Chapter 6

Molecular Mechanisms Involved in the Adaptive Evolution of Industrial Yeasts

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6.1 Introduction

Adaptation is obviously a key concept in modern biology, but its precise meaning has often been controversial (Mayr 1982). At the most basic level, the concept of adaptation is related with function. This way, some trait, or integrated suit of traits, of an organism is adaptive if it performs a function that is, in some way, beneficial to the organism to live in an environment. Adaptations can involve aspects of an organism's behavior, physiology, morphology, etc., or the ability of an individual to alter those properties depending on the environment (phenotypic plasticity). The originality of the theory of natural selection proposed by Charles Darwin lay in the fact that it provided a hypothesis to explain the origin of adaptations. Since then, adaptive traits have been considered the result of adaptive evolution, i.e., an evolutionary process directed by natural selection.

The neo-Darwinian theory of evolution by natural selection, also known as the new synthesis, was based on the idea that most natural populations contain enough genetic variation to respond to any sort of selection. Most of this genetic variation is due to the presence of different alleles generated by mutation and homologous recombination. Adaptation may then be explained by the gradual evolution resulting from small changes in the allele frequencies acted upon by natural selection.

However, with the advent of molecular methods, the potential importance of major, new mutations (novelties) in adaptive evolution has been emphasized (Nei 1987; Li 1997). Molecular studies have shown that mutations include not just the generation of new alleles by nucleotide substitution, but such important processes as the generation of new genes, not only by gene duplication (Long et al. 2003), or radically new alleles by unequal crossing over. The complete sequencing of different

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yeast genomes as well as the study of the molecular basis of the physiological properties of yeasts have provided unique tools to study the molecular mechanisms involved in the adaptive evolution of yeast traits of industrial interest.

In the present chapter, we are not going to deal with the procedures to identify, demonstrate or understand the adaptive significance of the traits and properties of industrial yeasts. Rather, we are going to review the different molecular mechanisms involved in the generation of these major genetic novelties that can explain the adaptive evolution of industrial yeasts.

6.2 The *Saccharomyces sensu stricto* Complex Includes the Most Important Industrial Yeasts

Yeasts are defined as unicellular ascomycetous or basidiomycetous fungi whose vegetative growth results predominantly from budding or fission, and which do not form their sexual states within or upon a fruiting body (Kurtzman and Fell 1998). Of the more than 700 known yeast species, several dozen are used in industrial processes, mainly in the production of fermented products and metabolites. Among them, the most useful and widely exploited species are those from the *Saccharomyces* genus, especially *S. cerevisiae* and its relatives, included in the *Saccharomyces sensu stricto* complex.

The *Saccharomyces sensu stricto* complex (Vaughan-Martini and Martini 1998) consists of three species associated with industrial fermentation processes, *S. bayanus*, *S. cerevisiae*, and *S. pastorianus*, and four species isolated from natural habitats, *S. cariocanus*, *S. kudriavzevii*, *S. mikatae*, and *S. paradoxus*.

S. cerevisiae has been found associated to very diverse fermentation processes, including baking, brewing, distilling, winemaking, and cider production, and also in different traditional fermented beverages and foods around the world. The origin of *S. cerevisiae* is controversial. Some authors propose that this species is a "natural" organism present in plant fruits (Mortimer and Polsinelli 1999). Others argue that *S. cerevisiae* is a "domesticated" species found only in association with human activities, because attempts to find this species in regions remote from human activities have been unsuccessful (Naumov 1996). Moreover, some authors suggested that this species could originate from its closest relative *S. paradoxus*, a wild species found all around the world (Vaughan-Martini and Martini 1995). This debate is important in postulating the original genome of *S. cerevisiae* and how the strong selective pressure applied since its first unconscious use in controlled fermentation processes has reshaped it.

The cryophilic *S. bayanus* has been found in nature in cold areas of Europe and also appears associated with different fermentation processes: winemaking, cider production, brewing, and as grape must contaminants. The type strain of this species, originally isolated from beer, has recently been described as a hybrid possessing also a nuclear genome from *S. cerevisiae* (Nguyen et al. 2000; de Barros Lopes et al. 2002; Nguyen and Gaillardin 2005), which led to the proposal of the reinstatement of *S. uvarum*, a former taxon included in *S. bayanus*, as a distinct species (Pulvirenti et al. 2000; Nguyen and Gaillardin 2005) or as a different variety within *S. bayanus* (Naumov 2000).

S. pastorianus (synonym *S. carlsbergensis*) is the bottom-fermenting yeast responsible of the production of lager beer, although it has also been found in musts and

wines. Different studies (Hansen and Kielland-Brandt 1994; Nguyen et al. 2000; Casaregola et al. 2001) have demonstrated that strains of this species correspond to natural hybrids between *S. cerevisiae*, and a *S. bayanus* like yeast. Chromosome sets from both parental species are present in strains of *S. pastorianus* (Tamai et al. 1998; Yamagishi and Ogata 1999), while the mitochondrial DNA (mtDNA) was inherited from the non *S. cerevisiae* parent (Piškur et al. 1998).

The wild yeast *S. paradoxus*, the closest relative to *S. cerevisiae*, according to phylogenetic reconstructions (Rokas et al. 2003), is a natural species distributed worldwide with a fortuitous presence in fermentation processes. However, it has recently been described as the predominant yeast in Croatian vineyards (Redzepović et al. 2002).

Finally, the *Saccharomyces sensu stricto* complex also includes three other wild species, *S. cariocanus*, *S. mikatae*, and *S. kudriavzevii*, whose description (Naumov et al. 2000a) was based on a few strains isolated from natural habitats in Brazil, the first one, and Japan, the other two.

Saccharomyces sensu stricto yeasts possess a series of unique characteristics that are not found in other genera (Vaughan-Martini and Martini 1998). One of these unique characteristics is their high capability to ferment sugars vigorously, both in the presence and in the absence of oxygen, to produce ethanol. This ability allows them to colonize sugar-rich substrates (plant saps and fruits) and compete with other yeasts, which are not so tolerant to alcohol. The aparition of angiosperm plants with sugar-rich saps and fruits introduced a new ecological niche with a different selection regime that likely imposed altered physiological demands on the ancestors of *Saccharomyces* yeasts (Wolfe and Shields 1997). Under such circumstances, adaptive evolution took place in this new ecological context favoring the acquisition of such high fermentative capability.

This capability has unconsciously been used by humans to produce fermented foods and beverages, which introduced new selective pressures on these yeasts. Neolithic human populations likely observed that fruit juice spontaneously ferments producing an alcoholic beverage (Mortimer et al. 1994). Since then, the yeast *S. cerevisiae* and related species have become an essential component of many important human activities, including baking, brewing, distilling, and winemaking.

In general, these industrial *Saccharomyces* strains are highly specialized organisms which have evolved to utilize the different environments or ecological niches that have been provided by human activity. This process can be described as "domestication" and is responsible for the peculiar genetic characteristics of the industrial yeasts. During the last few years, intensive research efforts have been focused on elucidating the molecular mechanisms involved in yeast adaptation to the industrial process, and the reshaping of genomic characteristics of the industrial yeast which have been unconsciously selected over billions of generations (Querol et al. 2003).

6.3 Adaptive Evolution by "Genome Renewal"

Although *Saccharomyces sensu stricto* yeasts are becoming ideal model organisms to test population genetics models (Zeyl 2000) and to study speciation mechanisms (Greig et al. 2002a), very little information is available about the genetic variability of natural *Saccharomyces* populations.

The analysis of natural populations of *S. cerevisiae* from spontaneous wine fermentations (Mortimer et al. 1994) showed that, although genetic diversity was high, almost all strains were homozygous for most of the genes analyzed. This observation, together with the high fertility of the strains and their homothallic character, led the authors of the study to propose a mechanism of evolution for natural wine yeasts, termed genome renewal. This hypothesis is based on the ability of homothallic haploid *S. cerevisiae* cells to switch their mating type and conjugate with cells of the same single-spore colony to produce completely homozygous diploids. Strains of *S. cerevisiae* accumulating heterozygous recessive mutations can change to completely homozygous diploids by sporulation and homothallic switching of individual haploid spores. This process would favor the action of selection, removing recessive deleterious genes and fixing recessive beneficial alleles, thereby enabling yeasts to adapt efficiently to changing environmental conditions. However, Puig et al. (2000) demonstrated that homozygosis could also be achieved by mitotic recombination or gene conversion during vegetative growth.

6.4 Molecular Mechanisms Involved in the Generation of Evolutionary Novelties

Decades of genetics research and the development of large-scale genomic approaches led to the complete sequencing of the genome of *S. cerevisiae* (Goffeau et al. 1996), the first eukaryote to have its genome sequenced. The available molecular techniques and the rapidly expanded genome data with recent publication of new genome sequences from yeasts (Cliften et al. 2003; Kellis et al. 2003, 2004; Dietrich et al. 2004; Dujon et al. 2004), including other *Saccharomyces sensu stricto* species, provided a new approach to decipher the molecular mechanisms involved in the generation of evolutionary novelties in yeasts. Also, molecular evolution and molecular population genetics have provided useful analytical tools for the detection of the processes and mechanisms that underlie the origin of these evolutionary novelties.

Recently, Long et al. (2003) reviewed the different molecular mechanisms that are known to be involved in the creation of new gene structures, the details of which are understood to varying degrees. In the next sections, we will provide evidence of the role of several molecular mechanisms in the adaptive evolution of yeasts.

6.4.1 Gene Duplication

Gene duplication as the most important source of new genes was postulated by Haldane (1933). He proposed that redundant gene copies generated by gene duplications (called paralogues, i.e., genes that are homologous by duplication of an ancestral gene, in contrast to orthologues, genes that are homologous by descent) are not constrained to maintain their original function and, hence, they can accumulate divergent mutations, resulting in new gene functions.

Gene duplications can be produced by different mechanisms resulting in the duplication of a single gene or a group of adjacent genes (Koszul et al. 2004), in the duplication of a chromosome, called aneuploidy (Hughes et al. 2000), or in the duplication of the whole genome content, called polyploidy (Wolfe and Shileds 1997).

In some cases, redundant genes could be retained if there is an evolutionary advantage to having extra dose repetitions. In others, one duplicate will be free to accumulate mutations because only one of the duplicates will be under purifying selection owing to the restrictions to maintain the ancestral gene function. The classical model of acquisition of new genes by duplication proposes that both paralogues could be preserved if one of them acquires a mutation with a new, beneficial function and the other retains the original function (a process called neofunctionalization). However, this process was assumed to be extremely rare (Wagner 1998), because most changes neutrally fixed in the unrestricted duplicate will be loss-of-function mutations, and, hence, this copy will become a pseudogene to be finally lost (a process known as nonfunctionalization). Accordingly, the classical model predicted that few duplicates should be retained in the genome over the long term, but the sequencing of complete genomes showed that retention of ancient duplicates is very common (Wagner 1998).

To explain the preservation of paralogous genes, Force et al. (1999) proposed an alternative process, called subfunctionalization, whereby both members of a pair acquire complementary degenerative mutations in independent subfunctions, originally present in the ancestral gene. This way, both duplicates are required to produce the full patterns of activity of the single ancestral gene, and subsequent adaptive evolution will promote their subfunctional specialization.

The *GAL1* and *GAL3* paralogous genes of the *Saccharomyces sensu stricto* species provide an example of subfunctionalization in yeasts (Hughes 1999). The galactose-inducible *GAL1* gene encodes a galactokinase that catalyzes the production of galactose-1-phosphate from galactose and ATP, whereas the galactose-inducible *GAL3* gene encodes a regulatory protein involved in the activation of both *GAL1* and *GAL3* genes in the presence of galactose and ATP. *Kluyveromyces lactis* contains a single *GAL1* gene encoding a protein with both regulatory and structural functions. The phylogenetic analysis of these genes indicates that *K. lactis GAL1* diverged from the *Saccharomyces sensu stricto GAL1-GAL3* genes before the gene duplication event, indicating that each paralogue specialized by subfunctionalization.

6.4.1.1 Polyploidization: Whole Genome Duplication in Yeasts

The importance of whole genome duplication in the evolution of higher eukaryotes was postulated by Ohno (1970). The complete sequencing of diverse eukaryote genomes revealed that whole genome duplications occurred several times during the evolution of certain eukaryotic lineages (some plants, fishes, amphibians, etc.).

One of the most striking results obtained from the sequencing of the *S. cerevisiae* complete genome was the presence of 376 gene pairs within 55 large duplicated regions. This observation led Wolfe and Shields (1997) to propose that a whole-genome duplication event, polyploidization, occurred in an ancestor of *S. cerevisie* after the split from *K. lactis*, some one hundred to two hundred million years ago. Polyploidization followed by extensive gene loss of most paralogues by pseudogenization and the accumulation of chromosomal rearrangement events explains the observed pattern of dispersed, large segmental duplications present in the *S. cerevisiae* genome (Keogh et al. 1998).

The hypothesis that *S. cerevisiae* is a paleopolyploid was initially very controversial. Other authors suggested that the duplicated segments could arise via independent local duplication events (Souciet et al. 2000), but the comparative analysis of gene order (Wong et al. 2002) in the genomes of different yeast species, partially sequenced by the Genolévures consortium (Souciet et al. 2000), corroborated this hypothesis and also allowed the location of the polyploidization event in the phylogeny of the hemiascomycetous yeasts. The complete, or almost complete, sequencing of genomes from yeast species of the *Saccharomyces* complex diverged before the genome duplication event (*Saccharomyces sensu stricto*, Kellis et al. 2003; Cliften et al. 2003; *S. castellii*, Cliften et al. 2003; *Candida glabrata*, Dujon et al. 2004) and after (*S. kluyveri*, Cliften et al. 2003; *Ashbya gossypii*, Dietrich et al. 2004; *K. waltii*, Kellis et al. 2004; *K. lactis*, Dujon et al. 2004) confirmed that the duplication event encompassed the entire genome, and was produced by polyploidization of an ancestor of *S. cerevisiae* and related species. The comparison of pre- and postduplication genomes allowed the conclusion to be drawn that the whole genome duplication event doubled the number of chromosomes in the *Saccharomyces* lineage, but subsequent gene-loss events, 88% of paralogous genes were lost, led to the current *S. cerevisiae* genome, which contains only about 500 more genes than the preduplication species, but distributed among 16 chromosomes instead of eight. The polyploid genome returned to functional normal ploidy, not by meiosis or chromosomal loss, but instead by a large number of deletion events of small size (average size of two genes), balanced between the two duplicated regions.

Polyploidization in yeasts can theoretically occur by different mechanisms: (1) an error during meiosis can lead to the production of diploid spores and subsequent conjugation between diploid cells, (2) an error during mitosis in unicellular organisms, (3) rare mating between two diploid yeasts of the same species that became mating-competent by interchromosomal mitotic recombination at the MAT locus (de Barros Lopes, 2002), (4) interspecific hybridization by conjugation of spores from different species, and subsequent genome duplication by errors during mitosis or meiosis, or (5) rare mating between two mating-competent diploid strains belonging to different species (de Barros Lopes 2002). In the first three cases, the result is an autotetraploid yeast, whose nucleus contains four allelic copies of each chromosome; however, in the last two cases, the result is a fertile allotetraploid (also called amphidiploid) yeast, containing pairs of "homeologous" chromosomes, i.e., homologous chromosomes coming from two different species. Examples of both types of polyploid yeasts have been described (Naumov et al. 2000b).

Andalis et al. (2004) demonstrated that isogenic autopolyploidy is accompanied by defects affecting viability and subsequent survival of the new organisms, and, hence, postulated that the entire genome duplication event that occurred in an ancestor of *S. cervisiae* was likely generated by allopolyploidization.

But the most important consequence of the whole genome duplication event was the sudden acquisition of extra copies of each gene in the genome. Wolfe (2001) suggested that these duplicated genes formed by polyploidy should be called "ohnologues", after Susumu Ohno, to distinguish them from other kinds of paralogues because they are all the same age.

The complete genome sequences of *K. waltii* (Kellis et al. 2004) and *A. gossypii* (Dietrich et al. 2004), species that diverged before the polyploidization event, were used to map and analyze the fate of the ohnologues during the evolution of the *S. cerevisiae* lineage. The different expected outcomes with respect to the fate of duplicated genes, described in Sect. 6.4.1, were observed. This way, nonfunctionalization was the most frequent process: 88% of paralogous genes generated by polyploidization were lost.

Of the approximately 460 surviving ohnologues, 60 pairs showed decelerated evolution and tend to be highly similar, even at the silent codon positions, suggesting that they may be subject to periodic gene conversion. Moreover, in about half of these cases, the two paralogues in *S. cerevisiae* are closer in sequence to each other than either is to its orthologue in *S. bayanus*, showing that gene conversion occurred after the relatively recent divergence of the two *Saccharomyces* species. These cases often involve proteins known to be highly constrained, such as ribosomal proteins, histone proteins, and translation initiation/elongation factors, indicating that they have likely been retained because of the advantage of having extra dosage of the genes.

The remaining ohnologues have diverged in sequence and often also in function. Kellis et al. (2004) found that more than 100 gene pairs show a higher rate of protein evolution relative to *K. waltii*, with one ohnologue accumulating significantly more amino acid replacements than the other. They also argue that, in many of these cases, accelerated evolution was confined to only one of the two paralogues, which strongly supports a process of neofunctionalization, the slowly evolving paralogue has probably retained the ancestral gene function and the rapidly evolving paralogue probably corresponds to the copy relieved of selective constraints, which is free to evolve more rapidly to acquire a derived function after duplication. Most of these ohnologues correspond to protein kinases and regulatory proteins, generally involved in metabolism and cell growth.

The other approximately 300 ohnologue pairs did not show significant differences in their rates of evolution. In some cases, the functional changes may be similar to those just described but subtler. In other cases, gene pairs may have been retained by subfunctionalization. Specialization to different ancestral subfunctions may explain the similar rates of evolution in both ohnologues. Moreover, this subfunctionalization may have occurred by divergence in regulatory sequences.

The polyploidization event suddenly provided new gene functions that have had a profound impact in the evolution of the *Saccharomyces sensu stricto* lineage (Piškur and Langkjær 2004; Wolfe 2004). The partitioned functions of most ohnologues, retained in the *Saccharomyces sensu stricto* lineage, indicate that the genome duplication provided new genes that played a direct role in the adaptation of these species toward a highly efficient fermentation performance under anaerobic conditions. Wolfe and Shields (1997) indicated that many ohnologue pairs are differentially regulated in the presence and absence of oxygen (DeRisi et al. 1997), including genes of proteins of the electron transport chain complexes (e.g., *CYC1*/*CYC7* encoding cytochrome c isoforms, or *COX5A*/*COX5B* encoding cytochrome c oxidase subunit 5 isoforms) and genes encoding enzymes of the glycolysis/gluconeogenesis pathway (e.g., *PYK1*/*PYK2* coding for pyruvate kinases, *ENO1*/*ENO2* for enolases, etc.).

The polyploidization also allowed the development of efficient glucose-sensing and glucose-repression pathways (Kwast et al. 2002). Ohnologues encoding regulatory proteins are involved in the development of the two glucose-sensing pathways of high affinity and low affinity, the Snf1 pathway of glucose-repression of gluconeogenesis and respiration, and in the glucose-responsive protein kinase A pathway (Wolfe 2004).

In conclusion, the polyploidization event provided the basis for the evolution of new gene functions during the competition to colonize sugar-rich substrates supplied by fruit-bearing plants. The competitive advantage of a fermentative metabolism, fast growth and the production of toxic ethanol put the ancestors of the industrial *Saccharomyces* yeasts in the pole position to become, under the selective pressures unconsciously imposed to improve controlled fermentation processes, the highly efficient mono- and oligosaccharide fermenters that exist today.

6.4.1.2 Aneuploidy: Chromosome Duplication

An alternative mechanism to provide potential new genes is by changing chromosome copy numbers, which is known as aneuploidy. However, the most important consequence of aneuploidy is the increase of gene dose.

Aneuploidy arises by nondisjunction, i.e., inaccurate chromosome segregation, during meiosis or mitosis. The increase in copy numbers for some genes results in an imbalance of the gene products and disruption of the regulatory interactions, which could be deleterious or even lethal for many organisms. Although aneuploidy is tolerated in industrial yeasts, it is one of the causes of the poor sporulation exhibited by some strains.

Wine *Saccharomyces* strains are frequently aneuploid, with disomies (two chromosome copies), trisomies and, less frequently, tetrasomies (Bakalinsky and Snow 1990). This aneuploidy, and also autopolyploidy, has been postulated as a mechanism that may confer advantages for adaptation to variable external environments by increasing the number of copies of beneficial genes or by protecting the yeasts against recessive lethal or deleterious mutations (Bakalinsky and Snow 1990; Guijo et al. 1997; Salmon 1997).

Hughes et al. (2000) observed that the deletion of a gene strongly favors the acquisition of a second copy of a whole chromosome or a chromosomal segment containing a paralogue of the deleted gene. About 8% of 300 yeast deletion mutants examined had acquired a detectable aneuploidy, and in six of the cases they examined, the amplified chromosome contained a close paralogue of the deleted gene, implying that characteristic aneuploidies can act as dominant suppressors and under some circumstances lead to increased fitness.

Kellis et al. (2004) correlated these deletion results with the identification of the ancestral and derived functions of paralogues (Sect. 6.4.1.1). Strikingly, deletion of the ancestral paralogue was lethal in 18% of cases, whereas deletion of the derived paralogue was never lethal. The derived paralogue is thus not essential under these conditions, either because it does not function in a rich medium or because the ancestral paralogue can complement its function. Along with possibly gaining a new function, the derived copy has lost some essential aspect of its function, and cannot typically complement deletion of the ancestral gene.

6.4.1.3 Single Gene and Segmental Duplications

Gene duplication can also involve either a single gene or a group of adjacent genes (segmental duplication). Genome sequencing projects have revealed that multigene families, i.e., groups of identical or similar genes generated by successive single gene or segmental duplications, are common components of all genomes. This way, the *S. cerevisiae* genome contains 265 multigene families with three or more paralogues, including a family with 108 members (Llorente et al. 2000), which indicates that successive gene duplications should have occurred.

Genome comparisons (Souciet et al. 2000; Dujon et al. 2004) showed that tandem repeated gene duplication is very common among yeasts and illustrates the importance of ancestral duplications that occurred before divergence of hemiascomycetous yeasts. Sequence divergence between paralogues in different yeast species shows bimodal distributions, with a fraction of multigene families showing high sequence identities, probably reflecting recent duplications and/or sequence homogenization by gene conversion, and an important fraction with low identities, corresponding to ancient duplications that occurred before species divergence.

Single-gene and segmental duplications mainly correspond to intrachromosomal direct tandem-repeat duplications. Although there are some examples of segmental duplications that are dispersed throughout the genome, most gene families are located in subtelomeric regions (adjacent to chromosome telomeres). Classical examples of redundant genes in subtelomeric regions are the *MEL*, *SUC*, *MGL* and *MAL* genes involved in the assimilation of sugars. Yeast strains differ by the presence or absence of particular sets of these genes, which could be attributed to selective pressure induced by human domestication, as it appears that they are largely dispensable in laboratory strains.

Clusters of duplicated genes have also been found internal to chromosomes. A typical example is the large gene cluster on chromosome VIII near *CUP*1. The *CUP*1 gene encoding copper metallothionein, is contained in a 2-kb repeat that also includes an open reading frame (ORF) of unknown function (Fogel and Welch 1982). The repeated region has been estimated to span 30 kb in laboratory strains, which could encompass 15 repeats, but the number of repeats varies among yeast strains.

Different mechanisms have been postulated to explain the origin of single-gene and segmental tandem duplications. The critical step is the origin of the first tandem duplication, which requires the presence of similar nucleotide sequences flanking the duplicated region. These similar sequences may also be provided by transposable elements. Ectopic recombination between homologous chromosomes or unequal sister chromatide exchange, at the similar sequences, will result in the duplication of the genome region. Subsequent duplications can occur by ectopic recombination between paralogous repeats.

The fate of the duplicated genes is discussed in Sect. 6.4.1. However, many tandemly duplicated genes exhibit identical or nearly identical sequences, indicating that these multigene families evolve in a concerted way to preserve gene function, and, hence, increase gene dosage. Ectopic recombination and gene conversion are the mechanisms postulated to explain the concerted evolution observed in the members of multigene families (Li 1997).

Another process, postulated to preserve identical function in the members of a gene family, is the birth-and-death model of multigene family evolution (Nei et al. 1997), in which repeated gene duplications are counterbalanced by gene degeneration or deletion (nonfunctionalization). A systematic analysis of *S. cerevisiae* intergenic regions revealed the presence of many degenerated pseudogenes, called gene relics, homologous to extant *S. cerevisiae* ORFs (Lafontaine et al. 2004). Gene relic distribution is mainly subtelomeric and related to multigene families. Thus, multigene family evolution by a gene birth-and-death mechanism is also compatible with the presence of new paralogues and relics in several yeast strains and the sequence polymorphism within the tandem *DUP240* family, one of the largest *S. cerevisiae* gene families (Leh-Louis et al. 2004a, b).

Many of the tandemly repeated genes, especially the subtelomeric multigene families, are involved in secondary metabolism. These genes are not essential, but they play an important role in the adaptation to new environmental conditions. For example, subtelomeric gene families in *S. cerevisiae* are often related to cell membrane and cell wall components, such as lectine-like proteins (the *FLO* family), sugar transporters (the *HXT* family), genes related to cell–cell fusion (the *PRM* family), and assimilation and utilization of nutrients (*GAL*, *MAL*, *SUC*, and *PHO* families) (Vega-Palas 2000; Harrison et al. 2002). Some dispersed gene families may also be related to adaptation to environmental conditions, such as the *CUP1* gene tandem repeats present in copper-resistant *S. cerevisiae* strains (Fogel and Welch 1982).

Other species, including those that diverged before the whole genome duplication event, also contain subtelomeric gene families that are probably involved in adaptation to changing environments. For example, the genome of *K. waltii* also contains several families of membrane proteins, hexose transporters, and flocculins (Kellis et al. 2004); and multigenic families encoding multidrug resistance proteins and hexose transporters are specifically more expanded in *Debaryomyces hansenii* than in the other yeasts (Dujon et al. 2004).

Many of these subtelomeric repeats were likely advantageous to industrial strains during selection for thousands of years of human biotechnology practices. Rapid changes in the gene composition of these families may increase the chances of acquiring a selective advantage and improving their industrial fitness. In fact there are several examples of spontaneous gene duplications selected as a response to limiting conditions (Brown et al. 1998).

6.4.2 Lateral Gene Transfer: Acquisition of New Genes from Another Species

Another possible way in which a genome can acquire new genes is to obtain them from another species. This process, known as lateral or horizontal gene transfer, has been proven to be very important in prokaryotes, but not so frequent in eukaryotes. In the case of eukaryotes, allopolyploidy and introgression due to interspecific hybridization could be considered as mechanisms of lateral gene transfer, and they will be treated in Sect. 6.4.3.

Genome sequencing has revealed the presence of a few genes occurring in a single yeast species that have close homologues in bacteria. These genes, most of them

encoding metabolic enzymes, are rare in the yeast genomes less than 1%), but do appear.

A recent study (Gojković et al. 2004) demonstrated that lateral gene transfer has played, together with the whole-genome duplication event, a major role in the evolutionary history of the *Saccharomyces* complex yeasts. These authors proposed that horizontal gene transfer promoted evolution of the ability to propagate under anaerobic conditions in *Saccharomyces* yeasts. In strict aerobic yeasts, the "de novo" pyrimidine biosynthesis, more precisely the fourth enzymic activity catalyzed by a mitochondrial dihydroorotate dehydrogenase (DHODase) is dependent on the active respiratory chain. However, the facultative anaerobic *Saccharomyces sensu stricto* yeasts have a cytoplasmic DHODase independent of the respiratory chain, which is phylogenetically related to a bacterial DHODase from *Lactococcus lactis*. Gojković et al. (2004) demonstrated that *S. kluyveri*, which separated from the *S. cerevisiae* lineage more than one hundred million years ago, represents an evolutionary intermediate, having both anaerobic cytoplasmic and aerobic mitochondrial DHODases. From these observations, they suggested that a *Saccharomyces* yeast ancestor, which originally had a eukaryotic-like mitochondrial DHODase, acquired a bacterial DHODase, which subsequently allowed cell growth gradually to become independent of oxygen.

6.4.3 Interspecific Hybridization and Introgression

In the case of *Saccharomyces sensu stricto*, one of the most interesting mechanisms observed in the adaptation of these yeasts to industrial process is the formation of interspecific hybrids. Allopolyploidy and introgression by interspecific hybridization are the main mechanisms of lateral gene transfer in eukaryotes.

Artificial interspecific hybridization experiments indicated that *Saccharomyces* "*sensu stricto*" interspecific hybrids can easily be formed (Naumov 1996), and, although sterile, they are viable and can be maintained by asexual reproduction. *Saccharomyces sensu stricto* species are present in the same ecological niche and could hence be involved in the formation of hybrids because haploid cells or spores of these species are able to mate with each other and form viable, but sterile, hybrids. Hybrids produce spores with extensive imbalance in chromosome number and low frequencies of genetic exchange. The mismatch repair system plays a major antirecombination role in these yeast hybrids. The ways in which yeast hybrids may escape this postzygotic barrier are achieved either by doubling of the chromosome number, which results in an allotetraploid (Naumov et al. 2000b), or by recovering euploidy by homothallic diploidization of spores, which results in a homoploid (Greig et al. 2002a).

The best described example of hybrid yeasts is the lager yeasts, included in the taxon *S. pastorianus* (synonym *S. carlsbergensis*) (Vaughan-Martini and Kurtzman 1985). This yeast is a partial allotetraploid hybrid between two species of the *Saccharomyces sensu stricto* group, *S. cerevisiae*, and a *S. bayanus* related yeast (Hansen and Kielland-Brandt 1994; Nguyen et al. 2000; Casaregola et al. 2001). Chromosome sets from both parental species are present in strains of *S. pastorianus* (Tamai et al. 1998; Yamagishi and Ogata 1999), while the mtDNA was inherited

from the non *S. cerevisiae* parent (Piskur et al. 1998). Extensive and variable aneuploidy is found in different *S. pastorianus* isolates (Casaregola et al. 2001), and many of them are chimerical, with part from each parent indicating recombination sometime in their history (Bond et al. 2004).

Moreover, the type strain of *S. bayanus*, originally isolated from beer, has recently been described as possessing also a nuclear genome from both *S. cerevisiae* and *S. bayanus* (Nguyen et al. 2000; de Barros Lopes et al. 2002; Nguyen and Gaillardin 2005).

New natural hybrids have been found in environments different from brewing. Masneuf et al. (1998) characterized a *S. bayanus* × *S. cerevisae* hybrid strain (S6U) isolated from Italian wine, and a triple hybrid present in a homemade French cider (CID1). This hybrid contained two copies of the nuclear gene *MET2*, one coming from *S. cerevisiae* and the other from *S. bayanus*, and the mitochondrial genome originated from a third species, which Groth et al. (1999) demonstrated corresponded to the type strain of the species *S. kudriavzevii*. This was the first report indicating that a rare *Saccharomyces sensu stricto* species, for which only two strains isolated from tree exudates in Japan were known (Naumov et al. 2000a), was involved in interspecific hybridization.

New hybrids *S. cerevisiae* × *S. kudriavzevii* isolated from both natural habitats and fermentation processes, and natural *S. cerevisiae* × *S. paradoxus* hybrids have also been postulated on the basis of their patterns of hybridization with repetitive elements (Liti et al. 2005). Natural hybrids are not restricted to the *Saccharomyces sensu stricto* complex: James et al. (2005) have recently described hybrids between species of the genus *Zygosaccharomyces*.

In two recent studies, new hybrids resulting from the hybridization between *S. cerevisiae* and *S. kudriavzevii* have been described among wine strains (González et al. 2005a) and among brewing yeasts (González et al. 2005b). These wine hybrid strains were predominant in spontaneous fermentations from eastern Switzerland (Schütz and Gafner 1994), and different brewing hybrids were isolated from three Belgian Trappist beers, and also from English, German and New Zealand beers. These authors also found a *S. bayanus* × *S. cerevisiae* × *S. kudriavzevii* hybrid strain, also isolated in Switzerland in 1951, that shows a different genome structure than the other triple hybrid CID1. The sequencing analysis of gene regions located at different chromosomes and the comparative genome hybridization to *S. cerevisiae* DNA microarrays showed that *S. kudriavzevii* hybrid strains contain aneuploidy differences and chimerical chromosomes resulting from recombination between "homeologous" chromosomes of different parental origin (S.S. González, A. Querol, J. García-Martínez, J.E. Pérez-Ortín, and E. Barrio, unpublished results).

The diversity of *Saccharomyces sensu stricto* hybrids, their distinct origins and their presence in different habitats indicate that, in spite of the homothallic character of most natural *Saccharomyces* strains and the persistence of their asci, interspecific hybridization is not so infrequent. Pulvirenti et al. (2002) proposed that yeast-feeding invertebrates may provide the appropriate conditions promoting intra- and interspecific hybridization, because these animals produce, in their digestive tracts, enzymes that hydrolyze the ascus wall, releasing free spores able to conjugate.

As an alternative to haploid cell conjugation, de Barros Lopes et al. (2002) proposed that rare mating between diploid strains of the *Saccharomyces sensu stricto* complex could be involved in the generation of interspecific hybrids. They demonstrated that rare mating is possible not only between nonhybrid diploid strains, but also between CID1, S6U, and lager hybrids with *S. paradoxus* and *S. cerevisiae* diploids, indicating that this mechanism may be involved as well in the generation of multiparental hybrids also from allopolyploids, such as S6U (Naumov et al. 2000b).

Natural interspecific hybridization in yeasts is more frequent that suspected and has probably been undervalued as an important mechanism in the evolution of yeasts by providing new gene combinations of adaptive value (Masneuf et al. 1998; Greig et al. 2002b), genetic robustness due to redundancy, new or specialized functions from divergence of redundant genes (Wolfe and Shields 1997), and also new species through allopolyploid (Naumov et al. 2000b) or homoploid (Greig et al. 2002b) speciation.

In fact, interspecies hybridization might have been a key event in evolution of the high fermentation capabilities of the species of the *Saccharomyces sensu stricto* complex. As mentioned in Sect. 6.4.1.1, Andalis et al. (2004) proposed that the whole-genome duplication in the ancestor of the *Saccharomyces sensu stricto* complex was probably generated by allopolyploid hybridization.

6.4.4 Recruited Autonomous Mobile Elements as a Source of New Genes

There are different examples in eukaryotic genomes indicating that an autonomous mobile element could be directly recruited by host genes to generate a new gene function (Long et al. 2003). In fact, 4% of new exons of human protein-coding genes correspond to recruited autonomous mobile elements.

In the case of yeasts, Butler et al. (2004) demonstrated that homothallic mating (self-fertility based on a mating type switch mediated by HO endonuclease) in the *Saccharomyces* complex originated through the acquisition of an intein-like sequence. Inteins are selfish DNA elements inserted in-frame and translated together with their host proteins (Gogarten et al. 2002). This precursor protein undergoes an autocatalytic protein splicing reaction resulting in two products: the host protein and the intein peptide, which exhibits endonuclease activity involved in the intein mobility.

The close resemblance between HO endonuclease and the endonuclease encoded by the VMA1 intein suggests that, shortly before the whole duplication event, an intein from an unknown origin invaded the *VMA1* gene of the ancestor of the *Saccharomyces sensu stricto* yeast, which gave rise to the HO endonuclase encoding gene after subsequent duplication (Butler et al. 2004). The *HO* mating type switching gene facilitated the change from a cell cycle with a major haploid phase to a cycle with a major diploid phase, which increased the level of genetic robustness of the yeast genome, at least owing to dominance, and promoted the evolution of a repair system based on efficient homologous recombination (Piškur and Langkjær 2004).

6.4.5 New Genes Generated by Retroposition

Retroposition may create duplicate genes in new genomic positions through the reverse transcription of expressed parental genes (Long et al. 2003). This way, messenger RNAs (mRNAs) can be retrotranscribed to complementary DNAs (cDNAs) by a retrotransposon reverse transcriptase and inserted in a new genome position. These retrotransposed genes differ from their parental genes in the absence of introns and the presence at the 3′ end of an A–T stretch coming from the retrotranscription of the mRNA poly(A) tail. As a retroposed protein-coding gene copy lacks internal promoter sequences, it has to recruit a new regulatory sequence to be functional or it will become a processed pseudogene.

Schacherer et al. (2004) recently described in yeasts experimental evidence for the recovery of a function involving duplication by retroposition. They used a positive selection screen of *S. cerevisiae URA2* mutants to isolate spontaneous revertants containing a duplication of the terminal part of the *URA2* gene.

The molecular characterization of the duplicated *URA2* regions showed that they were generally punctuated by a poly(A) tract and were always located in Ty1 sequences. Schacherer et al. (2004) demonstrated that the duplication mechanism involves the reverse transcription of *URA2* mRNA packed in Ty1 viruslike particles, and the subsequent integration of the cDNA into a Ty1 resident copy. Reverse transcription was initiated in the poly(A) region via the terminal part of the URA2 gene and switch at the level of the 5′ junction observed on a Ty element template, leading to the formation of the chimerical structure observed: a δ long terminal repeat (LTR) TyA segment in frame with the duplicated terminal part of the *URA2* gene. Integration was mediated by a homologous recombination event resulting from gene conversion between preexisting chromosomal Ty elements and the 5′ end of the cDNA. Finally, in order to be transcribed to mRNA, the chimerical gene was likely using the promoter located in the δ-LTR region.

6.4.6 Domain Shuffling: New Chimerical Genes Generated Unequal Crossing Over

The ectopic recombination either between similar short sequences (microhomology) present in nonhomologous genes or between divergent paralogous genes could generate new chimerical genes with a different function. An ectopic recombinational event that combines a gene with a new promoter may be a way to generate a dramatic change in the pattern of expression and, thus, may be important in adaptive evolution.

Experimental evolution with yeasts has shown that natural selection can rapidly favor new gene functions generated by ectopic recombination between paralogous genes and subsequent duplications. Brown et al. (1998) analyzed a population of *S. cerevisiae* yeasts that underwent 450 generations of glucose-limited growth. Relative to the ancestral strain, the evolved strain grew at significantly lower steady-state glucose concentrations and demonstrated enhanced cell yield per mole of glucose, significantly enhanced high-affinity glucose transport, and greater relative fitness in pairwise competition. The analysis of the evolved strain revealed the existence of more

than three tandem duplications of a chimerical gene, derived from unequal crossing over, containing the upstream promoter of *HXT7* and the coding sequence of *HXT6*, two adjacent highly similar genes encoding high-affinity hexose transporters originating from a recent duplication. Selection under low glucose concentrations favored a strain containing these duplicated *HXT7*/*HXT6* chimaeras, which increase the ability of *S. cerevisiae* to scavenge glucose at low substrate concentrations.

Another example comes form the study of *S. cerevisiae* yeasts present in spontaneous wine fermentations. Pérez-Ortín et al. (2002) found in several wine strains a new allele of *SSU1* (SSU1-R), a gene that mediates sulfite efflux and, hence, confers sulfite resistance. This new allele was the product of a reciprocal translocation between chromosomes VIII and XVI owing to unequal crossing over mediated by microhomology between very short sequences on the 5′ upstream regions of the *SSU1* and *ECM34* genes. This ectopic recombination put the coding sequence of *SSU1* under the control of the promoter upstream region of *ECM34*, which resulted in a significant increase of *SSU1* expression. They also showed that this chimerical gene (and the translocation) is only present in wine yeast strains, suggesting that the use for millennia of sulfite as a preservative in wine production could have favored its selection.

6.4.7 Domain Duplication: Gene Elongation Generated by Tandem Duplications

Internal duplications have occurred frequently in eukaryote evolution. This increase in gene size, or gene elongation, is an important mechanism to generate complex genes from simple ones (Li 1997).

In the case of yeasts, the most important source of gene elongation is the presence of codon repeats, i.e., trinucleotide microsatellite expansions in coding regions. The most abundant codon repeats found in yeasts are those coding for the amino acids glutamine, asparagine, aspartic acid, glutamic acid, and serine (Albà et al. 1999; Malpertuy et al. 2003).

In most cases, codon repeats show a significant bias toward long tracts of one of the possible codons, suggesting that "trinucleotide replication slippage" is the most important mechanism generating these reiterations (Albà et al. 1999). Replication slippage occurs when a template strand containing contiguous short repeats, in this case trinucleotide repeats, and its copy shift their relative positions during replication owing to mispairing between neighboring repeats, so that part of the template is either copied twice or missed out (Hancock 1999).

However, these different codon repeats are concentrated in different classes of proteins. Thus, acidic and polar amino acid repeats, particularly glutamine, are significantly associated with transcription factors and protein kinases (Richard and Dujon 1997). Changes in the length of repeats in such cellular components of the cell signaling system could alter their biochemical properties, and, hence, modify their interactions with DNA, with other DNA binding proteins, or with other transcription factors and contribute to their evolutionary diversification (Albà et al. 1999; Malpertuy et al. 2003). This modified protein can then be selected for its new function, allowing the cell to increase diversity among its transcription factors,

to specialize them, to adapt to a new environment, and eventually to speciate (Malpertuy et al. 2003). Such diversification could be relatively rapid on an evolutionary time scale because of the high mutation rates of microsatellites (Hancock 1999), which is congruent with the overrepresentation among these transcription factors containing trinucleotide repeats of hemiascomycete-specific genes, which were shown to diverge more rapidly during evolution (Malpertuy et al. 2000).

6.5 Gross Chromosomal Rearrangements in Yeast Evolution

It has largely been proposed that speciation frequently occurs when a population becomes fixed for one or more chromosomal rearrangements that reduce fitness when they are heterozygous. This way, chromosomal rearrangements induce the formation of multivalents during meiosis, resulting in a loss of gamete viability (50% reduction for each translocation).

In the case of *Saccharomyces sensu stricto* species, chromosomal rearrangements have been suggested to account for their postzygotic reproductive isolation (Ryu et al. 1998). However, Fischer et al. (2000) characterized the translocation differences in the species of the *sensu stricto* complex, and concluded that these rearrangements are not required for speciation, since translocations are present only in three species and are not shared between species, indicating that occurred after species divergence.

Delneri et al. (2003) used a reverse approach to determine the role of translocations in speciation. They engineered the genome of a *S. cerevisiae* strain to make it collinear with that of two different *S. mikatae* strains differing in one and two translocations, respectively, with respect to *S. cerevisiae*. Interspecific crosses between strains with collinear genomes resulted in hybrids showing an increase in spore viability (up to 30%). These results indicate that although chromosome rearrangements are not a prerequisite for yeast speciation, they may likely contribute to the reduction of gene flow by suppressing recombination.

The comparative analysis of genomes (Kellis et al. 2003) showed that paralogous genes, transposons, and transfer RNAs (tRNAs) are located at the rearrangement breakpoints, which indicates that ectopic recombination may have been involved in the origin of these chromosomal rearrangements. Indeed, Ty elements or δ-LTRs are well known to induce chromosomal deletion, duplication, translocation, and inversion events by allelic or ectopic recombination in yeasts (Kupiec and Petes 1988; Rachidi et al. 1999). Ectopic recombination, between similar sequences present in nonhomologous genes, between divergent paralogous genes, or between transposable elements could generate evolutionary novelties such as new chimerical genes with a different function of adaptive value (discussed in Sect. 6.4.6) or changes in gene regulation caused by transposable elements on nearby genes.

The fact that selected industrial yeast strains display differences in fitness and in phenotypic traits of industrial relevance that are associated with chromosomal variation (Codón and Benítez 1995) suggests that gross chromosomal rearrangements may be involved in the adaptive evolution of yeasts and account for the high

capacity of industrial yeasts to rapidly evolve. There are several studies whose conclusions support the role of chromosomal rearrangements in the adaptive evolution of yeasts.

Dunham et al. (2002) analyzed the karyotypic changes in six yeast strains, evolved after 100–500 generations of growth in glucose-limited chemostats. These strains contained different chromosomal rearrangements mediated by Ty and tRNA recombinations. Moreover, evolved strains from three independent cultures shared a similar translocation in a chromosome XIV region immediately adjacent to *CIT1*, which encodes the citrate synthase involved in the regulation of tricarboxylic acid cycle. The fact that the same genomic rearrangements recur in different strains suggests that they may be adaptive and responsible for the increased fitness of these strains. Dunham et al. (2002) also postulated that some of the approximately 300 transposon-related sequences found in the *S. cerevisiae* genome are in positions that may provide a selective advantage by allowing adaptively useful chromosomal rearrangements.

Colson et al. (2004) used *S. cerevisiae* strains with artificial translocations, introduced to make their genomes collinear with those of *S. mikatae* strains (see earlier; Delneri et al. 2003), in competition experiments under different physiological conditions. Their experiments showed that the translocated strains of *S. cerevisiae* consistently outcompeted the reference strain with no translocation, both in batch and chemostat culture, but especially under glucose limitation. These results also suggest that chromosomal translocations in yeasts may have an adaptive significance.

Another example comes from the analysis of natural strains. Pérez-Ortín et al. (2002; Sect. 6.4.6) demonstrated that the translocation between *S. cerevisiae* chromosomes VII and XVI, found very frequently in wine strains, was generated by ectopic recombination between genes *ECM34* and *SSU1*, resulting in a chimerical gene that confers a higher resistance to sulfite, a preservative used during winemaking.

Finally, Infante et al. (2003) used the method of comparative genome hybridization with DNA chips, to analyze the genomes of two variants of *S. cerevisiae* flor yeasts, which are adapted to grow aerobically on the surface of sherry wines by transforming ethanol into acetaldehyde. This analysis showed that both strains differ in 116 rearranged regions that comprise 38% of their genomes. These authors concluded that the presence of genes that confer specific characteristics to the flor yeast within these regions supports the role of chromosomal rearrangements as a major mechanism of adaptive evolution in *S. cerevisiae*.

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