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Cytology, Taxonomy and Ecology of Grape and Wine Yeasts

1.1 INTRODUCTION

Man has been making bread and fermented beverages since the beginning of recorded history. Yet the role of yeasts in alcoholic fermentation, particularly in the transformation of grapes into wine, was only clearly established in the middle of the nineteenth century. The ancients explained the boiling during fermentation (from the Latin *fervere*, to boil) as a reaction between substances that come into contact with each other during crushing. In 1680, a Dutch cloth merchant, Antonie van Leeuwenhoek, first observed yeasts in beer wort using a microscope that he designed and produced. He did not, however, establish a relationship between these corpuscles and alcoholic fermentation. It was not until the end of the eighteenth century that Lavoisier began the chemical study of alcoholic fermentation. Gay-Lussac continued Lavoisier’s research into the next century.
As early as 1785, Fabroni, an Italian scientist, was the first to provide an interpretation of the chemical composition of the ferment responsible for alcoholic fermentation, which he described as a plant–animal substance. According to Fabroni, this material, comparable to the gluten in flour, was located in special utricles, particularly on grapes and wheat, and alcoholic fermentation occurred when it came into contact with sugar in the must. In 1837, a French physicist named Charles Cagnard de La Tour proved for the first time that the yeast was a living organism. According to his findings, it was capable of multiplying and belonged to the plant kingdom; its vital activities were at the base of the fermentation of sugar-containing liquids. The German naturalist Schwann confirmed his theory and demonstrated that heat and certain chemical products were capable of stopping alcoholic fermentation. He named the beer yeast zucker-pilz, which means sugar fungus—Saccharomyces in Latin. In 1838, Meyen used this nomenclature for the first time.

This vitalist or biological viewpoint of the role of yeasts in alcoholic fermentation, obvious to us today, was not readily supported. Liebig and certain other organic chemists were convinced that chemical reactions, not living cellular activity, were responsible for the fermentation of sugar. In his famous studies on wine (1866) and beer (1876), Louis Pasteur gave definitive credibility to the vitalist viewpoint of alcoholic fermentation. He demonstrated that the yeasts responsible for spontaneous fermentation of grape must or crushed grapes came from the surface of the grape; he isolated several races and species. He even conceived the notion that the nature of the yeast carrying out the alcoholic fermentation could influence the gustatory characteristics of wine. He also demonstrated the effect of oxygen on the assimilation of sugar by yeasts. Louis Pasteur proved that the yeast produced secondary products such as glycerol in addition to alcohol and carbon dioxide.

Since Pasteur, yeasts and alcoholic fermentation have incited a considerable amount of research, making use of progress in microbiology, biochemistry and now genetics and molecular biology.

In taxonomy, scientists define yeasts as unicellular fungi that reproduce by budding and binary fission. Certain pluricellular fungi have a unicellular stage and are also grouped with yeasts. Yeasts form a complex and heterogeneous group found in three classes of fungi, characterized by their reproduction mode: the sac fungi (Ascomycetes), the club fungi (Basidiomycetes), and the imperfect fungi (Deuteromycetes). The yeasts found on the surface of the grape and in wine belong to Ascomycetes and Deuteromycetes. The haploid spores or ascospores of the Ascomycetes class are contained in the ascus, a type of sac made from vegetative cells. Asporiferous yeasts, incapable of sexual reproduction, are classified with the imperfect fungi.

In this first chapter, the morphology, reproduction, taxonomy and ecology of grape and wine yeasts will be discussed. Cytology is the morphological and functional study of the structural components of the cell (Rose and Harrison, 1991).
Yeast are the most simple of the eucaryotes. The yeast cell contains cellular envelopes, a cytoplasm with various organelles, and a nucleus surrounded by a membrane and enclosing the chromosomes. (Figure 1.1). Like all plant cells, the yeast cell has two cellular envelopes: the cell wall and the membrane. The periplasmic space is the space between the cell wall and the membrane. The cytoplasm and the membrane make up the protoplasm. The term protoplast or sphaeroplast designates a cell whose cell wall has been artificially removed. Yeast cellular envelopes play an essential role: they contribute to a successful alcoholic fermentation and release certain constituents which add to the resulting wine’s composition. In order to take advantage of these properties, the winemaker or enologist must have a profound knowledge of these organelles.

1.2 THE CELL WALL

1.2.1 The General Role of the Cell Wall

During the last 20 years, researchers (Fleet, 1991; Klis, 1994; Stratford, 1999; Klis et al., 2002) have greatly expanded our knowledge of the yeast cell wall, which represents 15–25% of the dry weight of the cell. It essentially consists of polysaccharides. It is a rigid envelope, yet endowed with a certain elasticity.

Its first function is to protect the cell. Without its wall, the cell would burst under the internal osmotic pressure, determined by the composition of the cell’s environment. Protoplasts placed in pure water are immediately lysed in this manner. Cell wall elasticity can be demonstrated by placing yeasts, taken during their log phase, in a hypertonic (NaCl) solution. Their cellular volume decreases by approximately 50%. The cell wall appears thicker and is almost in contact with the membrane. The cells regain their initial form after being placed back into an isotonic medium.

Yet the cell wall cannot be considered an inert, semi-rigid ‘armor’. On the contrary, it is a dynamic and multifunctional organelle. Its composition and functions evolve during the life of the cell, in response to environmental factors. In addition to its protective role, the cell wall gives the cell its particular shape through its macromolecular organization. It is also the site of molecules which determine certain cellular interactions such as sexual union, flocculation, and the killer factor, which will be examined in detail later in this chapter (Section 1.7). Finally, a number of enzymes, generally hydrolases, are connected to the cell wall or situated in the periplasmic space. Their substrates are nutritive substances of the environment and the macromolecules of the cell wall itself, which is constantly reshaped during cellular morphogenesis.

1.2.2 The Chemical Structure and Function of the Parietal Constituents

The yeast cell wall is made up of two principal constituents: β-glucans and mannoproteins. Chitin represents a minute part of its composition. The most detailed work on the yeast cell wall has been carried out on Saccharomyces cerevisiae—the principal yeast responsible for the alcoholic fermentation of grape must.

Glucan represents about 60% of the dry weight of the cell wall of S. cerevisiae. It can be chemically fractionated into three categories:

1. Fibrous β-1,3 glucan is insoluble in water, acetic acid and alkali. It has very few branches. The branch points involved are β-1,6 linkages. Its degree of polymerization is 1500. Under the electron microscope, this glucan appears fibrous. It ensures the shape and the rigidity of the cell wall. It is always connected to chitin.

2. Amorphous β-1,3 glucan, with about 1500 glucose units, is insoluble in water but soluble in alkalis. It has very few branches, like the preceding glucan. In addition to these few branches, it is made up of a small number of β-1,6 glycosidic linkages. It has an amorphous aspect under the electron microscope. It gives the cell wall its elasticity and acts as an anchor for the mannoproteins. It can also constitute an extraprotoplasmic reserve substance.
3. The β-1,6 glucan is obtained from alkali-insoluble glucans by extraction in acetic acid. The resulting product is amorphous, water soluble, and extensively ramified by β-1,3 glycosidic linkages. Its degree of polymerization is 140. It links the different constituents of the cell wall together. It is also a receptor site for the killer factor.

The fibrous β-1,3 glucan (alkali-insoluble) probably results from the incorporation of chitin on the amorphous β-1,3 glucan.

Mannoproteins constitute 25–50% of the cell wall of S. cerevisiae. They can be extracted from the whole cell or from the isolated cell wall by chemical and enzymatic methods. Chemical methods make use of autoclaving in the presence of alkali or a citrate buffer solution at pH 7. The enzymatic method frees the mannoproteins by digesting the glucan. This method does not denature the structure of the mannoproteins as much as chemical methods. Zymolyase, obtained from the bacterium Arthrobacter luteus, is the enzymatic preparation most often used to extract the parietal mannoproteins of S. cerevisiae. This enzymatic complex is effective primarily because of its β-1,3 glucanase activity. The action of protease contaminants in the zymolyase combine, with the aforementioned activity to liberate the mannoproteins. Glucanex, another industrial preparation of the β-glucanase, produced by a fungus (Trichoderma harzianum), has been recently demonstrated to possess endo- and exo-β-1,3 and endo-β-1,6-glucanase activities (Dubourdieu and Moine, 1995). These activities also facilitate the extraction of the cell wall mannoproteins of the S. cerevisiae cell.

The mannoproteins of S. cerevisiae have a molecular weight between 20 and 450 kDa. Their degree of glycosylation varies. Certain ones containing about 90% mannose and 10% peptides are hypermannosylated.

Four forms of glycosylation are described (Figure 1.2) but do not necessarily exist at the same time in all of the mannoproteins. They are linked to the peptide chain by O-glycosyl linkages on serine and threonine residues. These glycosidic side-chain linkages are α-1,2 and α-1,3.

The glucidic part of the mannoprotein can also be a polysaccharide. It is linked to an asparagine residue of the peptide chain by an N-glycosyl linkage. This linkage consists of a double unit of N-acetylglucosamine (chitin) linked in β-1,4. The mannan linked in this manner to the asparagine includes an attachment region made up of a dozen mannose residues and a highly ramified outer chain consisting of 150 to 250 mannose units. The attachment region beyond the chitin residue consists of a mannose skeleton linked in α-1,6 with side branches possessing one, two or three mannose residues with α-1,2 and/or α-1,3 bonds. The outer chain is also made up of a skeleton of mannose units linked in α-1,6. This chain bears short side-chains constituted of mannose residues linked in α-1,2 and a terminal mannose in α-1,3. Some of these side-chains possess a branch attached by a phosphodiester bond.

A third type of glycosylation was described more recently. It can occur in mannoproteins, which make up the cell wall of the yeast. It consists of a glucomannan chain containing essentially mannose residues linked in α-1,6 and glucose residues linked in α-1,6. The nature of the glycanc–peptide point of attachment is not yet clear, but it may be an asparaginyl–glucose bond. This type of glycosylation characterizes the proteins freed from the cell wall by the action of a β-1,3 glucanase. Therefore, in vivo, the glucomannan chain may also comprise glucose residues linked in β-1,3.

The fourth type of glycosylation of yeast mannoproteins is the glycosyl–phosphatidyl–inositol anchor (GPI). This attachment between the terminal carboxylic group of the peptide chain and a membrane phospholipid permits certain mannoproteins, which cross the cell wall, to anchor themselves in the plasmic membrane. The region of attachment is characterized by the following sequence (Figure 1.2): ethanolamine-phosphate-6-mannose-α-1,2-mannose-α-1,6-mannose-α-1,4-glucosamine-α-1,6-inositol-phospholipid. A C-phospholipase specific to phosphatidyl inositol and therefore capable of realizing this cleavage
was demonstrated in the *S. cerevisiae* (Flick and Thorner, 1993). Several GPI-type anchor mannoproteins have been identified in the cell wall of *S. cerevisiae*.

**Chitin** is a linear polymer of *N*-acetylglucosamine linked in β-1,4 and is not generally found in large quantities in yeast cell walls. In *S. cerevisiae*, chitin constitutes 1–2% of the cell wall and is found for the most part (but not exclusively) in bud scar zones. These zones are a type of raised crater easily seen on the mother cell under the electron microscope (Figure 1.3). This chitinic scar is formed essentially to assure cell wall integrity and cell survival. Yeasts treated with *D* polyoxine, an antibiotic inhibiting the synthesis of chitin, are not viable; they burst after budding.

The presence of lipids in the cell wall has not been clearly demonstrated. It is true that cell walls

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**Fig. 1.2.** The four types of glucosylation of parietal yeast mannoproteins (Klis, 1994). M = mannose; G = glucose; GN = glucosamine; GNAc = *N*-acetylglucosamine; Ins = inositol; Ser = Serine; Thr = threonine; Asn = asparagine; Xxx = the nature of the bond is not known.

**Fig. 1.3.** Scanning electron microscope photograph of proliferating *S. cerevisiae* cells. The budding scars on the mother cells can be observed.
prepared in the laboratory contain some lipids (2–15% for \textit{S. cerevisiae}) but it is most likely contamination by the lipids of the cytoplasmic membrane, adsorbed by the cell wall during their isolation. The cell wall can also adsorb lipids from its external environment, especially the different fatty acids that activate and inhibit the fermentation (Chapter 3).

**Chitin** are connected to the cell wall or situated in the periplasmic space. One of the most characteristic enzymes is the invertase (\(\beta\)-fructofuranosidase). This enzyme catalyzes the hydrolysis of saccharose into glucose and fructose. It is a thermostable mannoprotein anchored to a \(\beta\)-1,6 glucan of the cell wall. Its molecular weight is 270,000 Da. It contains approximately 50% mannose and 50% protein. The periplasmic acid phosphatase is equally a mannoprotein.

Other periplasmic enzymes that have been noted are \(\beta\)-glucosidase, \(\alpha\)-galactosidase, melibiase, trehalase, aminopeptidase and esterase. Yeast cell walls also contain endo- and exo-\(\beta\)-glucanases (\(\beta\)-1,3 and \(\beta\)-1,6). These enzymes are involved in the reshaping of the cell wall during the growth and budding of cells. Their activity is at a maximum during the exponential log phase of the population and diminishes notably afterwards. Yet cells in the stationary phase and even dead yeasts contained in the lees still retain \(\beta\)-glucanases activity in their cell walls several months after the completion of fermentation. These endogenous enzymes are involved in the autolysis of the cell wall during the ageing of wines on lees. This ageing method will be covered in the chapter on white winemaking (Chapter 13).

### 1.2.3 General Organization of the Cell Wall and Factors Affecting its Composition

The cell wall of \textit{S. cerevisiae} is made up of an outer layer of mannoproteins. These mannoproteins are connected to a matrix of amorphous \(\beta\)-1,3 glucan which covers an inner layer of fibrous \(\beta\)-1,3 glucan. The inner layer is connected to a small quantity of chitin (Figure 1.4). The \(\beta\)-1,6 glucan probably acts as a cement between the two layers. The rigidity and the shape of the cell wall are due to the internal framework of the \(\beta\)-1,3 fibrous glucan. Its elasticity is due to the outer amorphous layer. The intermolecular structure of the mannoproteins of the outer layer (hydrophobic linkages and disulfur bonds) equally determines cell wall porosity and impermeability to macromolecules (molecular weights less than 4500). This impermeability can be affected by treating the cell wall with certain chemical agents, such as \(\beta\)-mercaptoethanol. This substance provokes the rupture of the disulfur bonds, thus destroying the intermolecular network between the mannoprotein chains.

The composition of the cell wall is strongly influenced by nutritive conditions and cell age. The proportion of glucan in the cell wall increases

![Fig. 1.4. Cellular organization of the cell wall of S. cerevisiae](image-url)
with respect to the amount of sugar in the culture medium. Certain deficiencies (for example, in mesoinositol) also result in an increase in the proportion of glucan compared with mannanproteins. The cell walls of older cells are richer in glucans and in chitin and less furnished in mannanproteins. For this reason, they are more resistant to physical and enzymatic agents used to degrade them. Finally, the composition of the cell wall is profoundly modified by morphogenetic alterations (conjugation and sporulation).

1.3 THE PLASMIC MEMBRANE

1.3.1 Chemical Composition and Organization

The plasmic membrane is a highly selective barrier controlling exchanges between the living cell and its external environment. This organelle is essential to the life of the yeast.

Like all biological membranes, the yeast plasmic membrane is principally made up of lipids and proteins. The plasmic membrane of \textit{S. cerevisiae} contains about 40\% lipids and 50\% proteins. Glucans and mannans are only present in small quantities (several per cent).

The lipids of the membrane are essentially phospholipids and sterols. They are amphiphilic molecules, i.e. possessing a hydrophilic and a hydrophobic part.

The three principal phospholipids (Figure 1.5) of the plasmic membrane of yeast are phosphatidylethanolamine (PE), phosphatidylcholine (PC) and phosphatidylinositol (PI) which represent 70–85\% of the total. Phosphatidylerserine (PS) and diphosphatidylglycerol or cardiolipin (PG) are less prevalent. Free fatty acids and phosphatidic acid are frequently reported in plasmic membrane analysis. They are probably extraction artifacts caused by the activity of certain lipid degradation enzymes.

The fatty acids of the membrane phospholipids contain an even number (14 to 24) of carbon atoms. The most abundant are C\textsubscript{16} and C\textsubscript{18} acids. They can be saturated, such as palmitic acid (C\textsubscript{16}) and stearic acid (C\textsubscript{18}), or unsaturated, as with oleic acid (C\textsubscript{18}, double bond in position 9), linoleic acid (C\textsubscript{18}, two double bonds in positions 9 and 12) and linolenic acid (C\textsubscript{18}, three double bonds in positions 9, 12 and 15). All membrane phospholipids share a common characteristic: they possess a polar or hydrophilic part made up of a phosphorylated alcohol and a non-polar or hydrophobic part comprising two more or less parallel fatty acid chains (Figure 1.6). In an aqueous medium, the phospholipids spontaneously form bimolecular films or a lipid bilayer because of their amphiphilic characteristic (Figure 1.6). The lipid bilayers are cooperative but non-covalent structures. They are maintained in place by mutually reinforced interactions: hydrophobic interactions, van der Waals attractive forces between the hydrocarbon tails, hydrostatic interactions and hydrogen bonds between the polar heads and water molecules.

The examination of cross-sections of yeast plasmic membrane under the electron microscope reveals a classic lipid bilayer structure with a thickness of about 7.5 nm. The membrane surface appears sculped with creases, especially during the stationary phase. However, the physiological meaning of this anatomic character remains unknown. The plasmic membrane also has an underlying depression on the bud scar.

Ergosterol is the primary sterol of the yeast plasmic membrane. In lesser quantities, 24 (28) dehydroergosterol and zymosterol also exist (Figure 1.7). Sterols are exclusively produced in the mitochondria during the yeast log phase. As with phospholipids, membrane sterols are amphipathic. The hydrophilic part is made up of hydroxyl groups in C-3. The rest of the molecule is hydrophobic, especially the flexible hydrocarbon tail.

The plasmic membrane also contains numerous proteins or glycoproteins presenting a wide range of molecular weights (from 10,000 to 120,000). The available information indicates that the organization of the plasmic membrane of a yeast cell resembles the fluid mosaic model. This model, proposed for biological membranes by Singer and Nicolson (1972), consists of two-dimensional solutions of proteins and oriented lipids. Certain proteins are embedded in the membrane; they are called integral proteins (Figure 1.6). They interact...
strongly with the non-polar part of the lipid bilayer. The peripheral proteins are linked to the precedent by hydrogen bonds. Their location is asymmetrical, at either the inner or the outer side of the plasmic membrane. The molecules of proteins and membrane lipids, constantly in lateral movement, are capable of rapidly diffusing in the membrane.

Some of the yeast membrane proteins have been studied in greater depth. These include adenosine triphosphatase (ATPase), solute (sugars and amino acids) transport proteins, and enzymes involved in the production of glucans and chitin of the cell wall.

The yeast possesses three ATPases: in the mitochondria, the vacuole, and the plasmic membrane. The plasmic membrane ATPase is an integral protein with a molecular weight of around 100 Da. It catalyzes the hydrolysis of ATP which furnishes the necessary energy for the active transport of solutes across the membrane. (Note: an active
transport moves a compound against the concentration gradient.) Simultaneously, the hydrolysis of ATP creates an efflux of protons towards the exterior of the cell.

The penetration of amino acids and sugars into the yeast activates membrane transport systems called permeases. The general amino acid permease (GAP) contains three membrane proteins and ensures the transport of a number of neutral amino acids. The cultivation of yeasts in the presence of an easily assimilated nitrogen-based nutrient such as ammonium represses this permease.

The membrane composition in fatty acids and its proportion in sterols control its fluidity. The hydrocarbon chains of fatty acids of the membrane phospholipid bilayer can be in a rigid and orderly state or in a relatively disorderly and fluid state. In the rigid state, some or all of the carbon bonds of the fatty acids are trans. In the fluid state, some of the bonds become cis. The transition from the rigid state to the fluid state takes place when the temperature rises beyond the fusion temperature. This transition temperature depends on the length of the fatty acid chains and their degree of unsaturation. The rectilinear hydrocarbon chains of the saturated fatty acids interact strongly. These interactions intensify with their length. The transition temperature therefore increases as the fatty acid chains become longer. The double bonds of the unsaturated fatty acids are generally cis, giving a curvature to the hydrocarbon chain (Figure 1.8). This curvature breaks the orderly
stearic acid (C_{18}, saturated)

oleic acid (C_{18}, unsaturated)

Fig. 1.8. Molecular models representing the three-dimensional structure of stearic and oleic acid. The cis configuration of the double bond of oleic acid produces a curvature of the carbon chain stacking of the fatty acid chains and lowers the transition temperature. Like cholesterol in the cells of mammals, ergosterol is also a fundamental regulator of the membrane fluidity in yeasts. Ergosterol is inserted in the bilayer perpendicularly to the membrane. Its hydroxyl group joins, by hydrogen bonds, with the polar head of the phospholipid and its hydrocarbon tail is inserted in the hydrophobic region of the bilayer. The membrane sterols intercalate themselves between the phospholipids. In this manner, they inhibit the crystallization of the fatty acid chains at low temperatures. Inversely, in reducing the movement of these same chains by steric encumberment, they regulate an excess of membrane fluidity when the temperature rises.

1.3.2 Functions of the Plasmic Membrane

The plasmic membrane constitutes a stable, hydrophobic barrier between the cytoplasm and the environment outside the cell, owing to its phospholipids and sterols. This barrier presents a certain impermeability to solutes in function of osmotic properties.

Furthermore, through its system of permeases, the plasmic membrane also controls the exchanges between the cell and the medium. The functioning of these transport proteins is greatly influenced by its lipid composition, which affects membrane fluidity. In a defined environmental model, the supplementing of membrane phospholipids with unsaturated fatty acids (oleic and linoleic) promoted the penetration and accumulation of certain amino acids as well as the expression of the general amino acid permease (GAP), (Henschke and Rose, 1991). On the other hand, membrane sterols seem to have less influence on the transport of amino acids than the degree of unsaturation of the phospholipids. The production of unsaturated fatty acids is an oxidative process and requires the aeration of the culture medium at the beginning of alcoholic fermentation. In semi-anaerobic wine-making conditions, the amount of unsaturated fatty acids in the grape, or in the grape must, probably favor the membrane transport mechanisms of fatty acids.

The transport systems of sugars across the membrane are far from being completely elucidated. There exists, however, at least two kinds of transport systems: a high affinity and a low affinity system (ten times less important) (Bisson, 1991). The low affinity system is essential during the log phase and its activity decreases during the stationary phase. The high affinity system is, on the contrary, repressed by high concentrations of glucose, as in the case of grape must (Salmon et al., 1993) (Figure 1.9). The amount of sterols in the membrane, especially ergosterol, as well as the degree of unsaturation of the membrane phospholipids favor the penetration of glucose in the cell. This is especially true during the stationary and decline phases. This phenomenon explains the determining influence of aeration on the successful completion of alcoholic fermentation during the yeast multiplication phase.

The presence of ethanol, in a culture medium, slows the penetration speed of arginine and glucose into the cell and limits the efflux of protons
resulting from membrane ATPase activity (Alexandre et al., 1994; Charpentier, 1995). Simultaneously, the presence of ethanol increases the synthesis of membrane phospholipids and their percentage in unsaturated fatty acids (especially oleic). Temperature and ethanol act in synergy to affect membrane ATPase activity. The amount of ethanol required to slow the proton efflux decreases as the temperature rises. However, this modification of membrane ATPase activity by ethanol may not be the origin of the decrease in plasmic membrane permeability in an alcoholic medium. The role of membrane ATPase in yeast resistance to ethanol has not been clearly demonstrated.

The plasmic membrane also produces cell wall glucan and chitin. Two membrane enzymes are involved: β-1,3 glucanase and chitin synthetase. These two enzymes catalyze the polymerization of glucose and N-acetyl-glucosamine, derived from their activated forms (uridine diphosphates—UDP). The mannoproteins are essentially produced in the endoplasmic reticulum (Section 1.4.2). They are then transported by vesicles which fuse with the plasmic membrane and deposit their contents at the exterior of the membrane.

Finally, certain membrane proteins act as cellular specific receptors. They permit the yeast to react to various external stimuli such as sexual hormones or changes in the concentration of external nutrients. The activation of these membrane proteins triggers the liberation of compounds such as cyclic adenosine monophosphate (cAMP) in the cytoplasm. These compounds serve as secondary messengers which set off other intercellular reactions. The consequences of these cellular mechanisms in the alcoholic fermentation process merit further study.

1.4 THE CYTOPLASM AND ITS ORGANELLES

Between the plasmic membrane and the nuclear membrane, the cytoplasm contains a basic cytoplasmic substance, or cytosol. The organelles (endoplasmic reticulum, Golgi apparatus, vacuole and mitochondria) are isolated from the cytosol by membranes.

1.4.1 Cytosol

The cytosol is a buffered solution, with a pH between 5 and 6, containing soluble enzymes, glycogen and ribosomes.

Glycolysis and alcoholic fermentation enzymes (Chapter 2) as well as trehalase (an enzyme catalyzing the hydrolysis of trehalose) are present. Trehalose, a reserve disaccharide, also cytoplasmic, ensures yeast viability during the dehydration and rehydration phases by maintaining membrane integrity.

The lag phase precedes the log phase in a sugar-containing medium. It is marked by a rapid degradation of trehalose linked to an increase in trehalase activity. This activity is itself closely related to an increase in the amount of cAMP in the cytoplasm. This compound is produced by a membrane enzyme, adenylate cyclase, in response
to the stimulation of a membrane receptor by an environmental factor.

Glycogen is the principal yeast glucidic reserve substance. Animal glycogen is similar in structure. It accumulates during the stationary phase in the form of spherical granules of about 40 µm in diameter.

When observed under the electron microscope, the yeast cytoplasm appears rich in ribosomes. These tiny granulations, made up of ribonucleic acids and proteins, are the center of protein synthesis. Joined to polysomes, several ribosomes migrate the length of the messenger RNA. They translate it simultaneously so that each one produces a complete polypeptide chain.

1.4.2 The Endoplasmic Reticulum, the Golgi Apparatus and the Vacuoles

The endoplasmic reticulum (ER) is a double membrane system partitioning the cytoplasm. It is linked to the cytoplasmic membrane and nuclear membrane. It is, in a way, an extension of the latter. Although less developed in yeasts than in exocrine cells of higher eucaryotes, the ER has the same function. It ensures the addressing of the proteins synthesized by the attached ribosomes. As a matter of fact, ribosomes can be either free in the cytosol or bound to the ER. The proteins synthesized by free ribosomes remain in the cytosol, as do the enzymes involved in glycolysis. Those produced in the ribosomes bound to the ER have three possible destinations: the vacuole, the plasmic membrane, and the external environment (secretion). The presence of a signal sequence (a particular chain of amino acids) at the N-terminal extremity of the newly formed protein determines the association of the initially free ribosomes in the cytosol with the ER. The synthesized protein crosses the ER membrane by an active transport process called translocation. This process requires the hydrolysis of an ATP molecule. Having reached the inner space of the ER, the proteins undergo certain modifications including the necessary excising of the signal peptide by the signal peptidase. In many cases, they also undergo a glycosylation.

The yeast glycoproteins, in particular the structural, parietal or enzymatic mannoproteins, contain glucidic side chains (Section 1.2.2). Some of these are linked to asparagine by N-glycosidic bonds. This oligosaccharidic link is constructed in the interior of the ER by the sequential addition of activated sugars (in the form of UDP derivatives) to a hydrophobic, lipidic transporter called dolicholphosphate. The entire unit is transferred in one piece to an asparagine residue of the polypeptide chain. The dolicholphosphate is regenerated.

The Golgi apparatus consists of a stack of membrane sacs and associated vesicles. It is an extension of the ER. Transfer vesicles transport the proteins issued from the ER to the sacs of the Golgi apparatus. The Golgi apparatus has a dual function. It is responsible for the glycosylation of protein, then sorts so as to direct them via specialized vesicles either into the vacuole or into the plasmic membrane. An N-terminal peptidic sequence determines the directing of proteins towards the vacuole. This sequence is present in the precursors of two vacuolar-orientated enzymes in the yeast: Y carboxypeptidase and A proteinase. The vesicles that transport the proteins of the plasmic membrane or the secretion granules, such as those that transport the periplasmic invertase, are still the default destinations.

The vacuole is a spherical organelle, 0.3 to 3 µm in diameter, surrounded by a single membrane. Depending on the stage of the cellular cycle, yeasts have one or several vacuoles. Before budding, a large vacuole splits into small vesicles. Some penetrate into the bud. Others gather at the opposite extremity of the cell and fuse to form one or two large vacuoles. The vacuolar membrane or tonoplast has the same general structure (fluid mosaic) as the plasmic membrane but it is more elastic and its chemical composition is somewhat different. It is less rich in sterols and contains less protein and glycoprotein but more phospholipids with a higher degree of unsaturation. The vacuole stocks some of the cell hydrolases, in particular Y carboxypeptidase, A and B proteases, I aminopeptidase, X-propyl-dipeptidylaminopeptidase and alkaline phosphatase. In this respect, the yeast vacuole can
be compared to an animal cell lysosome. Vacuolar proteases play an essential role in the turn-over of cellular proteins. In addition, the A protease is indispensable in the maturation of other vacuolar hydrolases. It excises a small peptide sequence and thus converts precursor forms (proenzymes) into active enzymes. The vacuolar proteases also autolyze the cell after its death. Autolysis, while ageing white wine on its lees, can affect wine quality and should concern the winemaker.

Vacuoles also have a second principal function: they stock metabolites before their use. In fact, they contain a quarter of the pool of the amino acids of the cell, including a lot of arginine as well as S-adenosyl methionine. In this organelle, there is also potassium, adenine, isoguanine, uric acid and polyphosphate crystals. These are involved in the fixation of basic amino acids. Specific permeases ensure the transport of these metabolites across the vacuolar membrane. An ATPase linked to the tonoplast furnishes the necessary energy for the movement of stocked compounds against the concentration gradient. It is different from the plasmic membrane ATPase, but also produces a proton efflux.

The ER, Golgi apparatus and vacuoles can be considered as different components of an internal system of membranes, called the vacuome, participating in the flux of glycoproteins to be excreted or stocked.

1.4.3 The Mitochondria

Distributed in the periphery of the cytoplasm, the mitochondria (mt) are spherically or rod-shaped organelles surrounded by two membranes. The inner membrane is highly folded to form cristae. The general organization of mitochondria is the same as in higher plants and animal cells. The membranes delimit two compartments: the inner membrane space and the matrix. The mitochondria are true respiratory organelles for yeasts. In aerobiosis, the \textit{S. cerevisiae} cell contains about 50 mitochondria. In anaerobiosis, these organelles degenerate, their inner surface decreases, and the cristae disappear. Ergosterol and unsaturated fatty acids supplemented in culture media limit the degeneration of mitochondria in anaerobiosis. In any case, when cells formed in anaerobiosis are placed in aerobiosis, the mitochondria regain their normal appearance. Even in aerated grape must, the high sugar concentration represses the synthesis of respiratory enzymes. As a result, the mitochondria no longer function. This phenomenon, catabolic glucose repression, will be described in Chapter 2.

The mitochondrial membranes are rich in phospholipids—principally phosphatidylcholine, phosphatidylinositol and phosphatidylethanolamine (Figure 1.5). Cardiolipin (diphosphatidylglycerol), in minority in the plasmic membrane (Figure 1.4), is predominant in the inner mitochondrial membrane. The fatty acids of the mitochondrial phospholipids are in C16:0, C16:1, C18:0, C18:1. In aerobiosis, the unsaturated residues predominate. When the cells are grown in anaerobiosis, without lipid supplements, the short-chain saturated residues become predominant; cardiolipin and phosphatidylethanolamine diminish whereas the proportion of phosphatidylinositol increases. In aerobiosis, the temperature during the log phase of the cell influences the degree of unsaturation of the phospholipids- more saturated as the temperature decreases.

The mitochondrial membranes also contain sterols, as well as numerous proteins and enzymes (Guerin, 1991). The two membranes, inner and outer, contain enzymes involved in the synthesis of phospholipids and sterols. The ability to synthesize significant amounts of lipids, characteristic of yeast mitochondria, is not limited by respiratory deficient mutations or catabolic glucose repression.

The outer membrane is permeable to most small metabolites coming from the cytosol since it contains porine, a 29 kDa transmembrane protein possessing a large pore. Porine is present in the mitochondria of all the eucaryotes as well as in the outer membrane of bacteria. The intermembrane space contains adenylate kinase, which ensures interconversion of ATP, ADP and AMP. Oxidative phosphorylation takes place in the inner mitochondrial membrane. The matrix, on the other hand, is the center of the reactions of the tricarboxylic acids cycle and of the oxidation of fatty acids.
The majority of mitochondria proteins are coded by the genes of the nucleus and are synthesized by the free polysomes of the cytoplasm. The mitochondria, however, also have their own machinery for protein synthesis. In fact, each mitochondrion possesses a circular 75 kb (kilobase pairs) molecule of double-stranded AND and ribosomes. The mtDNA is extremely rich in A (adenine) and T (thymine) bases. It contains a few dozen genes, which code in particular for the synthesis of certain pigments and respiratory enzymes, such as cytochrome b, and several sub-units of cytochrome oxidase and of the ATP synthetase complex. Some mutations affecting these genes can result in the yeast becoming resistant to certain mitochondrial specific inhibitors such as oligomycin. This property has been applied in the genetic marking of wine yeast strains. Some mitochondrial mutants are respiratory deficient and form small colonies on solid agar media. These ‘petit’ mutants are not used in winemaking because it is impossible to produce them industrially by respiration.

1.5 THE NUCLEUS

The yeast nucleus is spherical. It has a diameter of 1–2 mm and is barely visible using a phase contrast optical microscope. It is located near the principal vacuole in non-proliferating cells. The nuclear envelope is made up of a double membrane attached to the ER. It contains many ephemeral pores, their locations continually changing. These pores permit the exchange of small proteins between the nucleus and the cytoplasm. Contrary to what happens in higher eucaryotes, the yeast nuclear envelope is not dispersed during mitosis. In the basophilic part of the nucleus, the crescent-shaped nucleolus can be seen by using a nuclear-specific staining method. As in other eucaryotes, it is responsible for the synthesis of ribosomal RNA. During cellular division, the yeast nucleus also contains rudimentary spindle threads composed of microtubules of tubulin, some discontinuous and others continuous (Figure 1.10). The continuous microtubules are stretched between the two spindle pole bodies (SPB). These corpuscles are permanently included in the nuclear membrane and correspond with the centrioles of higher organisms. The cytoplasmic microtubules depart from the spindle pole bodies towards the cytoplasm.

There is little nuclear DNA in yeasts compared with higher eucaryotes—about 14 000 kb in a haploid strain. It has a genome almost three times larger than in Escherichia coli, but its genetic material is organized into true chromosomes. Each one contains a single molecule of linear double-stranded DNA associated with basic proteins known as histones. The histones form chromatin which contains repetitive units called nucleosomes. Yeast chromosomes are too small to be observed under the microscope.

Pulse-field electrophoresis (Carle and Olson, 1984; Schwartz and Cantor, 1984) permits the separation of the 16 chromosomes in S. cerevisiae, whose size range from 200 to 2000 kb. This species has a very large chromosomic polymorphism. This characteristic has made karyotype analysis one of the principal criteria for the identification of S. cerevisiae strains (Section 1.9.3). The scientific community has nearly established the complete sequence of the chromosomic DNA of S. cerevisiae. In the future, this detailed knowledge of the yeast genome will constitute a powerful tool, as much for understanding its molecular physiology as for selecting and improving winemaking strains.

The yeast chromosomes contain relatively few repeated sequences. Most genes are only present
in a single example in the haploid genome, but the ribosomal RNA genes are highly repeated (about 100 copies).

The genome of *S. cerevisiae* contains transposable elements, or transposons—specifically, Ty (transposon yeast) elements. These comprise a central ε region (5.6 kb) framed by a direct repeated sequence called the δ sequence (0.25 kb). The δ sequences have a tendency to recombine, resulting in the loss of the central region and a δ sequence. As a result, there are about 100 copies of the δ sequence in the yeast genome. The Ty elements code for non-infectious retrovirus particles. This retrovirus contains Ty messenger RNA as well as a reverse transcriptase capable of copying the RNA into complementary DNA. The latter can reinsert itself into any site of the chromosome. The random excision and insertion of Ty elements in the yeast genome can modify the genes and play an important role in strain evolution.

Only one plasmid, called the 2 µm plasmid, has been identified in the yeast nucleus. It is a circular molecule of DNA, containing 6 kb and there are 50–100 copies per cell. Its biological function is not known, but it is a very useful tool, used by molecular biologists to construct artificial plasmids and genetically transform yeast strains.

### 1.6 REPRODUCTION AND THE YEAST BIOLOGICAL CYCLE

Like other sporiferous yeasts belonging to the class Ascomycetes, *S. cerevisiae* can multiply either asexually by vegetative multiplication or sexually by forming ascospores. By definition, yeasts belonging to the imperfect fungi can only reproduce by vegetative multiplication.

#### 1.6.1 Vegetative Multiplication

Most yeasts undergo vegetative multiplication by a process called budding. Some yeasts, such as species belonging to the genus *Schizosaccharomyces*, multiply by binary fission.

Figure 1.11 represents the life cycle of *S. cerevisiae* divided into four phases: M, G1, S, and G2. M corresponds with mitosis, G1 is the period preceding S, which is the synthesis of DNA and G2 is the period before cell division. As soon as the bud emerges, in the beginning of S, the splitting of the spindle pole bodies (SPB) can be observed in the nuclear membrane by electron microscopy. At the same time, the cytoplasmic microtubules orient themselves toward the emerging bud. These microtubules seem to guide numerous vesicles which appear in the budding zone and are involved in the reshaping of the cell wall. As the bud grows larger, discontinued nuclear microtubules begin to appear. The longest microtubules form the mitotic spindle between the two SPB. At the end of G2, the nucleus begins to push and pull apart in order to penetrate the bud. Some of the mitochondria also pass with some small vacuoles into the bud, whereas a large vacuole is formed at the other pole of the cell. The expansion of the latter seems to push the nucleus into the bud. During mitosis, the nucleus stretches to its maximum and the mother cell separates from the daughter cell. This separation takes place only after the construction of the separation cell wall and...
the deposit of a ring of chitin on the bud scar of the mother cell. The movement of chromosomes during mitosis is difficult to observe in yeasts, but a microtubule–centromere link must guide the chromosomes. In grape must, the duration of budding is approximately 1–2 hours. As a result, the population of the cells double during the yeast log phase during fermentation.

### 1.6.2 Sexual Reproduction

When sporiferous yeast diploid cells are in a hostile nutritive medium (for example, depleted of fermentable sugar, poor in nitrogen and very aerated) they stop multiplying. Some transform into a kind of sac with a thick cell wall. These sacs are called asci. Each one contains four haploid ascospores issued from meiotic division of the nucleus. Grape must and wine are not propitious to yeast sporulation and, in principal, it never occurs in this medium. Yet Mortimer et al. (1994) observed the sporulation of certain wine yeast strains, even in sugar-rich media. Our researchers have often observed asci in old agar culture media stored for several weeks in the refrigerator or at ambient temperatures (Figure 1.12). The natural conditions in which wild wine yeasts sporulate and the frequency of this phenomenon are not known. In the laboratory, the agar or liquid medium conventionally used to provoke sporulation has a sodium acetate base (1%). In *S. cerevisiae*, sporulation aptitude varies greatly from strain to strain. Wine yeasts, both wild and selected, do not sporulate easily, and when they do they often produce non-viable spores.

Meiosis in yeasts and in higher eucaryotes (Figure 1.13) has some similarities. Several hours after the transfer of diploid vegetative cells to a sporulation medium, the SPB splits during the DNA replication of the S phase. A dense body (DB) appears simultaneously in the nucleus near the nucleolus. The DB evolves into synaptonemal complexes—structures permitting the coupling and recombination of homologous chromosomes. After 8–9 hours in the sporulation medium, the two SPB separate and the spindle begins to form. This stage is called metaphase I of meiosis. At this stage, the chromosomes are not yet visible. Then, while the nuclear membrane remains intact, the SPB divides. At metaphase II, a second mitotic spindle stretches itself while the ascospore cell wall begins to form. Nuclear buds, cytoplasm and organelles migrate into the ascospores. At this point, edification of the cell wall is completed. The spindle then disappears when the division is achieved.

Placed in favorable conditions, i.e. nutritive sugar-enriched media, the ascospores germinate, breaking the cell wall of the ascus, and begin to multiply. In *S. cerevisiae*, the haploid cells have two mating types: a and α. The ascus contains two a ascospores and two α ascospores (Figure 1.14). Sign a (MATa) cells produce a sexual pheromone a. This peptide made up of 12 amino acids is called sexual factor a. In the same manner, sign α cells produce the sexual factor α, a peptide made up of 13 amino acids. The a factor, emitted by the MATa cells, stops the multiplication of MATα cells in G1. Reciprocally, the α factor produced by α cells stops the biological cycle of a cells. Sexual coupling occurs between two cells of the opposite sexual sign. Their agglutination permits cellular and nuclear fusion and makes use of parietal glycoproteins and a and α agglutinins. The vegetative diploid cell that is formed (aα) can no longer produce sexual pheromones and is insensitive to their action; it multiplies by budding.
**Fig. 1.13.** Meiosis in *S. cerevisiae* (Tuite and Oliver, 1991). SPB = spindle pole body; DB = dense body; SC = synaptonemal complexes. (a) Cell before meiosis; (b) dividing of SPB; (c) synaptonemal complexes appear; (d) separation of the SPB; (e) constitution of spindle (metaphase I of meiosis); (f) dividing of the SPB; (g) metaphase II of meiosis; (h) end of meiosis; formation of ascospores.

**Fig. 1.14.** Reproduction cycle of a heterothallic yeast strain (*a, α*: spore sexual signs)

Some strains, from a monosporic culture, can be maintained in a stable haploid state. Their sexual sign remains constant during many generations. They are **heterothallic**. Others change sexual sign during a cellular division. Diploid cells appear in the descendants of an ascospore. They are **homothallic** and have an *HO* gene which inverses sexual sign at an elevated frequency during vegetative division. This changeover (Figure 1.15) occurs in the mother cell at the G1 stage of the

**Fig. 1.15.** Sexual sign commutation model of haploid yeast cells in a homothallic strain (Herskowitz *et al.*, 1992) (* designates cells capable of changing sexual sign at the next cell division, or cells already having undergone budding). *S* = initial cell carrying the *HO* gene; F1, F2 = daughter cells of S; F1.1. = daughter cell of F1
biological cycle, after the first budding but before the DNA replication phase. In this manner, a sign $\alpha$ ascospore $S$ divides to produce two $\alpha$ cells ($S$ and the first daughter cell, $F1$). During the following cellular division, $S$ produces two cells ($S$ and $F2$) that have become $a$ cells. In the same manner, the $F1$ cell produces two $\alpha$ cells after the first division and two $a$ cells during its second budding. Laboratory strains that are deficient or missing the $HO$ gene have a stable sexual sign. Heterothallism can therefore be considered the result of a mutation of the $HO$ gene or of genes that control its functioning (Herskowitz et al., 1992).

Most wild and selected winemaking strains that belong to the $S. cerevisiae$ species are diploid and homothallic. It is also true of almost all of the strains that have been isolated in vineyards of the Bordeaux region. Moreover, recent studies carried out by Mortimer et al. (1994) in Californian and Italian vineyards have shown that the majority of strains (80%) are homozygous for the $HO$ character ($HO/\overline{HO}$); heterozygosis ($HO/\overline{ho}$) is in minority. Heterothallic strains ($ho/ho$) are rare (less than 10%). We have made the same observations for yeast strains isolated in the Bordeaux region. For example, the F10 strain fairly prevalent in spontaneous fermentations in certain Bordeaux growths is $HO/\overline{HO}$. In other words, the four spores issued from an ascus give monoparent diploids, capable of forming asci when placed in a pure culture. This generalized homozygosis for the $HO$ character of wild winemaking strains is probably an important factor in their evolution, according to the genome renewal phenomenon proposed by Mortimer et al. (1994) (Figure 1.16), in which the continuous multiplication of a yeast strain in its natural environment accumulates heterozygotic damage to the DNA. Certain slow-growth or functional loss mutations of certain genes decrease strain vigor in the heterozygous state. Sporulation, however, produces haploid cells containing different combinations of these heterozygotic characters. All of these spores become homozygous diploid cells with a series of genotypes because of the homozygosity of the $HO$ character. Certain diploids which prove to be more vigorous than others will in time supplant the parents and less vigorous ones. This very

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**Fig. 1.16.** Genome renewal of a homozygote yeast strain for the $HO$ gene of homothallism, having accumulated recessive mutations during vegetative multiplication (Mortimer et al., 1994) ($a$ and $b =$ mutation of certain genes)
tempting model is reaffirmed by the characteristics of the wild winemaking strains analyzed. In these, the spore viability rate is the inverse function of the heterozygosis rate for a certain number of mutations. The completely homozygous strains present the highest spore viability and vigor.

In conclusion, sporulation of strains in natural conditions seems indispensable. It assures their growth and fermentation performance. With this in mind, the conservation of selected strains of active dry yeasts as yeast starters should be questioned. It may be necessary to regenerate them periodically to eliminate possible mutations from their genome which could diminish their vigor.

1.7 THE KILLER PHENOMENON

1.7.1 Introduction

Certain yeast strains, known as killer strains (K), secrete proteinic toxins into their environment that are capable of killing other, sensitive strains (S). The killer strains are not sensitive to their toxin but can be killed by a toxin that they do not produce. Neutral strains (N) do not produce a toxin but are resistant. The action of a killer strain on a sensitive strain is easy to demonstrate in the laboratory on an agar culture medium at pH 4.2–4.7 at 20°C. The sensitive strain is inoculated into the mass of agar before it solidifies; then the strain to be tested is inoculated in streaks on the solidified medium. If it is a killer strain, a clear zone in which the sensitive strain cannot grow encircles the inoculum streaks (Figure 1.17).

This phenomenon, the killer factor, was discovered in *S. cerevisiae* but killer strains also exist in other yeast genera such as *Hansenula, Candida, Kloekera, Hanseniaspora, Pichia, Torulopsis, Kluyveromyces* and *Debaryomyces*. Killer yeasts have been classified into 11 groups according to the sensitivity reaction between strains as well as the nature and properties of the toxins involved. The killer factor is a cellular interaction model mediated by the proteinic toxin excreted. It has given rise to much fundamental research (Tipper and Bostian, 1984; Young, 1987). Barre (1984, 1992), Radler (1988) and Van Vuuren and Jacobs (1992) have described the technological implications of this phenomenon for wine yeasts and the fermentation process.

1.7.2 Physiology and Genetics of the Killer Phenomenon

The determinants of the killer factor are both cytoplasmic and nuclear. In *S. cerevisiae*, the killer phenomenon is associated with the presence of double-stranded RNA particles, virus-like particles (VLP), in the cytoplasm. They are in the same category as non-infectious mycovirus. There are two kinds of VLP: M and L. The M genome (1.3–1.9 kb) codes for the K toxin and for the immunity factor (R). The L genome (4.5 kb) codes for an RNA polymerase and the proteinic capsid that encapsulates the two genomes. Killer strains (K⁺R⁺) secrete the toxin and are immune to it. The sensitive cells (K⁻R⁻) do not possess M VLP but most of them have L VLP. The two types of viral particles are necessary for the yeast cell to express the killer phenotype (K⁺R⁺), since the L mycovirus is necessary for the maintenance of the M type.
There are three kinds of killer activities in *S. cerevisiae* strains. They correspond with the K1, K2 and K3 toxins coded, respectively, by M1, M2 and M3 VLPs (1.9, 1.5 and 1.3 kb, respectively). According to Wingfield *et al.* (1990), the K2 and K3 types are very similar; M3 VLP results from a mutation of M2 VLP. The K2 strains are by far the most widespread in the *S. cerevisiae* strains encountered in wine. Neutral strains (K\(^{-}\)R\(^{+}\)) are insensitive to a given toxin without being capable of producing it. They possess M VLPs of normal dimensions that code only for the immunity factor. They either do not produce toxins or are inactive because of mutations affecting the M-type RNA.

Many chromosomic genes are involved in the maintenance and replication of L and M RNA particles as well as in the maturation and transport of the toxin produced.

The K1 toxin is a small protein made up of two sub-units (9 and 9.5 kDa). It is active and stable in a very narrow pH range (4.2–4.6) and is therefore inactive in grape must. The K2 toxin, a 16 kDa glycoprotein, produced by homothallic strains of *S. cerevisiae* encountered in wine, is active at between pH 2.8 and 4.8 with a maximum activity between 4.2 and 4.4. It is therefore active at the pH of grape must and wine.

K1 and K2 toxins attack sensitive cells by attaching themselves to a receptor located in the cell wall—a β-1,6 glucan. Two chromosomic genes, *KRE1* and *KRE2* (*Killer resistant*), determine the possibility of this linkage. The *kre1* gene produces a parietal glycoprotein which has a β-1,6 glucan synthetase activity. The *kre1* mutants are resistant to K1 and K2 toxins because they are deficient in this enzyme and devoid of a β-1,6 glucan receptor. The *KRE2* gene is also involved in the fixation of toxins to the parietal receptor; the *kre2* mutants are also resistant. The toxin linked to a glucan receptor is then transferred to a membrane receptor site by a mechanism needing energy. Cells in the log phase are, therefore, more sensitive to the killer effect than cells in the stationary phase. When the sensitive cell plasmic membrane is exposed to the toxin, it manifests serious functional alterations after a lag phase of about 40 minutes. These alterations include the interruption of the coupled transport of amino acids and protons, the acidification of the cellular contents, and potassium and ATP leakage. The cell dies in 2–3 hours after contact with the toxin because of the above damage, due to the formation of pores in the plasmic membrane.

The killer effect exerts itself exclusively on yeasts and has no effect on humans and animals.

### 1.7.3 The Role of the Killer Phenomenon in Winemaking

Depending on the authors and viticultural regions studied, the frequency of the killer character varies a lot among wild winemaking strains isolated on grapes or in fermenting grape must. In a work by Barre (1978) studying 908 wild strains, 504 manifested the K2 killer character, 299 were sensitive and 95 neutral. Cuinier and Gros (1983) reported a high frequency (65–90%) of K2 strains in Mediterranean and Beaujolais region vineyards, whereas none of the strains analyzed in Tourraine manifested the killer effect. In the Bordeaux region, the K2 killer character is extremely widespread. In a study carried out in 1989 and 1990 on the ecology of indigenous strains of *S. cerevisiae* in several tanks of red must in a Pessac-Léognan vineyard, all of the isolated strains manifested K2 killer activity, about 30 differentiated by their karyotype (Frezier, 1992). Rossini *et al.* (1982) reported an extremely varied frequency (12–80%) of K2 killer strains in spontaneous fermentations in Italian wineries. Some K2 killer strains were also isolated in the southern hemisphere (Australia, South Africa and Brazil). On the other hand, most of the killer strains isolated in Japan presented the K1 characteristic. Most research on the killer character of wine yeasts concerns the species *S. cerevisiae*. Little information exists on the killer effect of the alcohol-sensitive species which essentially make up grape microflora. Heard and Fleet (1987) confirmed Barre’s (1980) observations and did not establish the existence of the killer effect in *Candida, Hanseniaspora, Hansenula* and *Torulaspora*. However, some killer strains of *Hanseniaspora uvarum* and *Pichia kluyveri* have been identified by Zorg *et al.* (1988).
Barre (1992) studied the activity and stability of the K2 killer toxin in enological conditions (Figure 1.18). The killer toxin only manifested a pronounced activity on cells in the log phase. Cells in the stationary phase were relatively insensitive. The amount of ethanol or SO2 in the wine has practically no effect on the killer toxin activity. On the other hand, it is quickly destroyed by heat, since its half-life is around 30 minutes at 32°C. It is also quickly inactivated by the presence of phenolic compounds and is easily adsorbed by bentonite.

Fig. 1.18. Yeast growth and survival curves in a grape juice medium containing killer toxin (Barre, 1992): *, 10% K2 strain active culture supernatant; ○, 10% supernatant inactivated by heat treatment. (a) White juice, pH 3.4; cells in exponential phase introduced at time = 0. (b) Same juice, cells in stationary phase introduced at time = 0. (c) Red juice extracted by heated maceration, pH 3.4; cells in exponential phase introduced at time = 0
Scientific literature has reported a diversity of findings on the role of the killer factor in the competition between strains during grape must fermentation. In an example given by Barre (1992), killer cells inoculated at 2% can completely supplant the sensitive strain during the alcoholic fermentation of must. In other works, the killer yeast/sensitive yeast ratio able to affect the implantation of sensitive yeasts in winemaking varies between 1/1000 and 100/1, depending on the author. This considerable discrepancy can probably be attributed to implantation and fermentation speed of the strains present. The killer phenomenon seems more important to interstrain competition when the killer strain implants itself quickly and the sensitive strain slowly. In the opposite situation, an elevated percentage of killer yeasts would be necessary to eliminate the sensitive population. Some authors have observed spontaneous fermentations dominated by sensitive strains despite a non-negligible proportion of killer strains (2–25%). In Bordeaux, we have always observed that certain sensitive strains implant themselves in red wine fermentation, despite a strong presence of killer yeasts in the wild microflora (for example, 522M, an active dry yeast starter). In white winemaking, the neutral yeast VL1 and sensitive strains such as EG8, a slow-growth strain, also successfully implant themselves. The wild killer population does not appear to compete with a sensitive yeast starter and therefore is not an important cause of fermentation difficulties in real-life applications.

The high frequency of killer strains among the indigenous yeasts in many viticultural regions confers little advantage to the strain in terms of implantation capacity. In other words, this character is not sufficient to guarantee the implantation of a certain strain during fermentation over a wild strain equally equipped. On the other hand, under certain conditions, inoculating with a sensitive strain will fail because of the killer effect of a wild population. Therefore, the resistance to the K2 toxin (killer or neutral phenotype) should be included among the selection criteria of enological strains. The high frequency of the K2 killer character in indigenous wine yeasts facilitates this strategy.

A medium that contains the toxin exerts a selection pressure on a sensitive enological strain. Stable variants survive this selection pressure and can be obtained in this manner (Barre, 1984). This is the most simple strategy for obtaining a killer enological strain. However, the development of molecular genetics and biotechnology permits scientists to construct enological strains modified to contain one or several killer characters. Cytoduction can achieve these modifications. This method introduced cytoplasmic determinants (mitochondria, plasmids) issued from a killer strain into a sensitive enological strain without altering the karyotype of the initial enological strain. Seki et al. (1985) used this method to make the 522M strain K2 killer. By another strategy, new yeasts can be constructed by integrating the toxin gene into their chromosomes. Boone et al. (1990) were able to introduce the K1 character into K2 winemaking strains in this manner. The K1 killer character among wine yeasts is rare, and so the enological interest of this last application is limited. The acquiring of multi-killer character strains presents little enological advantage. Sensitive selected strains and current K2 killer strains can already be implanted without a problem. On the other hand, the dissemination of these newly obtained multi-killer strains in nature could present a non-negligible risk. These strains could adversely affect the natural microflora population, although we have barely begun to inventory its diversity and exploit its technological potentials. It would be detrimental to be no longer able to select wild yeasts because they have been supplanted in their natural environment by genetically modified strains—a transformation that has no enological interest.

1.8 CLASSIFICATION OF YEAST SPECIES

1.8.1 General Remarks

As mentioned in the introduction to this chapter, yeasts constitute a vast group of unicellular fungi—taxonomically heterogeneous and very complex. Hansen’s first classification at the
beginning of this century only distinguished between sporiferous and asporiferous yeasts. Since then, yeast taxonomy has incited considerable research. This research has been regrouped in successive works progressively creating the classification known today. The last enological treaty of the University of Bordeaux (Ribéreau-Gayon et al., 1975) was based on Lodder’s (1970) classification. Between this monograph and the previous classification (Lodder and Kregger-Van Rij, 1952), the designation and classification of yeasts had already changed profoundly. In this book, the last two classifications by Kregger-Van Rij (1984) and Barnett et al. (2000) are of interest. These contain even more significant changes in the delimitation of species and genus with respect to earlier systematics.

According to the current classification, yeasts belonging to Ascomycetes, Basidiomycetes and imperfect fungi (Deuteromycetes) are divided into 81 genera, to which 590 species belong. Taking into account synonymy and physiological races (varieties of the same species), at least 4000 names for yeasts have been used since the nineteenth century. Fortunately, only 15 yeast species exist on grapes, are involved as an alcoholic fermentation agent in wine, and are responsible for wine diseases. Table 1.1 lists the two families to which enological yeasts belong: Saccharomycetaceae in the Ascomycetes (sporiferous) and Cryptococcaceae in the Deuteromycetes (asporiferous). Fourteen genera to which one or several species of grape or wine yeasts belong are not listed in Table 1.1.

### 1.8.2 Evolution of the General Principles of Yeast Taxonomy and Species Delimitation

Yeast taxonomy (from the Greek *taxis*: putting in order), and the taxonomy of other microorganisms for that matter, includes classification and identification. Classification groups organisms into *taxa* according to their similarities and/or their ties to a common ancestor. The basic taxon is species. A species can be defined as a collection of strains having a certain number of morphological, physiological and genetic characters in common. This group of characters constitutes the standard description of the species. Identification compares an unknown organism to individuals already classed and named that have similar characteristics.

Taxonomists first delimited yeast species using morphological and physiological criteria. The first classifications were based on the phenotypic differences between yeasts: cell shape and size, spore formation, cultural characters, fermentation and assimilation of different sugars, assimilation of nitrates, growth-factor needs, resistance to cycloheximide. The treaty on enology by Ribéreau-Gayon et al. (1975) described the use of these methods on wine yeasts in detail. Since then, many rapid, ready-to-use diagnostic kits have been

| Table 1.1. Classification of grape and wine sporogeneous and asporogeneous yeast genera (Kregger-Van Rij, 1984) |
|---------------------------------------------|---------------------------------------------|---------------------------------------------|
| **Saccharomycetaceae family** (sporogeneous) | **Spermophtoraceae family** (asporogeneous) | **Cryptococcaceae family** (asporogeneous) |
| **Sub-family** | **Sub-family** | **Sub-family** | **Genus** | **Genus** | **Genus** |
| Schizosaccharomycetoideae | Nadsonioideae | Saccharomycetoideae | Schizosaccharomyces | Saccharomycodes | Metschnikowia |
| Hanseniaspora | | | Kloeckera | Redlsonomyces | Brettanomyces |
| | | | | Candida | |
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proposed to determine yeast response to different physiological tests. Lafon-Lafourcade and Joyeux (1979) and Cuinier and Leveau (1979) designed the API 20 C system for the identification of enological yeasts. It contains eight fermentation tests, 10 assimilation tests and a cycloheximide resistance test. For a more complete identification, the API 50 CH system contains 50 substrates for fermentation (under paraffin) and assimilation tests. Heard and Fleet (1990) developed a system that uses the different tests listed in the work of Barnett et al. (1990).

Due to the relatively limited number of yeast species significantly present on grapes and in wine, these phenotypic tests identify enological yeast species in certain genera without difficulty. Certain species can be identified by observing growing cells under the microscope. Small apiculated cells, having small lemon-like shapes, designate the species *Hanseniaspora uvarum* and its imperfect form *Kloeckera apiculata* (Figure 1.19). *Saccharomycodes ludwigii* is characterized by much larger (10–20 µm) apiculated cells. Since most yeasts multiply by budding, the genus *Schizosaccharomyces* can be recognized because of its vegetative reproduction by binary fission (Figure 1.20). In modern taxonomy, this genus only contains the species *Schizosacch. pombe*. Finally, the budding of *Candida stellata* (formerly known as *Torulopsis stellata*) occurs in the shape of a star.

According to Barnett et al. (1990), the physiological characteristics listed in Table 1.2 can be used to distinguish between the principal grape and wine yeasts. Yet some of these characters (for example, fermentation profiles of sugars) vary within the species and are even unstable for a given strain during vegetative multiplication. Taxonomists realized that they could not differentiate species based solely on phenotypic discontinuity criteria. They progressively established a delimitation founded on the biological and genetic definition of a species.

In theory, a species can be defined as a collection of interfertile strains whose hybrids are themselves fertile—capable of producing viable spores. This biological definition runs into several problems

![Fig. 1.19. Observation of two enological yeast species having an apiculated form. (a) Hanseniaspora uvarum. (b) Saccharomycodes ludwigii](image)

![Fig. 1.20. Binary fission of Schizosaccharomyces pombe](image)
### Table 1.2. Physiological characteristics of the principal grape and wine yeasts (Barnett et al., 1990)

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+ : test positive; − : test negative; v: variable result.

*With these tests they cannot be differentiated from S. bayanus, S. paradoxus and S. pastorianus.*
when applied to yeasts. First of all, a large number of yeasts (Deuteromycetes) are not capable of sexual reproduction. Secondly, a lot of Ascomycetes yeasts are homothallic; hybridization tests are especially fastidious and difficult for routine identification. Finally, certain wine yeast strains have little or no sporulation aptitude, which makes the use of strain infertility criteria even more difficult.

To overcome these difficulties, researchers have developed a molecular taxonomy over the last 15 years based on the following tests: DNA recombination; the similarity of DNA base composition; the similarity of enzymes; ultrastructure characteristics; and cell wall composition. The DNA recombination tests have proven to be effective for delimiting yeast species. They measure the recombination percentages of denatured nuclear DNA (mono-stranded) of different strains. An elevated recombination rate between two strains (80–100%) indicates that they belong to the same species. A low recombination percentage (less than 20% of the sequences in common) signifies that the strains belong to different and very distant species. Combination rates between these extremes are more difficult to interpret.

1.8.3 Successive Classifications of the Genus Saccharomyces and the Position of Wine Yeasts in the Current Classification

Due to many changes in yeast classification and nomenclature since the beginning of taxonomic studies, enological yeast names and their positions in the classification have often changed. This has inevitably resulted in some confusion for enologists and winemakers. Even the most recent enological works (Fleet, 1993; Delfini, 1995; Bolton et al., 1995) use a number of different epithets (cerevisiae, bayanus, uvarum, etc.) attached to the genus name Saccharomyces to designate yeasts responsible for alcoholic fermentation. Although still in use, this enological terminology is no longer accurate to designate the species currently delimited by taxonomists.

The evolution of species classification for the genus Saccharomyces since the early 1950s (Table 1.3) has created this difference between the designation of wine yeasts and current taxonomy. By taking a closer look at this evolution, the origin of the differences may be understood.

In Lodder and Kregger-Van Rij (1952), the names cerevisiae, oviformis, bayanus, uvarum, etc. referred to a number of the 30 species of the genus Saccharomyces. Ribéreau-Gayon and Peynaud (1960) in the Treatise of Œnology considered that two principal fermentation species were found in wine: S. cerevisiae (formerly called ellipsoides) and S. oviformis. The latter was encountered especially towards the end of fermentation and was considered more ethanol resistant. The difference in their ability to ferment galactose distinguished the two species. S. cerevisiae (Gal+) fermented galactose, whereas S. oviformis (Gal−) did not. According to the same authors, the species S. bayanus was rarely found in wines. Although it possessed the same physiological fermentation and sugar assimilation characters as S. oviformis, its cells were more elongated, its fermentation was slower, and it had a particular behavior towards growth factors. The species S. uvarum was identified in wine by many authors. It differed from cerevisiae, oviformis and bayanus because it could ferment melibiose.

In Lodder’s following edition (Lodder 1970), the number of species of the genus Saccharomyces increased from 30 to 41. Some species formerly grouped with other genera were integrated into the genus Saccharomyces. Moreover, several species names were considered to be synonyms and disappeared altogether. Such was the case of S. oviformis, which was moved to the species bayanus. The treatise of Ribéreau-Gayon et al. (1975) considered, however, that the distinction between oviformis and bayanus was of enological interest because of the different technological characteristics of these two yeasts. Nevertheless, by the beginning of the 1980s most enological work had abandoned the name oviformis and replaced it with bayanus. This name change began the confusion that currently exists.

The new classification by Kregger-Van Rij (1984), based on Yarrow’s work on base percentages of guanine and cytosine in yeast DNA,
### Table 1.3. Evolution of the nomenclature for the *Saccharomyces* genus, 1952–1990

<table>
<thead>
<tr>
<th>1952: The Yeasts, a Taxonomic Study—I (Lodder and Kregger-Van Rij)</th>
<th>1970: The Yeasts, a Taxonomic Study—II (Lodder)</th>
<th>1984: The Yeasts, a Taxonomic Study—III (Yarrow)</th>
<th>2000: Yeasts (Barnett et al.)</th>
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### Group I

- Saccharomyces paradoxus (sensu stricto)
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- Saccharomyces cerevisiae
- Saccharomyces pastorianus
- Saccharomyces oviiformis
- Saccharomyces logos
- Saccharomyces chevalieri
- Saccharomyces fructuum
- Saccharomyces lactis
- Saccharomyces elegans
- Saccharomyces heterogenicus
- Saccharomyces fermentati
- Saccharomyces mellis
- Saccharomyces italicius
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- Saccharomyces telluris
- Saccharomyces transvaalensis
- Saccharomyces unispores
- Saccharomyces vafer
- Saccharomyces servazzi
brought forth another important change in the designation of the *Saccharomyces* species. Only seven species continued to exist, while 17 names became synonyms of *S. cerevisiae*. Certain authors considered them to be races or physiological varieties of the species *S. cerevisiae*. As with the preceding classification, these races of *S. cerevisiae* were differentiated by their sugar utilization profile (Table 1.4). However, this method of classification was nothing more than an artificial taxonomy without biological significance. Enologists took to the habit of adding the varietal name to *S. cerevisiae* to designate wine yeasts: *S. cerevisiae* var. *bayanus*, var. *uvarum*, var. *chevalieri*, etc. In addition, two species, *bailii* and *rosei*, were removed from the genus *Saccharomyces* and integrated into another genus to become *Zygosaccharomyces bailii* and *Torulaspora delbrueckii*, respectively.

The latest yeast classification (Barnett et al., 2000) is based on recent advances in genetics and molecular taxonomy—in particular, DNA recombination tests reported by Vaughan Martini and Martini (1987) and hybridization experiments between strains (Naumov, 1987). It has again thrown the species delimitation of the genus *Saccharomyces* into confusion. The species now number 10 and are divided into three groups (Table 1.3). The species *S. cerevisiae*, *S. bayanus*, *S. paradoxus* and *S. pastorianus* cannot be differentiated from one another by physiological tests but can be delimited by measuring the degree of homology of their DNA (Table 1.5). They form the group *Saccharomyces sensu stricto*. *S. pastorianus* replaced the name *S. carlsbergensis*, which was given to brewer’s yeast strains used for bottom fermentation (lager) and until now included in the species *cerevisiae*. The recently delimited *S. paradoxus* species includes strains initially isolated from tree exudates, insects, and soil (Naumov et al., 1998). It might constitute the natural common ancestor of three other yeast species involved in the fermentation process. Recent genomic analysis (Redzepovic et al., 2002) identified a high percentage of *S. paradoxus* in Croatian grape microflora. The occurrence of this species in other vineyards around the world and its winemaking properties certainly deserve further investigation.

### Table 1.4. Physiological Races of *Saccharomyces cerevisiae* regrouped under a single species *Saccharomyces cerevisiae* by Yarrow and Nakase (1975)

<table>
<thead>
<tr>
<th>Fermentation</th>
<th>Ga</th>
<th>Su</th>
<th>Ma</th>
<th>Ra</th>
<th>Me</th>
<th>St</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Saccharomyces</em></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>aceti</td>
<td>−</td>
<td>−</td>
<td>−</td>
<td>−</td>
<td>−</td>
<td>−</td>
</tr>
<tr>
<td>bayanus</td>
<td>−</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>−</td>
<td>−</td>
</tr>
<tr>
<td>capensis</td>
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<td>+</td>
<td>−</td>
<td>+</td>
<td>−</td>
<td>−</td>
</tr>
<tr>
<td>cerevisiae</td>
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<td>+</td>
<td>+</td>
<td>+</td>
<td>−</td>
<td>−</td>
</tr>
<tr>
<td>chevalieri</td>
<td>+</td>
<td>+</td>
<td>−</td>
<td>+</td>
<td>−</td>
<td>−</td>
</tr>
<tr>
<td>coreanus</td>
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<td>−</td>
<td>+</td>
<td>+</td>
<td>−</td>
</tr>
<tr>
<td>diastaticus</td>
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<td>−</td>
<td>+</td>
</tr>
<tr>
<td>globosus</td>
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<td>−</td>
<td>−</td>
<td>−</td>
<td>−</td>
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<td>heterogenicus</td>
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<td>+</td>
<td>+</td>
<td>−</td>
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<td>hienipiensis</td>
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<td>−</td>
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<tr>
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<td>−</td>
<td>−</td>
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<tr>
<td>oleaceus</td>
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<td>−</td>
<td>−</td>
<td>+</td>
<td>+</td>
<td>−</td>
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<tr>
<td>oleaginosus</td>
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<td>−</td>
<td>−</td>
<td>+</td>
<td>+</td>
<td>−</td>
</tr>
<tr>
<td>prostoserdovii</td>
<td>−</td>
<td>−</td>
<td>+</td>
<td>+</td>
<td>−</td>
<td>−</td>
</tr>
<tr>
<td>steineri</td>
<td>+</td>
<td>+</td>
<td>−</td>
<td>−</td>
<td>−</td>
<td>−</td>
</tr>
<tr>
<td>uvarum</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>−</td>
</tr>
</tbody>
</table>

Ga = D-galactose; Su = saccharose; Ma = maltose; Ra = raffinose; Me = melibiose; St = soluble starch.
A second group, Saccharomyces sensu largo, is made up of the species *exiguus*, *castelli*, *servazzi* and *unisporus*. The third group consists only of the species *kluyveri*. Only the first group comprises species of enological interest: *S. cerevisiae*, *S. bayanus*, and, possibly, *S. paradoxus*, if its suitability for winemaking is demonstrated. This new classification has created a lot of confusion in the language pertaining to the epithet *bayanus*. For taxonomists, *S. bayanus* is a species distinct from *S. cerevisiae*. For enologists and winemakers, *bayanus* (ex *oviformis*) designates a physiological race of *S. cerevisiae* that does not ferment galactose and possesses a stronger resistance to ethanol than *Saccharomyces cerevisiae* var. *cerevisiae*.

By evaluating the infertility of strains (a basic species delimitation criterion), Naumov *et al.* (1993) demonstrated that most strains fermenting melibiose (Mel*) isolated in wine, and until now classed as *S. cerevisiae* var. *uvarum*, belong to the species *S. bayanus*. Some strains, however, can be crossed with a reference *S. cerevisiae* to produce fertile descendants. They are therefore attached to *S. cerevisiae*. These results confirm, but nevertheless put into perspective, the past works of Rossini *et al.* (1982) and Bicknell and Douglas (1982), which were based on DNA recombination tests. The DNA recombination percentages are low between the *uvarum* and *cerevisiae* strains tested, but they are elevated between these same *uvarum* strains and the *S. bayanus* strain (CBS 380). In other words, most enological strains formerly called *uvarum* belong to the species *S. bayanus*. This relationship, however, is not complete. Certain *Saccharomyces* Mel*+* found in the spontaneous fermentations of grapes belong to *cerevisiae*. The yeasts that enologists commonly called *S. cerevisiae* var. *bayanus*, formerly *S. oviformis*, were studied to determine if they belong to the species *bayanus* or to the species *cerevisiae*, as the majority of *uvarum* strains. In this case, their designation only leads to confusion.

All of the results of molecular taxonomy presented above show that the former phenotypic classifications, based on physiological identification criteria, are not even suitable for delimiting the small number of fermentative species of the genus *Saccharomyces* found in winemaking. Moreover, specialists have long known about the instability of physiological properties of *Saccharomyces* strains. Rossini *et al.* (1982) reclassified a thousand strains from the yeast collection of the Microbiology Institute of Agriculture at the University of Perouse. During this research, they observed that 23 out of 591 *S. cerevisiae* strains conserved on malt agar lost the ability to ferment galactose. Twenty three strains ‘became’ *bayanus*, according to Lodder’s (1970) classification. They found even more frequently that, over time, strains acquired the ability to ferment certain sugars. For example, 29 out of 113 strains of *Saccharomyces oviformis* became capable of fermenting galactose, thus ‘becoming’ *cerevisiae*. According to these authors, this physiological instability is a specific property of strains from the *Saccharomyces* group sensu stricto. In the same collection, no noticeable change in fermentation profiles was observed in 150 strains of *Saccharomyces rosei* (today *Torulaspora delbrueckii*) or in 300 strains of *Kloeckera apiculata*. Genetic methods are therefore indispensable for identifying wine yeasts. Yet DNA recombination percentage measures or infertility tests between homothallic strains, a long and fastidious technique, are not practical for routine microbiological controls. The amplification of genome segments by polymerization chain reaction (PCR) is a quicker and easier method which has recently proved to be an excellent tool for discrimination of wine yeast species.

### Table 1.5. DNA/DNA reassociation percentages between the four species belonging to genus *Saccharomyces sensu stricto* (Vaughan Matini and Martini, 1987)

<table>
<thead>
<tr>
<th></th>
<th><em>S. cerevisiae</em></th>
<th><em>S. bayanus</em></th>
<th><em>S. pastorianus</em></th>
<th><em>S. paradoxus</em></th>
</tr>
</thead>
<tbody>
<tr>
<td><em>S. cerevisiae</em></td>
<td>100</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>S. bayanus</em></td>
<td>20</td>
<td>100</td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>S. pastorianus</em></td>
<td>58</td>
<td>70</td>
<td>100</td>
<td></td>
</tr>
<tr>
<td><em>S. paradoxus</em></td>
<td>53</td>
<td>24</td>
<td>24</td>
<td>100</td>
</tr>
</tbody>
</table>
1.8.4 Delimitation of Winemaking Species of *S. cerevisiae* and *S. bayanus* by PCR

Since its discovery by Saiki *et al.* (1985), PCR has often been used to identify different plant and bacteria species. This technique consists of enzymatically amplifying one or several gene fragments *in vitro*. The reaction is based on the hybridization of two oligonucleotides which frame a target region on a double-stranded DNA or template. These oligonucleotides have different sequences and are complementary to the DNA sequences which frame the strand to amplify. Figure 1.21 illustrates the different stages of the amplification process. The DNA is first denatured at a high temperature (95°C). The reactional mixture is then cooled to a temperature between 37 and 55°C, permitting the hybridization of these oligonucleotides on the denatured strands. The strands serve as primers from which a DNA polymerase permits the stage-by-stage addition of deoxyribonucleotidic units in the 5′–3′ direction. The DNA polymerase (Figure 1.22) requires four deoxyribonucleoside-5′

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**Fig. 1.21.** Principle of the polymerization chain reaction (PCR)
triphosphates (dATP, dGTP, dTTP, dCTP). A phosphodiester bond is formed between the 3′-OH end of the primer and the innermost phosphorus of the activated deoxyribonucleoside; pyrophosphate is thus liberated. The newly synthesized strand is formed on the template model. A thermoresistant enzyme, the TAQ DNA polymerase, is derived from the thermoresistant bacteria *Thermus aquaticus*. It permits a large number of amplification cycles (25–40) *in vitro* without having to add the DNA polymerase after each denaturation. In this manner, the DNA fragment amplified during the first cycle serves as the template for the following cycles. In consequence, each successive cycle doubles the target DNA fragment—amplified by a factor of $10^5$ to $10^6$ during 25–30 amplification cycles.

Hansen and Kielland-Brandt (1994) proposed *MET2* gene PCR amplification to differentiate between *S. cerevisiae* and *S. bayanus*, while working on strain types of the species *cerevisiae* and *bayanus* and on a strain of the variety *S. uvarum*. This gene, which codes for the synthesis of the homoserine acetyltransferase, has different sequences in the two species. Part of the gene is initially amplified by using two complementary oligonucleotides of the sequences which border the fragment to be amplified. The amplificat obtained, about 600 b.p, is the same size for the strains of the species *cerevisiae* and *bayanus* tested, as well as for the isolate designated *S. uvarum*. Different restriction endonucleases, which recognize certain specific DNA sequences, then digest the amplified fragment. Figure 1.23 gives an example of the mode of action of the EcoRI restriction endonuclease. This enzyme recognizes the base sequences GAATTC and cuts at the location indicated by the arrows. Electrophoresis is used to separate the obtained fragments. As a result, the restriction fragment length polymorphism (RFLP) can be appreciated. The restriction profiles obtained differ between *cerevisiae* and *bayanus*. They are identical for the strain types *bayanus* and *uvarum* tested.

This PCR–RFLP technique associated to the *MET2* gene has been developed and adapted for rapid analysis. The whole cells are simply heated in water (95°C), 10 minutes before amplification. Only two restriction enzymes are used: EcoRI and PstI (Masneuf et al., 1996a,b). The *MET2* amplificat (580 bp) is cut into two fragments (369 and 211 bp) by EcoRI in *S. cerevisiae*. PstI restriction creates two fragments for strain type *S. bayanus*. EcoRI does not cut the *MET2* amplificat of *S. bayanus*, nor does PstI cut the *S. cerevisiae* amplificat (Figure 1.24). Masneuf (1996) demonstrated that *S. paradoxus* can be identified by this method. Its *MET2* gene amplificat produces one fragment of the same size as with the two other species. This one, however, is not cleaved by EcoRI or PstI, but rather by Mae III.

![Recognition site and cutting mode of an EcoRI restriction endonuclease](image-url)
Fig. 1.24. Identification principles for the species *S. cerevisiae* and *S. bayanus* by the *MET* gene PCR-RFLP technique, after cutting the amplificat by EcoRI and PstI restriction enzymes.

![Identification principles](image)

**Fig. 1.25.** Agar gel electrophoresis (1.8%) of (a) *Eco*RI and *Pst*I digestions of the *MET* gene amplificats of the Mel+ strains studied by Naumov *et al.* (1993). Band 1: *S. bayanus* SCU 11; band 2: *S. bayanus* SCU 13; band 3: *S. bayanus* SCU 73; band 4: *S. bayanus* L19; band 5: *S. bayanus* L490; band 6: *S. cerevisiae* L 579; band 7: *S. cerevisiae* L 1425; band 8: *S. cerevisiae* VKM-Y 502; band 9: *S. bayanus* VKM-Y 1146. M = molecular weight markers.

Fig. 1.26. Agar gel electrophoresis (1.8%) of (a) *Eco*RI and *Pst*I digestions of the *MET* gene amplificats of the Mel+ strains studied by Naumov *et al.* (1993). Band 1: *S. bayanus* SCU 11; band 2: *S. bayanus* SCU 13; band 3: *S. bayanus* SCU 73; band 4: *S. bayanus* L19; band 5: *S. bayanus* L490; band 6: *S. cerevisiae* L 579; band 7: *S. cerevisiae* L 1425; band 8: *S. cerevisiae* VKM-Y 502; band 9: *S. bayanus* VKM-Y 1146. M = molecular weight markers.

By applying this relatively simple and quick technique to different enological strains of *S. uvarum* studied by Naumov *et al.* (1993), strains attached to the species *bayanus* by hybridization tests have been clearly demonstrated to present the same profile characteristic as *bayanus* (two bands after restriction with PstI, no restriction with EcoRI). On the other hand, the *uvarum* strains included in the species *cerevisiae*, according to hybridization tests, effectively have a restriction profile characteristic of *S. cerevisiae* (Figure 1.25 and Table 1.6). The delimitation of the species *cerevisiae* and *bayanus* by these two methods produced identical results for the 12 strains analyzed.

This type of PCR–RFLP analysis of the *MET*2 gene has been extended to different selected yeast strains available in the trade and currently used in winemaking. Depending on their ability to ferment galactose, wine professionals in the entire world still call these strains *cerevisiae* or *bayanus* (Table 1.7). For all of these strains, restriction profile characteristics of the species *S. cerevisiae* have been obtained.

In the same manner, the species of 82 indigenous *Saccharomyces* strains isolated in wines in fermentation and on grapes has been determined (Table 1.8). For the eight Gal+Mel− strains and the 47 Gal−Mel− strains analyzed, called *cerevisiae* and *bayanus* respectively by enologists, the restriction profiles of the *MET*2 gene amplificat are characteristic of the species *S. cerevisiae*. Similar results were obtained for 2 chevalieri strains fermenting galactose but not maltose (Ma−), as well as for the *capensis* strain (Gal−Ma−). Most of the Mel+ strains, called *uvarum* until now, (11 out of 12 for the isolates from Sauternes and 11 out of 11 for the isolates from Sancerre), belong to the species *S. bayanus*. Yet certain Mel+ are *S. cerevisiae* (one strain from Sauternes and two strains from the Lallemand collection). To summarize, the classification of the main winemaking yeasts (Section 1.8.3) has gone through three stages. Initially, several separate species were envisaged: *S. cerevisiae*, *S. bayanus* and/or *S. oviformis*, and *S. uvarum*. Subsequently, all of these were thought to belong to a single species: *S. cerevisiae*. The current classification identifies three distinct species on the basis of molecular biological data: *S. cerevisiae*, *S. bayanus*, and *S. paradoxus*. As the strains of *S. bayanus* used in winemaking belong exclusively to the *S. uvarum* variety (or sub-species), the remainder of this Handbook will consider just two species of winemaking yeasts, *S. cerevisiae* and *S. uvarum*. The involvement of *S. paradoxus* in grape fermentation microflora has yet to be confirmed.

Finally, PCR–RFLP associated with the *MET*2 gene can be used to demonstrate the existence of hybrids between the species *S. cerevisiae* and *S. bayanus*. This method has been used to prove the existence (Masneuf *et al.*, 1998) of such a natural hybrid (strain S6U var. *uvarum*) among dry yeasts commercialized by Lallemand Inc. (Montreal, Canada). Ciolfi (1992, 1994) isolated
### Table 1.6. Characterization by PCR–RFLP of the MET2 gene of 12 *S. uvarum* (Mel⁺) reclarified, after hybridization test by Naumov et al. (1993), as the species *S. cerevisiae* and *S. bayanus* (Masneuf, 1996)

<table>
<thead>
<tr>
<th>Strain</th>
<th>CLIB number</th>
<th>Origin</th>
<th>Author</th>
<th>Hybridization test</th>
<th>PCR–RFLP of the MET2 gene</th>
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</thead>
<tbody>
<tr>
<td>VKM Y-502</td>
<td>219</td>
<td>Russia</td>
<td>Naumov</td>
<td><em>S. cerevisiae</em> (control)</td>
<td><em>S. cerevisiae</em></td>
</tr>
<tr>
<td>VKM Y-1146</td>
<td>218</td>
<td>Russia grape</td>
<td>Naumov</td>
<td><em>S. bayanus</em> (control)</td>
<td><em>S. bayanus</em></td>
</tr>
<tr>
<td>58 l</td>
<td>—</td>
<td>FŒB must</td>
<td>Sapis-Domercq</td>
<td></td>
<td></td>
</tr>
<tr>
<td>SCU 11</td>
<td>101</td>
<td>ITVN wine</td>
<td>Poulard</td>
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<td><em>S. bayanus</em></td>
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<td>102</td>
<td>ITVN wine</td>
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<td>L 19</td>
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<td>Cuinier</td>
<td><em>S. bayanus</em></td>
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<td>Cuinier</td>
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<td><em>S. bayanus</em></td>
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<tr>
<td>DBVPG 1642</td>
<td>113</td>
<td>UPG grape</td>
<td>Vaughan Martini</td>
<td><em>S. bayanus</em></td>
<td><em>S. bayanus</em></td>
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<td><em>S. cerevisiae</em></td>
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<td>L 1425</td>
<td>95</td>
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<td>Cuinier</td>
<td><em>S. cerevisiae</em></td>
<td><em>S. cerevisiae</em></td>
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</table>

CLIB: Collection de Levures d’Intérêt Biotechnologique (collection of yeasts of Biotechnological Interest) INA-PG, Grignon, France.
FŒB: Faculté d’Onologie de l’Université de Bordeaux II, Talence, France.
ITVN: Institut Technique du Vin (Institut of Wine Technology), Centre d’expérimentation de Nantes, France.
ITVT: Institut Technique du Vin (Institut of Wine Technology), Centre d’expérimentation de Tours, France.
UPG: Universita degli Studi de Perugia, Italy.

### Table 1.7. Characterization by PCR–RFLP of the MET2 gene of various selected commercial strains used in winemaking

<table>
<thead>
<tr>
<th>Strains</th>
<th>Commercial brand</th>
<th>Origin</th>
<th>Enological designation</th>
<th>Species</th>
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<td>Dormstadt</td>
<td><em>S. cerevisiae</em></td>
<td><em>S. cerevisiae</em></td>
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<td>Actiflore primeur</td>
<td>INRA Narbonne</td>
<td><em>S. cerevisiae</em></td>
<td><em>S. cerevisiae</em></td>
</tr>
<tr>
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<td>FŒB</td>
<td><em>S. bayanus</em></td>
<td><em>S. cerevisiae</em></td>
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<td>R2</td>
<td>Vitlevure KD</td>
<td>n-a</td>
<td><em>S. bayanus</em></td>
<td><em>S. cerevisiae</em></td>
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<td>n-a</td>
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<td><em>S. cerevisiae</em></td>
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</table>

CLIB: Collection de Levures d’Intérêt Biotechnologique (Collection of yeast of biotechnological interest), INA-PG, Grignon, France.
FŒB: Faculté d’Onologie de l’Université de Bordeaux II, Talence, France.
UTM: Université de Trasos Montes, Portugal.
ŒC: Institut Onologie de Champagne, France.
CIVC: Comité Interprofessionnel des vins de Champagne (Interprofessional Champagne Committee), Epernay, France.
UB: Université de Bourgogne, Dijon, France.
n-a: not available.
Table 1.8. Characterization by PCR–RFLP of the MET2 gene of various species of wild Saccharomyces isolated on the grape and in wine (Masneuf, 1996)

<table>
<thead>
<tr>
<th>Number of different strain analyzed</th>
<th>Origin</th>
<th>Collection</th>
<th>Enological designation</th>
<th>Species</th>
</tr>
</thead>
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<tr>
<td>8</td>
<td>Sauternes wines</td>
<td>FŒB</td>
<td>cerevisiae</td>
<td>S. cerevisiae</td>
</tr>
<tr>
<td>2</td>
<td>Dry white Bordeaux wines</td>
<td>FŒB</td>
<td>bayanus</td>
<td>S. cerevisiae</td>
</tr>
<tr>
<td>9</td>
<td>Sauternes wines</td>
<td>FŒB</td>
<td>bayanus</td>
<td>S. cerevisiae</td>
</tr>
<tr>
<td>2</td>
<td>Dry white Bordeaux wines</td>
<td>FŒB</td>
<td>chevalieri</td>
<td>S. cerevisiae</td>
</tr>
<tr>
<td>1</td>
<td>Sauternes wines</td>
<td>FŒB</td>
<td>capensis</td>
<td>S. cerevisiae</td>
</tr>
<tr>
<td>36</td>
<td>Unknown</td>
<td>Lallemand</td>
<td>bayanus</td>
<td>S. cerevisiae</td>
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<tr>
<td>11</td>
<td>Sauternes wines</td>
<td>FŒB</td>
<td>uvarum</td>
<td>S. bayanus</td>
</tr>
<tr>
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<td>FŒB</td>
<td>uvarum</td>
<td>S. cerevisiae</td>
</tr>
<tr>
<td>10</td>
<td>Sancerre and Pouilly/Loire Valley wines</td>
<td>FŒB</td>
<td>uvarum</td>
<td>S. bayanus</td>
</tr>
<tr>
<td>1</td>
<td>Sancerre grapes</td>
<td>FŒB</td>
<td>uvarum</td>
<td>S. bayanus</td>
</tr>
<tr>
<td>2</td>
<td>Unknown</td>
<td>Lallemand</td>
<td>uvarum</td>
<td>S. cerevisiae</td>
</tr>
</tbody>
</table>

FŒB: Faculté d’Enologie de l’Université de Bordeaux II, Talence, France. Lallemand: Lallemand Inc. Montreal, Quebec, Canada.

1 2 3 4 5 6 7 8 9 M

(a) EcoRI and (b) PstI digestions of the amplificats of the MET2 gene of the strain hybrid. Bands 1, 2, 3: sub-clones of the hybrid strain; band 4: hybrid strain; band 5: S. cerevisiae VKM-Y 502; band 6: S. bayanus VKM-Y 1146. M = molecular weight marker

Fig. 1.26. Electrophoresis in agarose gel (1.8%) of (a) EcoRI and (b) PstI digestions of the amplificats of the MET2 gene of the strain hybrid. Bands 1, 2, 3: sub-clones of the hybrid strain; band 4: hybrid strain; band 5: S. cerevisiae VKM-Y 502; band 6: S. bayanus VKM-Y 1146. M = molecular weight marker

results. Furthermore, after sporulation of the strain in the laboratory, 10 tetrads were equally isolated by a micromanipulator after the digestion of the ascus cell wall. None of the 40 ascospores analyzed could germinate. The non-viability of the ascospores concurs with the hypothesis that this strain is an interspecific hybrid. Hansen of the Carlsberg laboratory (Denmark) sequenced two of the MET2 gene alleles from this strain. The sequence of one of the alleles is identical to that of the S. cerevisiae MET2 gene, with the exception of one nucleotide. The sequence of the other allele is 98.5% similar to that of S. bayanus. The presence of this allele is thus probably due to an interspecific cross.

Recent research (Naumov et al., 2000b) has shown that the S6U strain is, in fact, a tetraploid interspecific hybrid. Indeed, the percentage germination of spores from 24 tetrads, isolated using a micromanipulator, was very high (94%), whereas it would have been very low for a “normal” diploid interspecific hybrid. The monospore clones in this first generation (F1) were all capable of sporulating, while none of the ascospores of the second-generation tetrads were viable. The hybrid nature of the monospore clones produced by F1 was confirmed by the presence of the S. cerevisiae and S. uvarum MET2 gene, identified by PCR/RFLP. Finally, measuring the DNA content per cell using flux cytometry estimation confirmed that the descendants of S6U were interspecific diploids
and that S6U itself was an allotetraploid. Natural \textit{S. cerevisiae}/\textit{S. uvarum} hybrids have been isolated on grapes and in spontaneously fermenting musts in Alsace (Lejeune and Masneuf, unpublished results).

Several other methods using PCR/RFLP have been applied to typing \textit{Saccharomyces} itself. The fragments amplified were ribosomal DNA sequences (DNAr) (Guillamon \textit{et al.}, 1998; Nguyen \textit{et al.}, 2000).

\section*{1.9 IDENTIFICATION OF WINE YEAST STRAINS}

\subsection*{1.9.1 General Principles}

The principal yeast species involved in grape must fermentation, particularly \textit{S. cerevisiae}, comprise a very large number of strains with varied technological properties. The yeast strains which are involved during winemaking influence fermentation speed, the nature and quantity of secondary products formed during alcoholic fermentation, and the aromatic characters of the wine. The ability to differentiate between the different strains of \textit{S. cerevisiae} is required for the following fields: the ecological study of wild yeasts responsible for the spontaneous fermentation of grape must; the selection of strains presenting the best enological qualities; production and marketing controls; the verification of the implantation of selected yeasts used as yeast starter; and the constitution and maintenance of wild or selected yeast collections.

Bouix \textit{et al.} (1981) (cited in Van Vuuren and Van Der Meer, 1987) conducted the initial research on infraspecific differentiation within \textit{S. cerevisiae}. They attempted to distinguish strains by electrophoretic analysis of their exocellular proteins and later (1987) used the separation of intracellular proteins. Other teams proposed identifying the strains by the analysis of long-chain fatty acids using gas chromatography (Tredoux \textit{et al.}, 1987; Augustyn \textit{et al.}, 1991; Bendova \textit{et al.}, 1991; Rozes \textit{et al.}, 1992). Although these different techniques differentiate between certain strains, they are irrefutably less discriminatory than genetic differentiation methods. They also present the major inconvenience of depending on the physiological state of the strains and the cultural conditions, which must always be identical.

In the late 1980s, owing to the development of genetics, certain techniques of molecular biology were successfully applied to characterize wine yeast strains. They are based on the clonal polymorphism of the mitochondrial and genomic DNA of \textit{S. cerevisiae}. These genetic methods are independent of the physiological state of the yeast, unlike the previous techniques based on the analysis of metabolism byproducts.

\subsection*{1.9.2 Mitochondrial DNA Analysis}

The mtDNA of \textit{S. cerevisiae} has two remarkable properties: it is extremely polymorphous, depending on the strain; and its is stable (it mutates very little) during vegetative multiplication. Restriction endonucleases (such as \textit{EcoR}5) cut this DNA at specific sites. This process generates fragments of variable size which are few in number and can be separated by electrophoresis on agarose gel.

Aigle \textit{et al.} (1984) first applied this technique to brewer’s yeasts. Since 1987, it has been used for the characterization of enological strains of \textit{S. cerevisiae} (Dubourdieu \textit{et al.}, 1987; Hallet \textit{et al.}, 1988).

The extraction of mtDNA comprises several stages. The protoplasts obtained by enzymatic digestion of the cell walls are lysed in a hypotonic buffer. The mtDNA is then separated from the chromosomal DNA by ultracentrifugation in a cesium chloride gradient, in the presence of bisbenzimide which acts as a fluorescent intercalating agent. This agent amplifies the difference in density between chromosomal and mtDNA. The mtDNA has an elevated amount of adenine and thymine base pairs for which the bisbenzimide has a strong affinity. Finally, the mtDNA is purified by a phenolchloroform-based extraction and an ethanol-based precipitation.

Defontaine \textit{et al.} (1991) and Querol \textit{et al.} (1992) simplified this protocol by separating the mitochondria from the other cellular constituents before extracting the DNA. In this manner, they avoided
the ultracentrifugation step. The coarse cellular debris is eliminated from the yeast lysate by centrifuging at 1000 g. The supernatant is recentrifuged at 1500 g to obtain the mitochondria. The mitochondria are then lysed in a suitable buffer to liberate the DNA.

Unlike industrial brewer strains analyzed by Aigle et al. (1984), which have the same mtDNA restriction profile, implying that they are of common origin, the enological yeast strains have a large mtDNA diversity. This method differentiates between most of the selected yeasts used in winemaking as well as wild strains of *S. cerevisiae* found in spontaneous fermentations (Figure 1.27).

This technique is very discriminating and not too expensive, but it is long and requires several complex manipulations. It is useful for the subtle characterization of a small number of strains. Inoculation effectiveness can also be verified by this method. To verify an inoculation, a sample is taken during or towards the end of alcoholic fermentation. In the laboratory, the lees are placed in a liquid medium culture. The mtDNA restriction profile of this total biomass and of the yeast starter strain are compared. If the restriction profile of the sample has no supernumerary bands with respect to the yeast starter strain profile, the yeast starter has been properly implanted, with an accuracy of 90%. In fact, in the case of a binary mixture, the minority strain must represent around 10% of the total population to be detected (Hallet et al., 1989).

![Fig. 1.27. Restriction profile by *EcoR*5 of mtDNA of different strains of *S. cerevisiae*. Band 1: F10; band 2: BO213; band 3: VLI; band 4: 522; band 5: Sita 3; band 6: VL3c. M = marker](image)

### 1.9.3 Karyotype Analysis

*S. cerevisiae* has 16 chromosomes with a size range between 250 and 2500 Kb. Its genomic DNA is very polymorphic; thus it is possible to differentiate strains of the species according to the size distribution of their chromosomes. Pulse-field electrophoresis is used to separate *S. cerevisiae* chromosomes and permits the comparison of the karyotypes of the strains. This technique uses two electric fields oriented differently (90 to 120 degrees). The electrodes placed on the sides of the apparatus apply the fields alternately (Figure 1.28).

![Fig. 1.28. CHEF pulsed field electrophoresis device (contour clamped electrophoresis field)](image)
The user can define the duration of the electric current that will be applied in each direction (pulse). With each change in direction of the electric field, the DNA molecules reorientate themselves. The smaller chromosomes reorientate themselves more quickly than the larger ones (Figure 1.29).

Blondin and Vezhinet (1988), Edersen et al. (1988) and Dubourdieu and Frezier (1990) applied this technique to identify enological yeast strains. Sample preparation is relatively easy. The yeasts are cultivated in a liquid medium, collected during the log phase, and then placed in suspension in a warm agarose solution that is poured into a partitioned mold to form small plugs.

Figure 1.30 gives an example of the identification of S. cerevisiae strains isolated from a grape must in spontaneous fermentation by this method. Vezhinet et al. (1990) have shown that karyotype analysis can distinguish between strains of S. cerevisiae as well or better than the use of mtDNA restriction profiles. Furthermore, karyotype analysis is much quicker and easier to use than mtDNA analysis. In the case of ecological studies of spontaneous fermentation microflora, pulse-field electrophoresis of chromosomes is extensively used today to characterize strains of S. cerevisiae (Frezier and Dubourdieu, 1992; Versavaud et al., 1993, 1995).

Very little research on the chromosomic polymorphism in other species of grape and wine yeasts is currently available. Naumov et al. (1993) suggested that S. bayanus and S. cerevisiae karyotypes can be easily distinguished. Other authors (Vaughan-Martini and Martini, 1993; Masneuf, 1996) have confirmed his results. In fact, a specific chromosomic band systematically appears in S. bayanus. Furthermore, there are only two chromosomes whose sizes are less than 400 kb in S. bayanus but generally more in S. cerevisiae, in all of the strains that we have analyzed.

Species other than Saccharomyces, in particular apiculated yeasts (Hanseniaspora uvarum, Kloeckera apiculata), are present on the grape and are sometimes found at the beginning of fermentations. These species have fewer polymorphous karyotypes and fewer bands than in Saccharomyces. Versavaud et al. (1993) differentiated between strains of apiculated yeast species and Candida famata by using restriction endonucleases at rare sites (Not I and Sfi I). The endonucleases cut the chromosomes into a limited number of fragments, which were then separated by pulse-field electrophoresis.

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**Fig. 1.29.** Principle of DNA molecule separation by pulsed field electrophoresis

**Fig. 1.30.** Example of electrophoretic (pulsed field) profile of S. cerevisiae strain caryotypes
1.9.4 Genomic DNA Restriction Profile Analysis Associated with DNA Hybridization by Specific Probes (Fingerprinting)

The yeast genome contains DNA sequences which repeat from 10 up to 100 times, such as the $\delta$ or Y1 sequences of the chromosome telomeres. The distribution, or more specifically, the number and location of these elements, has a certain intraspecific variability. This genetic fingerprint is used to identify strains (Pedersen, 1986; Degre et al., 1989).

The yeasts are cultivated in a liquid medium. Samples are taken during the log phase, as in the preceding techniques. The entire DNA is isolated and digested by restriction endonucleases. The generated fragments are separated by electrophoresis on agarose gel and then transferred to a nylon membrane (Southern, 1975). Complementary radioactive probes (nucleotide sequences taken from $\delta$ and Y1 elements) are used to hybridize with fragments having homologous sequences. The result gives a hybridization profile containing several bands.

Genetic fingerprinting is a more complicated and involved method than mtDNA or karyotype analysis. It is, however, without doubt the most discriminating strain identification method and may even discriminate too well. It has correctly indicated minor differences between very closely related strains. In fact, in the Bordeaux region, S. cerevisiae clones isolated from spontaneous fermentations in different wineries have been encountered which have the same karyotype and the same mtDNA restriction profile. Yet their hybridization profiles differ according to sample origin (Frezier, 1992). These strains, probably descendants of the same mother strain, have therefore undergone minor random modifications, maintained during vegetative multiplication.

1.9.5 Polymerization Chain Reaction (PCR) Associated to $\delta$ Sequences

This method consists of using PCR to amplify certain sequences of the yeast genome (Section 1.8.4), occurring between the repeated $\delta$ elements, whose separation distance does not exceed a certain value (1 kb). Ness et al. (1992) and Masneuf and Dubourdieu (1994) developed this method to characterize S. cerevisiae strains. The amplification is carried out directly on whole cells. They are simply heated to make the cellular envelopes permeable. The resulting amplification fragments are separated according to their size by electrophoresis in agarose gel and viewed using ultraviolet fluorescence (Figure 1.31).

PCR profile analysis associated with $\delta$ sequences can distinguish between most S. cerevisiae active dry yeast strains (ADY) used in winemaking (Figure 1.32): 25 out of the 26 selected commercial yeast strains analyzed. Lavallée et al. (1994) also observed excellent discriminating power with this method while analyzing industrially produced commercial strains from Lallemand Inc. (Montreal, Canada). In addition, this method permits the identification of 25 to 50 strains per day; it is the quickest of the different strain identification techniques currently available. When used for
indigenous strain identification in a given viticultural region, however, it seems to be less discriminating than karyotype analysis. PCR profiles of wild yeasts isolated in a given location often appear similar. They have several constant bands and only a small number of variable discriminating bands. Certain strains have the same PCR amplification profile while having different karyotypes. In a given location, the polymorphism witnessed by PCR associated with $\delta$ sequences is less important than that of the karyotypes. This method is therefore complementary to other methods for characterizing winemaking strains. PCR permits a rapid primary sort of an indigenous population. Karyotype analysis refines this discrimination.

*S. bayanus* strains cannot be distinguished by this technique because their genome contains only a few Ty elements.

Finally, because of its convenience and rapidity, PCR associated with $\delta$ sequences facilitates verification of the implantation of yeast starters used in winemaking. The analyses are effectuated on the entire biomass derived from lees, placed beforehand in a liquid medium in a laboratory culture. The amplification profiles obtained are compared with inoculated yeast strain profiles. They are identical with a successful implantation, and different if the inoculation fails. Figure 1.33 gives examples of successful (yeasts B and C) and unsuccessful

(yeasts A, D, and E) implantations. Contaminating strains have a different amplification profile than the yeast starter. The detection threshold of a contaminating strain was studied in the laboratory by analyzing a mixture of two strains in variable proportions. In the example given in Figure 1.34, the contaminating strain is easily detected at 1%. In winery fermentations, however, several minority indigenous strains can coexist with the inoculated

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**Fig. 1.32.** Electrophoresis in agar gel (at 1.8%) of amplified fragments obtained from various commercial yeast strains. Band 1: F10; band 2: BO213; band 3: VL3c; band 4: UP30Y5; band 5: 522 D; band 6: EG8; band 7: L-1597; band 8: WET 136. M = molecular weight marker; T = negative control.

**Fig. 1.33.** Electrophoresis in agar gel (1.8%) of amplified fragments illustrating examples of verifying yeast implantation (successful: yeasts B and C; unsuccessful: yeasts A, D and E). Band 1: negative control; band 2: Lees A; band 3: ADY A; band 4: Lees B; band 5: ADY B; band 6: Lees C; band 7: ADY C; band 8: Lees D; band 9: ADY D; band 10: Lees E; band 11: ADY E. M = molecular weight marker.

**Fig. 1.34.** Determination of the detection threshold of a contaminating strain. Band 1: strain A 70%, strain B 30%; band 2: strain A 80%, strain B 20%; band 3: strain A 90%, strain B 10%; band 4: strain A 99%, band B 1%; band 5: strain A 99.9%, strain B 0.1%; band 6: strain A; band 7: strain B. M = molecular weight marker; T = negative control.
strain. When must in fermentation or lees is analyzed by PCR, the yeast implantation rate is at least 90% when the amplification profiles of the lees and the yeast starter are identical.

In light of various research, different DNA analysis methods should be combined to identify wine yeast strains.

**1.9.6 PCR with Microsatellites**

Microsatellites are tandem repeat units of short DNA sequences (1–10 nucleotides), i.e. in the same direction and dispersed throughout the eukaryote genome (Field and Wills, 1998). The number of motif repetitions is extremely variable from one individual to another, making these sequences highly polymorphous in size. These regions are easily identified, thanks to the full sequence of the *S. cerevisiae* genome, available on the Internet in the *Saccharomyces* Genome Database. Approximately 275 sequences have been listed, mainly AT dinucleotides and AAT and AAC trinucleotides (Perez et al., 2001). Furthermore, these sequences are allelic markers, transmitted to the offspring in a Mendelian fashion. Consequently, these are ideal genetic markers for identifying specific yeast strains, making it possible not only to distinguish between strains but also to arrange them in related groups. This technique has many applications in man: paternity tests, forensic medicine, etc. In viticulture, this molecular identification method has already been applied to *Vitis vinifera* grape varieties (Bowers et al., 1999).

The technique consists of amplifying the region of the genome containing these microsatellites, then analyzing the size of the amplified portion to a level of detail of one nucleotide by electrophoresis on acrylamide gel. This varies by a certain number of base pairs (approximately 8–40) from one strain to another, depending on the number of times the motif is repeated. A yeast strain may be heterozygous for a given locus, giving two different-sized amplified DNA fragments. Using 6 microsatellites, Perez et al. (2001) were able to identify 44 different genotypes within a population of 51 strains of *S. cerevisiae* used in winemaking. Other authors (Gonzalez et al., 2001; Hennequin et al., 2001) have shown that the strains of *S. cerevisiae* used in winemaking are weakly heterozygous for the loci studied. However, interstrain variability of the microsatellites is very high. The results are expressed in numerical values for the size of the microsatellite in base pairs or the number of repetitions of the motifs on each allele. These digital data are easy to interpret, unlike the karyotype images on agarose gel, which are not really comparable from one laboratory to another. Microsatellite analysis has also been used to identify the strains of *S. uvarum* used in winemaking (Masneuf and Lejeune, unpublished). As the *S. uvarum* and *S. cerevisiae* microsatellites have different amplification primers, this method provides an additional means of distinguishing between these species and their hybrids.

In future, this molecular typing method will certainly be a useful tool in identifying winemaking yeast strains, ecological surveys, and quality control of industrial production batches.

Finally, another technique has recently been proposed for identifying *Saccharomyces* strains with PCR by amplifying introns of the COX1 mitochondrial DNA gene, which varies in number and position in different strains. It is possible to amplify either purified DNA or fermenting must. This technique has been used to monitor yeast development during fermentation (Lopez et al., 2003).

**1.10 ECOLOGY OF GRAPE AND WINE YEASTS**

**1.10.1 Succession of Grape and Wine Yeast Species**

Until recently, a large amount of research focused on the description and ecology of wine yeasts. It concerned the distribution and succession of species found on the grape and then in wine during fermentation and conservation (Ribéreau-Gayon et al. 1975; Lafon-Lafourcade 1983).

The ecological study of grape and wine yeast species represents a considerable amount of research. De Rossi began his research in the 1930s (De Rossi, 1935). Castelli (1955, 1967) pursued
yeast ecology in Italian vineyards. Peynaud and Domercq (1953) and Domercq (1956) published the first results on the ecology of enological yeasts in France. They described not only the species found on the grape and during alcoholic fermentation, but also contaminating yeasts and diseases. Among the many publications on this theme since the 1960s in viticultural regions around the world, the following works are worth noting: Brechot et al. (1962), Minarik (1971), Barnett et al. (1972), Park (1975), Cuinier and Guerineau (1976), Soufleros (1978), Belin (1979, 1981), Poulard et al. (1980), Poulard and Lecocq (1981), Bureau et al. (1982), Rossini et al. (1982).

Yeasts are widespread in nature and are found in soils, on the surface of vegetables and in the digestive tract of animals. Wind and insects disseminate them. They are distributed irregularly on the surface of the grape vine; found in small quantities on leaves, the stem and unripe grapes, they colonize the grape skin during maturation. Observations under the scanning electron microscope have identified the location of yeasts on the grape. They are rarely found on the bloom, but multiply preferentially on exudates released from microlesions in zones situated around the stomatal apparatus. Botrytis cinerea and lactic acid and acetic acid bacteria spores also develop in the proximity of these peristomatic fractures (Figure 1.35).

The number of yeasts on the grape berry, just before the harvest, is between $10^3$ and $10^5$, depending on the geographical situation of the vineyard, climatic conditions during maturation, the sanitary state of the harvest, and pesticide treatments applied to the vine. The most abundant yeast populations are obtained in warm climatic conditions (lower latitudes, elevated temperatures). Insecticide treatments and certain fungicidal treatments can contribute to the rarefaction of indigenous grape microflora. Quantitative results available on this subject, however, are few. After the harvest, transport and crushing of the crop, the number of cells capable of forming colonies on an agar medium generally attains $10^6$ cells/ml of must.

The number of yeast species significantly present on the grape is limited. Strictly oxidative metabolism yeasts, which belong to the genus Rhodotorula and a few alcohol-sensitive species, are essentially found there. Among the latter, the apiculated species (Kloeckera apiculata and its sporiferous form Hanseniaspora uvarum) are the most common. They comprise up to 99% of the yeasts isolated in certain grape samples. The following are generally found but in lesser proportions: Metschnikowia pulcherrima, Candida famata, Candida stellata, Pichia membranefaciens, Pichia fermentans, Hansenula anomala.

All research confirms the extreme rarity of S. cerevisiae on grapes. Yet these yeasts are not totally absent. Their existence cannot be proven by spreading out diluted must on a solid medium prepared in aseptic conditions, but their presence on grapes can be proven by analyzing the spontaneous fermentative microflora of grape samples placed in sterile bags, then aseptically crushed and vinified in the laboratory in the absence of all contamination. Red and white grapes from the Bordeaux region were treated in this manner. At mid-fermentation in the majority of cases, S. cerevisiae represented almost all of the yeasts isolated. In some rare cases, no yeast of this species developed and apiculated yeasts began the fermentation.

Ecological surveys carried out at the Bordeaux Faculty of Enology from 1992 to 1999 (Nau mov et al., 2000a) demonstrated the presence of S. uvarum yeasts on grapes and in spontaneously fermenting white musts from the Loire Valley, Jurançon, and Sauternes. The frequency of the

Fig. 1.35. Grape surface under scanning electron microscope, with detail of yeast peristomatic zones. Department of Electronic Microscopy, University of Bordeaux I
presence of this species alongside *S. cerevisiae* varies from 4–100%. On one estate in Alsace, strains of *S. uvarum* were identified on grapes, in the press, and in vats, where they represented up to 90% of the yeasts involved throughout fermentation in two consecutive years (Lejeune, unpublished work). More recently, Naumov et al. (2002) showed that *S. uvarum*, identified on grapes and in fermenting must, was involved in making Tokay wine.

The adaptation of *S. uvarum* to relatively low temperatures (6–10°C) certainly explains its presence in certain ecological niches: northerly vineyards, late harvests, and spontaneous “cool” fermentation of white wines. In contrast, this strain is sensitive to high temperatures and has not been found in spontaneous fermentations of red Bordeaux wines.

Recent observations also report the presence of natural *S. cerevisiae*/*S. uvarum* hybrids on grapes and in wineries where both species are present (Lejeune, unpublished work).

Between two harvests, the walls, the floors, the equipment and sometimes even the winery building are colonized by the alcohol-sensitive species previously cited. Winemakers believe, however, that spontaneous fermentations are more difficult to initiate in new tanks than in tanks which have already been used. This empirical observation leads to the supposition that *S. cerevisiae* can also survive in the winery between two harvests. Moreover, this species was found in non-negligible proportions in the wooden fermenters of some of the best vineyards in Bordeaux during the harvest, just before they were filled.

In the first hours of spontaneous fermentations, the first tanks filled have a very similar microflora to that of the grapes. There is a large proportion of apiculated yeasts and *M. pulcherrima*. After about 20 hours, *S. cerevisiae* develops and coexists with the grape yeasts. The latter quickly disappear at the start of spontaneous fermentation. In red winemaking in the Bordeaux region, as soon as must density drops below 1.070–1.060, the colony samples obtained by spreading out diluted must on a solid medium generally isolate exclusively *S. cerevisiae* (10⁷ to 10⁸ cells/ml). This species plays an essential role in the alcoholic fermentation process. Environmental conditions influence its selection. This selection pressure is exhibited by four principal parameters: anaerobiosis; must or grape sulfiting; the sugar concentration; and the increasing presence of ethanol. In winemaking where no sulfur dioxide is used, such as white wines for the production of spirits, the dominant grape microflora can still be found. It is largely present at the beginning of alcoholic fermentation (Figure 1.36). Even in this type of winemaking, the presence of apiculated yeasts is almost nonexistent at mid alcoholic fermentation.

During dry white winemaking, the separation of the marc after pressing combined with clarification by racking strongly reduces yeast populations, at least in the first days of the harvest. The yeast population of a severely racked must rarely exceeds 10⁴ to 10⁵ cells/ml.

A few days into the harvest, the alcochegoous *S. cerevisiae* yeasts contaminate the harvest material, grape transport machinery and especially the harvest receiving equipment, the crusher-stemmer, and the wine press. For this reason, it is already largely present at the time of filling the tanks (around 50% of yeasts isolated during the first homogenization pumping-over of a red-grape tank). Fermentations are initiated more rapidly in the course of the winemaking campaign because

![Fig. 1.36. Comparison of yeast species present at the start of alcoholic fermentation (d = 1.06). (A) in a tank of sulfited red grapes in Bordeaux (Frezier, 1992); (B) in a tank of unsulfited white must, for the elaboration of Cognac (Versavaud, 1994)]
of this increased percentage of S. cerevisiae. In fact, the last tanks filled often complete their fermentations before the first ones. Similarly, static racking in dry white winemaking is becoming more and more difficult to achieve, even at low temperatures, from the second week of the harvest onward, especially in hot years. The entire installation inoculates the must with a sizeable alcoogenous yeast population. General weekly disinfection of the pumps, the piping, the wine presses, the racking tanks, etc. is therefore strongly recommended.

During the final part of alcoholic fermentation (the yeast decline phase), the population of S. cerevisiae progressively decreases while still remaining greater than 10^6 cells/ml. In favorable winemaking conditions, characterized by a rapid and complete exhaustion of sugars, no other yeast species significantly appears at the end of fermentation. In poor conditions, spoilage yeasts can contaminate the wine. One of the most frequent and most dangerous contaminations is due to the development of Brettanomyces intermedius, which is responsible for serious olfactive flaws (Volume 2, Section 8.4.5).

In the weeks that follow the completion of alcoholic fermentation, the viable populations of S. cerevisiae drop rapidly, falling below a few hundred cells/ml. In many cases, other yeast species (spoilage yeasts) can develop in wines during ageing or bottle storage. Some yeasts have an oxidative metabolism of ethanol and form a veil on the surface of the wine, such as Pichia or Candida, or even certain strains of S. cerevisiae—sought after in the production of specialty wines. By topping off regularly, the development of these respiratory metabolism yeasts can be prevented. Some other yeasts, such as Brettanomyces or Dekkera, can develop in anaerobiosis, consuming trace amounts of sugars that have been incompletely or not fermented by S. cerevisiae. Their population can attain 10^4 to 10^5 cells/ml in a contaminated red wine in which alcoholic fermentation is otherwise completed normally. These contaminations can also occur in the bottle. Refermentation yeasts can develop significantly in sweet or botrytized sweet wines during ageing or bottle storage; the principal species found are Saccharomycodes ludwigii, Zygosaccharomyces bailii, and also some strains of S. cerevisiae that are particularly resistant to ethanol and sulfur dioxide.

### 1.10.2 Recent Advances in the Study of the Ecology of S. cerevisiae Strains

The ecological study of the clonal diversity of yeasts, and in particular of S. cerevisiae during winemaking, was inconceivable for a long time because of a lack of means to distinguish yeast strains from one another. Such research has become possible with the development of molecular yeast strain identification methods (Section 1.9). This Section focuses on recent advances in this domain.

The alcoholic fermentation of grape must or grapes is essentially carried out by a single yeast species, S. cerevisiae. Therefore, an understanding of the clonal diversity within this species is much more important for the winemaker than investigations on the partially or non-fermentative grape microflora.

The analysis in this Section of S. cerevisiae strains in practical winemaking conditions in particular intends to answer the following questions:

- Is spontaneous fermentation carried out by a dominant strain, a small number or a very large number of strains?
- Can the existence of a succession of strains during alcoholic fermentation be proven? If so, what is their origin: grape, harvest material, or winery equipment?
- During winemaking and from one year to another in the same winery or even the same vineyard, is spontaneous alcoholic fermentation carried out by the same strains?
- Can the practice of inoculating with selected strains modify the wild microflora of a vineyard?

During recent research (Dubourdieu and Frezier 1990; Frezier 1992; Masneuf 1996), many samples of yeast microflora were taken at the vineyard and
the winery from batches of white and red wines spontaneously fermenting or inoculated with active dry yeasts. Several conclusions can be drawn from this research, carried out on several thousand wild strains of *S. cerevisiae*.

In the majority of cases, a small number of major strains (one to three) representing up to 70–80% of the colonies isolated, carry out the spontaneous fermentations of red and dry white wines. These dominant strains are found in comparable proportions in all of the fermentors from the same winery from start to end of alcoholic fermentation. This phenomenon is illustrated by the example given in Figure 1.37, describing the indigenous microflora of several tanks of red must in a Pessac-Léognan vineyard in 1989. The strains of *S. cerevisiae*, possessing different karyotypes, are identified by an alphanumeric code comprising the initial of the vineyard, the tank number, the time of the sampling, the isolated colony number and the year of the sample. Two strains, Fz Ib1 (1989) and Fz Ib2 (1989), are encountered in all of the tanks during the entire alcoholic fermentation process.

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**Fig. 1.37.** Breakdown of *S. cerevisiae* caryotypes during alcoholic fermentation in red grape tanks in Fz vineyard (Pessac-Léognan, France) in 1989 (Frezier, 1992) (b, c, and d designate the start, middle, and end of alcoholic fermentation, respectively). Tanks I and II (Merlot) and III and IV (Cabernet-Sauvignon) are filled on the 1st, 3rd, 7th and 23rd day of the harvest, respectively.
The spontaneous fermentation of dry white wines in the same vineyard is also carried out by the same dominant yeast strains in all of the barrels.

The tank filling order and the grape variety have little effect on the clonal composition of the populations of *S. cerevisiae* spontaneously found in the winery. The daily practice of pumping-over the red grape must with pumping equipment used for all of the tanks probably ensures the dissemination of the same strains in the winery. In white winemaking, the wine press installation plays the same role as an inoculator.

In addition, in Figure 1.37, all of the strains analyzed are K2 killer. The two dominant strains do not ferment galactose (phenotype Gal\(^{-}\)). Their former denomination was therefore *S. oviformis* or *S. cerevisiae* (race bayanus) in previous classifications. Domercq (1956) observed a lesser proportion of *S. oviformis* in the spontaneous microflora of Bordeaux region fermentations in the 1950s (one-fifth at the beginning of fermentation to one-third at the end). In the indigenous fermentative microflora of Bordeaux musts, certain strains of *S. cerevisiae* Gal\(^{-}\) which dominate from the start of alcoholic fermentation were selected over time. The causes of this change in the microflora, remain unknown. On the other hand, a systematic increase in the proportion of Gal\(^{-}\) strains during red or dry white wine fermentation has not been observed (Table 1.9). In botrytized sweet wines from Sauternes, the succession of strains is more distinct.

The same major strain is frequently encountered for several consecutive vintages in the same vineyard in spontaneous-fermentation red-grape must tanks. In 1990, one of the major strains was the same as the previous year in the red grape must tank of the Fz vineyard. Other strains appeared, however, which had not been isolated in 1989.

When sterile grape samples are taken, pressed steriley, sulfited at winemaking levels and fermented in the laboratory in sterile containers, one or several dominant strains responsible for spontaneous fermentations in the winery exist in some samples. These strains are therefore present at the vineyard. In practice, they probably begin to multiply as soon as the grapes arrive at the winery. A few days into the harvest, they infest the winery equipment which in turn ensures a systematic inoculation of the fresh grape crop.

The presence each year of the same dominant strain in the vineyard is not systematic (Table 1.10). In the Fz vineyard, the FzIb2-89 strain could not be isolated in 1991 although it was present in certain vineyard samples in 1990, 1992 and 1994. In 1993, another strain proved to be dominant in spontaneous fermentations of sterile grape samples.

<table>
<thead>
<tr>
<th>Stage of fermentation</th>
<th>Physiological race</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td><em>cerevisiae oviformis capensis chevalieri</em></td>
</tr>
<tr>
<td>Red wine(^a)</td>
<td></td>
</tr>
<tr>
<td>start</td>
<td>23</td>
</tr>
<tr>
<td>middle</td>
<td>10</td>
</tr>
<tr>
<td>end</td>
<td>14</td>
</tr>
<tr>
<td>middle</td>
<td>35</td>
</tr>
<tr>
<td>end</td>
<td>32</td>
</tr>
<tr>
<td>Sweet wine(^c)</td>
<td></td>
</tr>
<tr>
<td>start</td>
<td>37</td>
</tr>
<tr>
<td>middle</td>
<td>40</td>
</tr>
<tr>
<td>end</td>
<td>23</td>
</tr>
</tbody>
</table>

\(^a\)Isolation of 100 colonies from six tanks of a Pessac-Léognan vineyard (four tanks in 1989 and two tanks in 1990).

\(^c\)Isolation of 100 colonies in two barrels of a Sauternes vineyard in 1990.

<table>
<thead>
<tr>
<th>Year</th>
<th>Number of clones analyzed</th>
<th>Sample I</th>
<th>Sample II</th>
<th>Sample III</th>
</tr>
</thead>
<tbody>
<tr>
<td>1990</td>
<td>30</td>
<td>70%</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1991</td>
<td>60</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1992</td>
<td>85</td>
<td>25%</td>
<td>31%</td>
<td>3%</td>
</tr>
<tr>
<td>1993</td>
<td>74</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1994</td>
<td>79</td>
<td>87%</td>
<td></td>
<td>40%</td>
</tr>
</tbody>
</table>
The spontaneous microflora of *S. cerevisiae* seems to fluctuate. At present, the factors involved in this fluctuation have not been identified. In a given vineyard, spontaneous fermentation is not systematically carried out by the same strains each year; strain specificity does not exist and therefore does not participate in vineyard characteristics. Ecological observations do not confirm the notion of a vineyard-specific yeast. Furthermore, some indigenous strains, dominant in a given vineyard, have been found in other nearby or distant vineyards. For example, the FzIb2-89 strain, isolated for the first time in a vineyard in Pessac-Léognan, was later identified not only in the spontaneous fermentation of dry white and red wines of other vineyards in the same appellation, but also in relatively distant wineries as far away as the Médoc. This strain has since been selected and commercialized under the name Zymaflore F10.

In some cases (Figure 1.38), *S. cerevisiae* populations with a large clonal diversity carry out spontaneous must fermentation. Many strains coexist. Their proportions differ from the start to the end of fermentation and from one winery to another. In the Bordeaux region, this diversity causes slow fermentations and sometimes even stuck fermentations. No strain is capable of asserting itself. On the other hand, the presence of a small number of dominant strains generally characterizes complete and rapid spontaneous fermentations. These dominant strains are found from the start to the end of the fermentation.

In normal red winemaking conditions, the inoculation of the first tanks in a winery influences the wild microflora of non-inoculated tanks. The strain(s) used for inoculating the first tanks are frequently found in majority in the latter. Figure 1.39 provides an example comparing the microflora of a tank of Merlot from Pomerol, inoculated with an active dry yeast strain (522M) on the first day of the harvest, with a non-inoculated tank filled later. From the start of alcoholic fermentation, the selected strain is successfully implanted in the inoculated tank. Even in the non-inoculated tank, the same strain is equally implanted throughout the fermentation. It is therefore difficult to select the dominant wild strains in red winemaking tanks, when some of the tanks have been inoculated. An early and massive inoculation of the must, however, permits the successful implantation of

![Fig. 1.38. Breakdown of *S. cerevisiae* caryotypes in tank I of red grapes at LG vineyard (Pomerol, France) in 1989 (Frezier, 1992) (b, c, and d designate the start, middle, and end of alcoholic fermentation, respectively)](image-url)
different selected yeasts in several tanks at the same winery (Figure 1.40).

In white winemaking, inoculating rarely influences the microflora of spontaneous fermentations in wineries. For the most part, dominant indigenous strains in non-inoculated barrels of fermenting dry white wine are observed, whereas in the same wine cellar, other batches were inoculated

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**Fig. 1.39.** Breakdown of *S. cerevisiae* caryotypes in tank I and tank II P vineyard in 1990 (b, c, and d designate the start, middle, and end of alcoholic fermentation, respectively). Tank I is inoculated with 522M dry yeasts and tank II underwent spontaneous fermentation (b, c and d designate the start, middle and end of alcoholic fermentation, respectively)
with different selected yeasts. The absence of pumping-overs probably hinders the dissemination of the same yeasts in all of the fermenting barrels. This situation permits the fermentative behavior and enological interest of different selected strains to be compared with each other and with indigenous strains in a given vineyard. The barrels are filled with the same must; some are inoculated with the yeast to be compared. A sample of the biomass is taken at mid fermentation. The desired implantation is then verified by PCR associated with δ sequences. Due to the ease of use of this method, information on characteristics of selected strains and their influence on wine quality can be gathered at the winery.

Vezhinet et al. (1992) and Versavaud et al. (1995) have also studied the clonal diversity of yeast microflora in other vineyards. Their results confirm the polyclonal character of fermentative populations of S. cerevisiae. The notion of dominant strains (one to two per fermentation) is obvious in the work carried out in the Charentes region. As in Champagne and the Loire Valley, some Charentes region strains are found for several years in a row in the same winery. The presence of these dominant strains on the grape has been confirmed before any contact with winery equipment during several harvests.

Why do some S. cerevisiae strains issued from a very heterogeneous population become dominant during spontaneous fermentation? Why can they be found several years in a row at the same vineyard and wine cellar? Despite their practical interest, these questions have not often been studied and there are no definitive responses. It seems that these strains rapidly start and complete alcoholic fermentation and have a good resistance to sulfur dioxide (up to 10 g/hl). Furthermore, during mixed inoculations in the laboratory of either 8% ethanol or non-fermented musts, these strains rapidly become dominant when placed in the presence of other wild non-dominant strains of S. cerevisiae isolated at the start and end of fermentation. This subject merits further research. Without a doubt, it would be interesting to compare the genetic characteristics of dominant and non-dominant yeasts and their degree of heterozygosity. Considering the genome renewal theory of Mortimer et al. (1994) (Section 1.6.2), dominant strains are possibly more homozygous than non-dominant strains.

REFERENCES

Cytology, Taxonomy and Ecology of Grape and Wine Yeasts


