke et al. 1960; Wollett and Hedrick 1970; Wollett et al. 1970; Hagler and Mendonça-Hagler 1981). Selective methods have been suggested for human-associated yeasts based on incubation at an elevated temperature of 37°C (Buck 1975; Buck et al. 1977). However, this temperature was

not found sufficiently selective for tropical habitats and a method targeting especially *C. krusei* and *C. tropicalis* with 40 C incubation was suggested by Hagler et al. (1986). These two yeasts have been found to be especially associated with waters heavily polluted by domestic sewage (Cooke et al. 1960; Hagler and Mendonça-Hagler 1981). Hagler et al. (1986) made counts of fermentative yeasts with 1-ml volumes and decimal dilutions in aquatic yeast medium (2% glucose, 0.5% yeast extract, 0.5% ammonium sulfate, 0.2% monosodium phosphate, 400 mg/l chloramphenicol and pH adjusted to between 4.0 and 4.5 with HCl) with incubation under static conditions for 3 days. These MPN counts were found to correlate well with total yeast counts made using plating methods and also fecal coliform counts in polluted waters. That *C. krusei* and *C. tropicalis* are both highly typical of polluted water and associated sediments, both grow well at 43°C (a notably higher temperature than most yeasts) and both have strong fermentation, suggests that counts of fermentative yeasts at 43°C could be an effective method to monitor pollution by domestic wastes.

The use of total counts of "pink" carotenoid pigment producing yeasts was proposed by Simard (1971) and Simard and Blackwood (1971a, b) as a water quality indicator. Since they are easy to cultivate and their pigment formation allows presumptive identifications of colonies, the approach is attractive. Their method included a prefiltration of the sample through Whatman no. 1 filter paper to remove large particles and fungal hyphae before concentrating the yeasts on membrane filters. This method yielded higher yeast counts than was found in most other studies of yeasts in water. The preisolation filtration step may have eliminated some yeasts attached to particles or in filamentous form from the samples, but the elimination of a large portion of the molds that could overgrow and obscure yeast colonies on plates and possibly also disaggregating grouped yeasts should have increased the counts. Pink to red or orange pigmented yeasts are frequent in polluted waters at variable but often relatively high population levels. But, pigmented basidiomycetous yeasts can actually make up a higher portion of the yeast population of less polluted aquatic habitats (Hinzelin and Lectard 1978, 1979; Hagler and Mendonça-Hagler 1981). Spencer et al. (1974) noted that although pink yeasts were among the prevalent colonies from polluted freshwaters in Saskatchewan, Canada, their portion of the total yeast population varied from 0 to about 50%. A more recent study of carotenogenic yeasts isolated on membrane filters placed on acidified YM agar with 100mg/l chloramphenicol showed them to be present in temperate oligotrophic waters of glacial origin in Patagonia, Argentina (Libkind et al. 2003). Whereas *Rhodotorula muciliginosa* was present in the majority of the samples and made up about 50% of the isolates studied, this species was not detected in waters with very low anthropic influence. This suggests that if selective isolation media and more accurate identification now available were applied to allow monitoring of individual species of carotenoid-producing yeasts, they could serve as nonfecal pollution indicators at very low levels of contamination. The pink yeasts were among the first yeasts suggested as indicators of environmental quality and continue to show potential especially in more pristine environments.

The total yeast population is expected to have a stronger and more consistent general response to water pollution than pink yeasts, and has been suggested as an

indicator of the trophic state of aquatic environments (Rosa et al. 1990). *Trichosporon cutaneum* has been suggested as a fecal pollution indicator by Hinzelin and Lectard (1978) on the basis of a study of the Moselle River. By contrast, small tropical lakes in Minas Gerais, Brazil, were mostly populated by yeasts fluctuating between rainy and dry periods, in which constantly introduced transitory species dominated during rainy periods (Rosa et al. 1995; Morais et al. 1996). In contrast to the data from the temperate river, it was suggested that *T. cutaneum* is part of the indigenous aquatic microbiota of the dry period, meaning that the same species can indicate different things in different regions. Some apparently clean artificial lakes in rural areas of Slovakia had *C. krusei*, *C. lambica*, *C. tropicalis*, *C. guilliermondii*, *Pichia anomala*, *P. burtonii* and *Rh. glutinis* associated more with recreational areas having cottages, fishing and swimming areas (Slavikova et al. 1992). Yeast-like fungi were noted to have bioindicator properties in a Polish river because species including some potential pathogens, *C. albicans*, *P. guilliermondii*, *P. anomala*, *Rh. glutinis* and *T. beigelii*, were found in water with a high content of municipal sewage and altered chemical parameters, whereas *T. aquatile* was found in clean waters only (Dynowska 1997). Dabrowski et al. (1998) noted that in the Szezecin lagoon in Poland these species were not increased in their most polluted site although *Rh. glutinis* was frequent in all four stations in the study and *S. cerevisiae* had an unexplainable high frequency in one. The few yeasts isolated from lakes in a rural region of northwest Poland with low yeast populations were noted by Rózga et al. (1999) to not include *C. albicans* or the other commoner opportunistic pathogens except for *C. glabrata*. Although filamentous fungi can complicate enumeration of yeasts in polluted waters, counts of some *Rhodotorula* and *Trichosporon* species and also some fermentative species of the very diverse genera *Candida* and *Pichia* have been considered by authors from various regions as potential indicators of environmental quality.

C. albicans is an obvious target organism for pollution monitoring, but has not been isolated as frequently as expected on common yeast media in work with polluted water. This is apparently due to it being a poor competitor with other yeasts in mixed culture when growing on less selective culture media (Hagler and Ahearn 1987). This species is not comparable with coliforms or fecal streptococci as an indicator of recent fecal pollution, but it is an opportunistic pathogen present in feces and is also washed from body surfaces during bathing and can survive in natural waters (Valdez-Collazo et al. 1987). That makes it useful as a complement to counts of facal indicator bacteria in monitoring environmental quality. A culture medium for *C. albicans* based on YNB with maltose as a carbon source and cycloheximide to inhibit growth of many yeasts and other fungi was developed for membrane-filtered water samples (Buck and Bubucis 1978). With this methodology *C. albicans* was shown to be common in polluted waters and detectable in low pollution level samples. It could be used together with other media and germ tube formation to easily confirm the identity of this species (Cook and Schlitzer 1981). Application of this method in an extensive study of Lake Ontario bathing beaches showed a relation of *C. albicans* to elevated fecal pollution indicator levels observed in July and August in association with peak bather load at the beaches, although in only one instance the data suggested the beach was subjected to human fecal contamination (Sherry

et al. 1979). The application of a selective culture medium has allowed *C. albicans* counts to be applied as a valuable environmental quality monitoring tool for recreational quality of bathing waters and beach sands.

21.3.2 Specific Types of Industrial Contamination in Water

Industrial production strains like *S. cerevisiae* are not common in pristine habitats. If such yeasts are found in large populations it is clear evidence that pollution has occurred. In tropical aquatic habitats in south Florida (Ahearn et al. 1968) and also the North Paraiba River in Brazil (Oliveira 1990) large populations of *S. cerevisiae* were found present near sugar refineries. Yeasts responding to nutrients included in industrial wastes can also be useful. Xylose-assimilating yeasts of the *P. membranifaciens* clade, cellobiose-fermenting *Hanseniaspora uvarum*, sulfite-resistant *Trichosporon* strains and yeasts growing at 45°C were typical near pulp mills on Lake Champlain (Meyers et al. 1970). This contrasted with the low levels of yeasts and black yeasts dominated by *Rhodotorula*, *Cryptococcus* and *Aureobasidium* that were typical of most of Lake Champlain and are typical for pristine waters in general. These pulp mill waste associated yeasts were suggested as indicators of pulp mill residues in aquatic habitats.

Hydrocarbon degrading yeast populations can respond to pollution from petroleum sources. The species and densities of yeasts isolated from the North Sea before and after production of oil were different (Ahearn and Crow 1980). *Debaryomyces hansenii* was the prevalent species in both sets of samples, but after oil production the hydrocarbonoclastic yeast *C. guilliermondii* was commoner and the frequency of *A. pullulans* decreased. The species *C. lipolytica* (= *Yarrowia lipolytica*) has been suggested as an indicator of petroleum-related pollution in marine and estuarine environments. It uses relatively few carbon sources and is not of very common occurrence in water, but is able to grow in estuarine conditions, including the typical slightly alkaline pH that does not favor growth of most yeasts, and is a factor restricting many yeast species from marine habitats (Hagler and Mendonça-Hagler 1979). These characteristics make it a good target organism to monitor for petroleum pollution. We have observed a strong prevalence of *C. rugosa* in oxidation lagoons receiving petroleum refinery waste near mangrove ecosystems and also in intertidal estuarine sediments receiving high levels of urban sewage in Rio de Janeiro, suggesting it as another target species for environmental monitoring (unpublished data).

21.3.3 Yeasts in Aquatic Sediments

Also related to recreational use of water resources is the microbial quality of beach sand. This is of concern since the concentration of microbes in aquatic sediments is about 10 times higher than in water and most bathers actually spend much more time in contact with sand on the beach than they do in the water. Sediments in unpolluted estuarine environments have low yeast populations dominated by *Cryptococcus* and *Rhodotorula* species (Lazarus and Korburger 1974). More polluted sediments have higher levels of yeast populations in general and these

include a much more prevalent presence of fermentative ascomycetous species (Hagler et al. 1982; Soares et al. 1997). The use of *Candida* as an indicator to monitor beach sands has been suggested (Mendes et al. 1998). Studies of sands from marine recreational beaches in São Paulo, Brazil, have shown the presence of the opportunistic pathogen *C. albicans* (Sato et al. 2005). Also associated with littoral regions are filter-feeding aquatic invertebrates that can be sampled for their naturally accumulated microbes, including yeasts. Sampling mussels, for example, can allow increased sensitivity of yeast detection because they are concentrated about 10–100 times from those in the surrounding waters, and also reflect the quality of water at a site over longer periods of time during which the filtration by these animals occurred.

An example of a potential application of aquatic sediment yeasts as environmental quality indicators in aquatic sediments is the potential for combined use of *Kluyveromyces aestuarii* and *C. krusei* to indicate the environmental quality of mangrove ecosystems. Mangroves are typical of tropical estuaries and are important for the reproduction of many marine species, including species of economic importance (Mendonça-Hagler et al. 2001). Since coastal cities tend to be located in estuarine regions this type of habitat is frequently threatened by urban pollution and real-estate development. The species *K. aestuarii* has been found to be typical in or near mangrove regions (Fell et al. 1960; Fell 1961; Ahearn et al. 1968; Soares et al. 1997). It is found in association with marine and estuarine mangrove sediments under typical vegetation and within detritus-feeding animals, but not other animals and not in sediments a few meters outside the mangrove vegetation in adjacent tidal flats (Araujo et al. 1995; Araujo and Hagler 2005). It is rare or absent from more polluted mangrove areas in which *C. krusei* is typically a prevalent yeast species. It would seem an indicator system could be developed based on these two yeast species to monitor the environmental quality of mangrove ecosystems. For this application to be developed differential culture media or other means of accurate detection and population estimates of these species are needed and also environmental data to show the significance of their population levels relative to different levels of anthropic influence in mangrove systems.

21.3.4 Application of Yeasts as Indicators of Air Quality

There is considerable use of microbial counts in studies of air quality, but generally yeasts are noted only as colonies appearing on mycological culture media (Marchisio and Airaudi 2001; Shelton et al. 2002). Yeasts present in the air could be a direct threat to human health as a source of allergy-associated diseases. Unfortunately zymologists have not given much attention to isolation and identification of yeasts from air in spite of their conspicuous presence in many air samples. Ballistospore-forming yeast species are dispersed from plants and should be seen as normal components of air. By contrast, yeast species without such effective spores for air dispersal should be more expected to originate from material suspended during anthropic activities like harvesting or construction activities. As a result we should expect lower levels of yeasts like *Sporobolomyces* resulting from toxic chemicals in the air and a greater presence of other types of yeasts resulting from

suspension of dust from physical activities. Yeasts were included among the culturable fungi present in an extensive study using Anderson N6 samplers for air from 1,717 buildings and outdoors from different regions in the USA (Shelton et al. 2002). Although identification was limited to morphological characteristics in this work, the genera *Candida*, *Geotrichum*, *Rhodotorula*, *Sporobolomyces* and the black yeast *Aureobasidium* were specifically noted in addition to an unidentified yeast group after cultivation on Rose Bengal agar and malt extract agar. Median indoor fungal concentrations were about 80 CFU/m^3 but ranged from below detection levels to over 10,000 CFU/m^3. The median outdoor air fungal concentration was higher at about 500 CFU/m^3 but the range was similar. Fungal concentrations were highest in the fall and summer and indoor concentrations to outdoor concentrations did not vary substantially by season. Outdoor air is considered the dominant source for indoor fungi and median indoor fungal concentrations correlated with the corresponding outdoor concentrations in this study. Regional variations of fungal concentrations in air were substantial. The strength of the work by Shelton and colleagues was in the large number of samples allowing a significant statistical evaluation. It invites speculation that specific populations of yeasts in air could be more precise indicators of different kinds of contamination. For example, the opportunistic pathogen *Cr. neoformans* has been found associated with trees and deteriorating wood (Licea et al. 1999; Randhawa et al. 2001). Because of the relation between plant-associated yeasts and yeasts recovered from air it would seem advisable to target this species in air quality studies done with a more species specific medium and large sample volumes.

House dust settled from the air can be a practical measure of inside environmental quality. The yeast community structure was studied on indoor plants and house dust collected using a vacuum cleaner from 25 apartments in Moscow, Russia (Glushakova et al. 2004). This could be compared with data on yeast communities in other habitats of the region (Bab'eva and Chernov 1995). The yeasts found in house dust were not diverse and were mainly represented by epiphytic and eurybiotic basidiomycetous species that are most frequently found on live and dead plants in natural habitats. They noted the occurrence of the epiphytic basidiomycetous yeasts *Sporobolomyces roseus* in about 30% and *Rh. glutinis* in about 10% of the house dust samples, while the ascomycetous yeasts were dominated by *D. hansenii*. The abundance of the dominant yeast species varied greatly among the apartments. The mean abundance of yeasts on indoor plants was considerably lower than that on outdoor plants, whereas that in the soil of indoor flower pots was about the same as found in the upper horizons of soddy podzolic soils. The taxonomic composition of the epiphytic yeast communities of indoor plants and soil differed considerably from that of similar natural habitats and included opportunistic human pathogens in larger proportions. *Rh. muciliginosa* dominated in house dust and on the leaves of indoor plants. A previous study in Moscow had shown yeast species to be an important part of the fungi in house dust (Petrova et al. 2000). Glushakova et al. (2004) concluded that the anthropogenic yeast communities formed in domestic interiors differ from the respective natural communities in both abundance and species composition.

Yeasts have long been known to be significant in microbial phyllosphere communities (Phaff et al. 1978; Phaff and Starmer 1987). In spite of the importance

of this habitat, it has not received much attention from zymologists until recently. Extensive studies in the Asia–Pacific region have shown diverse balistosporous yeasts to be common in the phyloplane habitat (Nakase 2000). In our experience these yeasts were not frequently isolated from washing of plant surfaces, but when plant materials were suspended above the surface of culture media allowing ballistospores to be discharged and land on the surface, these yeasts were frequently isolated. Because of their prolonged exposure to air, it is reasonable to expect some effect of pollution on these communities. *Sporobolomyces* on leaves has been suggested and studied as a rough and indirect measure of air quality (Dowding 1987; Dowding and Richardson 1990). Ballistospore forming yeast counts were shown to have a relation with air pollution although the results were dependent on which types of leaves were sampled with differences expected depending on the species of plant, the age and position of the leaves and climatic conditions. A small study done in Costa Rica of the yeast community on leaves of the bromeliad *Tillandsia* is notable (Brighigna et al. 2000). This group of plants absorbs its nutrients through the leaves and seems to be especially prone to accumulating pollutants from the air to the extent that they have been used to sample for heavy metals in environmental monitoring. The *Tillandsia* leaves in city environments were noted to be different in appearance and a study of the yeast community also showed differences. Although the data were limited, yeasts were notably lacking on leaves from sites with air pollution accumulated on the leaves but were present on leaves from unpolluted sites. This approach seems promising especially in tropical regions where suitable leaves are more available throughout the year and especially if applied to epiphytes absorbing their nutrients from the air.

21.3.5 Anthropic Impacts on Forests

Measuring the yeast diversity of forests is a challenge because they represent a complex mosaic of many different types of microhabitats, including different plants, animals and soils (Phaff and Starmer 1987). Water running from a forest in pristine streams and rivers should include yeasts washed form the phylloplane, soils and other habitats included in the ecosystem of origin. Hagler et al. (1997) noted a high portion of diverse "atypical" yeasts many not fitting described species in streams running from uninhabited areas of the Tijuca Forest in Rio de Janeiro. This contrasted with higher levels of human-associated yeasts in the same stream as soon as these waters ran through populated areas. A similar situation was noted in the North American prairie in Saskatoon (Spencer et al. 1974). In neotropical forests many bromeliad species accumulate water in tanks formed by their rosettes of leaves. These bromeliad tanks maintain water for long periods of time and material degrading in them substitutes soil in providing nutrients for these plants. These small volumes of eutrophic waters attract many animal species to use their associated resources. They vector yeast species of various sources to these small but nonephemeral aquatic habitats spread throughout forests. The bromeliad tanks did have typical prevalent yeast species for the tank microhabitat independent of the plant species themselves and the ecosystem in which they were found. Ascomycesous yeasts including *C. intermedia, Debaryomyces* spp. and a *Saccharomyces* clade species similar to *Kazachstania martiniae* were typical in shaded tanks, but not in

unshaded plants that were dominated by basidiomycetous yeast species. However, the shaded tanks also had a diverse accumulation of yeast species present at lower population levels, as indicated by the lower isolation frequency that apparently reflected the ecosystem in which they were found (Hagler et al. 1993; Araujo et al. 1998). These should reflect the general yeast diversity of the forest habitats. Other types of forest microhabitat that can reflect surrounding yeast diversity are the fruit-associated populations of drosophilids or fruit flies involved in vectoring yeasts between degrading fruits. These fruits are natural yeast baits in the forest or other habitats and can accumulate different yeast populations typical of a site as they are vectored by the insects. The drosophilids can be attracted to fermenting banana bait in containers protected by sterile gauze and collected for analysis of associated yeasts they have collected from the habitat under study. Morais et al. (1992) found higher yeast diversity was associated with drosophilids from the least perturbed forests in and near the city of Rio de Janeiro, Brazil. The yeasts associated with degrading fruit change in time with an ecological succession involving different drosophilid vector species as well as changes in the fruit (Morais et al. 1995) so care must be taken to collect comparable samples. As studies of yeasts in forests yield more data we should expect to find some yeast species responding to anthropic influences and able to serve as environmental quality indicators for forests.

21.3.6 Soil Quality

Some species of yeasts have been known to be typical of soils for about as long as yeasts have been studied in natural habitats (di Menna 1957; Bab'eva and Chernov 1995). As with water, soils are expected to reflect yeast populations associated with various microhabitats. The upper soil layers have more degrading organic matter and eutrophic conditions supporting a wide diversity of species depending on specific conditions, whereas deeper layers have more oligotrophic conditions harboring yeasts like *Lipomyces* and *Cryptococcus* species at low population levels. Yeast isolations from soils are typically complicated by growth of many filamentous fungi that are more adapted to solid substrates than are yeast forms, and this has inhibited the enthusiasm of zymologists to study this important yeast habitat. Monitoring microbial populations following application of sewage or sewage sludge to soil for disposal is an obvious application. The potential of using yeasts of the genus *Candida* as an indicator for application of sewage sludge to soil has been suggested (Kacprzak and Stanczyk 2003) and yeasts with molds can be used to monitor composting of municipal solid wastes (Hassen et al. 2001). Application of enrichment methods and cultivation-independent methods should yield a more complete image of the yeast community in soil (Gomes et al. 2003). Focus on more specific groups of yeasts as eukaryotic indicator organisms for specific soil applications should yield useful results. Human-associated opportunistic pathogens could be as useful here as they have been in water. We have noted high levels of ascomycetous yeasts in soils of an experimental "organic" farm compared with uncultivated soils (unpublished results). Yeasts should be useful in monitoring the application of sewage sludge and organic fertilizers to soils and studies focused on more specific indicator species should yield positive results.

21.4 Conclusions

The use of yeasts to monitor water, air and soil quality of the environment has been limited. However, as the importance of environmental monitoring increases to allow better environmental management, yeasts should be considered more seriously for this task because of their ease of cultivation and our increasing knowledge of their distribution in nature.

Yeasts are amply but not uniformly distributed in nature and different habitats tend to harbor distinct yeast communities or guilds. Yeast species associated with man and other warm-blooded animals can be monitored to show their potential influence on environmental quality. Other yeasts can respond to habitat deterioration or enter the environment as part of industrial effluents. Yeasts have a large untapped potential to be employed as environmental quality indicators and community ecology data should be studied to identify potential environmental quality indicator yeasts.

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Chapter 22

Yeast Biodiversity and Biotechnology

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22.1 Introduction

Since time immemorial fermented food and beverages have represented practical examples of yeast-associated biotechnology. Nevertheless, even though beer-, wine-, bread-, kefir- and koumiss-making technologies are centuries old, it was not until the mid 1800s that Pasteur demonstrated the essential role of microorganisms in these processes.

To most people yeasts are exemplified by the species *Saccharomyces cerevisiae* and with the production of alcoholic beverages. This is in spite of the fact that this domesticated microorganism represents only a fragment of the vast biodiversity and variegated biotechnological potential of the yeast world. In recent decades, in fact, studies of the metabolic diversity of so-called nonconventional yeasts (NCYs) have revealed innumerable promising biotechnological properties (Wolf et al. 2003).

Yeast biotechnology encompasses an enormous variety of processes involving the activity of yeast cells and metabolites which include fermented foods and beverages, chemicals and pharmaceuticals, as well as important agricultural and environmental interactions. The impact of yeast biotechnology has been extensively documented in a number of reviews (Burden and Eveleigh 1990; Demain et al. 1998; Walker 1998). While many products and molecules are commercially produced, other technologies are still confined to the laboratory and perhaps will eventually be developed into profitable ventures.

The present chapter will review current and past studies on yeast metabolic biodiversity for industrial, medical and environmental applications. In addition, future developments involving the biotechnological potentialities of these essential eukaryotes will be discussed.

22.2 Yeasts in Alcoholic Fermentation

While the ability of *S. cerevisiae* to produce alcoholic beverages is well documented and has been exploited for centuries, there is currently a world-wide interest in an

alternative use of ethanol of fermentative origin (bioethanol) as a partial or total gasoline substitute in internal combustion engines. Interest in bioethanol as a renewable, nonpolluting energy source has varied significantly during most of the last century mainly in response to oil prices. In the 1930s to 1940s, 75% of US production was by fermentation, while after World War II ethylene derived from petroleum and natural gas provided a less expensive source, and fermentative ethanol production declined rapidly (Burden and Eveleigh 1990; Walker 1998). Nevertheless, the oil crises of the 1970s and in recent years have generated renewed interest in bioethanol production.

Although bioethanol has normally been obtained through a batch process, the use of continuous fermentation and high-gravity systems give higher yields (Burden and Eveleigh 1990; Nagashima 1990). Long-term (6-month) continuous cultures have been carried out without substrate sterilization as the ethanol produced can control unwanted microbial contamination (Nagashima 1990). Higher yields have also been achieved by using more ethanol-tolerant strains or through the use of immobilized cell systems (Margaritis and Merchant 1984; Burden and Eveleigh 1990). A pilot-plant-scale 20,000 l operation using calcium alginate immobilized cells of *S. cerevisiae* operated continuously for several months (Nagashima 1990).

As mentioned before, the use of bioethanol is economical only when there is a low-cost supply of fermentable substrates. Since the 1980s some countries have considered this technology a means for transforming agricultural produce or for disposing of large amounts of wastes or by-products of agro-industrial origin. In some Latin American countries, particularly Brazil, interest in bioethanol is directed at obtaining economic independence from petroleum imports. As a result, in 1990 an ambitious scheme (National Alcohol Program of Brazil) was initiated for the production of one trillion liters per year; and Brazilian Gasohol (100% ethanol) from sugarcane became available throughout the country (Burden and Eveleigh 1990; Panek and Panek 1990). In the USA and Canada less ambitious programs for the production of ethanol from corn starch or spent sulfite liquor resulted in the production of over three billion liters per year in the 1990s (Murthagh 1986) and this has grown significantly in recent years to almost equal the Brazilian production levels (Ingledew and Bellissimia 2004).

Other interesting possibilities for bioethanol production, studied so far only on a laboratory scale, could be the fermentation of lignocellulosic hydrolysates, which constitute the most abundant renewable raw feedstock on Earth (Walker 1998; Zaldivar et al. 2001). The conversion of both cellulose and hemicellulose into bioethanol has been intensively studied (Chandrakant and Bisaria 1998). *Candida shehatae*, *Pichia stipitis* and *Pachysolen tannophilus* can ferment lignocellulosic hydrolysates containing cellobiose and xylose (Bashir and Lee 1994; Szczodrak and Fiedurek 1996). Innovative approaches for the utilization of hemicellulosic hydrolysates involving the simultaneous isomerization and fermentation of xylose or a simultaneous isomerization and cofermentation of a glucose/xylose mixture by *S. cerevisiae* in the presence of xylose isomerase (Chandrakant and Bisaria 2000) have been recently described.

An additional possibility since the 1980s is constituted by genetically engineered xylose fermenting strains of *S. cerevisiae*. In the 1990s, the first transformed strain

was able to ferment a mixture of glucose and xylose to ethanol thanks to a carefully designed xylose fermentation pathway with modified carbon flow dynamics (Ho et al. 1999). In addition, the recent development of a strain of *P. stipitis* able to give higher ethanol yields from lignocellulose hydrolysates could make the commercialization of bioethanol economically feasible for some applications (Jeffries and Jin 2004). Table 22.1 lists some alternative carbohydrate sources that have been proposed for bioethanol production using various yeast species.

In addition to ethanol, other industrially useful alcohols, including higher and polyhydric alcohols, can be obtained through yeast fermentation. Higher alcohols

Table 22.1 Yeast bioethanol production from different substrates

Scale	Substrate[a]	Species	Yield (g/l)	Reference
Industrial	Corn and grain	*Saccharomyces cerevisiae*	69	Maiorella (1985)
	Sugarcane molasses	*S. cerevisiae*	69	Maiorella (1985)
Laboratory: wild-type strains	Cane syrup	*Kluyveromyces marxianus*	80	Freer and Destroy (1982)
	Cellobiose	*Candida wickerhamii*	14	Fein et al. (1984)
		C. intermedia (*K. cellobiovorus*)	19	Morikawa et al. (1985)
	Cellulose (cellulase)	*K. marxianus*	10	Barron et al (1995)
	Dextrin	*S. cerevisiae*	80	Laluce et al. (1988)
	Starch	*S.* (*diastaticus*) *cerevisiae*	52	Debnath et al. (1990)
	Starch (amylase)	*K. marxianus*	15	Ward et al. (1995)
	Whey hydrolysate	*S. cerevisiae*	70	Bailey et al. (1982)
	Xylose	*C. shehatae*	6.6	Toivola et al. (1984)
		C. tenuis	6.4	Toivola et al. (1984)
		C. intermedia (*K. cellobiovorus*)	22	Morikawa et al. (1985)
		Pachysolen tannophilus	13.5	Slininger et al. (1982)
		Pichia stipitis	5.9	Toivola et al. (1984)
	Xylose (xylose isomerase)	*S. cerevisiae*	110	Gong et al. (1981)
	Xylulose	*C. shehatae*	17	Yu et al. (1995)
Laboratory: transformed strains	Starch	*S. cerevisiae*	1.3	Compagno et al. (1995)
	Lactose	*S. cerevisiae*	0.6	Compagno et al. (1995)
	Xylose	*Schizosaccharomyces pombe*	21	Chan et al. (1989)

[a]Substrate pretreatment in *parentheses*

("fusel oils" such as isobutyl alcohol, isoamyl alcohol, phenylethanol and isopropyl alcohol) are produced during amino acid metabolism and several of these compounds are important flavor ("bouquet") components in wines or spirits (Walker 1998).

Many yeasts produce various polyhydroxy alcohols such as glycerol, erytritol, mannitol, xylitol and arabinitol in relatively high amounts (Burden and Eveleigh 1990; Granstrom et al. 2001; Costenoble et al. 2003; Lee et al. 2003) which have a variety of commercial applications (Walker 1998). The first industrial-scale production of glycerol (a constituent for TNT) by yeasts was carried out in Germany during World War II (Burden and Eveleigh 1990). Today glycerol produced by *S. cerevisiae* is widely used as a base for synthetic resins, drugs, cosmetics and toothpaste (Walker 1998).

Yeast production of other polyhydroxy alcohols is still limited to the laboratory. Some processes under study include mannitol produced by *C. magnoliae* and by genetically engineered strains of *S. cerevisiae* (Costenoble et al. 2003; Lee et al. 2003), xylitol obtained from *C. guilliermondii* (Roberto et al. 1996; Sene et al. 2001a; Silva and Roberto 2001) and arabinitol produced by *C. entomaea* and *P. guilliermondii* (Saha and Bothhast 1996). All three of these polyhydroxy alcohols can be used as substitute sweeteners for confectionery, biscuits, soft drinks and pharmaceutical coatings (Burden and Eveleigh 1990; Walker 1998).

22.3 Yeasts as Producers of Single-Cell Protein

One of the first uses of yeasts as a source of single-cell protein (SCP) was during World War I when Germany used cells of *S. cerevisiae* as a meat substitute for human consumption. In more recent years, yeast SCP was mainly used as an animal feed additive in former socialist countries of eastern Europe (Burden and Eveleigh 1990).

During the 1960s to 1970s some companies developed pilot- or industrial-scale SCP production processes based on the use of *Yarrowia lipolytica* or some species of *Candida*, of the former genus *Hansenula* and of *Saccharomycopsis* for growth on *n*-alkanes (Solomons 1983; Burden and Eveleigh 1990). Although considerable money and effort were allocated to the development of an economically feasible process using petroleum-derived hydrocarbons, the industry never really took off for a number of economical and health-related reasons (Tuse 1984; Demain et al. 1998). Alternatively, a certain interest has been manifested towards using methanol as a pure, water-soluble substrate for SCP production (Tuse 1984; Burden and Eveleigh 1990).

Finally, various agro-industrial residues have been studied as possible substrates for SCP production. Starch from potatoes was proposed for the coculture of amylolytic (*Saccharomycopsis fibuligera*) and nonamylolytic (*C. utilis*) yeasts (Tubb 1986), while milk whey could be a substrate for lactose-positive yeasts (Burden and Eveleigh 1990). Additional investigations on SCP production from different feedstocks are reported in Table 22.2.

Table 22.2 Single-cell protein production from different feedstocks

Substrate	Species	Reference
Ethanol	*Pichia jadini* (*Candida utilis*)	Litchfield (1979)
Inulin	*Kluyveromyces marxianus*	Boze et al. (1995)
Lignocellulosic	*C. shehatae*	Walker (1998)
biomass	*C. utilis*	Litchfield (1979)
	P. stipitis	Walker (1998)
Methanol	Former *Hansenula* species	Litchfield (1979)
	P. pastoris	Boze et al. (1995)
Molasses	*Saccharomyces cerevisiae*	Litchfield (1979)
n-Alkanes	*Yarrowia lipolytica*	Litchfield (1979)
n-Paraffins	*Y. lipolytica*	Boze et al. (1995)
Starch	*Debaryomyces occidentalis* (*Schwanniomyces castellii*)	Boze et al. (1995)
Sulfite waste liquor	*P. jadini* (*C. utilis*)	Solomons (1983)
Whey	*K. fragilis*	Boze et al. (1995)

22.4 Yeasts as Producers of Industrially Relevant Molecules

22.4.1 Enzymes

Compared with certain fungi (e.g., *Aspergillus niger*) and bacteria (e.g., *Bacillus* spp.), yeasts are not particularly rich sources of industrially useful enzymes. Nevertheless, some species have been tested on the laboratory scale as enzyme producers for potential industrial exploitations (Bilinski and Stewart 1990; Burden and Eveleigh 1990; de Mot 1990; Guiraud and Galzy 1990; Ratledge and Tan 1990; Walker 1998). In recent years several large-scale investigations studying yeasts from various environments have been undertaken in the quest for novel enzymes (Ray et al. 1992; Abranches et al. 1997; Braga et al. 1998; Buzzini and Martini 2002). Some of these studies are listed in Table 22.3.

Yeast proteases have been extensively studied for application in the food and beverage industries (Nelson and Young 1986; Bilinski and Stewart 1990; Dizy and Bisson 2000; Poza et al. 2001). For a considerable period of time attention to amylolytic yeasts did not extend beyond *S. fibuligera* (Walker 1998). However, the potential of using amylases for candy and jam manufacture renewed interest in searching for additional starch-degrading species. As a result, several α-amylases, glucoamylases and cyclodextrinases produced by species of the genera *Saccharomyces*, *Candida*, *Filobasidium*, *Lipomyces* and *Schwanniomyces* have been purified and characterized (Wilson and Ingledew 1982; Burden and Eveleigh 1990; de Mot 1990; Demain et al. 1998; Walker 1998). In addition, the use of recombinant DNA techniques allowed for the cloning of glucoamylase genes from *Schwanniomyces* spp. and *S.* (*diastaticus*) *cerevisiae* and their introduction into brewer's yeasts (Walker 1998).

The most economically interesting application of yeast inulinases could be for the synthesis of bioethanol or of fructose for use as a sweetener (Guiraud and Galzy

Table 22.3 Some enzymes produced by yeast

Enzyme	Species	Reference
Amylase	*Schwanniomyces alluvius*	Wilson and Ingledew (1982)
Carboxypeptidase	*Saccharomyces cerevisiae*	Shiba et al. (1998)
α-Galactosidase	*S. pastorianus*	Church et al. (1980)
	Pichia guilliermondii	Church et al. (1980)
	Kluyveromyces lactis	Ward (1985)
Glucoamylase	*S.* (*diastaticus*) *cerevisiae*	Tubb (1986)
β-Glucosidase	*P. farinosa* (*Candida cacaoi*)	Drider et al. (1993)
	P. capsulata (*C. molischiana*)	Freer and Skory (1996)
	Torulaspora pretoriensis	Oda et al. (1993)
Invertase	*Rhodotorula glutinis*	Rubio et al. (2002)
	Torulaspora pretoriensis	Oda and Tonomura (1994)
Lipase	*Cryptococcus curvatus*	Hassan et al. (1994)
Polygalacturonase	*K. marxianus*	Serrat et al. (2002)
Protease	*C. caseinolytica*	Poza et al. (2001)
Xylose dehydrogenase	*C. guilliermondii*	Sene et al. (2001b)
Xylose reductase	*C. guilliermondii*	Sene et al. (2001b)

1990). This activity in *Kluyveromyces marxianus*, *C. salmanticensis* and *Debaryomyces polymorphus* has been extensively reviewed (Vandamme and Deryche 1983; Burden and Eveleigh 1990; Demain et al. 1998). In addition, several pectic enzymes from *C. norvegensis*, *Cryptococcus albidus*, *K. marxianus*, *S. cerevisiae* and *S. pastorianus* have been studied for potential applications (Blanco et al. 1999).

Lipases from *Y. lipolytica*, *Cr*. (*Candida*) *curvata*, *Y. lipolytica* (*C. deformans*), and *Rhodotorula glutinis* have been extensively investigated (Montet et al. 1985; Muderhwa et al. 1985; Ratledge and Tan 1990) for application in the oil and fat industries, in laundry detergents and in the food industry (Burden and Eveleigh 1990). Although similar to lipases, little attention has been devoted to yeast esterases as only *Y. lipolytica* and *C. guilliermondii* (Lloyd et al. 1971; Basaran and Hang 2000) have been studied. Finally, additional enzymatic activities have been found in yeast and yeast-like fungi such as cellulase from *Aureobasidium pullulans*, β-glucosidase from *Dekkera intermedia* and *C. intermedia* (*K. cellobiovorus*) (Morikawa et al. 1985), and phenylalanine ammonium lyase from *Cryptococcus* and *Trichosporon* spp. (Walker 1998).

In recent years, genetically modified yeasts with industrially relevant enzymatic activities have been developed. Lipase-overproducing mutants of *C. rugosa* and *Y. lipolytica*, as well as invertase-overproducing strains of *S. cerevisiae* have been recently proposed, and are under study (Ferrer et al. 2001; Fickers et al. 2003; Rossi-Alva and Rocha-Leao 2003).

22.4.2 Lipids

A small number of yeasts belonging to the genera *Candida*, *Cryptococcus*, *Endomycopsis*, some species of the former genus *Hansenula*, *Lipomyces*,

Rhodosporidium, *Rhodotorula*, *Trichosporon*, *Trigonopsis* and *Yarrowia* have been found to accumulate intracellular lipids as microdroplets. These cellular reserves, sometimes exceeding 70% of biomass weight, are composed almost exclusively of triacylglycerols, typically C_{18}-saturated and unsaturated (oleic, palmitic) and C_{16}-saturated (linoleic) fatty acids (Burden and Eveleigh 1990). Although lipid production has traditionally been done in batch culture (Burden and Eveleigh 1990; Ratledge and Tan 1990), the use of continuous culture systems has been reported (Papanikolaou and Aggelis 2002).

Additional classes of lipids are also produced by yeasts such as sophorolipids from *R.* (*C.*) *bogoriensis* and *C. bombicola* (Spencer et al. 1979; McCaffrey and Cooper 1995), cocoa butter-like lipids from *Cr. curvatus* and *Y. lipolytica* (Hassan et al. 1994; Papanikolaou et al. 2002a, 2003) and polyol fatty esters from *R. graminis* and *A. pullulans* (Spencer et al. 1979). Recent studies have also described the use of a genetically modified *S. cerevisiae* for the production of lipid-derived compounds (Dyer et al. 2002).

22.4.3 Carotenoids

Owing to their well-known antioxidant properties, carotenoids represent a valuable class of molecules for applications in the pharmaceutical, chemical, food and feed industries (Olson 1989; Krinsky 1994; Burton 1989; Nishino et al. 1999). *Phaffia rhodozyma* is a pigmented basidiomycetous yeast that was isolated in the early 1970s from the slime fluxes of some deciduous trees (Johnson et al. 1980). This yeast is used industrially for the production of astaxanthin, a pigment employed as a feed additive for salmonid fish grown in aquaculture (Tangeras and Slinde 1994). Studies of the astaxanthin biosynthetic pathway have resulted in the development of several overproducing mutants (Johnson and Schroeder 1995).

Although the occurrence of astaxanthin in yeasts appears to be confined to *Ph. rhodozyma*, additional species have been found which produce other carotenoids. Basidiomycetous yeasts belonging to the genera *Rhodotorula* and *Sporobolomyces*, along with their teleomorphic states *Rhodosporidium* and *Sporidiobolus*, produce carotenoids such as β-carotene, γ-carotene, torulene and torularhodin (Johnson and Schroeder 1995). The biotechnological potential of these pigmented yeasts has been extensively studied on the laboratory scale in recent years (Frengova et al. 1994; Buzzini and Martini 1999; Buzzini 2000, 2001; Bhosale and Gadre 2001a,b; Bhosale 2004). Finally, astaxanthin-, β-carotene- and lycopene-producing strains have been obtained by transforming *C. utilis* with pertinent bacterial genes (Miura et al. 1998a,b; Shimada et al. 1998).

22.4.4 Flavor compounds

Volatile organic compounds (VOCs) belong to several chemical classes (aldehydes, alcohols, esters, lactones, terpenes, sulfur compounds). These are characterized by having a low molecular weight, a high volatility and the ability to interact with olfactory receptors (Cheetham 1997). Despite their high number, only a few are currently exploited by the flavor industry for chemical, pharmaceutical, cosmetic or food and feed applications (Berger and Drawert 1987; Cheetham 1997).

Yeasts are well documented VOC producers (Torner et al. 1992; Romano et al. 1997; Martin et al. 2001; Spinnler et al. 2001; Rojas et al. 2001; Buzzini et al. 2003). Among these, volatile organic sulfur compounds (VOSCs), detected below parts per billion levels (Cuer et al. 1979; Cheetham 1997), are essential determinants of the aroma of some foods and beverages (Berger et al. 1999; Mestres et al. 2000; Martin et al. 2001; Spinnler et al. 2001). The production of some VOSCs (thiols, thioalcohols, thioesters and sulfides) has been observed in ascomycetous yeasts of the species *D. hansenii*, *Geotrichum candidum*, *K. lactis*, *S. cerevisiae* and *Y. lipolytica* (Berger et al. 1999; Spinnler et al. 2001). In addition, the production of terpenes from *Ambrosiozyma monospora* (Demain et al. 1998) and lactones from *Candida* spp., *K. lactis*, *Torulaspora delbrueckii*, *Sporobolomyces odorus* and *Y. lipolytica* has recently been observed (Endrizzi et al. 1996; Wache et al. 2001).

22.4.5 Vitamins

Although yeast cells have generally been considered a good source of vitamins, only riboflavin has been produced industrially since the 1930s from *Eremothecium* (*Ashbya*) *gossypii* and *E. ashbyi* (Vandamme 1992; Demain et al. 1998; Stahmann et al. 2000). Demain et al. (1998) reported that a patented riboflavin-overproducing strain of *D. hansenii* (*C. famata*) has recently been obtained by protoplast fusion and mutation.

The production of other vitamins from yeasts, studied until now only on a laboratory scale, include ascorbic acid and D-*erythro*-ascorbic acid from *Candida* spp., *Clavispora lusitaniae*, *Cr. terreus*, *Kluyveromyces* spp., *P. fermentans* and *S. cerevisiae* (Onofri et al. 1997; Demain et al. 1998; Hancock et al. 2000; Hancock and Viola 2002). In addition, ergosterol (a precursor of vitamin D2) has been obtained from strains of *S. cerevisiae* (Ratledge and Boulton 1985).

22.4.6 Organic Acids

Owing to a faster growth rate and easier cultivation than filamentous fungi, yeasts could potentially be employed for the production of some organic acids such as citric, α-ketoglutaric, itaconic or gluconic acids for the food and pharmaceutical industries (Burden and Eveleigh 1990). Citric acid synthesized by species such as *Y. lipolytica*, *C. zeylanoides, C. boidinii* and *C.* (*citrica*) *tropicalis* has been extensively studied (Tani 1984; Walker 1998; Papanikolaou et al. 2002b).

Other organic acids obtained from yeast and yeast-like organisms include gluconic acid from *Saccharomycopsis* spp. and from *A. pullulans* (Milsom and Meers 1985), fumaric acid from *C.* (*hydrocarbofumarica*) *blankii* (Sinkey 1983) and isocitric acid from *C.* (*brumptii*) *catenulata* (Atkinson and Mavituna 1983). Finally, Witte et al. (1989) obtained lactic acid from *K. thermotolerans*, while malic acid can be produced by *C. utlis*, *P. membranifaciens* and *A. pullulans* (Sinkey 1983).

22.4.7 Extracellular Polysaccharides

Yeast polysaccharides include β-(1→3)- and β-(1→6)-glucans, mannans, galactomannans and pseudonigeran (Barreto-Bergter and Gorin 1983), while glycopro-

teins are also produced as additional components of the cell wall (Burden and Eveleigh 1990). In spite of their viscosity and gelling properties, however, industrial application of these water-soluble polymers has so far been hampered because of their high sensitivity to salt, pH, shearing and heat (Kang and Cottrell 1979; Sutherland 1986; Burden and Eveleigh 1990). Additional extracellular polysaccharide (EPS) produced by yeasts include phosphomannans from *Pichia*, former *Hansenula* spp. and *Pachysolen* spp. (Walker 1998), and glycolipids from *Y. lipolytica* and *C. bombicola* (Burden and Eveleigh 1990; Walker 1998). Recently, an acidic heteropolysaccharide from *Rh. glutinis* composed of sugars (85%) and uronic acid (15%) was characterized (Cho et al. 2001).

The synthesis of pullulan, an α-glucan with α-(1→6)-linked maltotriose units, by the yeast-like organism *A. pullulans*, commercially employed in film-packaging of foods (Burden and Eveleigh 1990), has been well documented (Kang and Cottrell 1979). Also from that species, a novel glucan-like EPS characterized by having α-1, 4-D-, β-1,6-D- and β-1,3-D-glycosidic bonds has been obtained in recent years (Yurlova and de Hoog 1997).

22.4.8 Miscellaneous Compounds and Bioconversions

As early as 1970 it was known that yeasts produce amino acids, the most relevant examples being lysine (using 5-formyl-2-ketovaleric acid as a precursor) from *S. cerevisiae* and *C. utilis*, and phenylalanine from *Rh. rubra* (Walker 1998). Amino acid overproducing strains of *S. cerevisiae* and *P. angusta* (*Hansenula polymorpha*) have been obtained by mutation (Harder and Brooke 1990; Walker 1998) or by protoplast fusion (Brigidi et al. 1988).

Yeasts have also been studied as agents for a number of bioconversions (de Mot and Verachert 1984) such as ketoreductions, hydroxyl group oxidation, ester hydrolysis, hydrogenation of double bounds in steroids and phenol degradation (Demain et al. 1998). Some organic chemists have even used *S. cerevisiae* cells as an enantioselective catalytic agent (Kometani et al. 1996; Stewart et al. 1996). Additional studies on yeast bioconversions, some of which have been scaled up to the industrial level, are reported in Table 22.4.

22.5 Yeasts as Probiotics and as Biotherapeutic Agents

Since antiquity the health benefits of consuming fermented dairy products has been known. This became officially recognized by Metschnikoff, who at the end of the 1800s attributed the long life of Bulgarian peasants to their high intake of fermented milk products containing *Lactobacillus* species. Since then multiple health-promoting properties of so-called probiotic microorganisms have been suggested, such as modulation of the immune system, protection of the host from invading bacteria and viruses (and infection), assistance in digestion and prevention or alleviation of diarrhea (Ouwehand et al. 2002).

The term probiotic, popularized by Fuller in the 1980s (Fuller 1989), is derived from a Greek term “for life”. Probiotic microbes are defined as those which after surviving the rigors of the human digestive system can compete with pathogens and

Table 22.4 Some bioconversions by yeast

Substrate	Product	Species	Reference
Cysteine–furfural	2-Furfurylthiol	*Saccharomyces cerevisiae*	Huynh-Ba et al. (2003)
Ethanol	Acetaldehyde	*Pichia angusta* (*Hansenula polymorpha*)	Moroz et al. (2000)
Ferulic acid	Vanillic acid + guaiacol	*Rhodotorula rubra*	Huang et al. (1993)
Fumaric acid	L-Malic acid	*Dipodascus magnusii*	Rosenberg et al. (1999)
Ketosulfone	*trans*-Hydroxysulfone	*Rh. rubra*	Lorraine et al. (1996)
L-Phenylalanine	2-Phenylethanol	*S. cerevisiae*	Stark et al. (2003)

assist in improving gut microbiota balance; or as living microorganisms which upon ingestion in adequate amounts confer health benefits on the host (Alvarez-Olmos and Oberhelman 2001). Such a definition does not comprise changes in intestinal microbiota or so-called "colonization"; however, as the probiotic organism can exert its effects locally or during transient passage through the gastrointestinal (GI) tract. While specific numbers are normally not mentioned in the definition, it is generally thought that at least 10^9 colony forming units (CFUs) need to be ingested per day for effective results.

Although the most traditionally employed probiotic microorganisms are members of the heterogeneous group of the lactic acid bacteria (LAB) (lactobacilli, enterococci), in recent years other bacterial groups (bifidobacteria) and even yeasts have been employed. The choice is often determined by the actual purpose for which the treatment is required.

The general requirements for a good probiotic include (1) resistance to intestinal enzymes, acid and bile so as to survive passage through the GI tract, (2) ability to adhere to intestinal mucosa in order to be active in pathogen exclusion and/or for enhancing healing of damaged mucosa, (3) a documented lack of pathogenicity and safety so as not to pose health risks for the consumer, and (4) positive technological properties including the possibility for large-scale production and strain stability with an acceptable shelf life (Ouwehand et al. 2002).

Today it is possible to find several commercially available supplements containing viable microorganisms with probiotic properties. These are sold either in lyophilized form or as part of fermented foods. The most commonly used are various LAB species, although some yeasts such as *K. lactis* (Bonekamp and Oosterom 1994) and more frequently *S. cerevisiae* or strains of that species referred to as "*S. boulardii*" have attracted attention.

A strain of *S.* (*boulardii*) *cerevisiae*, isolated from lychee fruit in Indochina, has been used for the prevention and treatment of antibiotic-associated diarrhea caused by *Clostridium difficile* since the 1950s (Nicod-Bertin and Panouse-Perrin 1984). It has been shown to be well suited as a treatment agent as it can quickly achieve high

concentrations and maintain constant levels in the colon. While it does not permanently colonize the organ, it can remain in the intestinal tract long enough to elicit benefits. In addition, since it is a yeast, it is not sensitive to antibacterial antibiotics and is therefore considered a "pharmaceutical probiotic" or "biotherapeutic agent" (BTA) (Surawicz et al. 1989; McFarland and Bernasconi 1993).

22.5.1 Mechanisms of Action of Yeast Probiotics

Although the mechanisms by which probiotics exert protective or therapeutic consequences is not fully understood, Fuller (1991) postulated four hypotheses regarding the possible effects of *S.* (*boulardii*) *cerevisiae* therapy:

1. Competition for nutrients. It has been demonstrated that probiotic bacteria prevent the colonization of pathogenic microorganisms (Ouwehand et al. 1999), although the actual mechanism of this activity has not been completely shown. It would appear logical that probiotic yeasts can also have this effect, and one of the hypotheses could be that the yeast consumes nutrients which are necessary for an overgrowth of undesirable germs.
2. Competition for adhesion receptors. Other studies have shown that *S.* (*boulardii*) *cerevisiae* can decrease in vitro attachment of *Entamoeba histolytica* trophozoites to erythrocytes (Rigothier et al. 1994).
3. Production of antitoxin and/or antireceptor substances. *S.* (*boulardii*) *cerevisiae* is probably responsible for a modification of the brush-border receptors (Czerucka et al. 1991), although it has been shown that mice are protected against the effects of *Cl. difficile* toxins only when the yeast is administered in a viable state prior to toxin introduction (Elmer and Corthier 1991). Studies have shown that treatment with living yeast cells reduces the quantities of free *Cl. difficile* toxins in the GI tract thanks to the production a 54-kDa protease which inhibits toxins A and B receptor binding and enterotoxicity (Castagliuolo et al. 1999). There is also a stimulation of intestinal membrane enzymes (hydrolases) as well as an endoluminal release of polyamines as a result of yeast intake which have antagonistic effects against overgrowth of pathogens in the intestine (Buts et al. 1986). More recent studies have shown that *S.* (*boulardii*) *cerevisiae* is also active against the cholera toxin in vitro (Czerucka and Rampal 2002).
4. Stimulation of immunity. Studies in mice have demonstrated that *S.* (*boulardii*) *cerevisiae* stimulates an increase in intestinal immunoglobin A secretion during a *Cl. difficile* toxin A challenge (Qamar et al. 2001); and also modulates proinflammatory cytokines (Rodrigues et al. 2000).

While a redundant number of studies have shown that the effectiveness of *S.* (*boulardii*) *cerevisiae* against the main causative agent of hospital diarrhea (*Cl. difficile)* is lost if it is not administered in a living state (Elmer and Corthier 1991; Pothoulakis et al. 1993; McFarland and Elmer 1995), other studies have shown that nonviable yeasts can have other effects. For example, a group from Japan showed that administration of mannan fractions from *S. cerevisiae* could have significant protective effects against intraperitoneal and intravenous infections by *Listeria*

monocytogenes and *Pseudomonas aeruginosa* in mice (Kobayashi et al. 1990). Another study showed that yeast glucans can have antitumoral activities (di Luzio et al. 1979; Sherwood et al. 1987).

The use of yeast as a probiotic is historically linked to animal feed. Surplus biomass from the fermentation industry, recycled as an additive to cattle, pig and poultry diets (Lyons et al. 1993), was shown to improve performance and product quality (Burnett and Neil 1977). This could be partly supported by a study showing that when live or autoclaved cells of *S. cerevisiae* were added to an *in vitro* coculture of rumen acetogenic and methanogenic prokaryotes, hydrogen utilization of the bacterium improved and methane emission by the methanogen diminished. It was hypothesized that the yeast in any state provides essential B vitamins and organic acids that can stimulate the acetogens.

Another investigation studied the stimulating effect of *S.* (*boulardii*) *cerevisiae* on LAB growth during yogurt production and their subsequent survival during shelf life (Lourens-Hattingh and Viljoen 2001). The results showed that the yeast was able to develop as a secondary microflora (reaching up to 10^6 CFU/ml) utilizing mainly lactic acid and galactose for growth. On the other hand, the addition to yeast in fruit yogurts presented problems of ethanol and gas production as a result of fructose and sucrose fermentation. Finally, a certain interest has been shown for other dairy-product-associated yeast species with specific enzymatic capabilities such as lipolytic or proteolytic activities (Jakobsen and Narvhus 1996).

22.5.2 Safety, Advantages and Disadvantages of Yeasts as Probiotics

A number of studies have demonstrated the general safety of oral administration of *S.* (*boulardii*) *cerevisiae* (McFarland et al. 1995; Bleichner et al. 1997). Nevertheless, disseminated fungemia, directly related to that strain, was found in hospitals where the BTA had been administered apparently having been transmitted through catheters (Piarroux et al. 1999; Cassone et al. 2003). Since this was observed almost exclusively in immunocompromised patients, this biotherapeutic is not recommended in those cases.

The future of probiotics and microbial BTAs depends upon further elucidation of basic mechanisms allowing scientists and clinicians to maximize their health benefits. This is very important since their use could offer an alternative to conventional antimicrobials to which many pathogenic microorganisms can develop resistance. Other advantages include low risk, relatively low production and formulation costs and the existence of multiple mechanisms of action. Finally, as stated earlier, yeasts represent the added advantage of inherently resisting the activity of antibacterials; and therefore can be used in parallel with these during therapies.

22.6 Yeasts for Biological Control of Postharvest Disease of Fruits and Grains

While rotting of stored fruits, vegetables and grains following harvest represents a significant loss to agriculture, controls through chemical treatment can introduce a

series of health and environmental risks. An alternative strategy for preventing fungal decay could be based on the surface treatment of the stored goods with antagonistic saprophytic yeasts. Studies have shown that molds exposed to cell-free extracts are not inhibited, but that effective protection appears to be the result of a competition for nutrients by living yeast cells (Spadaro and Gullino 2004). Strains of various yeast species, such as *C. oleophila*, *Cr. laurentii*, *D. hansenii*, *Metschnikowia pulcherrima*, *P. anomala* and *P. guilliermondii*, have been studied as biocontrol agents of fungal postharvest diseases of fruits and grains (Chalutz and Wilson 1990; Björnberg and Schnürer 1993; Filinow et al. 1996; Spadaro and Gullino 2004).

22.7 Yeasts as Bioremediation Agents

Although much interest for microbial bioremediation has been directed towards bacteria and filamentous fungi, some yeast species have been shown to be able to degrade synthetic pollutants originating from industrial activities. Some of the toxic molecules degraded by yeasts are reported in Table 22.5.

22.7.1 Yeasts for Cleaning Oil Spills

Hydrocarbon-assimilating species belonging to the genera *Candida*, *Clavispora*, *Debaryomyces*, *Leucosporidium*, *Lodderomyces*, *Metschnikowia*, *Pichia*, *Rhodosporidium*, *Rhodotorula*, *Sporidiobolus*, *Sporobolomyces*, *Stephanoascus*, *Trichosporon* and *Yarrowia* have been studied on a laboratory scale as possible microbial agents for the degradation of oil spills on land or water surfaces as a consequence of ecological disasters (Neujahr 1990; Demain et al. 1998).

Table 22.5 Biodegradation by yeast

Substrate	Species	Reference
4-Aminobutylphosphonate	*Kluyveromyces fragilis*	Ternan and McMullan (2000)
Benzonitrile	*Cryptococcus* spp.	Rezende et al. (2000)
Chlorobenzilate	*Rhodotorula gracilis*	Miyazaki et al. (1970)
Chloropropylate	*Rh. gracilis*	Miyazaki et al. (1970)
Diquat and paraquat	*Lipomyces starkeyi*	Demain et al. (1998)
Iminodiacetate	*K. marxianus*	Ternan and McMullan (2002)
Nitriles	*Candida guilliermondii*	Dias et al. (2001)
Nitrilotriacetate	*K. marxianus*	Ternan and McMullan (2002)
Phenols	*Trichosporon cutaneum*	Neujahr (1978)
Phthalic acid esters	*Saccharomyces cerevisiae*	Begum et al. (2003)
Plasticizers	*Rh. rubra*	Gartshore et al. (2003)
Tannin	*Zygosaccharomyces rouxii*	Demain et al. (1998)
Tetracyanonickelate (II)	*Cr. humicolus*	Kwon et al. (2002)

22.7.2 Yeasts as Biosorbents

Yeasts have also been studied as biosorbent agents of heavy or radioactive metals (Wenzl et al. 1990; Bustard et al. 1996; Omar et al. 1996; Singleton and Simmons 1996). Accordingly, innovative technologies have recently been investigated involving the use of *S. cerevisiae* as carriers in a crossflow microfiltration for the removal of heavy metal ions, Ni^{2+}, Cu^{2+} and Pb^{2+} (Bayhan et al. 2001).

22.7.3 Yeasts as Agents for the Degradation of Aromatic Compounds

A recent study showed that strains of *Aureobasidium*, *Rhodotorula* and *Trichosporon* isolated from industrial effluents were able to grow in the presence of phenol. Cell-free extracts from cultures grown on phenol exhibited catechol 1,2-dioxygenase and phenol hydroxylase activities, suggesting that catechol was oxidized by an *ortho*-type ring fission (Santos and Linardi 2001). Another study investigated the ability of a *T. cutaneum* strain to utilize various phenolic derivatives (resorcinol, 2.6-dinitrophenol. 3-nitrophenol, 4-nitrophenol and *m*-cresol) as sole carbon and energy sources. Results using yeast nitrogen base medium showed that all of these compounds except 4-nitrophenol were degraded. Resorcinol and 2,6-dinitrophenol were rapidly utilized, while 3-nitrophenol and *m*-cresol were only partly degraded (Aleksieva et al. 2002).

22.7.4 Yeast-Like Organisms for Dye Decolorization

The emission of synthetic dyes into the environment is causing significant ecological problems. Since many of these are poorly biodegradable, the treatments of choice tend to be physical or chemical, practices which often lead to the production of toxic substances. As a result, microbial approaches have been attempted as a more environmentally friendly solution to the problem. In a study carried out in Japan a strain of *Geotrichum candidum* able to decolorize 21 different azo and anthraquinone dyes was used (Kim et al. 1995). This broad spectrum of activity is due to the production of a novel extracellular peroxidase (DyP) of 60 kDa (Kim and Shoda 1999).

22.8 Improvement of Yeasts by Mutation, Protoplast Fusion and Recombinant DNA Technology

Despite some problems encountered with the appearance of unwanted secondary characteristics (Barney et al. 1980; Goodey et al. 1981), many properties of industrial relevance have been transferred from one yeast to another by protoplast fusion. Some of these include osmotolerance (Demain et al. 1998), ethanol tolerance (Legmann and Margalith 1983), flocculation (Panchal et al. 1982), lactose utilization (Farahnak et al. 1986) and killer activity (Young 1981; Bortol et al. 1986).

Many countries and international organizations devoted to food safety control are still reluctant to authorize the use of genetically modified organisms (GMOs) for food and beverage production, also in view of the fact that most consumers are hostile towards GMOs. In spite of these obstacles to their application outside of the

laboratory, there are many genetically modified yeast strains with improved characteristics of technological importance. Some examples which could be useful in the fermentation industry include: (1) the introduction of the *STA*2 glucoamylase gene from *S*. (*diastaticus*) *cerevisiae* enhances the ability of *S. cerevisiae* to ferment polysaccharides (Pretorius et al. 1986; Iserentant 1990), (2) the addition of an α-acetolactate decarboxylase gene from *Enterobacter aerogenes* allows the production of highly flavored beers by diacetyl-accumulating brewer's yeasts (Sone et al. 1988), and (3) the inclusion of a malolactic conversion encoding gene from *Lactobacillus delbrueckii* resulted in *S. cerevisiae* strains producing lower acidity and enhanced flavor in some wines (Demain et al. 1998; Pretorius 2000).

Finally, a strain of *S. cerevisiae* transformed with a plant α-glucosidase resulted in desirable properties. Fear of negative consumer response has so far prevented the commercial exploitation for bread making in the UK of an approved recombinant baker's yeast containing heterologous melibiase and maltose permease genes (Demain et al. 1998). Nevertheless, the future of this technology will inevitably include the commercial application of some of these strains when safety measures can be guaranteed, and when the consumer has been properly educated and assured. The use of recombinant DNA techniques for transforming non-*Saccharomyces* yeasts (NCYs) such as *Candida* spp., former *Hansenula* species, *Kluyveromyces* spp., *Pichia* and *Yarrowia* spp. has been extensively reviewed by Iserentant (1990).

22.9 Yeasts as Hosts for the Expression of Recombinant DNA

In spite of negative acceptance in the food industry, since the 1990s the possibility of expressing heterologous genes in yeasts for production of rare molecules of therapeutic value has represented a spectacular potential for the pharmaceutical industry. Although some products are now available on the market (Demain et al. 1998; Walker 1998), two principal reasons are responsible for the relatively low number of actual commercial applications. First of all, the factors inherent in heterologous gene expression may present significant obstacles to producing molecules with the appropriate structure and purity so as to insure biological activity as well as adequate safety guarantees. Secondly, scale-up to commercial production requires very large investments so as to meet both economic and regulatory criteria (Demain et al. 1998; Walker 1998).

The choice of a particular host for heterologous gene expression can be a very important element for the final outcome. Owing to their ease of cultivation and genetic manipulation, yeasts are the preferred hosts for introduction of genes encoding heterologous proteins. This is also due to their high product secreting ability, sometimes even in the glycosilated (active) form (Gellissen and Melber 1996; Walker 1998). Owing to its long history of use in industrial fermentations and its generally regarded as safe (GRAS) status, *S. cerevisiae* has been the most extensively studied host yeast. In fact, foreign genes encoding heterologous proteins are generally more accepted in pharmaceuticals and in food products for human consumption if synthesized by this species (Demain et al. 1998; Walker 1998). A considerable number of heterologous proteins of medical interest have been expressed in *S. cerevisiae* (de Wilde 1990; Gellissen and Melber 1996; Demain et al. 1998; Walker 1998).

The development of industrial-scale processes involving heterologous protein-producing *S. cerevisiae* strains has been reviewed by Mendoza-Vega et al. (1994).

Species other than *S. cerevisiae* such as *P. pastoris* and *P. angusta* (*H. polymorpha*) have received much attention for heterologous gene expression. Among the advantages that these methylotrophic yeasts display over *S. cerevisiae* is a higher protein expression efficiency (de Wilde 1990) and a reduced production of overglycosilated proteins (Demain et al. 1998; Walker 1998). Their ability to produce hormones, antigens and enzymes on an industrial or a laboratory scale has been reviewed by several authors (Gellissen et al. 1992; Subdery 1995). Some *P. angusta* (*H. polymorpha*) derived products are presently undergoing preclinical or clinical trials and are expected to reach the market in the near future (Gellissen and Melber 1996). Additional examples of studies focused on heterologous proteins expression in yeasts are reported in Table 22.6.

22.10 Conclusions

While *S. cerevisiae* will always be important for a wide variety of scientific, commercial and medical applications, it must be remembered that those who ignore the existence of the other 800-plus yeast species risk missing out on the benefits of an

Table 22.6 Some heterologous proteins expressed in yeast

Donor DNA	Protein expressed	Host Species	Reference
Viral			
Hepatitis B virus	Polymerase	*Pichia methanolica*	Choi et al. (2002)
Archea			
Pyrococcus furiosus	β-Glucosidase	*Saccharomyces cerevisiae*	Smith and Robinson (2002)
Bacteria			
Providencia rettgeri	Penicillin G amidase	*S. cerevisiae*	Sevo et al. (2002)
Sulfolobus solfataricus	β-Glucosidase	*S. cerevisiae*	Morana et al. (1995)
Thermomyces lanuginosus	β -1,4-Xylanase	*P. pastoris*	Damaso et al. (2003)
Thermus aquaticus	Aqualysin I	*P. pastoris*	Oledzka et al. (2003)
Protozoa			
Plasmodium falciparum	Malaria antigen	*S. cerevisiae*	Bathurst (1994)
Plant			
Cyamopsis tetragonoloba	α-Galactosidase	*Kluyveromyces lactis*	Hensing et al. (1995)
Human			
	α-Galactosidase A	*S. cerevisiae*	Chiba et al. (2002)
	Hepatitis B surface antigen	*S. cerevisiae*	McAleer et al. (1984)

enormous source of genetic and biotechnological biodiversity. As illustrated in this chapter, the so-called NCYs provide alternative biocatalysts to *S. cerevisiae* in applications that utilize a wide variety of inexpensive feedstocks.

Many genera of NCY have high osmotolerance to sugars and/or salts and significantly higher tolerance to organic acids such as acetate and lactate than *S. cerevisiae*. Although genetically engineered strains of that species are continuously being developed, many NCYs naturally exhibit significant tolerance to aromatic and phenolic compounds, ability to grow on methanol or *n*-alkanes and resistance to a wide variety of toxic compounds present in lignocellulosic hydrolysates or to commonly used food preservatives. The increased exploration of NCYs has fueled renewed research in their physiology, metabolism and genetics which will inevitably lead to a vast array of useful biotechnological and industrial applications in the years to come.

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In the last few decades more and more yeast habitats that were not investigated earlier, spanning cold climates to tropical regions and dry deserts to rainforests, have been explored. As a result, a large body of ecological data has been accumulated and the number of known yeast species has increased rapidly. This book provides an overview of the biodiversity of yeasts in different habitats. The recent advances achieved by the application of molecular biological methods in the field of yeast taxonomy and ecology are also incorporated in the book. Wherever possible, the interaction between yeasts and the surrounding environment is discussed.

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