Helmut König
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Biology of Microorganisms on Grapes, in Must and in Wine

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Cover illustration top: Sporangiophore with sporangia from *Plasmopara viticola*; Low-Temperature-Scanning-Electron-Microscopy (H.-H. Kassemeyer, State Institute for Viticulture and Oenology, Freiburg; S. Boso and M. Düggelin, University of Basel); *below*: Microscope image of a mixture of *Dekkera/Brettanomyces* yeast species (Christoph Röder, Institute of Microbiology and Wine Research, University of Mainz)

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Foreword

The ancient beverage wine is the result of the fermentation of grape must. This naturally and fairly stable product has been and is being used by many human societies as a common or enjoyable beverage, as an important means to improve the quality of drinking water in historical times, as therapeutical agent, and as a religious symbol.

During the last centuries, wine has become an object of scientific interest. In this respect different periods may be observed. At first, simple observations were recorded, and subsequently, the chemical basis and the involvement of microorganisms were elucidated. At a later stage, the scientific work led to the analysis of the many minor and trace compounds in wine, the detection and understanding of the biochemical reactions and processes, the diversity of microorganisms involved, and the range of their various activities. In recent years, the focus shifted to the genetic basis of the microorganisms and the molecular aspects of the cells, including metabolism, membrane transport, and regulation. These different stages of wine research were determined by the scientific methods that were known and available at the respective time.

The recent "molecular" approach is based on the analysis of the genetic code and has led to significant results that were not even imaginable a few decades ago. This new wealth of information is being presented in the Biology of Microorganisms on Grapes, in Must, and in Wine. The editors were lucky in obtaining the cooperation of many specialists in the various fields. This joint international effort has resulted in a comprehensive book presenting our present day knowledge of a specialized group of organisms that are adapted to the very selective habitat of wine. The various contributions of the book have the character of reviews and contain an extensive bibliography, mainly of the actual scientific papers.

I sincerely wish the editors and the authors that the presented book will be widely received by the scientific community and will be frequently used as a welcome source of information and a helpful means for further work on the microorganisms of wine. Furthermore, understanding the intricate microbiological and biochemical processes during the fermentation should be helpful in the production of wine.

Mainz, June 2008 Ferdinand Radler

Preface

"Ce sont les microbes qui ont le dernier mot"
(Louis Pasteur)

Archaeology, genetics, ancient literature studies (*Epic of Gilgamesh*, ca. 2000 BC), paleobotany and linguistics point to the Neolithic period (ca. 8000 BC) as the time when domestic grape growing (*Vitis vinifera vinifera*) and wine making began, most probably in Transcaucasia (P. E. McGovern, 2003). For ages wine has been an essential part of the gracious, cultured and religious way of life.

Starting at the heartlands of Middle East, winemaking techniques have been empirically improved since neolithic times, expanding into experimental and scientific viticulture and oenology in our days. Despite these long traditions in wine making it was only 1857 that significant contributions of Louis Pasteur on alcoholic and lactic acid fermentation, as well as on acetic acid formation, proved that the conversion of grape juice into wine was a microbiological and not a purely chemical process.

Up to now, bounteous knowledge about wine making techniques and procedures has been accumulated, which was already found in several books about wine microbiology, biotechnology and laboratory practices. Especially in the last two decades, our knowledge about the role of microbes and their application as starter culture has been greatly increased.

Therefore, the aim of this book is to focus on the ecological and biological aspects of the wine-associated microbiota, starting from grape-colonising to wine-spoiling microbes. Purely technical aspects of winemaking are not a subject of this publication.

Growth in the must and wine habitat is limited by low pH values and high ethanol concentrations. Therefore, only acid- and ethanol-tolerant microbial groups can grow in grape juice, must and wine, which include lactic acid and acetic acid bacteria, yeasts and fungi. The most important species for wine-making are *Saccharomyces cerevisiae* and *Oenococcus oeni*, which perform the ethanol and malolactic fermentation, respectively. These two species are also applied as starter cultures. However, the diverse other microorganisms growing on grapes and must have a significant influence on wine quality.

The book begins with the description of the diversity of wine-related microorganisms, followed by an outline of their primary and energy metabolism. Subsequently, important aspects of the secondary metabolism are dealt with, since these activities have an impact on wine quality and off-flavour formation. Then chapters about stimulating and inhibitory growth factors follow. This knowledge is helpful for the growth management of different microbial species. During the last twenty years, significant developments have been made in the application of the consolidated findings of molecular biology for the rapid and real-time identification of certain species in mixed microbial populations of must. Basic knowledge was acquired about the functioning of regulatory cellular networks, leading to a better understanding of the phenotypic behaviour of the microbes in general and especially of the starter cultures as well as of stimulatory and inhibitory cell-cell interactions during winemaking. In the last part of the book, a compilation of some modern methods round off the chapters.

This broad range of topics about the biology of the microbes involved in the vinification process could be provided in one book only because of the input of many experts from different wine-growing countries. We thank all the authors for offering their experience and contributions. Finally, we express our special thanks to Springer for agreeing to publish this book about wine microbes.

We hope that this publication will help winemakers as well as scientists and students of oenology to improve their understanding of microbial processes during the conversion of must to wine.

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June 2008

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Part I
Diversity of Microorganisms

Chapter 1
Lactic Acid Bacteria

Helmut König and Jürgen Fröhlich

1.1 Introduction

In 1873, ten years after L. Pasteur studied lactic acid fermentation (between 1857 and 1863), the first pure culture of a lactic acid bacterium (LAB) ("*Bacterium lactis*") was obtained by J. Lister. Starter cultures for cheese and sour milk production were introduced in 1890, while fermented food has been used by man for more than 5,000 years (Schlegel 1999; Stiles and Holzapfel 1997). The first monograph by S. Orla-Jensen appeared in 1919. A typical lactic acid bacterium grown under standard conditions (nonlimiting glucose concentration, growth factors and oxygen limitation) is gram-positive, nonsporing, catalase negative in the absence of porphorinoids, aerotolerant, acid tolerant, organotrophic, and a strictly fermentative rod or coccus, producing lactic acid as a major end product. It lacks cytochromes and is unable to synthesize porphyrins. Its features can vary under certain conditions. Catalase and cytochromes may be formed in the presence of hemes and lactic acid can be further metabolized, resulting in lower lactic acid concentrations. Cell division occurs in one plane, except pediococci. The cells are usually nonmotile. They have a requirement for complex growth factors such as vitamins and amino acids. An unequivocal definition of LAB is not possible (Axelsson 2004).

Lactic acid bacteria are characterized by the production of lactic acid as a major catabolic end product from glucose. Some bacilli, such as *Actinomyces israeli* and bifidobacteria, can form lactic acid as a major end product, but these bacteria have rarely or never been isolated from must and wine. The DNA of LAB has a G + C content below 55 mol%. LAB are grouped into the *Clostridium* branch of gram-positive bacteria possessing a relationship to the bacilli, while *Bifidobacterium* belongs to the Actinomycetes. They are grouped in one order and six families. From the 32 described genera, only 22 species belonging to five genera have been isolated from must and wine (Table 1.1).

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The homofermentative species produce lactic acid (<85%) as the sole end product, while the heterofermentative species produce lactic acid, CO_2 and ethanol/acetate. At least half of the end product carbon is lactate. Heterofermentative LAB utilizes the pentose phosphate pathway, alternatively referred to as the phosphoketolase or phosphogluconate pathway. Homofermentative wine-related LAB include pediococci and group I lactobacilli. Obligate heterofermentative wine-related LAB include *Leuconostoc*, *Oenococcus*, *Weissella* and group III lactobacilli (Tables 1.2–1.5).

Table 1.1 Current taxonomic outline of lactic acid bacteria[a] of the *Clostridium* branch

Phylum Class Order	Family	Genus	Species from Must and Wine
"Firmicutes" "Bacilli" "Lactobacillales"	I. Lactobacillaceae	I. *Lactobacillus*	*Lb. brevis*, *Lb. buchneri*, *Lb. casei*, *Lb. curvatus*, *Lb. delbrueckii*, *Lb. diolivorans*, *Lb. fermentum*, *Lb. fructivorans*, *Lb. hilgardii*, *Lb. jensenii*, *Lb. kunkeei*, *Lb. mali*, *Lb. nagelii*, *Lb. paracasei*, *Lb. plantarum*, *Lb. vini*
		II. *Paralactobacillus*	
		III. *Pediococcus*	*P. pentosaceus*, *P. parvulus*, *P. damnosus*
	II. "Aerococcaceae"	I. *Aerococcus*	
		II. *Abiotrophia*	
		III. *Dolosicoccus*	
		IV. *Eremococcus*	
		V. *Facklamia*	
		VI. *Globicatella*	
		VII. *Ignavigranum*	
	III. "Carnobacteriaceae"	I. *Carnobacterium*	
		II. *Agitococcus*	
		III. *Alkalibacterium*	
		IV. *Allofustis*	
		V. *Alloiococcus*	
		VI. *Desemzia*	
		VII. *Dolosigranulum*	
		VIII. *Granulicatella*	
		IX. *Isobaculum*	
		X. *Lactosphaera*	
		XI. *Marinilactibacillus*	
		XII. *Trichococcus*	

(continued)

Table 1.1 (continued)

Phylum Class Order	Family	Genus	Species from Must and Wine
	IV. "Enterococcaceae"	I. *Enterococcus*	
		II. *Atopobacter*	
		III. *Melissococcus*	
		IV. *Tetragenococcus*	
		V. *Vagococcus*	
	V. "Leuconostocaceae"	I. *Leuconostoc*	*Lc. mesenteroides*
		II. *Oenococcus*	*O. oeni*
		III. *Weissella*	*W. paramesenteroides*
	VI. Streptococcaceae	I. *Streptococcus*	
		II. *Lactococcus*	

[a]Garrity GM (2005). Principal genera of LAB are underlined (Axelsson 2004)

Table 1.2 Differential characteristics of the wine-related lactic acid genera

Genus	Morphology from Glc	Carbohydrate fermentation[a]	Lactic acid isomer
Lactobacillus	Rods, coccobacilli cells single or in chains	homo- or heterofermentative facultatively heterofermentative	D, L, DL
Leuconostoc[b]	Spherical or lenticular cells in pairs or chains	heterofermentative	D
Oenococcus[b]	Spherical or lenticular cells in pairs or chains	heterofermentative	D
Pediococcus	Spherical cells, pairs or tetrads	homofermentative or facultatively heterofermentative[c]	DL, L
Weissella	Spherical, lenticular, irregular cells	heterofermentative	D, DL

[a]nonlimiting concentration of glucose and growth factors, but oxygen limitation.
[b]Differentiation of wine-related species of *Leuconostoc* and *Oenococcus* cf. Table 1.4.
[c]Facultatively heterofermentative species: *P. pentosaceus, P. acidilactici, P. claussenii.*

Our present knowledge about LAB in general (Carr et al. 1975; Wood and Holzapfel 1995; Holzapfel and Wood 1998; Wood 1999; Wood and Warner 2003; Salminen et al. 2004) and their activities on grape or in must and wine (Fleet 1993; Dittrich and Großmann 2005; Ribéreau-Gayon et al. 2006a, b; Fugelsang and Edwards 2007) has been compiled in several books.

1.2 Ecology

In general, LAB occur in habitats with a rich nutrition supply. They occur on decomposing plant material and fruits, in dairy products, fermented meat and fish, beets, potatoes, mash, sauerkraut, sourdough, pickled vegetables, silage, beverages, plants, water, juices, sewage and in cavities (mouth, genital, intestinal and respiratory tract) of human and animals. They are part of the healthy microbiota of the

human gut. Apart from dental caries, lactobacilli are generally considered apathogenic. *Lb. plantarum* could be associated with endocarditis, septicemia and abscesses. Some species are applied as starter cultures for food fermentation. Because of the acidification they prevent food spoilage and growth of pathogenic microorganisms (Hammes et al. 1991). Some LAB are employed as probiotics, which are potentially beneficial bacterial cells to the gut ecosystem of humans and other animals (Tannock 2005).

Lactic acid bacteria can also be found on grapes, in grape must and wine, and beer. Undamaged grapes contain $<10^3$ CFU per g and the initial titer in must is low (Lafon-Lafourcade et al. 1983). Because of the acidic conditions (pH: 3.0–3.5) grape must provides a suitable natural habitat only for a few microbial groups which are acid tolerant such as LAB, acetic acid bacteria and yeasts. While many microbes are inhibited by ethanol concentrations above 4 vol%, ethanol tolerant species survive in young wine or wine. Besides yeasts, some *Lactobacillus* species (e.g. *Lb. hilgardii*) and *Oenococcus oeni* can grow at higher ethanol concentrations. While only a few LAB species of the genera *Lactobacillus* (*Lb.*), *Leuconostoc* (*Lc.*), *Pediococcus* (*P.*), *Oenococcus* (*O.*) and *Weissella* (*W.*) (Table 1.1 and 1.2) and the acetic acid genera *Acetobacter* and *Gluconobacter* can grow in must and wine, more than 90 yeast species have been found. Malolactic fermentation by lactic acid bacteria is occasionally desirable during vinification, but they can also produce several off-flavours in wine. The genera *Carnobacterium*, *Enterococcus*, *Lactococcus*, *Streptococcus* and *Bifidobacterium* have not been isolated from must and wine.

1.3 Phenotypic and Phylogenetic Relationship

The classification of LAB is largely based on morphology (rods, cocci, tetrads), mode of glucose fermentation, substrate spectrum, growth at different temperatures (15 and 45°C), configuration of lactic acid produced, ability to grow at high salt concentrations (6.5% NaCl; 18% NaCl), and acid, alkaline or ethanol tolerance, as well as fatty acid composition and cell wall composition, lactic acid isomers from glucose, behaviour against oxygen (anaerobic or microaerophilic growth), arginine hydrolysis, acetoin formation, bile tolerance, type of hemolysis, production of extracellular polysaccharides, growth factor requirement, presence of certain enzymes, growth characteristics in milk, serological typing, murein, teichoic acid and menaquinone type, fatty acid composition and electrophoretic mobility of the lactate dehydrogenases and DNA, PCR-based fingerprinting techniques, DNA-DNA homology and soluble protein pattern, 16S rDNA and gene sequencing (e.g. *recA*) (Axelsson 2004).

The genera and species of lactic acid bacteria occurring in must and wine can be differentiated by phenotypic features (Tables 1.2–1.5). The species can be identified by the API 50 CHL identification system (Bio-Mérieux) or the Biolog Microbial Identification System (Biolog, Inc.).

The first taxonomic outline given by Orla-Jensen (1919) is still of some importance. Based on physiological features Kandler and Weiss (1986) divided the genus *Lactobacillus* into the three groups (1) obligate homofermenters, (2) faculative heterofermenters and (3) obligate heterofermenters (Table 1.3). The phylogenetic relationship has been revealed by rRNA sequencing (Fig. 1; Collins et al. 1990, 1991,1993; Martinez-Murcia and Collins 1990; Dicks et al. 1995). According to the 16S rDNA analysis Collins et al. (1990, 1991, 1993) divided the genus *Lactobacillus* into three groups. Group I contains obligate homofermentative species and facultatively heterofermentative species. Group II contains more than 30 *Lactobacillus* species and five pediococcal species. The wine-related facultative heterofermenters *Lb. casei* and the obligate heterofermenters *Lb. brevis*, *Lb. buchneri* and *Lb. fermentum* belong to this group. Group III contains the genus *Weissella,* the leuconostocs (*Lc. mesenteroides)* and *O. oeni*. Schleifer and Ludwig (1995a, b) proposed the phylogenetic groups (1) *Lb. acidophilus* group, (2) *Lb. salivarius* group, (3) *Lb. reuteri* group (*Lb. fermentum*), (4) *Lb. buchneri* group (*Lb. buchneri*, *Lb. fructovorans*, *Lb. hilgardii*) and (5) *Lb. plantarum* group.

The *Leuconostoc* group can be clearly separated from other lactobacilli (Collins et al. 1991; Schleifer and Ludwig 1995a, b). The wine-related species *Lc. mesenteroides* forms a subgroup of the obligately heterofermentative *Leuconostoc* group. *Lc. oenos* was placed in the separate genus *Oenococcus* (Dicks et al. 1995) consisting of the two species *O. oeni* and *O. kitahareae* (Endo and Okada 2006). The latter was isolated from a composting distilled shochu residue. It does not grow at acidic conditions (pH 3.0–3.5) of must and lacks the ability to perform malic acid degradation.

Hammes and Hertel (2003) described seven phylogenetic groups, which were modified by Dellaglio and Felis (2005) (cf. Table 1.3).

1.4 Physiology

Carbohydrates are used as carbon and energy source by a homofermentative or heterofermentative pathway. Sugars or oligosaccharides are taken up by the phosphotransferase system (PTS, e.g. lactose: *Lb. casei*) or the permease system. Homofermentation of hexoses procedes via the Embden-Meyerhof-Parnas pathway, while heterofermentation is performed via the 6-P-gluconate/phosphoketolase pathway resulting in lactate, acetate/ethanol and CO_2 as endproducts or the Bifidus pathway (*Bifidobacterium*). Pentoses are fermented by 6-phosphocluconate/phosphoketolase pathway leading to lactic acid, acetic acid/ethanol and carbon dioxide. Some lactibacilli such as *Lb. salivarius* (Raibaud et al. 1973) or *Lb. vini* (Rodas et al. 2006) can ferment pentoses homofermentatively. Some strains can produce acetate, ethanol and formate from pyruvate under low substrate concentrations and strictly anaerobic conditions (Hammes and Vogel 1995). Lactic acid bacteria form D(−) or L(+) lactic acid or a racemic mixture of lactic acid isomers (Kandler 1983).

Table 1.3 Differential characteristics of wine-related species of the genus *Lactobacillus*

Characteristics	*Lb. brevis*	*Lb. buchneri*	*Lb. casei*[a]	*Lb. curvatus*	*Lb. delbrueckii*[d]	*Lb. diolivorans*	*Lb. fermentum*	*Lb. fructivorans*[b]
Phylogenetic group	I	A	D	G	C	A	F	A
Fermentation mode	III	III	II	II	I	III	III	III
Mol% G + C	44–47	44–46	45–47	42–44	49–51	40	52–54	38–41
Murein type	Lys-D-Asp	Lys-D-Asp	Lys-D-Asp	Lys-D-Asp	Lys-D-Asp	n.d.	Orn-D-Asp	Lys-D-Asp
Teichoic acid	glycerol	n.d.	n.d.	n.d.	n.d.	n.d.	ribitol or	n.d.
Lactic acid	DL	DL	L	DL	D	n.d.	DL	DL
Growth at 15/45 °C	+/–	+/–	+/–	+/-	–/+	+/–	–/+	+/–
NH_3 from Arg	+	+	n.d.	n.d.	d	n.d.	+	+
Fermentation of:								
Amygdalin	n.d.	n.d.	+	–	+	–	n.d.	n.d.
L-Arabinose	+	+	–	–	n.d.	+	d	–
Cellobiose	–	–	+	+	d	–	d	–
Esculin	d	d	+	+	n.d.	n.d.	–	–
Galactose	d	d	n.d.	n.d.	d	+	+	–
Gluconate	n.d.	n.d.	+	+	n.d.	+	n.d.	n.d.
Lactose	n.d.	n.d.	n.d.	n.d.	+	–	n.d.	n.d.
Maltose	+	+	n.d.	n.d.	+	+	+	d
Mannitol	n.d.	n.d.	+	+	–	–	n.d.	n.d.
D-Mannose	–	–	n.d.	n.d.	+	–	w	–
Melezitose	–	+	+	–	n.d.	–	–	–
Melibiose	+	+	–	–	–	+	+	–
D-Raffinose	d	d	–	–	–	w	+	–
Ribose	+	+	+	–	n.d.	+	+	w
Salicin	n.d.	n.d.	n.d.	n.d.	+	–		n.d.
Sorbitol	n.d.	n.d.	+	–	n.d.	–	n.d.	n.d.
Sucrose	d	d	+	+	+	–	+	d
Trehalose	–	–	n.d.	n.d.	+	n.d.	d	–
D-Xylose	d	d	–	–	n.d.	+	d	–

Characteristics	*Lb. hilgardii*[b]	*Lb. jensenii*	*Lb. kunkeei*	*Lb. mali*	*Lb. nagelii*	Lb. paracasei[e]	*Lb. plantarum*[c]	*Lb. vini*
Phylogenetic group	A	C	B	H	H	D	E	H
Fermentation mode	III	II	III	I	I	II	II	I
Mol% G + C	39–41	35–37	n.d.	32–34	n.d.	45–47	44–46	39
Murein type	Lys-D-Asp	Lys-D-Asp	Lys-D-Asp	mDAP direct	mDAP direct	Lys-D-Asp	mDAP direct	Lys-D-Asp
Teichoic acid glycerol	glycerol	n.d.	n.d.	n.d.	n.d.	n.d.	ribitol or	n.d.
Lactic acid	DL	D	L	L	DL	L	DL	DL
Gowth at 15/45 °C	+/–	–/+	+/–	+/ n.d.	+/+	+/n.d.	–/+	–/+
NH_3 from Arg	+	+	+	n.d.	–	n.d.	–	–
Fermentation of:								
Amygdalin	n.d.	+	–	n.d.	+	+	+	+
Arabinose	–	n.d.	–	–	–	–	d	+
Cellobiose	–	+	–	+	+	+	+	+
Esculin	–	n.d.	–	n.d.	n.d.	+	+	+
Galactose	d	+	–	n.d.	+	n.d.	n.d.	–
Gluconate	n.d.	n.d.	–	n.d.	–	+	+	–
Lactose	n.d.	–	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
Maltose	+	d	–	–	+	n.d.	n.d.	+
Mannitol	n.d.	d	+	+	+	+	+	–
D-Mannose	–	+	–	n.d.	+	n.d.	n.d.	+
Melezitose	d	–	–	n.d.	–	+	+	–
Melibiose	–	n.d.	–	n.d.	–	–	+	–
D-Raffinose	–	–	w	n.d.	–	–	+	–
Ribose	+	n.d.	–	n.d.	–	+	+	+
Salicin	n.d.	+	n.d.	n.d.	+	n.d.	n.d.	n.d.
Sorbitol	n.d.	n.d.	–	+	+	d	+	–
Sucrose	d	+	+	n.d.	+	+	+	+
Trehalose	–	+	–	n.d.	+	n.d.	n.d.	+
D-Xylose	+	n.d.	–	n.d.	–	–	d	–

+, ≥90% of the strains are positive; –, ≥90% of the strains are negative; *d* 11–89% of the strains are positive; *w* weak positive reaction (Hammes and Vogel 1995). Three phylogentic groups (Hammes and Vogel 1995; Schleifer and Ludwig 1995a, b) were described in 1995 (group A: *Lb. delbrueckii* group; group B: *Lb. casei-Pediococcus* group; group C: *Leuconostoc* group). Eight years later Hammes and Hertel (2003) described seven phylogenetic groups, which were modified by Dellaglio and Felis (2006) (wine-related species are given in brackets): A. *Lb. buchneri* group (group a: *Lb. buchneri*, *Lb. diolivorans*, *Lb. hilgardii*; group b: *Lb. fructivorans*). B. *Lb. kunkeei* group (*Lb. kunkeei*). C. *Lb. delbrueckii* group (*Lb. delbruechii*, *Lb. jensenii*). D. *Lb. casei* group (group a: *Lb. casei*, *Lb. paracasei*). E. *Lb. plantarum* group (group a: *Lb. plantarum*). F. *Lb. reuteri* group (group a: *Lb. fermentum*). G. *Lb. sakei* group (*Lb. curvatus*). H. *Lb. salivarius* group (*Lb. mali*, *Lb. nagelii, Lb. vini*). I. *Lb. brevis* group (*Lb. brevis*). Definition of the fermentative groups (Kandler and Weiss 1986; Hammes and Vogel 1995; Schleifer and Ludwig 1995a, b): Group I: Obligately homofermentative lactobacilli. Hexoses are almost exclusively (>85%) fermented to lactic acid by

(continued)

Table 1.3 (continued)

the Embden-Meyerhof-Parnas pathway (EMP). The organisms possess a fructose-1.6-bisphosphate aldolase, but lack a phosphoketolase. Gluconate of pentoses are not fermented. Group II: Facultatively heterofermentative lactobacilli. Hexoses are almost exclusively fermented to lactic acid by the Embden-Meyerhof-Parnas pathway (EMP). The species possess both a fructose-1.6-bisphosphate aldolase and a phosphoketolase. Consequently, the species can ferment hexoses and pentoses as well as gluconate. In the presence of glucose the enzymes of the phosphogluconate pathway are repressed. Group III: Obligately heterofermentative lactobacilli. Hexoses are fermented by the phosphogluconate pathway yielding lactic acid, ethanol/acetic acid and CO_2 in nearly equimolar amounts. Pentoses are fermented by the same pathway

[a]formation of acetate and formate from lactate or pyruvate, or acetate and CO_2 in the presence of oxidants;

[b]high tolerance to ethanol and acidity;

[c]nitrate reduction, presence of pseudocatalase;

[d]subsp. *Lactis*;

[e]subsp. *Paracasei*; *N.d.* no data given

The Embden-Meyerhof-Parnas pathway is used by lactobacilli (group I and II; Table 1.3) and pediococci, while group III of lactobacilli, leuconostocs and oenococci use the 6-phosphogluconate/phosphoketolase pathway (other designations: pentose phosphate pathway, pentose phosphoketolase pathway, hexose monophosphate pathway). Changes in the end product composition can be influenced by environmental factors. Depending on the growth conditions the end products of homofermenters can be changed largely. In addition to glucose, the hexoses mannose, fructose and galactose may be fermented after isomerisation and/or phosphorylation. Galactose is used via the tagatose pathway by e.g. *Lb. casei*.

Under anaerobic conditions pyruvate can be metabolized by *Lb. casei* to formate and acetate/ethanol (pyruvate formate lyase system) under glucose limitation. End produts are lactate, acetate, formate and ethanol (mixed acid fermentation). Under aerobic conditions *Lb. plantarum* can convert pyruvate to CO_2 and acetyl phosphate with a pyruvate oxidase (Sedewitz et al. 1984).

Flavin-containing enzymes such as NADH:H_2O_2 oxidase and NADH:H_2O oxidase (Condon 1987) can occur in lactic acid bacteria. Oxygen acts as external electron acceptor. Oxygen-dependent glycerol fermentation by *P. pentosaceus* and mannitol fermentation of *Lb. casei* are examples. An oxygen-dependent lactate metabolism has been proposed for *Lb. plantarum* involving NAD^+-dependent and/or NAD^+-independent lactate dehydrogenase, a pyruvate oxidase and an acetate kinase (Murphy et al. 1985).

Lactobacilli interact with oxygen. Some lactic acid bacteria use high intracellular manganese concentration for protection against superoxide (30–35 mM; Archibald 1986). Theobald et al. (2005) found a growth stimulation of *O. oeni* at concentrations of 68 µM or 34 mM manganese in the growth medium. In some strains 34 mM manganese could replace tomato juice. Other compounds are also stimulatory for oenococci (Theobald et al. 2007a, b).

Citrate can lead to diacetyl/actoin formation if the excess of pyruvate is reduced to lactic acid. Oxaloacetate can also function as electron acceptor leading to succinic acid formation when *Lb. plantarum* was grown on mannitol (Chen and McFeeters 1986). *Lb. brevis* and *Lb. buchneri* can use glycerol as electron acceptor in an anaerobic cofermentation with glucose leading to lactate, acetate, CO_2 and 1.3-propandiol (Schütz and Radler 1984a, b). Fructose can be fermented via the 6-phosphocluconate/phosphoketolase pathway and function as electron acceptor to yield mannitol by *Lb. brevis* (Eltz and Vandemark 1960). Malic acid can be used as sole energy source by *Lb. casei* yielding acetate, ethanol and CO_2 or it can be converted to L-lactate and CO_2 (malolactic fermentation) by e.g. *O. oeni* (Radler 1975). The biosynthesis of amino acids in lactic acid bacteria is limited. Some have peptidases and can hydrolyse proteins. Lactic acid bacteria can also perform chemical cell communication (Nakayama and Sonomoto 2002).

1.5 Genetics

The genome size of lactic acid bacteria varies (Morelli et al. 2004). The genome of *Lb. paracasei* consists of 3.4 Mb (Ferrero et al. 1996) and that of *Lb. plantarum* of 3.4 Mb (Chevallier et al. 1994). Restriction maps have been obtained from *O. oeni* (Ze-Ze et al. 2000). The total genome of more than 20 lactic acid bacteria is available, including the wine-related strains *Lc. mesenteroides*, *Lb. plantarum*, *Lb. brevis*, *Lb. paracasei*, *Lb. casei*, *O. oeni* and *P. pentosaceus* (Makarova et al. 2006).

Lactic acid bacteria possess circular as well as linear plasmids associated with carbohydrate fermentation and proteinase activities, bacteriocin production, phage defense mechanisms, and antibiotic resistance mechanisms (Morelli et al. 2004).

Phages have been found with the wine-related species of *Lactobacillus* (*Lb. casei*, *Lb. fermentum*, *Lb. plantarum*,), *Leuconostoc* (*Lc. mesenteroides*) and *Oenococcus* (*O. oeni*) (Josephsen and Neve 2004). They can cause stuck malolactic fermentation (Poblet-Icart et al. 1998).

1.6 Activities in Must and Wine

Lactic acid bacteria are involved in food and feed fermentation and preservation as well as food digestion in the intestinal tracts of humans and animals. Due to its tolerance against ethanol and acidic conditions, LAB can grow in must. Generally they are inhibited at ethanol concentrations above 8 vol%, but *O. oeni* tolerates 14

vol% and *Lb. brevis*, *Lb. fructivorans* and *Lb. hilgardii* can be found even in fortified wines up to an ethanol concentration of 20 vol%. Slime-producing strains of *P. damnosus* grow up to 12 vol% of ethanol. Lactic acid bacteria isolated from wine grow between 15 and 45°C in the laboratory with an optimal growth range between 20 and 37°C. Best growth in must during malolactic fermentation is obtained around 20°C. During the first days of must fermentation the CFU of LAB increases from 10^2 to 10^4–10^5 per ml. After the alcoholic fermentation and during the malic acid fermentation, the cell number can reach a titer of 10^7–10^8 CFU per ml (Ribérau-Gayan et al. 2006a, b). The titer of different lactic acid species during alcoholic fermentation has been determined by Lonvaud-Funel et al. (1991): *O. oeni*, 3.4×10^6 (day 13, alcohol content: 18 vol%); *Lc. mesenteroides*, 9.6×10^4 (day 6, alcohol content: 9 vol%); *P. damnosus*, 3.8×10^4 (day 3, alcohol content: 7 vol%); *Lb. hilgardii*, 8.0×10^4 (day 3, alcohol content: 7 vol%); *Lb. brevis*, 2.0×10^4 (day 3, alcohol content: 7 vol%) and *Lb. plantarum*, 2.0×10^4 (day 3, alcohol content: 7 vol%).

Lactic acid bacteria gain their energy mainly from sugar fermentation. They use both main hexoses of the wine, glucose and fructose, as energy and carbon source. In this respect they are competitors of the ethanol producing yeast *Saccharomyces cerevisiae*. The heterofermentative LAB in wine can also use the pentoses (arabinose, xylose, ribose), which occur in minor concentrations in wine.

Lactic acid bacteria also metabolize the three main acids of must: tartrate, malate and citrate. Citrate is converted to lactate, acetic acid, CO_2 and acetoin. Malate is converted to L-lactate and CO_2 (malolactic fermentation). Especially in northern countries, where must can have high acidity, the biological reduction with starter cultures of *O. oeni* is an important step in vinification. The malolactic enzyme has been found in many lactic acid bacteria occurring in wine (e.g. *Lb. casei*, *Lb. brevis*, *Lb. buchneri*, *Lb. delbruechii*, *Lb. hilgardii*, *Lb. plantarum*, *Lc. mesenteroides, and O. oeni*). *O. oeni* is applied for reduction of the malic acid content because of its high tolerance against ethanol and acidity. Malolactic fermentation and the use of sugars can lead to a more stable wine. Tartrate can be converted to lactate, acetate and CO_2 by the homofermentative lactic acid bacterium *Lb. plantarum* and to acetate and CO_2 or fumaric acid (succinic acid) by the heterofermentative lactic acid bacterium *Lb. brevis* (Radler and Yannissis 1972).

Lactic acid bacteria produce different biogenic amines. *O. oeni*, *P. cerevisiae* and *Lb. hilgardii* (Landete et al. 2005; Mangani et al. 2005) are examples of producers of biogenic amines. The most important is histamine, which is produced by decarboxylation of histidine. The COST Action 917 (2000–2001) of the EU "Biologically active amines in food" suggested prescriptive limits for histamine (e.g. France: $8\,mg\,l^{-1}$, Germany: $2\,mg\,l^{-1}$) in wines. Biogenic amines can cause health problems (Coton et al. 1998) and sensory defects in wine (Lehtonen 1996; Palacios et al. 2004). From arginine, ammonium is liberated by heterofermentative species such as *Lb. higardii* and *O. oeni*, but also by facultatively heterofermentative species like *Lb. plantarum*.

Lactic acid bacteria have an influence on the flavour of wine, because they can produce acetic acid, diacetyl, acetoin, 2,3- butandiol, ethyl lactate, diethyl succinate and acrolein. They cause a decrease in colour up to 30%. In German wines 1.08 g acetic acid per l white wine or 1.20 g acetic acid per l red wine are the upper limits for acetic acid, while e.g. "Beerenauslese" (German quality distinction) can even have higher concentrations. The natural value is 0.3–0.4 g l^{-1} and it becomes sensory-significant at concentrations above 0.6 g l^{-1}. Aerobic acetic acid bacteria, facultatively anaerobic heterotrophic lactic acid bacteria, yeast under difficult fermentation conditions and *Botrytis cinerea* on infected grapes are the potential producers. Fructose is reduced to mannitol or converted to erythrol and acetate. Heterofermentative lactic acid bacteria can produce higher concentrations of acetic acid (>0.6 g L^{-1}), especially in the absence of pantothenic acid (Richter et al. 2001). Lactic acid bacteria can convert sorbic acid, which is used because of its antifungal properties, to 2-ethoxy-3.5-hexadiene (geranium-like odour) (Crowel and Guymon 1975). Glycerol is converted to propandiol-1.3 or allylalcohol and acrolein leading to bitterness (Schütz and Radler 1984a, b). Off-flavour is produced by *O. oeni* from cysteine and methionine. Cysteine is transformed into hydrogen sulfide or 2-sulfanyl ethanol and methionine into dimethyl disulfide, propan-1-ol, and 3-(methasulfanyl) propionic acid. They increase the complexity of the bouquet. The latter has an earthy, red-berry fruit flavour (Ribéreau-Gayon et al. 2006a, b). Lactic acid bacteria may produce a smell reminiscent of mice (mousiness). Species of *Lactobacillus* such as *Lb. brevis*, *Lb. hilgardii* and *Lb. fermentum* produce 2-acetyltetrahydropyridine (perception threshold: 1.6 ng l^{-1}) from ethanol and lysine (Heresztyn 1986). Also 2-acetyl-1-pyrroline and 2-ethyltetrahydropyridine can contribute to this off-flavour (Costello and Henschke 2002). Ethyl carbamate is produced from urea and ethanol by *O. oeni* and *Lb. hilgardii* (Uthurry et al. 2006), which probably is carcinogenic.

Polysaccharide production (Claus 2007) leads to graisse of the must, which causes problems during filtration. *P. damnosus* increases viscosity. It produces a glucose homopolymer. The repeating unit is a β-1.3 linked glucose disaccharide carrying a β-1.2 linked glucose site group [3)-β-D-Glc*p*-(1.3)-[β-D-Glc*p*-(1.2)]-β-D-Glc*p*-(1] (Llaubères et al. 1990; Dueñas et al. 2003). The viscosity, which is influenced by many factors such as the ethanol concentration and temperature, becomes apparent at 10^7 colony forming units.

Lactic acid disease occurs at higher sugar concentrations when lactic acid bacteria grow during ethanolic fermentation at higher pH values and low nitrogen concentrations. Higher amounts of acetic acid can be produced, which hampers the activities of yeast. Most often, LAB do not multiply or disappear during alcoholic fermentation, except oenococci, which resist at low cell levels. It was found that fatty acids (hexanoic, octanoic and decanoic acid) liberated by growing yeast have a negative effect on bacterial growth (Lonvaud-Funel et al. 1988). Oenococci can grow during the stationary/death phase of the yeasts after alcoholic fermentation, when released cell constituents of yeasts stimulate bacterial growth. In this stage oenococci have an influence on yeast lysis by producing glycosidases and proteases.

The degradation of sugars and acids contributes to the microbial stabilisation of wine by removing carbon and energy substrates. Low concentrations of diacetyl increase the aromatic complexity. If the concentration of volatile acids increases 1 g l^{-1} the lactic disease becomes apparent, which can lead to a stuck alcoholic fermentation.

Lactic acid bacteria potentially produce antimicrobial components (Rammelberg and Radler 1990; Blom and Mörtvedt 1991) such as acetic acid, higher concentrations of carbon dioxide, hydrogen peroxide, diacetyl, pyroglutamic acid and bacteriocins, which inhibit the growth of other bacterial and yeast species. Brevicin from *Lb. brevis* inhibits growth of *Oenococus oeni* and *P. damnosus* (Rammelberg and Radler 1990).

The malolactic fermentation and the consumption of nutrients (hexoses and pentoses) as well as the production of bacteriocines (De Vuyst and Vandamme 1994) lead to a stabilization of wine.

1.7 Characteristics of Genera and Species of Wine-Related Lactic Acid Bacteria

1.7.1 Genus Lactobacillus

Lactobacillus is one of the most important genera involved in food microbiology and human nutrition, owing to their role in food and feed production and preservation, as well as their probiotic properties. In October 2008 this genus contained in total 174 validly described species (including subspecies) (DSMZ 2008). *Lactobacillus* species live widespread in fermentable material. Lactobacilli contribute to the flavour of fermented food by the production of diacetyl, H_2S and amines. They play a role in the production as well in the spoilage of food (sauerkraut, silage, dairy and meat as well as fish products) and beverages (beer, wine, juices) (Kandler and Weiss 1986; Hammes et al. 1991).

Lactobacilli are straight gram-positive non-motile or rarely motile rods (e.g. *Lb. mail*), with a form sometimes like coccobacilli. Chains are commonly formed. The tendency towards chain formation varies between species and even strains. It depends on the growth phase and the pH of the medium. The length and curvature of the rods depend on the composition of the medium and the oxygen tension. Peritrichous flagellation occurs only in a few species, which is lost during growth in artificial media. They are aciduric or acidophilic. The maximum for growth pH is about 7.2.

The murein sacculi possess various peptidoglycan types (Lys-D-Asp, m-Dpm-direct, Orn-D-Asp, Lys-Ala, Lys-Ala_2, Lys-Ala-Ser, Lys-Ser Ala_2) of group A. Polysaccharides are often observed. Membrane-bound teichoic acids are present in all species and cell wall-bound teichoic acids in some species (Schleifer and Kandler 1972).

The G + C content of the DNA ranges from 32 to 53 mol%.

Lactobacilli are strict fermenters. They can tolerate oxygen or live anaerobic. They have complex nutritional requirements for carbohydrates, amino acids, peptides, fatty acids, nucleic acid derivatives, vitamins and minerals.

Some species possess a pseudocatalase and some strains can take up porphorinoids and then exhibit catalase, nitrite reductase and cytochrome activities.

They gain energy by homofermentative or heterofermentative carbohydrate fermentation in the absence or presence of oxygen. An energy source is also the conversion of carbamyl phosphate to CO_2 and NH_3 during arginine degradation. They possess flavine-containing oxidases and peroxidases to carry out an oxidation with O_2 as the final electron acceptor. The pathways of sugar fermentation are the Embden-Meyerhof pathway converting 1 mol hexose to 2 mol lactic acid (homolactic fermentation) and the phosphoketolase pathway (heterolactic fermentation) resulting in 1 mol lactic acid, ethanol/acetate and CO_2. Pyruvate produced during hexose fermentaion may be converted to lactate, but also to other products such as diacetyl or acetic acid, ethanol and formate/CO_2. In the presence of oxygen, lactate can be converted to pyruvate and consequently to acetic acid and CO_2 or acetate and formate. The conversion of glycerol to 1,3-propanediol with glucose serving as electron donor was observed in *Lb. brevis* isolated from wine (Schütz and Radler 1984a, b). The homofermentative species possess an FDP aldolase, while the heterofermentative species have a phosphoketolase. The facultative heterofermenters possess an inducible phosphoketolase. Heterofermentative species can also use pentoses as substrate. Some homofermenters use pentores homofermentatively (Rodas et al. 2006)

Sucrose is also used for the formation of dextrans with the help of dextran sucrase. Fructose can serve as electron acceptor and mannitol is formed by heterofermentative species. Monomeric sugars and saccharides are taken up by permeases or the phosphotransferase system. They are split inside the cell by glycosidases. Galactose-6-phosphate from lactose phosphate is fermented via the tagatose-6-phosphate pathway (Kandler 1983). Several organic acids such as citric acid, tartaric acid or malic acid are degraded (Radler 1975). Several amino acids are decarboxylated to biogenic amines.

Depending on the stereospecificity of the lactate dehydrogenase or the presence of an inducible lactate racemase lactate may have the D(−) or L(+) configuration. The lactate dehydrogenases can differ with respect to electrophoretic mobility and kinetic properties. Some enzymes are allosteric with FDP and Mn^{2+} as effectors.

Plasmids linked to drug resistance or lactose metabolism are often found (Smiley and Fryder 1978). Double-stranded DNA phages have been isolated (Sozzi et al. 1981) and lysogeny is widespread (Yokokura et al. 1974). Strains producing bacteriocins (lactocins) have been found among the homo- and heterofermentative species (Tagg et al. 1976). Several serological groups have been designed. From the species in must, *Lb. plantarum* belongs to group D (antigen: ribitol teichoic acid), *Lb. fermentum* to group F and *Lb. brevis* to group E (Archibald and Coapes 1971).

The complete genome of eleven *Lactobacillus*-species has been sequenced; it includes the wine related species *Lb. casei* and *Lb. plantarum* (http://www.ncbi.nlm.nih.gov/genomes/lproks.cgi).

Some characteristics of the species are compiled in Table 1.3. A combination of physiological and biochemical as well as molecular tests are required for the unambiguous identification of *Lactobacillus* species (Pot et al. 1994; Hammes and Vogel 1995). Hundred and fifteen validly published species of the genus *Lactobacillus* can be assigned to nine groups (cf. Table 1.3) (Yang and Woese 1989; Collins et al. 1991; Hammes et al. 1991; Hammes and Vogel 1995; Dellaglio and Felis 2005). Out of about 174 described species/subspecies, sixteen have been found in must and wine (Table 1.3) (Ribéreau-Gayon et al. 2006 a, b; Fugelsang and Edwards 2007).

The type species is *Lb. delbrueckii* DSM 20074^T.

Lb. brevis
Morphology: Rods. 0.7–1.0 µm × 2.0–4.0 µm. Single or chains.
Isolation: Milk, cheese, sauerkraut, sourdough, silage, cow manure, mouth, intestinal tract of humans and rats, grape must/wine.
Type strain: DSM 20054.

Lb. buchneri
Morphology: Rods. 0.7–1.0 µm × 2.0–4.0 µm. Single or short chains.
Characteristics: As described for *Lb. brevis* except the additional fermentation of melezitose and the distinct electrophoretic behaviour of L-LDH and D-LDH.
Isolation: Milk, cheese, plant material and human mouth, grape must/wine.
Type strain: DSM 20057.

Lb. casei
Morphology: Rods. 0.7–1.1 µm × 2.0–4.0 µm.
Isolation: Milk, cheese, dairy products, sour dough, cow dung, silage, human intestinal tract, mouth and vagina, sewage, grape must/wine.
Type strain: DSM 20011.

Lb. cellobiosus
→ *Lb. fermentum*.

Lb. curvatus
Morphology: Bean-shaped rods. 0.7–0.9 µm × 1.0–2,0 µm. Pairs, short chains or close rings. Sometimes motile.
Characteristics: LDH is activated by FDP and Mn^{2+}. Lactic acid racemase.
Isolation: Cow dung, milk, silage, sauerkraut, dough, meat products, grape must/wine.
Type strain: DSM 20019 (subsp. *curvatus*).

Lb. delbrueckii
Morphology: Rods. 0.5–0.8 µm × 2.0–9.0 µm. Single or in short chains.
Isolation: Milk, cheese, yeast, grain mash, grape must/wine.
Type strain: DSM 20072 (subsp. *lactis*).

Lb. diolivorans
Morphology: Rods. 1.0 µm × 2.0–10.0 µm. Single, pairs and short chains.
Isolation: Maize silage, grape must/wine.
Type strain: DSM 14421.

Lb. fermentum
Morphology: Rods. diameter 0.5–0.9 µm, length variable. Single or pairs.
Isolation: Yeast, milk products, sourdough, fermenting plant material, manure, sewage, mouth and faeces of man, grape must/wine.
Type strain: DSM 20052.

Lb. fructivorans
Morphology: Rods. 0.5–0.8 µm × 1.5–4.0 µm (occasionally 20 µm). Single, pairs, chains or long curved filaments.
Isolation: Spoiled mayonnaise, salad dressing, vinegar preserves, spoiled sake, dessert wine and aperitifs.
Type strain: DSM 20203.

Lb. heterohiochii
→ *Lb. fructivorans*.

Lb. hilgardii
Morphology: Rods. 0.5–0.8 µm × 2.0–4.0 µm. Single, short chains or long filaments.
Isolation: Wine samples.
Type strain: DSM 20176.

Lb. jensenii
Morphology: Rods. 0.6–0.8µm × 2.0–4.0 µm. Single or short chains.
Isolation: Human vaginal discharge and blood clot, grape must/wine.
Type strain: DSM 20557.

Lb. kunkeei
Morphology: Rod. 0.5 µm × 1.0–1.5 µm.
Characteristics: Week catalase activity.
Isolation: Commercial grape wine undergoing a sluggish/stuck alcoholic fermentation.
Type strain: DSM 12361.

Lb. leichmannii
→ *Lb. delbrueckii* subsp. *lactis*

Lb. mali
Morphology: Slender rods. 0.6 µm × 1.8–4.2 µm, Single, in pairs, palisades and irregular clumps.
Characteristics: Motile by a few peritrichous flagella. Pseudocatalase activity in MRS medium containing 0.1% glucose. Menaquinones with predominantly eight or nine isoprene residues.

Isolation: Apple juice, cider and wine must.
Type strain: DSM 20444.

Lb. nagelli
Morphology: Rods. 0.5 µm × 1.0–1.5 µm.
Characteristics: Nitrate reduction.
Isolation: Partially fermented wine with sluggish alcoholic fermentation.
Type strain: DSM 13675.

Lb. paracasei
Morphology: Rods. 0.8–1.0 µm × 2.0–4.0 µm. Single or chains.
Isolation: Dairy products, silage, humans, clinical sources, grape must/wine.
Type strain: DSM 5622 (subsp. *paracasei*).

Lb. plantarum
Morphology: Rods. 0.9–1.2 µm × 3.0–8.0 µm. Single, pairs or short chains.
Characteristics: Nitrate can be reduced under glucose limitation and a pH above 6.0. A pseudocatalase may be produced especially under glucose limitation. A ribitol or glycerol teichoic acid can be present in the cell walls.
Isolation: Dairy products, silage, sauerkraut, pickled vegetables, sourdough, cow dung, human mouth, intestinal tract and stool, sewage and grape must.
Type strain: DSM 20174.

Lb. trichodes
→ *Lb. fructivorans*.

Lb. vermiforme
→ *Lb. hilgardii*.

Lb. vini
Morphology: Rods. 0.49–0.82 µm × 1.36–2.8 µm. Single, in pairs or in short chains. Motile.
Characteristics: Uses ribore and arabinose homofermentatively. Catalase-negative. Exopolysaccharide is not produced from sucrose.
Isolation: Fermenting grape must.
Type strain: DSM 20605.

Lb. yamanashiensis
→ *Lb. mali*

1.7.2 Genus Leuconostoc

Leuconostocs thrive on plants and sometimes in milk, milk products, meat, sugar cane and other fermented food products. One species, *Lc. mesenteroides,* has been isolated from must. It is nonhemolytic and nonpathogenic to plants and animals (Garvie 1986a). *Leuconostocs* are heterofermentative cocci producing only D-lactic

acid from glucose and are unable to produce ammonia from arginine (Björkroth and Holzapfel 2003).

Leuconostocs form spherical or lenticular cells, pairs or chains. The peptidoglycan belongs to type A. The interpeptide bridge of the peptidoglycan consists of Lys-Ser-Ala_2 or Lys-Ala_2.

Sugars are fermented by the 6-P-gluconate/phosphoketolase pathway with D-lactic acid, ethanol/acetate and CO_2 as end products. $NADP^+$ or NADP will serve as coenzyme of the glucose-6-phosphate dehydrogenase. During malolactic fermentation malate is degraded to L-lactate and CO_2. Cells are nonproteolytic. Nitrate is not reduced.

Cells grow in a glucose medium as elongated cocci. Cells are found singly or in pairs, and form short to medium length chains. On solid media, cells form short rods.

Leuconostocs share many features with the heterofermentative lactobacilli (Dellaglio et al. 1995).

Dextrans, which are of industrial importance, are produced by leuconoctocs, especially *Lc. mesenteroides,* from sucrose as substrate.

Leuconostoc species were divided by Garvie (1960) into six different groups according to the fermentation of 19 carbohydrates. Electrophoretic mobilities of enzymes e.g. LDHs, cell protein pattern, cellular fatty acids, DNA base composition and DNA homology are applied for differentiation of the species (Dellaglio et al. 1995). Citrate metabolisms of *Lc. mesenteroides* subsp. *mesenteroides* might be plasmid linked (Cavin et al. 1988). No other phenotypic features were found to be coded on plasmids, while plasmids of *Lactobacillus* and *Pediococcus* code for sugar utilisation, proteinase, nisin, bacteriocins production, drug resistance, slime formation, arginine hydrolysis and bacteriophage resistance (Dellaglio et al. 1995).

They play a role in the organoleptic quality and texture of food such as milk, butter, cheese, meat and wine. *Leuconostocs* can also spoil food, but often they contribute to the flavour of dairy products due to the production of diacetyl form citrate. These strains are used as starter cultures, for e.g., buttermilk and cheese production. They produce gas from glucose, which can change the texture of fermented food. Due to their slow growth and acidification properties, they represent

Table 1.4 Differential characteristics of wine-related species of the genera *Leuconostoc*, *Oenococcus* and *Weissella*

Characteristics	*Lc. mesenteroides*	*O. oeni*	*W. paramesenteroides*
Acid from sucrose	+	–	+
Dextran formation	+	–	–
Growth below pH 3.5	–	+	n.d.
Growth in 10% ethanol	–	+	n.d.
NAD^+-dependent Glc-6-P-DH	+	–	n.d.
Murein type	Lys-Ser-Ala_2	Lys-Ser_2, Lys-Ala-Ser	Lys-Ser-Ala_2, Lys-Ala_2

n.d. data not given

a minor percentage of the LAB in food. They can become predominant when antibiotic agents are present. They can influence the organoleptic behavior of wine. *Lc. mesenteroides* subsp. *mesenteroides* has been isolated from grape must during alcoholic fermentation (Wibowo et al. 1985).

The G + C content of the DNA ranges between 37 and 41 mol%.

The genus *Leuconostoc* contains in total: 24 (including subspecies; October 2008; DSMZ 2008). Only *Lc. mesenteroides* plays a role in must and wine. Some characteristics are compiled in Table 1.4.

The type species is *Lc. mesenteroides* DSM 20343^T.

Lc. mesenteroides subsp. *mesenteroides*
Morphology: Coccoid cells in milk, elongated cocci in glucose containing culture media. Single, pairs, short to medium chains. Often rod-shaped on solid media.
Characteristics: Production of excess of exopolysaccharides (dextran) from sucrose. Phages have been described (Sozzi et al. 1978).
Isolations: Silage, fermenting olives, sugar milling plants, meat, milk, dairy products, grape must/wine.
Type strain: DSM 20343

1.7.3 Genus Oenococcus

Oenococci have been isolated from must and wine (Garvie 1986a). They form spherical or lenticular cells, pairs or chains. Murein belongs to type A. The interpeptide bridge contains Lys-Ala-Ser or Lys-Ser-Ser. Only NAD^+ will serve as coenzyme of the glucose-6-phosphate dehydrogenase (Björkroth and Holzapfel 2003).

Oenococci have been separated from the genus *Leuconostoc* by 16S rDNA sequence analysis (Fig. 1.1; Dicks et al. 1995; Schleifer and Ludwig 1995a, b). Only two species *O. oeni* and *O. kitahareae* (Endo and Okada 2006) have been described, and can easily be distinguished. *O. kitaharae* (type strain: DSM 17330^T) has been isolated from a composting distilled shochu residue. L-Malate is not decarboxylated to L-lactate and CO_2 in the presence of fermentable sugars. Cells do not grow below pH 4.5 and in 10% ethanol. Growth is not stimulated by tomato juice. The DNA G + C content ranges from 41 to 43 mol%.

O. oeni can grow at pH 3.0 and 10% ethanol. Heat shock proteins and special membrane lipids are produced under these environmental conditions (Coucheney et al. 2005).

The DNA homology with other lactic acid genera is relatively low with a certain relationship to the genera *Leuconostoc* and *Weissella* (Stiles and Holzapfel 1997). The distinct pylogenetic position (Fig. 1.1) because of the quite different 16S rDNA sequence may indicate a quick evolving rRNA (Yang and Woese 1989), which could not be approved by a comparison of the gene sequences of the DNA-dependent RNA-polymerases (Morse et al. 1996). Oenococci can be distinguished from less acid tolerant *Leuconostoc* species by using saccharose, lactose and maltose as substrate (Garvie 1986a).

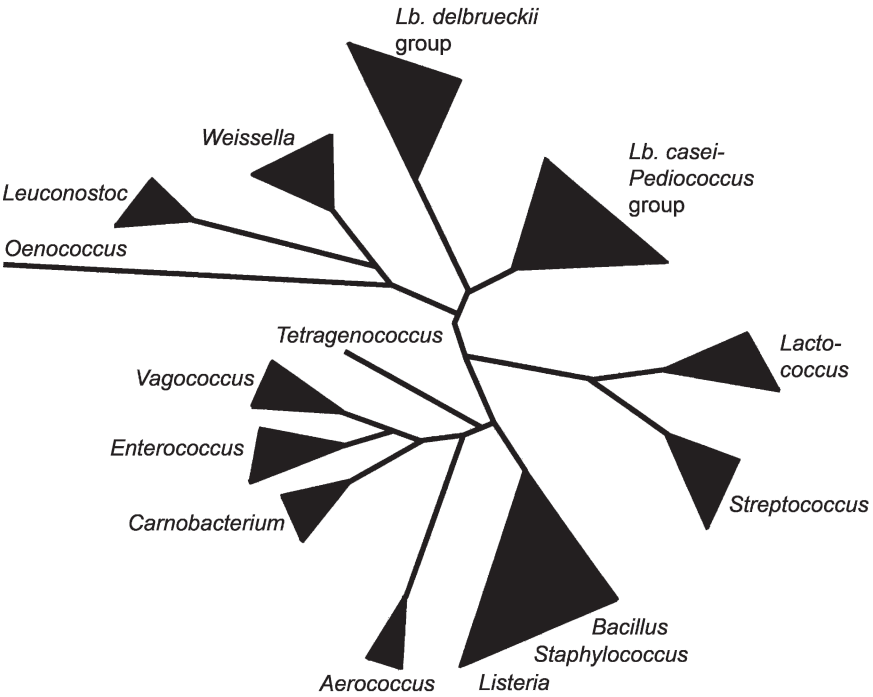

Fig. 1.1 Schematic unrooted phylogenetic tree of lactic acid bacteria and related genera (Axelsson 2004; with permission of the author and the publisher)

O. oeni can use the hexoses glucose and fructose, while not all strains use trehalose (Garvie 1986a). L-arginine can be degraded to carbon dioxide, ammonia and ornithine. *O. oeni* can perform a malolactic fermentation (Caspritz and Radler 1983), which is also found in the genera *Lactobacillus*, *Leuconostoc*, and *Pediococcus*. The malolactic fermentation leads to a membrane potential and a proton gradient. With the aid of an F_1F_0 ATPase energy can be gained (Poolman et al. 1991).

Oenococci exhibit a high mutability due to the lack of the mismatch repair genes *mutS* and *mutL* (Marcobal et al. 2008), which may facilitate the formation of strains. Specific methods for the rapid detection or differentiation of *O. oeni* strains in must and wine samples have been developed (Kelly et al. 1993; Viti et al. 1996; Zavaleta et al. 1997; Fröhlich 2002; Fröhlich and König 2004; Larisika et al. 2008).

The type species is *O. oeni* DSM 20252^T.

O. oeni

Morphology: Spherical, lenticular cells in pairs or chains.
Characteristics: Growth below pH 3.0 and 10% ethanol.
Isolation: must/wine.
Type strain: DSM 20252^T.

1.7.4 Genus Pediococcus

Pediococci occur on plant material, fruits and in fermented food. They are nonpathogenic to plants and animals. Cells are spherical and never elongated as it is the case with leuconostocs and oenococci. The cell size is 0.36–1.43 µm in diameter. Cell division occurs in two directions in a single plane. Short chains by pairs of cells or tetrads are formed (Garvie 1986b). Tetrad-forming homofermentative LABs in wine are pediococci. Pediococci are nonmotile and do not form spores or capsules (Simpson and Tachuchi 1995). The murein belongs to type A with an interpeptide bridge consisting of L-Lys-Ala-Asp (Holzapfel et al. 2003).

Glucose is fermented by the Embden–Meyerhof–Parnas pathway to DL or L-lactate. A wide range of carbohydrates is used such as hexoses, pentoses, disaccharides, trisaccharides and polymers such as starch. All wine-related species grow only in the presence of carbohydrates. The PTS system is used for glucose transport. Species producing DL-lactate possess an L- and D-LDH. Pyruvate can be converted mainly by *P. damnosus* to acetoin/diacetyl. *P. pentosaceus* and *P. damnosus* can degrade malate. They are nonproteolytic and nitrate is not reduced. Pediococci are catalase negative. Some strains of *P. pentosaceus* produce pseudocatalase. Pediococci do not reduce nitrate.

The G + C content of the DNA ranges between 34 and 44 mol%.

Pediococci can have plasmids, which code for production of bacteriocins or fermentation of carbohydrates. *P. pentosaceus* has three different plasmids for the fermentation of raffinose, melibiose and sucrose.

Pediococci are involved in beer spoilage (*P. damnosus*) and cause off-flavour in wine by production of diacetyl. *P. halophilus*, which has not been found in must/wine, is used to prepare soya sauce. Pediococci are used as starter culture in cheese production, silage and sausage production (*P. acidilactici*; *P. pentosaceus*). They play a role in cheese ripening. Pediococci (*P. acidilactici*; *P. pentosaceus*) can produce bacteriocins (pediocin) which can prevent meat spoilage. *P. damnosus* is a major spoilage organism in beer manufacture, since it may produce diacetyl resulting in a buttery taste.

The species are differentiated by their range of sugar fermentation, hydrolysis of arginine, growth at different pH levels (4.5, 7.0), the configuration of lactic acid produced (Axelsson 2004) and ribotyping (Satokari et al. 2000). *P. pentosaceus* produces a nonheme pseudocatalase (Engesser and Hammes 1994).

The genus *Pediococcus* contains 11 species (October 2008; DSMZ 2008). Four species have been found in must or wine (*P. damnosus*, *P. inopinatus*, *P. parvulus*, *P. pentosaceus*). Some characteristics of the species are compiled in Table 1.5).

The type species is *P. damnosus* DSM 20331^{T}.

P. damnosus
Morphology: Tetrades.
Characteristics: Ribose not fermented, arginine not hydrolysed. No growth at pH 8 or 35°C. DL-lactic acid produced from glucose.
Isolation: Beer and wine.
Type strain: DSM 20331

Table 1.5 Differential characteristics of wine-related species of the genus *Pediococcus*

Characteristics	*P. damnosus*	*P. parvulus*	*P. pentosaceus*
Mol% G + C	37–42	40.5–41.6	35–39
Growth at/in:			
35 °C	–	+	+
6% NaCl	–	+	+
pH 8.0	–	–	+
Arginine hydrolysis	–	–	+
Acid from:			
Arabinose	–	–	+

Pedicocci can be identified by multiplex PCR (Pfannebecker and Fröhlich 2008)

P. inopinatus
Morphology: Tetrades
Characteristics: *P. parvulus* and *P. inopinatus* can be distinguished by the electrophoretic mobility of the L- and D-LDHs.
Isolation: Fermenting vegetables, beer, wine.
Type strain: DSM 20285

P. parvulus
Morphology: Tetrades, 0.7 µm × 1.1 µm in diameter. Single, pairs, tetrads, irregular clusters.
Characteristics: Grows at pH 4.5. Lactose, starch and pentoses not utilized. Arginine not hydrolysed. DL-lactic acid produced from glucose.
Isolation: Plant material, sauerkraut, fermented vegetables, fermented beans, beer, cider and wine.
Type strain: DSM 20332

P. pentosaceus
Morphology: Tetrades.
Characteristics: Pentoses and maltose fermented. Arginine is hydrolysed. Growth up to 45°C. Used for the inoculation of semi-dry sausage, cucumber, green bean or soya milk fermentations and silage. Some strains produce pediocins.
Isolation: Plant material and wine.
Type strain: DSM 20336

1.7.5 Genus Weissella

Based on rDNA analysis *Lc. paramesenteroides* ("*Lc. paramesenteroides* group") was reclassified as *W. paramesenteroides*. F*ive heterofermentative lactobacilli* (*Lb. confusus*, *Lb. halotolerans*, *Lb. kandleri*, *Lb. minor*, *Lb. viridescens)* were also assigned to the genus *Weissella* (Collins et al. 1993; Björkroth and Holzapfel 2003). Weissellas are spherical, lenticular or irregular rods. They are heterofermentative species, which produce D, L-lactic acid, while *W. paramesenteroides* forms D-lactic acid from glucose. They have been isolated from food

and meat. Weissellas produce greenish oxidized porphyrins in meat products by H_2O_2 accumulation. The genus *Weissella* contained 12 validly described species (October 2008, DSMZ 2008). *W. paramesenteroides* is the only species of this genus isolated from must/wine.

The type species is *W. viridescens* DSM 20410^T.

W. paramesenteroides
Morphology: Sperical, lenticular
Characteristics: Pseudocatalase may be produced in the presence of low glucose content.
Isolation: must/wine
Type strain: DSM 20288^T

1.8 Conclusions

Lactic acid bacteria are widespread in habitats with complex nutritional supply such as plant material or fruit juice as well as animals. They influence the aroma, the quality, the consistency and safety of food. Since the 1900s, the production of fermented food and consequently the demand for starter cultures of lactic acid bacteria has been largely increased (Mäyrä-Mäkinen and Bigret 2004). They play an important role in the fermentation of sugar-containing food. Because of the acid formation and production of inhibitory components, they contribute to the preservation of food. On the other hand, they can produce off-flavour (e.g. diacetyl) and cause ropiness by exopolysaccharide production.

Especially in northern wine growing regions, grapes can contain high amounts of acid with unfavourable organoleptic properties. So far, mainly *O. oeni* and sometimes *Lb. plantarum* are used as starter cultures for wine making to reduce the malic acid content.

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Chapter 2
Acetic Acid Bacteria

José Manuel Guillamón and Albert Mas

2.1 Introduction

Acetic acid Bacteria (AAB) are a group of microorganisms included in the *Acetobacteraceae* family that have a very unique characteristic of oxidising the alcohol into acetic acid and this differential capacity originates their name. However, this metabolic ability derives in a high capacity of quick oxidation of alcohols and sugars yielding the corresponding organic acids, which can easily accumulate in the media. This feature makes AAB an especial group to be used in biotechnological applications such as production of ascorbic acid (vitamin C) or cellulose (Deppenmeier et al. 2002). In the food industry AAB are being used as main participants in the production of several foods and beverages, such as vinegar, cocoa, kombucha and other similar fermented beverages. However, their presence and activity can easily derive into spoilage of other foods or beverages such as wine, beer, sweet drinks and fruits.

In the environment, AAB occur in sugary elements, such as fruits or flowers. Additionally, naturally spoiled fruits, which might be partially fermented into alcohols, are an excellent medium for the proliferation of some AAB due to their tolerance to ethanol and the trasformation into acetic acid, both compounds highly restrictive for the proliferation of other microorganisms. However, these are especially abundant in the man-made environments where alcohol is produced.

As the AAB are specialised in rapid oxidation of sugars or alcohols, oxygen availability plays a pivotal role in their growth and activity. Their metabolic activity and growth is especially enhanced when oxygen is present or specifically added (for example in vinegar production). Their optimal pH is 5.5–6.3 (De Ley et al. 1984), however, they can survive and grow in the pH of the wine which can be as low as 3.0–4.0 (Du Toit and Pretorius 2002). In fact, acid resistance can be induced by prolonged and gradual exposure to low pH (Kösebalan and Özingen 1992). Finally, the optimal growth temperature is 25–30°C (De Ley et al. 1984), yet some strains can grow very slowly at 10°C (Joyeux et al. 1984a).

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2.2 From Grapes to Wine: an Adverse Environment

Yeasts, bacteria and filamentous fungi all contribute to the microbial ecology of wine production and the chemical composition of wine, although yeasts have the dominating influence because of their role in conducting the alcoholic fermentation (Fleet 1993). Many factors affect the microbial ecology of wine production, of which the chemical composition of the grape juice and the fermentation processes are the most significant. In complex microbial ecosystems, containing mixtures of different species and strains, there is the possibility that interactions between microorganisms will occur and that this will also determine the final ecology (Drysdale and Fleet 1989a; Ribereau-Gayon et al. 2000).

It is commonly known that grape juice presents extreme conditions for the growth of microorganisms, such as a low pH and a high sugar concentration. Overall, during the alcoholic fermentation, this sugar content is transformed into ethanol by yeasts, meaning an additional restriction for the development of microorganisms. Such environmental changes are responsible for the differences in the microbial ecology throughout the process, where tolerance to high concentrations of ethanol and low pH will be the main factors that select species occurrence in wine ecosystems (Fleet 1993). Additionally, the coexistence of different microorganisms in the media generates competition for the nutrients. Thus, early growth of yeasts in grape juice decreases the nutrient content, making the resulting wine less favourable as an environment for any further microbial growth. Moreover, such growth releases to the media different metabolites, some of which could be toxic to other species. Another factor that affects the development of some microorganisms is the carbon dioxide production that strips it of oxygen, thereby limiting the growth of the aerobic species, such as acetic acid bacteria (AAB). Therefore, if a vigorous onset of alcoholic fermentation by yeasts (or *Saccharomyces cerevisiae*) occurred, non-*Saccharomyces* yeasts and bacteria would show little growth (Gonzalez et al. 2005). However, if yeast growth is delayed, various species of lactic acid bacteria and acetic acid bacteria may grow, inhibit the growth of yeast, and cause sluggish or stuck fermentations (Ribéreau-Gayon et al. 2000; Fleet 2001).

The interaction among the different wine microorganisms may or may not favour a particular microbial group. For example, when the large amount of yeast biomass produced during fermentation dies, the autolysis releases to the media amino acids and vitamins which may encourage the growth of AAB and lactic acid bacteria species later in the process (Fleet 2001). Another concept that must be considered is quorum sensing as a mechanism by which microbial cells communicate with each other and regulate population growth. It is therefore evident that the development of microorganisms during the winemaking process depends on different parameters, such as the aforementioned microbial interactions, but also the media composition and the oenological practices.

A parameter that could affect the development of microorganisms during alcoholic fermentation is temperature. Lately, there has been a preference towards fermenting white wines at controlled temperatures (between 13 and 18°C) in order to enhance the production and retention of flavour volatiles (Llauradó et al. 2002), while in red wine fermentations, temperature is less controlled, and able to reach

temperature values of 25–30°C. It is known that fermentation temperature will affect the rate of yeast growth and, consequently, the duration of fermentation (Torija et al. 2003). Therefore, a delay in yeast growth, which will occur in low temperature fermentation, could help the development of indigenous yeasts and cause a sluggish fermentation (Llauradó et al. 2002). However, the wine bacterial population is more sensitive to lower temperatures.

Finally, the addition of sulphur dioxide to grape juice and wine is a commonplace winemaking practice in order to control oxidation reactions and prevent the growth of indigenous microflora, such as indigenous non-*Saccharomyces* yeasts or AAB and lactic acid bacteria. The antimicrobial effectiveness of the SO_2 is highly dependent on the pH and on the presence of reactive molecules which can bind to SO_2. With a lower pH in the must and wine, there will be more SO_2 in free molecular form, which is the active form against microorganisms (Ribereau-Gayon et al. 2000).

2.3 Isolation and Taxonomy

The physiological differences among microorganisms made it possible to develop differential culture media for isolating AAB whose carbon source is glucose, mannitol, ethanol, etc. Some of these media can also incorporate $CaCO_3$ or bromocresol-green as acid indicators (Swings and De Ley 1981, De Ley et al. 1984). Culture media are usually supplemented with pimaricin or similar antibiotics in the agar plates to prevent yeasts and molds from growing and with penicillin to eliminate Gram positive acidophilic bacteria such as lactic acid bacteria (Ruiz et al. 2000).

Some of the most widely used culture media are GYC (5% D-glucose, 1% yeast extract, 0.5% $CaCO_3$ and 2% agar (w/v)) and YPM (2.5% mannitol, 0.5% yeast extract, 0.3% peptone and 2% agar (w/v)). Plates must be incubated for between 2 and 4 days at 28°C under aerobic conditions. These culture media are suitable for wine samples (Bartowsky et al. 2003; Du Toit and Lambrechts 2002).

Nevertheless, some studies show that it is difficult to culture this bacterial group from some industrial samples, especially those originated in extreme media, such as vinegar (Sokollek et al. 1998). This problem has been partially solved by introducing a double agar layer (0.5% agar in the lower layer and 1% agar in the upper layer (w/v)) into the cultures and media containing ethanol and acetic acid in an attempt to simulate the atmosphere of the acetification tanks, such as AE medium (Entani et al. 1985). However, culturing AAB is still a limitation to proper studies of this group of microorganisms that is a critical point for ecological studies. Thus, culture independent molecular techniques are being developed to solve this problem.

The identification of AAB has gone in parallel with the changes in taxonomy and AAB classification. Since *Mycoderma* was first described by Persoon in 1822 and observed by Pasteur, Hansen and Beijerinck in the nineteenth century, the general consensus throughout the last part of the twentieth century is that there were two AAB genera: *Gluconobacter* and *Acetobacter*. The keys to the taxonomy of bacteria have been traditionally collected in Bergey's Manual of Systematic Bacteriology. In the 1984 edition (De Ley et al. 1984), it included such molecular

techniques as fatty acid composition, soluble protein electrophoresis, % of G + C content, and DNA–DNA hybridisation.

The taxonomy of AAB microorganisms, initially based on morphological and physiological criteria, has been continuously varied and reoriented, largely because of the application of molecular techniques. The most common techniques are

- DNA–DNA hybridisation: From a taxonomic point of view, this is the most widely used technique for describing new species within bacterial groups. The technique measures the degree of similarity between the genomes of different species.
- % Base ratio determination: This was one of the first molecular tools to be used in bacterial taxonomy. It calculates the percentage of G + C in a bacterial genome. Bergey's Manual of Systematic Bacteriology (De Ley et al. 1984) included these values to differentiate among *Acetobacteraceae* species.
- 16S rDNA sequence analysis: The 16S rDNA gene is a highly preserved region with small changes that characterise different species. Ribosomal genes are compared in most taxonomical studies of bacteria. However, the differences in 16S rDNA sequences are very limited and some species have few nucleotide pairs of difference.

The *Acetobacteraceae* family is no exception to this reorganization of species and genera. AAB are considered a lineage within the *Acetobacteraceae* family, which is characterised by the ability to produce acetic acid, although some of them are very weak producers. Eight new AAB genera have been added to the two traditional genera mentioned above: *Acidomonas*, *Gluconacetobacter*, *Asaia*, *Kozakia*, *Saccharibacter*, *Swaminathania, Neoasaia* and *Granulibacter* (an updated 2007 list can be seen in Table 2.1). As expected, some species have also been renamed (particularly some species of *Acetobacter* which were assigned to the *Gluconacetobacter* genus).

Table 2.1 Species of acetic acid bacteria

Acetobacter	*Gluconacetobacter*	*Gluconobacter*	*Acidomonas*
A. aceti	***Ga. liquefaciens***	***G. oxydans***	*Ac. methanolica*
A. pasteurianus	*Ga. diazotrophicus*	*G. frateurii*	
A. pomorum	***Ga. xylinus***	*G. assaii*	
A. peroxydans	***Ga. hansenii***	*G. cerinus*	*Kozakia*
A. indonesiensis	***Ga. europaeus***	*G. albidus*	*K. baliensis*
A. tropicalis	***Ga. oboediens***	*G. thailandicus*	
A. syzygii	***Ga. intermedius***		
A. cibinongenesis	*Ga. sacchari*	*Asaia*	*Saccharibacter*
A. orientalis	***Ga. entanii***	*As. bogorensis*	*Sa. floricola*
A. orleaniensis	***Ga. johannae***	*As. siamensis*	
A. lovaniensis	*Ga. azotocaptans*	*As. krugthepensis*	
A. estuniensis	*Ga. swingsii*		*Neoasaia*
A. malorum	*Ga. kombuchae*		*N. chiangmaiensis*
A. cerevisiae	*Ga. nataicola*		
A. oeni	*Ga. rhaeticus*	*Swaminathania*	*Granulibacter*
A. nitrogenifigens	*Ga. saccharivorans*	*S. salitolerans*	*Gr. Bethesdensis*

In *bold* are highlighted the species described on grapes, in wine or vinegar

2.4 Molecular Techniques for Routine Identification of AAB

The main objective of microbial classification is to identify an isolated microorganism up to the species level. However, discriminating or typing the different strains or genotypes of a species is gaining increasing importance from an industrial point of view. Not all the strains of a species have the same ability to oxidize ethanol into acetic acid. Therefore, it is important to be able to determine how well each technique can discriminate among strains and to know how many species or strains are involved.

Depending on the degree of polymorphism provided by the various molecular markers, they are more suitable for inter-specific or for intra-specific discrimination. Therefore, we divided the molecular techniques into two main groups: those that can discriminate up to species level and those that can discriminate up to strain level.

Species level

- PCR–RFLP of the rDNA 16S: This technique is appropriate for differentiating and grouping microorganisms on the basis of their phylogenetic relationships (Poblet et al. 2000; Ruiz et al. 2000). In eubacterial DNA, the rRNA loci include 16S, 23S and 5S rRNA genes, which are separated by internally transcribed spacer (ITS) regions. The technique consists of amplifying the 16S rDNA region and then digesting the amplified fragment with different restriction enzymes. The DNA fragments obtained are separated by electrophoresis. The resulting patterns are characteristic of every species and make it possible to characterize almost all the AAB species (Fig. 2.1) (Guillamon et al. 2002; Gonzalez et al. 2006a).
- PCR–RFLP of the 16S–23S rDNA Internally Transcribed Spacer (ITS): This technique consists of amplifying a region of the ITS (here it spans the 16S and 23S rRNA genes) and then digesting the amplified products with different restriction endonucleases (Sievers et al. 1996; Ruiz et al. 2000; Trcek and Teuber 2002; González et al. 2006a; Prieto et al. 2007). The sequences and lengths of the 16S–23S ITS region varies considerably among the species, and this region also contains conserved sequences with functional roles such as tRNA genes and antitermination sequences (Sievers et al. 1996). In other bacterial groups intergenic sequences are known to have higher variability than functional sequences, and they make it possible to distinguish below the species level. However, in AAB, the results obtained by Ruiz et al. (2000) and Trcek and Teuber (2002) only differentiated up to species level. Although it may be more resolutive than the previous one, in some cases it may give more than one pattern for the same species and also may give some different, unknown patterns for known species.
- PCR–RFLP of the 16S–23S–5S sequences: This technique consists, as in the two previous ones, of amplifying part of the ribosomal DNA; in this case the region compressed by the 16S, 23S and 5S rDNA genes, generating an amplified product of around 4,500 bp. This is then digested using *Rsa*I as a restriction endonuclease (Gullo et al. 2006). The results obtained were similar to the previ-

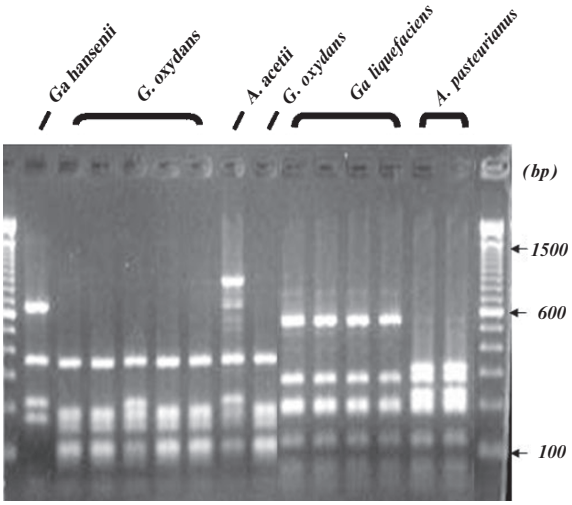

Fig. 2.1 *Taq*I restriction patterns obtained after amplification of 16S rDNA of different acetic acid bacteria strains isolated throughout the alcoholic fermentation. All the strains belonging to the same species showed the same restriction pattern. Size in bp (*right*) of 100-bp ladder (Gibco-BRL), used as markers, are indicated

ous techniques, although with higher and more resolutive polymorphism. However, the main limitation of this technique results from the amplification of such a long fragment.

- Denaturing gradient gel electrophoresis (DGGE): DGGE separation of bacterial DNA amplicons is a common method used to characterize microbial communities from specific environmental niches. This technique has been used by De Vero et al. (2006) to study the AAB population in vinegar (*Aceto Balsamico Tradizionale*) production. It does not require the microorganisms to be isolated. The most commonly used genes for the DGGE method are 16S and 23S rDNA because they are species specific. The band pattern obtained is indicative of the number of different species present in a sample. Each individual band can be recovered and used for sequencing, which can be an additional tool for species identification. A main limitation of this technique is that minor species are hardly detected, especially when other species constituted an overwhelming majority.
- Real Time PCR: This technique identifies and enumerates bacterial species without culturing. It has been successfully used to enumerate total populations of AAB in both wines and vinegars (González et al. 2006b) and for the enumeration of *Gluconobacter* and *Gluconacetobacter* species in soft drinks (Gammon et al. 2007). A clear advantage of this technique is its specificity to detect a specific family group, genera or species.

- PQQ-dependent Alcohol dehydrogenase gene targeting: This technique has been used to detect both generic AAB and specifically *A. aceti* from cider vinegar (Trcek 2005). The variable and conserved segments in partial *adhA* sequences allows the construction of generic PCR set of primers for all the AAB species and a specific PCR-primer for detection of *A. aceti*. The author claimed that the analysis of partial *adhA* sequences showed that this region was more discriminative for AAB species than 16S rRNA gene but less than 16S–23S rRNA intergenic regions.
- Fluorescence in situ hybridisation (FISH): This technique has been used to detect *Ga. sacchari* (Franke et al. 1999) and other wine-related microorganisms such as lactic acid bacteria (Blasco et al. 2003). FISH directly identifies and quantifies bacterial species at microscopic level without previous cultivation. It consists of DNA fluorescent labelled probes that will specifically hybridise each of the species or genera. The high content of different binding compounds in wine or vinegar can quench fluorescence and also limit the resolution.

Strain level

- Random amplified polymorphic DNA-PCR (RAPD-PCR): The RAPD fingerprint amplifies the genomic DNA with a single primer of arbitrary sequence, 9 or 10 bases in length, which hybridise with sufficient affinity to chromosomal DNA sequences at low annealing temperatures so that they can be used to initiate the amplification of bacterial genome regions. The amplification is followed by agarose gel electrophoresis, which yields a band pattern that should be characteristic of the particular bacterial strain. The technique was initially used with AAB by Trcek et al. (1997) in spirit vinegar and later by Nanda et al. (2001) to characterize rice vinegar AAB. Bartowsky et al. (2003) and Prieto et al. (2007) also used this technique to differentiate strains in spoiled wines.
- Enterobacterial Repetitive Intergenic Consensus-PCR (ERIC-PCR) and Repetitive Extragenic Palindromic-PCR (REP-PCR): ERIC and REP elements have been described as consensus sequences derived from highly conserved palindromic inverted repeat regions found in enteric bacteria. However, these sequences seem to be widely distributed in the genomes of various bacterial groups. The amplification of the sequences between these repetitive elements has generated DNA fingerprints of several microbial species. ERIC-PCR has already been used by Nanda et al. (2001) to identify AAB strains isolated from vinegar. Both techniques have been applied to AAB in wines (González et al. 2004) and used to follow the AAB population dynamics before and during alcoholic fermentation (González et al. 2005).
- A similar technique based on repetitive elements for genomic fingerprinting has been recently proposed by De Vuyst et al. (2008) using $(GTG)_5$ primers. These noncoding sequences are present in multiple copies in the genomes of most Gram-negative and several Gram-positive bacteria. This study reported a good discrimination method with a high degree of polymorphism in AAB.

2.5 AAB Ecology During Winemaking

2.5.1 AAB in Grapes and Musts

As the grapes mature the amount of sugars (glucose and fructose) increases and improves the chances for AAB growth. In healthy grapes, the predominant species is *Gluconobacter oxydans*, and the most common populations are around 10^2–10^5 cfu ml^{-1} (Joyeux et al. 1984a; Du Toit and Lambrechts 2002; Gonzalez et al. 2005; Renouf et al. 2005; Prieto et al. 2007). *Acetobacter* species have also been isolated from unspoiled grapes, albeit in very low amounts (Du Toit and Lambrechts 2002; Gonzalez et al. 2004; Prieto et al. 2007). On the other hand, damaged grapes contain larger AAB populations (Barbe et al. 2001), mainly belonging to the *Acetobacter* species (*Acetobacter aceti* and *Acetobacter pasteurianus*). In these conditions, the sugars released from the spoiled grapes can be metabolised by yeasts into ethanol, which is a preferred carbon source of the *Acetobacter* species that overgrow *Gluconobacter* (Joyeux et al. 1984b; Grossman and Becker 1984; Gonzalez et al. 2005). However, the descriptions of new AAB species will increase the number of species isolated from this substrate. In fact, the recently described *Acetobacter cerevisiae* (Cleenwerk et al. 2002) has already been isolated from Chilean grapes (Prieto et al. 2007).

Grape processing in the cellar (pressing, pumping, racking, etc.) may contaminate must since there is contact with cellar equipment, which contains resident AAB and will increase their population, mostly made up of *Acetobacter* species (Gonzalez et al. 2005). However, in the literature it is possible to find some exceptions to this ubiquitous presence of AAB. Subden et al. (2003) were not able to find AAB among the bacteria isolated from icewine musts. Curiously the predominant species isolated from this substrate was *Pantoea agglomerans*, which had never been reported as a contaminant in grape musts.

Most of the studies on AAB in winemaking have focused on the evolution of species during the process. Recently, we have also typed the different AAB isolates from grapes to wine at strain level (Gonzalez et al. 2005). We found important strain diversity in grapes (calculated as the percentage of different strains in the total isolates analysed) which ranged from 45 to 70%. A few of these grape strains were continuously isolated throughout the alcoholic fermentation. Prieto et al. (2007) have also typed isolates from Chilean grapes, confirming the high diversity of AAB strains in grapes, in particular among the *G. oxydans* isolates.

2.5.2 AAB During Fermentation

Studies concerning the evolution of AAB species along wine fermentations have established certain general trends (Table 2.2). *G. oxydans* is usually the dominant species in fresh must and the initial stages of fermentation and is rarely isolated

Table 2.2 Summary of publications that indicate the presence of AAB on grapes, in musts and wines

			Grapes/must			Mid fermentation		End fermentation
Reference	Source	Harvest yearw	cfu ml^{-1}	Main Species	cfu ml^{-1}	Main species	cfu ml^{-1}	Main species
Joyeux et al. (1984a)	White wine		10^6	*G. oxydans*	10^5	*A. pasteurianus*	10^2	*A. aceti*
	Semillon botrytized grapes			*A. pasteurianus*		*A. aceti*		*A. pasteurianus*
	Red wine		10^4	*G. oxydans*	10^2	*G. oxydans*	10^1	*A. pasteurianus*
	Cabernet-Sauvignon					*A. pasteurianus*		*G. oxydans*
Barbe et al. (2001)	Botrytized grapes	1995	10^6	*Gluconobacter sp.*				
		1996		*A. pasteurianus*				
		1997		*A. aceti*				
Du Toit and Lambrechts (2002)	Cabernet Sauvignon	1998	10^6–10^7	*G. oxydans*	10^3–10^4	*A. pasteurianus*	10^2–10^3	*A. pasteurianus*
						A. aceti		
		1999	10^4–10^5	*G. oxydans*	10^2–10^4	*A. pasteurianus*	10^2–10^3	*A. pasteurianus*
				A. pasteurianus		*Ga. liquefaciens*		*Ga. liquefaciens*
						G. oxydans		*Ga. hansenii*
						A. aceti		
Bartowsky et al. (2003)	Bottled red wine						10^2–10^3	*A. pasteurianus*
Gonzalez et al. (2004)	Red Grenache	2001	10^3	*G. oxydans*	10^3	*A. aceti*	50	*A. aceti*
						Ga. liquefaciens		
						Ga. hansenii		
Gonzalez et al. (2005)	Red Grenache	2002	10^4–10^6	*G. oxydans*	10^4–10^5	*A. aceti*	10^2–10^3	*A. aceti*
				A. aceti				
Prieto et al. (2007)	Carmenere, Cabernet Sauvignon Other varieties Chile	2004		*G. oxydans* *A. cerevisiae*				

from wines, while *A. aceti* is the major strain in the final stages of fermentation (Joyeux et al. 1984a; Drysdale and Fleet 1988; Gonzalez et al. 2005). However, we have also found *G. oxydans*, *Gluconacetobacter liquefaciens* and *Gluconacetobacter hansenii* in higher percentages as well as *A. pasteurianus* in the final stages of fermentation (Gonzalez et al. 2004) or recently, *Acetobacter oeni* has been proposed as a new species isolated in wine (Silva et al. 2006). Thus, this pattern of species evolution seems somewhat reductionist and may depend on multiple oenological factors such as SO_2, pH, ethanol, low temperature and yeast inoculation. All these factors have been reported as inhibitors of AAB growth, yet they can also modify the species distribution during the process. For instance, different studies have suggested that *A. pasteurianus* is more resistant to SO_2 (Du Toit and Lambrechts 2002), ethanol (De Ley et al. 1984), and low temperature than *A. aceti*. Otherwise, inoculation with high population of yeasts, which is a common practice in winemaking, will produce a rapid onset of alcoholic fermentation and a concomitant decrease of the AAB population (Fig. 2.2) (Guillamon et al. 2002). However little is known about the impact of these oenological factors or the interactions with other wine microorganisms on the selection and evolution of the AAB species during wine fermentation.

The growth of AAB during alcoholic fermentation is also linked to the number of bacteria and yeast in the must at the start of the fermentation (Watanabe and Lino 1984). The initial population of AAB, before the alcoholic fermentation starts, may determine the number of cells surviving during and after fermentation (Du Toit and Pretorius 2002). If AAB grow significantly during the initial stages of alcoholic fermentation, it may become stuck or sluggish, which might enhance the growth of AAB during wine storage, with a corresponding reduction in the quality of the wines (Joyeux et al. 1984b).

Even less is known about the AAB development during malolactic fermentation and their interaction with lactic acid bacteria (the main microorganisms during this process). Joyeux et al. (1984a) reported constant cell counts of AAB of approximately 10^2–10^3 cfu ml^{-1}, consisting mainly of *A. pasteurianus*, throughout malolactic

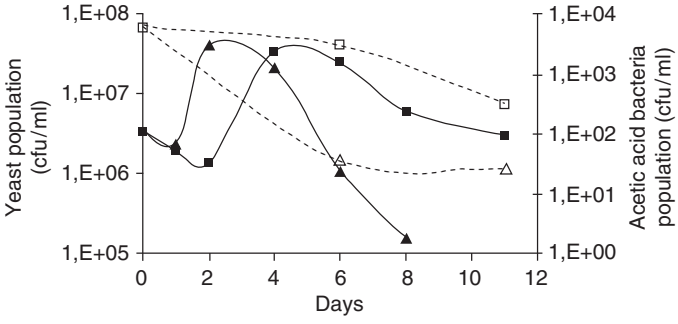

Fig. 2.2 Comparison of yeasts (*solid line* and *solid symbol*) and acetic acid bacteria populations (*dotted line* and *open symbol*) in inoculated (*filled triangle*) and spontaneous (*filled square*) wine fermentations

fermentation. Conversely we detected a major increase in the AAB population up to approximately 10^6 cfu ml^{-1} during this process, *A. aceti* being the main species found in this environment (Fig. 2.3) (Guillamon et al. 2002). This increase in the AAB population did not interfere with the simultaneous development of the lactic acid bacteria population up to cell densities of approximately 10^8 cfu ml^{-1}. A possible synergic mechanism between both bacterial groups may emerge from this result.

In our studies on typing AAB strains and monitoring strain evolution during alcoholic fermentations, we were able to conclude: (1) the origin of the strains isolated during wine fermentation are both the grape and wine cellar environment; (2) the high strain diversity detected at the beginning of the process decreased significantly during the final stages of the process. The anaerobic conditions and ethanol increasing concentrations clearly selected the most resistant strains; (3) Regardless of the degree of genotype diversity, there were clear dominant genotypes in all stages (Gonzalez et al. 2004, 2005).

2.5.3 AAB During Aging and Wine Maturation

Once the alcoholic fermentation has finished the pumping over and racking of wine may stimulate the growth of AAB and can lead to populations of up to 10^8 cells ml^{-1} (Joyeux et al. 1984b, Drysdale and Fleet 1985), owing to the intake of oxygen during these operations. During storage and ageing, the main species found belong to *Acetobacter* (*A. aceti* and *A. pasteurianus*). AAB have been isolated from the top, middle and bottom of the tanks and barrels, suggesting that AAB can actually survive under the semi-anaerobic conditions occurring in wine containers (Du Toit et al. 2005). This can be explained by the ability of AAB to use such compounds as quinones and reducible dyes as electron acceptors (Du Toit and Pretorius 2002). The number of bacteria usually decreases drastically after bottling, because of the relatively anaerobic conditions present within a bottle. However, excessive aeration

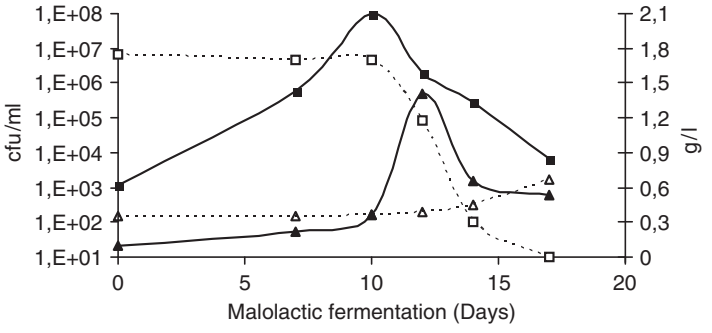

Fig. 2.3 Acetic acid bacteria (*dashed filled triangle*) and lactic acid bacteria (*dashed filled square*) growth during a malolactic fermentation. Malic acid consumption (*dotted open square*) and acetic acid (*dotted open triangle*) production are also indicated

during bottling can increase the number of AAB (Millet and Lonvaud-Funel 2000). Furthermore, the bottle position during storage, poor storage conditions or spoiled corks may facilitate AAB growth. In fact, wine spoilage in the bottle by AAB has also been reported, mostly due to *A. pasteurianus* (Bartowsky et al. 2003). It should be pointed out that the number of AAB in wine after fermentation can be underestimated because the counting of colonies grown in solid media does not take into account the VBNC status (Millet and Lonvaud-Funel 2000).

2.6 Acetic Acid Bacteria and Wine Spoilage

The presence and growth of acetic acid bacteria has generally been related to wine spoilage, mostly by increasing the acetic acid and, thus, the volatile acidity. However, the changes introduced by acetic acid bacteria in wine depend on the process stage involved.

Grape and must: The overall effect of the acetic acid bacteria growth in the grapes is considered as acid rot that can sometimes involve other microorganisms such as fungi like *Botrytis cinerea* (Barbe et al. 2001). In the grape or must the main carbon source used by AAB is glucose, which is readily oxidized to gluconic acid. In fact, gluconic acid in oenology is considered as an indicator of *Botrytis* infection, although it seems clear that most of the gluconic acid is produced by the AAB associated with the *Botrytis* infection (Barbe et al. 2001). Also, fructose can be oxidized to oxofructose, although glucose is preferred as a substrate. The production of gluconic acid and oxofructose is important not only because of the organoleptic changes that might take place, but also because of the binding and reduction of free SO_2. This will result in the need for a higher SO_2 dosage (Barbe et al. 2001; Du Toit and Pretorius 2002).

Although this is the main change induced by AAB in grapes, there are further changes relevant to oenology. The production of cellulose as a result of sugar metabolism (Kouda et al. 1997) can result in the production of fibres that can affect grape must and wine filterability (Drysdale and Fleet 1988).

Wine: Probably the best known transformation of AAB in general is the transformation of ethanol into acetic acid, which gives the group its name. Thus, during wine production this will be the main carbon source (Drysdale and Fleet 1988; Du Toit and Pretorius 2002). However, the AAB population decreases during wine making due to the anaerobic conditions exerted by yeast metabolism and produces only limited amounts of acetic acid, although in some cases high enough to be noticeable to consumers (0.8 g l^{-1}, vinegary taint). However, not all the acetic acid found in wines is due to AAB since yeasts and lactic acid bacteria can also produce it. Thus, even low AAB population counts can significantly affect the final quality of wines as they are strong acetic acid producers that will add to what is produced by other microorganisms. After alcoholic fermentation, even when ethanol concentrations of 5–10% are toxic for AAB, some strains are able to survive in very high ethanol concentrations of up to 15% (De Ley et al. 1984). The production of acetic acid by AAB requires oxygen and it is

directly related to oenological practices that may produce an increase in dissolved oxygen (aeration, pumping over, fining, etc.).

During the transformation of alcohol into acetic acid both acetaldehyde (Drysdale and Fleet 1989a) and ethyl acetate (as a result of yeast and AAB alcohol acetyl transferease activity) (Plata et al. 2005) are produced, which are also noticeable in the final wines due to their low perception threshold (Drysdale and Fleet 1989a). Beyond the effect of acetaldehyde upon aroma and taste, acetaldehyde is the most reactive species to bind SO_2, and therefore reduce its free form (Ribereau-Gayon et al. 2000).

After ethanol, glycerol is also a main alcoholic fermentation product and can be a substrate for AAB oxidation. The resulting product is dihydroxyacetone, which does not give the mouth the smoothness of glycerol and also binds free SO_2. Other wine minority compounds, such as organic acids, can be used as an oxidisable substrate. The levels of malic acid, tartaric acid and citric acids decrease after wine AAB contamination (Drysdale and Fleet 1989b). All these changes affect the sensory perception of the final wine.

2.7 Conclusions: Prevention of Wine Spoilage and Oenological Practices

Although oxygen availability is low during winemaking, AAB can survive such conditions (Drysdale and Fleet 1989a), either cultivable or VBNC status (Millet and Lonvaud-Funel 2000). Thus an AAB risk-free oenological practice is impossible, although some practices will reduce the risks by minimisation of the AAB population or by limitation of its metabolism and activity. Among these, it is important to pay special attention to

- Control of grape production, aimed at obtaining an appropriate acidic pH (Holt et al. 1994) and maintenance of that low pH during wine production and ageing. Although AAB survive at wine and grape pH of 3–4, their populations are reduced after lowering pH (Joyeux et al. 1984a; Du Toit and Lambrechts 2002). This low pH also favours the presence of SO_2 in free form (Ribereau-Gayon et al. 2000).
- Healthy grape status and care during handling and pressing. The presence of AAB, rotten or damaged grapes should be avoided as much as possible. Also, entrance to the cellar should be well controlled and quick, as this stage can be an excellent source for winery-resident AAB (González et al. 2005). At this stage, SO_2 addition, cold settling, and clarification could be highly recommended practices in order to reduce the population size and prevent unwanted microorganisms (Ribereau-Gayon et al. 2000). However, it has to be emphasized that AAB could survive high SO_2 concentrations (Du Toit et al. 2005).
- A quick start of fermentation is advisable as this will produce both ethanol and CO_2 that can reduce the AAB population and metabolic activity. Thus, inoculations with ADWY or equivalent practices are advisable to ensure this quick start (Guillamon et al. 2002).

- A well controlled process of aeration or oxygenation. The O_2 need for AAB growth has already been discussed, yet some oxygen supply is needed for ageing while other oxygen supply is unavoidable in certain oenological practices (pumping over, racking, bottling, etc.). The current microoxygenation practice may enhance the growth of AAB, however Perez-Magariño et al. (2007) reported that low levels of oxygen supply do not affect volatile acidity. During ageing in wooden barrels some oxygen penetrates through the wood, enough to maintain a population of viable AAB (Millet and Lonvaud-Funel 2002). Pumping over and bottling can be an additional source of oxygen. Thus, filtering through 0.45 µm mesh prior to bottling will prevent the presence of AAB in bottled wine, although this drastic filtration may imply an important loss of compounds that are important for the quality and aroma of the wines.
- The optimal growing temperatures of AAB are between 25 and 35°C, with variations according to strains and species. Lowering storage temperatures to 10–15°C will inhibit growth to a large extent (Joyeux et al. 1984a).
- Finally, good cellar hygiene practices are a must. The risk of AAB contamination in the cellars is very high as demonstrated by the incorporation of different AAB strains that can be considered as cellar resident in grape musts (Gonzalez et al. 2005). Low alcohol content and low pH wines ageing in barrels have the highest risk of AAB spoilage in cellars, since AAB develop well in porous solid materials like wood. Regular practices such as equipment and barrel sanitization with SO_2 or hot water could be very effective in keeping AAB counts low.

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Chapter 3
Yeasts

Linda F. Bisson and C.M. Lucy Joseph

3.1 Introduction

Numerous yeast genera and species are found during the production of wine. The low pH of wine, high sugar content, rapidly generated anaerobic conditions, and presence of phenolic compounds creates the ideal environment to support the growth of yeasts and to enrich these organisms over other microbes. The metabolic activities of yeast can have a profound impact on the composition of the wine, and therefore on its aroma and flavor properties (Fleet 2003; Gil et al. 1996; Lema et al. 1996; Romano et al. 2003). Some wine styles, in fact, depend upon the metabolites of specific yeasts for their characteristic compositions. The yeasts that impact the composition of the wine can come in with the grapes from the vineyard, can be residents of the winery flora, or can be spread by insect vectors such as fruit flies, bees, and wasps (Fleet et al. 2002). The organisms found in wine can also derive from direct inoculation using commercial yeast preparations (Boulton et al. 1996).

Over twenty yeast genera have been identified from wines (Renouf et al. 2007). In addition to this species diversity, there is also significant biodiversity within a given species (Cavalieri et al. 1998; Sabate et al. 1998; Schuller et al. 2005; Sipiczki 2002, 2006; Valero et al. 2007; Versavaud et al. 1995; Vezinhet et al. 1992). The extent and persistence of the diverse yeast populations are influenced by the winemaking conditions employed. For example, holding of the must at low temperatures to increase extraction from the skins, termed a "cold soak," results in a bloom of yeast species tolerant of low temperatures (Fleet and Heard 1993). The presence of these yeasts can then influence the metabolic behavior of the principal agent of yeast fermentation, *Saccharomyces,* in addition to directly contributing aroma impact compounds to the wine. This review will cover the breadth of the biodiversity of wine yeasts found on grapes and winery surfaces and those emerging during aging that may be agents of spoilage, and will note the major variables of wine production impacting the nature of the organisms present and their persistence.

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3.2 Methods of Diversity Assessment

A critical factor in the analysis of yeast biodiversity concerns the methodology used to identify the microbes present. Often, yeasts are cultured prior to identification by physiological or molecular analyses. The act of growing yeast colonies in isolation prior to identification may result in failure to detect some species that are present or skew the relative numbers of different yeasts as minor populations are missed, given their under-representation among the colonies. Direct plating on non-selective rich media favors the faster growing yeasts such as *Saccharomyces*, and may limit the growth of more slow-growing yeasts so that they are not observed. Inclusion of conditions or inhibitors to prevent or limit the growth of fast-growing yeasts often prevents or limits the growth of other yeast species and strains present.

3.2.1 Direct Culturing Methods of Identification

One of the most frequently used methods to identify *Saccharomyces* versus non-*Saccharomyces* yeasts is plating on Lysine Agar (Egli et al.1998; Fleet 1993; Ganga and Martinez 2004). *Saccharomyces* should not grow on lysine as a sole nitrogen source, therefore only non-*Saccharomyces* yeasts will grow on these plates. In our experience, many "wild" *Saccharomyces* strains will grow slowly on lysine and some other non-*Saccharomyces* yeast may not grow well on lysine. One can also use a direct selection, such as plating a wine on media containing cycloheximide, which is a standard selection for *Brettanomyces* yeast (Boulton et al. 1996), but has also been used to select for other non-*Saccharomyces* yeast (Renouf et al. 2006b). Researchers attempt to select for a wide range of organisms by plating on non-selective media such as Wallersteins Nutrient agar (WL) and identifying yeast by colony morphology and dye uptake (Pallmann et al. 2001). However, this may select against yeast that grow slowly on WL. To get around these issues, people often plate on several different media that select for different types of yeast (Nadal et al. 1999; Nisiotou and Nychas 2007). Patterns of nutrient utilization and production of secondary metabolites, as well as sporulation and morphological characteristics, were traditionally used to identify organisms after isolation (Kurtzman and Fell 1998). However, given the extent of the natural diversity within species, spontaneously arising mutations can alter phenotypic properties so that the yeast is misidentified. As a consequence, these methods have been almost entirely supplanted in the last decade by molecular techniques.

3.2.2 Molecular Methods of Identification

Initially, molecular techniques were used to identify yeast isolates after isolation and growth in pure culture. Many different techniques have been used for this purpose,

including polymerase chain reaction (PCR) of the 26S ribosomal DNA (Kurtzman and Robnett 1998) and sequencing, and PCR and restriction enzyme digestion of internal transcribed spacers (ITS) from the 5.8S ribosomal DNA (Guillamon et al. 1998). These techniques still contain the bias inherent in the initial plating and isolation of the organism to be identified. To get around this type of bias, direct DNA sampling methods coupled to molecular characterization of the consortium DNA and identification of different marker sequences are being used to determine the numbers and types of yeast in an environmental sample (Prakitchaiwattana et al. 2004). Techniques such as PCR combined with denaturing gradient gel electrophoresis (DGGE) (Cocolin et al. 2000) and quantitative PCR (q-PCR) (Phister and Mills 2003) have been used with great success to study the ecological succession of microbes during fermentations and to identify spoilage organisms in wine. These methods allow the identification of organisms that do not grow on a given medium under given conditions. However, these methods also have their limitations. Analysis of DNA cannot distinguish between viable and nonviable cells, the methods often are limited to finding organisms only if they occur above a certain threshold frequency in the population, and are frequently limited to finding only those types of organisms that have previously been identified. PCR-based methods typically rely upon specific primers that select only organisms of a certain genus and/or species. If an organism that is not expected to occur in a specific environment being examined is present, it may not be detected using specific primers.

3.3 Biodiversity of Grape Surfaces

The diversity of yeast species on grapes has been investigated in vineyards worldwide (Barnett et al. 1972; Bureau et al. 1982; Combina et al. 2005; Davenport 1974; Goto and Yokotsuka 1977; Martini et al. 1996; Nisiotou and Nychas 2007; Parish and Carroll 1985; Prakitchaiwattana et al. 2004; Raspor et al. 2006; Renouf et al. 2007; Rosini et al. 1982; Sapis-Domercq et al. 1977; Yanagida et al. 1992) and previous reviews have covered this topic (Fleet et al. 2002; Fleet 1993; Kunkee and Bisson 1993). Using aggressive washing and analytical techniques, a concentration of 3×10^5 yeast cells cm^{-2} of the berry surface has been estimated (Rosini et al. 1982). Other studies suggest a range of 10^4–10^6 cells cm^{-2} (Fleet et al. 2002).

The factors impacting which genera and species are found have also been evaluated. The methodologies have differed, but there is a striking similarity of the main genera and species found. There are three principal genera found on grapes: *Hanseniaspora uvarum* (anamorph: *Kloeckera apiculata*), *Metschnikowia pulcherrima* (anamorph: *Candida pulcherrima*), and *Candida stellata*. In some reports, *Hanseniaspora* is the dominant species (Beltran et al. 2002; Combina et al. 2005; Hierro et al. 2006) and in others it is *Candida* (Clemente-Jimenez et al. 2004; Torija et al. 2001). *Candida* has been shown to complete the alcoholic fermentation in some cases (Clemente-Jimenez et al. 2004). Several of the *Candida stellata* isolates from wine are actually *Candida zemplinina* (Csoma and Sipiczki 2008). In one study of grapes from cooler climates (Yanagida et al. 1992), the basidiomycetes *Cryptococcus* and *Rhodotorula* dominated

in number over the ascomycete yeasts. In another, the dimorphic fungus, *Aureobasidium*, was found as the dominant yeast on grape surfaces in addition to *Cryptococcus*, followed by *Rhodotorula* and *Rhodosporiduim*, depending upon the grape variety (Prakitchaiwattana et al. 2004). A key factor determining the species present on the surface of grape appears to be the amount of damage to the fruit. The leakage of sugar substrates either through physical damage mediated by insects, birds, or invasive fungal species, or as a consequence of berry aging and shrivel on the vine due to dehydration, enriches for the ascomycetes (Fleet et al. 2002; Parish and Carroll 1985; Prakitchaiwattana et al. 2004). The amount of natural seepage varies with different grape varieties and the tightness of the clusters, so it is not surprising that some studies have seen a strong correlation of the variety with the biodiversity of the fruit surface (Yanagida et al. 1992). The first of the ascomycetous yeasts to appear are *Hanseniaspora*, *Candida*, and *Metschnikowia* (Prakitchaiwattana et al. 2004). These yeasts dominate the grape surface flora as the grapes ripen (Rosini et al. 1982; Prakitchaiwattana et al. 2004). Thus, some of the variations in species identified in comparing different published reports is a function of the physiological ripeness and integrity of the grapes when harvested for the analysis.

Other yeasts can be commonly found, although they are not as universal. *Saccharomyces* can be detected, but is present on grape surfaces at very low levels (Prakitchaiwattana et al. 2004; Martini et al. 1996), and has been undetectable in some studies (Combina et al. 2005; Raspor et al. 2006). In a comprehensive study using direct DNA profiling of grape surface microbes, 52 species of yeast were identified from the following 22 genera: *Aureobasidium, Auriculibuller, Brettanomyces, Bulleromyces, Candida, Cryptococcus, Debaryomyces, Hanseniaspora, Issatchenka, Kluyveromyces, Lipomyces, Metschnikowia, Pichia, Rhodosporidium, Rhodotorula, Saccharomyces, Sporidiobolus, Sporobolomyces, Torulaspora, Yarrowia, Zygoas cus*, and *Zygosaccharomyces* (Renouf et al. 2007). Other researchers have also found *Hansenula* (Heard and Fleet 1985; Longo et al. 1991; Mora and Mulet 1991) and *Saccharomycodes* (Combina et al. 2005). *Saccharomyces* is more commonly isolated from heavily damaged grapes (Mortimer and Polsinelli 1999). The change in species on the surface of grapes that occurs during ripening follows a pattern of early dominance by the basidiomycetous yeasts, *Aureobasidium*, *Cryptococcus*, *Rhodosporidium,* and *Rhodotorula* pre-veraison, and during early ripening, giving way, as the fruit ripens, to the ascomycetous yeast, particularly *Hanseniaspora*, *Metschnikowia,* and *Candida*, with berry damage that occurs later in ripening due to physical or biological factors enriching for these yeasts, as well as fermenting yeasts such as *Saccharomyces*. The presence of other yeast genera depends upon regional and climactic influences, the grape variety, disease pressure and level of damage of the grapes, and vineyard practices.

A direct comparison of plating to obtain viable isolates to total DNA extraction analysis of species present on the surface of grapes indicated that different organisms were obtained by the two methods, most likely due to differences in relative sensitivities and abilities to grow on the selective medium (Prakitchaiwattana et al. 2004). The major species identified using either methodology were the same, but a greater number and diversity of yeasts were detected in the direct DNA isolation studies.

In addition to stage of ripening, many factors have been identified that impact the presence and numbers of yeasts on the surface of grapes (Kunkee and Bisson 1993). In general, the number of yeasts present on grapes increases with ripening, and the numbers are higher by one or two orders of magnitude nearer the peduncle (Rosini et al. 1982). Seasonal variation has also been observed with warmer and dryer years yielding increased yeast populations (Rementeria et al. 2003). Infection with molds such as *Botrytis,* that can penetrate the berry surface, releasing nutrients, can impact the microbial flora of the surface of the grape (Nisiotou and Nychas 2007; Sipiczki 2006). Infection with *Botrytis* was found to increase the numbers of yeasts by three orders of magnitude (Nisiotou and Nychas 2007). Another study of *Botrytis*-infected grapes demonstrated the presence of *Metschnikowia* strains that were then inhibitory to other yeasts, fungi, and bacteria (Sipiczki 2006). The mechanism of inhibition was thought to be the sequestration of iron (Sipiczki 2006).

The insect pressure in a vineyard is also an important factor. Bees, wasps, and the fruit fly *Drosophila* have all been shown to be vectors of yeast species in vineyards (Benda 1982; Parle and DiMenna 1965; Stevic 1962). Microorganisms can adhere to the surfaces of the insects and be deposited on other fruit surfaces as the insect travels about the vineyard. As the insects are attracted to damaged fruit, they can spread the yeasts from the surface of the damaged fruit to other sectors of the vineyard. The application of fungicides such as elemental sulfur in the vineyard may also impact the yeast species present (Schutz and Kunkee 1977). The regional climate and altitude of the vineyard can affect the yeasts found (Castelli 1957). The type of grape variety may also impact the yeast species found on the grape surface (Nisiotou and Nychas 2007).

It was thought that the higher levels of *Saccharomyces* seen in some vineyards may be due to the practice of placing yeast lees from the fermentation in the vineyard as a source of vine fertilization (Boulton et al. 1996). To test this hypothesis, the effect of deliberate inoculation of vineyards with *Saccharomyces* on the presence of *Saccharomyces* at the time of harvest has been investigated (Comitini and Ciani 2006; Valero et al. 2005). The winery residents and vineyard inocula did not become established in the berry flora in spite of high inoculation levels. Puncturing the grapes to induce berry seepage and damage did not improve the chances of colonization by the *Saccharomyces* inoculum (Comitini and Ciani 2006).

3.4 Biodiversity of Wineries

Significantly fewer studies have been conducted of the yeast flora found on winery surfaces and equipment. It has been demonstrated that the winery flora represent a significant source of inoculation for the juice, must, and wine (Fleet and Heard 1993; Renouf et al. 2007). Following grape processing, the numbers of *Saccharomyces* found per unit volume can increase by three orders of magnitude or more (Boulton et al. 1996). Biofilms readily form on winery surfaces (Joseph et al. 2007). Stainless steel is commonly used for fermentation, but juices are also fermented in more porous

containers such as wooden barrels and vats. These are notoriously difficulty to clean, let alone sanitize, and cannot be sterilized without loss of integrity. Microbial flora often also coat walls, outer barrel surfaces, hoses, and drains, particularly during barrel ageing, as this is typically done under conditions of humidity to prevent evaporative loss of wine volume. Sanitation practices vary widely, as does the practice of supplementation with nutrients. All of these factors impact winery flora.

Only a few studies of the flora found on winery surfaces have been conducted (Martini 2003; Renouf et al. 2007). Analysis of the surfaces of barrels indicated high numbers of *Saccharomyces*, with *Candida*, *Cryptococcus,* and *Brettanomyces* also commonly present, although in lower concentrations (Renouf et al. 2006a, 2007). Bacteria and molds can be more commonly found on winery surfaces except during active fermentation, when the populations of yeasts can be high. There is considerable diversity of mold species present in wineries (Picco and Rodolfi 2004).

A current controversy concerns the origin of the *Saccharomyces* species that arise during a spontaneous or uninoculated fermentation (Martini et al. 1996; Torok et al. 1996). A direct analysis of the presence of *Saccharomyces* isolates on grape surfaces was undertaken using aseptically harvested grapes, immediately processed under sterile fermentation conditions without benefit of possible inoculation by contaminated winery surfaces (Valero et al. 2007). In this study, 68% of the vineyard samples were able to initiate fermentation. However, only 42% of the completed fermentations or 28% of the total aseptic samples taken from the vineyard were dominated by *Saccharomyces*. In another study that also used aseptic grape handling techniques, the major species found during the alcoholic fermentation was *Candida stellata,* with *Saccharomyces* only rarely found, and often not in high numbers (Clemente-Jimenez et al. 2004). These studies demonstrate that *Saccharomyces* can indeed be found in vineyards and that, in some cases, the level of *Saccharomyces* yeasts coming in with the grapes is sufficient to initiate fermentation. However, this is not always the case, and it is also true that the yeast conducting the fermentation may derive from the winery flora. As there can be a significant *Saccharomyces* bioflora on winery surfaces (Martini 2003), if the number of *Saccharomyces* yeasts derived from the winery surfaces dominates the number of those coming from the vineyard, the winery yeasts will be the major species present during fermentation. This is also true if an inoculum is used (Querol et al. 1992). Thus, whether the grapes or the winery flora are the major source of the fermentation flora depends upon the relative numbers of *Saccharomyces* coming from the surface of the grapes versus those derived from the surfaces of the winery and winery equipment.

3.5 Biodiversity of Wine Fermentations

Many analyses of the yeast flora found during wine fermentation have been conducted. Wine fermentations can be divided into two types: directly inoculated and uninoculated. Uninoculated fermentations are also called native flora or spontaneous or natural fermentations, and rely on the indigenous flora of the grapes and winery for fermentation. In both cases, following crushing of the grapes, the must (grape solids and accompanying

juice) generally displays high concentrations of the yeasts present on the grape berry (Clemente-Jimenez et al. 2004; Fleet et al. 2002; Schuller et al. 2005). These yeasts initiate the bioconversion of grape juice into wine. How long the non-*Saccharomyces* yeasts persist depends upon the winemaking conditions and relative levels of the major species present. The factors affecting the yeasts found in fermentations are similar to those affecting the flora on the berry, such as the maturity of the fruit, age of the vineyard, variety, use of antifungal agents, climate, and vineyard location (Fleet et al. 1984; Ganga and Martinez 2004; Longo et al. 1991; Martini et al. 1980; Parish and Carroll 1985; Regueiro et al. 1993; Rosini et al. 1982; Van der Westhuizen et al. 2000b). The use of antifungal agents in the vineyard results in increased populations of *Metschnikowia* (Regueiro et al. 1993) and decreased populations of *Saccharomyces* (Valero et al. 2007). In addition, harvesting techniques can also impact the yeasts present in the fermentation, particularly if the berries are damaged during harvest and microbial growth occurs during shipping to the winery (Boulton et al. 1996).

Numerous studies have categorized the changes and persistence of non-*Saccharomyces* flora during uninoculated fermentations (Beltran et al. 2002; Constanti et al. 1997; Gutierrez et al. 1997; 1999; Hierro et al. 2006; Querol et al. 1994; Renouf et al. 2006a; Schutz and Gafner 1994; Torija et al. 2001; Van der Westhuizen et al. 2000a; Van Keulen et al. 2003; Vezinhet et al. 1992; Xufre et al. 2006). These studies all demonstrate a similar pattern of species evolution during fermentation. In the beginning, the species present on the surface of the grape appear to dominate the species found in the fermentation, including the basidiomycetous yeasts and the oxidative ascomycetes. As fermentation progresses, the levels of these yeasts decrease, while that of *Saccharomyces* increases (Fleet and Heard 1993). By the end of fermentation, *Saccharomyces* is the majority of the yeasts found, and often the only yeast isolated.

Several additional factors have been found to affect the persistence of the non-*Saccharomyces* yeasts during fermentation. Sanitation practices can have a dramatic effect on the organisms present during fermentation. In one study, wineries with poorer sanitation practices had higher levels of the fermentative yeasts, presumably because these yeasts had colonized winery equipment (Regueiro et al. 1993). Surprisingly, sulfur dioxide, used as an antimicrobial agent typically added to juice upon crushing of the fruit, does not show a significant effect on the wild fermentative yeast species (Henick-Kling et al. 1998). Other studies have seen a slight effect in the decrease in yeast cell numbers with use of sulfite, but have not seen an effect on the aroma profile of the resulting wines (Egli et al. 1998). In contrast, the basidiomycetous yeasts seem to show a greater sensitivity to sulfite, with one study reporting decreases of these yeasts up to 90% (Rementeria et al. 2003).

Factors such as pH and temperature of fermentation can impact the persistence of the yeast species present (Charoenchai et al. 1998; Heard and Fleet 1985). Incubation of the juice at low temperatures to settle solids has been shown to impact yeast populations. In one study, the genera *Hansenula*, *Issatchenkia,* and *Saccharomyces* decreased dramatically, while *Hanseniaspora* and *Candida* species increased (Mora and Mulet 1991). In a similar study using a cold soak of must from a red grape variety, again *Hanseniaspora* and *Candida* species persisted during this incubation at

low temperature; however, these species showed a greater dominance during the alcoholic fermentation (Hierro et al. 2006). Interestingly, this study also showed that, during the fermentation, *Pichia* emerged along with *Saccharomyces*. Thus, the changes in flora accompanying the cold settling altered the microbial dynamics much later during the fermentation. The variation in persistence of yeast species during fermentation is also dependent upon the variety (Clemente-Jimenez et al. 2004). One factor that does impact the persistence of non-*Saccharomyces* flora is the inoculation with commercial strains of *Saccharomyces*. Inoculation with *Saccharomyces* leads to a faster domination of the fermentation and more rapid inhibition of the other yeasts present (Egli et al. 1998; Ganga and Martinez 2004).

3.6 Biodiversity of *Saccharomyces* Strains

Two principal species of *Saccharomyces* are found during alcoholic fermentation: *Saccharomyces cerevisiae* and *Saccharomyces bayanus* (formerly *S. uvarum*) (Sipiczki 2002). Occasionally, *S. pastorianus* can be found (Naumov 1996). The *S. bayanus* group includes cryptophilic strains that are able to ferment melibiose (Naumov 1996). *S. cerevisiae* has recently been divided taxonomically into six groups: cerevisiae, cheresanus, diastaticus, ellipsoideus, logos, and oviformis (Naumov 1996). Strains previously designated as *S. cerevisiae* var *bayanus* are now classified in the oviformis group. Although yeasts from all six groups have been found in wine, the major wine yeasts are from the ellipsoideus and oviformis groups. Hybrid strains of *S. cerevisiae* and *S. bayanus* as well as *S. cerevisiae* and *S. kudriavzevii* have recently been found in fermentations (Gonzalez 2006).

In addition to the diversity of non-*Saccharomyces* yeasts, genetic diversity within *Saccharomyces cerevisiae* has been well documented (Khan et al. 2000; Lopes et al. 2002; Querol et al. 1994; Sabate et al. 1998; Schuller et al. 2005; Schutz and Gafner 1994; Valero et al. 2007; Van der Westhuizen et al. 2002a, b; Versavaud et al. 1995). In one comprehensive study, over 1,600 isolates from 54 spontaneous fermentations were examined and found to comprise 297 unique strains (Schuller et al. 2005). An even higher ratio of unique genotypes (91) to total isolates (104) was found in a similar analysis (Valero et al. 2007). In one study that examined yeast biodiversity over two vintages, 60 and 65 different yeast strains as determined by analysis of mitochondrial DNA were found with only 21 of these common for the two vintages (Sabate et al. 1998). A study in Argentina found similar results: 9 out of 29 genotypes were dominant during fermentation and, of these, only 5 were common across vintages (Lopes et al. 2002). Other studies have found less, but still significant, yeast diversity (Lopes et al. 2002; Vezinhet et al. 1992; Versavaud et al. 1995). Most of these studies find the greatest number of genotypes are represented by a single isolate, indicating that the true extent of the diversity present is still being underestimated. Some studies have found that one or a few strains dominate throughout fermentation (Versavaud et al. 1995), while others have seen different strains dominate at different stages of the fermentation (Sabate et al.

1998). Other studies have seen no clear dominance of one strain during fermentation, and several strains of *Saccharomyces* appear to be simultaneously present in equivalently high numbers (Torija et al. 2001; Vezinhet et al. 1992). In cases where a single strain dominates, it has been shown to carry the killer phenotype (Schuller et al. 2005; Versavaud et al. 1995). Significant diversity among strains of *S. bayanus* has also been found (Sipiczki 2002).

The diversity of wine yeasts has been documented using genomic sequence comparisons and functional genomic analysis of transcript profiles (Fay et al. 2004; Townsend et al. 2003; Winzeler et al. 2003). Even strains that are similar in genetic composition may show changes in important enological phenotypes if the genetic differences are targeted to high impact genes (such as transcription factors), or genes involved in flavor modification or production. The biodiversity of wine strains of *Saccharomyces* is possibly a consequence of both natural selection and random mutagenesis and accumulation of mutations. Wild yeasts show elevated rates of spontaneous mutagenesis, which, if followed by sporulation and diploidization, can rapidly lead to the creation of significant diversity across a population. The return to a homozygous state has been termed "genome renewal" (Mortimer et al. 1994), and is likely a key feature of life in the wild for *Saccharomyces*.

3.7 The Biodiversity of Yeasts During Wine Aging

Yeasts are also present during the aging of wines, and can play an important role in the evolution of wine composition throughout the aging process. The type of flora present during aging depends upon the type of vessel used and winery sanitation practices. Both stainless steel and barrel surfaces can support yeast biofilm formation (Joseph et al. 2007). Stainless steel is easier to sanitize than porous wooden surfaces, which tend to build up significant numbers of yeast over the years of use. The *Saccharomyces* and non-*Saccharomyces* yeasts found during the fermentation can persist through aging, although these yeasts are usually not biologically active (Boulton et al. 1996). Species of *Candida*, *Pichia,* and particularly *Brettanomyces,* can be found in wines in barrels and can lead to cosmetic (film) or organoleptic defects in the wine (Kunkee and Bisson 1993; Heresztyn 1986; Rankine 1966; Renouf et al. 2007). Significant diversity is found among isolates of *Brettanomyces* as well (Conterno et al. 2006). *Zygosaccharomyces*, due to its tolerance of both sulfur dioxide and sorbate, can also be found as a contaminant of wine (Thomas and Davenport 1985).

3.8 Conclusions

A comprehensive understanding of the biodiversity of yeasts associated with grapes and the production of wine exists, due to the large number of studies that have been conducted worldwide and the important role of biodiversity in the evolution of aroma and flavor compounds of wine. The biodiversity within the genus *Saccharomyces* has

only recently come to be appreciated, and this diversity is likewise important in creating the desired chemical constitution of the finished wine. The flora found on grapes from vineyards across the globe shows a striking similarity, with differences in the dominant species being influenced by climate, altitude, vectors, grape variety, age of the grapes at harvest, disease pressure in the vineyard and intactness of the berries, and vineyard practices and seasonal conditions. The yeasts present initially during fermentation reflect the diversity of the species present on the grape surface at the time of harvest. The non-*Saccharomyces* yeasts can persist throughout fermentation or can be eliminated early on, depending upon winemaking conditions, grape juice composition, winery practices, and type of inoculation used. Judicious management of the flora is desirable, in order to better control the metabolites appearing in the wine. The development of rapid, real-time tools to monitor wine flora will ultimately result in the ability to better direct the development of the flora, which will represent an important advance for winemakers.

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Chapter 4
Fungi of Grapes

Hanns-Heinz Kassemeyer and Beate Berkelmann-Löhnertz

4.1 Introduction

Grapevine can be attacked by a number of fungi and fungus-like organisms which affect the berries and cause loss of quality and influence the taste of the wine. Due to attack of the grapevine by pathogens' the infected plant tissue is destroyed and necrotization occurs. When large areas of the canopy are affected by grapevine diseases, the assimilation capacity of the vine is reduced and as a result the berry quality decreases. Aside from leaves, most grapevine pathogens also infect inflorescences, clusters and berries so that the yield can be reduced. Berry infections result in decay of fruit tissue, however specific effects on berry quality depend on the ripening stage at which the infection occurs. Some pathogens directly destroy the fruit tissue enzymtically; others impede ripening, and a number of fungi produce off flavours or myctoxins. Grapevine diseases can spread rapidly under favourable conditions and cause more or less severe epidemics. To avoid loss of quality and yield, the pathogens have to be controlled by appropriated culture techniques and targeted application of fungicides. Besides the pathogenic fungi causing grapevine diseases, berries are also colonized by ubiquitous epiphytic fungi which use sugar and amino acids leaking out of berries as nutrient source. In general grapevine pathogens can be sub-divided into main pathogens of high economical importance which are pre-dominant, like downy mildew (*Plasmopara viticola*), powdery mildew (*Erysiphe necator*) and bunch rot (*Botrytis cinerea*) and those which occur only locally or temporary. Moreover other important grapevine diseases are caused by wood decaying fungi which pre-dominantly attack the trunk and canes (Fischer and Kassemeyer 2003). In the present chapter such fungi and oomycetes are regarded which colonize grapevine berries and consequently may influence the must and wine. All fungi reported to colonize grapevine berries are listed in Table 4.1, however some of them can be regarded as harmless epiphytes, others actually as antagonists

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Table 4.1 Fungi taxa detected on grapevine clusters and berries (Hall and Emmett 2001; Serra et al. 2005; Serra et al. 2006; Uyovbisere et al. 2007; Whitelaw-Weckert et al. 2007); the taxonomy of the Ascomycetes is according to Gams et al. 1998 and Mc Laughlin et al. 2001

Kindom	Class	Subclass	Order	Genus	Species
Chromista	Peronosporomycetes		Peronosporales	*Plasmopara*	*P. viticola* (Berk. & Curt.) Berl. & De Toni
Mycota	Zygomycetes		Mucorales	*Cunninghamella*	*C. spec*. Matr.
				Mucor	*M. mucedo* Fresen
					M. hiemalis Wehmer
					M. piriformis A. Fisch
				Rhizopus	*R. stolonifer* (Ehrenb.) Lind.
				Syncephalastrum	*S. spec*. J. Schröt.
	Ascomycetes	Plectomycetes	Eurotiales	*Aspergillus*	*A. aculeatus* Iizuka
					A. alliaceus Thumb & Church
					A. auricomus Saito
					A. candidus Link
					A. carbonarius Bainier
					"A. ibericus"
					A. carneus Blochwitz
					A. clavatus Desm.
					A. flavipes Thom & Church
					A. flavus Link
					A. fumigatus Fresen
					A. japonicus Saito
					A.niger aggregate Tiegh.
					A. ochreaceus G. Wilh.
					A. ostinianus Wehmer
					A. terreus Thom
					A. ustus Thom & Church

(continued)

Table 4.1 (continued)

Kindom	Class	Subclass	Order	Genus	Species
					A. versicolour Tirab.
					A. wentii Wehmer
				Emericella	*E. spec*. Berk.
				Eurotium	*E. amstelodami* L. Mangin
					E. chevalieri L. Mangin
				Paecilomyces	*Paecilomyces variotii* Bain
				Penicillium	*P. aurantiogriseum* Dierckx
					P. bilaiae Chalabuda
					P. brevicompactum Dierckx
					P. canescens Sopp
					P. chrysogenum Thom
					P. citrinum Thom
					P. corylophilium Dierckx
					P. crustosum Thom
					P. echinulatum Fassatiova
					P. expansum Link
					P. fellutanum Biourge
					P. funiculosum Thom
					P. glabrum/spinulosum
					P. griseofulvum Dierckx
					P. implicatum Biourge
					P. janczeweskii K.M. Zalessky
					P. miczynskii Zelaski
					P. minioluteum Dierckx
					P. novae-zeelandiae J.F.M. Beyma

(continued)

Table 4.1 (continued)

Kindom	Class	Subclass	Order	Genus	Species
					P. olsonii Bainier & Sartory
					P. oxalicum Currie & Thom
					P. pinopilum Hedgcock
					P. purpurogenum Stoll
					P. raistrickii G. Sm.
					P. restrictum J.C. Gilman & E.V. Abott
					P. roquefortii Thom
					P. rugulosum Thom
					P. sclerotiorum van Beyma
					P. simplicissimum Thom
					P. solitum Westling
					P. thomii Maire
					P. variabile Sopp
					P. verruculosum Peyronel
					P. waksmannii Zalski
			Onygenales	*Histoplasma*	*H. spec*. Darling
		Pyreno mycetes	Hypocreales	*Acremonium*	*A. spec*. Link
				Beauveria	*B. spec*. Vuill.
				Fusarium	*F. spec*. Link
				Gliocladium	*G. spec*. Corda
				Trichoderma	*T. spec*. Pers
				Trichoth ecium	*T. roseum* (Pers.) Link
			Diaporthales	*Coniella*	*C. petrakii* B.Sutton
				Phomopsis	*Ph. viticola* (Sacc.) Sacc
			Sordariales	*Arthrinium*	*A. spec*.Kunze
				Chaetomium	*Ch. spec*. Kunze
				Chrysonilia	*C. spec*. Arx
				Neurospora	*N. spec*. Shear & Dodge

(continued)

Table 4.1 (continued)

Kindom	Class	Subclass	Order	Genus	Species
			Xylariales	*Pestaltiopsis*	*P. spec*. Steyeart
				Truncatella	*T. spec*. Steyeart
			Erysiphales	*Erysiphe*	*Erysiphe necator* Schwein.
		Loculoasco mycetes	Dothideales	*Aureobasidium*	*A. spec*. Viala & Boyer
				Guignardia	*G. bidwellii* (Ellis) Viala & Ravaz
			Pleosporales	*Alternaria*	*A. alternata* (Fr.: Fr.) Keissler
				Curvularia	*C. spec*. Boedijn
				Drechslera	*D. spec*. S. Ito
				Epicoccum	*E. spec*. Link
				Periconia	*P. spec*. Tode ex Fr.
				Phoma	*Ph. spec*. Sacc.
				Pithomyces	*P. spec*. Ellis
				Stemphylium	*S. spec*. Wallr.
				Ulocladium	*U. atrum* Pers.
			Capnodiales	*Cladosporium*	*C. herbarum* (Pers.) Link
			Myrangiales	*Elsinoë*	*E. ampelina* Shear
		Disco mycetes	Helotiales	*Botrytis*	*B. cinerea* Pers.: Fr.
				Pseudopezicula	*P. tracheiphila* (Müll.-Thurg.) Korf & Zhuang

Table 4.2 Taxa producing mycotoxins considered relevant for human health (Serra et al. 2005)

Taxon	Mycotoxin
Aspergillus ochraceus	Ochratoxins
Aspergillus alliaceus	
Aspergillus niger aggregate	
Aspergillus carbonarius	
Penicillium verucosum	
Trichothecium roseum	Trichothecene
Penicillium expansum	Patulin

of pathogenic fungi, e.g. *Trichoderma* and *Ulocladium* (Schoene and Köhl 1999; Li et al. 2003). Some of the fungi produce mycotoxins (Table 4.2) which are more or less human-toxic and some may release compounds which are toxic to yeasts. In addition numerous fungi colonizing the berry surface during different stage of berry development and ripening have been identified (Table 4.3).

Table 4.3 Taxa identified on grapes in Portugal at different phaenological stages (Serra et al. 2005)

	Phenologial stage of the berry colonization		
Taxon	Pea size	Veraison	Harvest
Acremoniella Sacc.	X	X	X
Acremonium Link	X	X	X
Arthrinium Kunze.	X	X	0
Aspergillus Fr.:Fr.	X	X	X
Aureobasidium Viala & Boyer	X	X	X
Beauveria Vuill.	X	0	0
Chaetomium Kunze	X	X	0
Chrysonilia Arx	0	X	X
Cunninghamella Matr.	X	X	X
Curvularia Boedijn	X	X	X
Drechslera S. Ito	X	X	X
Emericella Berk.	X	X	X
Epicoccum Link	X	X	X
Eurotium Link: Fr.	X	X	X
Fusarium Link	X	X	X
Geotrichum Link: Fr.	X	0	0
Gliocladium Corda	X	X	X
Histoplasma Darling	X	0	0
Neurospora Shear & Dodge	0	X	X
Nigrospora Zimm.	0	X	X
Periconia Tode ex Fr.	X	0	0
Pestalotiopsis Steyeart	X	X	0
Phoma Sacc.	X	X	X
Pithomyces Ellis	X	X	X
Rhizopus Ehrenb.	X	X	X
Scytalidium Pesante	X	X	0
Sremphylium Wallr.	X	X	X
Syncephalastrum J. Schröt.	X	0	X
Trichoderma Pers.	X	X	X
Truncatella Steyeart	X	0	0
Ulocladium Preuss	X	X	X

4.2 Peronosporomycetes

4.2.1 Plasmopara viticola (Berk. & Curt.) Berl. & De Toni: Grapevine Downy Mildew

General aspects. Downy mildew is the most serious disease of grapevine, particularly in warm and humid climates. The pathogen is indigenous on wild grapevine species, e.g. *Vitis aestivalis* in the south-east of USA. The European cultivars of *Vitis vinifera* first came in contact with this pathogen roughly around 1878 where

first symptoms were found in the Bordeaux region. Due to high susceptibility of European cultivars, grapevine downy mildews spread within a few years and caused a pandemic in the viticultural regions of the whole of Europe. To date grape downy mildew occurs in all viticultural regions that are warm and wet during the vegetative growth of vine (e.g. Europe, Eastern part of North America, New Zealand, China, and Japan). The absence of rainfall in spring and summer limits the spread of the disease in certain areas (e.g. Australia, California, and Chile).

The disease affects all green parts of the vine, particularly leaves, inflorescences and young berries. Depending on grape cultivar and leaf age, lesions are yellowish and oily. After a damp night, oilspots and sporulation occurs on the lower leaf surface visible as dense, white patches. Later on the sporulation sites become necrotic and severely infected leaves generally drop. Such defoliation reduces sugar accumulation in berries and decreases frost hardiness of shoots and over-wintering buds. Inflorescences and clusters with young berries are highly susceptible which finally turn brown, dry up, and drop. Although berries become less susceptible as they mature, infection of the rachis can spread into older berries which turn into a dry brown rot, without sporulation.

Taxonomy. The causal agent of grapevine downy mildew, *Plasmopara viticola* (Berk. & Curt.) Berl. & De Toni, belongs to the Oomycetes, and according to current taxonomy is a member of the family of Peronsporomycetes (Dick 2002). This family is different from the kingdom of true fungi (Mycota) and is a part of Chromista, a kingdom which comprises heterogeneous microorganisms among others the autotrophic Chrysophyceae (golden algae) and Bacillariophyceae (diatoms). Like all Chromista the cell wall of *P. viticola* consists of glucanes and biflagellated zoospores are formed. Within the family of Peronosporomycetes *P. viticola* shows some primary characteristic of Chromista; among others, parts of the life cycle of the organism are bound to water. Like *P. viticola*, majority of Peronosporomycetes are plant pathogens such as the causal agent of potato late blight, *Phytophthora infestans*.

Biology and Epidemiology. P. viticola is a biotrophic pathogen strongly adapted to members of the genus *Vitis*. It develops in the inter cellular space within the colonized tissues of the vine in the form of tubular, coenocytic hyphae, developing globular haustoria. The haustoria penetrate the cell wall and invaginate the outer membrane to take nutrients from the host cell. Asexual reproduction occurs by formation of lemon shaped sporangia formed on branched sporangiophores during humid nights. Each sporangium gives rise to four to ten biflagellate zoospores which are released as soon as the sporangium is incubated in water (Kiefer et al. 2002). Asexuell developed zoospores as well vegetative hyphae are diploid.

Sexual reproduction begins in the summer by developing of gametangia. In the male antheridium as well as in the female oogonium, meiosis runs and the haploid nucleus of the antheridium fuse with that of the oogonium forming a diploid oospore. *P. viticola* is heterothallic and therefore fertilization occurs only between two different mating types. The thick walled oospore over-winter in fallen leaves, becomes mature in spring and germinates in free water forming a primary sporangium, which produces 30–60 zoospores. Germination occurs during the vegetation

period from spring to midsummer as soon as temperatures reach 10°C and rainfall ensures required wetness (Hill 1989). From the primary sporangium the zoospores are dispersed during intensive rainfall.

The released zoospores both from oospores and asexual sporangia swim within a water film covering the surface of the host plant after precipitation and dew, and attach around the stomata. They shed their flagella and encyst forming a cell wall (Riemann et al. 2002). Subsequently an infection tube emerges from each encysted spore (Fig. 4.1) which penetrates the stoma and forms a sub-stomatal vesicle in the sub-stomatal cavity where it dilates into a primary hyphae (Kiefer et al. 2002). Under optimal conditions, the period from the release of zoospores to penetration is less than 90 min. From the sub-stomatal vesicle a hyphae grows in the intercellular space of the host tissue. The hyphae branch and form a mycelium that colonizes the host tissue (Unger et al. 2007). The period from infection to first appearance of oilspots–the incubation period- depends on temperature and humidity. In general sporulation takes place at the end of the incubation period, in the first night when conditions for sporulation mentioned above occur (Rumbolz et al. 2002). Under favourable conditions, incubation period is very short and *P. viticola* is able to sporulate three to four days after infection. As soon as the host tissue is totally colonized by the mycelium of *P. viticola,* sporulation takes place. Sporulation requires 95–100% relative humidity and at least 4 h of darkness at temperatures >12.5°C; the optimal temperature for sporulation is 18–22°C and therefore an outbreak of the disease, visible in the morning after a warm and damp night yields maximum sporangia. Induction of sporulation is influenced by the photoperiod and sporangiophores and sporangia differentiate only during the night (Rumbolz et al. 2002). At the beginning of sporulation a secondary sub-stomatal

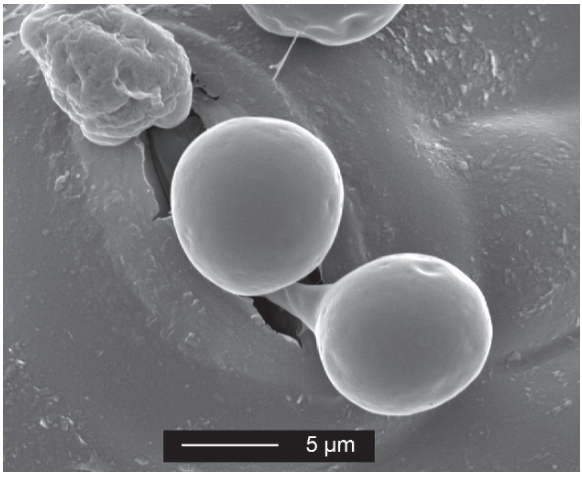

Fig. 4.1 Encysted zoospores with a penetration peg from *Plasmopara viticola* attached at a stoma; Low-Temperature-Scanning-Electron-Microscopy (Kassemeyer H.-H. and Düggelin M., University of Basel)

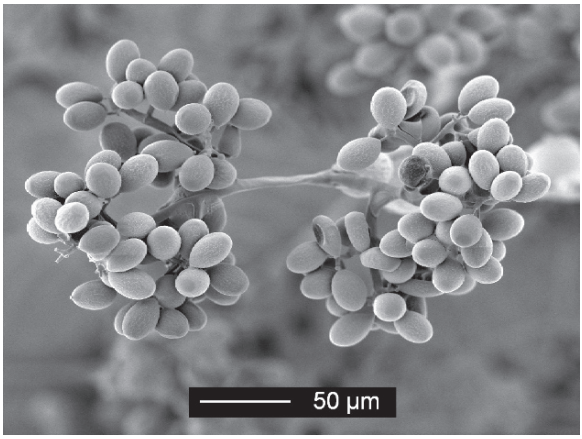

Fig. 4.2 Sporangiophore with sporangia from *Plasmopara viticola*; Low-Temperature-Scanning-Electron-Microscopy (Kassemeyer H.-H., Boso S. and Düggelin M., University of Basel)

vesicle is formed from which hyphae grow out of the stoma. The emerging hyphae, branch and form typical sporangiophores (Fig. 4.2). Finally, sporangia develop at the tips of the branches and around seven hours after the beginning of sporulation, mature sporangia are present. Immediately after formation, sporangia are detached from sporangiophores and spread by wind. Successful infection conditions can be calculated using the relation between temperature and duration of leaf wetness (Huber et al. 2003).

4.3 Ascomycetes

4.3.1 Erysiphe necator Schwein. (emend. Uncinula necator (Schw.) Burr) (Erysiphales): Grapevine Powdery Mildew

General Aspects. Grapevine powdery mildew occurs worldwide in all viticultural regions and causes severe losses of yield and quality especially in warm and dry weather conditions. This grapevine disease was introduced from North America and detected first in Europe in the middle of the 19th century. The disease spread within a short time in Europe and gave rise to economically relevant epidemics. After bud burst, first symptoms are visible as white or grey powdery patches on leaves and shoot tips between the three- and six-leaf stages on leaves. These “flag-shoots” strike on susceptible cultivars such as Chardonnay, Cabernet Sauvignon, Carignane, Portugieser, Vernatsch (Trollinger); they occur more or less pronounced also on the vast majority of European cultivars. Young leaves, inflorescences and young berries are highly susceptible immediately after the bloom; however older

leaves and berries up to the veraison are also infected (Ficke et al. 2002). Young leaves and berries can be totally covered with white powdery patches, whereas on older leaves small colonies occur on the upper leaf side. Infected leaves remain green over a longer period but the assimilation efficiency of the leaves is reduced. Shoot tips, inflorescences and young clusters are also covered with whitish or greyish patches. Infected inflorescences become curled and necrotize. Shoots become stunted and leaves appear yellowed. As a result of impeded growth of the berry skin on infected berries from the pea-size stage, cracking and splitting occurs. The splits are entrance ports for secondary invaders such as acetate acid producing yeasts and bacteria (Fig. 4.3). Fully expanded berries can be colonized by the pathogen up to the beginning of veraison. Berries with these late infections ripen but the mycelium of powdery mildew can affect wine quality by its mouldy taste. Additionally the pathogen penetrates the berry skin and facilitates infections by bunch rot.

Taxonomy. The agent causing grapevine powdery mildew, *Erysiphe necator* Schwein. (emend. *Uncinula necator* (Schw.) Burr) is an Ascomycete belonging to the Erysiphales which comprise a broad range of plant pathogens (Bélanger et al. 2002). In all Ascomycetes, the cell wall of *E. necator* consists of chitin, a polymere of N-acetylglucosamine.

*Biology and Epidemiology***.** Like all powdery mildew fungi, *E. necator* is a biotrophic fungus with limited spectrum of host plants, infecting only grapevine (*Vitis*) species. The fungus grows epiphytically on the epidermis of green plant tissue forming a dense white mycelium. *E. necator* over-winters as hyphae hidden in the buds, or as ascospores in fruit bodies (Rügner et al. 2002; Rumbolz and Gubler 2005). Both over-wintered hyphae and ascospores act as primary inoculum. During the formation of winter buds in spring, hyphae colonize the inner bud scales and remain dormant up to the following spring. After bud burst, over-wintered hyphae colonize young leaves and shoots forming more or less striking

Fig. 4.3 Berry infection by *Erysiphe necator* with splitting of the berry skin

"flagshoots". The powdery cover of this "flagshoots" pre-dominatly consists of conidiophores with chains of conidia (Pearson and Goheen 1988; Agrios 1997). The ascospores are formed after karyogamie in an ascogenic hyphae, during dry and warm weather in late summer and autumn. *E. necator* is heterothallic and, two different mating types have to combine for sexual reproduction. The ascospores are located in asci which are embedded in cleistothecia. These possess hooked appendices responsible for the attachment of the fruit bodies at the bark of canes and trunks. In the spring during rainfall, the cleistothecia open and by mean of a special mechanism the ascospores are ejected out of the asci. Ascspores as well as conidia attach actively on the surface of host plants and germinate under optimal temperatures between 20 and 27°C within 4 h (Rumbolz et al. 2000). No water is necessary for germination, but higher humidity favours this process. The germ tube forms an apressorium which strengthens the attachment of the pathogen on the host epidermis (Fig. 4.4). Beneath the apressorium, a penetration peg penetrates encymatically the cuticle and epidermis cell wall (Rumbolz et al. 2000). At the tip of the penetration peg, a lobed haustorium is formed which invaginates the epidermis cell and deprive nutrients from the host. As soon as nutrient uptake is ensured, a second hyphae emerges from the conidia and colonization of the host surface commences. Temperatures ranging from 18 to 28°C promote hyphae growth and mycelium formation. Within five to six days after infection, conidiophores are formed projecting at a right angle from the host surface. From a basal cell in the conidiophore, conidia develop and are cut off permanently. Conidia are adapted to transport by wind and spread over long distances. High temperatures and humid nights are favourable for the production of high amount of conidia. In most cases, the epidemic starts in spring from ascospore infection or "flagshoots"

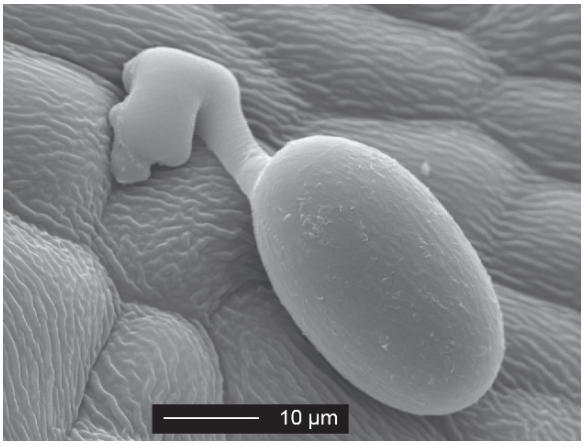

Fig. 4.4 Germinated conidia from *Erysiphe necator* on the surface of a grapevine leaf; Low-Temperature-Scanning-Electron-Microscopy (Rumbolz, J., Kassemeyer H.-H., Düggelin M. and R. Guggenheim, University of Basel)

when three to six leaves are unfolded. Under warm and dry conditions disease incidence and severity increase up to berry set, due to high susceptibility of young leaves, inflorescences and young berries (Ficke et al. 2002).

4.3.2 Botrytis cinerea Pers.:Fr. (Helotiales): Botrytis Bunch Rot

General Aspects. Botrytis cinerea is a plant pathogen of economical importance causing rot in a broad range of crops, fruits and ornamental plants. In viticulture *B. cinerea* may cause both serious loss and enhancement of quality. Injury and profit, respectively, depends not only on the stage of ripening in which berries are infected but also on weather conditions. Under dry and warm conditions infections of ripe berries may raise the quality especially of white cultivars. In this case berry ingredients are concentrated due to the perforation of the berry skin by the fungus. In addition *B. cinerea* produces gluconic acid which confers a pronounced tastiness to the wine. Consequently late infections of mature berries facilitate the production of dessert wines like "Trockenbeerenauslesen", "Sauternes" and "Tokay". On the other hand, berry infection at an early stage of ripening and during long lasting wetness of the clusters reduces the quality due to berry decay. Infestation of clusters with berry moth enhances bunch rot because the feedings sites of the larvae on berries set entrance ports for *B. cinerea*. At the beginning of infection by *B, cinerea* berries from white varieties become light-coloured from pinkish to light brown; those from red variety changes from red to purple. Later on a light grey mycelium occurs on the surface (Fig. 4.5), and in a proceeded infection stage berries become brownish and rotten. On infected berries *B. cinerea* produces high amounts of laccases which oxidase the anthocyanes and flavonoids to brown oxidation products. Laccases are very stable and can pass over in must and wine and as a result, wine becomes brownish and red wines especially lose their characteristic red colour.

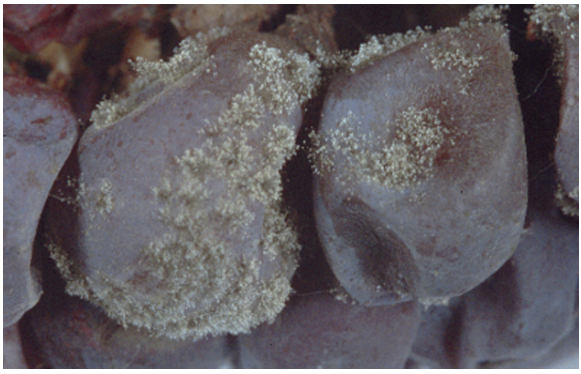

Fig. 4.5 Bunch rot caused by *Botrytis cinerea*; conidiophore emerge from pores and cracks in the rotten berry skin and form a grey pad

Taxonomy. The teleomorph of *Botrytis cinerea* Pers.:Fr., *Botryotinia fuckeliana* (de Bary) Whetzel is a member of the Heliotales (Ascomycetes). *B. cinerea* occurs mainly in its anamorph form, whereas teleomorph *B. fuckeliana* is very rare (Gams et al. 1998; Elad et al. 2004).

Biology and Epidemiology. B. cinerea is an ubiquitous fungus and has a broad range of host plants. The fungus can live saprophytically on organic debris and produce sclerotia as long-term survival form. *B. cinerea* over-winters both as mycelium and as sclerotium on canes and leaf litter on the ground. The conidia produced on sclerotia during periods with raising temperatures in the early spring are considered the main source of primary inoculum. Conidia are short-lived propagules during the season, and are spread by wind, rain and also insects. On the host plant surface, the conidia germinate 1–3 h after inoculation forming various penetration structures. In the presence of sugar the germ tubes of *B. cinerea* forms a multilobed appressorium (Elad et al. 2004). To penetrate the host tissue *B. cinerea* prefers wounds and natural openings, e.g. specialised structures of flowers on which sugar and other nutrient are available (Keller et al. 2003; Viret et al. 2004). When spores germinate on floral tissue of inflorescences or later in the season on ripening berries, *B. cinerea* can change from saprophytic to necrotrophic life style. The fungus expresses a set of enzymes such as lipases, cutinases and pectinases that enables the pathogen to penetrate the epidermis of the host tissue. The penetration of the host cuticle by *B. cinerea* mediated by cuteolytic enzymes triggers a programmed cell death in the epidermis and the underlying cells before they are invaded by hyphae. Effector proteins of *B. cinerea* acting as pathogenicity factors and the induction of the programmed cell death facilitate invasion and are essential for successful infection. So the pathogen is able to complete its disease and life cycle (Elad et al. 2004). Flowers are susceptible to infection because the receptacle constitutes natural openings and provides sugar that facilitate flower colonization by the pathogen (Keller et al. 2003; Viret et al. 2004). Increasing susceptibility of ripening berries relies on several factors: (1) host defence, e.g. expression of stilbenes, weakens with ongoing ripening, (2) amount of fungistatic protoanthocyanidins reduces after veraison, (3) structure of the cuticle and epidermis changes with advanced seed maturation and micro cracks occur which allow the leakage of sugars (Kretschmer et al. 2007). Conidia germination, germ tube growth, penetration and colonization of the host tissue are crucial processes of the infection cycle. Conidia germination and infections occur under high humidity (> 94% relative humidity) even on dry berries; however long wetness period favours development of *B. cinerea* and increases disease incidence. At 20–24°C and humid conditions, a germ tube arises within four to eight hours and under this condition hyphae grow up to 4 mm per day (Fig. 4.6). After penetration of the host tissue hyphae grow and after branching, a dense grey mycelium is formed in which conidiophores with conidia develop (Person and Goheen 1988; Agrios 1997). Conidia germination and growth of mycelium and conidia formation also occur at lower temperatures up to 5°C; however infection and development of the pathogen is delayed. Epidemics with severe infections and high disease incidence arise under continuing rainfall after veraison.

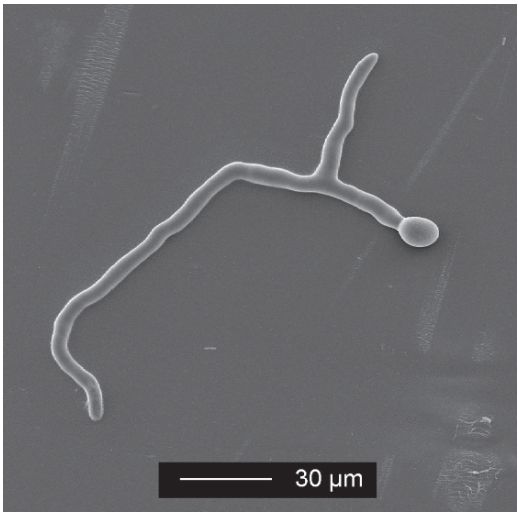

Fig. 4.6 First stage of development of *Botrytis cinerea* 17 h after inoculation, Low-Temperature-Scanning-Electron-Microscopy (Jäger, B., Jacków, J., Kassemeyer H.-H. and Düggelin M., University of Basel)

4.3.3 Pseudopezicula tracheiphila (Müll.- Thurg.) Korf & Zhuang (Helotiales): Rotbrenner

General Aspects. Rotbrenner is locally confined and occurs primarily in warm vineyards with stony soil. In some areas the disease results in severe losses annually, whereas in others it occurs only sporadically or not at all. Lesions on leaves are initially yellow on white and bright red to reddish brown on red cultivars. Subsequently a reddish brown necrosis develops in the center of the lesion, leaving only a thin margin of yellow or red tissue between the necrotic and green areas of the leaf. The lesions are typically confined to the major veins and the edge of the leaf and are several centimeters wide. Early infections occur on the first to the sixth leaf of young shoots, resulting in minor losses. Later infections attack leaves up to the 10th or 12th position on the shoot which result in severe defoliation. In addition, fungus attacks inflorescences and berries causing them to rot and dry out (Mohr et al. 2005).

Taxonomy. The causing fungus of Rotbrenner, *Pseudopezicula tracheiphila* (Müll.-Thurg.) Korf & Zhuang (syn. *Pseudopeziza tracheiphila* Müll.-Thurg.) belongs to its teleomorph *Phialophora tracheiphila* (Sacc. & Sacc.) Korf to the Helotiales (Ascomycetes) (Korf et al. 1986).

Biology and Epidemiology. The source of inoculum of the disease in spring is ascospores which are formed sexually in asci. P. *tracheiphila* appears to be composed of two mating types which exhibit a bipolare heterothallic mating system. Apothecia formed primarily on fallen leaves in the spring, hold the asci with the ascospore. Apothecia may also develop on current-season infected leaves in late

summer or fall. Depending on weather conditions, apothecia with mature ascospores may be present throughout the season (Perarson et al. 1991). The primordia of the apothecia mature as soon as the temperature rises at the end of winter. Apothecia development requires sufficient wetness of fallen leaves. Under wet and warm conditions ascospores are released already before bud burst. Heavy rainfall and prolonged surface wetness favour infection and lead to severe disease. Young leaves are susceptible after they reach a width of about 5 cm but the probability of infections increases from the 6-leaf stage. After an incubation period of two to four weeks, the fungus invades the vascular elements of infected leaves, causing symptom development (Reiss et al. 1997). The fungus remains latent if it is unable to invade the vessel elements, in which case it can be isolated from green leaves showing no symptoms. Conditions required for fungus to invade the vascular system are not well understood; however, soil conditions and water supply that place the vine under temporary stress appear to be important factors. Disease incidence and severity depend on the abundance of apothecia on fallen leaves on the ground of the vineyards and on released ascospores. Monitoring of the ascospore release by means of spore traps enables forecast of the disease situation. On malt agar, the anamorph may be formed, with hyaline, septate, short conidiophores that are coarser than vegetative hyphae. Conidiogenous cells are monophialidic and lageniform, with well-defined but thin-walled collarettes. Conidia are ellipsoid, hyaline, and unicellular. Hyphae grow in a characteristic sine-wave pattern that, when observed in the vessel elements of diseased tissue, are considered diagnostic.

A disease very similar to Rotbrenner, called angular leaf scorch, has been described in New York State (Person et al. 1988). The fungus causing angular leaf scorch in North America produces smaller apothecia than *P. tracheiphila,* and its broadly clavate asci has four spores in contrast to the eight-spored European fungus. The American counterpart has been described as a distinct species, *P. tetraspora* Korf, Pearson & Zhuang (anamorph *Phialophora*-type).

4.3.4 Phomopsis viticola (Sacc.) Sacc (Diaporthales): Phomopsis Cane and Leaf Spot

General Aspect. Phomopsis cane and leaf spot first observed in 1935 in California is also widespread in Europe for more than 50 years. Actual loss of quality due to the disease in most years is insignificant. However in rainy spring years, severe infections occur and cause lesions on shoots. In addition shoot infections affect the formation of basal buds and in consequence in the following year buds on the base of the canes especially do not sprout. Repeated infections affect the fertility of the basal parts of the canes and shorten life span of the vine. Under cool and rainy conditions during berry ripening berry infections occasionally occur. The first symptoms on shoots are dark brown to black spots on the shoot base visible from the three- to six-leaf stage. The spots elongate and the cortex crack due to secondary growth of the shoots. Large numbers of spots at the shoot base become scabby and

black. Heavy infected shoots can be dwarfed and may die. During winter, infected canes bleach and black pustules occur. Cluster infections show black spots on the rachis. However these lesions become inactivate in the course of cluster development. Rainfall in autumn rarely reactivate the lesions and cause berry infections. Infected berries show brown spots which enlarge quickly and cause a bunch rot.

Taxonomy. Phomopsis viticola (Sacc.) Sacc. (*Sphaeropsis viticola* Cooke) belongs to the Diaporthales (Ascomycetes). The teleomorph *Diaporthe* according to current knowledge is very rare in viticulture (Agrios 1997; Gams et al. 1998).

Biology and Epidemiology. Ph. viticola overwinters on infected canes and black pustules on bleached canes occurring during dormancy are pycnidia (Fig. 4.7) where pycnospores develop. Generally infections occur in spring as soon as pycnospores mature in the pycnidia and green shoots sprout. During rainfall pycnospores emerge in large quantities from the pycnidia embedded in vermiform cirri. Pycnospores (Fig. 4.8) are dispersed by splashing raindrops onto the sprouting shoots and infections occur when water remains on the green host tissue for a longer time. Prolonged wetness of sprouts and young shoots from bud break up to the six-leaf stage, favour infections by *Ph. viticola*. The number of basal buds affected by fungus vary according to frequency of rainfall and wetness of the host surface. After infection, mycelium growth in the infected host tissue but mainly shoots and buds are colonized. During summer *Ph. viticola* is less active, but in wet autumn mycelium may be reactivated and berry infections may occur (Agrios 1997; Mohr et al. 2005).

4.3.5 Elsinoë ampelina Shear (Myringiales): Anthracnose

General Aspects. Anthracnose was widespread in earlier times in European viticulture and before downy mildew was identified as the most dangerous of grapevine disease.

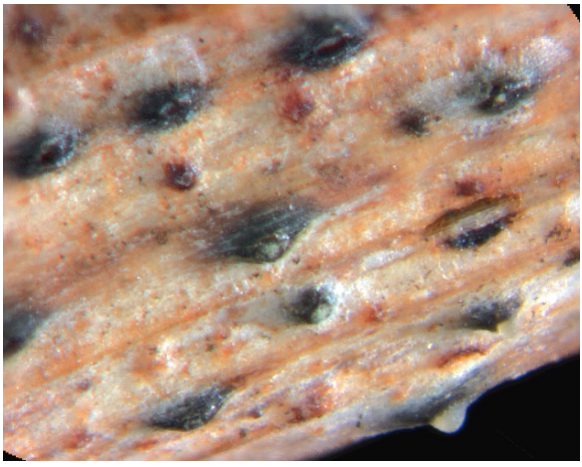

Fig. 4.7 Cane with *Phomopsis viticola* pyknidia

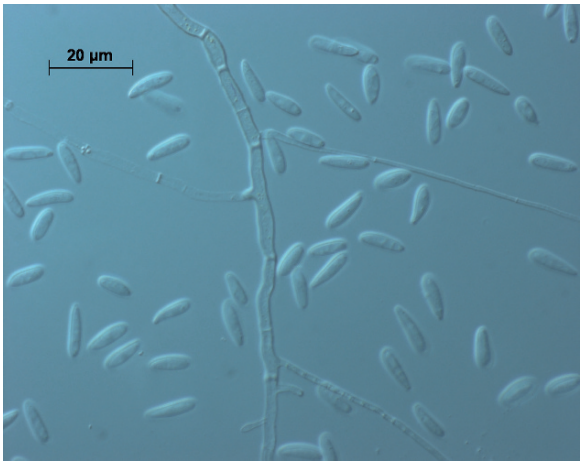

Fig. 4.8 Pycnospores from Phomopsis viticola and hyphae with characteristic septae; Differential Interference Contrast (63x)

Due to regular application of fungicides, anthracnose occurs only sporadically under very humid conditions in untreated vineyards. Infected shoots show light brown spots with black-violaceous edges. Black circular lesions occur on the leaves that necrotize and over time gives rise to small holes like a shot gun effect. Affected berries show sunken circular lesions with black-violaceous edges ("bird's eyes") which crack and finally decay. Infections of the rachis cause necrosis of the cluster with "bird's eyes" on the stems (Mohr et al. 2005). Shoots and leaf infections reduce the vigour of vine, yield and quality and shorten the life span of the plant. Decayed berries have to be removed because they can influence the quality of must and wine (Magarey et al. 1993; Sosnowski et al. 2007)).

Taxonomy. The causing fungus of the anthracnose, *Elsinoë ampelina* Shear (syn. *Gloesporium ampelophagum* (Pass) Sacc., *Ramularia ampelophagum* Pass., *Sphaceloma ampelinum* de Bary) is a member of the Elsinoaceae family which comprises ten genera (Gams et al. 1998). Elsinoaceae and Myrangiaceae belong together to the order of Myrangiales which is a member of the larger class of the Dothideomycetes (Ascomycetes).

Biology and Epidemiology. E. ampelina overwinters as sclerotia on the canes which are formed in the autumn at lesions on shoots. The sclerotia develop stromata on which under humid conditions shell-like acervuli with conidia emerge in the spring (Agrios 1997). The conidia are covered with a gelatinous layer and provide primary inoculum at the beginning of the vegetation period. Conidia propagation is favoured by rainfall and for conidia germination, wetness of the host surface for 12 h is necessary. At times fruiting bodies with asci and ascospores develop on the lesion. The propagules are transported during rainfall over a short distance; thus the disease initiates on more ore less widespread spots within the vineyards (Brook 1992).

4.3.6 *Guignardia bidwellii* (Ellis) Viala & Ravaz (Dothideales): Black Rot

General Aspects. Black rot originated from North America and has been in Europe for nearly 30 years. The disease occurs particularly in abandoned vineyards and also on resistant cultivars which are not treated with fungicides. To date black rot is restricted to some viticultural regions but the disease is becoming more common. Typical symptoms on leaves are light brown necrotic lesions with black edges up to 10 mm in diameter. Within the necrotic spots black dots are barely visible to the naked eye. On shoots, petiols and the rachis black sunken lesions appear. Infected young berries primarily show pale spots which enlarge to concentric red-brown lesions. Within a view days the affected berry gets blue-black and is covered with black pustules (Fig. 4.9). The berries finally wrinkle and dry, but remain as mummies fixed on the rachis. Frequently originated from some infected berries, the whole cluster can be infected. High infestation of black rot defoliate the canopy and as a result decrease the quality of grapes seriously. Cluster infections have an effect on yield and berry quality and affected grapes are not suitable for wine production (Pearson and Goheen 1988; Mohr et al. 2005).

Taxonomy. Black rot is caused by *Guignardia biwellii* (Ellis) Viala & Ravaz (syn. *Greeneria uvicola* (Berk. & M.A. Curtis) Punith., *Botryosphaeria bidwellii* (Ellis) Petr.) which belongs to the Dothideales an order within the Dothideomycetes (Ascomycetes) comprising some other plant pathogens such as *Ascochyta*, *Didymella*, *Botryosphaeria* and *Phoma* (Agrios 1997; Gams et al. 1998).

Biology and Epidemiology. G. bidwellii overwinters mainly in the mummified clusters and berries remaining on the shoot and also on infected canes. Asci with ascospore develop in perithecia on infected berries in spring. The ascospore are

Fig. 4.9 Berry affected by black rot (*Guignardia bidwellii*)

ejected actively from the asci during low rainfall and spread by wind. For ascospore germinate, prolonged wetness of host surface is necessary. All young green grapevine tissue including shoots, inflorescences and berries may be infected. At the beginning, infections are hard to detect but with progressed development of the fungus, necrotic spots are visible, and finally necrotic lesions occur. Within necrotic lesions on leaves, shoots and berries pyknidia with pyknospores develop during the season. Pyknospores are released during rainfall and cause infections on berries. In late summer the sexual cycle initiates on infected berries and perithecia are formed which overwinter on the infected mummified berries and clusters (Jermini and Gessler 1996; Hoffman et al. 2002; Longland and Sutton 2008).

4.3.7 Penicillium expansum Link (Eurotiales): Green Mold

General Aspects. Green mould is a secondary disease on mature berries, after wounding or bunch rot infections. Green mould occurs in warm and humid years when berries enter into ripening stage precociously. Recently incidence of green mould increases may be due to high temperature in summer and frequent precipitation during berry ripening. White pads occur on the edges of wounds and cracks which enlarge and change to glaucous (Fig. 4.10). Infected berries soften and change colour from olive-green to light-brown. In an advanced stage of infection berries decay and shrink under dry conditions. Due to the squeezing of berries and related wounds closed bunches are more frequently affected by green mould. These clusters show nests inside with decayed berries (Mohr et al. 2005). Therefore cultivars with close bunches are more susceptible than those with loose bunches. Besides in years with high berry set resulting in dense clusters green mould occurs more frequently. From single infected berries the whole cluster

Fig. 4.10 Berry infected by *Penicillium expansum*, the fungus colonizes pores in the epidermis on which nutrients leak from the berry

may be affected causing mummified clusters covered with green mould. Green mould produce mycotoxins (Abrunhosa et al. 2001; La Guerche et al. 2004; Serra et al. 2006; Pardo et al. 2006) for example patuline which is however degraded during fermentation and by sulphurization. Berries affected by green mould have an off-flavor and even a small amount of infected berries add a mouldy taste to the wine.

Taxonomy. The causal agent of green mould is mainly *Penicillium expansum* Link; other species of *Penicillium* can also be detected on affected berries (Serra and Peterson 2007). The genus *Penicillium* is regarded as a member of the Deuteromycotina but according to current taxonomy it belongs to the Eurotiales (Ascomycetes) (Agrios 1997; Gams et al. 1998; Mc Laughlin et al. 2001).

Biology and Epidemiology. P. expansum is ubiquitous and propagates by conidia which are formed abundantly on conidiophores. The conidiophores of *P. expansum* consist of two asymmetric branches with a number of flask-shaped phialides at the tip of each branch. Phialides are conidiogenous cells which produce masses of conidia in short intervals under humid and warm conditions. The phialides appear as clusters on each tip of the conidiophore and the conidia are formed in chains on each phialide. Conidiophores with the mass of conidia are visible as white to glaucous pad on infected berries (Gams et al. 1998). *P. expansum* is a typical airborne pathogen and the long-living conidia are transported by wind. In consequence conidia are released even by a gentle movement while removing infected clusters. Conidia germinate on wet surface of berries as soon as a sugary medium is available. Possibly vigorous berry development due to high amount of water supply and high temperatures causes micro cracks in the berry skin and consequently sugar runs off the slow berries. The temperature range of *P. expansum* for conidia germination, growth of the mycelium and sporulation is relatively broad, but optimal development of the fungus occur at 25°C and high humidity. Under cool and dry conditions *P. expansum* is rare even on berries whose skin is not intact. Slow and consequently late ripening cultivars and those with a strong epidermis are less susceptible to colonization by *P. expansum*.

4.3.8 Aspergillus spec. (Eurotiales): Aspergillus Rot

General Aspects. Aspergillus rot is widespread on substrates containing a disposable source for carbohydrates such as mono- and polysaccharides. Rot is common in crops and fruits and contaminate also sugary and starchy foods. At present aspergillus rot occurs on grapevine particularly in warm climate (Leong et al. 2007). The symptoms of aspergillus rot are visible as soon as sugar leaks from ripening berries after the beginning of veraison. The surface of infected berries is covered by a black mould and the berries decay. Aspergillus rot produces ochratoxins (Samson et al. 2004; Pardo et al. 2006) and contaminate must and wine with this mycotoxin suspected to be carcinogenic. For this reason clusters affected with aspergillus rot have to be sorted at harvest.

Taxonomy. Aspergillus rot is caused by different members of the genus Aspergillus which are widely distributed worldwide. On grapes particularly *A. alliaceus* Thom & Church, *A. carbonarius* (Bainier) Thom, *A. niger* aggregate Tiegh. and *A. ochraceus* G. Wilh. occur. Totally the genus *Aspergillus* comprises more than 200 taxa including species with numerous sub-species and is a genetically heterogenous group. Therefore the current taxonomy can change in the course of new findings on the phylogeny of this group. Some teleomorphs associated with *Aspergillus* are known for instance, *Emericella* Berk & Br. and *Eurotium* Link and allow integration in the Eurotiales (Mc Laughlin et al. 2001).

Biology and Epidemiology. Aspergillus species sporulate asexually by forming conidia without fruit bodies. Conidia develop on conidiophores which are sometimes aggregated and visible as a black powdery pad. The unbranched conidiophores terminate in vesicle on which phialides arise (Gams et al. 1998; Domsch et al. 2007). At the tip of the flask-shaped phialides, conida develop in chains which are spread by wind. The optimum temperature range for development of *Aspergillus* is 17–42°, minimum temperature for growth is 11–13°C.

4.3.9 Coniella petrakii B. Sutton (Diaporthales): White Rot

General Aspects. White rot occurs sporadically in southern viticultural regions while in cool and moderate climate viticulture the disease appears very rarely. Wounds, mainly from hail, favour infections by white rot. Above all damages appear on affected rootstocks showing brown spots. Rootstocks infected by white rot are not suitable for grafting and may disseminate the disease. Infected berries become yellowish, shrink and have brown pustules. Due to the development of the pustules on the berry skin the cuticle detaches from the epidermis and as a result the berry becomes pale. Clusters affected by white rot should be sorted at harvest because otherwise they may influence the quality of must and wine. In most cases white rot is controlled by regular treatments against downy mildew and bunch rot.

Taxonomy. Coniella petrakii B.Sutton is the causal agent of white rot and belongs to the order of Diaporthales (Ascomycetes) (Tiedemann 1985).

Biology and Epidemiology. The mycelium of *C. petrakii* is frequently septated and abundant branched. Globose and ostiolate pycnidia are formed by a stroma below the cuticle. The elliptical or ovate shaped pycnospores are single-celled and light brown and arise from a basal stroma in the pycnidia from the pycnidial wall (Sutton and Waterston 1966; Locci and Quaroni 1972; Tiedemann 1985). *C. petrakii* is soil borne and splash events are necessary to transport the propagules onto the host surface (Aragno 1973). High temperatures between 24 and 27°C favour conidia development and infection. After infection of wounded host tissue, the incubation period varies from 3 to 8 days (Bisiach 1988). Masses of pycnidia forming pycnospores arise from the berry surface and overwinter on the ground and are source of inoculum over years. High temperature and simultaneous wetness of the host surface necessary for successful infections exclude in most cases, infestation in cool or moderate climate viticulture.

4.3.10 *Alternaria alternata* (Fr.: Fr.) Keissler (Pleosporales): Alternaria Rot

General Aspects. Alternaria rot is ubiquitous and distributed world-wide. A number of fruit and crops may be affected; also foodstuffs and organic material like textiles, leather and paper. Alternaria rot causes merely marginal losses of berry quality in viticulture and colonize mainly ripe berries with leaked sugar. Colonized berries show a black smut on the surface. Only occasionally injured berries are infected. In this case Alternaria rot raises a mouldy taste of grapes and wine and produces mycotoxins. Therefore infected clusters have to be sorted at harvest.

Taxonomy. To the taxon *Alternaria* belongs to numerous species of which *Alternaria alternata* (Fr.: Fr.) Keissler is most common on grapevine. The genus *Lewia* is described as teleomorph for *Alternaria* (Pleosporales, Ascomycetes) (Gams et al. 1998; Mc Laughlin et al. 2001).

Biology and Epidemiology. The conidiophores of *Alternaria alternata* produce pale to medium brown conidia in long, often branched chains. The brown to olive-green conidia have transverse and longitudinal septae and a cylindrical or short conical beak (Samson and Reenen-Hoekstra 1988). The fungus has a saprophytic lifestyle and prefers a sugary substrate but occasionally it become parasitic. For setting an infection, high relative humidity is necessary (98–100%). Under these conditions the germination peg of the conidia is able to penetrate the epidermis directly. Therefore, frequent rain in late summer and autumn is favourable for the infection process (Hewitt 1988; Valero et al. 2007).

4.3.11 *Cladosporium herbarum* (Pers.) Link (Capnodiales): Cladosporium Rot

General Aspects. Cladosporium rot is widespread and very common in temperate regions on dead or dying plant substrates and other organic matter. Cladosporium rot is typically a post-harvest disease on fruits and crops. Late harvested grapes and table grapes may be infested by the rot and berries can decay. No major mycotoxins of concern are produced (Frisvad 1988; Northolt and Soentoro 1988), however volatile organic compounds are accumulate conferring a mouldy off-flavour to the affected clusters.

Taxonomy. The genus *Cladosporium* comprises numerous species of which some are the most common indoor and outdoor moulds. On grapevine clusters and berries mainly *C. herbarum* (Pers.) Link occurs (Whitelaw-Weckert et al. 2007). According to its teleomorph *Davidiella tassiana* (De Not.) Crous & U. Braun (emend. *Mycosphaerella tassiana* (de Not.) Johanson) the fungus belongs to the Capnodiales (Ascomycetes) (Gams et al. 1998; Mc Laughlin et al. 2001).

Biology and Epidemiology. Colonies of *C. herbarum* are velvety- powdery and the colour is olivaceous to brown due to the abundant mass of conidia formed on the mycelium. Smooth-walled conidiophores have terminal and intercalary swellings. At the tip of the conidiophores one-, two- or more-celled conidia are formed in sim-

ple or sometimes branched chains. The conidia are ellipsoidal to cylindrical and pigmented. They germinate on moist surfaces and lesions are black and circular ranging from several millimeters in diameter to up to two-thirds of the berry surface. If the lesion turns olivaceous, sporulation starts and conidiophores with numerous conidia are present. The fungus has a broad temperature range (4–30°C), the optimum lies between 20 and 24°C (Hewitt 1988; Whitelaw-Weckert et al. 2007).

4.3.12 Trichothecium roseum (Pers.) Link (Hypocreales): Pink Rot

General Aspects. Pink rot occurs under high relative humidity and rain on berries after veraison. Normally the rot is associated with wounds and may occur on berries infected by *Botrytis* cinerea which acts as a primary invader. Infected berries show white to pink pads, shrink and decay. Expended infections cause mummified clusters with pinkish covering. While harvesting, rotten parts of the clusters should be sorted out as mycotoxins such as crotocin, trichothecin and roseotoxin may be produced (Frisvad 1988). Additionally the rot causes an unsavoury, bitter taste in affected parts of the cluster and can be responsible for off flavour in the wine.

Taxonomy. Trichothecium roseum (Pers.) Link belongs to the order of Hypocreales (Ascomycetes); however the current Taxonomy is *incerta sedis* (Gams et al. 1998; Mc Laughlin et al. 2001).

Biology and Epidemiology. On berry surfaces, pinkish erect, unbranched conidiophores arise from the mycelium. Conidiophores are often septate near the base and more or less rough-walled. At the apex, chains of ellipsoidal to pyriform conidia develop by retrogressive division (Gams et al. 1998). Young conidia are aseptate, and when they mature, one septum in the middle of the conidia is formed. After conidia removal from the conidiophore an obliquely truncate basal scar is present where the conidia have been inserted into the conidiophore. Fungus develop also at lower temperature but optimal conidia germination and hyphae growth occurs at 25°C (Samson and Reenen-Hoekstra 1988).

4.4 Zygomycetes

4.4.1 Rhizopus stolonifer (Ehrenb.) Lind. (Mucorales): Rhizopus Rot

General Aspects. Rhizopus rot is common on soft fruits, more abundant in warm, humid climates than in cool climate viticulture. In several fruits and crops such as strawberry, tomato, cucumber and table grapes Rhizopus rot causes a soft rot during transport and storage (Hallmann et al. 2007). Rhizopus rot also affect bread and is known as black bread mould.

Taxonomy. The causal agent *Rhizopus stolonifer* (Ehrenb.) Lind is a member of Mucorales which belong to the Zygomycetes a phylum of the kingdom of Mycota distinct from the Ascomycetes (Mc Laughlin et al. 2001).

Biology and Epidemiology. R. stolonifer is heterothallic and sexual reproduction occurs exclusively when opposite mating types fuse (Schipper and Stalpes 1980). The young mycelium is whitish, becoming greyish-brown due to brownish sporangiophores and brown-black sporangia. Sporangiophores stand alone or in groups of usually 3–4. They are extremely tall, often over 20 mm high, erect and unbranched. At the opposite side of the globose sporangia branched rhizoids are obvious, a typical formation of *R. stolonifer* among most other *Rhizopus* species (e.g. *R. oryzae*). The columella is of globose to ovoid shape. The sporangiospores are irregularly formed (polygonal, globose, ovoid) with numerous striations on the spore surface (Samson and Reenen-Hoekstra 1988). Rhizopus rot is not restricted to berry infection alone. Under humid weather conditions, the fungus may spread to other berries in a cluster, causing a bunch rot (Hewitt 1988).

4.4.2 Mucor Spp (Mucorales): Mucor Rot

General Aspects. In general Mucor rot occurs as a post-harvest disease on fruits including table grapes (Hallmann et al. 2007). The rot is very rare on grapes for wine production.

Taxonomy. The three species *Mucor mucedo* Fresen, *M. hiemalis* Wehmer and *M. piriformis* A. Fisch. are characteristic species of the Mucorales (Zygomycetes) (Mc Laughlin et al. 2001).

Biology and Epidemiology. The thallus of *Mucor* spp. is white or coloured. The sporangiophores end in a globose sporangium in which spores are formed. The wall of the sporangiophore bursts for spore release (Gams et al. 1998). *Mucor* spp. can even grow and develop at cool storage conditions, In the case of *M. hiemalis* the optimum temperature for growth and sporulation is 5–25°C (Samson and Reenen-Hoekstra 1988).

4.5 Conclusions

The majority of fungi causing berry rot need mono- and oligosaccharides for spore germination and formation of a mycelium which is at disposal in the substrate. Consequently berry rot is favoured by sugar leaking from the ripening berries. In addition all these fungi develop at high temperature with an optimum from 22 to 24°C. Precocious veraison and consequently early onset of sugar import in the berry under high temperature in the summer particularly provide optimal conditions for berry infection. Susceptibility of berries to fungi is also triggered by the structure of the berry skin. An intact berry skin with a dense layer of epicuticular waxes

and a compact cuticle provide a constitutive barrier against invaders. On the other hand a weak skin is permeable for both the infection structures of the invading fungus and sugars from inside the berries. The structure of the berry skin depends on numerous factors- among others on the weather conditions, water supply and nitrogen uptake of the vine. Warm and humid conditions favouring the development of fungi may also reduce reinforcement of the berry skin. These interactions between susceptibility of berries for fungi and the epidermal tissue including the cuticle have to be noted for the control of berry rot. Another aspect of berry colonizing fungi is the off-flavour and the production of mycotoxins which can devastate the yield of the affected vineyard. To raise the quality and to avoid remarkable economical loss all measures have to be taken to control fungi colonizing berries and clusters. An important prerequisite for that is the knowledge of the biology, biochemistry and epidemiology of fungi.

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Chapter 5
Phages of Yeast and Bacteria

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5.1 Introduction

For many years, stuck and sluggish fermentations have been – and still remain – a major problem for winemakers. While stuck fermentations can usually be characterized by high residual sugar contents at the end of the alcoholic fermentation, sluggish fermentations are accompanied by a low rate of sugar utilization (Bisson 1999). In both scenarios, malfermentations can be caused by a variety of factors, most of which lead to a decrease in the metabolism of the fermenting yeast strain associated, by a decrease in biomass production, cell viability, and/or fermentation rate. One such factor potentially causing a variety of oenological problems during wine fermentation is the production of toxic proteins by certain yeasts: soon after the initial discovery of toxin-secreting killer strains in the wine yeast *S. cerevisiae* in the early 1960s of the last century (Bevan and Makower 1963), it became evident that killer yeasts and their secreted protein toxins (so-called killer toxins) can cause severe stuck fermentations, particularly under conditions when yeast starter cultures become suppressed by wild-type killer strains present on the grapes (Bussey 1981; Young 1987; Perez et al. 2001; Medina et al. 1997).

Bacteriophage attack of food-fermenting bacteria has always been a major cause of economic losses, particularly in the dairy industry (Sanders et al. 1987; Everson 1991). Research on phages and phage–host interactions in lactic acid bacteria (LAB) has thus developed, with the ultimate goal of preventing phage-induced lysis of starter strains. In the wine industry, the LAB bacterium *Oenococcus oeni* (formerly *Leuconostoc oenos*) is the organism of choice to promote malolactic fermentation (MLF), a process of major importance for the oenological properties of most wines. The economical importance of MLF and of its favored promoting agent (*O. oeni*), combined with the experience coming from the dairy industry, has prompted the research of bacteriophages – in this case, oenophages – as a potential cause of MLF failure.

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In the following sections, we will focus first on the yeast killer phenotype and its relationship to dsRNA viruses, and then move to the state-of-the-art research on phages infecting *Oenococcus*.

5.2 Killer Yeasts and Wine Fermentation

The negative effect of a particular killer strain on wine fermentation critically depends on: (a) the initial ratio of killer to non-killer yeast cells in the must, (b) toxin sensitivity of the fermenting yeast strain, and (c) the presence of protein adsorbers such as bentonite (Petering et al. 1991; Radler and Schmitt 1987; Carrau et al. 1993; Van Vuuren and Wingfield 1986; Van Vuuren and Jacobs 1992). Many studies have demonstrated that killer interactions negatively affect yeast strain activity during fermentation (Jacobs and Van Vuuren 1991; Shimizu 1993; Musmanno et al. 1999). In addition, killer spoilage yeasts spontaneously occurring in wine fermentation can compete with commercial starter cultures, in particular if the starter is susceptible to killer toxins. *Vice versa*, killer toxin-secreting strains of *S. cerevisiae* with desirable enological properties have been employed as starter cultures to improve wine quality by preventing the outgrowth of spoilage yeasts during the early onset of fermentation (Seki et al. 1985; Ciani and Fatichenti 2001; Du Toit and Pretorius 2000; Marquina et al. 2002; Schmitt and Breinig 2002; Comitini et al. 2004; Golubev 2006; Schmitt and Schernikau 1997).

5.3 Viral Infections Can Cause a Killer Phenotype in Yeast

Although double-stranded (ds)RNA viruses in yeast were originally discovered as determinants of the killer phenomenon in *Saccharomyces cerevisiae*, they are now known to be associated with the presence of cytoplasmically inherited members of the totivirus family, which are frequently found in different yeast genera (Wickner 1996; Schmitt and Breinig 2002). Among these, killer strains of *S. cerevisiae*, *Zygosaccharomyces bailii*, *Hanseniaspora uvarum,* and *Ustilago maydis* are best studied. Characteristic of all killers is the secretion of protein toxins that are lethal to sensitive (susceptible) strains of different species and genera. Cell killing is usually achieved in a receptor-mediated process, requiring initial toxin binding to components of the yeast cell surface (such as β-1,6-D-glucans, α-1,3-mannoproteins, or chitin) and subsequent toxin transfer to a secondary plasma membrane receptor. Depending on the toxin, final lethality can be caused by plasma membrane damage, cell cycle arrest, and/or inhibition of DNA synthesis (Magliani et al. 1997; Bruenn 2005; Schmitt and Breinig 2006).

In the yeasts *S. cerevisiae*, *Z. bailii,* and *H. uvarum,* as well as in the corn smut fungus *U. maydis,* the killer phenotype is cytoplasmically inherited and caused by an infection with dsRNA viruses of the family *Totiviridae,* which spread vertically

by cell–cell mating and/or heterokaryon formation (Bruenn 2005). Diploid yeasts formed by mating of a killer with a sensitive strain are likewise killers, as are all haploid progeny of subsequent meiosis. In contrast, virus-free strains are usually sensitive non-killers, while those containing ScV-L-A and a toxin-encoding M-dsRNA are killers (see below). Sensitive strains survive mating with killers, and cytoplasmic mixing of the multiple M-dsRNA copies during zygosis, accounts for the inheritance pattern during meiosis. Extracellular spread of yeast virions is generally hampered by the rigid yeast and fungal cell wall barrier and fungal viruses, therefore, have adopted a strategy of transmission *via* mating and hyphal fusion, which frequently occurs in nature, making an extracellular route of spread dispensible. While some of these viruses can be associated with adverse phenotypic effects on the fungus (like La France disease in *Agaricus bisporus*, plaque formation in *Penicillium*, and hypovirulence in *Endothia*), dsRNA viruses and their satellite dsRNAs in yeasts are responsible for a killer phenotype that is based on the secretion of a polypeptide toxin (killer toxin) that is lethal to a variety of sensitive yeast and fungal strains. With the exception of toxin-secreting strains of *Z. bailii*, killer toxin production is usually associated with specific immunity, protecting killer yeasts against their own toxin (Schmitt and Neuhausen 1994).

5.3.1 dsRNA Viruses and Killer Phenotype Expression in Wine Yeast

On the basis of killing profiles and the lack of cross-immunity, three major killer types (K1, K2, and K28) have so far been identified in *S. cerevisiae*. Each of them produces a specific killer toxin and a self-protecting immunity component. Killer phenotype expression correlates with the presence of two types of dsRNAs stably persisting in the cytoplasm of the infected host: the genomic dsRNA of the helper virus, ScV-L-A, and one of three toxin-coding satellite dsRNAs (ScV-M1, ScV-M2, or ScV-M28). ScV-L-A and ScV-M are separately encapsidated into capsids encoded by ScV-L-A dsRNA, and are present in high copy number in the yeast cell cytoplasm. In vivo, ScV-L-A does not confer a phenotype, nor does it lead to host cell lysis or cell growth slowing. While the killer phenotype can be transmitted to sensitive yeast cell spheroplasts (harboring ScV-L-A) by transfection with an ScV-M preparation (El-Sherbeini and Bostian 1987), extracellular transmission occurs rarely in nature, if at all. The survival strategy adopted by these dsRNA viruses appears to be a balanced host interaction, resulting in stable maintenance, little if any growth disadvantage, and vertical transmission. Mechanisms of exiting and entering the host cell through its rigid cell wall are rendered unnecessary by efficient horizontal transmission during frequent zygosis events in yeast. Acquisition of a toxin-encoding M satellite dsRNA provides positive selection, as virus-free segregants are killed.

As summarized in Table 5.1, the linear dsRNA genome of ScV-L-A contains two open reading frames (ORF) on its (+) strand RNA: ORF1 encodes the major capsid protein Gag necessary for encapsidation and viral particle structure, and the second

Table 5.1 Double-stranded (ds)RNA viruses involved in killer phenotype expression in yeast

dsRNA virus	Virus host	dsRNA (kb)	Encoded protein(s)
ScV-L-A	*Saccharomyces cerevisiae*	L-A (4.6)	Gag, major capsid protein; Gag-Pol, RDRP[a]
ScV-M1	*Saccharomyces cerevisiae*	M1 (1.6)	K1 preprotoxin
ScV-M2	*Saccharomyces cerevisiae*	M2 (1.5)	K2 preprotoxin
ScV-M28	*Saccharomyces cerevisiae*	M28 (1.8)	K28 preprotoxin
UmV-P1	*Ustilago maydis*	M-P1 (1.4)	KP1 preprotoxin
UmV-P4	*Ustilago maydis*	M-P4 (1.0)	KP4 toxin
UmV-P6	*Ustilago maydis*	M-P6 (1.2)	KP6 preprotoxin
HuV-L	*Hanseniaspora uvarum*	L-Hu (4.6)	Gag, major capsid protein; Gag-Pol, RDRP[a]
HuV-M	*Hanseniaspora uvarum*	M-Hu (1.0)	KT470 toxin precursor
ZbV-L	*Zygosaccharomyces bailii*	L-Zb (4.6)	Gag, major capsid protein; Gag-Pol, RDRP[a]
ZbV-M	*Zygosaccharomyces bailii*	M-Zb (2.1)	Prepro-zygocin

[a]*RDRP* RNA dependent RNA polymerase

gene (ORF2) represents the RNA-dependent RNA polymerase Pol, which is expressed in vivo as a Gag-Pol fusion protein by a −1 ribosomal frameshift event. In contrast to L-A, each M-dsRNA genome contains a single open reading frame coding for a preprotoxin (pptox) representing the unprocessed precursor of the mature and secreted toxin that also gives functional immunity. As each toxin-coding ScV-M dsRNA depends on the coexistance of ScV-L-A for stable maintenance and replication, the killer viruses resemble classical satellites of ScV-L-A. Although the presence of all three M-dsRNAs with different killer specificities in a single cell is excluded at the replicative level of the M genomes, this limitation can be bypassed by introducing cDNA copies of the K2 and K28 pptox genes into a natural K1 killer, resulting in stable triple-killers producing three different toxins at a time and simultaneoulsy expressing triple immunity (Schmitt and Schernikau 1997).

5.3.2 Viral Preprotoxin Processing and Toxin Maturation

In virus infected killer yeasts, the toxin-encoding M(+)ssRNA transcript is translated on cytosolic ribosomes into a preprotoxin (pptox) precursor that is posttranslationally imported into the secretory pathway for further processing, maturation, and toxin secretion. Interestingly, intracellular pptox processing in killers of either *S. cerevisiae* (K1, K2, K28), *Z. bailii* (zygocin) or *U. maydis* (KP4) is mechanistically conserved, resulting in the secretion of a cytotoxic monomeric or heterodimeric virus toxin. In case of K28 killers containing ScV-M28, pptox is processed into a heterodimer whose subunits are covalently linked by a single disulfide bond. As the unprocessed toxin precursor resembles a secretory protein, it contains a hydrophobic signal sequence for pptox import into the ER lumen, followed by the toxin subunits α (10.5 kDa) and β (11.0 kDa), which are interrupted by an N-glycosylated γ sequence. During passage through the yeast secretory pathway, the K28 toxin precursor is enzymatically processed to the biologically active heterodimer in a way that is highly homologous to prohormone

conversion in mammalian cells. In a late Golgi compartment, the N-glycosylated γ-sequence is removed by the action of the furin-like endopeptidase Kex2p, the C terminus is trimmed by carboxypeptidase Kex1p cleavage, and the biologically active protein is secreted as a 21 kDa heterodimer, the C terminus of which carries a four amino acid epitope (HDEL) that represents a classical ER retention signal. As this signal is initially masked by a terminal arginine residue (HDELR), ER retention of the toxin precursor is prevented until the protoxin enters a late Golgi compartment, where Kex1p cleavage uncovers the HDEL signal of the toxin (Fig. 5.1).

5.3.3 Endocytosis and Intracellular Toxin Transport

In contrast to most virally encoded yeast killer toxins, which do not enter their host but rather kill a sensitive cell by disrupting plasma membrane function, K28 is

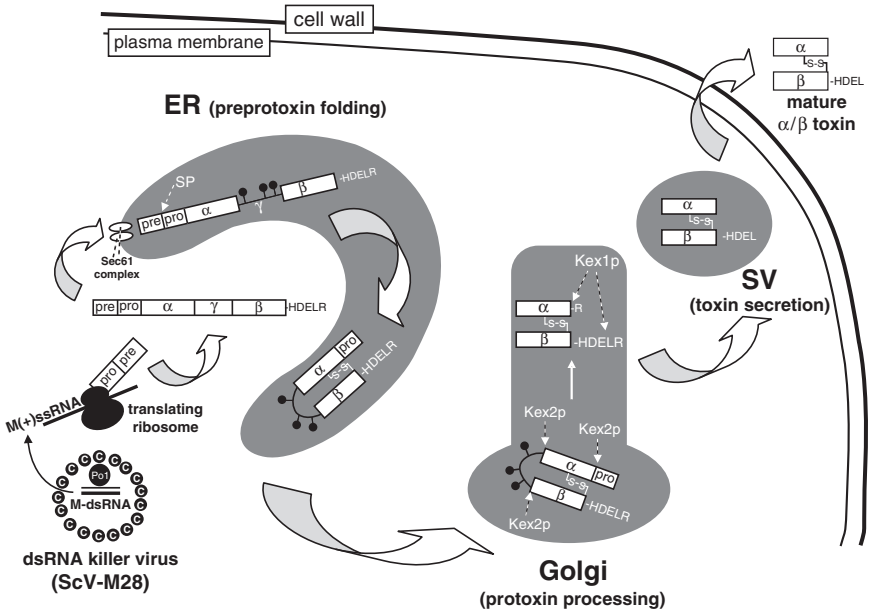


Fig. 5.1 Preprotoxin processing and toxin secretion in a ScV-M28-infected killer yeast. After in vivo translation of the preprotoxin coding killer virus transcript, the toxin precursor is posttranslationally imported into the endoplasmic reticulum (ER) through the Sec61 complex. Signal peptidase (SP) cleavage in the ER lumen removes the N-terminal signal sequence (pre-region) and protoxin folding is mediated by lumenal ER chaperones. The intervening γ-sequence is N-glycosylated and a single disulfide bond between α and β is generated. In a late Golgi compartment, Kex2p endopeptidase cleaves the pro-region, removes the γ-sequence, and carboxypeptidase Kex1p cleavage trims the C termini of both subunits, leading to the secretion of mature α/β toxin, whose β-C-terminal HDEL motiv is uncovered and thus accessible for interaction with the HDEL receptor of the target cell (*SV* secretory vesicle; adapted from Schmitt and Breinig (2002) with permission from Blackwell)

taken up by receptor-mediated endocytosis and subsequently targeted to the secretory pathway (Eisfeld et al. 2000). Once it has reached an early endosomal compartment, the toxin travels the secretion pathway in reverse, and translocates into the cytosol. Responsible and essential for this retrograde transport is a short amino acid motif at the carboxyterminus of K28-β (HDEL), which functions as an ER targeting/retention signal that is normally present at the C terminus of lumenal ER chaperones such as Kar2p/BiP and Pdi1p, the protein disulfide isomerase. In yeast and higher eukaryotes, H/KDEL-carrying proteins are recognized by a membrane-bound H/KDEL receptor, ensuring efficient recycling of H/KDEL-proteins from an early Golgi compartment back to the ER. In case of the K28, this sequence allows retrograde toxin transport from early endosomes (*via* Golgi) to the ER, from where the toxin enters the cytoplasm and transduces its lethal signal into the nucleus. Endocytotic uptake and retrograde transport is a common strategy realized in certain prototypes of bacterial toxins such as *Pseudomonas* exotoxin A, *E. coli* heat-labile toxin (HLT), or even Shiga toxin. All these protein toxins are family members of microbial A/B toxins, which are usually internalized by receptor-mediated endocytosis, followed by reverse secretion *via* Golgi and ER. Interestingly, many of these toxins contain putative ER retention signals at their C termini and H/KDEL-dependent mechanisms have, therefore, been postulated to be of major importance for toxin entry into mammalian cells. In this respect, a major difference between the yeast K28 virus toxin and bacterial A/B toxins is that K28 itself is produced and secreted by a eukaryotic (yeast) cell, and therefore the C-terminal ER-targeting signal in the toxin precursor is initially masked by a terminal arginine residue, which ensures successful pptox passage through the early secretory pathway. Once the toxin has reached a late Golgi compartment, Kex1p cleavage removes the β-C-terminal arginine residue and thereby uncovers the ER targeting signal of the virus toxin. To ensure proper access of the ER targeting signal to the K/HDEL receptor of the sensitive target cell, many A/B toxins (including the yeast K28 virus toxin) contain a unique disulfide bond at or near the C terminus. Consequently, mutant toxin variants with altered disulfide bonding are non-toxic in vivo due to the incapability to reach their intracellular target. Thus, disulfide bond formation in microbial and viral A/B toxins is of major importance to ensure interaction competence of the toxins with the K/HDEL-receptor of the target cell.

During host cell penetration, the heterodimer of K28 retrotranslocates from the ER into the cytosol and dissociates into its subunit components; while β is subsequently ubiquitinated and proteasomally degraded, the cytotoxic α subunit enters the nucleus and causes cell death (Fig. 5.1). ER exit of the heterodimeric toxin is mediated by the Sec61 complex, which functions as major transport channel (translocon) in the ER membrane of yeast and higher eukaryots. Besides being the major channel for co- and posttranslational protein import into the ER, Sec61p is also involved in the export and removal of malfolded and/or misassembled proteins from the secretory pathway to initiate their proteasomal degradation in the cytosol. In addition to its central function in protein quality control in the ER, Sec61 is also responsible for ER retrotranslocation of certain plant, microbial, and viral A/B toxins such as ricin, cholera toxin, *Pseudomonas* exotoxin A, and the yeast K28 virus toxin. In contrast to

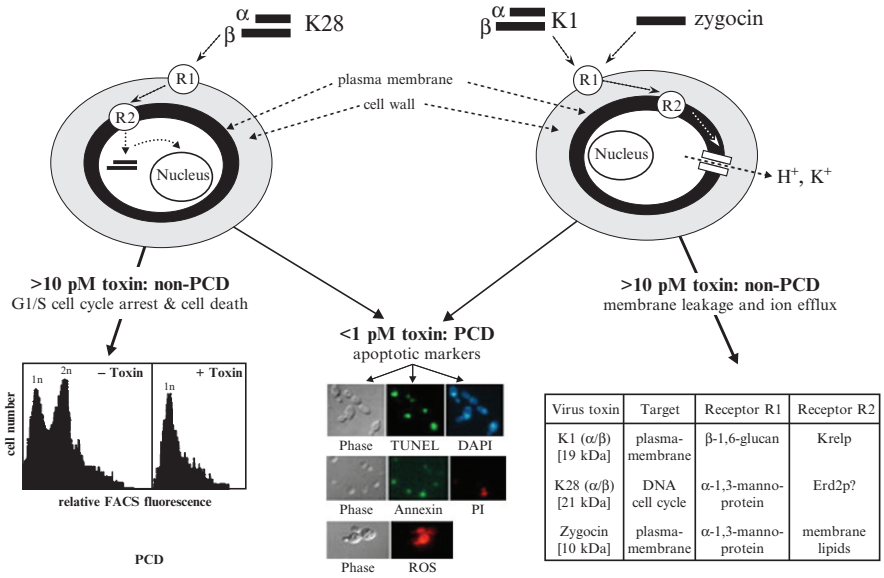
microbial and plant A/B toxins, however, retrotranslocation of K28 from the ER lumen is independent of ubiquitination and proteasome activity, and classical components normally involved in ER-associated protein degradation (ERAD) are not required for ER exit of this virus toxin (Heiligenstein et al. 2006).

5.3.4 K28 Affects DNA Synthesis and Cell Cycle Progression, and Induces Apoptosis

Although the cytotoxic component of K28 (10.5 kDa) can enter the nucleus by passive diffusion, its extension by a classical nuclear localization sequence (NLS) significantly enhances its in vivo toxicity, due to faster and more efficient nuclear import mediated by importins of the host. Within the nucleus, it interacts with host proteins of essential function in eukaryotic cell cycle control and initiation of DNA synthesis. Thus, as the virus toxin targets evolutionary highly conserved proteins with basic and central function, toxin resistance mechanisms based on mutations in essential chromosomal host genes hardly occur in vivo, indicating that the toxin has developed an amazing intelligent strategy to penetrate and kill its target cell. Most interestingly, while high toxin concentrations (10 pM or higher) cause necrotic cell killing *via* cell cycle arrest and inhibition of DNA synthesis, treatment with low doses of viral killer toxins (<1 pM) results in an apoptotic host cell response triggered by the accumulation of reactive oxygen species, ROS (Fig. 5.2). As toxin concentration is usually low in the natural environment of a killer yeast, toxin-induced apoptosis is probably an important prerequisite for efficient cell killing (Reiter et al. 2005). Furthermore, as apoptosis is also important in the pathogenesis of virus infections in mammals, it is not surprising that toxin encoding yeast killer viruses can also induce a programed suicide pathway in non-infected yeast (Ivanovska and Hardwick 2005). Although viral killer toxins were shown to be primarily responsible for this phenomenon, yeast killer viruses are not solely responsible for triggering a cell death pathway in yeast.

5.3.5 Lethality of Membrane-Damaging Viral Killer Toxins

Yeast viral killer toxins kill sensitive cells in a receptor-mediated fashion, by interacting with receptors at the level of the cell wall and the cytoplasmic membrane. The initial step involves rapid toxin binding to a primary receptor R1, which is localized within the mannoprotein or β-1,6-glucan fraction of the cell wall. In the second step, the toxin translocates to the plasma membrane and interacts with a secondary receptor, R2 (Fig. 5.2). To date, only the membrane receptor for the K1 virus toxin has been identified: Kre1p, an O-glycosylated cell surface protein initially GPI-anchored to the plasma membrane and involved in β-1,6-glucan biosynthesis and K1 cell wall receptor assembly. Once bound to the plasma membrane, ionophoric



Virus toxin	Target	Receptor R1	Receptor R2
K1 (α/β) [19 kDa]	plasma-membrane	β-1,6-glucan	Krelp
K28 (α/β) [21 kDa]	DNA cell cycle	α-1,3-manno-protein	Erd2p?
Zygocin [10 kDa]	plasma-membrane	α-1,3-manno-protein	membrane lipids

Fig. 5.2 Receptor-mediated toxicity of the viral killer toxins K1, zygocin, and K28. Killing of a sensitive yeast is envisaged in a two-step process involving initial toxin binding to receptors within the cell wall (R1) and the cytoplasmic membrane (R2). After interaction with the plasma membrane, ionophoric toxins such as K1 and zygocin disrupt cytoplasmic membrane function, while K28 enters the cell by endocytosis and diffuses into the nucleus to cause cell death (note that the cell surface receptors R1 and R2 are different in all three toxins; see also *table inset*). At high toxin doses (>10 pM), sensitive cells arrest in the cell cycle with pre-replicated DNA (1*n*; *left panel*), while cells treated with K28 in low concentrations (<1 pM) respond with apoptosis, as shown by typical apoptotic markers such as chromosomal DNA fragmentation (TUNEL positive cells), accumulation of reactive oxygen species (ROS), and phosphatidylserin exposure at the external surface of the plasma membrane detected by annexin V staining (*right panel*)

virus toxins (such as K1 and zygocin) disrupt cytoplasmic membrane function by forming cation-selective ion channels, while K28 enters the cell and acts in the nucleus. DNA synthesis is rapidly inhibited and cells arrest at the G1/S boundary of the cell cycle (Fig. 5.2).

Ion channel formation induced by the K1 virus toxin in yeast membranes as a result of direct toxin action was initially reported using patch-clamping techniques (Martinac et al. 1990). However, this observation is inconsistent with the complete resistance seen in immune yeast cell spheroplasts and, so far, receptor-independent channels have not been observed, either in yeast membranes or in *Xenopus laevis* oocytes. Similar to K1 (and probably K2 as well), zygocin represents a membrane-damaging virus toxin that is produced and secreted by ZbV-M infected killer strains of the osmotolerant spoilage yeast *Zygosaccharomyces bailii*. Zygocin itself is a monomeric, non-glycosylated protein toxin with an unusual broad killing spectrum, being equally active against phytopathogenic as well as human pathogenic yeasts, including *Candida albicans*, *C. glabrata*, *C. tropicalis,* and *Sporothrix schenkii*. As

even filamentous fungi such as *Fusarium oxysporum* and *Colletotrichum graminicola* are effectively killed by the toxin, zygocin represents a virus toxin with significant antimycotic potential. Similar to K1 but significantly more efficient, zygocin disrupts plasma membrane integrity and causes rapid cell killing. Its ionophoric mode of action has been reinforced by in silico sequence analysis, identifying a stretch of potential α-helical conformation that forms an amphipathic structure characteristic for membrane-disturbing antimicrobial peptides such as alamethicin, melittin, and dermaseptin. In addition, this feature is accompanied by a transmembrane helix at the C terminus of zygocin, which is predicted to favor a membrane permeabilizing potential, not by activating native ion channels, but rather by establishing pores by itself after toxin oligomerization. It is therefore assumed that the hydrophobic part in zygocins' amphipathic α-helix is responsible for toxin binding to the target cell. The postulated model of zygocin action resembles that of human α-defensins. In analogy to alamethicin, toxicity of zygocin is probably mediated by incorporation of its transmembrane helix into the plasma membrane, a process solely driven by the natural transmembrane potential of the energized yeast and fungal plasma membrane. Thus, zygocins' mode of action portrays the lethal mechanism of antimicrobial peptides that are produced by virtually all higher eukaryotes. Mechanisms of resistance against antimicrobial peptides are rare, and often limited to changes in the composition of the cytoplasmic membrane. In major contrast to mammalian cells, the outer leaflets of yeast and fungal membranes are enriched in negatively charged lipids. Due to the cationic net charge of antimicrobial peptides (including zygocin), an affinity to these lipids facilitates toxin adsorption to the target membrane. Consistent with that, deletion of chromosomal genes whose gene products affect plasma membrane lipid composition (such as *PDR16* and *PDR17*) cause a dramatic decrease in zygocin sensitivity, because toxin binding to the plasma membrane is largely prevented in the genetic background of a yeast *pdr16/17* mutant. In contrast to K1, a zygocin-specific membrane receptor is not required for its in vivo toxicity, as the physicochemical properties of zygocin allow efficient plasma membrane interaction independent of any membrane receptor or docking protein (Weiler and Schmitt 2005).

5.4 Toxin Immunity Ensures Self-Protection in Killer Virus Infected Yeast

For many decades, how a killer virus-infected yeast protects itself against its own secreted toxin was completely unknown. In killer yeast, functional immunity is essential for survival, as the toxins often target and inhibit central eukaryotic cell functions. This is in major contrast to bacterial toxins such as cholera toxin and Shiga toxins, which selectively kill eukaryotes, thus making immunity dispensable in a prokaryotic host. Recently, the mechanism of protecting immunity against the K28 virus toxin has been elucidated (Breinig et al. 2006). It is now known that immune cells take up external toxin (either produced by itself or by other K28

killers) and translocate it back to the cytosol, where the re-internalized toxin rapidly forms a complex with the pptox precursor that has not yet been imported into the ER. Within this complex, the K28 heterodimer is selectively ubiquitinated and proteasomally degraded, while the pptox moiety of the complex is in part released, to be either imported into the ER (to give active virus toxin) or to complex a newly internalized K28 heterodimer. In this process, the amount of cytosolic ubiquitin is critical for immunity and over-expression of mutant ubiquitin (blocked in polyubiquitin chain formation) results in a significant decrease in toxin secretion and a suicidal phenotype based on non-functional immunity. Vice versa, decreasing cytosolic ubiquitin causes an increase in toxin secretion, while immunity is not impaired as sufficient pptox is available for K28 complex formation. This simple and highly efficient mechanism ensures that a toxin-producing killer yeast is fully protected against the lethal action of its own toxin. In contrast to K28 immunity, the precise mechanism (or mechanisms) of self-protection against the ionophoric virus toxins K1 and zygocin is still obscure and remains largely unknown.

5.5 Phages in Wine and Malolactic Conversion

The presence of phage particles in wine was first evidenced by Sozzi et al. (1976) through electron microscopy analysis of Suisse white wine samples. A few years later, several papers by the same and other groups reported the isolation of phages from wine with undesirable MLF properties, which were capable of lysing different strains of *O. oeni* (Sozzi et al. 1982; Gnaegi and Sozzi 1983; Gnaegi et al. 1984; Davis et al. 1985; Henick-Kling et al. 1986a, b). However, the real impact of phages on MLF and growth of *O. oeni* in the wine environment has been somewhat controversial. On the one hand, some authors argued that irregular MLF and the consequential development of undesirable LAB strains (ex. *Pediococcus* sp.) could be the result of phage attack against *O. oeni*. On the other hand, it appeared that the ability of oenophages to lyse *O. oeni* in wine and perturb MLF was greatly dependent both on the type of phage and the bacterial strain, which responded differently to the properties of the wine, such as the pH and the composition in SO_2, ethanol, bentonite, and phenolic compounds (Davis et al. 1985; Henick-Kling et al. 1986b). Although it is still a matter of some debate, the current perception is that phage attack seems not to represent a critical problem in winemaking (Poblet-Icart et al. 1998), contrary to the scenario found in the dairy industry.

5.6 General Properties of Oenophages

Following the pioneer studies referred to above, oenophage research shifted toward the study of lysogeny in *O. oeni* and the molecular characterization of the isolated phages. A large number of phages were recovered upon treatment of potential *O. oeni* lysogenic strains with mitomycin C (see next section). These phages were subsequently characterized

not only with respect to their morphology and lytic spectrum, but also through the analysis of virion structural proteins and phage DNA (restriction patterns and DNA homology). Although the data collected from these studies allowed the definition of phage genetic groups according to their relatedness (for example, see Nel et al. 1987; Arendt and Hammes 1992; Santos et al. 1996), the general picture that emerged was a close relationship between the isolated oenophages with respect to morphology, DNA homology, and structural proteins.

All isolated oenophages belong to the *Siphoviridae* family (Ackermann 2005), whose virions are characterized by the presence of an icosahedral nucleocapsid (containing a double-stranded DNA genome) attached to a long, non-contractile tail. Morphologically, all phages are of the morphotype B1 (Bradley 1967; Ackermann and DuBow 1987). Frequently, a base plate can be distinguished in the tail extremity distal to the phage head (Fig. 5.3). The reported capsid diameters vary between 33 and 75 nm and the tails are 179–308 nm long and 6–15 nm wide. Baseplates can be up to 24 nm in diameter. The vast majority of the studied oenophages seems to be of temperate nature, i.e., they are phages that can either replicate through the lytic pathway or be propagated as prophages integrated in the host chromosome. Some temperate oenophages might have become virulent as a result of point-mutational events (Arendt et al. 1991a). The oenophage genome size has been shown to vary between 28 and 43 kb and the DNA extremities typically present cohesive ends (*cos*, Becker and Murialdo 1990).

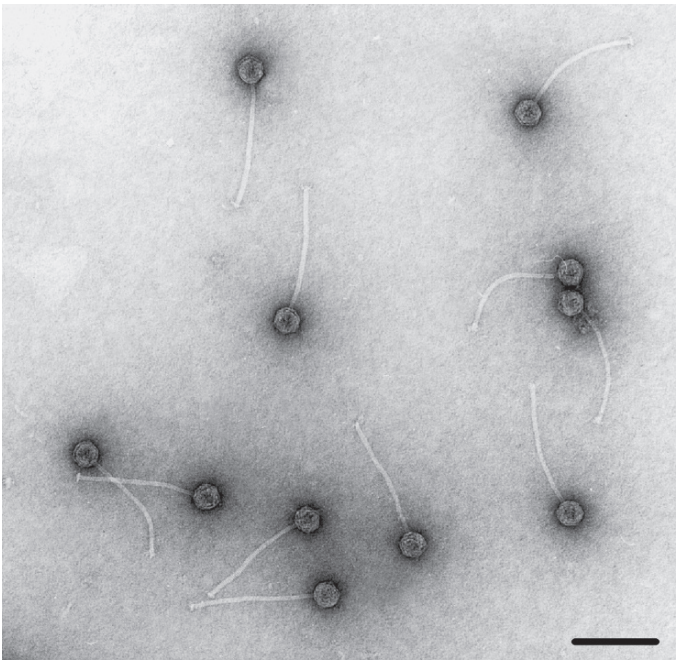


Fig. 5.3 Transmission electron microscopy photograph of negatively stained *O. oeni* phage fOg30. Magnification ×60,000; scale bar represents 170 nm

5.7 Oenophage–Host Interactions

A number of studies have revealed that lysogeny is widespread among *O. oeni*. In 1991, two independent groups (Arendt et al. 1991b; Cavin et al. 1991) reported the induction of prophages from *O. oeni* lysogenic strains upon addition of mitomycin C (MitC). The lysogeny incidence was as high as 63%, and indicator strains were found for 17 of the induced phages (Arendt et al. 1991b). This work suggested, for the first time, that spontaneous induction of prophages harbored by lysogenic strains could be a major source of bacteriophage contamination in wine. Subsequent studies have confirmed the high incidence of lysogeny in *O. oeni* (Arendt and Hammes 1992; Tenreiro et al. 1993). Later authors have examined lysogeny in 29 *O. oeni* strains, of which 22 were isolated from Portuguese wines. Phages could be detected in the supernatants of 19 different induced cultures (66% lysogeny). In a subsequent study, the Portuguese group further characterized 17 of the original isolated oenophages, and lysogenization of a phage-cured derivative of *O. oeni* strain PSU-1 was achieved with 16 phages (Santos et al. 1996). Analysis of the lysogens showed that phage DNA could integrate in one or two loci of the host chromosome.

In addition to lysogeny, some reports have indicated that pseudolisogeny may also occur in *O. oeni*. In pseudolysogeny, cells harbor unintegrated copies of phage DNA in a so-called carrier state, without being lysed significantly. In contrast to true lysogeny, serial subculturing of single colonies frequently results in the loss of contaminating phages from pseudolysogens. Studies conducted by Arendt et al. (1991a, b) suggested strongly that *O. oeni* strain 58N harbored a phage in such a carrier state. This unstable phage–host interaction might explain the lack of superinfection immunity of the original strains from which phages were initially isolated, the relatively high spontaneous induction of phages (10^3–10^5 pfu per ml of culture supernatant), and the emergence of virulent oenophages (Arendt et al. 1991a; Santos et al. 1996). Moreover, it was also observed later that infection of strain ML34-C10 with phage fOg44 at high multiplicities favored the isolation of lysogens carrying, at the same time, integrated and unintegrated forms of phage DNA (Type I lysogens, Parreira et al. 1999). Type I lysogens spontaneously released phages in relatively high titers, and exhibited a pattern of sensitivity to various oenophages that differed from strains carrying a stable prophage (Type II lysogens). It was proposed that Type I lysogens could result from cytoplasmic maintenance of unintegrated phage DNA following simultaneous penetration of several genomic copies. Type I strains would thus resemble phage-carrying strains, with the exception that, in this case, prophage DNA was also detected.

5.8 Sequencing and Functional Genomics

The first report on the partial nucleotide sequence of a phage infecting *O. oeni* was published by Sutherland et al. (1994), which sequenced an *Eco*RI–*Hind*III DNA fragment with 3.2 kb from the phage L10 genome. At the time, it was not possible

to ascertain the function of the proteins encoded by this DNA fragment. Presently, with the increasing number of phage sequences deposited in databases, we can now predict that the sequenced region harbors genes involved in phage morphogenesis.

The nucleotide sequence of a central 5.2 kb *Eco*RI DNA fragment of the phage ϕ10 MC genome was also partially determined. Analysis of the sequence (3.992 kb) allowed the recognition of the elements involved in site-specific integration (the integrase gene and the *attP* site, Gindreau et al. 1997) and the genes mediating lysis of the host cell at the end of the lytic cycle (*lys*, encoding a peptidoglycan hydrolase and *P163*, coding for a putative holin, Gindreau and Lonvaud-Funel 1999).

Santos et al. (1998) have mapped the restriction sites of six enzymes in six oenophage genomes and performed a comparative analysis of whole phage DNA. Phages were separated into two distinct groups (α and β), based on restriction site conservation and DNA–DNA cross-hybridization results. In spite of the heterogeneity in the restriction site profiles, hybridization results clearly evidenced homology between α and β phages in the central part of their genomes. This study has proven to be a useful starting point for studying specific regions of the phage DNA. A particular phage (fOg44, β-group) was studied in more detail through the determination of the nucleotide sequence of the central and of the *cos*-containing genomic regions (see next section).

5.8.1 General Outline of Oenophage Genome Organization

The nucleotide sequence of two regions of the phage fOg44 genome was determined (Parreira et al. 1999; São-José 2002): a 7.810-kb segment spanning the *cos* site (Fig. 5.4b, Acc. Number AJ421942) and a 10.858-kb central fragment (Fig. 5.4c, Acc. Number AJ421943). The total sequence covered about 50% of the fOg44 genome. Figure 5.4 summarizes the relevant data emerging from the analysis of the two sequenced regions.

The region encompassing the *cos* site of the fOg44 genome (Fig. 5.4b) carries genes involved in DNA metabolism and packaging, and head morphogenesis. Of the genes located upstream the *cos* site, we highlight *orf74* (*orf* stands for open reading frame), encoding a putative glutaredoxin-like protein, and *orf176*, which codes for an HNH-type endonuclease. These proteins are thought to be involved in the maintenance of a deoxyribonucleotides pool for phage DNA synthesis (Gleason and Holmgren 1988) and in DNA packaging (Dalgaard et al. 1997), respectively. Downstream the *cos* site (Fig. 5.4b), we find the genes coding for the small (*orf128*) and large (*orf640*) subunits of the terminase oligomeric complex, which mediate phage genome maturation and encapsidation (Feiss 1986; Becker and Murialdo 1990). The putative products of *orf64*, *orf389,* and of the incomplete *orfY*correspond to the head-tail joining, portal, and ClpP proteins. In all studied phages, the portal protein forms a ring structure composed of 12 subunits in one vertex of the phage capsid, which serves as an entrance and exit port for the phage DNA (Bazinet and King 1985). The role of ClpP-type endopeptidases in maturation

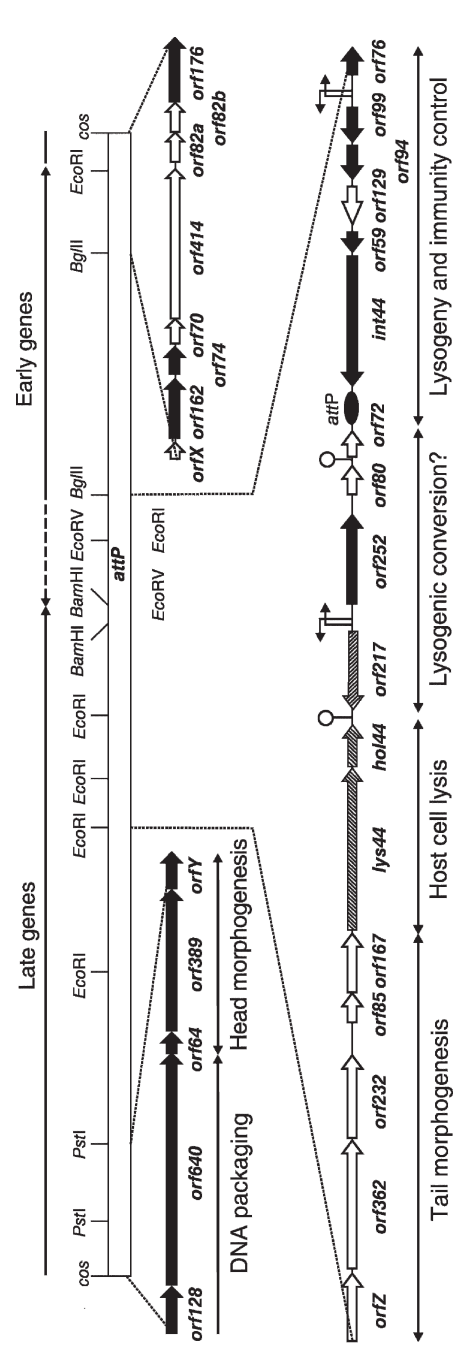


Fig. 5.4 Gene organization in two genomic regions of fOg44. (**a**) The fOg44 genome is represented by a *white rectangle* and the position of relevant restriction sites is indicated. The *thin arrows* above the map evidence the regions that are early and late-transcribed during the phage lytic cycle (São-José 2002). The *dashed arrow* indicates a genome segment showing a low level of transcription (harboring *orf217* to *orf59*). (**b**) The *arrows* represent the *orfs* identified by nucleotide sequence analysis of the *cos*-containing region. The *orfs X* and *Y* denote 5′ and 3′ truncated open reading frames, respectively. The *orfs* coding for putative products with and without homologues in the databases are colored in *black* and *white*, respectively. Putative gene clusters involved in a particular function are indicated below the corresponding *orfs*. (**c**) Organization of *orfs* identified in the genome central fragment. *Black* and *white arrows* as in (**b**) *orfZ* denotes a 5′ truncated open reading frame. *Slashed arrows* indicate genes whose homology-based predicted functions have been confirmed experimentally. Putative transcription terminators and divergent transcriptional promoters are depicted as *hairpins* and *bent arrows*, respectively. Gene sets involved in specific functions are indicated below the corresponding *orfs*

of the phage head has been experimentally shown for phage HK97 (Duda et al. 1995; Hendrix and Duda 1998).

The relative position of the *orfs* identified in the central region of fOg44 DNA (Fig. 5.4c) and the homology found for some of its encoded proteins allowed the identification of gene sets involved in specific functions: tail morphogenesis (based only in gene position), host cell lysis, lysogenic conversion, and lysogeny and immunity control.

The cluster defined by *orf217*, *orf252*, *orf80,* and *orf72* is not essential (at least under laboratory conditions) for the ability of fOg44 to complete its lytic cycle or to form lysogens (Parreira et al. 1999). LacZ-fusion experiments have shown that *orf217* is a transcriptional regulator that represses both its own promoter and that of *orf252* (encoding a putative permease, São-José et al. 2004). The location of this module between the lysis and integration elements and its dispensable nature suggests that, like other "morons," it may confer selective advantage to the host bacteria when the phage is residing as the prophage (Juhala et al. 2000; Desiere et al. 2001).

In the lysogeny and immunity control cluster, we find the elements involved in phage DNA integration in the host chromosome, i.e., the *attP* site and the *int44*gene (Parreira et al. 1999; São-José et al. 2004). The genes located upstream of *int44* regulate immunity and lysogeny functions (São-José 2002). In the prophage state, the putative products of *orf129* and *orf94* (a membrane protein and a metalloproteinase) most probably compose a system that excludes heteroimmune, superinfecting phages. *Orf99* is the λ-CI repressor-like protein regulating the lysis/lysogeny decision and conferring immunity to homoimmune phages when expressed from the prophage.

More recently (São-José et al. 2004), the central genomic region topologically equivalent to that spanning from *orf167* to *orf59* in fOg44 was sequenced in two other oenophages, fOgPSU1 (Acc. Number AJ629109) and fOg30 (Acc. Number AJ629110), and compared to the sequences of phages fOg44 and ϕ10MC. fOgPSU1 and fOg44 share the same lysis cassette, whereas in the other two phages the putative holin gene is replaced by *orf163*. With the exception of ϕ10MC where the putative holin and integrase genes are separated exclusively by the *attP* site, several *orfs* (five in fOgPSU1 and six in fOg30) could be found between the lysis and integration regions, with *orf217*, *orf252,* and *orfr80* being common to the three fOg phages. The integration elements of fOg30 and fOg44 were almost identical at the nucleotide level, and differed significantly with respect to the fOgPSU1 and the ϕ10MC sequences. These differences are responsible for the targeting of different loci in the *O. oeni* chromosome for phage DNA integration (see next section).

The overall gene organization in the sequenced regions of oenophage genomes was found to closely follow that described for equivalent regions in dairy phages belonging to the "λ supergroup" of *Siphoviridae* (Brüssow and Desiere 2001). It is thus possible to envisage a general outline of oenophage genome organization, with functional gene clusters arranged from the left to the right side of the genome as follows: DNA packaging/head morphogenesis/head-tail joining/tail morphogenesis/host lysis/lysogenic conversion/lysogeny and immunity control/DNA replication and recombination/transcription regulation.

5.8.2 Multiple tRNA Loci as Sites for Phage DNA Integration in the Host Chromosome

The viral and bacterial DNA attachment sites (*attP* and *attB*, respectively), containing the identical core sequences that are involved in the site-specific recombination event that leads to phage DNA integration (Campbell 1962), were determined at the nucleotide sequence level for three oenophages. Phage fOg44 was shown to integrate its DNA at the 3´ end of an *O. oeni* $tRNA^{Glu}$ gene, whereas fOgPSU1 targets a gene for $tRNA^{Lys}$ (São-José et al. 2004). Phage ϕ10MC integrates its genome at a $tRNA^{Leu}$ gene (Gindreau et al. 1997). In all cases, the intact tRNA gene sequence was reconstituted upon phage DNA integration. When the available DNA sequences of fOg44 and ϕ10MC were used for homology searches in the draft genome sequence of *O. oeni* strain PSU-1 (complete genome sequence published in 2005, Mills et al. 2005), two additional tRNA genes ($tRNA^{Glu}$ and $tRNA^{Leu}$) were found as potential integration sites (São-José et al. 2004). One of these sites may correspond to the secondary integration locus described by Santos et al. (1996).

5.8.3 The Lysis Region and a New Mechanism of Phage-Induced Host Lysis

Double-stranded DNA phages induce lysis of infected cells in order to release progeny virions. Until recently, it was assumed that these phages promoted host cell lysis following the same basic mechanism, which was extensively studied for the *Escherichia coli* phage λ (for a review, see Young et al. 2000). According to this mechanism, lysis is accomplished by the concerted action of a peptidoglycan hydrolase (referred to as endolysin) and a small hydrophobic protein (holin) that forms nonspecific lesions in the membrane at a precise scheduled time. The latter function was considered essential to allow access of the cytoplasm-accumulated endolysin to the cell wall at the correct time.

Interestingly, it was demonstrated that the fOg44 endolysin (Lys44) is endowed with a typical N-terminal signal peptide that mediates its export to the cell wall through the general bacterial secretion pathway (the Sec-system, São-José et al. 2000). All known oenophage endolysins share this property (São-José et al. 2004). Although not required for Lys44 export, the fOg44 holin was able to complement a nonsense mutation of the λ holin gene, proving its functionality (São-José et al. 2004). This result, associated to the fact that the Lys44 active form is detected about half-way through the fOg44 latent period (São-José et al. 2000), strongly argued that lysis regulation in fOg44 differed substantially from that described for λ.

A lysis model was proposed (São-José et al. 2000), in which the activity of the exported endolysin would be restrained by a molecular mechanism dependent on the membrane proton motive force (pmf). Holin-mediated dissipation of the pmf at a defined time would then activate the secreted enzyme. This model has recently

gained support through experiments showing that oenococcal and lactococcal cells are intrinsically resistant to Lys44 when added from without or expressed from within, respectively (Nascimento et al. 2008). Moreover, Lys44-mediated lysis of both cells could only be efficiently triggered upon addition of nisin, the only antimicrobial among those tested that promoted ion-nonspecific dissipation of the pmf, an event that should be undertaken by the fOg44 holin in the phage infection context (Nascimento et al. 2008).

Most interestingly, phages producing secreted endolysins were later revealed to be more common than initially suspected. Several phages infecting both Gram-positive and Gram-negative hosts have been suggested to produce Sec-exported endolysins (São-José et al. 2003, 2007). As far as we know, this has been confirmed experimentally for the lytic enzymes of the lactococcal phage ϕAM2 (São-José et al. 2000; São-José 2002), the *Lactobacillus* phage ϕg1e (Kakikawa et al. 2002), and for the coliphages P1 and N4 (Xu et al. 2004, 2005; Stojković and Rothman-Denes 2007).

5.9 Conclusions

In spontaneous wine fermentations, infection with a killer toxin-producing yeast can cause pronounced malfermentation, accompanied by low sugar consumption, high residual sugar content, and, eventually, by cell death of the desired wine yeast at the end of the process. Killer toxin-induced stuck fermentations can also occur under conditions when yeast starter cultures become suppressed by wild-type killer yeasts present on the grape. The inhibitory effects of a given killer yeast on the winemaking process largely depend on the initial ratio of killer to non-killer yeasts in the must, on toxin sensitivity of the fermenting yeast, as well as on the presence of protein adsorbers such as bentonite. However, since most of these factors cannot be tightly controlled during wine fermentation, toxin-secreting yeasts with desirable enological properties could be used as a starter culture to prevent outgrowth of spoilage yeasts during fermentation and thus to improve and ensure final wine quality.

Reports on the failure of malolactic fermentation, possibly due to the presence of bacteriophages in the must, led to several lines of investigation on phages infecting *Oenococcus oeni*. Molecular biology studies have so far been focused mainly on the central region of the genome of a few temperate phages, highlighting the elements involved in prophage integration, genes possibly involved in lysogenic conversion phenotypes, and genes essential for lysis of the host. Knowledge about the sequence of *int* and *attP* and the finding of several tRNA genes as potential sites for site-specific recombination may lead to the future construction of integrative vectors useful for the genetic manipulation of *Oenococcus*. A novel mechanism for bacterial cell lysis was discovered when studying lytic functions of oenophage fOg44, relying on secretion signals in the endolysin enzyme. Bioinformatic analysis indicates that this mechanism may be operative in several other systems as well.

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Part II
Primary and Energy Metabolism

Chapter 6
Sugar Metabolism by *Saccharomyces* and non-*Saccharomyces* Yeasts

Rosaura Rodicio and Jürgen J. Heinisch

6.1 Introduction

The predominant characteristic of the entire wine-making process is the conversion of must sugars into ethanol. This is almost exclusively carried out by unicellular eukaryotes which divide by budding – the yeasts. As outlined in Chap. 3, a large variety of non-*Saccharomyces* yeast species are present in the early stages of fermentation. Although these may contribute to ethanol production and produce important flavour compounds (Boulton et al. 1999), they are generally considered a problem due to the production of acetate and other off-flavours. Therefore, starter cultures of the wine yeast *Saccharomyces cerevisiae* are generally employed in all large scale wine production plants (reviewed in Chap. 29).

Even before the enormous advances of biochemistry in the first half of last century, *S. cerevisiae* drew much attention due to its capacity for alcoholic fermentation, for which it has been used since thousands of years by mankind (see Barnett 2003). Moreover, with the advances in molecular genetics since the early 1980s, it has evolved into the best studied eukaryotic organism. Consequently, the majority of this chapter will present data obtained from *S. cerevisiae*, with occasional references to similarities and differences to other wine yeasts. The interested reader is referred to other excellent reviews on yeast sugar metabolism (Fraenkel 1982; Gancedo and Serrano 1989; Zimmermann and Entian 1997; Dickinson 1999; Boulton et al. 1999).

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6.2 Available Sugars, Pasteur and Crabtree Effect

The ripe grape contains a considerable amount of sugar, represented mainly by glucose, fructose and minor amounts of sucrose. Being ideal carbon and energy sources for yeasts and other microorganisms, they are usually shielded from their attack by the intact berry skin. Yeasts present on the grapes thus only thrive in and around local lesions. This drastically changes in the early stages of wine making, when sugars are liberated at high concentrations, i.e. approximately 110 g l^{-1} each of glucose and fructose in a typical must.

Consequently, yeasts are actively dividing in the first phases of fermentation and degrade the sugars. Glucose utilization starts right away and is slightly faster, so that the proportion of fructose increases as fermentation progresses (Fig. 6.1; Fleet 1998). This constant increase in the fructose/glucose ratio has been suggested to be a major cause for stuck fermentations (Berthels et al. 2004). The preferential degradation of glucose has been attributed to the characteristics of hexose transporters as well as to the sugar phosphorylating enzymes (see Sect. 6.3). Yeasts like *Zygosaccharomyces bailii* may be fructophilic.

The rate of sugar catabolism decreases continuously in the course of fermentation. This phenomenon has been attributed to ethanol toxicity, declining transport of solubles and a general lack of nutrients. Later stages of alcoholic fermentations are then conducted by non-growing cells at high alcohol and low nutrient concentrations (Bauer and Pretorius 2000). It should be noted that *S. cerevisiae* ferments sugars to ethanol and carbon dioxide even with aeration. Thus, the "Pasteur effect" (a term coined by biochemists in the first half of last century), which states that an organism switches to a respiratory mode of metabolism in the presence of oxygen, *does not occur* in the very

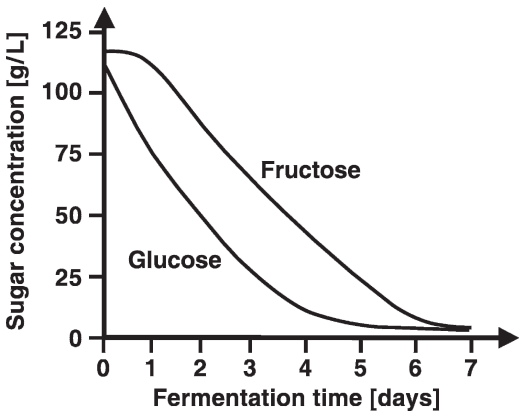

Fig. 6.1 Degradation of glucose and fructose in must. A typical kinetics of sugar degradation in must fermentations is presented. Note that significant differences from these *idealized curves* may occur with individual musts, yeast strains employed and varying fermentation conditions. Actual data from different must fermentations can be retrieved from references cited in the text

Table 6.1 Physiological categories of wine yeasts

Mode of fermentation	
Crabtree-positive	**Crabtree-negative**
Saccharomyces cerevisiae	*Hanseniaspora uvarum*
Zygosaccharomyces bailii	*Pichia anomala*
Brettanomyces intermedius	*Candida utilis*
Torulopsis glabrata	*Hansenula neofermentans*
Schizosaccharomyces pombe	*Kluyveromyces marxianus*
Hanseniaspora guilliermondii	*Debaryomyces hansenii*
Candida stellata	*Torulaspora delbrueckii*

yeast Pasteur was investigating (Lagunas 1981). Instead, at sugar concentrations above approximately 2 g l^{-1}, *S. cerevisiae* channels most of the substrate into alcoholic fermentation. This mode of metabolism has been called the "Crabtree effect" and yeasts can be classified, accordingly (Table 6.1). However, one should bear in mind that such metabolic differences become vain soon after the onset of vigorous fermentation, when in fact anaerobic conditions are achieved by the production of carbon dioxide and the shielding of musts from access to molecular oxygen.

6.3 Biochemistry and Physiology of Yeast Alcoholic Fermentation

Utilization of sugars as carbon- and energy source first requires their uptake and activation into a glycolytic intermediate. Glucose-6-phosphate is a metabolite of central importance and distributes carbohydrates into metabolism (Fig. 6.2). The main course through glycolysis was the first biochemical pathway to be elucidated. In fact, the experiments of Eduard Buchner in the late nineteenth century demonstrated the possibility of fermentation in cell-free yeast extracts and thus founded the science of biochemistry (Greek "enzymon" = "in yeast"). From then until the molecular cloning of the encoding genes in the 1980s, considerable detail has been gathered on the chain of reactions and their importance in yeast alcoholic fermentations, which will be briefly discussed in the following.

6.3.1 Hexose Transport

Early biochemical analyses indicated two basic types of hexose transport systems, so-called "high affinity" and "low affinity" transporters. Several carriers with intermediate affinities were described, later on. They have been shown to transport glucose, fructose and mannose (Bisson and Fraenkel 1983; Özcan and Johnston 1999). The K_m for both the low and high affinity transporters was shown to be lower for glucose than for fructose, while the V_{max} of fructose transport was higher

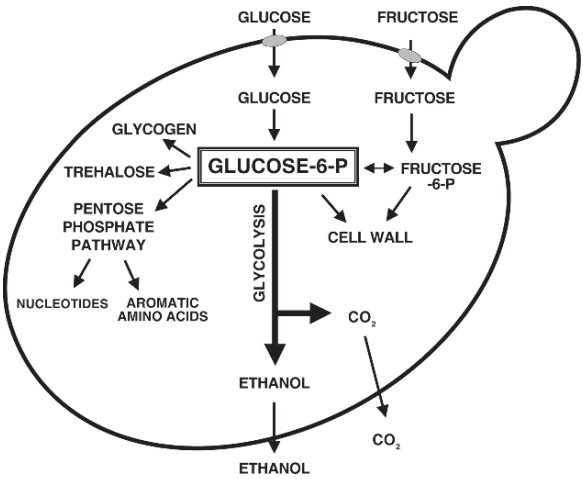

Fig. 6.2 Central role of glucose-6-phosphate in yeast sugar metabolism. A very simplified view of alcoholic fermentation is presented and the pathways for glucose-6-phosphate metabolism are highlighted. Synthesis and degradation of reserve carbohydrates is discussed in Chap. 15

than that for glucose. These differences in kinetic parameters have been correlated with the preferential degradation of glucose in wine fermentations (Bisson 1999; Berthels et al. 2008). It was also noted that transport does not need to be energized, but occurs by facilitated diffusion in *S. cerevisiae* (Lagunas 1993). However, sugar transporters of other wine yeasts have been shown to function as proton symporters and need to be energized.

With the completion of the yeast genome sequencing project, it turned out that *S. cerevisiae* encodes a total of 18 putative hexose transporters, which belong to the major facilitator superfamily (Saier et al. 1999). Though some of the encoded proteins may have different functions, such as being involved in glucose sensing (Snf3 and Rgt2) or serving as multidrug transporters (Hxt9 and Hxt11; Nourani et al. 1997), the majority is in fact capable of transporting glucose and fructose across the plasma membrane. A strain lacking seven of the genes (*hxt1–hxt7*) is not able to grow on glucose or fructose, anymore (Reifenberger et al. 1997). Growth of the mutant could be restored by re-introducing any one of the encoding wild-type genes. These seven proteins thus carry out most of the hexose transport in vivo. Yet, a strain lacking all putative hexose-transporter genes is now also available (Wieczorke et al. 1999). Both mutant strains turned out to be extremely useful to study the function of each single homolog in vivo. Specific subsets of hexose transporters are synthesized in response to the external abundance of hexoses and enable *S. cerevisiae* to use sugars over an extremely broad concentration range. Thus, the low affinity transporters have been shown to be expressed at high glucose concentrations, whereas the high affinity ones predominate when the sugar is scarce (Table 6.2; Özcan and Johnston 1999; Perez et al. 2005).

Table 6.2 Characteristics of sugar transporters in yeasts

Hexose transporter	Glucose affinity[a]	Regulation by glucose[b] (laboratory strains)	Expression during fermentation[c] (wine strains)
Hxt1	Low	Induced by high [glucose]	Start of fermentation
Hxt2	Moderate	Induced by low [glucose] Repressed by high [glucose]	Lag phase
Hxt3	Low	Induced by high and low [glucose]	Throughout fermentation
Hxt4	Moderate	Induced by low [glucose] Repressed by high [glucose]	Induced during growth phase
Hxt5	Moderate High	Not regulated by glucose Regulated by growth rate	Not induced
Hxt6	High	Induced by low [glucose] Repressed by high [glucose]	Induced in stationary phase
Hxt7	High	Induced by low [glucose] Repressed by high [glucose]	Induced in stationary phase

[a]Reifenberger et al. (1997), Maier et al. (2002), Verwaal et al. (2002)
[b]Boles and Hollenberg (1997), Özcan and Johnston (1999)
[c]Luyten et al. (2002) Perez et al. (2005)

A mutant lacking the major hexose-transporter genes (*hxt1–hxt7*) was also obtained from a wine strain (Luyten et al. 2002). Expression of different transporter gene combinations in this genetic background suggested that Hxt3 plays a predominant role during must fermentations. The high affinity carriers Hxt6 and Hxt7 are involved in hexose transport at the end of fermentation, while Hxt2 may play a role only at the beginning.

Recently, Guillaume et al. (2007) characterized a commercial strain which had a higher fructose fermenting capacity than other wine yeasts. This feature was attributed to the expression of a mutant *HXT3* allele. The importance of the hexose transport in sugar metabolism was also demonstrated by the construction of a yeast strain which expressed a chimeric carrier composed of the amino terminal half of Hxt1 and the carboxy terminal half of Hxt7. With this strain, a re-direction of the glucose-flux from alcoholic fermentation into respiration was observed, even in the presence of high glucose concentrations (Henricsson et al. 2005).

6.3.2 Glycolysis

Hexose uptake appears to be the primary step controlling the glycolytic rate in *S. cerevisiae*. The remaining enzymatic reactions are probably not rate limiting, since overproduction of key enzymes does not result in an increased flux to ethanol (Schaaff et al. 1989). In the following, we will briefly discuss the individual steps of glycolysis leading to the production of pyruvate in *S. cerevisiae* as the model yeast (Table 6.3).

Once inside the cell, glucose and fructose are phosphorylated by the action of kinases, which catalyze the first irreversible step of glycolysis (Entian and Barnett

1992). Consequently, intracellular free sugar concentrations are extremely low (<2.5 mM). Three kinases, glucokinase (Glk1), hexokinase 1 (Hxk1) and hexokinase 2 (Hxk2) have been identified (Fig. 6.3, Table 6.3). Glucokinase uses glucose or mannose as substrates, whereas both hexokinases can phosphorylate glucose, fructose or mannose. The three enzymes also differ in their kinetic parameters. Studies on the transcriptional regulation demonstrated that Hxk2 is the predominating isoform in cells growing on glucose and fructose, as found in the must (Moreno et al. 2005). After shifting the cells to a non-fermentable carbon source, *HXK2* expression ceases and *HXK1* and *GLK1* are rapidly derepressed.

Rossignol et al. (2003) monitored the transcriptome of a wine strain under production conditions. They found that in the first phase of fermentation *HXK2* is highly expressed. In the second phase, when growth ceases but fermentation still proceeds, Hxk2 transcription decreases and *HXK1* and *GLK1* become expressed. This supports the regulation patterns observed for laboratory strains. It may also explain the in vivo functions of Hxk1 and Glk1, which have remained a mystery: As stated above, the fructose/glucose ratio rises at the end of must fermentations. Considering the kinetic parameters of the enzymes, a shift from Hxk2 to Hxk1 would be favourable, as the latter displays a higher V_{max} for fructose (Rossignol et al. 2003). On the other hand, the very high affinity of the glucokinase for glucose (with a K_m of 0.03 mM) would facilitate its utilization when glucose levels are very low. Consistent with this, Berthels et al. (2008) found that a lower glucose preference correlates with a higher fructose/glucose phosphorylation ratio and a lower K_m for both sugars.

Early studies on the regulation of yeast hexokinase indicated an inhibition by ATP. However, the data presented above show that Hxk2 is mainly active at high sugar concentrations such as found in the must, which suggests that ATP inhibition may not be important for the in vivo activity (Golbik et al. 2001). Rather, a potent allosteric inhibitor of Hxk2 has been described with trehalose-6-phosphate (reviewed in Gancedo and Flores, 2004). Hexokinases from other yeasts may differ in these allosteric properties. Thus, for *Schizosaccharomyces pombe* is has been shown that SpHxk2 is not at all inhibited by trehalose-6-phosphate and SpHxk1 shows only a marginal effect. In *S. cerevisiae* a deficiency in trehalose-6-phosphate synthase (Tps1) results in growth inhibition. This was first explained by the deregulation of the initial part of glycolysis but may be more complex.

The reversible interconversion of glucose-6-phosphate and fructose-6-phosphate is performed by phosphoglucose isomerase, encoded by *PGI1* in *S. cerevisiae. PGI1* mutants grow on fructose, but are dependent on trace amounts of glucose in the medium (Aguilera 1986), underlining the importance of glucose-6-phosphate for feeding different routes of carbohydrate metabolism (Fig. 6.2). In *K. lactis*, the constitutively expressed *RAG2* gene encodes the phosphoglucose isomerase. In contrast to *S. cerevisiae*, null-mutants are still able to grow on glucose as a sole carbon source (Goffrini et al. 1991), due to a higher capacity of the pentose phosphate pathway.

The phosphorylation of fructose-6-phosphate to fructose-1,6-bisphosphate is the first irreversible reaction specific for glycolysis (Heinisch and Hollenberg 1993).

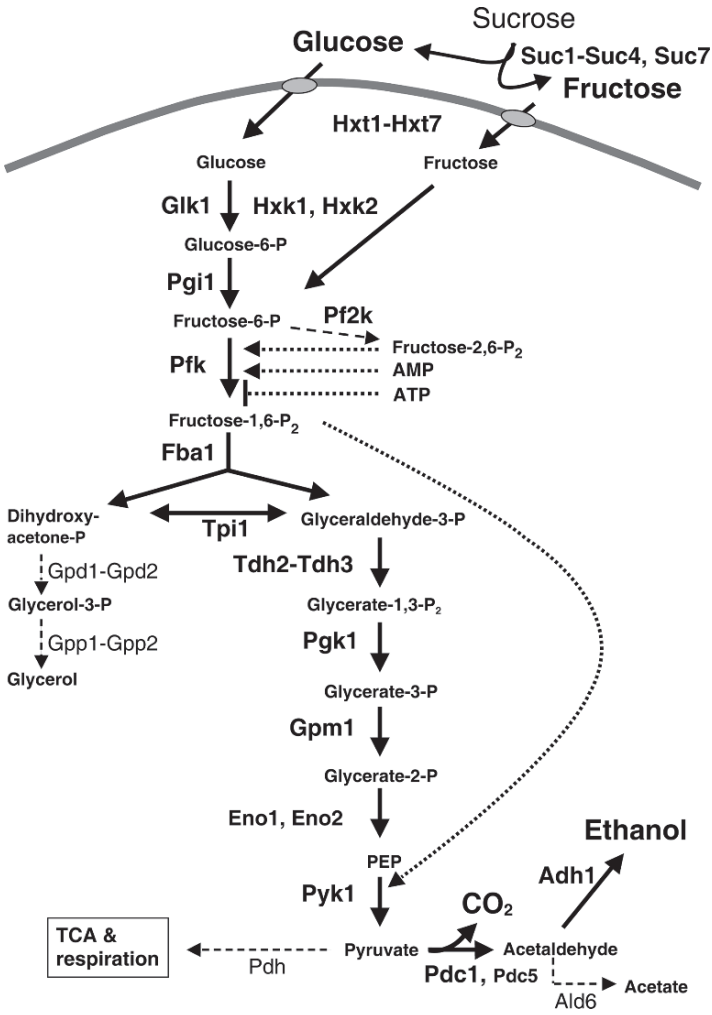

Fig. 6.3 Pathway of alcoholic fermentation in yeasts. The carbon flow from hexoses to ethanol in the yeast *S. cerevisiae* is presented by the *solid black arrows. Arrows with dashed lines* indicate minor diversions from the main flux. *Arrows with dotted lines* designate positive regulatory mechanisms, *dotted lines ending in a solid bar* mark inhibitory effects. Full names and physiological characteristics of the enzymes designated here in the *three letter code* are given in Table 6.3. *Pdh* pyruvate dehydrogenase complex; *Pfk* 6-phosphofructo-1-kinase (also see comment in Table 6.3); *Pf2k* 6-phosphofructo-2-kinase.The pathways to glycerol and acetate are shown in more detail in Fig. 6.4

Table 6.3 Genetic and biochemical features of enzymes involved in alcoholic fermentation in *S. cerevisiae*

Protein/abbreviation	Genes	Structure	Effectors/coenzymes[a]	Mutants/phenotypes[b]	Regulation[c]
Hexokinase1,2 glucokinase Hxk1 Hxk2 Glk1	*HXK1* *HXK2* *GLK1*	Homodimer (2 × 54 kDa)	Hxk2: (−) trehalose-6-P	*hxk1*Δ *hxk2* Δ: Fru^- *hxk1*Δ *hxk2*Δ *glk1*Δ: Glu^-, Fru^-	*HXK2*: only expressed on glucose; participates in glucose repression *HXK1*: not expressed on glucose *GLK1*: not expressed on glucose
Phosphoglucose isomerase Pgi1	*PGI1*	Homotetramer (2 × 61 kDa)		*pgi*Δ: Glu^-, Fru^-	Expressed on glucose and non-fermentable carbon sources
Phosphofructokinase Pfk[d]	*PFK1* + *PFK2*	Heterooctamer (4α × 108 kDa + 4β × 105 kDa)	(+) AMP, (+) Fru-2,6-bisphosphate (−) ATP	*pfk1*Δ *or pfk2*Δ: Glu^+ *pfk1*Δ *pfk2*Δ: Glu^-	Expressed on glucose and non-fermentable carbon sources
Fructose-1,6-bisphosphate aldolase Fba1	*FBA1*	Homodimer (2 × 40 kDa)		*fba1*Δ: Glu^-	Expressed on glucose and non-fermentable carbon sources
Triosephosphate isomerase Tpi1	*TPI1*	Homodimer (2 × 27 kDa)		*tpi1*Δ: Glu^-	Expressed on glucose and non-fermentable carbon sources
Glyeraldehyde-3-phosphate dehydrogenase GAPDH1-3	*TDH1* *TDH2* *TDH3*	Homotetramer (4 × 36 kDa)	NAD^+	*tdh2*Δ *or tdh3*Δ: Glu^+ *tdh2*Δ *tdh3*Δ: Glu^-	*TDH2,TDH3*: expressed in exponential phase *TDH1*: expressed in stationary phase
Phosphoglycerate kinase Pgk1	*PGK1*	Monomer (45 kDa)		*pgk1*Δ: Glu^-	Increased expression on glucose
Phosphoglycerate mutase Gpm1 Gpm2 Gpm3	*GPM1* *GPM2* *GPM3*	Homotetramer (*GPM1*: 4 × 28 kDa)	2,3-Bisphosphoglycerate	*gpm1*Δ: Glu^- *gpm2*Δ *gpm3*Δ: Glu^+	*GPM1*: expressed on glucose and non-fermentable carbon sources Gpm2, Gpm3: no function in glycolysis

Enolase Eno1 Eno2	*ENO1* *ENO2*	Homodimer (2 × 47 kDa)		*eno1*Δ *or eno2*Δ: Glu^+ *eno1*Δ *eno2*Δ: Glu^-	*ENO2*: strongly increased expression on glucose *ENO1*: expressed on glucose and non-fermentable carbon sources
Pyruvate kinase Pyk1 Pky2	*PYK1* *PYK2*	Homotetramer (4 × 55 kDa)	Pyk1: (+) Fru-1,6-bisP (−) ATP	*pyk1*Δ: Glu^- *pyk2*Δ: Glu^+	*PYK1*: increased expression on glucose *PYK2*: repressed by glucose
Pyruvate decarboxylase Pdc1 Pdc5 Pdc6	*PDC1* *PDC5* *PDC6*	Homotetramer (4 × 62 kDa)	TPP	*pdc1*Δ, *pdc5*Δ *or pdc6*Δ: Glu^+ *pdc1*Δ *pdc5*Δ *or pdc6*Δ: Glu^- in presence of antimycin A	*PDC1*: strongly increased expression on glucose *PDC1* and *PDC5* expression require the Pdc2 *PDC1, PDC5*: expression induced in *pdc1* Δ mutants *PDC6*: repressed by glucose
Alcohol dehydrogenase Adh1–5	*ADH1* *ADH2* *ADH3* *ADH4* *ADH5*	Homo/heterotetramer (4 × 37–41 kDa) Adh4: Homodimer (2 × 51 kDa)	NAD^+	*adh1*Δ: Glu^- in presence of antimycin A	*ADH1*: expressed on glucose and non-fermentable carbon sources *ADH2*: repressed by glucose Adh3-5: also participate in glucose fermentation

[a](+) indicates allosteric activation, (−) indicates allosteric inhibition; *TPP* thiamine pyrophosphate

[b]Glu^-, Fru^-: failure to grow on glucose or fructose as a sole carbon source, respectively

[c]The term "increased expression on glucose" refers to a more than twofold higher expression as compared to non-fermentable carbon sources; strongly increased expression indicates an at least fivefold increase

[d]Abbreviations for enzymes are usually according to the gene names. For Pfk we adopted a different approach, since the enzyme is a heterooctamer whose subunits are encoded by *PFK1* and *PFK2*. In the mammalian nomenclature PFK1 designates the 6-phosphofructo-1-kinase and PFK2 designates the 6-phosphofructo-2-kinase. To avoid misunderstandings, Pfk in this treaty designates the first, Pf2k the second enzyme. Isozymes of the latter in yeast are encoded by the genes *PFK26* and *PFK27*

In *S. cerevisiae*, phosphofructokinase is a heterooctameric enzyme (4α- and 4β-subunits; encoded by *PFK1* and *PFK2*, respectively; Kopperschläger and Heinisch 1997). In vitro activity can only be detected for the intact heterooctamer, yet each subunit is capable of catalysis in vivo, if the other one is missing (Arvanitidis and Heinisch 1994).

The genetics of *Kluyveromyces lactis* may be informative for the importance of this reaction in its close wine yeast relative *Kluyveromyces marxianus*. The enzyme is also a heterooctamer, but in contrast to the *pfk1 pfk2* mutant of *S. cerevisiae*, *Klpfk1 Klpfk2* null mutants still grow on glucose as a sole carbon source. Further investigation showed that glycolysis can be by-passed by the pentose phosphate pathway in *K. lactis* (Jacoby et al. 1993), and may thus not be essential for sugar degradation in all wine yeasts.

Regarding its biochemistry, phosphofructokinase (Pfk) is a paradigm for allosteric regulation. A number of small molecules affect enzyme activity, with ATP being the most potent inhibitor and AMP and most of all fructose-2,6-bisphosphate serving as activators (reviewed in Kopperschläger and Heinisch 1997). Linking carbon metabolism with nitrogen availability in must fermentations, ammonium has been shown to also stimulate Pfk activity in yeast (Afting et al. 1972). All these allosteric effectors are apparently sensed by each of the yeast Pfk subunits (Heinisch et al. 1996; Rodicio et al. 2000). Other wine yeast may be quite similar, since only Pfk from the distant non-wine yeast *Yarrowia lipolytica* apparently lacks allosteric properties (Flores et al. 2005).

Fructose-1,6-bisphosphate is then reversibly cleaved by aldolase (encoded by *FBA1*) into glyceraldehyde-3-phosphate and dihydroxyacetone phosphate (Schwelberger et al. 1989). Both triosephosphates are interchanged with the help of triosephosphate isomerase (encoded by *TPI1*), the most evolved enzyme known by biochemists (cited in Heinisch and Rodicio 1997). A lack of this enzyme causes accumulation of dihydroxyacetone phosphate which is then channelled into glycerol production (Compagno et al. 1996).

Further conversion of glyceraldehyde-3-phosphate through glycolysis depends on the availability of NAD^+ as an electron acceptor, which is regenerated from NADH at the last step of alcoholic fermentation. If the capacity for reoxidation at this step is limited, NAD^+ can be regenerated either by respiration or by conversion of dihydroxyacetone phosphate to glycerol. This is the major source of glycerol present in wine, whose production may also be triggered by the osmotic stress during the primary stages of must fermentation (Heinisch and Rodicio 1997).

All but the last of the following glycolytic reactions are also reversible: *S. cerevisiae* contains three isozymes of glyceraldehyde-3-phosphate dehydrogenases (encoded by the genes *TDH1-TDH3*) to generate glycerate-1,3-bisphosphate with the addition of an inorganic phosphate and the mentioned production of NADH. More than half of the isozymes present are encoded by *TDH3*, whereas *TDH1* contributes only 10–15% of the total dehydrogenase activity (McAlister and Holland 1985a, b). Consequently, *tdh2 tdh3* double mutants fail to grow on glucose. Moreover, *TDH1* expression studies indicate, that it is involved in the response to oxydative stress (Valadi et al. 2004).

Phosphoglycerate kinase (Pgk1) catalyzes the first reaction which generates ATP in glycolysis. *PGK1* is the glycolytic gene most strongly expressed and its strong promoter has been frequently employed for heterologous gene expression (Chambers 1997).

In the following, 3-phosphoglycerate is converted to 2-phosphoglycerate by a phosphoglycerate mutase (encoded by *GPM1*; Rodicio et al. 1993). The yeast genome sequencing project revealed the presence of two further homologs, which are not involved in sugar metabolism (Heinisch et al. 1998).

Phosphoenolpyruvate (PEP) is produced by the enolase reaction. Two isozymes are encoded by *ENO1* and *ENO2*. Whereas expression of the first gene is constitutive, that of the second is induced 20-fold by the presence of sugars (Cohen et al. 1987). Thus, Eno2 predominates in early wine fermentation.

The final step of glycolysis, mediated by pyruvate kinase (encoded by *PYK1*), generates the second ATP in the pathway and is essentially irreversible. Therefore, the enzyme serves as a second control point for glycolysis, and is also allosterically regulated. Fructose-1,6-bisphosphate, produced by Pfk, is a potent activator of pyruvate kinase (Morris et al. 1986). It is the intracellular metabolite found at the highest concentrations during growth on glucose (Heinisch and Rodicio 1997). Pyk1 is also subject to a cAMP-dependent phosphorylation by PKA (see Sect. 6.4.1), as a prerequisite for allosteric activation by fructose-1,6-bisphosphate (Portela et al. 2006). Pyruvate kinase activity determines the subsequent carbon flux. Thus, decreasing pyruvate concentrations lead to re-routing into respiration (Pearce et al. 2001). This indicates that allosteric control at this step is not only important for the speed of fermentation, but also for ethanol yield.

6.3.3 Pyruvate Decarboxylase and Alcohol Dehydrogenase

S. cerevisiae is employed in the production of beverages and bread because it produces ethanol and carbon dioxide. These are generated in the two final steps of alcoholic fermentation, which are catalyzed by pyruvate decarboxylase and alcohol dehydrogenase. The main isozymes are encoded by *PDC1* and *ADH1*, respectively (Schmitt and Zimmermann 1982; Ciriacy 1975). Other homologs present in the yeast genome are of minor importance for fermentation (Table 6.3). Yeast also contains a mitochondrial pyruvate dehydrogenase complex, which can introduce pyruvate into respiration (Fig. 6.3) if medium sugar concentrations are low.

Other yeast species also employ glycolysis, but it may not be essential for glucose degradation, as discussed above for the mutants of *K. lactis*. Moreover, it is to be expected, that less redundancy in the encoding genes will be found in non-*Saccharomyces* species. Strictly aerobic yeasts are likely to divert the carbon-flux from pyruvate predominantly through the pyruvate dehydrogenase into respiration, as long as oxygen is available.

One should note that glycolytic enzymes in *S. cerevisiae* account for at least 30% of the total soluble protein (Gancedo and Serrano 1989), consistent with transcriptome data indicating high level gene expression throughout alcoholic fermentation (Rossignol et al. 2003).

6.3.4 Glycerol and Acetate as Fermentation By-Products

Glycerol is considered a valuable by-product of yeast alcoholic fermentation in wine production. It originates from glycolysis at the step of the triosephosphates (Fig. 6.4). Two glycerol-3-phosphate dehydrogenases, encoded by *GPD1* and *GPD2*, can convert dihydroxyacetone phosphate to glycerol-3-phosphate, from which glycerol is produced by the irreversible reaction of phosphatases encoded by *GPP1* and *GPP2*. The inverse reactions are catalyzed by the kinase Gut1 and the dehydrogenase Gut2. In contrast to ethanol, glycerol does not freely diffuse through the plasma membrane and is excreted by the Fps1 transporter (Hohmann 2002). Glycerol import could be mediated by a redundant pair of transporters, encoded by *GUP1* and *GUP2*, but recent data indicate that Stl1 (a proton symporter for glycerol) may be more important (Ferreira et al. 2005).

Glycerol production is probably triggered by two main mechanisms: (1) The initial lack of alcohol dehydrogenase, which causes an imbalance of reduction equivalents (Gancedo and Serrano 1989). (2) The high initial sugar content in the must (20%), which causes osmotic stress and glycerol production in response (Tamás et al. 2003; see also Chap. 15). Depending on the wine, an increase in glycerol levels is frequently desired, but wine strains constructed to this end usually also generate increased acetate concentrations (Remize et al. 1999). Of the other yeast species present in musts, *Candida stellata* has been reported to be a potent producer of glycerol, both under aerobic and anaerobic growth conditions, with an increased content under the latter conditions (Ciani et al. 2000).

Acetate as the main component of volatile acidity adds a negative organoleptic property (Remize et al. 2000). It originates from the acetaldehyde produced by the pyruvate decarboxylase reaction if used by aldehyde dehydrogenases (Fig. 6.4). These function together with acetyl-CoA synthetase (ACS) as an essential cytosolic Pdh by-pass and ensure availability of acetyl-CoA, e.g. for lipid biosynthesis (Remize et al. 2000). The cytosolic aldehyde dehydrogenase isoforms are encoded by *ALD2, ALD3* and *ALD6. ALD4* and *ALD5* encode mitochondrial isoforms, which are employed when ethanol is the carbon source. Expression *ALD2* and *ALD3*, encoding cytosolic isoforms, is glucose-repressed, leaving Ald6 as the relevant enzyme in fermentative acetate production (Saint-Prix et al. 2004). A limited capacity of the acetyl-CoA synthetase probably causes the acetate overflow (van Urk et al. 1990). Of the two synthetase isoforms (encoded by *ACS1* and *ACS2*), only the second is expressed on glucose.

Metabolic activities of wine bacteria are usually regarded as the major cause of off-flavors, which includes the production of acetic acid (see Chaps. 7 and 11).

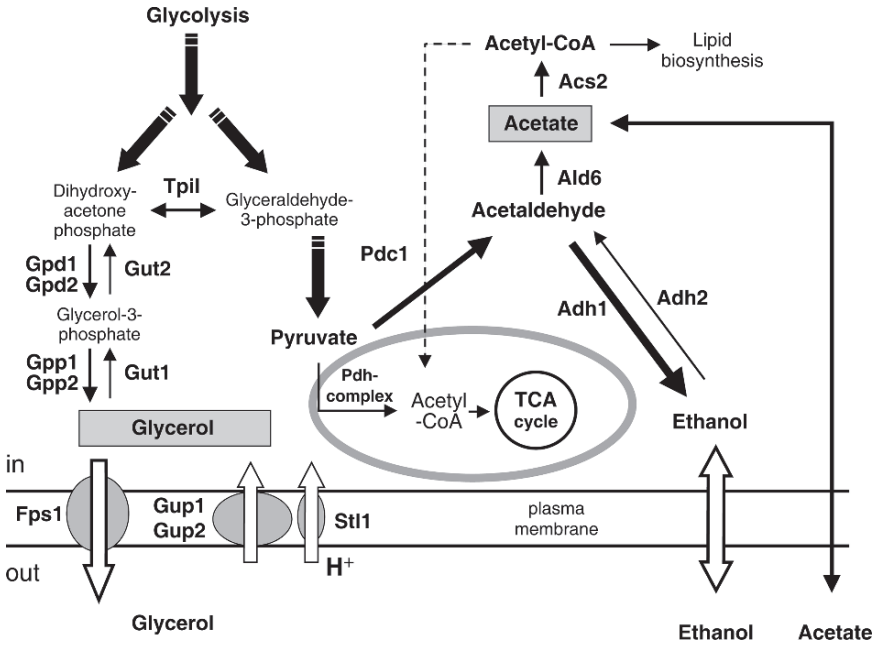

Fig. 6.4 Pathways to glycerol and acetate in *S. cerevisiae*. Glycerol originates from the glycolytic metabolite dihydroxyacetone phosphate and is exported by a special permease (Fps1). Ethanol originates from pyruvate and is believed to freely diffuse through the plasma membrane. The "Pdh bypass" ensures production of acetyl-CoA in the cytoplasm for lipid biosynthesis. *TCA* tricarboxylic acid cycle. *In* and *out* refer to the cytoplasmic and the periplasmic side of the plasma membrane, respectively. See text for further details

Nevertheless, *S. cerevisiae* employed in starter cultures can contribute significantly. Other wine yeasts, such as *Hanseniaspora uvarum* (*Kloeckera apiculata*), are also frequently claimed to produce detrimental amounts of acetate (Fleet 2003).

Positive aroma compounds derived from yeast acetate metabolism (e.g. ethyl- and isoamyl acetate) are produced by an alcohol acetyltransferase (encoded by *ATF1*), which localizes to lipid particles (Lilly et al. 2006). These may also come from other yeast species in the must, as *Pichia anomala* has been reported to increase ethylacetate production by tenfold under oxygen limitation (Fredlund et al. 2004).

Clearly, the presence of non-*Saccharomyces* yeasts in the early stages of fermentation also broadens the range of carbohydrates to be degraded. For example, cellobiose, trehalose, lactose, mannitol, ribose, xylose, and even xylitol can be used, and in some cases also fermented, by different wine yeast species (Barnett et al. 2000). It remains to be investigated if and which of the encoding genes in these yeasts are subject to catabolite repression.

Two forms of reserve carbohydrate compounds have been described to naturally accumulate in yeasts: Glycogen (a polysaccharide composed of α-1,4-glucose

chains branched by some α-1,6-linkages) and trehalose (a disaccharide of two α-1,1-linked glucose molecules). Metabolism of these carbohydrates is reviewed in Francois and Parrou (2001) and will be discussed in detail in Chap. 15.

6.4 Regulation of Carbohydrate Metabolism in Yeasts: Glucose Signalling

All organisms, especially unicellular eukaryotes such as yeasts, have evolved sophisticated mechanisms to appropriately adjust their metabolism to varying environmental conditions. *S. cerevisiae* has adapted best to the needs of alcoholic fermentation, where sugar concentrations constantly decrease, while those of ethanol rise. It can use glucose under all these conditions. Thus, the molecular mechanisms which allow the yeast cells to sense and adjust to the extracellular glucose concentration have been extensively studied and several signalling systems have been discovered. Figure 6.5 presents a broad overview of the pathways to be discussed in the following. The interested reader is referred to a number of excellent reviews for further details (Gancedo and Gancedo 1997; Gancedo 1998; Breunig et al. 2000; Gonzalez-Siso et al. 2000; Flores et al. 2000; Thevelein et al. 2000; Rolland et al. 2002; Schüller 2003; Holsbeeks et al. 2004; Verstrepen et al. 2004; Johnston and Kim 2005; Santangelo 2006; Hedbacker and Carlson 2008).

6.4.1 The Snf1 Kinase Complex (Glucose Repression)

Metabolism is tightly controlled at the transcriptional level by glucose. Since it was assumed that an intermediary metabolite would trigger this regulation, it was first called "carbon catabolite repression." In the early stages of must fermentation, transcription of genes whose products are involved in respiration and the utilization of alternative carbohydrates (e.g. sucrose) are repressed by the high levels of glucose and fructose. Derepression after sugar depletion depends on the activity of the Snf1 kinase complex, named after its α-catalytical subunit Snf1 (=Cat1). It forms a complex with the γ regulatory subunit Snf4 (=Cat3), which stimulates the kinase activity by blocking the auto-inhibition of Snf1. The β-subunit in *S. cerevisiae* can be encoded by either of three genes (*SIP1*, *SIP2* or *GAL83*), and may mediate intracellular localization of the complex. If activated the Snf1-complex phosphorylates transcription factors, which regulate target gene expression. Among the most important target transcription factors of the Snf1 complex are the Mig1 repressor, and the Cat8 and Sip4 activators. Mig1 is a member of a zinc finger family and is part of a complex which controls the utilization of alternative sugars such as galactose, sucrose and maltose. It also contributes to a minor extent in the repression of respiration and gluconeogenesis. The zinc finger transcription factors Cat8 and

Sip4 activate expression of genes encoding gluconeogenic and glyoxylate cycle enzymes. Interestingly, *CAT8* transcription is repressed by Mig1, whereas *SIP4* expression is induced by Cat8 (Fig. 6.5).

Repression of respiration is also indirectly mediated by this system: A heteromultimeric complex consisting of Hap2–Hap5 binds to elements present in the respective target gene promoters and activates transcription. *HAP4* is expressed only in the absence of glucose, since it requires the transcriptional activator Cat8. Thus, respiration and gluconeogenesis become coordinately regulated by glucose.

6.4.2 Signalling Through the cAMP/PKA Pathway

To inhibit respiration in favour of fermentation, yeast metabolism is also controlled at the post-transcriptional level. Early studies indicated that glucose triggers catabolite inactivation (i.e. phosphorylation and proteolytic degradation) of key gluconeogenic and respiratory enzymes such as fructose-1,6-bisphosphatase, isocitrate lyase and malate dehydrogenase. In contrast, phosphofructo-2-kinase (Pf2k), which produces a potent activator of Pfk, is activated by phosphorylation in response to glucose addition.

The major impact of glucose on catabolite inactivation/activation in *S. cerevisiae* is mediated by the cAMP/PKA signalling pathway (Fig. 6.5). This pathway also seems to mediate proteolytic degradation of the gluconeogenic enzymes fructose-1,6-bisphosphatase and malate dehydrogenase in the vacuole upon glucose addition. These enzymes apparently can also be degraded in a cAMP-independent manner in the cytosol via ubiquitin-conjugation and the proteasome.

6.4.3 Glucose Induction Mediated by the Snf3/Rgt2 Sensors: Sugar Transport

Despite the high must sugar concentrations, intracellular glucose does not exceed 2.5 mM in *S. cerevisiae* wild-type cells (Ernandes et al. 1998). Regulation of the sugar transport across the plasma membrane is therefore crucial and has to be tightly controlled by the physiological state of the cells. Glucose promotes transcription of the encoding *HXT* genes by a signal transduction pathway which ultimately regulates the Rgt1 repressor and uses the plasma membrane sensors Snf3 and Rgt2 (Fig. 6.5). This mechanism controls expression of the *HXT3* gene, encoding the hexose transporter predominating under must fermentation conditions. Transcription of other hexose transporter genes requires further regulation. In addition to the control of their transcription, some of the hexose transporters are also regulated by proteolytic inactivation in *S. cerevisiae* (Lagunas 1993).

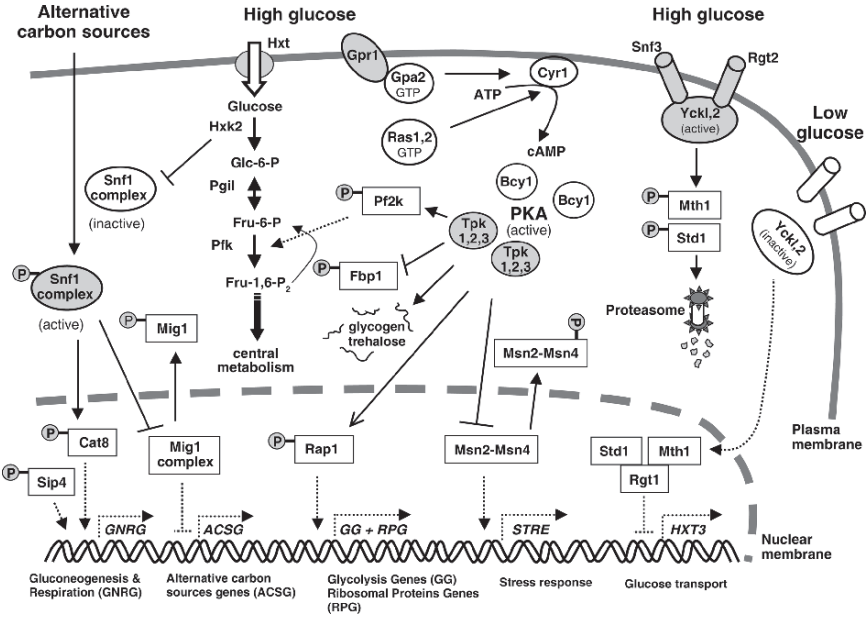

Fig. 6.5 Pathways of glucose signalling in *S. cerevisiae*. The three major glucose signalling pathways in *S. cerevisiae* are depicted in a simplified scheme. *Snf1-kinase pathway* (*left*): At high glucose concentrations, the Snf1 complex is rendered inactive by the Reg1-Glc7 phosphatase (not shown), which is regulated by Hxk2. The positive transcription factors do not reach their target promoters. Non-phosphorylated Mig1 is located in the nucleus (as a part of a complex also containing Hxk2), where it binds to the promoters of its target genes to prevent transcription. Without glucose and in the presence of non-fermentable carbon sources, Snf1 gets phosphorylated and activated by specific kinases. Phosphorylation of Mig1 inactivates the repressor and leads to its translocation to the cytoplasm. The positive transcription factors Cat8 and Sip4 are also phosphorylated by Snf1 and can activate transcription of their target genes. Note that the subcellular distribution of the Snf1 complex(es) is highly regulated under different growth conditions. These details have been omitted for clarity. *The cAMP/PKA pathway* (*in the middle*): Glucose addition to stationary cultures or cells grown on non-fermentable carbon sources results in a rapid and transient increase of cAMP (produced by the adenylate cyclase, Cyr1) and activation of protein kinase A (PKA). The latter enzyme is kept inactive by association of two identical inhibitory subunits (encoded by *BCY1*) with two catalytic subunits (encoded by either of the genes *TPK1*, *TPK2* or *TPK3*). Binding of cAMP to the Bcy1 subunits promotes the dissociation of the tetramer and the now catalytically active Tpk subunits phosphorylate their target proteins, such as Fbp1 and Pf2k. Extracellular glucose is sensed through the Gpr1–Gpa2 system. In addition, adenylate cyclase, can also be activated through the Ras pathway. *Glucose sensing through Snf3 and Rgt2* (*right*): High glucose concentrations are detected by the surface sensors Rgt2 or Snf3, which activate the protein kinases Yck1 and Yck2. The active kinases then phosphorylate the co-repressor proteins Mth1 and Std1, providing a signal for their ubiquitin-mediated degradation by proteasomes. In the absence of Mth1 and Std1, Rgt1 cannot bind to its promoter elements and transcription of the *HXT* genes occurs. At low glucose concentrations Rgt1 forms a complex with the proteins Mth1 and Std1 and binds to the promoters of the *HXT* genes to repress transcription. For further details see text and the reviews cited at the beginning of Sect. 6.4

6.4.4 General Transcription Factors for High Level Expression of Glycolytic Genes

Most yeast enzymes involved in alcoholic fermentation are abundant under all growth conditions, even if ethanol is used as the sole carbon source (Fraenkel 1982). However, some isozymes are only produced when glucose is depleted (see Sect. 6.3.2) and gene expression has to be controlled accordingly (Table 6.3). This is exemplified by the first step of glycolysis. Only *HXK2* is transcribed at high sugar concentrations. Expression of *GLK1* and *HXK1* is only activated upon sugar limitation. Interestingly, Hxk2 is required for repression of *HXK1* and *GLK1* as well as for expression of its own gene on glucose. The transcriptional repressor Rgt1 governs repression of *HXK2* after glucose depletion (Fig. 6.5).

Finally, most glycolytic genes contain binding sites for the general transcriptional factor Rap1 and the regulatory protein Gcr1 (CT-box) in their promoters. Thus, *gcr1* mutants have low levels of glycolytic enzymes and fail to grow on glucose. Another protein, Gcr2, is also required for high level expression. According to the current model, Rap1 and Gcr1 collaborate to stimulate transcription of glycolytic genes by binding directly to their promoter elements. Gcr2 interacts with Gcr1 to enhance transcription. Interestingly, Rap1 gets activated on high glucose concentrations by a cAMP/PKA dependent phosphorylation.

6.5 Conclusions and Perspectives

Yeast sugar metabolism remains the central metabolic process determining both the final alcohol content and, at least to a large extent, the organoleptic characteristics of wine. Interestingly, the vast amount of data obtained for laboratory strains of the predominating yeast species, *S. cerevisiae* fit well with the observed properties of its wine yeast varieties under real must fermentation conditions. Carbohydrate metabolism of non-*Saccharomyces* yeasts also contributes substantially to the wine quality, either positively or as “spoilage” yeasts. Hence the growing interest in mixed-culture fermentations with specific yeast combinations (see Nissen et al. 2003, and references therein).

The molecular genetic techniques established are more and more adapted to non-*Saccharomyces* yeasts as well, such as *S. pombe*, *K. lactis*, *K. marxianus*, *C. albicans*, *P. stipitis*, *H. polymorpha* and *Y. lipolytica*. Of those, only *K. marxianus* and *S. pombe* have been reported to also occur in must fermentations. Literature on the molecular genetics of wine yeast species is still extremely limited. There are a few reports on specific cloning and expression studies in *Torulaspora delbrueckii*, *Z. bailii*, *D. hansenii* and *P. anomala*. Surprisingly, a systematic genetic approach towards the most abundant natural wine yeast, *H. uvarum (K. apiculata)* has apparently not yet been initiated. We strongly believe that research efforts on non-*Saccharomyces* yeasts will strongly increase within the upcoming decade.

Finally, the use of *S. cerevisiae* as starter cultures will remain a common practice in industrial wine production. In this respect, it has frequently been claimed that laboratory strains would be less efficient in alcoholic fermentation and/or would perform worse than the commercial strains in the production of flavour compounds. Thus, the enormous arsenal of strain varieties and mutants available to basic research would not be of use. At least for the production of spirits we could recently demonstrate that this assumption is not true for a genetically defined, diploid laboratory strain (Schehl et al. 2004). This opens the possibility to employ classical yeast genetics with laboratory strains (if necessary without the involvement of genetic engineering) for breeding of desired wine strains. It will be exciting to see if what is true for spirit production can be adapted to must fermentations for high-quality wines.

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Chapter 7
Metabolism of Sugars and Organic Acids by Lactic Acid Bacteria from Wine and Must

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Heterofermentative lactic acid bacteria (LAB) which are common in plant associated environments are found also in grape must and wine. In this environment specific strains predominate which are adapted to the low pH and high alcohol contents. Must and wine harbour the strictly heterofermentative *Oenococcus oeni, Lactobacillus hilgardii* and *Lactobacillus brevis*, and the facultatively heterofermentative *Lactobacillus plantarum* and *Lactobacillus pentosus* (Rodas et al. 2005). In addition homofermentative lactic acid bacteria of the Pediococcus group are able to grow in wine and must, but are normally found at low cell densities.

The growth of lactic acid bacteria in wine depends largely on sugars and organic acids which are found in grape must. Like most heterofermentative LAB, *O. oeni* is able to degrade hexoses, pentoses and other sugars from must, which can result in the excretion of undesirable products. The metabolic activities related to the degradation of organic acids are responsible for positive effects attributed to *O. oeni* in wine (Mayer 1974). Beside malate degradation which leads to deacidification of wine, the bacteria degrade other organic acids.

Grape must contains in addition to hexoses (glucose, fructose, galactose, mannose) considerable amounts of pentoses (arabinose, xylose, ribose, rhamnose). Glucose and fructose are the most abundant sugars and are present in an approximate 1:1 molar ratio. The concentrations depend on the ripeness and type of grapes, but are typically ≥100 g L^{-1} for glucose and fructose, followed by much lower contents of galactose (<200 mg L^{-1}) and mannose (<50 mg L^{-1}) (Würdig and Woller 1989). Pentoses are derived from hydrolysis of plant cell wall pectins and are present in small amounts (L-arabinose, <1 g L^{-1}; D-xylose and D-ribose, each <0.1 g L^{-1}; L-rhamnose <0.4 g L^{-1}). Disaccharides (maltose, raffinose, trehalose) and oligosaccharides (stachyose) are found in small amounts (few mg L^{-1} each) (Würdig and Woller 1989). Excess hexoses are used also for the production of exopolysaccharides like dextran, levan and fructan by various groups of LAB (Cerning 1990). *Pediococcus damnosus* forms an extracellular polysaccharide (1,3:1,2-β-D-glucan) from glucose

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which increases the viscosity of wine (Llaubères et al. 1990). Hexoses and pentoses are fermented by the heterofermentative LAB by the phosphoketolase (or oxidative pentose-phosphate) pathway. The major products of glucose fermentation are D-lactate, ethanol and CO_2. Ethanol formation represents a limiting step in the heterofermentative hexose fermentation which can be overcome by modifications in the fermentation or by the use of external electron acceptors like fructose, O_2, pyruvate or citrate. The modified fermentation reactions produce also undesirable products like acetate or mannitol. The variations in the pathways and the biochemical background will be described.

Many heterofermentative LAB are able to metabolize organic acids which are common in grape must. Malate, citrate and pyruvate are degraded efficiently. Malate and pyruvate are metabolized without the need for cosubstrates, whereas the other acids are used mostly as co-substrates or electron sink during fermentation of hexoses which serve as the electron donor. Organic acids like L-tartrate, fumarate or galacturonic acid are fermented only under specific conditions. L-Malate (<20 g L^{-1}) and L-tartrate (<10 g L^{-1}) are found in high concentrations. The contents of citrate (<300 mg L^{-1}), gluconic acid (<300 mg L^{-1}), galacturonic acid (<250 mg L^{-1}) and fumarate (traces) are much lower in must from healthy grapes (Würdig and Woller 1989; Dittrich and Großmann 2005).

7.1 Special Features of the Phosphoketolase Pathway of *O. Oeni*: Use of Alternative Reactions for NAD(P)H Reoxidation

7.1.1 Phosphoketolase Pathway and Limitation of Ethanol Formation

O. oeni is able to use glucose, fructose and ribose as the substrates for growth. In addition, various strains show growth on further hexoses (galactose, mannose), pentoses (xylose, arabinose) and disaccharides (trehalose, cellobiose, sucrose, melibiose) (Beelman et al. 1977; Garvie 1986; Zhang and Lovitt 2005). Growth on further oligosaccharides (lactose, maltose, and raffinose) has been observed, but is unstable and can be lost (Beelman et al. 1977). The sugars are fermented by the phosphoketolase pathway resulting in the formation of pyruvate (derived from glyceraldehyd-3P, or GAP) and acetyl-P (Fig. 7.1). From pentoses only 1 NADH is produced during conversion to pyruvate, which is used as the acceptor for NADH reoxidation, and the acetyl-P is converted to acetate. Hexoses are first converted to a pentose by oxidation and decarboxylation (Figs. 7.1 and 7.2), yielding four extra [H] (or 2 NAD(P)H)), followed by one further NADH derived from pentose degradation to pyruvate. Formally, the latter NADH (equivalent to the NADH from GAP oxidation) is reoxidized by reduction of pyruvate, similar to growth on pentoses. Lactate formation therefore is a constant part of pentose and hexose fermentation

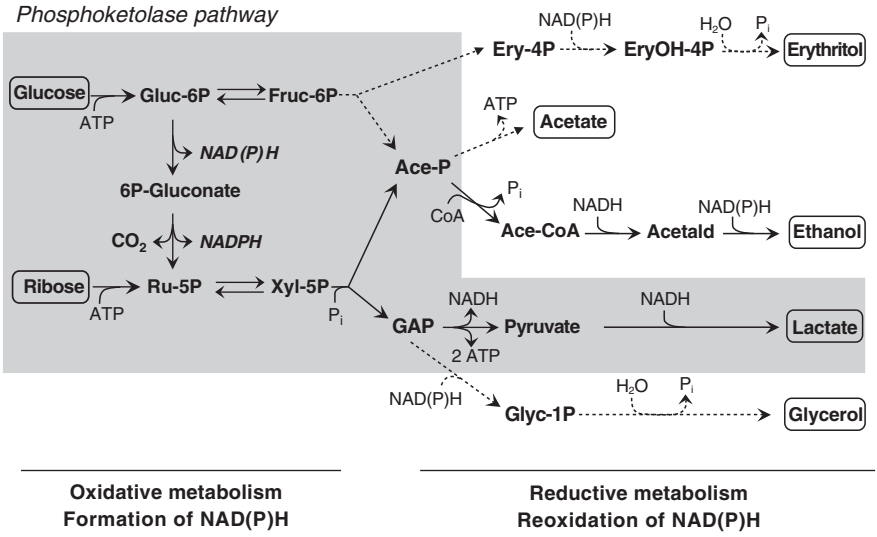

Fig. 7.1 Fermentation of hexoses and pentoses by the phosphoketolase (or oxidative pentose-P) pathway by *O. oeni*. The central phosphoketolase pathway and lactate formation (NAD(P)H reoxidation) which are constant parts of the metabolism are *boxed*. The major routes resulting in the formation of lactate and ethanol are shown with *solid lines*, alternative pathways resulting in the formation of mannitol, erythritol, acetate and glycerol with *broken lines*. Phosphoketolase (Xfp, genes OEOE_1812 and OEOE_1183 of *O. oeni* PSU-1) cleaves pentose-5P (xylulose-5P) or fructose-5P. Acetaldehyde and ethanol dehydrogenase reactions are catalyzed by the bifunctional AdhE enzyme (Koo et al. 2005). The genes encoding the enzymes of the phosphoketolase pathway and for ethanol and lactate formation are given by Mills et al. (2005), Zaunmüller et al. (2006) and in http://jgi.doe.gov/. The genome contains candidate genes for glycerol-1P dehydrogenase (gene OEOE_0562) and glycerol-1P phosphatase (gene OEOE_0563). *Non-standard abbreviations*: *Ery-4P* erythrose-4 phosphate; *EryOH-4P* erythritol-4P; *Ace-P* acetylphosphate; *Ace-CoA* acetyl-CoA; *Acetald* acetaldehyde; *Glyc-1P* glycerol-1 phosphate

(Fig. 7.1). The extra two NAD(P)H are loaded onto acetyl-P (or acetyl-CoA) with the formation of ethanol (ethanol pathway) instead of acetate. Shifting from pentoses to hexoses causes a drop in the growth rate by a factor of approximately 3 (Zaunmüller et al. 2006; Richter et al. 2003a), and a decrease in growth yields by a factor of about two in agreement with the lower ATP yield (two ATP/pentose versus one ATP/hexose).

The slow growth on glucose is caused by the low activity of the ethanol pathway in the reoxidation of the extra 2 NAD(P)H (Maicas et al. 2002; Richter et al. 2001, 2003a), compared to the high activities of the enzymes of the phosphoketolase pathway like glucose-6P dehydrogenase (Richter et al. 2001). The acetaldehyde dehydrogenase activity of the bifunctional acetaldehyde/ethanol dehydrogenase (AdhE), on the other hand, is low. When the cellular contents of HSCoA, and thus of acetyl-CoA are low, the activity of acetaldehyde dehydrogenase drops further and limits growth of the bacteria. Shortage of D-pantothenate, an essential precursor and growth factor for HSCoA synthesis in *O. oeni* and other LAB (Garvie 1967; http://jgi.doe.gov/),

reduces the HSCoA contents (Richter et al. 2001). The bacteria contain the enzymes for the conversion of D-pantothenate to HSCoA, but not for the synthesis of D-pantothenate from central intermediates (Zaunmüller et al. 2006; http://jgi.doe.gov/). The ethanol pathway appears to be the first site responding to D-pantothenate limitation (Richter et al. 2001). 4´-O-(β-D-glucopyranosyl)-D-pantothenic acid, which is found in tomato and fruit juice was suggested as the specific source ('tomato juice growth factor') for synthesis (Amachi et al. 1970), but free D-pantothenate has the same biological activity (Zaunmüller et al. 2006). D-Pantothenate therefore plays a specific role in ethanol formation by *O. oeni* (Richter et al. 2001). During growth on pentoses, when the ethanol pathway is not required, D-pantothenate depletion has no significant effect on the fermentation pattern.

7.1.2 Endogenous Alternative Pathways for [H] Reoxidation

The limitation in the ethanol pathway results in decreased production of ethanol and a partial shift to alternative pathways for NAD(P)H reoxidation (Veiga-Da-Cunha et al. 1992, 1993; Richter et al. 2001) (Fig. 7.1). Part of the extra NAD(P)H is consumed by reduction of erythrose-4P to erythritol-4P and erythritol by *O. oeni* and other heterofermentative LAB (Veiga-Da-Cunha et al. 1993; Stolz et al. 1995; Richter et al. 2001). Erythrose-4P is derived from fructose-6P by phosphoketolase Xfp. Phosphoketolase Xfp of *O. oeni* and *Bifidobacterium* accepts fructose-6P in addition to xylulose-5P (Veiga-Da-Cunha et al. 1993; Meile et al. 2001; Yin et al. 2005; Mills et al. 2005). The activity of the erythritol pathway is low and does not increase the rate of glucose fermentation significantly. Under pantothenate limitation or in resting cells up to 0.2 mol erythritol is formed per mol glucose. The enzymes (or corresponding structural genes) for the conversion of erythrose-4P to erythritol have not been identified in the genomes of *O. oeni* and *L. mesenteroides* (Zaunmüller et al. 2006).

Glycerol is a further minor product of NAD(P)H reoxidation. It is obtained by reduction of GAP to glycerol-1P followed by dephosphorylation (Fig. 7.1) (Veiga-Da-Cunha et al. 1993). The genome of *O. oeni* contains candidate genes for glycerol-1P dehydrogenase and phosphatase (http://jgi.doe.gov/) (Fig. 7.1). Biochemically, the reactions for erythritol and glycerol formation are similar, and both products might be formed by the same enzymes.

When fructose is used as the substrate for growth, part of the fructose is used as an electron sink, resulting in mannitol formation (see following section).

7.2 Modified Hexose Fermentation by the Use of External Electron Acceptors

Pyruvate, citrate, O_2, or fructose can be used by *O. oeni* as external acceptors for reoxidation of the extra NAD(P)H from hexose oxidation (Fig. 7.2). The external acceptors are more efficient electron acceptors than acetyl-CoA, and acetyl-P is used for ADP

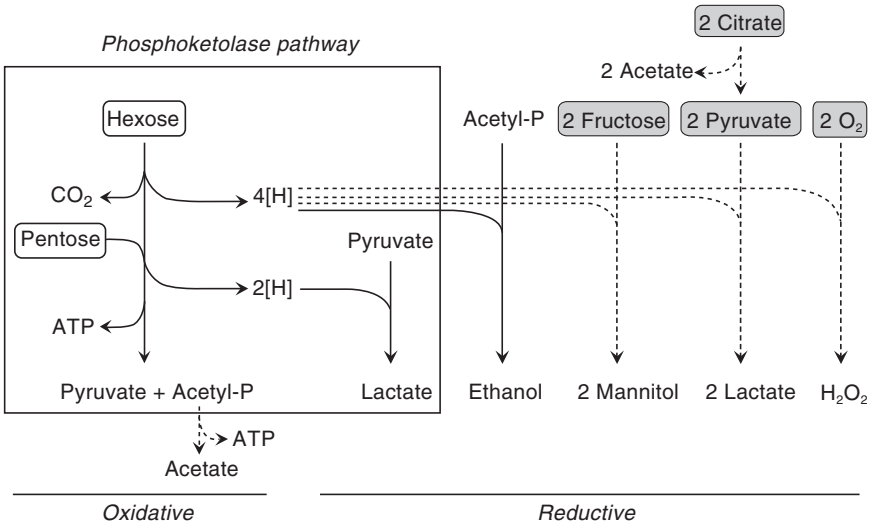

Fig. 7.2 Alternative routes for the reoxidation of NAD(P)H by *O. oeni* during growth on hexoses in the presence of external electron acceptors (fructose, pyruvate, citrate, O_2). The extra 2 NAD(P)H or 4 [H] which are derived from the conversion of hexose to pentose in the phosphoketolase pathway can be transferred to the external acceptors (*broken lines*). The oxidative (NAD(P)H producing) and reductive (NAD(P)H consuming) parts of metabolism are indicated. The following fermentation balances are found for growth on glucose (idealized reactions): (**a**) Glucose → 1 lactate + 1 EtOH + 1 CO_2; (**b**) Glucose + 2 fructose → 1 lactate + 1 acetate + 2 mannitol + 1 CO_2; (**c**) Glucose + 2 pyruvate → 3 lactate + 1 acetate + 1 CO_2; (**d**) Glucose + 2 citrate → 3 lactate + 3 acetate + 3 CO_2; (**e**) Glucose + 2 O_2 → 1 lactate + 1 acetate + 1 CO_2 + 2 H_2O_2

phosphorylation and excreted as acetate. As a consequence the ATP and molar growth yields increase by a factor of up to 2 per glucose (Zaunmüller et al. 2006). In addition, the growth rates increase by factors of 2–3 and approach those of ribose.

In the presence of external pyruvate, the pyruvate is reduced as additional electron acceptor by the highly active lactate dehydrogenase (Nuraida et al. 1992; Richter et al. 2003a). Citrate is used in a similar way after its conversion to pyruvate by citrate lyase and oxaloacetate decarboxylase Mae (see Fig. 7.3) (Salou et al. 1994; Hache et al. 1999; Stolz et al. 1995). Molecular O_2 is reduced by an oxidase to H_2O_2 (Maicas et al. 2002), and fructose to mannitol (Salou et al. 1994; Richter et al. 2003a, b). During cofermentation of fructose with glucose by *O. oeni*, fructose is used essentially as an electron acceptor and excluded from the phosphoketolase pathway, whereas glucose is channelled to the phosphoketolase pathway (Richter et al. 2003b).

When fructose is supplied as the only substrate, it is metabolized by the phosphoketolase pathway, or by combined action of the phosphoketolase and mannitol pathway (Richter et al. 2003a, b). With limiting supply of fructose, most of the fructose is fermented by the phosphoketolase pathway similar to glucose. At high fructose concentrations and in resting cells up to 2/3 of the fructose are used as electron acceptor and large amounts of mannitol are produced. Channelling of fructose in either pathway is regulated at the level of phosphoglucose isomerase (Richter et al. 2003b).

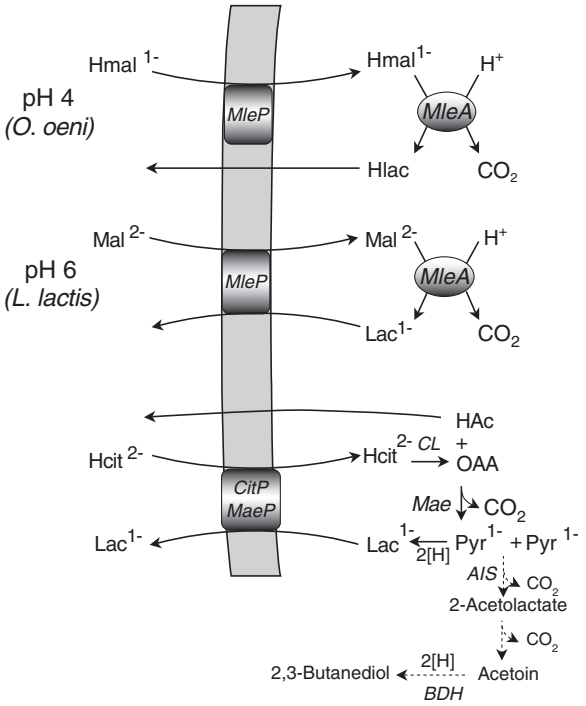

Fig. 7.3 Pathways and carriers for the fermentation of malate and citrate by *O. oeni*, *L. mesenteroides* and *Lactobacillus lactis*. Important enzymes and carriers (*MleP* malate carrier; *MleA* malolactic enzyme; *CitP* or *MaeP* citrate/lactate antiporter; *Mae* oxaloacetate decarboxylase; *CL* citrate lyase; *AlS* acetolactate synthase; *BDH*, butandiol dehydrogenase) and intermediates ($HMal^{1-}$ malate anion; Mal^{2-} malate dianion; *Hlac* lactic acid; *HAc* acetic acid; *OAA* oxaloacetate; Pyr^{-1} pyruvate; Lac^{-1} lactate) are indicated

Overall, growth of *O. oeni* and other heterofermentative LAB on hexoses is stimulated by the presence of electron acceptors which provide a by-pass to NAD(P)H reoxidation in the ethanol pathway. The erythritol and glycerol pathways are of limited capacity, whereas the pathways using the external acceptors O_2, pyruvate, citrate and fructose have a much higher capacity and increase the growth rate of the bacteria significantly. The redox reactions are cytoplasmic without involvement of electron transport or generation of a proton potential.

7.3 Fermentation of Organic Acids

Fermentation of organic acids plays an important role in the energy metabolism of heterofermentative LAB like *O. oeni* (Radler 1958, 1966; Radler and Brohl 1984; Stolz et al. 1995). From the organic acids found in grapes, citrate and malate are used by many LAB, including *O. oeni* and *L. mesenteroides*. Fumarate, tartrate and

pyruvate are used only by a limited number of LAB. Citrate is used only in co-fermentation with hexoses, whereas malate, pyruvate and L-tartrate can be degraded without need for a co-substrate. However, pyruvate appears to be the only organic acid which supports growth of *O. oeni* when supplied as the sole substrate.

7.3.1 Malate (or 'Malolactic') Fermentation

Fermentation of malate (L-malate → L-lactate + CO_2) by heterofermentative LAB is of physiological significance in wine and fruit juice which contain high amounts of this C_4-dicarboxylic acid. The malolactic enzyme (Caspritz and Radler 1983) catalyzes the key reaction (L-malate → CO_2 + L-lactate). The free energy of the reaction is conserved by a chemiosmotic mechanism (Salema et al. 1996) which depends on an electrogenic malate transport (Lolkema et al. 1995; Poolman et al. 1991; Konings 2002) (Fig. 7.3). In *O. oeni* (growing around pH 4) the transport is effected by a carrier-mediated uptake of mono-anionic malate versus a carrier-independent efflux of lactic acid. In *Lactococcus lactis* growing at less acidic conditions, the transport is mediated by the malate^{2-}/lactate^{-} antiporter. Both transport processes result in the net translocation of one charge per malate and energization of the membrane. In addition, one proton is consumed by the decarboxylation in the cytoplasm, generating a ΔpH. The proton motive force (1 H^+/malate) derived from both processes is used by the bacteria for maintenance of pH homeostasis and for the uptake of nutrients. Malolactic fermentation stimulates growth of the bacteria, but is not sufficient as the sole energy source for growth (Pilone and Kunkee 1976; Salema et al. 1996). The reaction results in raising the pH of the medium by conversion of a divalent to a monovalent carboxylic acid. The process is used in winemaking by applying starter cultures or by spontaneous fermentation of *O. oeni* in wine or must (Mills et al. 2005; Moreno-Arribas and Polo 2005; Liu 2002; Coucheney et al. 2005; Lonvaud-Funel 1999).

7.3.2 Pyruvate Fermentation

Pyruvate can be used as an electron acceptor for NAD(P)H reoxidation, but supports also growth of *O. oeni* and *L. mesenteroides* as the sole substrate (Wagner et al. 2005):

2 Pyruvate + H_2O → acetate + CO_2 + lactate ($\Delta G_0' = -95.1$ kJ/reaction)

The growth rates by pyruvate fermentation are comparable to those for glucose fermentation. Pyruvate is decarboxylated by pyruvate dehydrogenase (PDH) to acetyl~CoA and NADH. Acetyl~CoA is used for ATP formation (via acetyl~P), and the NADH is transferred by lactate dehydrogenase to a second molecule of pyruvate. *O. oeni* and *L. mesenteroides* contain a gram-positive type PDH which is annotated as acetoine/pyruvate dehydrogenase complex. PDH is regarded as a typical enzyme of aerobic metabolism, but alternative anaerobic enzymes like pyruvate: ferredoxin

oxidoreductase, pyruvate decarboxylase or pyruvate–formate lyase are not encoded by the bacteria (Wagner et al. 2005; http://jgi.doe.gov/). NADH produced by PDH is reoxidized by lactate dehydrogenase using a second molecule of pyruvate.

7.3.3 Citrate Fermentation

Many LAB including *O. oeni* and *L. mesenteroides* use citrate as an electron acceptor in co-metabolism with sugars like glucose, fructose, lactose or xylose which provide NADH (citrate + 2 [H] → lactate + acetate + CO_2) (Salou et al. 1994; Schmitt et al. 1997; Hache et al. 1999; Starrenburg and Hugenholtz 1991; Drinan et al. 1976) (see Fig. 7.2). Some other LAB are able to grow on citrate as the sole substrate (Medina de Figueroa et al. 2000). Transport of citrate and of the products lactate and acetate plays an important role in citrate fermentation (Fig. 7.3). The secondary transporters CitP or MaeP catalyze an electrogenic precursor/product ($Hcitrate^{2-}$/$lactate^{-}$) exchange resulting in an electrochemical gradient over the membrane (Ramos et al. 1994; Marty-Teysset et al. 1995, 1996; Konings 2002). The electrochemical gradient is not sufficient to support growth of the bacteria on its own.

The bacteria cleave citrate by citrate lyase. Oxaloacetate is decarboxylated by a cytoplasmic oxaloacetate decarboxylase which is related to (soluble) malate decarboxylase (Marty-Teysset et al. 1996; Mills et al. 2005; Sender et al. 2004). In the presence of sufficient NADH (e.g. from hexose oxidation) most of the pyruvate is reduced to lactate (Ramos et al. 1994) which drives the citrate/lactate antiport (Salou et al. 1994; Konings 2002). Part of the pyruvate is condensed and converted to acetoin and 2,3-butanediol (Fig. 7.3) (Ramos et al. 1994; Nielsen and Richelieu 1999). Chemical (non-enzymatic) oxidation of acetoin by O_2 yields diacetyl. The acetoin pathway is significant in LAB, when the bacteria are incubated with citrate in the absence of other carbon sources. Diacetyl, a flavour compound in products treated by LAB, is tolerated in wine only in low concentrations (Mills et al. 2005; Nielsen and Richelieu 1999; Schmitt et al. 1997; Bartowsky and Henschke 2004).

The *cit* gene clusters of *L. mesenteroides* and *O. oeni* comprise genes for citrate lyase (*citDEF*), citrate lyase ligase (*citC*), oxaloacetate decarboxylase (*mae* gene) and the citrate carrier (*maeP* or *citP*) (Mills et al. 2005; Martin et al. 2000). The clusters contain in addition the *citX citG* genes which are homologous to the corresponding genes of *Klebsiella* for the synthesis of the phosphoribosyl-dephospho-SCoA prosthetic group of citrate lyase (Schneider et al. 2002). Genes for acetolactate synthase and acetolactate decarboxylase are present in *O. oeni* and *L. mesenteroides* (Fig. 7.3).

7.3.4 Fumarate Fermentation

The small amounts of fumarate present in wine or must are degraded by yeast after conversion to malate or succinate (Radler 1986). Fumarate inactivates LAB from wine, but malate fermenting bacteria are able to degrade fumarate to lactate and

CO_2 when incubated with the organic acid (Radler 1986). It was assumed that fumarate is hydrated by the bacteria to malate and decarboxylated by the malolactic enzyme to lactate and CO_2. The genome of *O. oeni* PSU-1 encodes a fumarase (gene OEOE_0029, http://jgi.doe.gov/), therefore all of the enzymes required for the pathway appear to be present in the bacterium.

7.3.5 L-Tartaric Acid Fermentation

L-Tartaric acid is degraded only under specific conditions after degradation of the other organic acids. Only few LAB are able to degrade L-tartrate (Radler and Yannissis 1972), and tartrate degradation is found only in spoilt wine. *L. plantarum* degrades L-tartrate by a dehydratase to oxaloacetate which is decarboxylated to pyruvate (Fig. 7.4). In a reaction similar to pyruvate fermentation of *O. oeni*, half of the pyruvate is converted to acetate + CO_2, the other half to lactate. Tartrate fermentation does not support growth although the pathway is supposed to allow ATP formation from acetyl~CoA (or acetyl-P). The genome of *L. plantarum* contains structural genes for L-tartrate dehydratase TtdAB, oxaloacetate/malate decarboxylase Mae, and pyruvate dehydrogenase (http://img.jgi.doe.gov/and Fig. 7.4). The pathway is different from L-tartrate fermentation found in bacteria like *E. coli* which is linked to fumarate respiration (Kim and Unden 2007).

L-Tartrate fermentation by *Lactobacillus brevis* and other heterofermentative *Lactobacillus* species occurs by a branched pathway (Radler and Yannissis 1972). Two third of the tartrate is fermented to acetate and CO_2 as described for *L. plantarum*. The residual L-tartrate is converted from oxaloacetate via malate and fumarate to succinate, similar to L-tartrate fermentation by *E. coli*. By combining a NADH supplying and a consuming branch the overall redox reactions are balanced (Fig. 7.4). The enzymes have not been demonstrated in detail for *L. brevis*, but the genome of *L. brevis* ATCC 367 contains structural genes for a fumarase (gene LVIS_0714), pyruvate dehydrogenase (genes LVIS_1407-1410) (and pyruvate oxidase, gene LVIS_0313) and a potential malate dehydrogenase (gene LVIS_1406 annotated as malate/lactate dehydrogenase). Genes for L-tartrate dehydratase, oxaloacetate decarboxylase and fumarate reductase have not been identified yet.

7.4 Conclusion

Oenococcus oeni and other lactic acid bacteria from wine are able to grow at the expense of sugars (hexoses and pentoses) using the phosphoketolase pathway. The genes and enzymes of the central reactions of the phosphoketolase pathway are known, whereas the genes and (most of) the enzymes of erythritol formation and of the use of alternative sugars are unknown. Fermentation of the hexoses is stimulated in the presence of substrates (fructose, citrate, pyruvate, O_2) which are able to reoxidize NAD(P)H, resulting in a shift of the fermentation pattern. *O. oeni* degrades

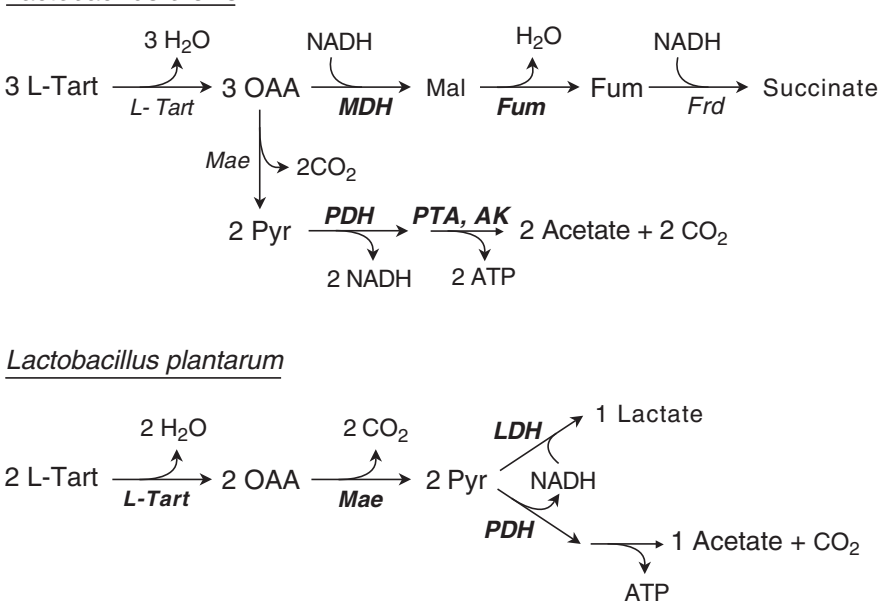

Fig. 7.4 Degradation of L-tartrate by *Lactobacillus brevis* (3 L-tartrate → 1 succinate + 2 acetate + 4 CO_2) and *L. plantarum* (2 L-tartrate → acetate + lactate + 3 CO_2). *L-Tart* L-tartarte dehydratase; *MDH* malate DH; *Fum* fumarase; *Frd* fumarate reductase; *Mae* oxaloacetate decarboxylase; *PDH* pyruvate dehydrogenase. Enzymes for which the corresponding structural genes were not identified in the genomes of *L. brevis* and *L. plantarum*, are printed in *normal type*, others in *bold type*. The enzymes and corresponding genes for *L. brevis* are: *MDH* (LVIS_1406); *Fum* (LVIS_0714); *PDH* (genes LVIS_1407, 1408, 1409, 1410); *Pox* (LVIS_0313), phosphotransacetylase (lvis_0674), and acetate kinase (lvis_129, 1601, 1190), and for *L. plantarum*: *L-Tart* (LP_1090 and 1089 for *TtdAB*), *Mae* (LP_1105); *PDH* (LP_2151 to 2154); *LDH* LP_2057 (D-lactate deyhdrogenase)). Abbreviations for substrates and intermediates: *L-Tart* T-tartrate; *OAA* oxaloacetate; *Mal* L-malate; *Fum* fumarate; *Pyr* pyruvate. Pathways according to Radler and Yannissis (1972)

also organic acids from wine or must (citrate, malate and pyruvate) by pathways for which enzymes and structural genes are known. The regulation underlying the use of alternative substrates like sugars, organic acids and electron acceptors is largely unknown. Some heterofermentative lactic acid bacteria from wine metabolize L-tartrate and fumarate by reactions for which the enzymes and encoding genes have been identified only in parts. The latter pathways appear to be strain specific and some of the postulated genes or enzymes were not identified in sequenced genomes. Identification of the unknown genes, enzymes and carriers for the alternative substrates, and regulation of the fermentation pathways remains an important goal for understanding energy metabolism of *O. oeni* and other lactic acid bacteria from wine and must.

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Chapter 8
Transport of Sugars and Sugar Alcohols by Lactic Acid Bacteria

Tanja Zaunmüller and Gottfried Unden

Lactic acid bacteria (LAB) play an important role in the fermentation of beverages like wine and beer, and in the production of dairy products, sour dough, sausages and cheese. The knowledge of the genome sequence offers an insight into the metabolism of the bacteria and provides means to optimize the manufacturing of the products. By now genomes of several lactic acid bacteria are sequenced, including wine related bacteria *Oenococcus oeni* PSU-1, *Pediococcus pentosaceus* ATCC 25745, *Leuconostoc mesenteroides* ATCC 8293 (http://www.jgi.doe.gov/; Klaenhammer et al. 2002; Mills et al. 2005) and *Lactobacillus plantarum* WCFS1 (http://www.cmbi.ru.nl/plantarum/; http://www.lacplantcyc.nl/; Kleerebezem et al. 2003). *Lactococcus lactis* ssp. *lactis*, formerly *Streptococcus lactis* (Schleifer et al. 1985), was the first fully sequenced and annotated lactic acid bacterium (Bolotin et al. 2001). *L. lactis* is mainly used in the dairy industry for the production of cheese. It serves as a model organism for studies on the physiology and genetics of lactic acid bacteria and is included in the genome comparison.

Lactic acid bacteria ferment carbohydrates with lactate as the main product by the homo- and heterofermentative pathways. Homofermentative LAB ferment hexoses by glycolysis to lactate as the only product (>90%). *P. pentosaceus* is classified as homofermentative, but metabolizes pentoses by the phosphoketolase pathway to lactic acid and acetate typical for heterofermentative LAB. *Lactobacillus delbrueckii* ssp. *bulgaricus* is obligately homofermentative and is not able to grow on pentoses (Axelsson 2004; Klaenhammer et al. 2002). The homofermentative *Lactococcus lactis* shifts under specific conditions from homofermentative to mixed-acid fermentation with formate as an additional product (Garrigues et al. 1997). Heterofermentative lactic acid bacteria like *O. oeni* and *L. mesenteroides* ferment hexoses and pentoses by the phosphoketolase pathway (Kandler 1983; Veiga-da-Cunha et al. 1993; Mills et al. 2005). Main products are lactate, ethanol and CO_2 from hexoses, or lactate and acetate from pentoses. In the presence of alternative acceptors, heterofermentative LAB replace ethanol by varying amounts of acetate (Kandler 1983; Zaunmüller et al. 2006; Stolz et al. 1995; Unden and

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Zaunmüller 2008). *Lactobacillus plantarum* is facultatively heterofermentative and ferments hexoses by the glycolytic and pentoses by the phosphoketolase pathway (Kleerebezem et al. 2003; Kandler 1983; Kandler and Weiss 1986). *L. plantarum* is also capable of mixed acid fermentation and ferments hexoses under specific conditions to lactate, acetate, formate and ethanol (Kleerebezem et al. 2003; Boekhorst et al. 2004).

Most lactic acid bacteria use a variety of sugars and sugar alcohols as substrates for growth and contain carriers and enzymes for conversion of non-conventional sugars to the intermediates of glycolysis or phosphoketolase pathway.

8.1 Classification of Transporters for Sugars in Bacteria

In bacteria generally three types of transporters are responsible for the uptake of sugars and of sugar alcohols like glycerol and mannitol (Konings 2002). The transport systems are classified according to the type of energy used for transporting the substrate across the membrane (Fig. 8.1). Primary transporters like the ATP-binding cassette (ABC) superfamily of transporters drive transport of substrates into the cells by ATP hydrolysis (Schneider and Hunke 1998; Boos and Lucht 1996). ABC transporters comprise uptake and efflux systems (Saurin et al. 1999). They consist of two membrane integral proteins or domains and two membrane associated ATPase subunits, and each of the domains or proteins is present as dimer. Uptake systems contain in addition an extracellular substrate binding protein that specifically binds the substrate.

Secondary carriers are driven by H^+, Na^+ or other gradients across the cytoplasmic membrane, and catalyze uniport, antiport or symport of solutes. Most of these transporters belong to the Major Facilitator Superfamily (MFS, Pao et al. 1998). The members of the MFS transport a variety of substrates, including sugars. The sugar porter family, the fucose: H^+ symporter family, the oligosaccharide: H^+ symporter family, the glycoside-pentoside-hexuronide (GPH): cation symporter family and the polyol permease family (Saier 2000; Poolman et al. 1996) are subfamilies of the MFS and responsible for sugar and sugar alcohol transport. Members are characterized by a length of 400–800 amino acids and typically 12 predicted transmembrane helices (Pao et al. 1998). The substrate specificity of transporters of the MFS cannot be predicted from the sequence due to low sequence identity of the members and poor conservation of substrate binding sites. So far the crystallization of only two members of the MFS was successful, namely the lactose permease LacY (Abramson et al. 2003) and the glycerol-3-phosphate transporter GlpT (Huang et al. 2003).

The third class are group translocators or phosphotransferase systems (PTS) which catalyze the uptake of sugars and sugar alcohols into the cells at the expense of PEP (Kundig et al. 1964; Postma et al. 1993; Deutscher et al. 2006). PTS transporters use hexoses and disaccharides as substrates whereas pentoses are not transported (Lengeler and Jahreis 1996). PTS transporters are composed of an

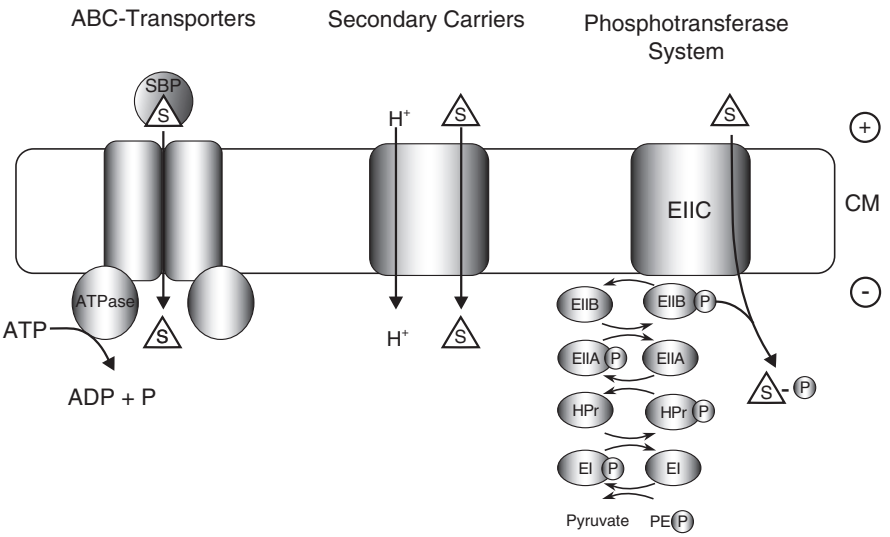

Fig. 8.1 Classification and organisation of transport systems. In bacteria three different types of transporters are responsible for the uptake of substrates: ABC-transporters, secondary carriers and phosphotransferase systems. ABC-transporters consist of an extracellular substrate binding protein (SBP), an integral membrane protein (permease) and a cytoplasmic ATPase subunit. The permease and ATPase domains or proteins are organised as homo- or heterodimers. Secondary carriers consist of single proteins with mostly 12 transmembrane helices. The phosphotransferase systems consist of a membrane protein (EIIC) which translocates the substrate. A phosphoryl residue is transferred from phosphoenolpyruvate (PEP) by a cascade of four proteins (EI, HPr, EIIA and EIIB) to the substrate which is phosphorylated after transport. EII proteins or domains may be fused in different ways (EIIABC, EIIBC; EIIAB). *Abbrevations CM* cytoplasmic membrane; *S* substrate (sugar or sugar alcohol); *P* phosphoryl residue

integral membrane protein (EIIC) which translocates the substrates across the cytoplasmic membrane, and several cytoplasmic proteins that transfer the energy delivering phosphoryl residue from phosphoenolpyruvate (PEP) to the substrate. The latter is phosphorylated during transport after crossing the membrane. The proteins HPr, EI, EIIA and EIIB are involved in the phosphotransfer cascade. HPr and EI are universal proteins which are used by all PTS of a cell, whereas EIIABC proteins are specific for one transport system. The sugars (hexoses and disaccharides) and sugar alcohols enter metabolism in the phosphorylated state. Some systems comprise an additional EIID component which is located in the membrane. The EIID protein is characteristic for the mannose-fructose-sorbose-PTS family. The members of this family usually transport a broad range of substrates. *E. coli* mannose PTS is a member of this family and transports in addition to mannose and fructose also glucose, glucosamine and *N*-acetylglucosamine (Huber and Erni 1996).

It has been suggested that only LAB with glycolytic hexose metabolism use PTS systems for sugar uptake in energy metabolism (Romano et al. 1979). Fermentation of hexoses by the phosphoketolase pathway supplies only one PEP per hexose. Consumption of PEP for PTS transport would leave no PEP for biosynthetic path-

ways in heterofementative bacteria (Romano et al. 1979; Reizer et al. 1988). However, some heterofermentative LAB like *Lactobacillus brevis* contain a fructose-specific PTS which is induced under anaerobic conditions in the presence of fructose (Axelsson 2004; Saier et al. 1996). Furthermore enzymes for fructose degradation (fructose-1-phosphate-kinase) and of glycolysis (fructose-1,6-bisphosphate aldolase) are expressed under the same conditions (Saier et al. 1996). In the heterofermentative *Lactobacillus fermentum* PTS activity for sucrose and mannose uptake was detected (Nagasaki et al. 1992).

The genome analysis of sequenced and annotated LAB genomes should allow further insight into the capacity and variety of systems for the transport of sugars and sugar alcohols.

8.2 Genome Comparison on Transporters Associated with the Uptake of Carbon Substrates

The genomes of the five LAB were analyzed for putative transporters for sugars (hexoses, pentoses, disaccharides and polysaccharides) and sugar alcohols (glycerol and mannitol) by the use of available annotation and by sequence comparison.

COGs (*C*lusters of *O*rthologous *G*roups) comprise orthologous genes, proteins or orthologous groups of paralogs. Orthologous genes are from different species. They show sequence similarity and are supposed to originate from a common ancestor. For the genes similar or same function is expected. Paralogs developed from duplication of one gene within the same genome. Paralogs usually have different functions. The system of orthologous and paralogous genes of Koonin and co-workers (Tatusov et al. 1997) allows assigning potential functions to genes and corresponding proteins of newly sequenced genomes. The present COGs are summarized in the Clusters of Orthologous Groups Database (http://www.ncbi.nlm.nih.gov/COG/) and are accessible via the IMG (Integrated Microbial Genome) database (http://img.jgi.doe.gov/). The IMG database is a platform to analyze microbial genomes which is provided by the Joint Genome Institute (Markowitz et al. 2006). The database allows screening of all sequenced microbial genomes for genes of specific functions, e.g., COGs and Pfams.

The Pfam (*P*rotein *fam*ily) database comprises a collection of protein domains and families. In proteins each functional domain is assigned to a Pfam (Finn et al. 2006). For proteins of unknown function, functions similar to those of their Pfam can be assumed. Proteins with several functional domains are assigned to more than one Pfam. Similar protein domain families are summarized to a superior clan. Thus, the clan of the major facilitator superfamily consists of 20 protein families including the family of sugar transporters. The database (http://pfam.sanger.ac.uk/) comprises currently 9,318 families (Pfam Version 22.0; July 2007).

The assignment of proteins to COGs and Pfams offers a possibility to predict their (putative) functions. Each gene belongs to one COG and to one or several Pfams.

8.3 Selection of COGs and Pfams of Sugar and Sugar Alcohol Carriers

The genomes of the five LAB were screened for clusters of orthologous groups of proteins (COGs) and protein families (Pfams) associated with the transport of sugars and sugar alcohols. The genes belong to the class of ABC transporters, secondary carriers or PTS. The genes for secondary transporters were selected from COGs and Pfams of the major facilitator superfamily which are known to transport sugars or sugar alcohols, for example COG2814 of arabinose efflux permeases, COG4975 of putative glucose uptake permease or Pfam00083 of sugar transporters. The latter belongs to the major facilitator superfamily clan and comprises sugar transporting proteins of the MFS. The protein components of PTS are found in well defined COGs and Pfams. Since PTS systems transport only sugars and sugar alcohols, all genes of the respective COGs and Pfams are candidates for transporters of these substrates. Therefore all COGs and Pfams comprising genes of PTS EIIA, EIIB and EIIC proteins were selected, such as COG1299 for fructose-specific EIIC components and Pfam03609 for sorbose-specific PTS EIIC proteins. Within the ABC transporter family prediction of the substrate is difficult, since the substrate of the reference carriers is often not known. For this study only COGs and Pfams of ABC transporters were used that are known to transport sugars. Only genes that are part of an operon coding for a complete ABC transport system consisting of an extracellular binding protein, a permease and an ATPase component were selected.

8.4 Sugar Transporters of the Heterofermentative *O. oeni* and *L. mesenteroides*

Oenococcus oeni PSU-1 is able to grow on the hexoses glucose and fructose, on the pentose ribose, and on the disaccharides trehalose and cellobiose (Beelman et al. 1977). Some strains of *O. oeni* use other sugars (Garvie 1986b; Zhang and Lovitt 2005) in addition to glucose, fructose and ribose which are degraded by most *O. oeni* strains. The majority of predicted sugar and sugar alcohol carriers are secondary carriers. The genome of *O. oeni* PSU-1 reveals 40 genes for secondary carriers of the major facilitator superfamily which could be responsible for the uptake of sugar substrates (Table 8.1). Twenty-three of the transporters are assigned to COG2814 of arabinose efflux permeases (Table 8.1). A well characterized member of this group is the arabinose transporter AraE of *E. coli* which catalyzes the uptake of arabinose (MacPherson et al. 1981). Four genes belong to COG2211 of Na^+/melibiose symporters which includes the melibiose transporter MelB of *E. coli* (Yazyu et al. 1984) and the xyloside transporter XylP of *Lactobacillus pentosus* (Chaillou et al. 1998; Heuberger et al. 2001).

O. oeni encodes eight complete PTS (Table 8.2) which resemble uptake systems for fructose, mannose, galactitol and cellobiose. Three of the PTS systems are related to

Table 8.1 Genome search for COGs and Pfams of secondary carriers for the uptake of sugars and sugar alcohols in *Lactococcus lactis (L. l.), Lactobacillus plantarum* (*L. p.*), *Pediococcus pentosaceus* (*P. p.*), *Leuconostoc mesenteroides* (*L. m.*) *and Oenococcus oeni* (*O. o.*). The total number gives the overall number of candidate genes for secondary carriers. The list shows the COGs and Pfams used for the genome screening and the number of corresponding genes found in each organism. Each gene can be assigned to one COG and/or one Pfam, therefore neither the number of COGs or Pfams, nor the sum of both sum up to the total number given

COG/Pfam	Description	*L. l.*	*L. p.*	*P. p.*	*L. m.*	*O. o.*
Total number		31	64	32	41	40
COG0580	Glycerol uptake facilitator and related permeases (Major Intrinsic Protein Family)	4	6	4	2	1
COG0697	Permeases of the drug/metabolite transporter (DMT) superfamily	1	1	0	0	0
COG0738	Fucose permease	0	1	0	0	1
COG2211	Na^+/melibiose symporter and related transporters	3	7	5	8	4
COG2270	Permeases of the major facilitator superfamily	0	1	0	0	0
COG2271	Sugar phosphate permease	2	1	1	1	0
COG2814	Arabinose efflux permease	16	33	16	23	23
COG4975	Putative glucose uptake permease	1	3	2	1	1
Pfam00083	Sugar transporter (of the MFS)	1	3	3	5	5
Pfam00230	Major Intrinsic Protein Family (e.g. glycerol facilitator protein GlfP)	4	6	4	2	1
Pfam04215	SgaT_UlaA: Putative sugar specific permease	0	2	0	1	0
Pfam06800	Sugar transport proteins	1	3	2	1	1
Pfam07690	MFS_1: Major Facilitator Superfamily	22	49	24	31	34

Table 8.2 Genome search for COGs and Pfams of PTS systems for the uptake of sugars and sugar alcohols in *Lactococcus lactis (L. l.), Lactobacillus plantarum* (*L. p.*), *Pediococcus pentosaceus* (*P. p.*), *Leuconostoc mesenteroides* (*L. m.*) *and Oenococcus oeni* (*O. o.*). The total number gives the overall number of candidate genes for phosphotransferase systems. It is indicated when genes encode a complete PTS consisting of the protein or domains EIIA, EIIB and EIIC or if the systems are incomplete. A complete system can be encoded by one to three genes depending on whether domains or proteins are fused. A system is designated as incomplete if a gene for one of the three components is missing in the close proximity of the cluster. Listed below are the COGs and Pfams used for the genome screening and the number of corresponding genes in each organism. Each gene can be assigned to one or several COGs and Pfams since domains may be fused. Thus, the sum of genes does not correspond to the total number of transporters

COG/Pfam	Description	*L. l.*	*L. p.*	*P. p.*	*L. m.*	*O. o.*
Number of PTS genes		16	65	35	22	28
Number of complete systems		6	25	11	7	8
Number of incomplete systems		4	19	9	4	3
COG1263	PTS IIC components, glucose/maltose/*N*-acetylglucosamine-specific	3	14	5	3	1
COG1264	PTS IIB components	0	2	1	0	1
COG1299	PTS fructose-specific IIC component	1	2	2	0	0
COG1440	PTS cellobiose-specific component IIB	1	3	2	2	3
COG1445	PTS fructose-specific component IIB	1	2	2	0	0
COG1447	PTS cellobiose-specific component IIA	1	3	2	2	3
COG1455	PTS cellobiose-specific component IIC	3	9	6	3	3

(continued)

Table 8.2 (continued)

COG/Pfam	Description	*L. l.*	*L. p.*	*P. p.*	*L. m.*	*O. o.*
COG1762	PTS mannitol/fructose-specific IIA domain (Ntr-type)	1	7	2	2	4
COG2190	PTS IIA components	3	18	7	6	2
COG2213	PTS mannitol-specific IIBC component	0	1	0	0	0
COG2893	PTS mannose/fructose-specific component IIA	1	3	3	2	3
COG3414	PTS galactitol-specific IIB component	0	4	0	1	2
COG3444	PTS mannose/fructose/*N*-acetylgalactosamine-specific component IIB	1	3	4	2	2
COG3715	PTS mannose/fructose/*N*-acetylgalactosamine-specific component IIC	1	2	5	2	2
COG3716	PTS mannose/fructose/*N*-acetylgalactosamine-specific component IID	1	2	5	2	2
COG3730	PTS sorbitol-specific component IIC	0	2	0	0	0
COG3731	PTS sorbitol-specific component IIA	0	3	1	0	0
COG3732	PTS sorbitol-specific component IIBC	0	2	0	0	0
COG3775	PTS galactitol-specific IIC component	0	2	0	0	1
COG4668	Mannitol/fructose-specific PTS, IIA domain	1	1	0	0	0
Pfam00358	PTS_EIIA_1	3	16	7	5	2
Pfam00359	PTS_EIIA_2	2	7	3	1	2
Pfam00367	PTS_EIIB	3	14	5	3	2
Pfam02255	PTS_IIA: Lactose/Cellobiose specific	1	2	2	2	3
Pfam02302	PTS_IIB: Lactose/Cellobiose specific	1	8	2	3	5
Pfam02378	PTS_EIIC	7	25	12	5	3
Pfam02379	PTS_IIB_fructose specific subunit IIB	1	2	2	0	0
Pfam03608	EII-GUT: PTS sorbitol specific factor	0	2	0	0	0
Pfam03609	EIIC: sorbose specific component	1	2	5	1	2
Pfam03610	EIIA-man PTS IIA fructose component	2	5	5	2	3
Pfam03611	EIIC-GAT: Galactitol specific IIC	0	2	0	0	1
Pfam03612	EIIBC-GUT_N: Sorbitol PTS N terminus	0	2	0	0	0
Pfam03613	EIID-AGA: PTS mannose/fructose/sorbose family IID component	1	2	5	2	2
Pfam03829	PTS IIA_gutA: PTS glucitol/sorbitol specific IIA component	0	2	0	0	0
Pfam03830	PTS IIB_sorb: PTS sorbose subfamily IIB	1	3	4	2	2
Pfam07663	EIIBC-GUT_C: Sorbitol PTS C terminus	0	2	0	0	0

cellobiose uptake systems. Two of the operons are accompanied by adjacent genes encoding putative β-glucosidases supporting their role as cellobiose or general disaccharide uptake system. The remaining five systems are annotated as fructose/manntitol, galactitol or mannose PTS. Two of these systems comprise an EIID protein which supports their function in the uptake of hexoses like fructose and mannose.

Seven complete ABC transport systems are encoded by *O. oeni* (Table 8.3) which may be related to the uptake of sugars, but only four of them are annotated as sugar transporters (see http://www.jgi.doe.gov/). Since *Oenococcus* ferments sugars by the phosphoketolase pathway supplying only one ATP per glucose, the ABC carriers cannot be used in hexose catabolism for energetic reasons. The ABC transporters may have a role in uptake of sugars not related to energy metabolism.

Table 8.3 Genome search for COGs and Pfams of candidate genes for ABC carriers for sugars or sugar alcohols in *Lactococcus lactis (L. l.), Lactobacillus plantarum* (*L. p.*), *Pediococcus pentosaceus* (*P. p.*), *Leuconostoc mesenteroides* (*L. m.*) *and Oenococcus oeni* (*O. o.*). The total number of candidate genes for ABC carriers for uptake of sugars and sugar alcohols is given. It is indicated if the genes belong to an operon encoding a complete transport system consisting of a carrier, an ATPase and an extracellular binding protein. Only few of the systems are annotated as sugar transporting system in the IMG database or corresponding genome publication. Additionally the number of genes for incomplete (potential export systems) is given. Therefore the sum of genes does not correspond to the total number of transporters. The list shows the COGs and Pfams used for the genome screening and the number of corresponding genes in each organism

COG/Pfam	Description	*L. l.*	*L. p.*	*P. p.*	*L. m.*	*O. o.*
Total number of genes		22	27	7	8	24
Complete ABC transport systems		4	11	2	3	7
Annotated sugar transport systems		2	4	1	1	4
Genes of incomplete systems		13	6	1	2	5
COG0395	ABC-type sugar transport system, permease component	2	0	0	4	1
COG1129	ABC-type sugar transport system, ATPase component	0	0	0	0	1
COG1134	ABC-type polysaccharide/polyol phosphate transport system, ATPase component	1	1	0	0	2
COG1172	Ribose/xylose/arabinose/galactoside ABC-type transport systems, permease components	0	0	0	0	1
COG1175	ABC-type sugar transport systems, permease components	2	0	0	4	1
COG1653	ABC-type sugar transport system, periplasmic component	2	0	0	2	1
COG1869	ABC-type ribose transport system, auxiliary component	1	1	0	1	1
COG1879	ABC-type sugar transport system, periplasmic component	0	0	0	0	1
COG2182	Maltose-binding periplasmic proteins/domains	1	0	0	1	1
COG3822	ABC-type sugar transport system, auxiliary component	0	0	0	0	0
COG3833	ABC-type maltose transport systems, permease component	1	0	0	0	1
COG3839	ABC-type sugar transport systems, ATPase components	3	0	0	3	1
COG4158	Predicted ABC-type sugar transport system, permease component	0	0	0	0	0
COG4209	ABC-type polysaccharide transport system, permease component	1	0	0	0	1
COG4211	ABC-type glucose/galactose transport system, permease component	0	0	0	0	0
COG4213	ABC-type xylose transport system, periplasmic component	0	0	0	0	0
COG4214	ABC-type xylose transport system, permease component	0	0	0	0	0
Pfam01061	ABC2_membrane (transport of carbohydrates)	4	3	0	2	4
Pfam01547	SBP_bac_1(bacterial extracellular solute-binding protein, e.g., for maltose)	8	1	3	7	5
Pfam02653	BPD_transp_2 (branched chain amino acid transport system, permease component; family also contains a galactose and ribose transport system	4	1	5	3	3

The large number of putative carriers for sugars and sugar alcohols (40 secondary, 8 PTS and 4 ABC transporters) exceeds the number of the corresponding substrates (glucose, fructose, ribose, trehalose and cellobiose). Therefore *O. oeni* might be able to use further substrates of this type, or contains isoforms of carriers for some substrates which are used under specific conditions.

Leuconostoc mesenteroides shows a similar number of genes associated with the transport of sugars as *O. oeni*. By the use of COGs and Pfams 41 genes for secondary carriers, 7 complete PTS and for 3 ABC transport systems related to sugar transport systems were found. Only one of the ABC systems is annotated as sugar transporting (see http://www.jgi.doe.gov/).

Overall, there is only very little knowledge about transport of sugars in *Oenococcus* and *Leuconostoc* species. Neither the biochemistry and energetics of sugar transport has been characterized nor is there experimental correlation of the sugar transport to specific carriers or genes, which is required to understand this important part of sugar metabolism. This lack of knowledge in sugar transport is contrasted by the detailed information on the biochemistry and genetics of organic acids (malate, citrate) and amino acids (glutamate, arginine) transport (for review see Konings 2002).

8.5 Sugar Transporters in the Homofermentative *Pediococcus pentosaceus*

P. pentosaceus uses in addition to glucose (Blickstad and Molin 1981) sugars like arabinose, xylose, fructose, galactose, mannose and the disaccharides maltose and trehalose (Teuber and Geis 1981). Growth on sugar alcohols like glycerol and mannitol is only poor (Teuber and Geis 1981; Garvie 1986a).

The genome of *P. pentosaceus* contains 32 genes for secondary carriers, 11 complete PTS and two ABC transport systems only one of which is annotated as sugar transporting (see http://www.jgi.doe.gov/). Thus, the number of potential sugar transporters of each type is similar to the numbers in the heterofermentative *O. oeni* and *L. mesenteroides*. In the homofermentative *P. pentosaceus* PTS are good candidates for sugar uptake. ABC transporters could be used for energetic reasons for uptake of sugars as well. The small number of ABC transporters argues against the latter possibility, and PTS and secondary carriers might be the major carriers. Similar to *O. oeni* and *L. mesenteroides* physiology and genetics of sugar transport has not been studied and it is not possible to relate the genes to specific transport systems.

8.6 Sugar Transport Capacities of the Facultatively Heterofermentative LAB *L. plantarum*

L. plantarum has the largest genome (3.3 Mb) among the sequenced LAB and contains the largest number of genes related to sugar transport. The genome size is nearly twice that of *O. oeni* and *L. mesenteroides* (about 1.8 Mb) (Table 8.4).

Table 8.4 General characteristics of the screened genomes and the candidate genes of transporters for the uptake of sugars and sugar alcohols in *Lactococcus lactis (L. l.), Lactobacillus plantarum* (*L. p.*), *Pediococcus pentosaceus* (*P. p.*), *Leuconostoc mesenteroides* (*L. m.*) *and Oenococcus oeni* (*O. o*)

	L. l.	L. p.	P. p.	L. m.	O. o.
Homo- or heterofermentative metabolism	*Homo*	*Facultative hetero*	*Homo*	*Hetero*	*Hetero*
General features of the genome					
Genome size (Mb)	2.3	3.3	1.8	1.8	1.8
Protein coding genes	2,310	3,052	1,755	1,970	1,691
Number of genes for transporters (% of total)	254 (11)	473 (8)	n. d.	n. d.	n. d.
Transporters associated with sugar uptake					
Secondary carriers	31	64	32	41	40
Complete PTS (total number of genes)	6 (16)	25 (65)	11 (35)	7 (22)	8 (28)
Complete ABC transporters (total number of genes)	4 (22)	11 (27)	2 (7)	3 (8)	7 (24)
Total number of genes for transporters (% of total number of genes of genome)	69 (3)	156 (5)	74 (4)	71 (4)	92 (5)

It encodes 25 complete PTS (Kleerebezem et al. 2003). The PTS systems (Table 8.2) are predicted to catalyze the uptake of fructose, mannose, mannitol, sorbitol, galactitol, sucrose, cellobiose, other β-glucosides and *N*-acetylglucosamine (Kleerebezem et al. 2003). The PTS EII^{man} protein of *L. brevis* catalyses passive diffusion of xylose across the membrane if expressed in *L. plantarum*. In the heterologous system xylose is transported without phosphorylation (Chaillou et al. 1999), but *L. plantarum* is not able to ferment xylose (Chaillou et al. 1998). One of the sucrose PTS systems (gene lp_0185) is predicted as an uptake carrier for fructose oligosaccharides (Saulnier et al. 2007).

In addition genes for 64 secondary carriers (Table 8.1) and 11 complete ABC transporters (Table 8.3), four of which are annotated as sugar ABC transports (see http://img.jgi.doe.gov/) were found using the COGs and Pfams search. *L. plantarum* grows on a wide variety of sugars and degrades hexoses (glucose, mannose, galactose), pentoses (ribose, arabinose, rhamnose), sugar alcohols (mannitol, sorbitol), and disaccharides (sucrose, lactose, maltose, trehalose, melibiose) (Kleerebezem et al. 2003).

In a comparative study (Boekhorst et al. 2004) the gene sequences of putative transporters of *L. plantarum* and *L. johnsonii* were compared to the sequences of the Transport Classification Database (Saier et al. 2006). The search revealed the presence of 286 genes associated with the transport in *L. johnsonii* and of 473 genes in *L. plantarum*. This corresponds to 15 and 13% of the total number of proteins in the bacteria (Boekhorst et al. 2004). In *Lactococcus lactis* (Bolotin et al. 2001; Boekhorst et al. 2004) and *Streptococcus mutans* (Ajdic et al. 2002) 11 and 8% of the proteins are predicted transporters. In *L. plantarum* transport proteins represent also by far the largest class of proteins (Klereebezem et al. 2003). The putative sugar or sugar alcohol transporters amount to 2–5% of the total carriers (Table 8.4). The large numbers support the significance of transport and sugar transport in particular.

8.7 *Lactococcus lactis* ssp. *lactis* IL1403 as Model Organism for Homofermentative LAB

The genome of *L. lactis* IL1403 reveals 31 genes for secondary carriers (Table 8.1), six PTS systems (Table 8.2) and six complete ABC transport systems (Table 8.3) two of which are annotated as sugar transporting (see http://img.jgi.doe.gov/).

L. lactis is able to grow on a variety of sugars (Bolotin et al. 2001). Glucose transport is catalyzed by the mannose specific PTS (Papagianni et al. 2007; Neves et al. 2005; Thompson 1987). Beside this high affinity uptake system a second low affinity system is active (Papagianni et al. 2007). Earlier results proposed an additional permease for glucose (Thompson and Chassy 1985). The gene for a secondary glucose carrier has not been identified yet (Neves et al. 2005). Galactose is fermented mostly in a mixed acid fermentation (Melchiorsen et al. 2002). The uptake of galactose is catalyzed by two galactose transport systems, a PTS and a secondary carrier (Thompson 1980). The carriers have similar activities, but the secondary carrier shows higher affinity. Only the gene *galP* for the secondary carrier is known (Neves et al. 2005; Grossiord et al. 2003). Sucrose is transported by a PTS (Thompson and Chassy 1981). The closely related *L. lactis* ssp. *cremoris* grows on fructose which is transported by a constitutively expressed mannose carrier and by an inducible fructose PTS (Benthin et al. 1993). Maltose is proposed to be transported via an ABC transporter (Neves et al. 2005; Law et al. 1995; Sjöberg and Hahn-Häerdahl 1989).

L. lactis uses the homofermentative pathway when rapidly metabolizable sugars like glucose are fermented. Sugars which are slowly degraded, like galactose, maltose, lactose, or glucose when supplied at limiting concentrations, are metabolized by mixed acid fermentation resulting in the products lactate, ethanol, formate and acetate (Melchiorsen et al. 2002; Garrigues et al. 1997). The parameters triggering the shift between both fermentation types are still under debate, and it has been suggested that either the rate of sugar consumption and the resulting $NADH/NAD^+$ ratio (Melchiorsen et al. 2002; Garrigues et al. 1997; Palmfeldt et al. 2004), or the type of sugar transport (Bolotin et al. 2001) govern the fermentation pathway. Thus, homofermentative metabolism is observed for sugars that are transported by PTS (fructose, glucose and mannose), whereas transport of sugars by secondary and ABC transporters is related to mixed acid (galactose, lactose, maltose) or heterofermentative (ribose) fermentation.

Genome annotation suggested permeases for xylose, lactose and glycerol and PTS for the transport of cellobiose, β-glucosides, mannose, fructose (Bolotin et al. 2001). ABC transporters were predicted for ribose and sugars. Transport activities and their relation to individual carriers or genes have not been studied yet. It is assumed, however, that for each substrate several transporters are available and that for different metabolic situations alternative carriers are used (Cocaign-Bousquet et al. 1996).

8.7.1 Outlook

Despite the large number of studies on sugar metabolism in LAB the knowledge on transport systems for sugars and sugar alcohols is very limited. The genome sequences of wine related LAB *O. oeni*, *L. mesenteroides, P. pentosaceus* and *L. plantarum* and comparison to *L. lactis* ssp. *lactis* shows that the LAB contain a large number of genes which are candidates for secondary carriers, PTS and ABC carriers for sugars and sugar alcohols. The carriers represent one of the largest gene families supporting the significance of sugar transport for the bacteria. Homo- and heterofermentative LAB contain the same type of carriers and in approximately the same relative amounts, suggesting that the type of carrier used is similar or of the same type in the bacteria independent of the metabolic pathways for sugar degradation (glycolysis vs. phosphoketolase pathway). In *L. lactis*, however, homofermentative lactic acid fermentation appeared to be related to transport by PTS. The number of putative carrier genes exceeds the number of substrate sugars or sugar alcohols largely, suggesting that the bacteria are able to use further sugar and sugar alcohol substrates, and that isoforms or alternative carriers are present for the same substrates which might be used under different metabolic conditions. The available genome sequences allow identification of candidate genes for the carriers, which should enable biochemical and genetic identification of sugar and sugar alcohol carriers in future work.

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Useful Databases and Websites

IMG Database (http://img.jgi.doe.gov/)
JGI Database (http://www.jgi.doe.gov/)
Genome sequence of Lactobacillus plantarum (http://www.cmbi.ru.nl/plantarum/http://www.lacplantcyc.nl/)
Transport Classification Database (http://www.tcdb.org/)
COG Database (http://www.ncbi.nlm.nih.gov/COG/)
Pfam Database (http://pfam.sanger.ac.uk/)

Part III
Secondary Metabolism

Chapter 9
Amino Acid Metabolisms and Production of Biogenic Amines and Ethyl Carbamate

Massimo Vincenzini, Simona Guerrini, Silvia Mangani, and Lisa Granchi

9.1 Introduction

The aminoacidic composition of a grape must is acknowledged to have noticeable implications with regard to quality and safety of the resulting wine, because both pleasant and unpleasant or even quite undesirable compounds can be produced by the microflora participating in the winemaking process – yeasts and lactic acid bacteria – especially as a consequence of their catabolic activity on the available amino acids. Indeed, microbial catabolism of amino acids mainly occurs through the activities of five groups of enzymes (i.e. aminotransferases, decarboxylases, dehydratases, lyases and deaminases) which, intracellularly, convert amino acids into a range of volatile and non-volatile compounds, such as α-keto acids, aldehydes, hydroxy acids, alcohols and amines, all playing an important role in determining the organoleptic qualities of wine. Among these catabolites from amino acids, the latter class of compounds, often referred to as biogenic amines (BA) to underline their main biological origin, is receiving much attention in wine science because of their potential implication for human health (Silla Santos 1996).

BA are nitrogenous low molecular weight organic bases that can have an aliphatic, aromatic or a heterocyclic structure and are widely present in foods, especially in fermented foods, mostly as a consequence of the decarboxylation of their respective free precursor amino acids, through the action of substrate-specific microbial decarboxylases. Hence, the amines histamine, tyramine, putrescine, cadaverine, 2-phenethylamine, agmatine and tryptamine originate from the precursor amino acids histidine, tyrosine, ornithine, lysine, phenylalanine, arginine and tryptophane, respectively.

Other amines, possibly present in fermented foods and wines, include the aliphatic volatile amines (methylamine, ethylamine and isoamylamine), that can be originated by the amination of non-nitrogen compounds, such as aldehydes and

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ketones (Bauza et al. 1995), and the polyamines, spermine and spermidine, that can be produced from putrescine (1,4-diaminobutane), through methylation reactions involving *S*-adenosyl-methionine.

As above mentioned, all these molecules have the potential to cause physiological distress in the human organism if ingested in relatively high concentration by sensitive people. More specifically, the heterocyclic amine histamine, the most toxic and studied biogenic amine, may induce headaches, hypotension, heart palpitation, cutaneous and gastrointestinal disorders; the aromatic amines, tyramine and 2-phenethylamine (a volatile amine), are known to cause migraines and hypertensive crises because they may originate the vasoconstrictor hormones noradrenaline and norephedrine; the polyamines (putrescine, agmatine, cadaverine, spermine and spermidine), although not toxic themselves, potentiate the effects of the toxic amines, being also able to inhibit enzymes such as the amino-oxidases, catalysing the oxidative deamination of amines, constituting the main detoxifying system of BA in humans (ten Brink et al. 1990); the volatile monoamines, in spite of their poor physiological significance, are active as irritants and, thus may negatively affect the sensorial profile of foods (Lehtonen 1996).

Fresh musts usually contain low levels of BA, almost entirely represented by spermidine and putrescine. This diamine that in plants is implicated in many physiological processes (Halasz et al. 1994), is reported to be synthesized by the vine in response to stress conditions, such as a potassium deficiency in the vineyard soil and then accumulated in the grapes.

Wines are usually characterized by a significantly higher content of BA than their respective fresh musts, red wines being generally characterized by BA content significantly higher than white wines (Table 9.1). The phenomenon was unambiguously ascribed to the fact that the winemaking process for red wine production usually includes a secondary transformation, widely known as malolactic fermentation (MLF) that does not occur or is not essential in white wine production. Consequently, BA presence in wine has been roughly considered as a consequence of MLF, but the matter also includes contradictory data.

In any case, it is without any doubt that BA formation in wine requires the presence of both precursor amino acids and microorganisms with amino acid decarboxylase activity, besides environmental conditions allowing microbial growth and enzyme activity.

Among the factors that have been suggested as favouring the abundance of amines in wine, some winemaking practices, such as nutrient addition and duration of wine contact with both grape skins and yeast lees, seem to play a major role because they can directly affect the content of the precursor amino acids of BA. However, it is worth mentioning that the amount of total amino acids in fresh grape musts generally is several dozen-fold higher than the total amount of amines in wines, so that it is difficult to imagine winemaking conditions where precursor amino acids are quite absent. Therefore, if the presence of available precursors is practically unavoidable, BA accumulation in wine is strongly related to the microbial ecology of wine fermentations, characterized by a complex mixture of different species and strains of yeasts and lactic acid bacteria (LAB).

Table 9.1 Concentration of the major biogenic amines found in wine, according to recent publications (values reported as range, minimum–maximum, or as mean ± SD)

Wine (origin)	n^a	Histamine (mgL^{-1})	Tyramine (mgL^{-1})	Putrescine (mgL^{-1})	Reference
White (Greece)	47	nd – 5.95	nd – 2.54	nd – 3.22	Soufleros et al. (2007)
Red (Greece)	45	nd – 2.11	nd – 3.65	nd – 5.23	Soufleros et al. (2007)
Rosé (Greece)	8	nd – 4.43	nd – 1.64	nd – 1.85	Soufleros et al. (2007)
Red (Italy)	33	6.1 ± 5.3	3.5 ± 2.8	15.5 ± 17.5	Mangani et al. (2006)
Red (Spain)	224	4.46 ± 5.7	3.13 ± 4.42	6.05 ± 10.6	Marcobal et al. (2006)
Red (Turkey)	30	nd – 1.97	nd – 0.29	nd	Anli et al. (2004)
Red (Spain)	6	2.75 ± 1.54	2.91 ± 1.92	9.59 ± 5.61	Romero et al. (2002)
Rosè (Spain)	7	1.81 ± 1.31	1.31 ± 0.71	6.04 ± 2.98	Romero et al. (2002)
White (Spain)	6	1.17 ± 0.99	0.48 ± 0.67	4.31 ± 4.21	Romero et al. (2002)
Red (USA)	59	5.4 ± 5.6	1.3 ± 2.3	20.9 ± 32.3	Gloria et al. (1998)
Red (France)	54	6.7 ± 13.5	3.7 ± 2.3	10.8 ± 6.7	Bauza et al. (1995)
Rosè (France)	15	22.0 ± 7.3	2.3 ± 1.7	2.5 ± 0.9	Bauza et al. (1995)
White (France)	15	3.7 ± 8.9	2.2 ± 1.4	1.9 ± 0.7	Bauza et al. (1995)

nd Not detectable

[a]Number of samples

This chapter will focus on the current knowledge on yeast and LAB capabilities to decarboxylate amino acids into BA and will give up to date information on accumulation of these molecules during winemaking, with an additional note on ethyl carbamate, a carcinogen compound originating in an alcoholic environment from precursors produced by microbial catabolism of amino acids.

9.2 Biogenic Amine Production by Wine Yeasts

Although yeasts have been long considered among the possible biological agents of BA formation in wine, their capability to decarboxylate precursor amino acids has been poorly investigated. If volatile amines, mainly synthesized from non-nitrogenous compounds, are excluded, the few available papers agree in indicating that BA production by wine yeasts is a practically negligible phenomenon, the concentration of most amines being at non-detectable or very low levels (Torrea and Ancin 2001; Caruso et al. 2002; Landete et al. 2007a). Nevertheless, some published results are, in some cases, contradictory.

According to the recent findings of Landete et al. (2007a), none of the studied yeast strains was able to produce at least one of the assayed amines (histamine, tyramine, 2-phenethylamine, putrescine, cadaverine and tryptamine), in both synthetic medium and grape must. On the contrary, according to a previous paper (Caruso et al. 2002), several strains of wine yeasts, assayed in sterilized grape must under laboratory conditions, showed a diffuse capability to produce 2-phenethylamine and agmatine, at concentrations variable within each species from non-detectable to more than 10 mg L^{-1}. In particular, both these amines were pro-

duced by strains of *Kloeckera apiculata*, *Metschnikowia pulcherrima*, *Brettanomyces bruxellensis* and *Saccharomyces cerevisiae*, whereas the strains of *Candida stellata* proved to be able to produce only agmatine. In spite of the contradictory data on 2-phenethylamine producing capability of wine yeasts, possible consequence of a high strain dependent variability of this metabolic feature, agmatine formation deserves some comments. This polyamine should be produced by decarboxylation of arginine, one of the most abundant amino acids of grape musts and an important nitrogen source for yeast growth. However, in all known yeasts, arginine breakdown usually proceeds through the arginase pathway. According to this metabolic route, arginine, transported into the cell through specific and/or general amino acid permeases, is cleaved by arginase into ornithine and urea, which are further metabolised or, at least in the case of urea, excreted (Fig. 9.1). Hence, agmatine production from arginine should represent an alternative route for arginine catabolism in yeast cells, but no information is available on the regulatory mechanism of this metabolic pathway as well as on its physiological significance.

9.3 Biogenic Amine Production by Lactic Acid Bacteria

BA accumulation in wine was long considered as index of poor hygiene in the winery and/or the result of wine spoilage from strains of lactic acid bacteria belonging to the genera *Pediococcus, Lactobacillus* and *Leuconostoc*. Indeed, several strains of different species of these three genera, isolated from BA containing wines, proved to be able to produce BA in the presence of their precursor amino acids (Landete et al. 2007a, Moreno-Arribas et al. 2003), *Pediococcus* strains being long considered among the major responsible for histamine accumulation in wine. However, it was soon demonstrated that also *Oenococcus oeni*, the bacterial species most commonly found in wines and most frequently associated with malolactic fermentation (MLF), was capable to decarboxylate histidine to histamine (Lonvaud-Funel and Joyeux 1994). More recently, several *O. oeni* strains, isolated from different Italian wines and assayed in synthetic media under laboratory conditions, demonstrated to be qualitatively and quantitatively variable in their capability to produce BA: more than 60% of the 44 assayed strains was able to produce histamine, at concentrations ranging from 1.0 to 33 mgL^{-1} and about 16% showed the additional capability to form both putrescine and cadaverine, in variable relative proportions but with the constant prevalence of the former diamine (Guerrini et al. 2002). In the same study, no production of tyramine, spermine, spermidine or 2-phenethylamine was observed.

However, it is to underline that published results on the capability of wine lactic acid bacteria to produce individual amines continue to be contradictory and make the origin of BA complex. Most likely, the controversial results reflect a situation where the BA producing capability is strain rather than species dependent and the

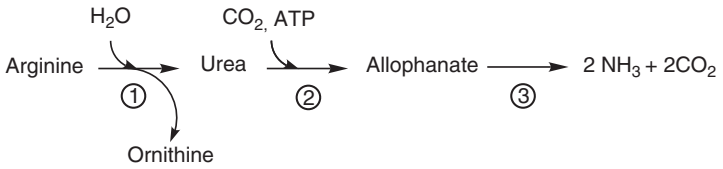

Fig. 9.1 Pathway of arginine breakdown by wine yeasts. (1) arginase; (2) urea carboxylase; (3) allophanate hydrolase

extent of BA accumulation is quite variable owing to the incidence of several factors that affect the concentration of precursor amino acids.

In any case, since histamine, tyramine and putrescine are the most abundant amines in wine, their formation by wine LAB will be examined in more details, with the preliminary remarks that a 100% correlation has been demonstrated between BA producing capability of LAB strains in synthetic medium and in wine as well as between the strain ability to produce individual amines and the presence of the genes encoding the corresponding amino acid decarboxylases (Landete et al. 2007a).

9.3.1 Histamine Production

According to recent studies, *O. oeni* is the species showing the highest percentage of histidine decarboxylase positive strains, but the levels of histamine production are generally low, so that strains belonging to other genera should be considered as mainly responsible for high histamine concentrations in wine. Indeed, in spite of the low frequency of the histidine decarboxylating activity among the strains of both lactobacilli and pediococci, some strains showed the capability of producing high levels of histamine. In particular, some strains of *Pediococcus parvulus* and *Lactobacillus hilgardii*, among the numerous species of wine origin tested produced the highest histamine concentration (Landete et al. 2007a).

In histamine producing strains, histamine production is regulated by both histidine and histamine, since the expression of the *hdc* gene, encoding the histidine decarboxylase enzyme, is induced by the presence of the amino acid (the substrate of the enzyme) and repressed by the presence of the final product from the enzyme activity, the amine (Landete et al. 2006). Other low molecular weight compounds usually present in wine, such as malic and citric acids, seems to affect *hdc* expression, demonstrating that the gene is highly regulated.

Based on the nucleotide sequence of *hdc*A gene from *Lactobacillus* sp. 30a, oligonucleotide primers able to reveal the presence of this gene in LAB by PCR amplification were initially developed (Le Jeune et al. 1995) and now both PCR primers and DNA probes are available and currently used for rapid detection of bacteria harbouring *hdc*A gene and thus the potential producers of histamine in wine (Landete et al. 2007c).

More recently, a histamine producing strain (*Lactobacillus hilgardii* IOEB 0006) proved to retain or to lose the ability to produce histamine, depending on the culture conditions (Lucas et al. 2005). Indeed, it was demonstrated that the *hdc*A gene in this strain was located on an unstable 80-kb plasmid, suggesting an acceptable cause for the great variability of histamine producing character among lactic acid bacteria.

The histidine decarboxylating enzyme (HDC) from LAB strains, differently from other HDC enzymes from Gram-negative bacteria that require pyridoxal-phosphate as cofactor, uses a covalently-bound pyruvoyl moiety as a prosthetic group in the reaction (Coton et al. 1998).

9.3.2 Tyramine Production

The ability to form tyramine is not a common feature of wine LAB. The character is highly variable among strains belonging to the genus *Lactobacillus* and very likely absent or quite rare within the species *O. oeni* and among strains of pediococci (Moreno-Arribas et al. 2000; Landete et al. 2007b). Among the lactobacilli of wine origin so far tested, this metabolic trait is held by most strains of the species *Lactobacillus brevis* and by some strains of *L. hilgardii*, the former species showing the highest tyramine producing activity. Consequently, lactobacilli might be primarily responsible for high concentration of tyramine in wine. However, in a recent paper, a strain of *O. oeni*, isolated from a spontaneous MLF, proved to be able to convert decarboxylate tyrosine into tyramine in a model system (Gardini et al. 2005). In the same paper, emphasis was given to the fact that tyramine production by resting cells of the studied *O. oeni* strain is markedly affected by several variables (pH, arabinose and pyridoxal-5-phosphate concentrations, ethanol percentage and SO_2 level), so that it is possible that the actual capability of *O. oeni* to decarboxylate tyrosine is highly underestimated.

The tyrosine decarboxylating enzyme (TDC) from *Lactobacillus brevis* IOEB 9809 of wine origin proved to be a protein with two subunits of 70 kDa each, requiring pyridoxal-phosphate as cofactor and showing maximum activity at pH 5.0 (Moreno-Arribas and Lonvaud-Funel 2001). In some cases, tyramine producing capability has been found to be associated with a low but significant 2-phenethylamine production, as a confirmation that TDC is able to use also phenylalanine as a substrate for its activity. However, it has been also reported that only the best tyramine producers are able to generate both amines simultaneously (Moreno-Arribas et al. 2000).

9.3.3 Putrescine Production

In general, putrescine is the biogenic amine most abundantly found in wines and putrescine producing capability may be considered widespread among LAB strains

of oenological interest (Moreno-Arribas et al. 2003). The ornithine decarboxylase gene *odc*, firstly sequenced from *Lactobacillus* sp. 30a, (Hackert et al. 1994) has been recently sequenced also from *O. oeni* (Marcobal et al. 2004). Many bacteria contain two forms of the decarboxylating enzyme (ODC): a constitutive enzyme, expressed when bacteria are grown at neutral pH in minimal culture media, and a form induced mainly under low pH conditions in rich media and suggested to play a role in maintaining pH homeostasis. Among LAB, an inducible ODC, structured as a dodecamer of about 1,000 kDa and requiring pyridoxal-phosphate as cofactor, has been described in *Lactobacillus* sp. 30a (Momany et al. 1995; Vitali et al. 1999).

The origin of putrescine deserves a more detailed description because high concentrations of this diamine cannot depend only on the amount of free ornithine, since the concentration of this amino acid in both musts and wines is usually very low. Indeed, ornithine may also be produced from the catabolism of the amino acid arginine, one of the major amino acids found in grape juices and mostly metabolised by yeasts during alcoholic fermentation but generally still present in wines at significant amounts before the onset of MLF. This amino acid is catabolized by several strains of lactic acid bacteria, both lactobacilli and oenococci, via the arginine-deiminase pathway (Liu and Pilone 1998). This metabolic route consists of three enzymes acting in series: arginine deiminase (ADI), ornithine transcarbamylase (OTC) and carbamate kinase (CK), as drawn in Fig. 9.2. As a result, bacterial cells catabolizing arginine excrete ornithine, ammonia and CO_2, besides small amounts of citrulline, in a molar ratio among the main products close to 1:2:1.

In a recent paper, some *O. oeni* strains demonstrated capability to produce putrescine only from ornithine, but other strains were able to produce putrescine also from arginine, proving to possess the necessary enzyme system to degrade arginine to ornithine and then to decarboxylate this amino acid to putrescine, with the additional formation of ammonia and useful ATP (Mangani et al. 2005). In the same paper, it was demonstrated that putrescine can be produced from arginine also by an association of strains possessing a complementary enzyme system: a co-culture of one *O. oeni* strain, capable to metabolize arginine to ornithine but unable to decarboxylate ornithine to putrescine and another strain capable to produce putrescine from ornithine but unable to degrade arginine giving rise to putrescine in the presence of the sole arginine, as a practical consequence of an exchange of ornithine between the two strains. Consequently, the occurrence of a metabiotic association of this type might really contribute to the increase in concentration of putrescine in wines.

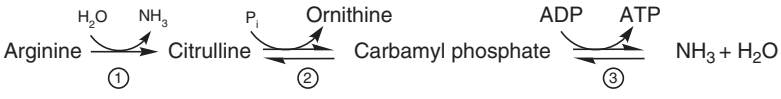

Fig. 9.2 Pathway of arginine breakdown by malolactic bacteria. (1) arginine deiminase; (2) ornithine transcarbamylase; (3) carbamate kinase

9.3.4 Production of Other Amines

Even if histamine, tyramine and putrescine have received major attention owing to their toxicity and abundance in wine, other amines can be produced by bacterial amino acid decarboxylases. However, information on the actual capability of wine LAB to produce agmatine, cadaverine and tryptamine is far too exhaustive. Most studies deal with chemical data on the presence of these amines during winemaking or in wines rather than with microbial populations responsible for amine formation.

Some *O. oeni* strains proved to be able to produce significant amounts of cadaverine, always coupled with a putrescine producing activity (Guerrini et al. 2002). In the same study, it was observed that almost all *O. oeni* strains that produced cadaverine and putrescine were low producers of histamine and some low producers of histamine were unable to produce cadaverine or putrescine.

With regard to agmatine production by wine LAB, it can be stated that this polyamine could originate from decarboxylation of arginine, but the reaction has been ascertained only in *L. hilgardii* strain X_1B (Arena and Manca de Nadra 2001). This unique finding suggests that the strain might possess an anomalous pathway for catabolism of arginine, the amino acid being usually metabolised by wine LAB via the ADI pathway. Finally, no information is available on the LAB capability to produce tryptamine.

9.3.5 Physiological Role of Amine Production

If BA production is of concern to winemakers and to wine-consumers, amino acid decarboxylation may play an important physiological role in microbial cells that might take advantage of this catabolic feature. Indeed, detailed studies have demonstrated that BA production is enhanced when growth conditions become less favourable owing to the absence of fermentable substrates and the presence of ethanol at low pH values. These findings have suggested that amino acid decarboxylases might function as an additional mechanism for energy generation, as it happens with the decarboxylation of malic acid by the action of malolactic enzyme.

In the case of histidine decarboxylation, the energetic advantage for the strain might be due to an electrogenic exchange between histidine and histamine, as it was found in *Lactobacillus buchneri*: the amino acid enters the cell in the neutral form while histamine is excreted with one positive charge, generating a proton motive force of sufficient value to drive ATP synthesis (Moolenar et al. 1993). This proton consuming decarboxylation also generates a trans-membrane pH gradient that enables the cells to protect themselves against the adverse effects of the acid environment (Fig. 9.3).

In the case of tyrosine decarboxylation as well as other amino acid decarboxylations, it has been envisaged an energetic advantage through a mechanism similar to that described above, but studies on this matter are still incomplete and more detailed

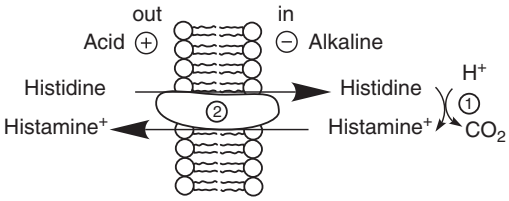

Fig. 9.3 Electrogenic exchange of histidine for charged histamine, produced by proton consuming decarboxylation of histidine. (1) histidine decarboxylase; (2) permease

investigations are required to establish the physiological importance of these reactions. Nevertheless, the possible advantages of bacterial cells able to produce putrescine deserve some comments because of a possible involvement of arginine catabolism in putrescine production, with arginine acting as a remote precursor of the polyamine.

In LAB strains able to catabolise arginine via the ADI pathway to ornithine, ammonia and CO_2 (Fig. 9.2), physiological advantages are due to a carbamate kinase dependent phosphorylation at the substrate level, since the uptake of arginine is coupled with the excretion of ornithine through an electroneutral antiport system that is sustained by concentration gradients and does not require energy (Driessen et al. 1987; Poolman et al. 1987). On the other hand, in LAB strains able to decarboxylate ornithine to form putrescine, physiological advantages, as above reported, are due to an electrogenic substrate/product exchange. In addition, both metabolic routes cause an increase in cytoplasmic pH, protecting cells against acid damages. In LAB strains able to catabolise arginine via the ADI pathway and to decarboxylate ornithine to putrescine, as demonstrated for some *O. oeni* strains (Mangani et al. 2005), energetic advantages could take place only if ornithine produced by the ADI pathway is at first excreted, in order to satisfy the electroneutral exchange with arginine, and then reutilized, in order to sustain the electrogenic exchange with putrescine. However, experimental evidence of such mechanism is lacking as well as it is to be proven that strains capable to catabolise arginine to putrescine take advantages over strains possessing either metabolic route.

9.4 Biogenic Amine Accumulation During Wine Fermentations

After some years of controversy about the origin of BA in wines, a wide experimental evidence indicates MLF as the winemaking phase responsible for most BA production and accumulation in wines, at least for the most frequently found amines, namely histamine, tyramine and putrescine (Soufleros et al. 1998; Marcobal et al. 2006).

However, in all these studies, microbiological data have been only rarely included, so that it is difficult to ascertain possible relationships between the changes that occur in individual amine concentrations and those occurring in microbial populations during the winemaking process. Moreover, it has been suggested that amino acid decarboxylation may take place for a long time period

independently of cell viability (Moreno-Arribas and Lonvaud-Funel 1999). Consequently, as stated by some researchers, no general rule for the time course of BA accumulation during winemaking is available yet. Nevertheless, by taking into consideration all the papers dealing with this matter as well as personal observations, some aspects appear elucidated.

During alcoholic fermentation, carried out by either indigenous or selected yeast strains, no remarkable increase in the BA concentration usually occurs, the levels of individual amines remaining at the values occurring in fresh grape must. With the onset of malolactic fermentation (MLF), after the development of the bacterial population to more than 10^5 CFU mL^{-1}, BA concentration begins to increase. The extent to which individual amines increase during MLF is unpredictable, each amount depending on both availability of precursor amino acid and catabolic properties of the participating bacterial strains. However, the BA producing rate usually shows a remarkable increase toward the final phases of MLF, as a confirmation that BA production is enhanced as the growth conditions become less favourable owing to the progressive consumption of an important energy source such as malic acid. As soon as malic acid is exhausted, BA production process enters a delicate phase, determined by two main factors: (1) malolactic population might be still highly active and at high cell densities and (2) catabolic activities of LAB population on some precursor amino acids might be delayed in comparison to MLF, as it has been demonstrated in the case of arginine breakdown by oenococci (Mangani et al. 2005). After completion of MLF, wines are generally sulfited in order to avoid growth or survival of undesirable bacteria, but it is known that, at usual concentrations, SO_2 does not immediately stop all the biochemical activities of the bacterial population that, otherwise, could survive for a long time after sulfitation, even if at a reduced cell density. The survival of the malolactic population and the availability of precursor amino acids might account for the increase in BA concentration often observed after completion of MLF or during wine ageing.

Other investigations, carried out to correlate the amount of BA in wines with factors suspected to play a significant role in affecting the level of BA accumulation, such as grape variety and degree of grape maturation, kind of soil and N-fertilizer, content of BA precursors, addition of nutrients, pH, ethanol concentration, levels of sulphur dioxide, duration of wine contact with yeast lees and other post-fermentative treatments (Bauza et al. 1995; Soufleros et al. 1998; Leitao et al. 2005; Herbert et al. 2005; Landete et al. 2005; Pramateftaki et al. 2006; Alcaide-Hidalgo et al. 2007), appear still too fragmentary, and sometimes contradictory, to be used to draw a general picture.

9.5 Ethyl Carbamate Formation in Wine

Besides BA, another health concern in wine is the presence of ethyl carbamate (EC), also referred to as urethane. This compound has attracted the attention of many researchers because of its animal carcinogen potential and its possible origin

from precursors produced by microbial catabolism of amino acids. Indeed, EC could originate in wine as a consequence of a spontaneous, non-enzymatic reaction between ethanol and a compound containing a carbamyl group, such as urea (produced from arginine breakdown by yeasts), citrulline and carbamylphosphate (produced from arginine breakdown by LAB). This acid catalysed alcoholysis of carbamyl compounds has been shown to be directly dependent on both concentration of reactants and temperature (Ough et al. 1988), the reaction occurring readily in the case of carbamyl phosphate and slowly in the cases of urea and citrulline at the normal wine storage temperatures. Consequently, EC levels are usually low or non-detectable in young wines and variable to different extent in aged or stored wines, depending on cellar or storage temperature.

With reference to the reactants, urea is considered the major precursor for EC in wine (Ough et al. 1988) and it is well known that it can be released by wine yeasts as metabolic intermediate from arginine breakdown. However, urea excretion by yeast cells is affected by a variety of environmental factors, including nitrogen availability, and is variable from strain to strain (Ough et al. 1991). In *S. cerevisiae*, the enzyme arginase, encoded by the gene CAR1, degrades arginine into ornithine and urea, that is further degraded into CO_2 and NH_4^+ by the bi-functional enzyme urea amidolyase, encoded by the DUR1,2 gene (Genbauffe and Cooper 1986). The expression of these two genes follows distinctive regulatory mechanisms, even if both genes are subject to nitrogen catabolite repression, a regulatory mechanism that causes the repression of genes encoding enzymes which degrade poor N-sources in the presence of good N-source. Since both arginine and urea are considered as secondary nitrogen sources, urea excretion should be the consequence of a lack or lower level of DUR1,2 expression in comparison to the expression of the CAR1 gene (Coulon et al. 2006). In any case, urea excretion is favoured by conditions of high availability of promptly assimilable nitrogen sources and mainly occurs during the first stages of wine fermentation.

As for the other reactants of microbial origin, citrulline has been suggested as the main EC precursor produced by LAB in wine (Azevedo et al. 2002). Citrulline is an intermediate product of arginine breakdown by LAB through the ADI pathway and can be excreted by the bacterial cells to variable extent, depending on the strain (Granchi et al. 1998; Mira de Ordu a et al. 2000). Carbamyl phosphate, the other intermediate of arginine catabolism, is usually excreted to a much lower extent than citrulline and, although it is highly reactive to ethanolysis, does not seem to play a significant role in EC formation in wine.

9.6 Conclusions

The increasing attention given in recent years to BA and EC in wines seems not only due to a general demand for healthier foods and beverages, but also dependent on the alcoholic nature of wine. Indeed, ethanol, besides being a reactant in EC formation, is known to be among the most active inhibitors of amine oxidases, so that wine consumers might really suffer the toxic effects of BA presence, even at low concentration.

The risks associated with the ingestion of BA containing wines led some European Countries to recommend maximum tolerable limits for histamine in wine: Germany, 2 mg L^{-1}; Holland, 3 mg L^{-1}; Finland and Belgium, 5 mg L^{-1}; France, 8 mg L^{-1}; Switzerland and Austria, 10 mg L^{-1} (Busto et al. 1996; Lehtonen 1996). On the other hand, the perceived risk for EC led Canadian Government to impose legal limit for this compound in imported wines (30 and 100 μ g L^{-1} in table and fortified wines, respectively) and later, in 1988, the Food and Drug Administration in the US brought to rule more stringent limits (15 and 60 μg L^{-1}, respectively, starting from the 1989 harvest).

In order to overcome any potential risk, different strategies have been tested or envisaged in the last 10 years, such as (1) to discourage spontaneous processes in favour of fermentations induced by selected yeast or malolactic strains with known biochemical properties; (2) to manage grape must pH, so that growth of BA producing lactobacilli and pediococci is hampered; (3) to eliminate bacterial populations by means of lysozyme treatment suddenly after MLF completion, so that BA production is broken down; and (4) to add preparations of acidic urease of bacterial origin as well as to make use of metabolically engineered urea-degrading yeast strains in alcoholic fermentation of grape musts so that the presence of a major reactant in EC formation is eliminated.

However, in spite of these efforts, the challenge of BA and EC exclusions from wines is still to be won.

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Chapter 10
Usage and Formation of Sulphur Compounds

Doris Rauhut

10.1 Introduction

Sulphur is important for the growth of all microorganisms due to the formation of sulphur containing amino acids. Among the wine-related microorganisms, extensive data has been accumulated for yeast of the species *Saccharomyces cerevisiae*. In recent years also more and more research work is focused on the sulphur metabolism of Non-*Saccharomyces* yeasts and lactic acid bacteria.

The goal of this review has been to assemble the literature concerning the usage and biosynthesis of sulphur amino acids and glutathione as well as on the formation of the high flavour-active volatile sulphur compounds during alcoholic and malolactic fermentation.

10.2 Sulphur Metabolism of Yeast

10.2.1 Sulphur Amino Acid Biosynthesis

Yeast has the ability to use various sulphur compounds in contrast to many other microorganisms due to the sulphur pathway in yeast, which allows it to use various organic and inorganic sulphur compounds as sole sulphur source.

The element sulphur can occur in a variety of stable compounds in which it can range from −2 in its most reduced form (sulphide) to +6 in its most oxidised form (sulphate). For all microorganisms, the biosynthesis of sulphur amino acids requires the ability to accumulate sulphur atoms from the growth medium and then the transformation of the transported intermediate compounds into the

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reduced form of the sulphur atom (S^{2-}). Basic research work to investigate sulphur metabolism in *Saccharomyces cerevisiae* was done by the research group of Surdin-Kerjan, which characterised more than 15 genes encoding enzymes of the sulphur amino acid pathway (Surdin-Kerjan 2003). Figure 10.1 gives a simplified overview on the metabolism of sulphur amino acids and glutathione in *Saccharomyces cerevisiae*.

The main sulphur source for yeasts during wine-making is sulphate, which occurs in grape must in a range of 160–400 mg L^{-1} or even more (Lemperle and Lay 1989), because the levels of methionine and cysteine are normally very low in grape

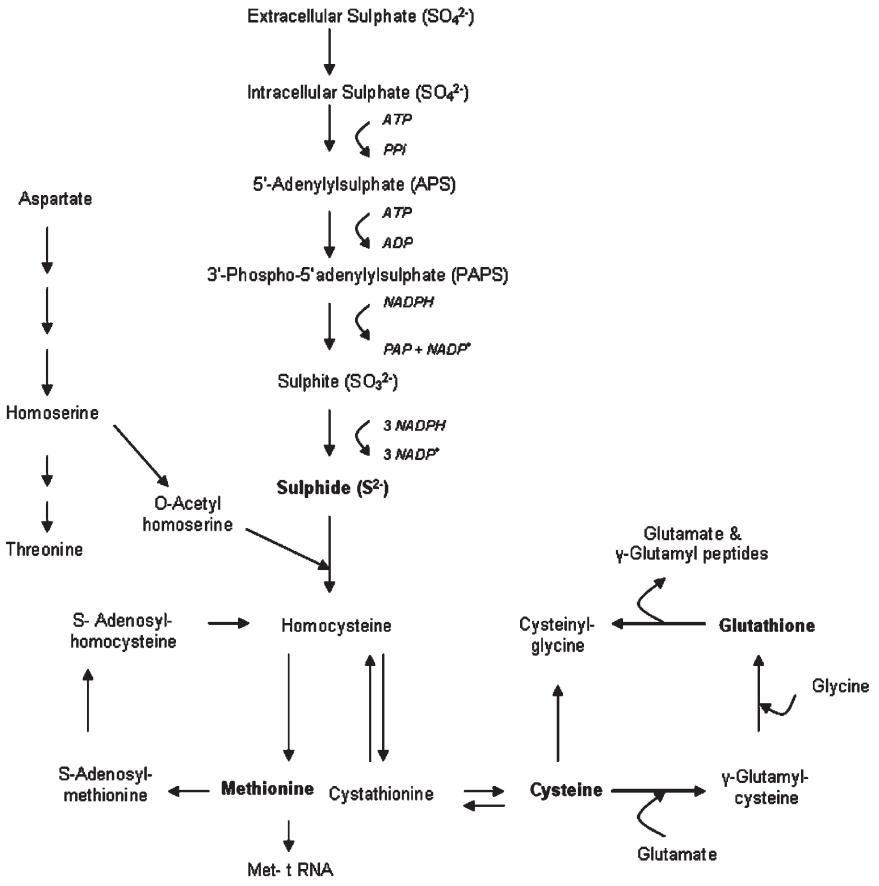

Fig. 10.1 Metabolism of sulphur amino acids in *Saccharomyces cerevisiae* (according to De Robichon-Szulmajster and Surdin-Kerjan (1971), Jones and Fink (1982), Henschke and Jiranek (1993), Rauhut (1993), Thomas and Surdin-Kerjan (2003), Wang et al. (2003), Linderholm et al. (2008))

musts (in most cases less than 10 mg L^{-1}methionine and cysteine) in comparison to other amino acids (Henschke and Jiranek 1991, 1993).

The first step of the sulphate reduction sequence (SRS) is the transport of extracellular sulphate from the environment into the cell through the enzyme sulphate permease. Cherest et al. (1997) have isolated and characterised two sulphate transporters. After that sulphate is activated by an adenylation and reduced by two successive reactions (requiring four molecules of NADPH + H^+ and two of ATP). The adenylation of sulphate lowers the electro-potential of sulphate that the subsequent reduction into sulphite and sulphide by means of NADPH + H^+ oxidation is possible. Activation of sulphate takes place by the transfer of the adenosyl-phosphoryl-moiety of ATP to sulphate and the formation of 5′-adenylylsulphate (APS). These reactions are catalysed through ATP sulphurylase. In a further step, APS is phosphorylated and 3′-phospho-5′-adenylylsulphate (PAPS) is formed through APS kinase. PAPS is then reduced through PAPS reductase to sulphite, which is reduced from sulphite reductase to form sulphide.

Sulphide is incorporated into amino acids through several more enzymatic steps.

The enzyme *O*-acetyl homoserine-*O*-acetyl serine sulphhydrylase is incorporating the sulphide, along with *O*-acetylhomoserine, into homocysteine. Cystathionine β-synthase converts homocysteine into cystathionine. γ-Cystathionase is then conducting the reaction to cysteine. Cysteine is only formed through the transsulphuration pathway. No direct synthesis of cysteine from sulphide occurs in yeast (Thomas and Surdin-Kerjan 1997). *O*-Acetylhomoserine is the amino acid precursor to form methionine with the reduced sulphur atom over homocysteine through the activity of *O*-acetylserine sulphhydrylase and homocysteinemethyltransferase. Methionine is not only involved in protein synthesis. It is also an indispensable intermediate of the one carbon metabolism. *S*-Adenosylmethionine is the methyl donor in various transmethylation reactions of nucleic acids, proteins and lipids. It is involved in the biosynthesis of polyamines and is one of the substrates used in a number of reactions, including vitamin biosynthesis and nucleotide modifications (Surdin-Kerjan 2003).

It is expected that the equilibrium between methionine and *S*-adenosylmethionine plays a central role in the overall cellular homeostasis. The ratio of methionine and *S*-adenosylmethionine seems to be controlled through two recycling pathways that operate on the products of *S*-adenosylmethionine catabolism (Surdin-Kerjan 2003).

Excellent reviews on metabolism of sulphur amino acids in *Saccharomyces cerevisiae* from Jones and Fink (1982), Hinnebush (1992) and Thomas and Surdin-Kerjan (1997) will offer more details also on the regulation of sulphur amino acids biosynthesis.

Further sulphur sources for yeasts during the wine-making process are sulphur dioxide, which is used due to its antimicrobial and antioxidative effect as well as its reaction with ethanal (acetaldehyde) to avoid an oxidative character and its inactivation of enzymes, and elemental sulphur from residues of the application of wettable sulphur (fungicide) on grapes (Ribéreau-Gayon et al. 2000a, b; Romano and Suzzi 1993; Dittrich 1987; Wucherpfennig 1984; Wenzel and Dittrich 1978; Schütz and Kunkee 1977).

10.2.2 Sulphite Production

The production of sulphite by *Saccharomyces cerevisiae* is strain characteristic (Fig. 10.2) and is also affected by the grape must composition. Strains of *Saccharomyces cerevisiae* normally produce sulphite in the range of 10–30 mg L^{-1} (Eschenbruch 1974; Dott et al. 1976; Dittrich 1987). 'SO_2-forming yeasts' or 'yeasts with high sulphite formation' can form sulphite in amounts exceeding 100 mg L^{-1} (Eschenbruch 1974). Suzzi et al. (1985) could demonstrate under comparable conditions that among 1,700 strains of *Saccharomyces cerevisiae*, the majority (80%) produced less than 10 mg L^{-1} SO_2 and only four strains synthesised more than 30 mg L^{-1}. Dott and Trüper (1976, 1978), Dott et al. (1976, 1977), Eschenbruch (1972, 1974) and Würdig (1985) reported substantial variation in the activity of sulphate permease, ATP-sulphurylase and sulphite reductase in the regulation of sulphur metabolism in high and low sulphite-producing yeast strains (reviewed by Rauhut 1993; Pretorius 2000).

An extreme formation of sulphite can be caused by defects in sulphate uptake and reduction, which is normally regulated by methionyl-t-RNA and *S*-adenosyl-methionine (Henschke 1997). It could be demonstrated that in high-sulphite-producing strains sulphate-permease is not repressed by methionine. In addition, ATP-sulphurylase is not regulated by sulphur-containing intermediates in high and low-sulphite-producing strains. Yeasts with low-sulphite production showed an increased biosynthesis of NADPH-dependent sulphite reductase during the exponential

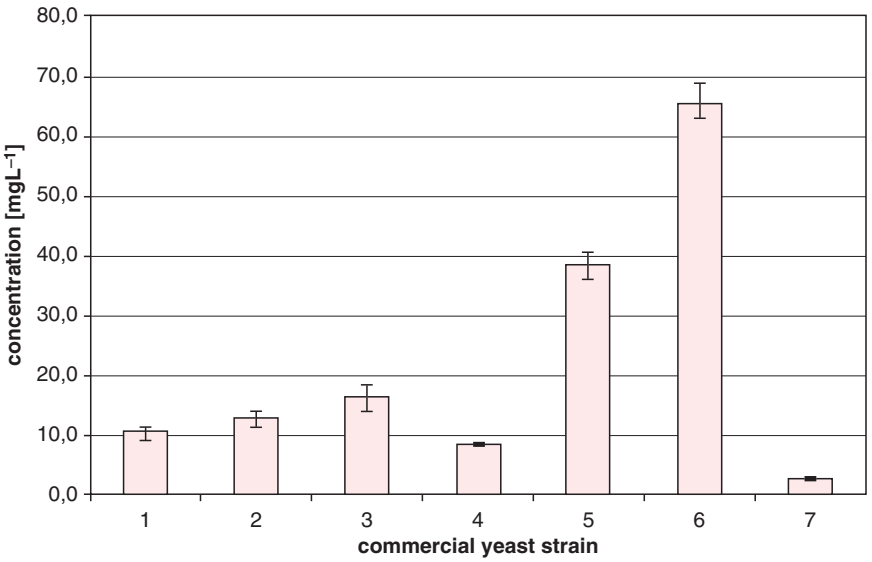

Fig. 10.2 Production of SO_2 by commercial yeast strains during alcoholic fermentation (Werner and Rauhut 2007, unpublished)

growth phase in comparison to yeasts with high-sulphite production. Sulphite production is very energy-dependent and the cellular metabolism of high SO_2-forming yeast strains is reduced, which explains a decreased production of biomass and a slow fermentation rate (Rauhut 1993; Pretorius 2000).

Higher levels of methionine and cysteine can diminish the levels of sulphite reductase (Rauhut 1993; Pretorius 2000). Sulphite production by wine yeasts is also influenced by nutrient composition of grape musts and in addition by the concentration of sulphate, must clarification, initial pH-value, temperature and other environmental conditions (Minarik 1977; Larue et al. 1985; Bakalinsky 1996; Larsen et al. 2003; Fleet 2007).

10.2.3 Importance of Glutathione

One of the major antioxidants in living cells is the tripeptide, glutathione (L-γ-glutamyl-L-cystinyl-glycine). It is formed through the reaction of cysteine with glutamate and glycine (Fig. 10.1). Glutathione prevents cellular destruction by maintaining certain thiols in their reduced stage due to its SH-group of the cysteine molecule. Furthermore, it can react with heavy metals and other toxic compounds (Duncan and Derek 1996; Field and Thurman 1996; Penninckx 2002; Du Toit et al. 2007). Glutathione is involved in the oxidative stress response through glutathione peroxidise and in detoxification processes. Vacuolar transport of metal derivatives of the tripeptide ensures resistance to metal stress (Penninckx 2000).

Glutathione was discovered in yeasts by Hopkins and Kendall in 1921 (Kocková-Kratochvílová 1990). It may account for 0.5–1% of the dry weight in the yeast *Saccharomyces cerevisiae*, represents more than 95% of the low-molecular-mass thiol pool and occurs in high concentrations up to 10 mM in yeast cells (Elskens et al. 1991; Mehdi and Penninckx 1997; Penninckx 2002).

Cheynier et al. (1989) showed that glutathione ranged from 14–102 mg L^{-1} in grape musts from different varieties. Park et al. (2000a, b) found glutathione up to 1.3 mg L^{-1} in grape musts and up to 5.1 mg L^{-1} in wines. Du Toit et al. (2007) detected up to about 35 mg L^{-1} glutathione in wines. Final wine concentration of glutathione was correlated with both total nitrogen and assimilable amino acid concentration and an increase of glutathione towards the end of fermentation was observed.

Lavigne et al. (2007) investigated that the amount of glutathione after fermentation depends on the yeast strain. Glutathione levels in the wine can be increased through the choice of an adequate yeast strain and to store the wine on lees.

Glutathione may be involved to maintain mitochondrial and membrane integrity in *Saccharomyces* and non-*Saccharomyces* yeasts and can be mobilised during nitrogen and sulphur starvation and/or reproduction. About 50% of the glutathione was in the yeast cytoplasm and the remaining in the central vacuole during growth on nitrogen-sufficient medium. Glutathione stored in the yeast cell is used as an endogenous sulphur source in case of total sulphur deficiency. More than 90% of

the cellular glutathione was transported to the central vacuole of the yeast, when *Saccharomyces cerevisiae* was subjected to nitrogen starvation (Mehdi and Penninckx 1997).

Glutathione in the must can be taken up by the yeast cell through two transport systems. Sulphur from glutathione is transferred to other metabolites along the sulphur metabolic pathway (Penninckx 2002). This explains that the supplementation of must with glutathione in a concentration of more than 50 mg L^{-1} can lead to unpleasant volatile sulphur compounds under certain conditions (Rauhut et al. 2001; Rauhut 2003).

Glutathione can prevent white musts against oxidation (Singleton 1987; Cheynier et al. 1986, 1989). Dubourdieu and Lavigne-Cruège (2002) proposed that glutathione seems to play an important role in protecting volatile thiols that are responsible for the varietal flavour of wines during the ageing of bottled white wines. Yeast strains with a higher formation and release of glutathione during fermentation and also during storage of wines on lees can contribute to the stabilization of volatile thiols and can prevent against browning to a certain extent.

Further research in glutathione formation and metabolism might be profitable for enology and biotechnology to take advantage of the differences among strains during the winemaking process and storage, in order to obtain strains better adapted to the frequent environmental stresses occurring before, during and after alcoholic fermentation (Penninckx 2002).

10.2.4 Volatile Sulphur Compounds

10.2.4.1 Hydrogen Sulphide and Related Volatile Sulphur Compounds

Volatile sulphur compounds are essential aroma compounds for a huge amount of different foods and play a considerable role in the sensory characteristics of wine. This is related to their high volatility, reactivity and impact at very low concentrations. Sulphur compounds in wine can be classified as thiols (mercaptanes), sulphides, thioesters and heterocyclic compounds. Some of the sulphur substances supply to the overall quality of wine, while others are the cause of strong objectionable flavours (rotten eggs, cooked cabbage, cauliflower, burnt rubber, cooked meat etc.), even at extremely low concentrations (e.g. hydrogen sulphide (H_2S), methanethiol (MeSH), ethanethiol (EtSH)). Because of their occurrence in very low concentrations (nanogram or microgram levels) and the high reactivity of thiols with metal residues (e.g. copper and silver) and their fast oxidation with traces of oxygen, it was very difficult to study the occurrence and formation of volatile sulphur compounds. After the development of soft analytical extraction methods and high sophisticated gas chromatographic systems in connection with high sensitive sulphur detectors, it was possible to get more information on their biosynthesis and presence in the last two decades. Sulphur aroma compounds were often separated in low and high volatile sulphur substances due to the broad range of different boiling points and the need of different analytical methods to enrich the sulphur compounds from wines

(Fedrizzi et al. 2007; Ferreira et al. 2007; Lopez et al. 2007; Mateau-Vivaracho et al. 2008; Rauhut et al. 2005, 2007; Schneider et al. 2006; Thibon et al. 2008). In accordance with the new rules for the international nomenclature of chemical compounds (IUPAC), the prefix 'methyl-sulphanyl' should replace the prefix 'methylthio' and 'ethyl-sulphanyl' should replace the prefix 'ethylthio'; furthermore the prefix 'sulphanyl' must replace the prefix 'mercapto'. In the following, the more familiar chemical names of the sulphur compounds are mainly used to avoid confusion.

Volatile sulphur compounds are formed through several pathways involving enzymatic and/or non-enzymatic processes. Yeast fermentation biochemistry with sulphate-, sulphite-, sulphur-containing amino acids (methionine and cysteine) and oligopeptides (e.g. glutathione) plays a crucial role among the enzymatic processes.

Non-enzymatic processes involve chemical, photochemical and thermal reactions during winemaking and storage. Other factors for an increased development of unpleasant sulphur substances are an increase of wettable sulphur (used as fungicide) residues and other sulphur containing pesticides in grape musts. Relevant overviews on the formation and occurrence of sulphur compounds in wine with a different focus are given from Maujean (2001), Rauhut (1993, 1996, 2003), Ribéreau-Gayon et al. (2000a, b), Swiegers et al. (2005) and Vermeulen et al. (2005). Most important sulphur compounds, their aroma impression, concentration in wine and aroma threshold are listed in Table 11.1 (Chap. 11).

In the following, a review is presented on the formation of hydrogen sulphide (H_2S) and related sulphur compounds through yeast sulphur and nitrogen metabolism during the wine-making process.

Sulphide is usually formed through the sulphate reduction sequence as indicated in Fig. 10.1 in response to a metabolic requirement, such as that induced by growth, for organic sulphur compounds like cysteine, methionine, *S*-adenosyl methionine and glutathione (Rankine 1963, 1964; Hallinan et al. 1999; Spiropoulos and Bisson 2000; Spiropoulos et al. 2000; Bell and Henschke 2005). Under certain conditions, mainly if insufficient or unsuitable nitrogen sources are available, a surplus of sulphite is released from the cell. It is converted spontaneously to H_2S as a consequence of the reductive conditions established in the anaerobic fermentation at low pH (Linderholm et al. 2008). The information on the threshold value for H_2S is in a range of 11–80 $\mu g\ L^{-1}$. The difference is depending on the used media for determination of the odour threshold value (Amoore and Hautala 1983; Wenzel et al. 1980). Concentrations exceeding these values cause an undesirable off-flavour that is reminiscent of rotten eggs. Lower levels in young wines contribute to the 'yeast' flavour or fermentation bouquet. Slight increased values take part in the so-called reductive off-odour in wines (Dittrich and Staudenmayer 1968; Monk 1986; Dittrich 1987).

One of the most influencing factors on H_2S production is the strain of *Saccharomyces cerevisiae*. Some strains produced amounts up to 1 mg L^{-1} H_2S (Acree et al. 1972; Eschenbruch 1974, 1978; Eschenbruch et al. 1978; Vos and Gray 1979; Giudici and Kunkee 1994; Jiranek et al. 1995a, b; Mendes-Ferreira et al. 2002). The variation among strains is genetically based, but also influenced by environmental flavours. Some yeast strains are constantly high or constantly low H_2S producers.

Most strains showed a strong influence on growth conditions and medium composition of sulphide formation. Genetic variation seen indicates a complex inheritance, meaning multiple genes are likely involved. Linderholm et al. (2008) identified several genes that have an impact on H_2S formation. Five genes (*MET17, CYS4, HOM2, HOM6 and SER33*) encode proteins directly involved in the biosynthesis of sulphur containing amino acids, whereas other genes or their substrates and products may have key regulatory effects in the reduction of sulphate or play a more indirect role. Accumulation of acetaldehyde and the elongator histone complex are suggested as two cellular activities that have an impact on sulphide production during anaerobic fermentation.

H_2S is produced during the early to middle stages of fermentation and responds to nutrient composition and concentration (Vos and Gray 1979; Vos 1981; Monk 1986; Henschke and Jiranek 1991; Jiranek et al. 1995a, b). Ammonium salts like diammoniumhydrogenphosphate (DAP) are widely used to compensate nitrogen deficiencies in grape musts and to control H_2S formation. Ammonium represses the *Met10* gene, which encodes the alpha-subunit of sulphite reductase (Hansen et al. 1994), but not in all cases its addition is effecting H_2S production due to other factors, e.g. methionine and other nitrogen sources that regulate amino acid transport into the yeast cell and sulphur metabolism (Spiropoulos et al. 2000; Spiropoulos and Bisson 2000).

H_2S formation can also occur in a second phase at the final stage of fermentation. Henschke 1996 observed limited evidence for a response to aeration and vitamin addition. Nutrient levels are very low at the end of fermentation, for that reason it is proposed that a deficiency of vitamins or the degradation of sulphur reserves like glutathione are involved (Eschenbruch et al. 1978; Elskens et al. 1991; Hallinan et al. 1999). Insufficiencies in vitamins and micronutrients (pantothenate) and vitamin B_6 (pyridoxine) essential for the synthesis of sulphur containing amino acids may also contribute to H_2S production (Jiranek et al. 1995a, b; Spiropoulos et al. 2000; Wainright 1970; Wang et al. 2003).

H_2S can be produced by the degradation of glutathione and the release of cysteine, which is then degraded by cysteine desulphhydrase, when nitrogen is limited (Tokuyama et al. 1973).

It is approximated that glutathione, which is accumulated in the yeast cell, can participate in up to 40% of the sulphide in cells with nitrogen starvation (Hallinan et al. 1999).

There are many other causes that increase H_2S production and the formation of other undesirable sulphur compounds, e.g. residues of elemental sulphur from wettable sulphur treatment of the vines and other sulphur containing pesticides and their breakdown products, clarification level, concentration of SO_2, fermentation temperature, residues of copper ions and storage on lees (Bell and Henschke 2005; Henschke and Jiranek 1993; Maujean 2001; Rauhut 1993, 2003; Ribéreau-Gayon et al. 2000a, b).

An accelerated H_2S production leads to a higher formation of volatile sulphur compounds, especially if it is not carried out with the carbon dioxide produced during fermentation and if increased amounts remain in the young wine.

It has been suggested that ethanethiol is formed by the reaction of H_2S and ethanal (acetaldehyde) via a cyclic trithioethanal intermediate (Rankine 1963, 1968; Tanner 1969), but this reaction could not be demonstrated in model solutions and in wine (Bobet 1987; Rauhut and Kürbel 1994). On the contrary, it could be demonstrated that H_2S and ethanal is reacting to 1,1 ethanedithiol, which has a sulphury and rubbery flavour note, at wine-like conditions and in wine (Rauhut 1993, 1996). Further reaction products were identified. The same compounds were measured in yeast extract (Werkhoff et al. 1991). Therefore a contribution of these sulphur compounds to sulphur-related off-flavours can be expected (Rauhut 1993). It has also been suggested that ethanol and H_2S react to ethanethiol, but this could not be confirmed up to now (Rankine 1963, 1968; Rauhut 1993, 2003).

A huge amount of all volatile sulphur substances is produced during the alcoholic fermentation. It could be demonstrated that yeast strains differ in their formation of sulphur compounds (Fig. 10.3; Rauhut and Kürbel 1994; Rauhut et al. 1995, 2000). An accelerated formation of H_2S leads to an increase of thioacetic acid esters. Matsui and Amaha (1981) supposed that high concentration of methanethiol and H_2S can hinder the growth of yeasts. It is therefore assumed that the formation of thioacetic acid esters is a detoxification process to transform sulphur substances with a free SH-group, which can inhibit enzymes, to non-affecting compounds like the thioacetic acid esters. High H_2S formation in the early phase of fermentation leads to a high formation of thioaceticacid-*S*-methyl ester (MeSAc) and high formation of H_2S at the end of fermentation increased the formation of thioacetic acid-S-ethyl ester (EtSAc)

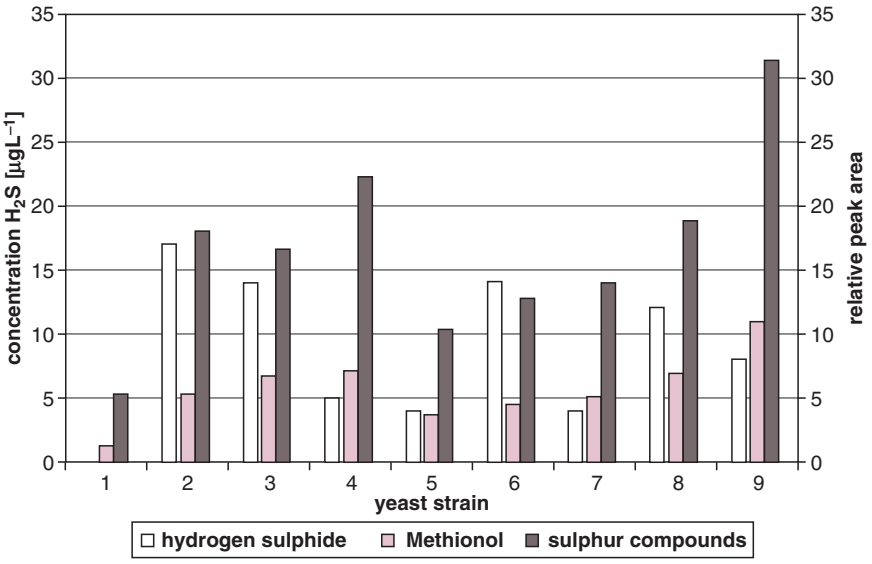

Fig. 10.3 Formation of hydrogen sulphide and the total sum of high volatile sulphur compounds and methionol in wine through nine different strains of *Saccharomyces cerevisiae* (according to Rauhut 1996, modified)

(Rauhut 1996). Therefore, an increased formation of these two thioacetic acid esters can be used as an indicator for a high H_2S formation during the fermentation process. Thioaceticacid esters were probably produced through the reaction of the thiols and acetyl Coenzym A (Matsui and Amaha 1981, Walker and Simpson 1993). The addition of methanethiol and ethanethiol during fermentation leads to the corresponding thioacetic acid esters (Rauhut 1996). Furthermore, an increase of 3-(methylthio)-1-propanol (methionol) and 3-(ethylthio)-1-propanol (ethionol) was observed. Increased levels of thioacetic acid ester can be seen as an indicator for a high H_2S production during fermentation. MeSAc can be detected in normal wine in a concentration up to about 20 µg L^{-1} (Leppänen et al. 1979, 1980). In off-flavour wines more than 130 µg L^{-1} could be detected (Rauhut 1996).

Thioacetic acid esters can hydrolyse during wine storage like other acetic acid esters after fermentation due to the chemical equilibrium (Rapp 1989), which is influenced from pH value, storage temperature, etc. The hydrolysis of thioacetic acid esters leads to free thiols and acetic acid. The thiols have very low threshold values (<2 µg L^{-1}) in comparison to the thioacetic acid esters (>40 µg L^{-1}). A treatment of wine with copper sulphate has no influence on the concentration of the thioacetic esters, because copper ions mainly react with H_2S and thiols (Rauhut 1996, 2003). A reoccurrence of off-flavours in wines after a copper fining, bottling and storage is related to a release of unpleasant volatile compounds from non-volatile or volatile precursors like the thioacetic acid esters.

Thiols can be oxidised to disulphides or trisulphides, which contribute to odours like ‘rubber’ or ‘garlic’. Disulphides or trisulphides cannot be removed by copper fining (Maujean 2001).

Cordente et al. (2007) used classical biological non-GMO techniques to develop *Saccharomyces cerevisiae* strains that are not able to produce increased H_2S amounts in grape musts with low assimilable nitrogen levels (additional information is available at Sect. 11.4.2 of Chap. 11).

Methionol is the main produced volatile sulphur compound through yeast metabolism. This sulphur containing higher alcohol can give a raw potato or cauliflower aroma in concentrations exceeding 2,000 µg L^{-1} (Meilgaard 1981). It was detected in wines up to concentrations of 6,300 µg L^{-1} (Keck 1989). Methionol production is linked to sulphur and nitrogen metabolism of yeast. Figure 10.4 indicates the formation of the total sum of high volatile sulphur compounds and methionol in two different synthetic media (AAI is low in assimilable nitrogen, whereas AAII is high in assimilable nitrogen) through two *Saccharomyces cerevisiae* strains with a different ability to produce volatile sulphur compounds. It could be demonstrated that the addition of inorganic nitrogen in form of 0.3 g L−1 diammoniumhydrogenphosphat (DAP) can decrease the total sum of volatile sulphur compounds and methionol in both media.

In synthetic media with ammonium as only sulphur source, methionol was produced as one of the main volatile sulphur compounds. This indicates that methionol can be synthesised like other higher alcohols from an α-keto acid, 3-(methylthio)-propanoic acid, derived from sugars via glycolysis. 3-(Methylthio)-propanoic acid is then decarboxylised to 3-(methylthio)-propanal (methional), which is reduced to the

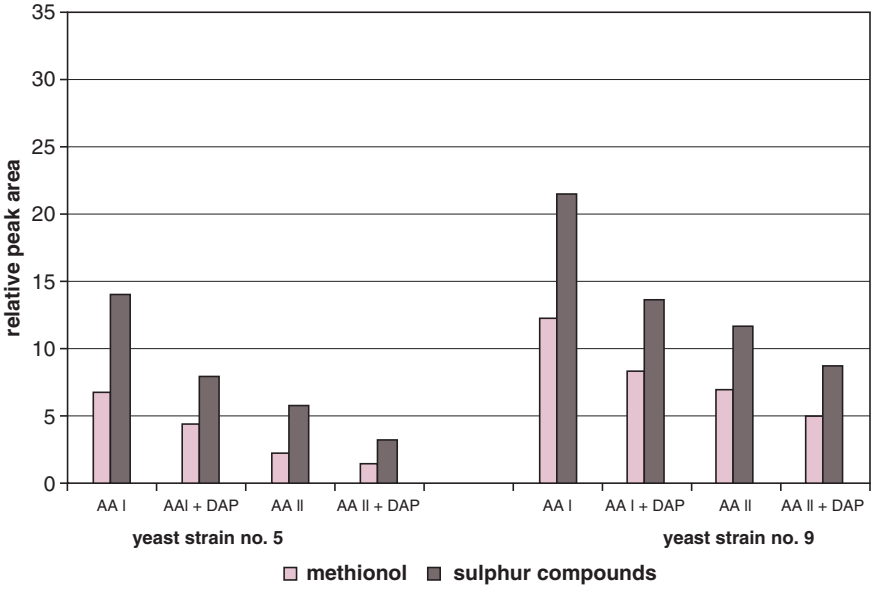

Fig. 10.4 Differences in the formation of the total sum of high volatile sulphur compounds and methionol in two different synthetic wine-like media (AAI (low in assimilable nitrogen) and AAII (high in assimilable nitrogen)) with and without addition of 0.3 g L^{-1} Diammoniumhydrogenphosphat (DAP) through two different strains of *Saccharomyces cerevisiae* (No. 5 and 9) (according to Rauhut 1996, modified)

alcohol. Methionol can be also produced from methionine through the Ehrlich pathway, which involves a deamination to the corresponding keto acid, decarboxylation to the aldehyde and enzymatic reduction to the alcohol. A supplementation of methionine before fermentation increased methionol, its corresponding acid and its acetic acid ester. These observations suggest that amounts of methionol in wine may be influenced by the levels of methionine as well as the assimilable nitrogen concentrations in the must (Rauhut 1996; Moreira et al. 2002; Bell and Henschke 2005).

The intermediate compound, methional, can only be detected in traces in wines after fermentation. Increased levels of methional are investigated in aged wines. There is also evidence that an accelerated level of methional and other sulphur compounds is involved in the so-called atypical ageing of wines (Rauhut 1996, 2003).

Further reaction products of methional like acetic acid-3-methylthiopropylester have an odour like 'mush-room' or 'garlic'. It has been also suggested that 4-methylthio-1-butanol and 2-mercapto-1-ethanol, which have both an unpleasant odour ('onion'/'garlic' and 'poultry'/'farmyard') can be probably formed by yeast in the same way like methionol via the amino acids homocysteine and cysteine, respectively (Moreira et al. 2002; Mestres et al. 2000; Swiegers et al. 2005).

Sulphur residues of more than 2.5 mg L^{-1} in the grape must from wettable sulphur applications in the vineyard can lead to an increased H_2S formation during the fermentation process (Thomas et al. 1993a–c; Rauhut 1996). The addition of elemental

sulphur in amounts over 5 mg L^{-1} leads to an increased level of unpleasant sulphur compounds (EtSH, MeSAc, EtSAc, etc.) and extreme off-flavours (Fig. 10.5).

A similar effect could be observed with the supplementation of glutathione to must, which indicated that higher concentrations of this tripeptide can increase the development of unpleasant volatile sulphur compounds during fermentation (Rauhut et al. 2001; Rauhut 2003). It is assumed that the increased glutathione levels (>50 mg L^{-1}) in grape musts can lead to a higher H_2S formation, which can cause a further production of other undesired sulphur compounds like ethanethiol (EtSH), MeSAc and EtSAc (Fig. 10.6) through yeast sulphur metabolism. This seems to be affected by the yeast strain and the nutrient composition of the grape must.

Several thiols were detected that seem to be synthesised in the presence of accelerated H_2S concentrations. Bernath (1997) detected increased levels of 2-methyl-3-furanthiol, a very powerful aroma compound with a very low threshold value. This thiol and its disulfide, bis(2-methyl-3-furyl)disulfide, contribute to the typical flavour of 'cooked meat'. It is supposed that these compounds are formed through the hydrolysis of thiamine (Belitz and Grosch 1992). Tominaga and Dubourdieu (2006) measured up to 100 ng L^{-1} of 2-methyl-3-furanthiol in different white and red wines. In off-flavour wines, concentrations of more than 300 ng L^{-1} were detected (Bernath 1997).

Furfurylthiol (2-furanmethanethiol) has a perception threshold of 0.4 ng L^{-1} (in water) and elicit odours like roasted coffee, meat, wheat bread and popcorn. It was found in Bordeaux red wines, white Petit Manseng and also in toasted barrel staves (Tominaga et al. 2000a). Blanchard et al. (2001) demonstrated that the addition of nitrogen can decrease the amount of furfurylthiol; therefore, it is assumed that its

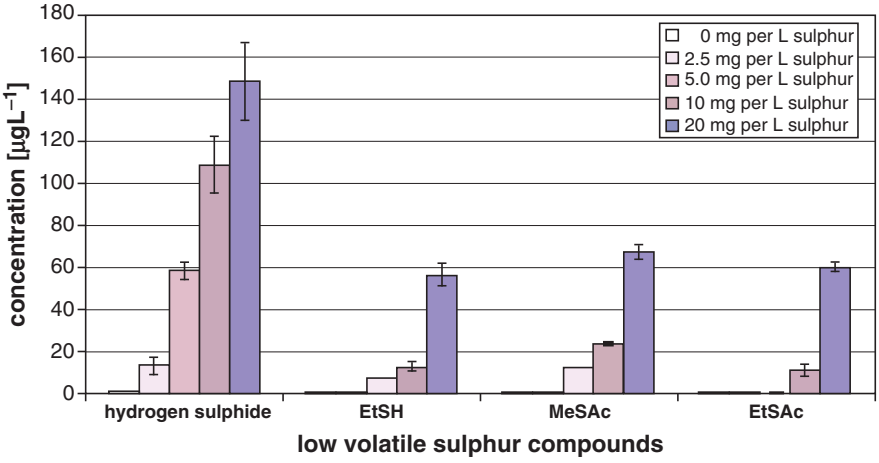

Fig. 10.5 Effect of different concentrations of elemental sulphur (added to must before fermentation) on the formation of hydrogen sulphide, ethanthiol (EtSH), thioacetic acid-*S*-methylester (MeSAc), and thioacetic acid-S-ethylester (EtSAc) during fermentation (Werner and Rauhut 2007, unpublished)

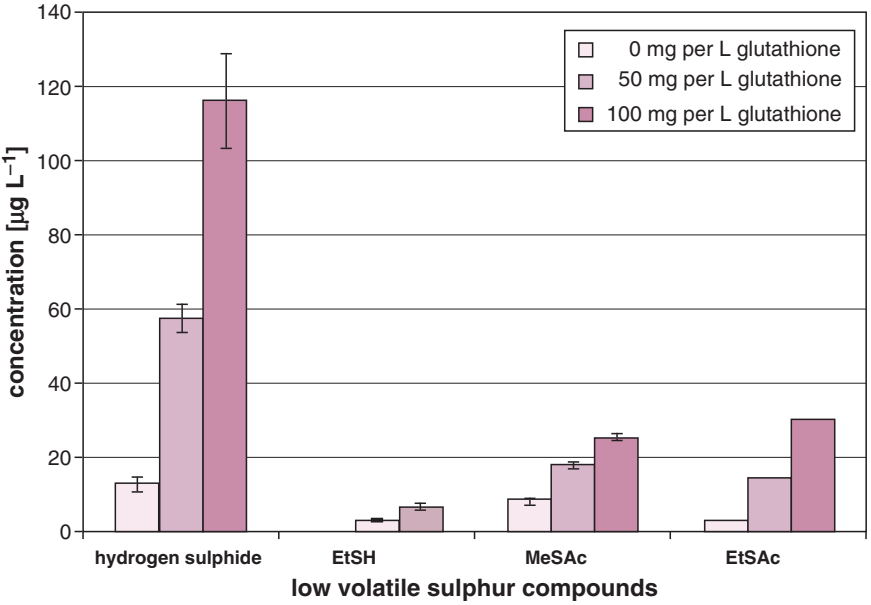

Fig. 10.6 Influence of different levels of glutathione addition to grape must on the formation of hydrogen sulphide, ethanthiol (EtSH), thioacetic acid-*S*-methylester (MeSAc) and thioacetic acid-S-ethylester (EtSAc) during fermentation (Werner and Rauhut 2007, unpublished)

production is related to the formation of H_2S. Furfurylthiol was detected in wines in a concentration up to about 50 ng L^{-1} (Tominaga and Dubourdieu 2006).

Benzylthiol (benzenemethanethiol) contributes to the 'smoky' and 'flintstone' character of wines and has an odour threshold of about 0.3 ng L^{-1} (water–ethanol model solution) and was detected in wines in an amount up to about 15 ng L^{-1}. Furfurylthiol and Benzylthiol can increase during the ageing of wines, probably in the presence of H_2S (Tominaga et al. 2003).

A release of H_2S can also take place during the autolysis of yeasts (Suomalainen and Lehtonen 1979). It has been suggested that apart from glutathione, sulphur containing amino acids are degraded during autolysis, but the mechanism involved is unclear (Henschke and Jiranek 1993). Berry and Watson (1987) proposed that yeast with a low vitality such as in other sluggish or stuck fermentations can tend to autolyse faster. Furthermore, low alcohol tolerant non-*Saccharomyces* yeasts in certain spontaneous fermentations lose viability through inhibition of the increasing levels of alcohol and presumably autolyse during the early to mid phases of fermentation (Henschke and Jiranek 1991; Fleet and Heard 1993; Swiegers et al. 2005).

Lavigne-Cruège (1996) demonstrated that H_2S and methanethiol that are present at the end of fermentation decrease during barrel ageing. The dropping off happens more rapidly in new barrels, probably due to a higher oxygen dissolution and the oxidizing effect of new wood tannins (Ribéreau-Gayon et al. 2000a). Objectionable flavours can occur if an addition of sulphite (SO_2) is carried out after fermentation and if the wines are stored on lees. This is due to the activity of sulphite reductase,

which can last up to about four weeks after fermentation. Sulphite reductase is producing H_2S from the added SO_2. It seems that the compacting of the lees under the pressure exerted at the bottom of high-capacity tanks is enhancing 'reductive' off-flavours in white wines after sulphite addition. Therefore, it is recommended to rack the wines after sulphite addition and to store the lees in barrels. This avoids the development of off-odours from gross lees. The separated lees should be reincorporated into the wine after approximately one month when the sulphite reductase activity is diminished, so that there is no longer a risk for the development of off-flavours. Yeasts have the opportunity to adsorb thiols due to their reaction with cell wall mannoproteins. A disulphide bond is formed between the cysteine of the cell wall mannoproteins and the thiols during aeration. More details on these enological practice can be taken from Lavigne-Cruège (1996) and Ribéreau-Gayon et al. (2000a).

Only a few research groups studied the contribution of non-*Saccharomyces* yeasts on the formation of volatile sulphur compounds (Romano et al. 1997, 2003). Trials in yeast–malt medium with mixed yeast cultures of *Hanseniaspora apiculata*, *Hanseniaspora guilliermondii* and *Saccharomyces cerevisiae* indicated that similar levels of 3-methylthiopropionic acid and acetic acid-3-(methylthio)propyl ester were produced like in the variant with pure inoculation of *Saccharomyces cerevisiae*. Non-*Saccharomyces* yeasts can also favour the formation of methionol production in fermented media (Moreira et al. 2005, Landaud et al. 2008).

Information on further volatile sulphur compounds that are determined in wines can be taken from Du Toit and Pretorius (2000), Dittrich and Großmann (2005), Keck (1989), Marchand et al. (2000), Pripis-Nicolau et al. (2000), Rauhut (1993, 1996), Ribéreau-Gayon et al. (2000a, b) and Segurel et al. (2004).

10.2.4.2 Occurrence and Formation of Thiols Involved in the Varietal Flavour of Wines

At the beginning of the 1980s, it was already proposed that certain volatile thiols contribute to the characteristic aromas of Chenin and Sauvignon wines (Augustyn et al. 1982; Marais 1994).

The first thiol identified in Sauvignon wines was 4-mercapto-4-methyl-pentan-2-one (4-MMP), whose odour threshold is very low (0.1–0.8 ng L^{-1} in water and model solution) and it elicits aromas like blackcurrant, box tree and broom (Darriet et al. 1991; Murat et al. 2001). Characteristic nuances of the variety Sauvignon blanc were observed with concentrations of 4-MMP close to 40 ng L^{-1}. Increased concentrations of 4-MMP cause a 'catty' note in wines.

Apart from 4-MMP, further thiols, e.g. 3-mercaptohexanol (3-MH), 4-mercapto-4-methylpentan-2-ol (4-MMPOH), acetic acid-3-mercaptohexylester (3-MHA), have been identified as major contributors to the varietal aroma of Sauvignon blanc wines (Darriet et al. 1995; Tominaga et al. 1995, 1998; Dubourdieu et al. 2000). Tropical fruit flavours such as passion fruit, grapefruit, citrus zest, lychee and guava in some of the Sauvignon wines are mainly related to 3-MH and its acetic acid ester. These compounds have a similar threshold value like 4-MMP.

4-MMP, 3-MH, 3-MHA and other related thiols have also been detected in wines made from other varieties like Petit and Grand Manseng, Arvine, Colombard, Chenin blanc, Alsace Muscat, Gewürztraminer, Riesling, Scheurebe, Bacchus, Cabernet Sauvignon, Merlot, etc. (Tominaga et al. 2000a; Murat et al. 2001; Guth 1997a, b; Ribéreau-Gayon et al. 2000b).

The volatile thiols are almost not occurring in the grape must and develop during the fermentation process. Tominaga et al. (1998) identified odourless sulphur–cysteine conjugates as precursors for the high odour-active thiols. The release of the thiols was investigated by the use of a cell-free enzyme extract of the bacterium *Eubacterium* limosum that contain carbon-sulphur lyase enzymes. It could be shown that carbon–sulphur lyases can release 4-MMP from its precursor *S*-4-(4-methylpentan-2-one)-L-cysteine. Therefore, it was suggested that a yeast cysteine β-lyase releases a thiol, pyruvic acid and ammonium from the corresponding sulphur–cysteine conjugate as it is shown for 4-MMP in Fig. 10.7 (Tominaga et al. 1995, 2004; Masneuf 1996; Peyrot des Gachons et al. 2000, 2002a, b).

Peyrot des Gachons et al. (2002a, b) identified the precursor of 3-MH, S-3-(hexan-1-ol)-glutathione, in must of Sauvignon blanc for the first time. It is assumed that a glutathione transferase is involved in the synthesis of the sulphur–glutathione-conjugates, which are probably transported with the help of a glutathione-conjugate-pump to the cell vacuole. The sulphur–cysteine-conjugates are almost certainly formed through the activity of a γ-glutamyltranspeptidase and a carboxypeptidase (Wüst 2003).

Research work of Murat et al. (2001) indicated that yeast strains vary in the ability to release 4-MMP, 3-MH and 4-MMPOH. It could be also demonstrated that commercial yeast strains differ in the release of the various volatile thiols. Strains of *Saccharomyces bayanus* and their hybrids created with *Saccharomyces cerevisiae* released even higher concentrations of the thiols. The activity of the enzymes involved in the release of the different thiols is strain dependent. A variation of the release of the enzymes and of the thiols can be achieved by the use of specific yeast strains. Dubourdieu et al. (2000) demonstrated in model fermentations that a synthesised precursor decreased in concentration, while the corresponding thiol increased, but only a small faction (1.6% at day 6 of fermentation) of the cysteine-bound precursor was transferred to the thiol. The same effect was shown with Cabernet Sauvignon and Merlot musts (Murat et al. 2001). Only 3.2% (average value) of the precursor was released during the fermentation.

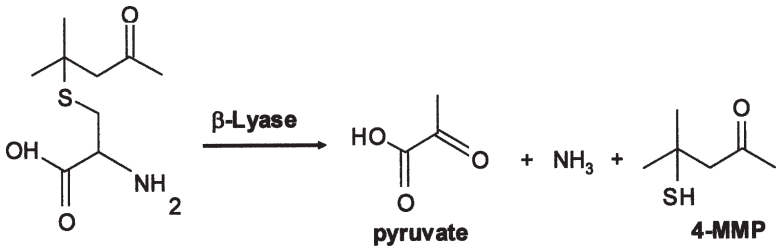

Fig. 10.7 Release of 4-MMP from the corresponding sulphur–cysteine-conjugate (according to Peyrot des Gachons et al. 2000 and Wüst 2003, modified)

Therefore, the choice of the yeast strain is of considerable importance to enhance flavour complexity of wines and to use the huge potential of odourless sulphur–cysteine-conjugate precursors to release the desired volatile thiols and to create specific wine styles (Swiegers et al. 2005). On the contrary, it is very crucial to avoid an overproduction of 'tropical' fruit flavours through specific yeast strains in wines from varieties that normally do not release this kind of aroma notes. Therefore, the adequate application of the yeast strains is very important to develop the 'typical' flavours of certain varieties and to activate the release of certain aromas for specific wine styles.

Howell et al. (2005) showed with a laboratory strain that four genes are involved in the release of 4-MMP. This points out that the mechanism of release probably involves multiple genes (Swiegers et al. 2005, 2006). Swiegers et al. (2005) demonstrate the link between ester and volatile thiol metabolism in yeast for the first time. It could be demonstrated that 3-MH is transformed to 3-MHA by the ester-forming alcohol acetyltransferase, encoded by the *ATF1* gene. Large differences in the ability of commercial strains were noticed to form 3-MHA. The ability to produce high levels of 3-MHA did not correspond with the ability to release 4-MMP (additional information is available at Sect. 11.3.5).

Swiegers et al. (2000) demonstrated the power of yeast in enhancing the aroma of wine due to the development of a prototype wine yeast able to release significantly more thiols than conventional yeast. The *Escherichia coli* tryptophanase gene, *tnaA*, was overexpressed in a commercial wine yeast by the use of genetic modification technology. The transformant producing the bacterial cysteine-β-lyase had more than a 10-fold increase in 4-MMP concentration in comparison to the commercial control strain. Lilly et al. (2006) indicated that the overexpression of *ATF1* in a wine yeast resulted in increased 3-MHA levels.

On the basis of these studies, alternative strategies with conventional techniques are studied to optimise the release of thiols and the formation of 'tropical fruit' esters. For example, a large number of hybrid yeasts or natural selections of yeasts could be proved for their ability to release thiols or for the capacity of ester formation. Furthermore, co-inoculated fermentations of certain yeast strains have been applied and will be investigated more intensively in future as an appropriate enological tool to generate specific aroma characteristics in wines (Swiegers et al. 2006). It can be also expected that apart from other species of the *Saccharomyces* genus also species and strains of non-*Saccharomyces* yeasts have different abilities to release thiols (Wakabayashi 2004; Kagli et al. 2006; Sourabié et al. 2008), which offers a broad field for future research.

Schneider et al. (2006) demonstrated a new pathway leading to 3-MH and 4-MMP, starting from conjugated carbonyl compounds, alternative to the already known release from cysteinylated precursors.

Finally, it has to be pointed out that recent research work of Sarrazin et al. (2007) indicated a contribution of the identified and quantified thiols, 3-sulphanylpentan-1-ol, 3-sulphanylheptan-1-ol, 2-methyl-3-sulphanylbutan-1-ol and probably 2-methyl-3-sulphanylpentan-1-ol, to the overall aroma of sweet wines made from *Botrytis*-infected grapes.

10.3 Sulphur Metabolism of Lactic Acid Bacteria

Lactic acid bacteria conduct malolactic fermentation, which usually occurs in wine a few days after alcoholic fermentation. Malolactic fermentation is forced in certain wines to degrade malic acid to lactic acid. This process softens the wine by decreasing its acidity and improves its organoleptic quality due to certain transformations. The lactic acid bacteria associated with grape must and wine mainly belong to the following genera: *Lactobacillus*, *Oenococcus* and *Pediococcus. Oenococcus oeni* mostly grows spontaneously in wine after the alcoholic fermentation or after commercial starters are added. There is a huge amount of literature available on the improvement of malolactic fermentation, on the formation of certain aroma compounds such as diacetyl and on health-related compounds such as biogenic amines (Bartowsky 2005; Henick-Kling 1993). Sulphur metabolism was only studied for lactic acid bacteria that are involved in cheese production for a great extent (Bonnarme et al. 2000; Dias and Weimer 1998; Sourabié et al. 2008).

Pripis-Nicolau et al. (2003, 2004) started to investigate the methionine catabolism of *Oenococcus oeni* and certain species of *Lactobacillus* at wine-making conditions for the first time. It could be indicated that in a laboratory media several lactic acid bacteria are able to metabolise methionine. The following sulphur compounds were detected: methanethiol, dimethyl disulfide, 3-(methylsulphanyl)propan-1-ol (methionol) and 3-(methylsulphanyl)propionic acid. Methionol and 3-(methylsulphanyl)propionic acid were formed in higher concentrations by *Oenococcus oeni* than by *Lactobacillus* species. It was observed that strains differ in their ability to produce the volatile metabolites. Figure 10.8 is giving an overview on the possible metabolites of methionine catabolism by *Oenococcus oeni* according to the research of Pripis-Nicolau et al. (2004). After malolactic fermentation in various red wines, only the level of 3-(methylsulphanyl)propionic acid increased in some cases significantly. It is assumed that this sulphur compound, which gives the impression of 'chocolate' and 'roasted odours' and a perception threshold of 50 $\mu g\ L^{-1}$, can probably contribute to the aromatic complexity often noticeable after malolactic fermentation. It is important to point out that no off-flavour compounds like methanethiol and dimethyl disulphide could be measured after malolactic fermentation conducted in wines.

Vallet et al. (2007) demonstrated that 2-oxo-4-(methylthio)butyric acid plays a central role in volatile sulphur compound synthesis.

The addition of low amounts of glutathione can have a positive effect on the growth of *Oenococcus oeni* in wine under certain conditions (Rauhut et al. 2004). The addition of methionine and glutathione seemed to accelerate the speed of malolactic fermentation a little bit at lower pH-values. The catabolism of glutathione can lead to increased levels of H_2S in wine-like synthetic media, if the supplemented concentrations are far over the normal levels in wines. Furthermore, it could be shown that the catabolism of methionine to volatile sulphur compounds seems to depend on the pH-value of the media (Rauhut et al. 2008). According to Pripis-Nicolau et al. (2004), no increase of volatile sulphur compounds that are related to off-flavours (e.g. H_2S, methanethiol, dimethyldisulphide, etc.) could be detected in wine-like model solutions and wine after

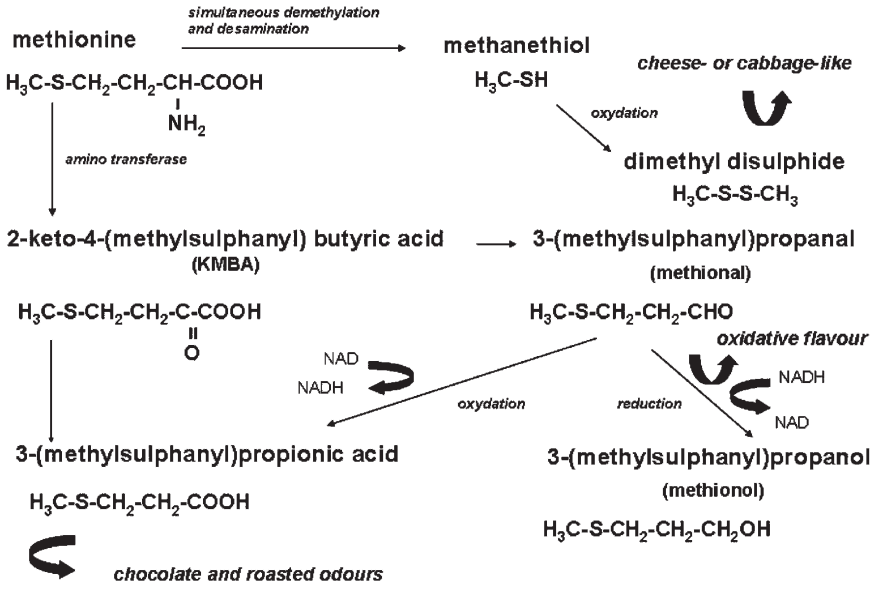

Fig. 10.8 Proposed metabolism of methionine by *Oenococcus oeni* in wine-like synthetic media (according to Pripis-Nicolau et al. 2003, 2004, modified)

addition of methionine and glutathione in amounts that can be usually expected in wines. As a result of the investigations, it is proposed that other factors such as the chemical or biochemical transformation of other volatile or non-volatile sulphur precursors in wine are the cause for 'reductive' sulphur off-flavours that can be sometimes noticed after malolactic fermentation and/or storage and ageing of wines.

10.8 Conclusions

Considerable research over more than two decades indicated that yeast sulphur metabolism influences to a high extent the wine flavour due to the occurrence of sulphur related off-flavours that mainly occur due to deficiencies of assimilable nitrogen and other nutrients in grape musts. Research on an adequate and focused nutrient support of yeasts during fermentation has to be continued to fully understand the complex process of nutrient composition and formation of volatile sulphur compounds. This is of high interest especially with the regard to global climate change, which can decrease the nutrient composition in grapes as a result of increased stress conditions such as water deficiencies in certain years.

Investigations of different research groups pointed out that yeast plays a great role in the characteristic of varietal aromas, in particular for wines from certain grape varieties like Sauvignon blanc, Muscat, Gewürztraminer, Scheurebe, etc.

Volatile sulphur aroma compounds are the best example to demonstrate that a comprehensive knowledge is necessary to avoid the formation of objectionable flavours and to optimise the release of specific thiols that offer wines specific desired 'tropical' fruit aromas. Any treatment or fining to get rid of developed 'reductive' sulphur off-flavours will also affect the varietal flavour triggered by volatile thiols. Ongoing research is necessary to process strategies to optimise the desired aromas through certain thiols and to avoid or to minimise the occurrence of malodorous sulphur compounds through yeast metabolism.

In addition, the interaction of yeasts from the same and/or from different species has to be studied to investigate their influence on the formation of volatile and non-volatile sulphur compounds. In a further step, other microorganisms like lactic acid bacteria should be integrated in these research investigations.

Appropriate wine yeast strains should be further on selected and scanned for a very low formation of sulphite, because a decrease of sulphites is generally required in food.

Acknowledgements Finally, I express my sincere thanks to the Geisenheim Research Center for offering me the opportunity to be engaged in flavour research, especially in the formation and importance of volatile sulphur compounds. Furthermore, I thank all members of the aroma analysis team of the Section Microbiology and Biochemistry for their support and interest.
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Chapter 11
Microbial Formation and Modification of Flavor and Off-Flavor Compounds in Wine

Eveline J. Bartowsky and Isak S. Pretorius

11.1 Introduction

The production of alcoholic beverages has a long history among humankind, with winemaking, as an example, dating back over 7,000 years. Even though the concept of transforming grape juice into wine does not appear difficult, it can be a complex process to ensure a delicious and stable product that does not spoil during storage. The true challenge for winemakers is to blend an ever-changing "menu" of grapes, soil, and climate with an evolving science of yeast and bacterial metabolism to produce the best possible expression of their chosen wine style. The vinification process used today is not vastly different from that used in the time of the ancient Egyptians and Greeks; however, modern-day winemakers have much more control at the various critical stages from the time and method of grape-picking to innovative maturation techniques. Many winemakers today use commercial yeast and bacteria starter cultures for alcoholic and malolactic fermentations. Selection of a "fit-for-purpose" starter strain has a pivotal role in optimizing flavor and aroma, and bottle and closure choice is another contributing factor to the characteristics of the finished wine.

To produce the best wine with the desired style, flavor formation must be optimized and off-flavors development minimized. Wine aroma and flavor originate from the grape, yeast and bacterial metabolism of grape juice and wood (if used), and chemical reactions during maturation and storage. Several recent reviews cover numerous aspects of these areas (Swiegers et al. 2005; Swiegers and Pretorius 2005). Most of the yeast and bacterial metabolism discussed in this chapter is not necessarily specific to production of red or white wine and is applicable to all winemaking styles. An overview of the winemaking pathway for red and white grapes is given in Fig. 11.1.

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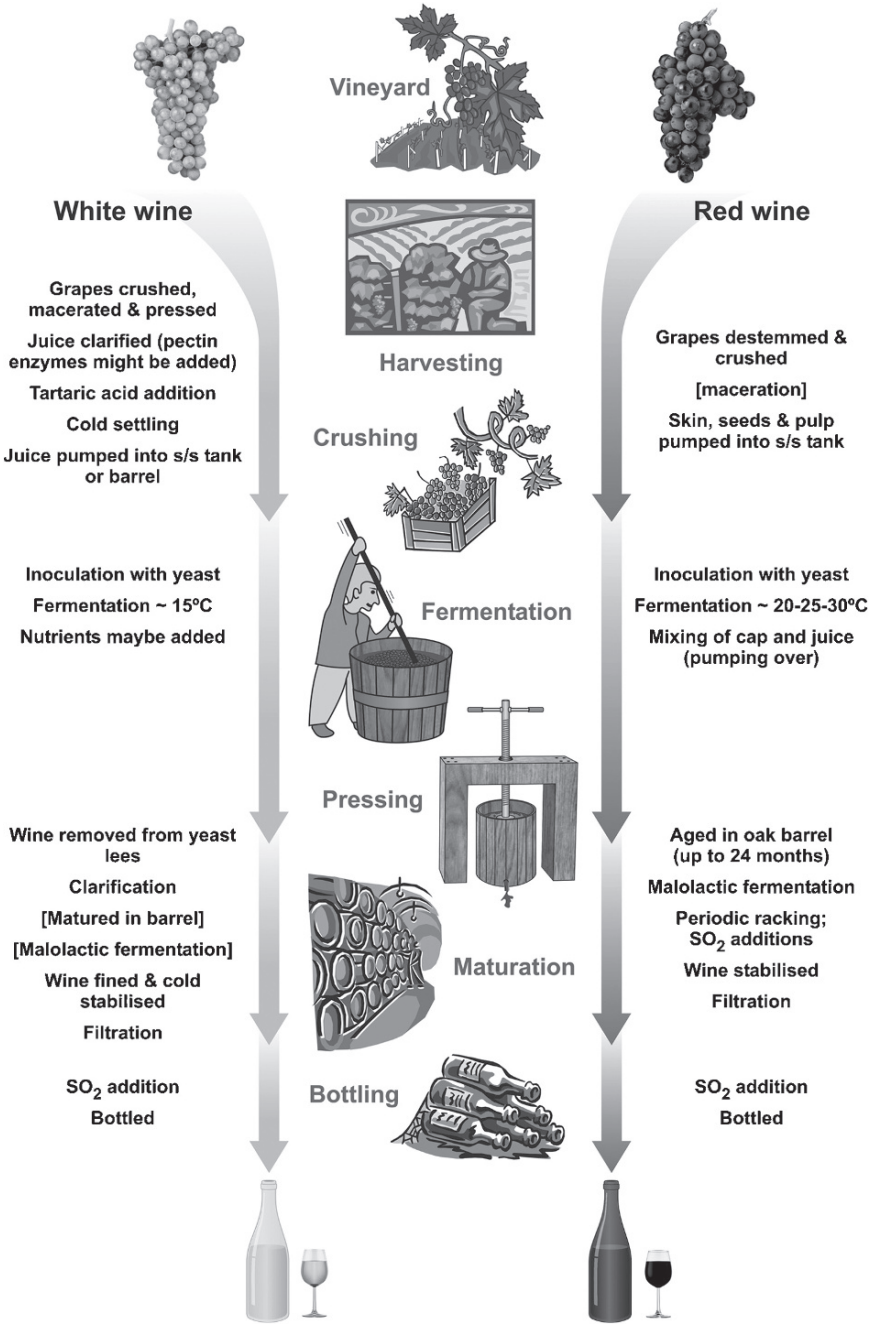

Fig. 11.1 A general summary of the main steps in the red and white grape vinification

11.2 Metabolism of Yeast and Lactic Acid Bacteria in Wine

Both eukaryotic and prokaryotic microorganisms can be isolated from wine. Their main role is to convert grape sugars into alcohol, reduce the acidity of wine, and introduce interesting and desirable aromas and flavors. Even though grape must is relatively complete in nutrient composition, it can support only a limited number of microorganisms; wine is even less inviting with limited nutrients available. The strongest selection pressures on yeast and bacteria proliferating in grape must and wine environments are the high sugar content and low pH in grape must and high ethanol and SO_2 content, and limited nutrients in wine.

Yeasts are predominant during the complex process of winemaking. Of the 100 yeast genera representing more than 700 species, 16 are associated with winemaking: *Brettanomyces* (*Dekkera)*, *Candida*, *Cryptococcus*, *Debaryomyces*, *Hanseniaspora* (*Kloeckera*), *Kluyveromyces*, *Metschnikowia*, *Pichia*, *Rhodotorula*, *Saccharomyces*, *Saccharomycodes*, *Schizosaccharomyces*, *Torulaspora*, and *Zygosaccharomyces* (Pretorius et al. 1999). Acetic acid and lactic acid bacteria are the only families of bacteria commonly found in grape juice and wine: lactic acid bacteria play a more important role in winemaking, whereas the acetic acid bacteria are considered to be only spoilage organisms. Only four genera of the lactic acid bacteria – *Lactobacillus*, *Leuconostoc*, *Oenococcus* and *Pediococcus*, and two genera of acetic acid bacteria – *Acetobacter* and *Gluconobacter*, are associated with grape must and wine.

Saccharomyces cerevisiae is the predominant yeast in winemaking and is the model yeast when describing its metabolic reactions. The glycolytic pathway linked to ethanol production is the essential key for production of wine (Fig. 11.2). This pathway drives the enzymatic reactions of the main grape sugars, glucose and fructose, through to pyruvate, which is the key for numerous other pathways, including to acetaldehyde through to ethanol. At the early stages of alcoholic fermentation, glycolysis occurs under aerobic conditions, but soon with the production of carbon dioxide the grape must/wine environment becomes quickly anaerobic.

Oenococcus oeni is the most adapted wine-associated lactic acid bacteria and is almost exclusively used for the induction of malolactic fermentation in red, white, and sparkling base wines (Wibowo et al. 1985), usually following alcoholic fermentation. In contrast to homolactic fermentation of hexoses where lactate is the only product, *O. oeni* uses heterolactic fermentation resulting in a mixture of products; for each glucose molecule fermented, approximately one each of lactate, ethanol, and carbon dioxide results (Fig. 11.3).

11.3 Flavor Compounds Produced by, or Liberation of Grape-Derived Flavor Compounds by, Yeast and Bacteria

The grape has an undeniable importance in wine aroma and flavor, and the creation of a distinctive wine style. However, wine has more flavor than the grape juice it is fermented from, and it is the metabolism of grape compounds by yeast and bacteria that is central to the development of wine flavor.

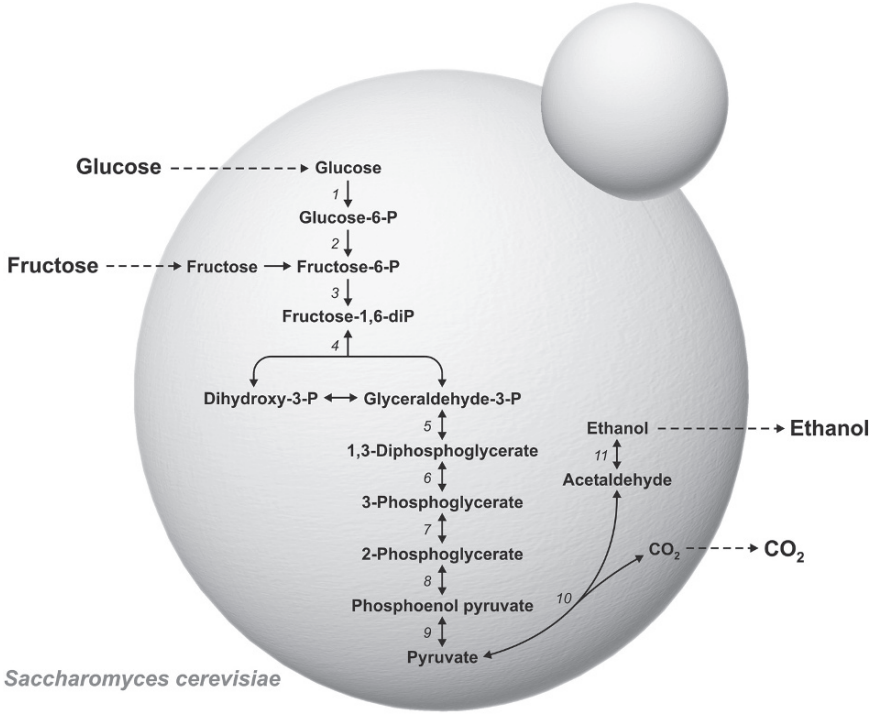

Fig. 11.2 Glycolysis and alcoholic fermentation pathway in *Saccharomyces cerevisiae*. Enzymes for the different stages in the pathway: 1, hexokinase; 2, phosphoglucose isomerase; 3, phosphofructokinase; 4, aldolase; 5, glyceraldehyde-3-phosphate dehydrogenase; 6, phosphoglycerate kinase; 7, phosphoglycero mutase; 8, enolase; 9, pyruvate kinase; 10, pyruvate decarboxylase; 11, alcohol dehydrogenase

Many biosynthetic pathways in wine yeast and malolactic bacteria are involved in the formation of wine aromas and flavors (Figs. 11.4 and 11.5). The undesirability of an aroma or flavor compound in wine is not only dependent upon its sensory descriptor but also on its concentration in wine. The final concentration of sensorially important compounds is dependent upon numerous factors, including the yeast and bacterial species proliferating during vinification, the maturation process, and the finishing process of the wine, wine filtration, bottling and closure type. The odor active compounds of wine have been grouped according to their main chemical structure and will be discussed in each category.

11.3.1 Esters

The majority of wine esters are produced by yeast during alcoholic fermentation and can have a significant effect on the fruity flavors in wine. The most significant esters are ethyl acetate (fruity, solvent-like), isoamyl acetate (isopentyl acetate,

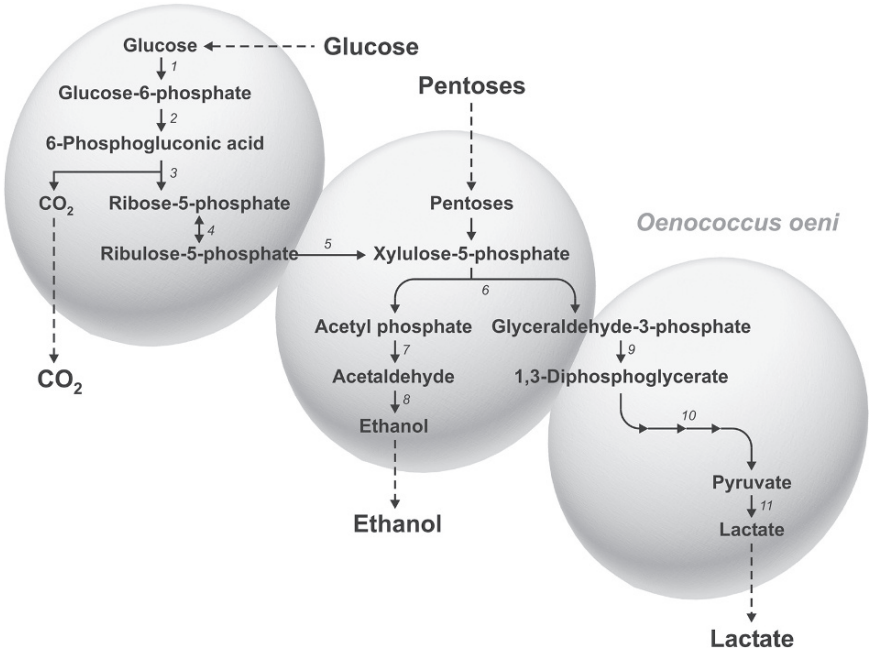

Fig. 11.3 Metabolic pathway of heterolactic fermentation of glucose by *Oenococcus oeni*. Enzymes for the different stages of the pathway: 1, hexokinase; 2, glucose 6-phosphate dehydrogenase and lactonase; 3, 6-phosphogluconate dehydrogenase; 4, phosphopentose isomerase; 5, phosphopentose epimerase; 6, xylulose 5-phosphoketolase; 7, acetaldehyde dehydrogenase; 8, alcohol dehydrogenase; 9, transaldolase; 10, triose-phosphate-dehydrogenase system; 11, lactate dehydrogenase

pear-drops aromas), isobutyl acetate (banana aroma), ethyl caproate (ethyl hexanoate, apple aroma), and 2-phenylethyl acetate (honey, fruity, flowery aromas) (Table 11.1 and Fig. 11.4) (Thurston et al. 1981). Yeast metabolism of esters is through lipid and acetyl-CoA metabolism or by chemical esterification of alcohols and from acids during wine-ageing (Lambrechts and Pretorius 2000).

Commercial *S. cerevisiae* wine strains produce variable amounts of esters, such as isoamyl acetate, hexyl acetate, ethyl hexanoate, and ethyl octanoate, which have a potential impact on the aroma profile (Lambrechts and Pretorius 2000; Marais 2001); however, several non-*Saccharomyces* wine yeasts can contribute to the ester aromas of wine. Mixed culture fermentations by wild yeasts, such as *Hanseniaspora guilliermondii* and *Pichia anomala*, together with *S. cerevisiae*, have shown increased acetate ester concentrations, compared with fermentations with *S. cerevisiae* alone (Rojas et al. 2003).

Ester concentrations differ among wine types, and there appears to be a synergy between the grape and the yeast metabolism in establishing the characteristic ester blueprint of different grape varieties. In Pinot Noir wines, the characteristic fruity flavors of plum, cherry, strawberry, raspberry, blackcurrant, and blackberry charac-

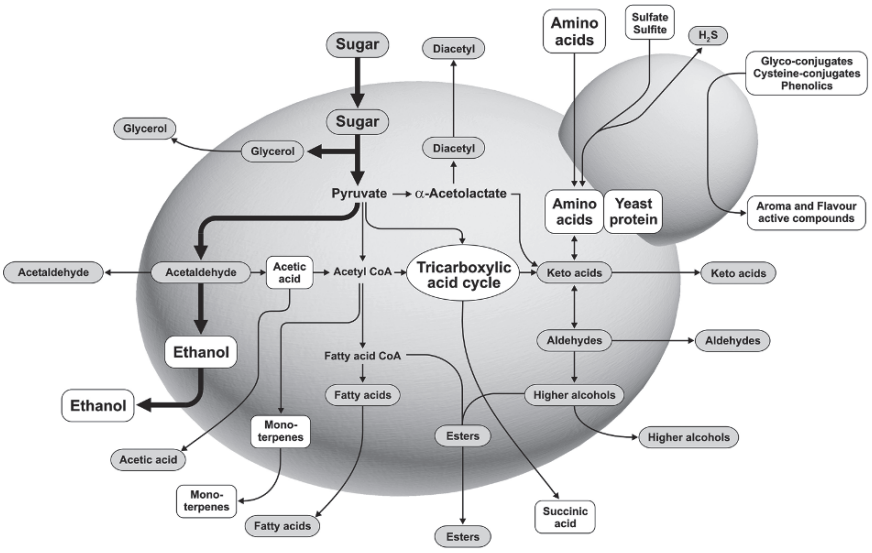

Fig. 11.4 A schematic representation of derivation and synthesis of flavor-active compounds from sugar, amino acids, and sulfur metabolism by wine yeast, *Saccharomyces cerevisiae* (reprinted with permission from Swiegers et al., 2005)

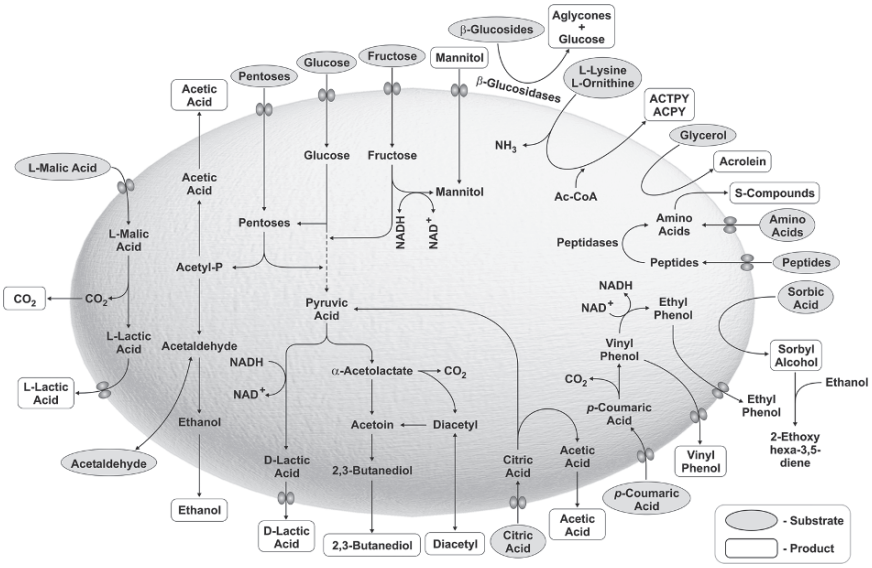

Fig. 11.5 A schematic representation of the biosynthesis and modulation of flavor-active compounds by malolactic bacterium, *Oenococcus oeni* (reprinted with permission from Swiegers et al., 2005)

Table 11.1 Yeast and bacterial metabolites and sensory descriptor

Compound	Aroma descriptor	Concentration in wine	Aroma threshold
Ethyl esters			
Ethyl butanoate	Floral, fruity	0.01–1.8 mg L^{-1}	0.02 mg L^{-1} [a]
Ethyl hexanoate	Green apple	0.03–3.4 mg L^{-1}	0.05 mg L^{-1} [a]
Ethyl octanoate	Sweet soap	0.05–3.8 mg L^{-1}	0.02 mg L^{-1} [a]
Ethyl decanoate	Floral, soap	0–2.1 mg L^{-1}	0.2 mg L^{-1} [e]
Ethyl propanoate	Fruity		1.8 mg L^{-1}
Ethyl 2-methyl propanoate	Fruity		15 μg L^{-1}
Ethyl 2-methyl butanoate	Sweet fruit		1 μg L^{-1}
Ethyl 3-methyl butanoate	Berry		3 μg L^{-1}
Ethyl lactate	Strawberry		14 mg L^{-1}
Cinnamic esters			
Ethyl 2,3-dihydrocinnamate	Flowery, fruity	0.21–3.02 μg L^{-1}	1.6 μg L^{-1}
Ethyl cinnamate	Cherry, plum, honey, cinnamon	0.1–8.89 μg L^{-1}	1.1 μg L^{-1}
Methyl anthranilate	Fruit, grape		
Ethyl anthranilate	Sweet, fruity, grape-like		
Acetates			
Ethyl acetate	Volatile acidity, solvent nail polish, fruity	22.5–63.5 mg L^{-1}	7.5 mg L^{-1} [a]
Isoamyl acetate	Banana, pear	0.1–3.4 mg L^{-1}	0.03 mg L^{-1} [a]
2-Phenylethyl acetate	Flowery, rose, honey, fruity	0–18.5 mg L^{-1}	0.25 mg L^{-1} [a]
Isobutyl acetate	Banana, fruity	0.01–1.6 mg L^{-1}	1.6 mg L^{-1} [d]
Hexyl acetate	Sweet, perfume	0–4.8 mg L^{-1}	0.7 mg L^{-1} [b]
2-Methyl propyl acetate	Banana, fruity		1.6 mg L^{-1}
2-Methyl butyl acetate	Banana, fruity		0.160 mg L^{-1}
3-Methyl butyl acetate	Banana		30 μg L^{-1}
2-Phenylethyl acetate	Flowery		0.25 mg L^{-1}
Alcohols			
Propanol	Stupefying	9.0–68 mg L^{-1}	500 mg L^{-1} [b]
Butanol	Fusel, spirituous	0.5–8.5 mg L^{-1}	150 mg L^{-1} [a]
Isobutanol	Fusel, spirituous	9.0–174 mg L^{-1}	40 mg L^{-1} [a]
Isoamyl alcohol	Harsh, nail polish	6.0–490 mg L^{-1}	30 mg L^{-1} [a]
Hexanol	Green, grass	0.3–12.0 mg L^{-1}	4 mg L^{-1} [b]
2-Phenylethyl alcohol	Floral, rose	4.0–197 mg L^{-1}	10 mg L^{-1} [a]
2-Methyl propanol	Fusel, spirituous		40 mg L^{-1}
2-Methyl butanol	Nail polish		65 mg L^{-1}
3-Methyl butanol	Harsh, nail polish		30 mg L^{-1}
Tyrosol		20–30 mg L^{-1}	
Tryptophol		0–1 mg L^{-1}	
Acids			
Acetic acid	Volatile acidity, vinegar	110–1,150 mg L^{-1}	280 mg L^{-1} [a]
Propanoic acid	Vinegar		8.1 mg L^{-1}
2-Methyl propanoic acid	Cheese, rancid		200,000 mg L^{-1}
Butanoic acid	Cheese, rancid		2.2 mg L^{-1}
2-Methyl butanoic acid	Cheese, sweaty		3 mg L^{-1}
3-Methyl butanoic acid	Blue cheese		3 mg L^{-1}

(continued)

Table 11.1 (continued)

Compound	Aroma descriptor	Concentration in wine (mg L^{-1})	Aroma threshold (mg L^{-1})
Hexanoic acid	Cheese, sweaty		8 mg L^{-1}
Octanoic acid	Rancid, harsh		8.8 mg L^{-1}
Decanoic acid	Fatty		6 mg L^{-1}
3-(Methylsulfanyl) propionic acid	Chocolate, roasted		
Polyols			
Glycerol	Odorless (slightly sweet taste)	5–14 g L^{-1}	5.2 g L^{-1}[b]
Mannitol	Sweet		
Erythritol	Sweet		
Carbonyls			
Acetaldehyde	Sherry, nutty, bruised apple	10–75 mg L^{-1}	100 mg L^{-1} [b]
Diacetyl	Buttery	<5 mg L^{-1}	0.2 mg L^{-1} [b]/2.8 mg L^{-1} [c]
Acetoin	Butter, cream	0.6–253 mg L^{-1}	150 mg L^{-1}
Monoterpenes			
Linalool	Rose	0.0017–0.010 mg L^{-1}	0.0015[f]/0.025 mg L^{-1} [e]
Geraniol	Rose-like	0.001–0.044 mg L^{-1}	5[f]/30 mg L^{-1} [a]
Citronellol	Citronella	0.015–0.042 mg L^{-1}	8[f]/100 mg L^{-1} [a]
Lactones			
cis-Oak lactone	Coconut, flowery	n.d. – 589 µg L^{-1}	67 µg L^{-1}
N-cyclic compounds			
2-Acetyl-1-pyrroline (ACPY)	Mousy	Trace	0.0001 mg L^{-1} [f]
2-Acetyltetrahydropyridine (ACPTY)	Mousy	0.0048–0.1 mg L^{-1}	0.0016 mg L^{-1} [f]
Phenols			
4-Ethylphenol	Medicinal, barnyard	0.012–6.5 mg L^{-1}	0.14[a]/0.6 mg L^{-1} [c]
4-Ethylguaiacol	Phenolic, sweet	0.001–0.44 mg L^{-1}	0.033[a]/0.11 mg L^{-1} [c]
4-Vinylphenol	Pharmaceutical	0.04–0.45 mg L^{-1}	0.02 mg L^{-1} [f]
4-Vinylguaiacol	Clove-like, phenolic	0.0014–0.71 mg L^{-1}	10 mg L^{-1} [f]
Sulfur containing			
Hydrogen sulfide	Rotten egg	Trace to >80 µg L^{-1}	10–80 µg L^{-1}
Methanethiol (methyl mercaptan)	Cooked cabbage, onion, putrefaction, rubber	5.1, 2.1 µg L^{-1}	0.3 µg L^{-1}
Ethanethiol (ethyl mercaptan)	Onion, rubber, natural gas	1.9–18.7 µg L^{-1}	1.1 µg L^{-1}
Dimethyl sulfide	Asparagus, corn, molasses	1.4–61.9 µg L^{-1}	25 µg L^{-1}
Diethyl disulfide	Garlic, burnt rubber	Trace–85 µg L^{-1}	4.3 µg L^{-1}
Dimethyl disulfide	Cooked cabbage, intense onion	2 µg L^{-1}	15, 29 µg L^{-1}

(continued)

Table 11.1 (continued)

Diethyl sulfide	Cooked vegetables, onion, garlic	4.1–31.8 μg L^{-1}	0.93 μg L^{-1}
3-(Methylthio)-1-propanol (methionol)	Cauliflower, cabbage, potato	140–5,000 μg L^{-1}	500 μg L^{-1}
Benzothiazole	Rubber	11 μg L^{-1}	50 μg $^{-1}$
Thiazole	Popcorn, peanut	0–34 μg L^{-1}	38 μg L^{-1}
4-Methylthiazole	Green hazelnut	0–11 μg L^{-1}	55 μg L^{-1}
Thiols			
Thiophene-2-thiol	Burned, burned rubber, roasted coffee	0–11 μg L^{-1}	0.8 μg L^{-1}
4-Mercapto-4-methylpentan-2-one (4MMP)	Cat urine, box tree/black-currant, broom	0–30 ng L^{-1}	3 ng L^{-1}
3-Mercaptohexan-1-ol (3MH)	Passion-fruit, grapefruit	50–5,000 ng L^{-1}	60 ng L^{-1}
3-Mercaptohexyl acetate (3MHA)	Riesling-type note, passion-fruit, box tree	1–100 ng L^{-1}	4 ng L^{-1}
2-Furanmethanethiol	Roasted coffee, burnt rubber	0–350 ng L^{-1}	1 ng L^{-1}
Furfuryl thiol	Roasted coffee, meat, wheat bread, popcorn		0.4 ng L^{-1}

Thresholds determined in
[a]10% ethanol,
[b]wine,
[c]red wine,
[d]beer,
[e]synthetic wine,
[f]water
Summarized from Swiegers et al. (2005) and Francis and Newton (2005)

ters were shown to be influenced by the synthesis of four distinct esters: ethyl anthranilate, ethyl cinnamate, 2,3-dihydrocinnamate, and methyl anthranilate (Moio and Etievant 1995). Chardonnay wines have been shown to characteristically contain ethyl esters such as ethyl-2-methyl propanoate, ethyl-2-butanoate, 3-methyl butanoate, ethyl hexanoate, ethyl octanoate, ethyl decanoate, and the acetate esters hexyl acetate, 2-methylbutyl acetate, and 3-methylbutyl acetate, whereas Riesling wines contained similar esters, which were found to be unimportant to the final aroma of the wines (Smyth et al. 2005).

The balance between ester-synthesizing enzymes (alcohol acetyltransferases) and ester-degrading enzyme (esterases) is important for the net rate of ester accumulation and overall flavor profile of wine (Verstrepen et al. 2003; Lilly et al. 2006a). The overall wine ester composition can be altered by manipulating the expression of these enzymes. For example, the acetate ester composition of wine produced with the commercial wine yeast strain VIN13 overexpressing the alcohol acetyltransferases had a pronounced effect on the solvent, fruity and flowery characters of the wines (Lilly et al. 2000). These investigations on flavor-active esters

represent progress towards laying the foundation for the possible development of wine yeast starter strains with optimized ester-producing capabilities.

Esterase activity of wine-associated bacterial species is not well understood; esterases of dairy-associated species of *Lactobacillus* and *Pediococcus* have been observed, and it appears that their growth in grape juice or wine might modify the ester profile of wine (Matthews et al. 2004). Increases in ethyl ester concentration in wine following malolactic fermentation, including ethyl acetate, ethyl hexanoate, ethyl lactate, and ethyl octanoate, as well as decreases in some esters have been observed (Laurent et al. 1994; de Revel et al. 1999; Delaquis et al. 2000; Gambaro et al. 2001).

11.3.2 Alcohols

It is becoming common practice to harvest fully ripened grapes, producing wines with high flavor intensity but also resulting in high ethanol content, often above 15% (v/v) (De Barros Lopes et al. 2003). Ethanol is essential to enhance the sensory attributes of other wine components, but when in excess, it can produce a perceived "hotness" and mask the overall aroma and flavor of the wine (Guth and Sies 2002). However, heightened health consciousness, stricter drinking-and-driving laws, and increased tax rates associated with high-ethanol wines have augmented the demand for wines with reduced alcohol concentrations (Day et al. 2002). The reduction of alcohol in wine can be achieved by various physical processes; however, these methods often involve expensive equipment and processing. Loss or modification of aroma and flavor compounds during processing is an important consideration when using several of these techniques.

Biological solutions are being developed to overcome some of the limitations imposed by the physical techniques to lower ethanol content of wines. As the initial sugar concentration of grape must is an important target for achieving wines with lower alcohol content, glucose oxidase (GOX) provides one approach for reducing the glucose content of must (Pickering et al. 1999a–c). Conversion of glucose by an *Aspergillus niger GOX1*-encoded glucose oxidase in yeast to D-glucono-δ-lactone and gluconic acid has resulted in a decrease of almost 2% in ethanol concentration (Malherbe et al. 2003). Similarly, a significant decrease in ethanol concentration (up to 2%) and a concomitant increase in extracellularly accumulated glycerol have been achieved by the overexpression of glycerol-3-phosphate dehydrogenase isozymes (*GPD1* and *GPD2*) of *S. cerevisiae* (De Barros Lopes et al. 2000). Theoretically, a combination of these two strategies should lower the alcohol content by more than 4% (v/v). This research is in progress.

Higher alcohols (also known as fusel alcohols) are secondary yeast metabolites, and can have both positive and negative impacts on the aroma and flavor of wine (Fig. 11.4). Excessive concentrations of higher alcohols (exceeding 400 mg L^{-1}) can result in a strong, pungent smell and taste, whereas optimal levels (below 300 mg L^{-1}) impart fruity characters (Table 11.1) (Lambrechts and Pretorius 2000; Swiegers and Pretorius 2005).

The use of different *S. cerevisiae* strains during fermentation contributes considerably to variations in higher alcohol profiles in wine (Giudici et al. 1990). The concentration of amino acids (precursors for higher alcohols) in grape must also influences higher alcohol production (Schulthess and Ettlinger 1978). Non-*Saccharomyces* yeast also can contribute to the levels of higher alcohols. For example, mixed fermentation with *Pichia fermentans* and *S. cerevisiae* produced a substantial increase in higher alcohols such as 1-propanol, *n*-butanol, and 1-hexanol, compared with fermentation with *S. cerevisiae* alone (Clemente-Jimenez et al. 2005).

Branched-chain higher alcohols, isoamyl alcohol, active amyl alcohol, and isobutanol, are synthesized in the yeast cell through the Ehrlich pathway. Increased yeast branched-chain amino acid transaminase activity, in particular Bat1p and Bat2p, influences the production of higher alcohols in the flavor profiles of wine. Sensory analyses indicated that these wines produced with the strains in which the *BAT1* and *BAT2* genes were overexpressed had more fruity characteristics (peach and apricot aromas) than did the wines produced by the wild-type control strains (Lilly et al. 2006b).

Glycerol, a major product of alcoholic fermentation, is a colorless, odorless polyol that tastes slightly sweet and has an oily and heavy mouth-feel. Sensory tests have shown that glycerol imparts sweetness at a threshold of about 5.2 g L^{-1} in dry white wine. Glycerol metabolism by yeasts plays several important roles during the anaerobic fermentation of sugars: synthesis of phospholipids, maintenance of the cell's redox balance, and protection from high osmotic shock (Pronk et al. 1996). Construction of prototype yeast strains to redirect carbon flow away from ethanol to glycerol production has shown promise to produce low-ethanol wines (Remize et al. 2000; Eglinton et al. 2002). Bacteria can modulate the concentrations of alcohols such as glycerol, mannitol, and erythritol, and influence wine flavor; however, these are considered spoilage (see Sect. 11.4.5).

11.3.3 Carbonyl Compounds

Acetaldehyde is the major carbonyl compound found in wine (Table 11.1). It contributes to flavor with aroma descriptors such as "bruised apple" and "nutty" but can also be a sign of wine oxidation. The presence of acetaldehyde in white wines is an indication of wine oxidation. The process of converting ethanol to acetaldehyde in the presence of oxygen is also referred to as "madeirization," producing a slightly "almondy" flavor that resembles the fortified sweet wine, Madeira. Acetaldehyde in red wines can contribute to aroma complexity as long as the concentration does not exceed 100 mg L^{-1}.

As one of the major metabolic intermediates in yeast fermentation (Fig. 11.2), the conversion of acetaldehyde to ethanol is crucial for maintaining a redox balance in the cell (Pronk et al. 1996). During fermentation, the most rapid accumulation of acetaldehyde occurs when the rate of carbon dissimilation is at its maximum, after which it falls to a low level at the end of fermentation and then slowly increases over time. Acetaldehyde concentration is also yeast-strain-dependent (Then and Radler 1971).

The ability of *O. oeni* to metabolize acetaldehyde bound to sulfur dioxide can inhibit the growth of bacteria by releasing sulfur dioxide, which accumulates to form an inhibitory concentration (Osborne et al. 2006). The chemical and sensory impact of ethanol and acetic acid formed by the metabolism of acetaldehyde by lactic acid bacteria is believed to be limited, but the reduction in the acetaldehyde pool in wine is believed to influence final wine color. However, the conversion of acetaldehyde to acetic acid by *Acetobacter* species has important sensory implications (see Sect. 11.4.4).

Another important carbonyl compound in wine is diacetyl (2,3-butanedione), which is described in a wine as a "butter" or "butterscotch" aroma. At low concentrations it can be described as "nutty" or "toasty," but it becomes objectionable at concentrations over 4 mg L^{-1} (Martineau et al. 1995). Although yeasts synthesize some diacetyl (0.2–0.3 mg L^{-1}) in wine, most of it originates from the metabolic activities of lactic acid bacteria (Fig. 11.5) (Bartowsky and Henschke 2004b). The sensory perception of diacetyl in wine is also highly dependent upon the presence of other compounds in the wine, and is influenced by the age, style, and origin of the wine (Martineau and Henick-Kling 1995; Bartowsky et al. 2002). A variety of factors, including some that the winemaker can control, affect the concentration of diacetyl in wine, including oxygen exposure, fermentation temperature, sulfur dioxide levels, and duration of malolactic fermentation (reviewed by Bartowsky and Henschke 2004b).

11.3.4 Acids

The acidity of grape juice and wine has a direct impact on its sensory quality and physical, biochemical, and microbial stability (Boulton et al. 1998). This acidity, particularly pH, influences numerous wine parameters, including the survival and growth of all microorganisms, and the freshness of some wine styles. Wine contains a large number of organic and inorganic acids. The predominant nonvolatile organic acids are tartaric acid and malic acid, accounting for 90% of the titratable acidity of grape juice. Citric acid and lactic acid also contribute to the acidity of grape juice; succinic and keto acids are present only in trace amounts in grapes, but concentrations are higher in wines as a result of fermentation (Boulton et al. 1998).

The decarboxylation of malic acid to lactic acid forms the basis of malolactic fermentation (Fig. 11.5). All wine lactic acid bacteria are able to perform the malolactic reaction; however, *O. oeni* is the preferred species. The increase of lactic acid content in the wine results in a softer mouth-feel.

Most yeasts can utilize significant concentrations of malic acid. Wine strains of *S. cerevisiae* typically degrade 3–45% of malic acid during fermentation, and most strains of *Schizosaccharomyces pombe* and *Schizosaccharomyces malidevorans* can completely degrade it to ethanol and carbon dioxide (Radler 1993). Several groups of researchers have explored the genetic engineering of wine yeast to degrade malate simultaneously during alcoholic fermentation as a means to alter wine acidity; malic acid to ethanol (malo-ethanolic) or to lactic acid (malolactic wine yeast)

(Dequin and Barre 1994; Ansanay et al. 1996; Volschenk et al. 1997). These approaches are discussed further in Chap. 23.

The other major organic acid, tartaric acid, is relatively stable to microbial activity and when metabolized by some bacteria it is often part of a general spoilage scenario (see Sect. 11.4.4).

The production of succinic acid is highly variable among strains of *S. cerevisiae* but *Saccharomyces uvarum* or *Saccharomyces bayanus* strains tend to produce higher concentrations (Eglinton et al. 2000). Succinic acid has been reported to have an "unusual salty, bitter taste" in wine (Whiting 1976).

Citric acid is commonly present in wine in the range of 0.1–0.7 g L^{-1}, and most strains of *O. oeni* are able to metabolize this acid during malolactic fermentation. The metabolism of citric acid normally occurs after that of malic acid, and results in the formation of acetic acid and diacetyl (Fig. 11.5). As some yeasts can metabolize citric acid, unexpected changes in the concentration of diacetyl and titratable acidity could result.

Volatile acidity describes a group of volatile organic acids of short carbon chain-length. The volatile acid content of wine is usually between 500 and 1000 mg L^{-1} (10–15% of the total acid content); of this, acetic acid usually constitutes about 90% of the volatile acids (Henschke and Jiranek 1993; Radler 1993). Other volatile acids, principally propionic and hexanoic acids, are produced as a result of fatty acid metabolism by yeast and bacteria. Acetic acid at elevated concentrations imparts a vinegar-like character to wine and becomes objectionable at concentrations of 0.7–1.1 g L^{-1} (Corison et al. 1979) (Table 11.1). Although *Saccharomyces* can produce acetic acid, excessive concentrations in wine are largely the result of the metabolism of ethanol by acetic acid bacteria (Sect. 11.4.4). A small increase in volatile acidity (acetic acid) is often observed after the completion of malolactic fermentation usually from citric acid metabolism (Ramos and Santos 1996) (Figs. 11.5 and 11.7).

11.3.5 Sulfur-Containing Compounds

Wine yeast and bacteria can metabolize sulfur compounds which can impact on the sensory quality of wine. The important volatile sulfur compounds found in wine are (1) hydrogen sulfide; (2) methanethiol; (3) dimethylsulfide, dimethyldisulfide, and dimethyltrisulfide; (4) methylthioesters (*S*-methyl thioacetate, *S*-methyl thioproonanoate, and *S*-methyl thiobutanoate); and (5) the "fruity volatile thiols" in wine (Table 11.1) (Swiegers et al. 2005). The more desirable fruity volatile thiols will be discussed here, and those sulfur-containing compounds that are considered as off-flavors will be discussed in Sect. 11.4.2.

The volatile thiols are one of the most potent groups of aroma compounds found in wine, some imparting negative aromas, others contributing positively. Volatile thiols 4-mercapto-4-methylpentan-2-one (4MMP), 3-mercaptohexan-1-ol (3MH), and 3-mercaptohexyl acetate (3MHA) are of particular importance to wine aroma, par-

ticularly varietal character such as in Sauvignon Blanc wines (Dubourdieu et al. 2000). Thiol compounds 4MMP and 3MH are present as nonvolatile, cysteine-bound conjugates, and yeast are responsible for the cleaving of thiol from the precursor (Darriet et al. 1995). Four genes have been shown to influence the release of the volatile thiol 4MMP (Howell et al. 2005). Overexpression of the *Escherichia coli tnaA* gene (encoding cysteine-β-lyase) in a commercial wine yeast resulted in up to a 25-fold increased release of 4MMP and 3MH from the precursor in model ferments. Sauvignon Blanc wine made with this modified yeast displayed an intense passion-fruit aroma, compared with the relatively neutral aromas produced by the control strain (Swiegers et al. 2007). 3MHA is formed by yeast from 3MH by the action of the ester-forming alcohol acetyltransferase, encoded by the *ATF1* gene (Swiegers et al. 2005). This research established for the first time a link in ester and volatile thiol metabolism in yeast. There are large variations in the ability of commercial wine yeast to convert 3MH and, in most cases, this did not correspond to the ability to release 4MMP (Swiegers et al. 2005).

11.3.6 Monoterpenoids

Non-odorous and nonvolatile glycoconjugates, aglycons bound to one or two sugars, represent an important source of fragrant compounds in grape must. The aglycons are grouped broadly by structure: norisoprenoids (e.g. damascenone), volatile phenols and other benzene derivatives (e.g. raspberry ketone), monoterpenes (e.g., linalool, nerol, geraniol α-terpineol, and citronellol), and aliphatics (e.g., hexanol, 2-phenylethanol, and benzylalcohol) (Günata et al. 1985) (Table 11.1). Liberation of the bound aglycon during winemaking by yeast or bacterial enzymatic hydrolysis involves the sequential release of the sugar moieties (Günata et al. 1990; D'Incecco et al. 2004). Release of the aglycon, such as the monoterpenes, influences the floral and fruity notes of wine.

11.4 Off-Flavor Compounds Produced by Yeast and Bacteria

Many secondary metabolites produced by yeast and bacteria affect the sensory qualities of wines. Those compounds that are less desirable are discussed in this section.

11.4.1 Volatile Phenols

Volatile phenols are formed from the hydroxycinnamic acid precursors present in grape must predominantly by yeast during fermentation and contribute to off-flavors such "Band-aid," "barnyard," or "stable" (Dubois 1983) (Fig. 11.6). Several bacteria

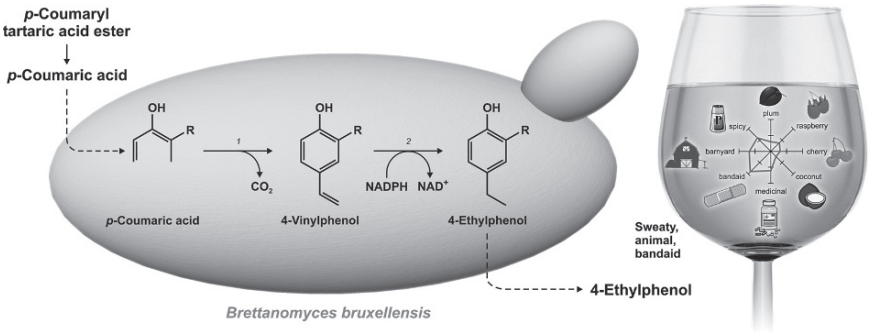

Fig. 11.6 Pathway for the formation of the volatile phenol, 4-ethylphenol, the main aroma compound responsible for the "Brett" character in red wine. The phenolic acid precursor, *p*-coumaric acid, is transported into the cell by active transport, decarboxylated to the vinyl derivative by hydroxycinnamic acid decarboxylases, and enzymatically reduced to the ethyl derivative. Some yeast and bacteria can undertake the first enzymatic reaction; however, it is the second step that *Brettanomyces bruxellensis* undertakes, resulting in the typical "Brett" aroma characters in wine. Enzymes involved in the pathway: 1, phenol decarboxylase; 2, vinyl phenol reductase

and fungi have the genes encoding phenolic acid decarboxylases; however, the *Brettanomyces/Dekkera* yeast species that undertake the final step to form volatile ethylphenol in wine are the main contributors to these aromas (Chatonnet et al. 1995; Du Toit and Pretorius 2000). Another non-flavonoid hydroxycinnamic acid, ferulic acid, is converted to 4-vinylguaiacol and 4-ethylguaicol with distinctive aromas phenolic, clove-like, and sweet (Table 11.1), in a manner similar to *p*-coumaric acid.

11.4.2 Sulfur-Containing Compounds

Sulfur-containing flavor compounds typically occur in wine at very low concentrations, have very low detection thresholds, and generally confer a negative sensory contribution to wine with sensory descriptors such as "cabbage," "rotten egg," "sulfurous," "garlic," "onion," and "rubber" (Vermeulen et al. 2005) (Table 11.1). However, some sulfur-containing compounds can contribute positive aromas to wine, such as strawberry, passion-fruit, and grapefruit, as mentioned in Sect. 11.3.5. The development of sulfur compounds by yeasts has been reviewed recently (Swiegers and Pretorius 2007).

Hydrogen sulfide is probably the best-known sulfur compound in wine, a highly volatile thiol that imparts a "rotten egg" aroma (Table 11.1 and Fig. 11.4). Owing to the frequent occurrence of this compound and the low aroma threshold (50–80 μg L^{-1}), it is probably one of the most common problems associated with the winery (Henschke and Jiranek 1991). However, the problem is relatively easily dealt with through the use of copper or aeration (Monk 1986).

Hydrogen sulfide is a highly reactive species, which can take part in a range of reactions to generate compounds that impact on the flavor of a wine, such as

mercaptans (ethanethiol), dimethyl sulfide, and polysulfides (dimethyl disulfide, dimethyl trisulfide, and dimethyl tetrasulfide) (Table 11.1). Unlike hydrogen sulfide, these compounds cannot be removed by copper-fining. To address the issue of hydrogen sulfide production during winemaking, classical biological non-GMO techniques were recently used to develop several *S. cerevisiae* strains that do not produce hydrogen sulfide in low nitrogen grape juices (Cordente et al. 2007). Trials in Chardonnay juice fermented with these three *S. cerevisiae* and the original parent (PDM) strains showed that the developed strains were able to ferment the juice as efficiently as the parent did with no detectable hydrogen sulfide production. These yeasts are currently being commercially produced and trialed.

Methionine can be metabolized by yeast to form sulfur-containing fusel alcohol, methionol or 3-methylthio-1-propanol, which has "cauliflower" and "cabbage" odors, and by *O. oeni* and *Lactobacillus* species to methanethiol, dimethyl sulfide, 3-(methylsulfanyl)propan-1-ol, and 3-(methylsulfanyl)-propanoic acid (Table 11.1) (Pripis-Nicolau et al. 2004). Cysteine can be the precursor of S-containing heterocycles, such as thiazoles, and there is evidence that *O. oeni* is able to metabolize these compounds, resulting in aroma descriptors, including "sulfury," "floral," "fruity," "toasted," and "roasted" (Pripis-Nicolau et al. 2004).

11.4.3 N-Heterocyclic Compounds

The metabolism in wine of certain amino acids, notably lysine and ornithine, can lead to the formation of several extremely potent and unpleasant nitrogen-heterocycle "mousy" off-flavor compounds (Costello et al. 2001) (Fig. 11.7). The compounds are perceived on the back palate as a persistent aftertaste reminiscent of caged mice. The heterofermentative lactic acid bacteria, *O. oeni*, *Leuconostoc mesenteroides*, and some *Lactobacillus* species are capable of synthesizing the three known sensorially important nitrogen-heterocycle compounds: 2-acetyltetrahydropyridine (ACTPY), 2-acetyl-1-pyrroline (ACPY), and 2-ethyltetrahydropyridine (ETPY) (Costello et al. 2001). The homofermentative lactic acid bacteria species, *Pediococcus* and *Lactobacillus plantarum* produced little or no detectable off-flavor compounds. Wine-associated *Brettanomyces* have also been shown to produce compounds responsible for mousy off-flavor (Grbin and Henschke 2000); however, they do not appear to be the major source of this spoilage.

11.4.4 Metabolism of Acids

The metabolism of wine organic acids, particularly by bacteria, tends to appear as part of a general spoilage scenario because of the production of undesirable secondary metabolites. These pathways are shown in Figs. 11.7 and 11.8.

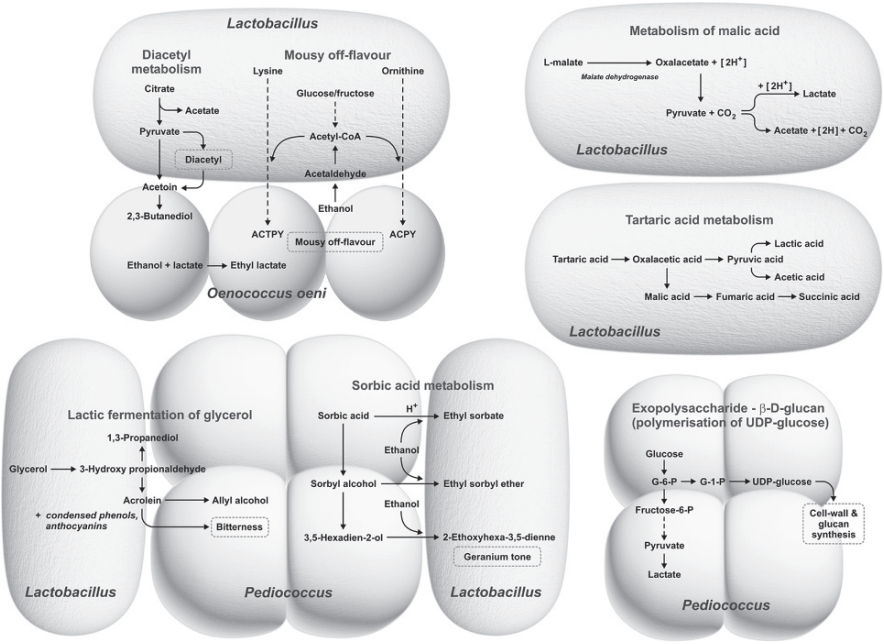

Fig. 11.7 Metabolism of organic acids and sugars by different genera of lactic acid bacteria and the formation of various undesirable aromas and flavors in wine

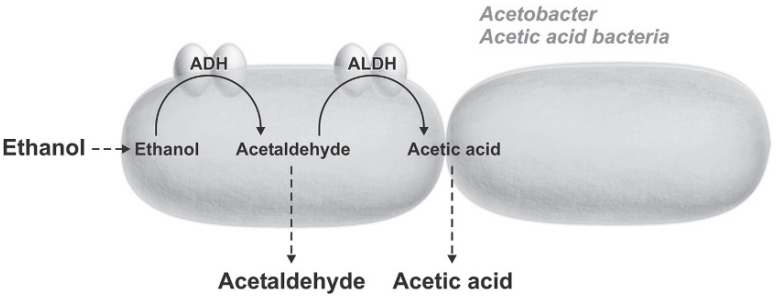

Fig. 11.8 Acetic acid formation from ethanol by acetic acid bacteria. *ADH* alcohol dehydrogenase, *ALDH* acetaldehyde dehydrogenase

Tartaric acid is relatively stable to microbial activity and can only be metabolized aerobically by some *Lactobacillus* species with the production of acetic acid, lactic acid, and succinic acid (Dittrich 1995). Citric acid can be metabolized by numerous genera of lactic acid bacteria, resulting in the production of acetic acid and diacetyl, both of which can have an important effect on wine flavor as desirability is dependent on concentration and wine style (Fig. 11.7) (Bartowsky and Henschke 2004b).

Sorbic acid, a short-chained unsaturated fatty acid, can be used as a chemical preservative in sweetened wines at bottling to prevent fermentation occurring in the bottle. It can be metabolized by lactic acid bacteria, including *O. oeni*, resulting in the formation of 2-ethoxyhexa-3,5-diene, which has an odor reminiscent of geranium leaves (Riesen 1992) (Fig. 11.7).

As mentioned in Sect. 11.3.4, acetic acid is an important component of wine volatile acidity. Its concentration can be increased due to the metabolism of citric acid, particularly during malolactic fermentation, malic acid via malate dehydrogenase, or tartaric acid metabolism (Figs. 11.5 and 11.7). Acetic acid bacteria are well known for their use in the production of vinegar, and are considered spoilage organisms during grape vinification. Under favorable conditions, such as in the presence of even low concentrations of oxygen, *Acetobacter* species are able to metabolize ethanol via acetaldehyde to acetic acid (Fig. 11.8) (recently reviewed by Bartowsky and Henschke 2008). Wines spoiled because of the metabolism of *Acetobacter* species have dulled fruity characters, such as bruised apple and sherry-like aromas (acetaldehyde) and vinegary tones (acetic acid) (Table 11.1). Increased concentrations of ethyl acetate are commonly associated with this type of spoilage (Bartowsky et al. 2003; Bartowsky and Henschke 2004a). This group of bacteria is the most likely cause of high acetic acid concentrations in wine.

11.4.5 Sugar Metabolism

Several sugars and polyols present in wine can be a source of spoilage, particularly because of the growth of bacteria. These spoilage scenarios tend to be rare and are avoidable with good cellar hygiene, and effective wine pH and sulfur management.

Metabolism of glycerol is not widespread among the wine lactic acid bacteria (some *Lactobacillus* and *Pediococcus* species) and results in the formation of acrolein, which in turn reacts with red wine phenolics to form a bitter complex (Fig. 11.7) (Sponholz 1993; Vizoso Pinto et al. 2004).

Fructose reduction by both heterolactic and homolactic bacteria results in the formation of mannitol, a six-carbon sugar alcohol (polyol), perceived as sliminess with a vinegary-estery, slightly sweet taste (also known as mannite disease) (von Weymarn et al. 2002; Wisselink et al. 2002) (Fig. 11.5). In general, the homofermentative lactic acid bacteria will produce only small amounts of mannitol, whereas some heterofermentative lactic acid bacteria produce substantial amounts (Wisselink et al. 2002). In *O. oeni*, fructose can be metabolized by two different pathways: heterolactic fermentation or mixed heterolactic/mannitol fermentation (Richter et al. 2003). The switch from one fermentation type to the other occurs at the metabolic level and is related to the growth rate.

The production of exopolysaccharides is almost exclusively due to *Pediococcus* growth and metabolism of glucose in wine. The pathway for the production β-D-glucan and the polymerization is well characterized (Fig. 11.7) (Walling et al. 2005a). Wines spoiled because of exopolysaccharide production are referred to as “ropy,”

are viscous, and have a thick texture. The presence of a plasmid carrying the *dps* gene (glucosyltransferase responsible for the polymerization of glucan residues) is required for *Pediococcus* production of β-D-glucan and ropy wines (Walling et al. 2005b). Some strains of *O. oeni* have been shown to carry the *dps* gene (Walling et al. 2005b). Recent trials in our laboratory have demonstrated that *O. oeni* strains vary in their ability to produce polysaccharides in a chemically defined wine medium (Costello, personal communication, 2007).

11.5 Conclusion

Continued investigation into the causes and interactions that give rise to desirable and undesirable flavors in wine must be regarded as more than a challenging preoccupation for wine scientists. In a world where consumers are becoming increasingly knowledgeable – and even concerned – about the origin, production, and quality of food and beverages, wine science has an invaluable role.

Clearly, the best wine is achieved by maximizing the desired aromas and flavor characteristics and minimizing off-odors and off-flavors. While grape juice is the starting point, it is the metabolism of grape compounds by yeast and bacteria that is central to the development of wine flavor. This chapter has sought to summarize the yeast, bacterial, and chemical interactions that contribute most significantly to wine aromas and flavors. It is evident that while much is known, science is still on a journey of discovery to fully understand the complex process of turning grapes into wine that is consistently pleasing to consume and sufficiently stable for transport and storage in what has become a global market.

Today's winemakers must negotiate a far more complex world than did their predecessors with rising consumer demands for consistency of quality, affordability, and enjoyability. In today's health-conscious society, even the perceived "hotness" of many high-alcohol wines has become cause for investigation as wine scientists evaluate biological processes to maintain desirable flavor and aroma characteristics while lowering the ethanol content.

As more and more beverage options come onto the market, it is becoming increasingly challenging to connect with consumers. Putting high-quality, affordable wine onto retail shelves does not necessarily deliver sales or, more importantly, repeated business.

As a product of natural origin, wine is subject to seasonal variability, which is seen as a positive by many wine consumers. However, the variability inherent in the fermentation of grapes and even the winemaker's choice of bottle and closure can all have a potentially negative impact on aroma and flavor, as perceived by the consumer. Ongoing research into the development of new starter strains and other biological processes that can enhance desirable characters and reduce or eliminate off-flavors is part of the restless innovation of the wine sector.

In future, greater understanding and control over all aspects of fermentation, maturation, and storage of wine will be essential to retain consumer confidence, enjoyment, and sales.

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Chapter 12
Pyroglutamic Acid: A Novel Compound in Wines

Peter Pfeiffer and Helmut König

12.1 Introduction

Wine contains a complex mixture of nearly 1,000 compounds, which include sugars, acids, alcohols, ketones, aldehydes, esters, amino acids, amines, phenol carbonic acids, flavonoids, anthocyanidins and cathechins. Many of these compounds have an influence on the aroma and bouquet of wine. Identification of wine-related compounds is by far not complete. Recently, pyroglutamic acid has been detected as a so far overlooked novel compound in wine samples, which is described below.

12.2 Formation of Pyroglutamic Acid

Pyroglutamic acid (5-oxoproline, 5-pyrrolidone-2-carboxylic acid, PCA) can variably change the aroma of food and beverages. Pyroglutamic acid is formed by cyclization of glutamic acid or glutamine, which can proceed chemically or enzymatically. In acidic food or beverages pyroglutamic acid is produced in significant amounts at elevated temperatures and increased glutamic acid or glutamine contents. Favourable conditions are found in commercial tomato puree, which is finally autoclaved before filling. Considerable amounts of pyroglutamic acid are also found in pasteurized grape juice, when grapes are harvested from grapevines with sufficient nitrogen fertilisation. (Pfeiffer and Orben 2000).

Pyroglutamic acid is determined by isocratic HPLC equipped with a refractometric detector (Fig. 12.1) (Pfeiffer and Radler 1985; Pfeiffer and Orben 2000).

As it is the case with hydroxymethylfurfural, the presence of pyroglutamic acid in food was previously believed to be caused by intensive heating treatment (Kern 1964). Certain processes of wine production include heating of grape mash before starting fermentation in order to prevent growth of undesirable microorganisms or increase the

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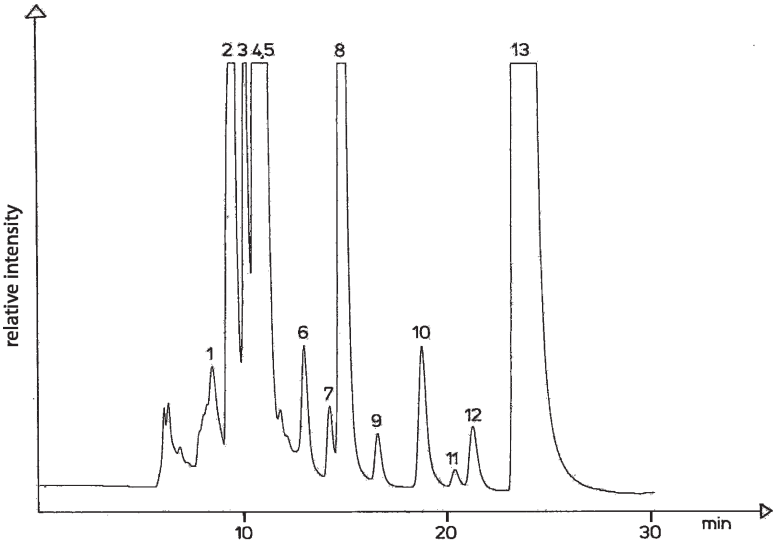

Fig. 12.1 Chromatogram of a Riesling wine containing pyroglutamic acid (0.61 g L^{-1}). (Column: HPX-87H, Bio-Rad). Citrate 1, tartrate 2, glucose 3, malate 4, fructose 5, succinate 6, lactate 7, glycerol 8, acetate 9, pyroglutamate10, meso-2,3-butandiol 11, 2,3-butandiol 12, ethanol13

depth of colour in red wines. This is also the case with unfermented grape juice for sweetening. For risk assessment prior to a required heating treatment, the content of amino acids can be determined. In the case of elevated glutamic acid or glutamine concentrations alternative sterilization treatments should be applied instead of a heating treatment. Of course, the heating treatment is under favourable conditions not the only reason for formation of pyroglutamic acid. PCA was also found in wine samples, which were produced without any heating treatment. Therefore, an additional possibility for formation of PCA has to be considered (Pfeiffer and Grünewald 2001).

To elucidate this possiblility we investigated the influence of temperature and environment on the production of PCA. Different wine samples and exemplary solutions were incubated at different temperatures between 4 and 30°C for 4 weeks with and without added glutamic acid or/and glutamine. The subsequent analysis revealed that in aqueous solutions no pyroglutamic acid was formed in the presence of glutamic acid or glutamine solely. In the presence of a mixture of both amino acids a spontaneous production of PCA was observed at 20°C, which was even enhanced in wines. The PCA concentration in red wines was 1.0 g L^{-1} after an incubation time of 4 weeks at 30°C in the presence of added glutamine (1.0 g L^{-1}). At concentrations of 1 g glutamine and 1 g glutamic acid per L the PCA concentration was additionally increased by 10% (Table 12.1). It was obvious that glutamic acid or glutamine was chemically converted to pyroglutamic acid within a few weeks (Fig. 12.2). This reaction is accelerated by increasing the temperature and acid content of wines. The conversion of glutamine is much faster than that of glutamic acid (Pfeiffer and Grünewald 2001).

The potential for formation of sensory relevant concentrations of PCA depends primarily on the content of glutamic acid or glutamine in must. There is also an impact on the consumption rate of yeasts during ethanol fermentation. A previous

Table 12.1 Production of pyroglutamic acid from glutamine (1 g L^{-1}) or glutamic acid (1 g L^{-1})

Sample	Pyroglutamic acid [g L^{-1}]					
	4°C		20°C		30°C	
	1. d	28. d	1. d	28. d	1. d	28. d
H_2O with glutamine	0,0	0,0	0,0	0,1	0,0	0,0
H_2O with glutamic acid	0,0	0,0	0,0	0,0	0,0	0,0
H_2O with glutamine and glutamic acid	0,0	0,0	0,0	0,3	0,0	0,5
White wine	0,0	0,1	0,0	0,1	0,0	0,1
White wine with glutamine	0,0	0,2	0,0	1,0	0,0	1,1
White wine with glutamic acid	0,0	0,1	0,0	0,1	0,0	0,1
white wine with glutamine and glutamic acid	0,0	0,2	0,0	1,0	0,0	1,2
Red wine	0,1	0,0	0,1	0,0	0,1	0,1
Red wine with glutamine	0,1	0,1	0,1	0,7	0,1	1,0
Red wine with glutamic acid	0,1	0,1	0,1	0,0	0,1	0,0
Red wine with glutamine and glutamic acid	0,1	0,1	0,1	0,6	0,1	1,1

d = day

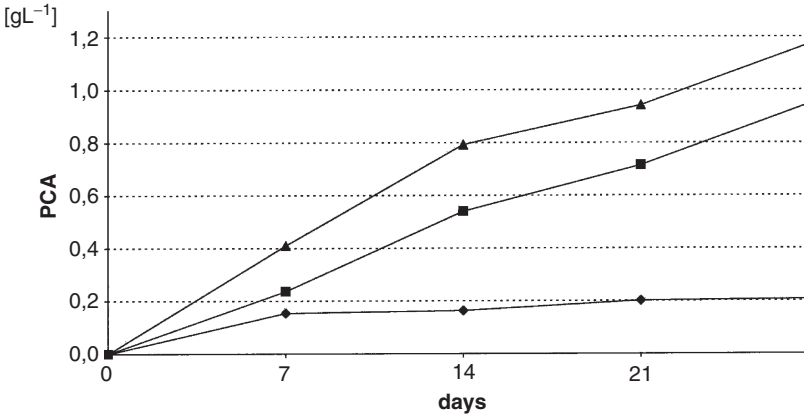

Fig. 12.2 Formation of pyroglutamic acid in white wine after addition of glutamin (1 g/L) and glutamic acid (1 g/L) at different temperatures. *Filled square* = 20°C, *filled diamond* = 20°C, *filled triangle* = 30°C

heating treatment is not a prerequisite for formation of PCA, since it can be formed under conditions applied during ethanol fermentation. A formation of PCA from glutamic acid by lactic acid bacteria seems to be possible and was demonstrated, e.g. in cheese (5.0 g L^{-1}) (Mucchetti et al. 2000, 2002).

12.3 Distribution of Pyroglutamic Acid and Sensory Evaluation

In order to get an overview of the frequency and concentration of PCA in fermented beverages 58 samples (45 white wines, 10 red wines; 1 sparkling wine, 1 cidre, 1 beer) from different EC countries were analyzed (Table 12.2). The highest

Table 12.2 Determination of pyroglutamic acid in alcoholic beverages

Sample	Number	Pyroglutamic acid [g L^{-1}]
White wines	11	<0,05
White wines	8	0,05–0,10
White wines	5	0,11–0,20
White wines	12	0,21–0,30
White wines	3	0,31–0,40
White wines	4	0,41–0,50
White wine	1	0,57
White wine	1	0,61
Sparkling wine	1	0,32
Red wines	6	<0,10
Red wines	3	0,11–0,20
Red wine	1	0,22
Cidre	1	0,05
Beer	1	0,16
Total number	58	

PCA concentrations were found in Kabinett (German quality distinction) (0.57 g L^{-1}) and late picking wines (0.61 g L^{-1}) of the wine variety Riesling (Fig. 12.2). In addition, the PCA content of 4 white wines was above 0.4 g L^{-1} and 3 white wines above 0.3 g L^{-1}. Forty percent of the white wines (19) and 60% of the red wines (6) as well as the cidar sample had a maximal concentration of 0.1 g l^{-1}, while in the case of 16% of white wines (7) and 10% of red wines (1) PCA could not be detected. The beer contained 0.16 g PCA per L, most probably caused by thermal treatment of the mash (Pfeiffer et al. 2002).

To evaluate the influence of PCA on wine aroma, samples containing different amounts of PCA were subjected to organoleptic tests. The tasting of tomato puree revealed an aroma shift at 0.5 g PCA per kg of product. An aqueous solution of PCA (1 g L^{-1}) had a penetrative metallic odour. The sample had a salty tang, the finish was bitter. The same was true for commercial grape juice. However, the wine tasting showed differences depending on vine variety and quality distinction. In the case of dry fruity white wines (Riesling) a penetrative odour with a bitter taste and finish was observed. A sample of the vine variety Gutedel exhibited an aroma shift at a concentration of 0.1–0.2 g L^{-1}. The corresponding controls with glutamine instead of PCA exhibited no significant shift of aroma, but they appeared less fruity. Sensory testing of wine samples containing more than 0.3 g PCA per L confirmed these observations. It seemed that the influence on aroma depended on vine variety and age of the wine. The sensory impact of PCA was masked particularly in fruity wines. Higher concentrations of PCA have a negative influence on sensory quality of wines. Therefore, production of higher amounts should be prevented. So far, the influence of pyroglutamic acid on wine quality was neglected, since it was not detected during conventional amino acid or amine analysis using ninhydrin and OPA as derivatization reagent, because it possesses a secondary nitrogen atom (Pfeiffer and Orben 2000).

12.4 Formation of Pyroglutamic Ethyl Ester and Influence on the Aroma Profile

Besides causing a shift in the tasting of wines, PCA also had an influence on the bouquet in the presence of ethanol. In some cases the sensory threshold was 0.2 g/L. It was shown that PCA formed a pyroglutamic acid ethyl ester (5-oxo-proline ethylester, ethyl (S)-2-pyrrolidone-5-carboxylate, EPC with ethanol). A method for the detection of pyroglutamate ethyl ester by gas chromatography was developed (Pfeiffer et al. 2007). EPC was enriched from wine samples by solid phase extraction using C_{18}-cartridges. Figure 12.3 shows a separation of EPC from a Riesling containing 1.6 mg EPC per L. The detection limit was 0.4 mg EPC per L wine (Pfeiffer et al. 2007).

Investigations were carried out, whether EPC was formed chemically or enzymatically. Pyroglutamic acid and ethanol were incubated at pH 3.5 (citrate buffer) for 4 weeks at 20°C. The subsequent analysis showed that no EPC was formed under these conditions. The same result was obtained when PCA was added to wine. Therefore, a spontaneous formation was not very likely. In contrary, EPC could be detected when PCA and yeast cells were added to wine samples. In this case *Saccharomyces cerevisiae* (10^8 cells ml^{-1}) formed EPC. The formed EPC content was not directly dependent on concentration of PCA. Other factors also played a role.

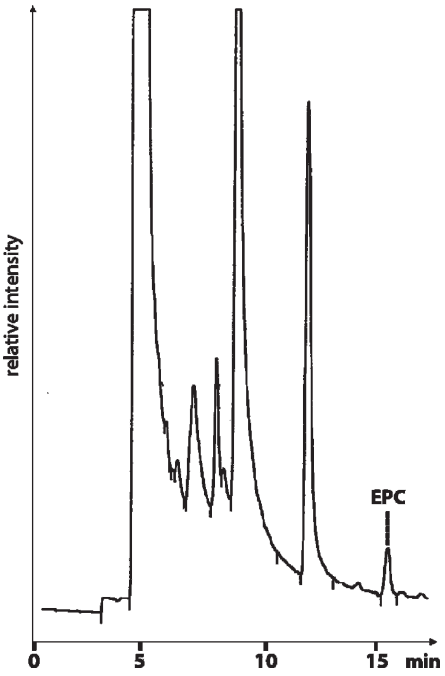

Fig. 12.3 Chromatogram of a Riesling wine containing pyroglutamic acid ethyl ester (EPC; 1.6 mg L^{-1}). (Column: Zebron-Wax, Phenomenex)

Sensory tasting revealed that wines containing 2 mg EPC per litre had a bitter taste as well as a bitter finish. The earlier observed aroma shift of wines containing PCA could be partly caused by EPC (Pfeiffer et al. 2007).

12.5 Metabolization of Pyroglutamic Acid by Yeasts

The potential risk of formation of measurable concentrations of PCA or EPC in wine depends primarily on content of glutamine or glutamic acid in the grape must as well as on consumption by wine-associated microorganisms.

To answer the question, whether yeast can degrade PCA, different grape must samples containing 2 g PCA were inoculated with 29 yeast strains belonging to different genera (Pfeiffer et al., 2004). The incubation was performed anaerobically at 20°C for 4 weeks. The culture supernatants were analyzed by HPLC (Table 12.3; Fig. 12.4). Out of 20 strains of *Saccharomyces cerevisiae* only 3 could degrade PCA. Under aerobic conditions 85% of added PCA amount was removed; under anaerobic conditions only 30%. Eight strains of the remaining 9 yeasts strains belonging to other genera degraded PCA up to 100% under aerobic conditions, while without oxygen only 4 were able to use PCA. Mixed cultures (9 non-*Saccharomyces* yeasts and *Saccharomyces cerevisiae*) were able to degrade 90% of the PCA under aerobic conditions and 25–30% under anaerobic conditions The aerobic degradation rate was higher than that of *S. cerevisiae* alone (Table 12.3; Fig. 12.4). Therefore, degradation of PCA under natural conditions by non-*Saccharomyces* yeasts in the must is most likely. It may be worthwhile to use PCA-degrading yeast strains for removal of PCA in must in order to avoid a negative influence on the aroma profile (Pfeiffer and Grünewald 2001).

Table 12.3 Degradation of pyroglutamic acid (g L^{-1}) by different yeast strains

Culture[a,b]	Strain designation	Aerobic conditions	Anaerobic conditions
Saccharomyces cerevisiae	H1/6–4	0,7	0,3
Saccharomyces cerevisiae	H1/6–32	1,7	0,6
Saccharomyces cerevisiae	H1/6–48	1,2	0,5
Zygosaccharomyces bailii	H1/6–6	2,0	1,2
Zygosaccharomyces bailii	H2/3–77	1,8	0,0
Schizosaccharomyces pombe	H1/6–13	1,6	1,2
Klyveromyces marxianus	H1/6–50	1,3	0,6
Klyveromyces lactis	H2/4–48	2,0	0,0
Debaryomyces hansenii	H1/2–72	0,1	0,0
Cryptococcus laurenti	H2/4–43	1,1	0,0
Torulaspora pretoriensis	H1/5–56	2,0	0,3
yeast mixture	A	1,8	0,5
yeast mixture	B	1,8	0,6

Non degrading yeast strains: H1/5–22, H1/5–47, H1/5–57, H1/6–25, H1/6–27, H1/6–29, H1/6–30, H1/6–34, H1/6–40, H1/6–42, H1/6–46, H1/6–49, H1/6–52, H1/6–54, H1/6–55, H1/6–72, H2/3–40, H1/3–1.Mixed yeast cultures A und B were parallel incubations. g per l

[a]Initial PCA concentration: 2 g L^{-1}

[b]Incubation: 4 weeks at 20°C.

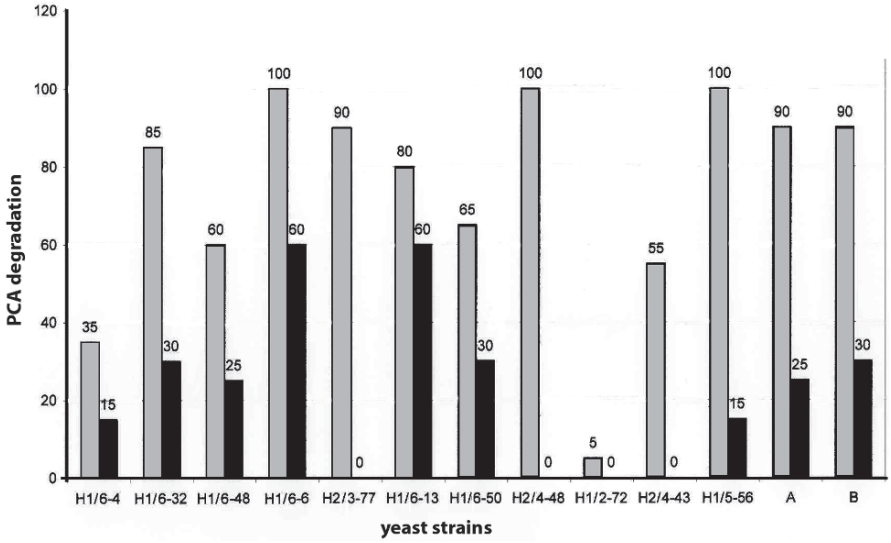

Fig. 12.4 Degradation of PCA (%) by different yeast strains. *Filled grey square* = aerobic conditions, *Filled black square* = anaerobic conditions. A and B = yeast mixture (cf. Table 12.3)

12.6 Pyroglutamic Acid and Health Aspects

Pyroglutamic acid occurs naturally in vegetables, fruits, diary products and meat. It is also found in human brain, the cerebrospinal fluid and in blood. It is a component of the thyrotropin-releasing factor (TRF), which stimulates the adenohypophysis to release the hormon thyrotropin, and of the gonadotropin-releasing factors (GnRF), which produces lutropin and follitropin (Voet and Voet 1995).

Pyroglutamic acid is believed to improve learning and memory like the chemically related nootropic drug piracetam (2-oxo-1-pyrrolidineacetamide) (Pepeu and Signoli 1989; Drago et al. 1988). The recommended daily allowance of a preparation of arginine-L-pyroglutamic acid is 400–1,000 mg according to the producer. The arginine salt is applied in the case of senility, mnemonic difficulties, mental problems connected with alcoholic disease and dyslexia. Whether consumption of PCA containing wine has similar effects has to be investigated.

12.7 Conclusions

Although pyroglutamic acid (PCA) can occur in significant amounts in wine from different grape varieties, its presence was overlooked for a long time. It is obvious that it has an impact on wine aroma at elevated concentrations, which is also true for the corresponding ethyl ester (EPC). Whether it has also a nootropic effect for wine consumers has not been investigated so far.

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Chapter 13
Polysaccharide Production by Grapes, Must, and Wine Microorganisms

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13.1 Introduction

Several species of fungi, yeasts, and bacteria develop successively throughout the winemaking process, starting with the grapes themselves, and contribute, via their own metabolic pathways, to the final chemical composition of the wine. Polysaccharides (PS) form a class of molecules produced by microbial metabolisms which affect wine quality. They constitute the highest molecular weight component of wine and consist of repeated sugar units, composed of a single monosaccharide (homopolysaccharides) or several different ones (heteropolysaccharides). The type of glycosidic bond, chain length, and degree of branching are also important characteristics of the molecular structure.

The PS content in must and wine varies throughout the winemaking process because of synthesis and hydrolysis reactions. Only the more soluble grape PS are extracted in must (pectins and arabinogalactan). From picking until the end of alcoholic fermentation, pectins are gradually degraded into smaller PS, because of the action of endogenous or exogenous pectolytic enzymes (Pellerin and Cabanis 1998). The first microbial event that significantly modifies the wine's final PS composition is when the grapes are infected by *Botrytis* (Sect. 13.2): the pectins are hydrolysed and specific neutral polymers are formed (Dubourdieu 1982). In the next stage, during alcoholic fermentation and aging on the lees, yeasts release mannoproteins (MP). These molecules constitute the second group of wine PS in quantitative terms, after those originating from grapes (Sect. 13.3) (Ribereau-Gayon et al. 2000). Pectolytic yeast species may also hydrolyse certain grape PS, thus providing substrates for the subsequent growth of other microbial species (Louws et al. 2006). As a result of the natural selection among bacteria occurring during alcoholic fermentation, *Oenococcus oeni* becomes dominant for the subsequent malolactic fermentation (MLF). MLF produces many changes in wine PS composition, indicating that, similar to *Botrytis* and yeast, *O. oeni* modifies both neutral and pectic PS

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content (Sect. 13.4) (Dols-Lafargue et al. 2007). Nevertheless, for a long time, bacterial PS in wine were associated only with a spoilage phenomenon known as "ropiness" (Sect. 13.5).

This chapter focuses on PS synthesis by microorganisms in grapes and wine, describing the structures of the polymers produced, as well as the metabolic pathways identified, including molecular aspects and regulation. PS are usually, at least partially, linked to the cells, thus forming a capsule, while the remainder is released into the surrounding medium (Sutherland 1993). The putative or demonstrated physiological benefits linked to capsular PS formation are discussed and, finally, the impact of the released PS on wine quality is examined.

13.2 PS Produced by *Botrytis cinerea*

Botrytis cinerea is a deuteromycete (Hyphomycete) fungus. It is an important plant pathogen with an exceptionally broad host range. Its development on grapes may be dreaded (gray rot) or desired ("noble rot") (Ribereau Gayon et al. 2000).

In terms of PS, must extracted from rotten grapes no longer contains pectic PS, and its galactose and mannose concentrations are modified. Moreover, these musts contain exopolysaccharides (EPS), specifically produced by *B. cinerea*. When the fungus is cultivated on liquid medium, it is possible to separate two groups of soluble PS by alcohol precipitation (Dubourdieu 1982):

- The more alcohol-soluble fraction consists of heteropolysaccharides.
- The less alcohol-soluble polymer is a glucose (glucan) homopolysaccharide, known as cinerean. This is also the only polymer observed with certain strains of *B. cinerea* (Leal et al. 1976; Stahmann et al. 1992). Most of this extracellular polymer is attached to the hyphal cell wall, forming capsules (60%) while the rest (40%) is released as slime (Pielken et al. 1990).

13.2.1 Structure of the PS Produced

The heteropolysaccharide fraction has been less studied than the β-glucan. It consists of mannose, galactose, glucose, and rhamnose (60/30/5/5), with molecular weights between 10 and 50 kDa (Dubourdieu 1982).

Cinerean has a linear backbone of glucosidic residues β-1,3, with branched chains, consisting of a single β-1,6-linked glucosidic residue, attached to every second or third glucose molecule (Fig. 13.1) (Dubourdieu et al. 1981). The chains are linked by low energy bonds. This increases the apparent molecular weight and leads to the trapping of a black pigment, melanin, by the glucan. The molecular weight of the glucan was estimated at 10^5 to 10^6 Da by size exclusion chromatography and 10^9 to 10^{10} by low-angle laser light scattering. Ultrasound treatment was used to separate

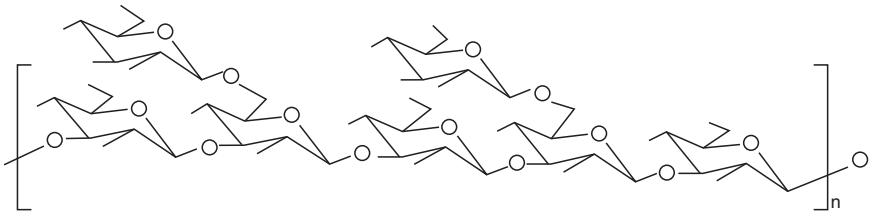

Fig. 13.1 Schematic representation of the repeating unit of *Botrytis cinerea* β-glucan (after Dubourdieu et al. (1981))

the polymer from the melanin, resulting in glucan fibrils of 50–250 kDa (Dubourdieu et al. 1981; Dubourdieu 1982; Stahmann et al. 1995; Doss et al. 2003).

13.2.2 PS Production Kinetics

The two families of PS are produced during active growth on glucose: 300 mg L^{-1} cinerean and about 50 mg L^{-1} heteropolysaccharides (Dubourdieu 1982). In batch fermentation, a decrease in cinerean is observed after glucose exhaustion, leading to a striking decrease in viscosity. Indeed, *B. cinerea* produces several β-1,3 glucanases. Cinerean may be considered an external carbon reserve (Leal et al. 1976; Dubourdieu et al. 1980; Martinez et al. 1983; Stahmann et al. 1992). The PS content of wines produced from botrytized musts is up to 750 mg L^{-1} higher than that in wines obtained from uncontaminated musts (Dubourdieu et al. 1978).

The genes and enzymes responsible for PS synthesis in *B. cinerea* have not been studied. However, Schmid et al. (2006) showed that the synthesis of epiglucan (another β-1,3 β-1,6 branched fungal glucan) occurred via the transfer of glucosyl residues (probably from UDP-glucose) to the nonreducing end of the growing chain. Furthermore, they proposed two PS formation mechanisms involving either (1) a single transmembrane glycosyltransferase (gtase), as proposed for *Streptococcus pneumoniae* and *Pediococcus parvulus* β-glucans (Sect. 13.5.2), or (2) a complex gtase set, as described for lactic acid bacteria EPS synthesis (Sect. 13.4.2). The identification of single or multiple genes associated with β-glucan formation would clarify which mechanism is actually responsible.

13.2.3 Benefit for the Fungus

As already stated, most of the β-glucan produced sticks to the cells, thus forming a thick capsule (Pielken et al. 1990). This capsule protects them from drought and assists in cell attachment on grapes (Dubourdieu 1982; Doss et al. 1995). Gil-ad et al. (2001) showed that the presence of the glucan sheath strongly modifies the morphology of the

fungus, protecting it from host responses by slowing the diffusion of host secretions. In addition, the glucan sheath traps enzymes (peroxidase, laccase, and catalase), which thus constitute an "arsenal" outside the cells (Doss 1999). Eventually, it undoubtedly plays a key role in the biofilm established on the grape berry, containing yeasts, bacteria, and other fungi.

13.2.4 Impact on Wine Quality

B. cinerea PS are detrimental to wine quality (Dubourdieu 1982). On one hand, cinerean is responsible for the high viscosity of musts produced from rotten grapes. After alcoholic fermentation, this glucan tends to form aggregates in the presence of ethanol, which block filters. This problem may be treated with efficient commercial glucanases (Dubourdieu et al. 1985). Moreover, the direct pressing of rotten grapes without crushing them reduces the amount of glucan released into the must. On the other hand, adding the heteropolysaccharide fraction to *Saccharomyces cerevisiae* or *S. bayanus* growth medium slows down alcoholic fermentation and stimulates the glyceropyruvic fermentation pathways, leading to simultaneous excretion of glycerol and acetate (Dubourdieu 1982).

13.3 Yeast Mannoproteins

Mannoproteins (MP) constitute the outer part of the yeast cell wall PS layer, playing an important role in its porosity. During alcoholic fermentation and aging on the lees, some of these MP are released into the wine, where they interact with many other wine components.

13.3.1 Yeast Cell-Wall Organization and MP Structure

The cell wall of *S. cerevisiae* makes up 15–30% of the cell's dry weight. It consists of separate, interconnected PS layers (Fig. 13.2). The outer layer is made of MP, connected to a matrix of amorphous β-1,3 glucan, while the inner layer consists of fibrous β-1,3 glucan over a small quantity of chitin. Small amounts of β-1,6 glucan probably link the components of the inner and outer walls (Kollar et al. 1997; Klis et al. 2002).

In the genus *Saccharomyces*, the carbohydrate component of the MP consists of mannose (about 90%), *N*-acetyl glucosamine, and mannosylphosphate (0.1–1%), in varying proportions, depending on the strain and growth phase (Ballou 1976, 1990; Jigami and Odani 1999; Klis et al. 2002). Their molecular weights vary from 20 to 450 kDa. Four forms of glycosylation have been described for *S. cerevisiae* MP, but

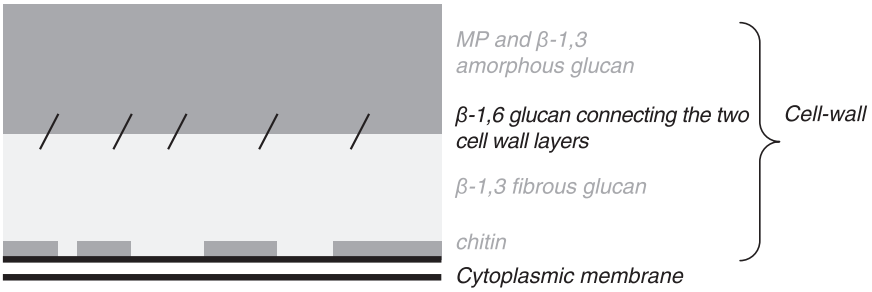

Fig. 13.2 *Saccharomyces cerevisiae* cell-wall organization

they do not necessarily coexist in all of the MP (Fig. 13.3). First, the glycosyl-phosphatidyl-inositol anchor attaches the carboxylic group of the peptide chain of certain MP, which cross the cell wall, to the plasma membrane. The second form of glycosylation consists of mainly α-1,6-linked glucomannan chains, but their peptide point of attachment has not yet been clearly identified. The third form of glycosylation consists of small α-1,2- and α-1,3-linked mannooligosaccharide chains, which are sometimes phosphorylated. These small chains are attached to the peptide chain via *O*-glycosyl bonds on serine or threonine residues. The last form of glycosylation is an *N*-linked PS attached to the peptide chain via an asparagine residue. The core of this PS consists of a double unit of β-1,4-linked *N*-acetyl glucosamine, to which an α-1,2-, α-1,3-, and α-1,6-linked branched, phosphorylated mannooligosaccharide is attached. A highly ramified outer chain (150–250 mannose units) is then attached to the core. This consists of a skeleton of α-1,6-linked mannosyl units, supporting short side chains of α-1,2- and α-1,3-linked mannosyl residues and phosphodiester-branched mannosyl residues.

The core of the PS fraction occurs in several yeast species, while the external PS chain is strain-specific (Ballou 1976).

13.3.2 Physiology of MP Release

The cell-wall construction is a dynamic, tightly regulated process, involving a large number of genes (Luissier et al. 1997; Smits et al. 1999; de Groot et al. 2001). The growing cells produce β-glucanases and other enzymes that partially degrade the β-1,3/β-1,6 glucan network, weakening the cell wall and facilitating cell division, budding, or mating. No mannosidase or *N*-Ac-glucosaminidase are detected (Llaubères 1987; Klis et al. 2002; Gonzales-Ramos and Gonzales 2006). As a result, yeasts release PS, and especially MP, from the cell wall during active growth. In a model medium, 100–250 mg L^{-1} MP is released, depending on the yeast strain, contact time, temperature, and agitation of the yeast biomass. This phenomenon slows down when cells enter the stationary phase as the walls become

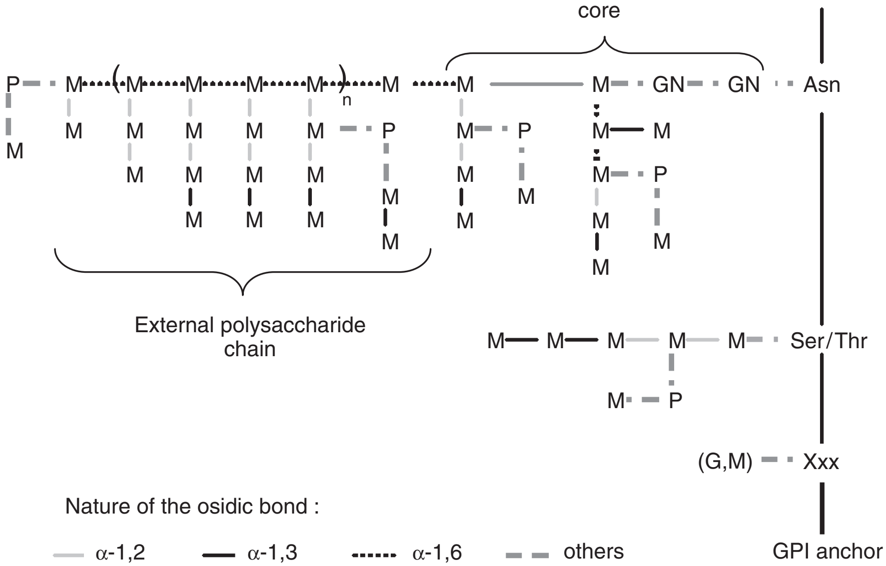

Fig. 13.3 Schematic representation of the *O*-linked oligosaccharide fraction and *N*-linked polysaccharide fraction of *S. cerevisiae* mannoproteins (MP) (n = 0–10) (adapted from Ballou (1990) and Jigami and Odani (1999)). *GNAc N*-acetyl glucosamine, *M* mannose, *P* phosphate, *Asn* asparagine, *Ser* serine, *Thr* threonine

thicker and more resistant to β-glucanases, while the level of MP phosphorylation increases (Llaubères 1987; de Nobel et al. 1989, 1990; Shimoi et al. 1998; Jigami and Odani 1999).

The same phenomena occur during alcoholic fermentation in wine. MP are mainly released during the early stages in alcoholic fermentation. However, the β-glucanases present in the cell wall maintain some residual activity a few months after cell death. As a result, aging on the lees further raises the MP level by 150–200 mg L^{-1}, depending on the yeast strain, especially when the lees are stirred and consist of fermented yeast rather than additional dried yeasts (Llaubères, 1987; Ribereau-Gayon et al. 2000; Guilloux-Benatier and Chassagne 2003).

The structure of the MP released into wine depends on the yeast strain, but is always similar to that of the yeast cell wall, with a molecular mass between 50 and 500 kDa (Villetaz et al. 1980; Llaubères 1987).

13.3.3 Benefit for the Yeasts

MP in the outer cell-wall layer play an important role in controlling the exchange of macromolecules (proteins, etc.) between the periplasmic space and the environment (de Nobel et al. 1989, 1990; Kapteyn et al. 1996). Several enzymes are

thereby retained in the periplasmic space (Klis et al. 2002). Moreover, the external PS fraction of MP, which emanates from the cell surface, is involved in cell–cell recognition events.

MP are also involved in cell protection and survival in hostile environments, e.g., water retention and drought protection (Klis et al. 2002). Furthermore, various studies have shown that mannosylphosphorylation or modified MP patterns help the cells to overcome stress and contribute to yeast flotation during velum formation (Jigami and Odani 1999; Parascandola et al. 1997; Martinez et al. 1997; Alexandre et al. 1998, 2000).

13.3.4 Impact on Wine Quality

Most studies report that the presence of MP is beneficial to wine quality (for a review, see Caridi 2006), although in specific cases, they may be responsible for a decrease in wine color intensity or lower filterability (Vernhet et al. 1999; Morata et al. 2003; Rizzo et al. 2006).

In the pH range of wine, MP are negatively charged and establish interactions with other components in wine, especially phenolic compounds (anthocyanins and tannins) and aroma molecules, thus increasing color stability, decreasing astringency, and modulating aroma intensity and volatility (Lubbers et al. 1994; Vernhet et al. 1996; Escot et al. 2001; Riou et al. 2002; Caridi et al. 2004; Chalier et al. 2007). This property is used to stabilize wine via the legally authorized addition of purified MP (mannostab™) (Dubourdieu and Moine 1996). MP also inhibit the crystallization of tartrate salts (Lubbers et al. 1993; Gerbaud et al. 1996), as well as prevent protein haze and adsorption of molecules that would otherwise be implicated in oxidation reactions, thus explaining the stabilization of white wines aged on lees (Chatonnet et al. 1992; Waters et al. 1994; Escot et al. 2001; Charpentier et al. 2004). Some MP have been shown to significantly adsorb ochratoxin A, a dangerous mycotoxin sometimes reported in grapes, must, and wine (Caridi 2006). In addition, MP contribute to yeast flocculation during sparkling wine production, as well as the co-flocculation of yeasts and bacteria (Suzzi et al. 1984; Peng et al. 2001; Fleet 2003). Finally, MP have been reported to stimulate the growth of malolactic bacteria (Guilloux-Benatier et al. 1995; Guilloux-Benatier and Chassagne 2003).

13.4 *O. oeni* PS Metabolism

MLF is mainly described as the bacterial conversion of L-malic acid into L-lactic acid, although many other metabolic conversions occur during this winemaking process. Among the malolactic bacteria, *O. oeni* is the most tolerant to wine conditions and the least implicated in wine spoilage (Van Vuuren and Dicks 1993; Lonvaud Funel 1999; Versari et al. 1999). In this section, we provide evidence that *O. oeni* produce EPS.

13.4.1 Evidence that O. oeni Produce PS During MLF

The complexity of wine makes it difficult to observe the formation of PS. However, we recently showed that soluble PS concentrations increased or decreased during MLF, depending on the wine considered, indicating that *O. oeni* both produced and degraded PS in nonropy red wines. Unfortunately, it was impossible to distinguish the PS released into the wine by *O. oeni* from those initially present by their size or chemical composition. However, they are expected to contain mannose, galactose, arabinose, rhamnose, glucose, and galacturonic acid (Dols-Lafargue et al. 2007).

Furthermore, PS production by *O. oeni* in model media had not previously been studied in detail. However, 15 years ago, Van Vuuren and Dicks (1993) reported that in grape juice medium most *O. oeni* strains "produced a silkiness in the liquid" without any increase in viscosity. They considered that this was due to the excretion of PS, but did not isolate or quantify the corresponding molecules. In addition, some *O. oeni* strains have been shown to produce the β-glucan that causes ropiness in wine (see Sect. 13.5), as well as two distinct heteropolysaccharides containing galactose, glucose, and rhamnose. Irrespective of the *O. oeni* strain studied, the level of PS production was low (between 50 and 200 mg L^{-1}) (Walling 2003; Ibarburu et al. 2007).

In addition, the *O. oeni* PSU-1 and ATCC BAA-1163 genomes display specific gene clusters of ~12 kb, similar to those described for EPS formation by other lactic acid bacteria, comprising genes annotated as Gtase, polymerase, or flippase genes (cluster of orthologous genes group M, Makarova et al. 2006). However, these two strains have never been implicated in wine ropiness.

All these data support the view that *O. oeni* produce PS, which do not necessarily increase wine viscosity. The structure of these nonropy PS has not yet been completely elucidated. However, the fact that many distinct monomers are produced by PS acid hydrolysis and the presence of complex *eps* gene clusters indicate that they are heteropolysaccharides, built up of successive, regularly repeated units.

13.4.2 Putative Metabolic Pathways

The synthesis of heteropolysaccharides by lactic acid bacteria has been widely studied and appears to involve a common mechanism. Numerous enzymes are implicated. Some are specific to EPS synthesis, while others are shared by several metabolic pathways. The specific enzymes are encoded by genes located in large *eps* gene clusters (10–15 kb), often located on large plasmids, but sometimes chromosomal (Kolkman et al. 1998; Van Kranenburg et al. 1999; Garcia et al. 1999; Kleerebezem et al. 1999; Boels et al. 2001; Levander and Radstrom 2001; Degeest et al. 2001; Laws et al. 2001; Jolly and Stingele 2001; Mozzi et al. 2006).

The heteropolysaccharide synthesis mechanism is described in Fig. 13.4. It involves sugar-nucleotides, such as UDP-glucose, produced in the central metabolic pathways. The repeating unit is assembled on a lipid-carrier molecule, anchored in the cytoplasmic membrane. The first monomer is linked to the lipid carrier by the priming

Gtase. Then, the following monomers are linked by other specific Gtases. Each Gtase uses the energy of the UDP-osyl bond to transfer the osyl to the growing repeating unit, forming a specific osidic bond. After completion, the repeating unit is assumed to be exported and polymerized on the outer face of the cell membrane, via a mechanism similar to that described for Gram-negative bacteria. The lipid carrier is externalized by a flippase and the repeating unit is added to the nonreducing end of the growing PS chain by a polymerase. A chain length determination factor limits the extension of the molecule and the PS is released.

13.4.3 Putative Benefit for the Bacteria and Possible Impact on Wine Quality

As shown for other lactic acid bacteria, part of the EPS produced by *O. oeni* may remain attached to the cell, forming a capsule, while the other part is released into

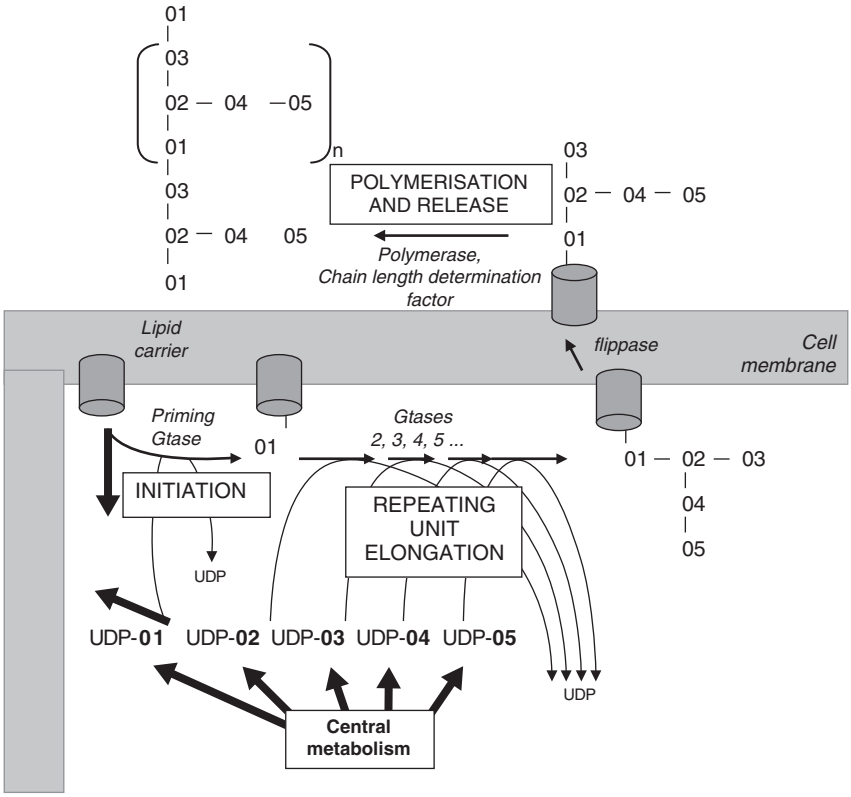

Fig. 13.4 Schematic representation of heteropolysaccharide biosynthesis by lactic acid bacteria. *O* osyl (e.g., glucosyl, rhamnosyl, galactosyl, etc.), *Gtase* glycosyltransferase

the surrounding medium (Sutherland 1993). Most of the time, the lactic acid bacteria EPS are not degraded by their producers and do not act as external carbon sources (Boels et al. 2001; Mozzi et al. 2006). The capsular EPS of *O. oeni* may help these bacteria to overcome the various stresses encountered in wine (toxic metal ions, sulfur dioxide, ethanol, acidity, etc.). It may also protect the cells from desiccation or help them adhere to the grapes or cellar equipment (Guzzo et al. 1994; Lonvaud-Funel 1999; Versari et al. 2003).

On the other hand, in the food industry, bacterial PS have been developed as an alternative class of biothickeners or stabilizers (de Vuyst and Degeest 1999; Ruas-Madiedo and de Los Reyes-Gavilan 2005). However, most *O. oeni* strains do not induce any increase in viscosity (Walling 2003). The soluble PS released by *O. oeni* may interact with many other molecules, inducing a stabilizing effect, as described for wine MP, thus contributing to the positive impact of MLF on wine quality. These points will require further study once the *O. oeni* PS structure has been elucidated.

13.5 Bacterial Production of PS Causing Ropiness

"Ropiness" or "oiliness" is one of the four major types of bacterial spoilage in wine (Pasteur 1866), giving them an oily, ropy texture.

13.5.1 Bacterial Species Isolated from Ropy Beverages

Streptococcus, *Leuconostoc*, *Pediococcus*, and *Lactobacillus* species, and even *O. oeni*, have been isolated from ropy wines or ciders (Luthi 1957; Van Oevelen and Verachtert 1979; Lonvaud Funel and Joyeux 1988; Manca de Nadra and Strasser de Saad 1995; Duenas et al. 1995; Fernandez et al. 1995; Walling et al. 2005b; Werning et al. 2006). However, the metabolic pathways leading to ropiness are not the same in all cases (Walling et al. 2005b). The most frequently incriminated and the most studied species is *P. parvulus*. These bacteria have been isolated from ropy red and white wines, ciders, and beers, and have been shown to cause ropiness in model media (Van Oevelen and Verachtert 1979; Lonvaud Funel and Joyeux 1988; Duenas et al. 1995; Walling et al. 2005b). These Pediococci were first considered to be *P. cerevisiae*, but, were later identified as *P. damnosus* by DNA/DNA hybridization, and finally as *P. parvulus*, thanks to 16 S RNA sequencing.

13.5.2 The β-Glucan of P. parvulus: Structure and Concentrations

The increase in viscosity is due to the production of a high molecular weight (500–2,000 kDa) β-glucan by *P. parvulus*. This polymer consists of a trisaccharide

repeating unit with a β-1,3-linked glucosyl backbone and branches made up of a single β-1,2-linked D-glucopyranosyl residue. This polymer is structurally related to the capsular PS of *S. pneumoniae* type 37, although the latter has twice as many branches (Fig. 13.5). In fact, antibodies targeting the type 37 capsule also agglutinate the ropy Pediococci (Adeyeye et al. 1988; Llaubères et al. 1990; Duenas-Chasco et al. 1997; Walling et al. 2005b). This β-glucan is also produced by certain *O. oeni* and *Lactobacillus diolivorans* strains. However, unlike Pediococci and Lactobacilli, which only produce β-glucan, *O. oeni* produces both β-glucan and soluble heteropolysaccharides (Duenas-Chasco et al. 1998; Ibarburu et al. 2007).

All the *Pediococcus* strains studied produce larger amounts of β-glucan when grown on glucose rather than on other carbon sources: up to 140–200 mg L^{-1} β-glucan may be observed, whereas 20–30 mg L^{-1} is sufficient to induce visible texture modification. Depending on the strain, β-glucan production is also observed with fructose, maltose, galactose, xylose, and arabinose as the carbon source. It may be stimulated by adding malic acid or ethanol to the growth medium. β-glucan production is not directly linked to cell growth. However, an efficient preliminary growth phase is essential for subsequent "large-scale" EPS production (Llaubères 1987; Lonvaud-Funel and Joyeux 1988; Duenas et al. 2003; Walling et al. 2005a; Velasco et al. 2006, 2007).

13.5.3 Metabolic Pathway for β-Glucan Synthesis by P. parvulus

A single glucosyltransferase gene *(gtf)* is associated with β-glucan synthesis. It is located on a 5.5-kb plasmid for red wine Pediococci (pF8801, Genebank AF196967), a 35-kb plasmid for cider Pediococci (pPP2, GeneBank AY999683), another 5.5-kb plasmid for *L. diolivorans* (GeneBank AY999684), and the chromosome of certain *O. oeni* strains (GeneBank AY999685). *gtf* displays over 98% identity from one

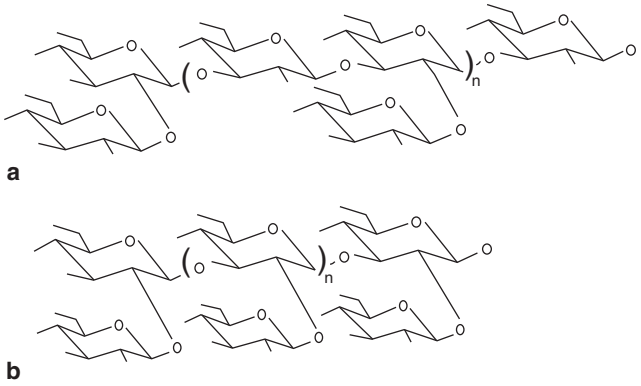

Fig. 13.5 Schematic representation of the chemical structure of (**a**) *P. damnosus* β-glucan and (**b**) *S. pneumoniae* β-glucan (after Llaubères et al. (1990) and Adeyeye et al. (1988))

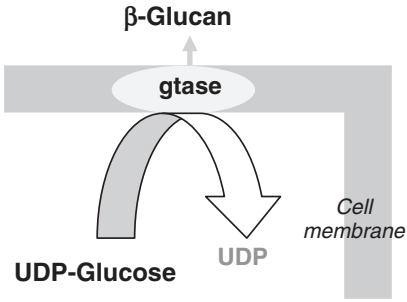

Fig. 13.6 Biosynthesis of *P. parvulus* β-glucan. A single glucosyltransferase catalyses the synthesis of the β-1,3 backbone and β-1,2 side chains, as well as the export of the growing polymer (after Llull et al. (2001) and Werning et al. (2006))

bacterial species to another. In all cases, it codes a 567-amino-acid, 65-kDa protein (Gtf). The *gtf* gene of *P. parvulus* 2.6 was cloned and its transmembrane Gtf product functionally expressed in *S. pneumoniae*, causing it to synthesize the β-glucan causing ropiness from UDP-glucose (Werning et al. 2006) (Fig. 13.6). The role of Gtf in ropiness was thus clearly demonstrated. This enzyme, similar to the glucosyltransferase of *S. pneumoniae* type 37, is a bifunctional transmembrane protein that catalyses the synthesis of two distinct glucosidic bonds, as well as the export of the branch polymer (Lonvaud Funel et al. 1993; Fernandez et al. 1995; Llull et al. 2001; Walling 2003; Werning et al. 2006).

13.5.4 Benefit for the Bacteria

β-glucan is not consumed by the bacteria and does not constitute an external carbon source. Moreover, *P. parvulus* cells are surrounded by a refringent capsule, and immunoagglutination by anti-type-37 antibodies confirms the presence of β-glucan around the cells. This capsule may explain the extreme resistance of ropy strains to SO_2, ethanol, and low pH, compared with their nonropy mutants (Lonvaud-Funel and Joyeux 1988; Lonvaud-Funel et al. 1993; Walling et al. 2005a,b; Dols-Lafargue et al. 2008).

The polymer probably also contributes to biofilm formation and cell adhesion on grapes and winemaking equipment. These biofilms are known to favour cell survival under extreme conditions, as well as genetic exchanges between species (Mah and O'Toole 2001). Recent gene transfers probably explain the high level of identity between the *gtf* genes in the various species.

13.6 Impact on Wine Quality

Ropiness occurs all over the world in red and white wines, as well as in beer and cider, in vat, barrel, or even bottle. This wine spoilage has no impact on human health, but the wine's viscosity makes it impossible to market without prior intensive mixing and

sulfuring. However, these rough treatments do not ensure the permanent elimination of bacteria and ropiness, especially as ropy bacteria are highly resistant to sulfur dioxide (Ribereau-Gayon et al. 2000, Dols-Lafargue et al. 2008). An alternative solution relies on bacterial detection and preventive treatment prior to the formation of ropiness. PCR-based methods were therefore developed to detect the presence of the *gtf* gene in wine microflora, as early as possible in the winemaking process (Gindreau et al. 2001; Delaherche et al. 2004; Walling et al. 2005b; Werning et al. 2006).

13.7 Conclusion

All the microorganisms on grapes and in must and wine produce exocellular PS. Some of these PS remain attached to the cell, forming a capsule, which constitutes a response to the hostile environment, especially in the final stages in winemaking. The remainder of the PS is released into the surrounding medium. Depending on the PS structure, this may be beneficial or detrimental to wine quality and/or subsequent growth of other species. Genetic exchanges between species are probably responsible for the present high diversity of microbial PS structure. As a result, microbial PS represents an important element for future investigation of wine microbial ecology.

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Chapter 14
Exoenzymes of Wine Microorganisms

Harald Claus

14.1 Introduction

The production of wine from grape juice is predominantly the result of enzymatic reactions. The enzymes originate from the grape itself, from epiphytic fungi like *Botrytis cinerea* colonizing the grape surface and finally from yeasts and bacteria growing in the must until termination of alcoholic fermentation. Especially non-*Saccharomyces* yeasts, also called "wild" yeasts, belonging to the genera *Kloeckera*, *Candida*, *Debaryomyces*, *Rhodotorula*, *Pichia*, *Zygosaccharomyces*, *Hanseniaspora*, *Kluyveromyces*, and *Metschnikowia* produce and secrete several enzymes (esterases, glycosidases, lipases, glucanases, proteases, cellulases, etc.) to the periplasmatic space and the medium where they may interact with grape precursor compounds to produce aroma active compounds (Charoenchai et al. 1997). Apart from yeasts, lactic acid bacteria play an import for vinification, i.e., the genera *Oenococcus*, *Leuconostoc*, *Pediococcus*, *Lactobacillus*, and *Weissella* (Wibowo et al. 1985; Fugelsang and Edwards 2007; see also Chap. 1).

Because of its vitality under the extreme life conditions of wine (pH 3.0–4.0; alcohol concentration 10–15%), *Oenococcus oeni* is the primary species responsible for the malolactic fermentation. After completion of alcoholic fermentation, the malolactic enzyme catalyzes the conversion of the dicarbonic acid L-malate to the monocarbonic acid L-lactate and CO_2. The result is a biological acid reduction which is often preferable in white wines. On the opposite, *Lactobacillus* and *Pediococcus* are more active in the early stages of vinification often in connection with stuck fermentations and wine spoilage (Fugelsang and Edwards 2007).

A considerable number of publications emphasize the importance of hydrolytic enzymes from lactic acid bacteria for the winemaking process and its control (Matthews et al. 2004, 2006, 2007; Claus 2007). Proteases, e.g., have been found in wine-associated strains of *Lactobacillus* and *Oenococcus* (Manca de Nadra et al. 1997,

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1999; Farías and Manca de Nadra 2000). Davis et al. (1988) detected lipases and esterases in different wine-relevant lactic acid bacteria. Vaquero et al. (2004) demonstrated the existence of tannases in some *Lactobacillus plantarum* isolates. A group of enzymes of special interest are glycosidases, as they deliberate flavor compounds from the nonvolatile glycosidic bound state. Their occurrence has been reported for the wine-relevant genera *Oenococcus*, *Pediococcus*, and *Lactobacillus* (Boido et al. 2002; Barbagallo et al. 2004; D'Incecco et al. 2004; Grimaldi et al. 2000, 2005a, b). According to recent studies of Matthews et al. (2004, 2006, 2007), also esterases, lipases, tannases, cellulases, β-glucanases, and lichenases are produced by these genera (Table 14.1). All these enzymatic activities can have profound impacts on wine flavor and quality.

14.2 Effect of Exoenzymes on Different Wine Ingredients

14.2.1 Proteins

Wine contains between 100 and 600 mg L^{-1} of nitrogen, mainly composed of peptides and free amino acids, which originate from grapes and yeast, are essentially glycosylated and may account for up to 2% of total nitrogen (Folio et al. 2008). Proteins of grapes and wine are considered unstable in the finished wine and can precipitate to produce undesirable haze. Proteases have been searched from a variety of sources as alternative to bentonite treatment to remove unwanted proteins while possibly also liberating assimilable nitrogen for the growth of wine microorganisms. Most commercial preparations failed as they did not work under wine-making conditions (low pH and temperatures). In contrast to *Saccharomyces cerevisiae* itself, non-*Saccharomyces* wine yeasts are important sources of extracellular enzymes including proteases. In the study of Fernández et al. (2000), 53 from 141 isolates of "wild yeasts" hydrolyzed casein. The positive strains were identified as *Metschnikowia pulcherrima* and *Pichia membranaefaciens*. In a similar study with 245 yeast isolates, ten strains of *Candida stellata*, *C. pulcherrima*, and *Kloeckera apiculata* and one strain of *Debaryomyces hansenii* showed proteolytic activity (Strauss et al. 2001).

There are some studies which document the occurrence of proteolytic activities in wine lactic acid bacteria (Table 14.1). The presence of amino acids in the medium is essential for the development of *O. oeni* due to its numerous auxotrophies. This bacterium is able to use small peptides and to hydrolyze wine proteins via extracellular enzyme activities. Rollán et al. (1993, 1995, 1998) described two proteases I and II, which are produced by several strains of *O. oeni* during the early and final stages of growth, respectively. Protease I displayed optimum activity at pH 4.0 and 30°C, and protease II at pH 5.5 and 40°C. Both proteases were repressed by ammonium, trypton, and casein hydrolysate, were induced by nutrient starvation, and hydrolyzed protein and polypeptide extracts from red and white wines. A third extracellular protease EprA of *O. oeni* has been recently expressed in *Escherichia coli*

(Folio et al. 2008). This protease differed from all lactic acid bacteria proteases identified so far. With a molecular mass of 21.3 kDa and a pH of 5.3, the enzyme showed maximum activity at pH 7.0 and 45°C. These features appear not compatible with winemaking conditions and the question arises whether there is a nutritional benefit for the bacterium.

14.2.2 Oligo- and Polysaccharides

Polysaccharides in must and wine are directly derived from the grape berries (cellulose, hemicellulose, pectins), but also by the growth and autolysis of yeasts like *S. cerevisiae* (β-glucans, chitin, mannoproteins; see also Chap. 13). Strains of the lactic acid bacterium *Pediococcus* sp. (Llaubéres et al. 1990; Manca de Nadra and Strasser de Saad 1995; Velasco et al. 2007) and the fungus *B. cinerea* produce glucan slimes (Dubourdieu et al. 1981) causing filtration problems during winemaking. In addition, fermentation in presence of *Botrytis* glycans leads to yeast inhibition coupled with increased levels of acetic acid and glycerol (Fugelsang and Edwards 2007).

β-Glucanases hydrolyze the glycosidic bonds of the glucan chains deliberating glucose and oligosaccharides. These enzymes, e.g., from *Trichoderma reesei* can be used to dissolve *Botrytis* slime (Villetaz et al. 1984). Also in wine-relevant lactic acid bacteria, such enzyme activities have been found (Table 14.1). A strain of *O. oeni* exhibited extracellular β-(1→3)-glucanase activity (Guilloux-Benatier et al. 2000), which increased when cells were cultivated with cell wall glucans. In addition, the culture supernatant of the organism effectively lysed viable or dead cells of *S. cerevisiae*. This lytic activity appeared in the early stationary phase of bacterial growth. Yeast cells at the end of the log phase of growth were the most sensitive. The optimum temperature for lysis of viable yeast cells was 40°C, which is very different from the temperatures observed under enological conditions (15–20°C). Moreover, the rate of the lytic activity was significantly lower in comparison with yeast cell wall-degrading activities previously measured in various other microorganisms. Therefore, yeast cell death that is sometimes observed during the alcoholic fermentation could hardly be attributed to the lytic activity of *O. oeni*.

Lichenan is a linear 1,3–1,4-β-D-glucan similar to the polysaccharide of *B. cinerea* (Strauss et al. 2001). Lichenases (endo-β-1,3–1,4-glucanases) of lactic acid bacteria (Table 14.1) could also be useful to treat ropy wines.

The exo-β-glucanase EXG1 of *S. cerevisiae* (Gil et al. 2005) attacks besides laminarin also pustulan and small substrates such as laminaribiose, *p*-nitrophenyl-β-D-glucoside (pNPG), and 4-methylumbelliferyl-β-D-glucoside (4-MUG) (Nebreda et al. 1986; Suzuki et al. 2001). Results of Ridruejo et al. (1989) supported the idea that yeast exo-1,3-β-glucanases (EC 3.2.1.58) are glucosidases that also attack laminarin, rather than glucanases capable of attacking pNPG. Suzuki et al. (2001) suggested that the yeast EXG1 may be classified as a new type of β-glucanase or β-glucosidase that has not been described before.

Table 14.1 Exoenzymes detected in wine-relevant lactic acid bacteria

Enzyme	*Lactobacillus*	*Oenococcus*	*Pediococcus*
Protease	+	+	+
Cellulase	+	+	+
Xylanase	–	–	–
β-Glucanase	+	+	+
Lichenase	+	+	+
Glucosidase	+	+	+
Lipase	+	+	+
Esterase	+	+	+
Tannase	+	+	+
Phenoloxidase	ND	ND	ND

For references, see Matthews et al. (2004, 2006, 2007) and Grimaldi et al. (2000, 2005a, b); ND: no data

The exo-β-glucanase activity of *S. cerevisiae* is produced constitutively, independently of the carbon source (Olivero et al. 1985) and is first secreted to the periplasmic space and then released into the culture medium (Cid et al. 1995). Overproduction of this major exo-β-glucanase in *S. cerevisiae* led to a moderate release of certain volatiles from grape glycosides (Gil et al. 2005). Therefore, screening for high exo-β-glucanase activity in *Saccharomyces* strains could be an approach for obtaining a flavor-enhancing strain.

Nevertheless, the major source of polysaccharide-degrading exoenzymes are non-*Saccharomyces* wine yeasts. In a study of Strauss et al. (2001), 245 yeast isolates – representing 21 species belonging to the genera *Kloeckera*, *Candida*, *Debaryomyces*, *Rhodotorula*, *Pichia*, *Zygosaccharomyces*, *Hanseniaspora*, and *Kluyveromyces* – were positively screened for the production of extracellular pectinases, β-glucanases, lichenases, cellulases, xylanases, and amylases.

Many aliphatic and aromatic compounds such as terpenes, anthocyanins, and phenolics in grapes are stored in a water-soluble glycosidic bound form.

Glycosidases that cleave the sugar moieties from the glycosides can therefore have a major impact on the sensory profile of wine, as they release the more volatile aroma compounds. The glycoside substrates contain either mono- or disaccharides. The terminal sugar can be either β-D-glucopyranoside, α-L-rhamnopyranoside, α-L-arabinofuranoside, β-D-apiofuranoside, or β-D-xylopyranoside, and the additional central sugar in disaccharides is always a β-D-glucopyranoside (Winterhalter and Skouroumounis 1997). Cleavage of these sugars requires a glycosidase specific for the terminal sugar and in the case of disaccharides a β-D-glucopyranosidase. In wine-relevant lactic acid bacteria, β- and α-D-glucopyranosidases have been demonstrated (Table 14.1).

Most research on β-glucosidases in yeasts has focused on species indigenous to winemaking and high activities were demonstrated intra- and extracellulary, especially in non-*Saccharomyces* yeasts (Rosi et al. 1994; Fernández et al. 2000; Ferreira et al. 2001; Strauss et al. 2001; Rodriguez et al. 2004; Fia et al. 2005; Villena et al. 2005). The majority of *Saccharomyces* isolates do not show β-glucosidase activity on a

natural substrate like arbutin (Rosi et al. 1994; Spagna et al. 2002; Rodriguez et al. 2004) and no gene in the genome of *S. cerevisiae* is known for coding a 1,4-β-glucosidase (EC 3.2.1.21) (Cherry et al. 1997). However, a β-glucosidase gene of a *S. cerevisiae* strain AL41 isolated on arbutin by Spagna et al. (2002) was recently partially sequenced by Quatrini et al. (2006). The translated amino acid sequence contained one of the conserved patterns, namely FGYGLSY, which is typical for most yeast β-glucosidases. This pattern was found in the C-terminal part of the β-glucosidase(s) from *Saccharomycopsis fibuligera*, *Candida pelliculosa*, and *Kluyveromyces fragilis* (Rojas and Romeu 1996). Apparently, only some *S. cerevisiae* yeasts possess a gene coding for β-glucosidase whereas the majority does not. Mateo and DiStefano (1997) demonstrated the hydrolysis of grape glycosides by crude extracts of *Saccharomyces* strains. This activity was suggested to be related to the major exo-β-glucanase EXG1 of *S. cerevisiae* (see above; Gil et al. 2005).

Considering the regulation of the enzymes, the β-glucosidase from *S. cerevisiae* strains appears to be repressed by glucose and induced by cellobiose (Duerksen and Halvorson 1958; Daenen et al. 2008). Attempts were undertaken for a technical application of glucosidases to improve the organoleptic quality of wine by enzymatic hydrolysis of glycosidically bound flavor compounds (van Rensburg and Pretorius 2000; Ugliano et al. 2006). Delcroix et al. (1994) investigated the use of *Saccharomyces* strains with higher β-glucosidase activity but noted only few differences in the concentrations of terpenes and the sensory quality of the product. Selected species of *Candida*, *Kloeckera*, *Pichia*, and *Metschnikowia* with high β-glucosidase could potentially contribute different aromas to wine (Fernández et al. 2000; Ferreira et al. 2001; Rodriguez et al. 2004). Although *Brettanomyces* has glucosidase activity (Daenen et al. 2008), the enzyme was not active against glycosides from grapes (Mansfield et al. 2002). On the other hand, wines treated with a β-glucosidase from *D. hansenii* showed increased concentrations of terpenoids (Yanai and Sato 1999).

14.2.3 Lipids

Lipids in wine originate from all parts of the grape berry; their composition (neutral lipids, glycolipids, phospholipids) and concentration are determined by factors like the variety, maturation, and climate (Gallander and Peng 1980; Izzo and Muratore 1993). Also, autolysis of wine yeasts deliberates lipids, including tri-, di-, and monoacylglycerols and sterols (Pueyo et al. 2000). A significant number of biologically active lipids have been detected in greek white wines and musts with antithrombotic and antiatherogenic properties in vitro (Fragopoulou et al. 2002). The authors found that the active lipids in must and wine have a glycerol backbone with some interesting differences. The lipids in must are phosphoglycolipids, while in wine it is a glycolipid. These findings suggest that the lipids of grape or yeast are subjected to chemical modification during fermentation and moreover that the biologically active lipids come from the grape, since almost the same structure was found in must and wine.

Several studies have provided information about lipase activities in genera that are of interest in winemaking, namely *Lactobacillus*, *Pediococcus*, and *Leuconostoc* (Table 14.1). Because lipases are located extracellulary or cell associated, these bacteria have the ability to influence the wine lipid content when they are grown in grape juice or wine. By their action, wine lipids are cleaved rendering different volatile compounds and fatty acids. Whereas the former (esters, ketones, aldehydes) may have a positive effect on wine flavor; the odors of fatty acids are usually not desirable.

14.2.4 Esters

Esters (e.g., ethyl acetate, isoamyl acetate, ethyl hexanoate, ethyl octanoate, ethyl decanoate) are qualitative one of the most important flavors in wine, where they contribute to a mostly desirable fruity taste (Fugelsang and Edwards 2007). Esters originate directly from the grapes (Rapp and Mandery 1986) as well as from the activity of yeasts during the alcoholic fermentation (Younis and Stewart 1998; Lambrechts and Pretorius 2000; Pfeiffer et al. 2007; Saerens et al. 2008). In the course of the malolactic fermentation, a concentration change of single esters has been observed, e.g., an increase of ethyl acetate (Maicas et al. 1999; Delaquis et al. 2000), isoamyl acetate (Maicas et al. 1999), and ethyl lactate (Delaquis et al. 2000). On the other hand, a decrease of different esters has been observed after completion of the malolactic fermentation (Zeemann et al. 1982). Esterase activities have been found in different wine-relevant lactic acid bacteria, especially in *O. oeni* strains (Table 14.1). Their possible involvement in the synthesis and hydrolysis of wine esters has been studied by Matthews et al. (2007). The enzymes were generally found to have a broad pH activity range, with the majority of strains showing a maximum close to pH 6.0. Exceptions included an *O. oeni* strain that retained most activity down to a pH of 4.0. Most strains exhibited highest activity across the range 30–40°C. Increasing alcohol concentration stimulated activity up to a maximum ethanol concentration of around 16%. Generally, strains were found to have greater activity toward short-chained esters (C2–C8) compared with long-chained esters (C10–C18). Even though the optimum physicochemical conditions for enzyme activity differed from those found in wine, these findings are of potential importance to enology because significant activities remained under wine-like conditions.

14.2.5 Phenolic Compounds

Wine contains a plenty of phenolic compounds deriving from the grape berries (Eder and Wendelin 2002; Adams 2006; Kennedy et al. 2006). Phenols contribute to the red pigmentation, the brown-forming substrates, the bitter and astringent components, and, to a small extent, the taste in grapes and wine. The primary constituents

of the phenolic compounds are flavonoids which make up approximately 85% of the total phenols and contain anthocyanins, 3-flavanols, and tannin polymers. The nonflavonoid phenolic acids include the hydroxycinnamates and hydroxybenzoates. Derivates of cinnamic acid (including caffeic, *p*-coumaric, and ferulic acids) can form an esteric bond with tartaric acid. These three primary acids are responsible for the golden color of white wines. The hydroxybenzoates, including *p*-hydroxybenzoic, protocatechuic, vanillic, gallic, and syringic acids, are more prevalent in maturing wines and wine that contain mold (Jacobsen 2006).

The total phenolic content of red wines is in the range of 10 μmol L^{-1}, and thus about ten times higher than that of white wines (Eder and Wendelin 2002). The importance of phenolic components in wine has got increasing scientific interest, especially in context with their antiangiogenic properties which can, at least in part, be attributed to their antioxidative effects (Espín and Wichers 2000; Eder and Wendelin 2002; Oak et al. 2005). In this respect, trans-resveratrol, quercetin, catechin, and epicatchin are among the best studied substances.

Oxidative reactions, both spontaneous and enzymatic catalyzed, have a dramatic effect on the final phenol composition from the grape berry up to the bottled wine. Once the berry integrity has been comprised, oxidative enzymes (phenoloxidases) and their phenolic substrates are exposed to air resulting in enzymatic browning (Macheix et al. 1991). During wine storage, oxidation of phenolic ingredients leads to discolorization and taste alterations especially of red wines (maderization). Among the responsible enzymes are the copper-containing enzymes tyrosinase and laccase which use molecular oxygen to oxidize a wide spectrum of phenolic compounds (Mayer and Staples 2002; Claus 2004; Claus and Decker 2006). Tyrosinase (EC 1.14.18.1) hydroxylates monophenols to *ortho*-diphenols, and the latter to *ortho*-quinones. Laccase (EC 1.10.3.2) has no monohydroxylase activity, but oxidizes a wide spectrum of different polyphenols and other compounds by a radical mechanism. The resulting quinones polymerize often under formation of brown pigments. Wine-relevant substrates of laccases are, e.g., the phenolics *p*-coumaric acid, caffeic acid, quercetin, catechin, and tannin as well as the biogenic amines tyramine and dopamine.

The occurrence of phenoloxidase activities in wine-related lactic acid bacteria is rather speculative (Matthews et al. 2004; Minussi et al. 2007), e.g., a gene encoding for a multicopper oxidase (Acc. ZB 00320225) can be found in the genome of *O. oeni* PSU-1. Tyrosinase is produced by the grape berry and is present in the skin, whereas laccases originate from epiphytic fungi. The secreted laccase of the fungal pathogen *B. cinerea*, which infects the fruits, flowers, or green tissues of at least 235 plant species and is the main cause of bunch rot, probably inactivates antifungal compounds produced by the infected berries such as resveratrol (Mayer and Staples 2002). In the course of the infection process, it may enter the must. Unlike grape oxidases, which are inhibited by SO_2, even at low concentrations, fungal laccases tend to be resistant and tolerate ethanol and could be detected in wine after 12 months of storage (Somers 1984). Although the presence of laccase can be considered as indicator of grape contamination by *B. cinerea* (Grassin and Dubourdieu 1989), there is controversy surrounding laccase activity and degree of fungal infection (Minussi et al. 2002;

Dewey et al. 2008). One strategy to limit the effects of laccases would be to minimize oxygen exposure during the processing of infected musts or application of heat. Bentonite fining of juice can help to reduce laccase activity, although this procedure may not completely remove the enzyme (Claus and Filip 1988).

On the other hand, an alternative to polyvinylpyrrolidone (PVPP) and sulfur dioxide is the technical use of enzymes that selectively target specific polyphenols during the maderization process. These phenolic compounds are oxidized by the enzyme, polymerized, and then removed during clarification. Many publications in the literature report that laccase treatment promotes wine stabilization. Laccase characteristics such as an optimum pH between 2.5 and 4.0, stability in acid medium, and reversible inhibition with sulfite are all favorable for its use at the removal of phenolic compounds from wines. A laccase of *Trametes versicolor* removed more than 90% of ferulic acid from a model solution and 34% of phenolic compounds from wines. A mutant laccase from *Polyporus versicolor* (optimum pH 2.7) eliminated up to 70% catechin and 90% of anthocyanidins in a model solution within 3 h of treatment. In addition, it removed 50% of the total phenols from a red grape juice.

Maier et al. (1990) evaluated the polyphenol content, color, haze stability, and sensorial quality of Riesling wines prepared with and without enzymatic treatment of the must. Enzymatic pretreatment of the musts yielded the best wines, suggesting that a stable and high-quality wine could be made with little or no added SO_2. Since the use of laccase as a food additive is still not allowed, this enzyme has been used in wine production in an immobilized form (Brenna and Bianchi 1994), which not only ensures its elimination from the must, but also aids its reutilization.

A contentious issue arises due to laccase removing or transforming certain phenolic compounds in wines. In recent years, the moderate consumption of red wines has been shown to benefit human health. The effect has mainly been attributed to the antioxidant properties of polyphenols that are present in red wines, especially resveratrol (Espín and Wichers 2000; Eder and Wendelin 2002; Oak et al. 2005). In view of this, it is necessary to analyze the possible interactions of laccase with these potentially beneficial components in wine. Espín and Wichers (2000) found that laccase did not modify the free-radical scavaging ability of resveratrol. However, according to Minussi et al. (2007), the treatment of a red must with laccase from *T. versicolor* mainly affected the phenolic compounds responsible for the antioxidant properties. They did observe that the treatment of white musts with the same laccase showed a greater reduction in total phenols than in total antioxidant potential, indicating the feasibility for the treatment of white wine musts. Further applications of laccases in wine industries are described elsewhere (Strong and Burgess 2007).

14.2.6 Tannins

Tannins are the most abundant class of soluble polyphenolics found in the skins and seeds of grape berries (Adams 2006). They vary in size from dimers and trimers up to oligomers with more than 30 subunits. The larger skin tannins contain

epigallocatechin subunits, whereas in the smaller seed tannins usually epicatechin gallate residues dominate. With the grapes and by leaching out from new oak barrels (Barrique), they enter must and wine. Especially in red wines, they are responsible for some bitterness and astringency. Tannases (tannin acyl hydrolases, EC 3.1.1.20) cleave the ester and depsid bonds in hydrolyzable gallotannins, yielding gallic acid and D-glucose. The existence of such activities has been detected in wine bacteria (Vaquero et al. 2004; Table 14.1). The release of gallic acid may have a beneficial effect as it stimulates growth and malolactic fermentation of *O. oeni* (Vivas et al. 1997).

14.3 Conclusions

Enzymes play a key role in the production of wine, which could be seen as the result of enzymatic transformations of grape juice (van Rensburg and Pretorius 2000). The enzyme activities do originate not only from the grape itself, but also from indigenous wine microorganisms. *S. cerevisiae*, the principal wine yeast, is not a significant producer of extracellular enzymes, unlike non-*Saccharomyces* yeasts and lactic acid bacteria. The production of extracellular hydrolytic enzymes by wine microorganisms needs to be understood and managed to the benefit of wine production.

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Part IV
Stimulaling and Inhibitary Growth Factors

Chapter 15
Physical and Chemical Stress Factors in Yeast

Jürgen J. Heinisch and Rosaura Rodicio

15.1 Introduction

With the use of starter cultures, the yeast *Saccharomyces cerevisiae* predominates must fermentations from the beginning and is virtually exclusive in later stages. This chapter will therefore concentrate on various stresses encountered by *S. cerevisiae* and on its counteracting strategies. Stress has been defined as any environmental factor which impairs yeast growth (Ivorra et al. 1999). Albeit yeasts are continuously exposed to changes in such factors (Table 15.1) growth kinetics in must fermentations basically follow those of laboratory batch cultures: after inoculation yeasts encounter high sugar concentrations (osmotic stress). Cells adapt during a lag-phase and then start to divide rapidly. Sugars are converted to carbon dioxide and ethanol (ethanol stress). The phase of vigorous fermentation can first cause a temperature rise and then a significant drop, if not controlled (temperature stress). Upon ethanol production, stationary phase is entered and respiratory functions become derepressed (oxidative stress). Nutrient supplies become limited or exhausted (nutrient stress). Moreover, yeasts may be exposed to sulphite added for microbiological containment, as well as to other must components (e.g. heavy metal ions).

Different environmental stresses may trigger some overlapping cellular responses: (1) Production of glycerol as a compatible solute involving the HOG pathway. (2) Changes in membrane- and cell wall constituents to enforce surface protection (CWI pathway). (3) Increase in heat shock proteins for targeting of proteins to refolding or degradation (HSR pathway). (4) Synthesis of detoxification enzymes and protectants against oxidative stress (OSR pathway). (5) Production of trehalose as a protectant mediated by the GSR pathway.

Each of these pathways has been covered by a variety of excellent reviews, which will be cited in corresponding sections. Yeast stress responses under beer brewing conditions have been summarized, recently (Gibson et al. 2007).

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H. König et al. (eds.), *Biology of Microorganisms on Grapes, in Must and in Wine*,

Table 15.1 Stress factors frequently encountered by *S. cerevisiae*

Stress	Condition	Response[a]	Section
Hypoosmolarity	On grape skins (after rain); Yeast production (washing steps)	CWI	15.2.2
Hyperosmolarity	First stages of must fermentations	HOG	15.2.1
Ethanol	Later stages of fermentation; Biological aging	HSR, OSR	15.2.3 15.2.4
Sugar limitation	Late stages of vinification	GSR, TOR	15.2.5 15.3.1
Nitrogen depletion	Later stages of vinification; Different stages of "natural" fermentations (FAN[b])	GSR, TOR	15.2.5 15.3.1
Sulfite	Throughout some must fermentations (if used for microbiological containment)	OSR	15.3.4
Low pH (Acetic acid)	Later stages of vinification; Biological aging	GSR	15.3.3 15.2.5
Oxidative stress	Later stages of vinification; Yeast production (biomass, dry yeast)	OSR	15.2.4
Temperature	Throughout must fermentation (if not controlled); Yeast production (dry yeast)	HSR	15.2.3

[a]*CWI* cell wall integrity pathway; *GSR* general stress response pathway; *HOG* high osmolarity glycerol pathway; *OSR* oxidative stress response; *HSR* heat shock response; *TOR* target of rapamycin pathway. See text for further details

[b]*FAN* free amino nitrogen, i.e. ammonium and free amino acids present in the must; applies primarily in cases without additional supplementation

15.2 Stress-Response Signalling Pathways

In the following sections we will first describe basic components of major stress response signalling pathways and then summarize how different signals may be integrated. The effect of wine-specific stress factors will be discussed in subsequent sections.

15.2.1 The High Osmolarity Glycerol (HOG) Pathway (Hyperosmotic Stress Pathway)

Yeasts in vinification first encounter high sugar concentrations (> 200 g L^{-1} total; Chap. 6). The resultant osmotic stress is counteracted by accumulation of intracellular glycerol as a compatible solute. This is achieved by an increased activity of enzymes which divert glycolytic flux from triosephosphates (see Chap. 6). Signalling in this context is mediated by the HOG pathway, as one of at least four MAPK pathways (for Mitogen Activated Protein Kinase) in yeast. These are highly conserved in eukaryotes and contain a central module, composed of three consecutive protein kinases (MAPKKK, MAPKK, MAPK). The HOG pathway stimulates glycerol accumulation, but also induces gene expression in response to heat shock (see below and Varela et al. 1992).

The main components of the HOG pathway are depicted in Fig. 15.1: The MAPK Hog1 is activated upon a hyperosmotic shock by phosphorylation by the MAPKK Pbs2. Pbs2 gets activated by upstream MAPKKK's, representing either of two branches. One is negatively controlled by the sensor Sln1, whereas the other branch receives signals through the Sho1 complex. The first branch resembles bacterial two-component systems. Under normal osmotic conditions Sln1 (containing an extracellular sensor domain, a cytosolic histidine kinase- and a receiver domain) transfers the phosphogroup to a histidine in Ypd1 and from there to Ssk1 (response regulator). Phosphorylated, the latter is incapable of activating a redundant pair of MAPKKK's (Ssk2/Ssk22). A hyperosmotic shock inhibits this chain, so that Ssk1 can interact with the MAPKKK's and trigger pathway activation.

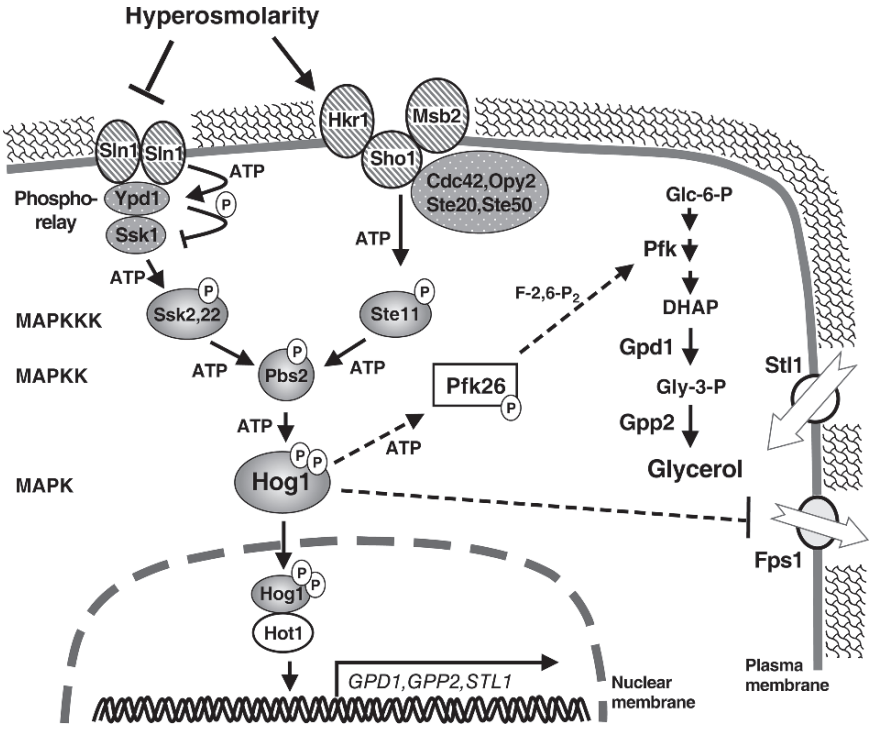

Fig. 15.1 High osmolarity glycerol (HOG) pathway. Two branches (Sho1- and Sln1-branch) sense high medium osmolarity and ultimately activate the MAPK Hog1. Hog1 mediates target gene expression by interacting with transcription factors like Hot1 (only some targets are presented). It also regulates different processes in the cytosol (*dashed lines*). Signal receptor proteins are designated by hatched, signal mediators by dotted patterns. Protein kinases are grey shaded and transcription factors are indicated by oval shaped, non-filled circles. ATP is only shown where it serves as a phosphate donor for protein phosphorylation, further indicated by phosphate groups attached to respective proteins. *Pfk26* Phosphofructo-2-kinase (*Pf2k*); *Pfk* Phosphofructokinase; *Gpd1* Glycerol-3-phosphate dehydrogenase; *Gpp2* Glycerol-3-phosphate phosphatase; *Glc-6-P* Glucose-6-phosphate; *DHAP* Dihydroxyacetone phosphate. See Chap. 6 for glycerol metabolism

In the second branch, Hkr1, Msb2 and Sho1 collaborate in sensing a high external osmolarity and generate the cytosolic signal by recruiting proteins, such as Ste20 kinase (Fig. 15.1). This activates MAPKKK Ste11, which phosphorylates MAPKK Pbs2 to trigger downstream events.

Activated Hog1 is partially translocated into the nucleus and induces target gene expression by interacting with transcription factors (Hot1, Msn1, Msn2, Msn4, Sko1). It also participates in transcription elongation (Proft et al. 2006) and regulates different processes in the cytosol (glycolytic flux, translation of mRNAs and cell cycle control). Glycerol accumulation is controlled at three levels by Hog1 (Hohmann et al. 2007): (1) Phosphofructo-2-kinase gets activated by phosphorylation, which produces an allosteric activator of phosphofructokinase (Pfk) and thereby ensures triosephosphate supply. (2) Glycerol export is inhibited by closing of the Fps1 channel, followed by its endocytosis and degradation. (3) Expression of genes encoding enzymes of glycerol synthesis is induced. Thus, genes encoding isozymes of glycerol-3-phosphate dehydrogenase (*GPD1*) and glycerol-3-phosphate phosphatases (*GPP2*) are transcribed under osmotic stress, as is *STL1,* which encodes a glycerol/H^+-symporter.

15.2.2 The Cell Wall Integrity (CWI) Pathway (Hypoosmotic Stress Pathway)

Another MAPK pathway signals low osmolarity, which occurs in production of dry yeast and preparation of starter cultures, and ensures cell wall reconstruction. The CWI pathway is also activated by cell wall or plasma membrane perturbations (Fig. 15.2; Straede et al. 2007). Its central kinase module is composed of Bck1 (MAPKKK), a dual pair of MAPKK's (Mkk1,2) and MAPK Mpk1 (=Slt2; Heinisch et al. 1999). Plasma membrane sensors of the Wsc-family (for Wall Stress Component) interact with Rom2, a GDP/GTP exchange factor (GEF) for the small GTPase Rho1. In its active, GTP-bound state, it interacts with yeast protein kinase C (Pkc1), which then activates the MAPK module by phosphorylation of upstream MAPKKK Bck1. The main target of downstream MAPK Mpk1 is the transcription factor Rlm1, which triggers expression of genes encoding proteins important for cell wall construction (e.g. glucan synthase and various cell wall proteins; Levin 2005). A re-enforcement of cell wall increases resistance to environmental stress conditions, as well as survival in stationary phase (Gray et al. 2004). Interestingly, CWI signalling is conserved in *Kluyveromyces lactis*, a close relative of the wine yeast *K. marxianus* (Rodicio et al. 2007). This indicates that non-*Saccharomyces* yeasts present in the early stages of must fermentation may use similar strategies to cope with surface stress.

15.2.3 The Heat Shock Response (HSR) Pathway

Sudden temperature changes do not occur in modern wine production. Yet, in smaller wineries (and in the millenniums yeast has been employed for vinification)

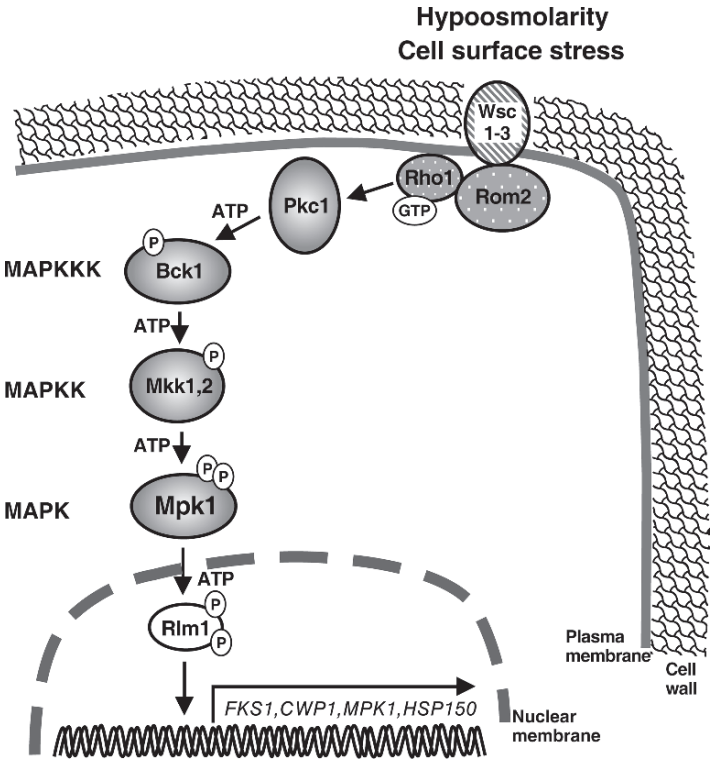

Fig. 15.2 Cell wall integrity (CWI) pathway. Low medium osmolarity and stresses affecting either cell wall structure or plasma membrane are detected by Wsc-family sensors. One effector of Pkc1 is the MAPK cascade, activating transcription factor Rlm1, which triggers expression of genes required for cell wall construction. Patterns used to designate protein functions are as explained in legend of Fig. 15.1

temperature may rise above 30°C during vigorous fermentation and drop dramatically thereafter. Further, in dry yeast production high temperatures are applied (Attfield 1997). Temperature shifts are also encountered by yeasts in their natural environment, i.e. in the vineyard.

Heat mainly increases plasma membrane fluidity and causes unfolding and aggregation of cytosolic proteins. The HSR pathway leads to an increase in heat shock proteins (Hsp's) which mediate either refolding of denatured proteins or their ubiquitin- and proteasome-dependent degradation (Estruch 2000). Expression of more than 50 genes is up-regulated upon a shift of yeast cells to higher temperatures (38 °C). The main components involved in yeast HSR are shown in Fig. 15.3. Two major transcription factors mediate the response: Hsf1 and general stress mediators Msn2 or Msn4. The former binds as a trimer to heat shock elements (HSE) in the promoters of its target genes, the latter to the STRE sequences (Sect. 15.2.5). Both overlapping and Hsf1-specific gene expression can be observed.

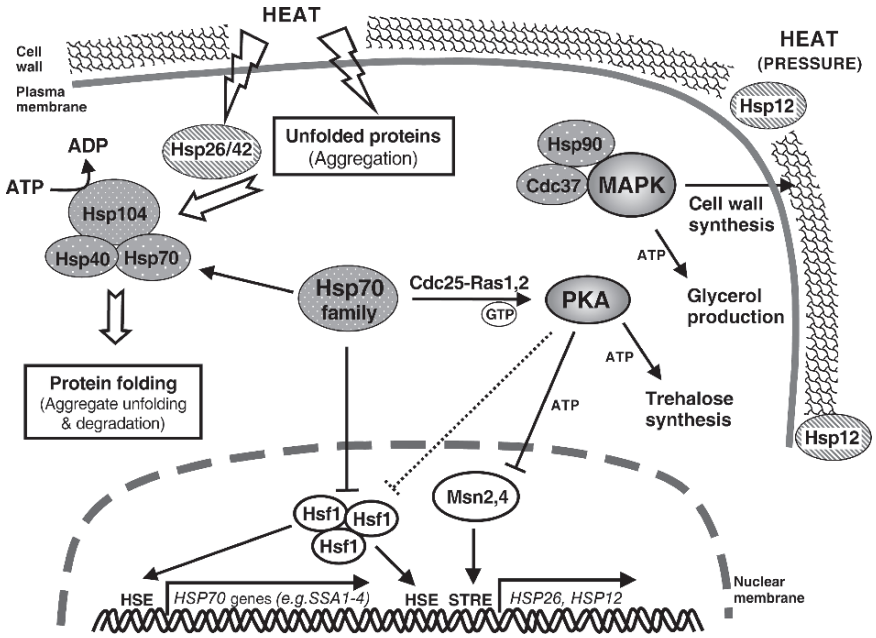

Fig. 15.3 The heat shock response (HSR). Upon heat shock, Hsp70 is recruited to the Hsp104/70/40 complex. This leads to activation of transcription factor Hsf1 and synthesis of heat shock proteins. Connections to other stress responses pathways are indicated (see section 15.2.6). *HSE* Hsf1 response element; *STRE* stress response element. Patterns used to designate protein functions are as explained in legend of Fig. 15.1

Hsf1 is negatively controlled by Hsp70 proteins (of which there are 14 homologs in yeast, amongst them Ssa1 and Ssa2), i.e. the latter interact with Hsf1 and inhibit its transcription activation function. On a heat shock, sensors Hsp26 and Hsp42 change their conformation and form large oligomers. These deliver unfolded proteins to the Hsp104/Hsp70/Hsp40 complex for refolding (Haslbeck et al. 2005). The recruitment of Hsp70's to the complex relieves inhibition of Hsf1 and results in induction of HSR genes including the ones for Hsp70 isoforms. Thus an auto-regulatory circle has been proposed (Fig. 15.3; Estruch 2000).

15.2.4 The Oxidative Stress Response (OSR) Pathway

During the respiratory process reactive oxygen species (ROS) are produced, causing severe cell damage, apoptotic cell death (apoptosis) and aging (Gibson et al. 2007; Kaeberlein et al. 2007). In vinification, ROS are produced in the early phases of yeast propagation, during prolonged exposure to ethanol (if oxygen is available), and in the presence of heavy metals. Under laboratory conditions, hydrogen peroxide is frequently employed to apply oxygen stress.

The main components involved in OSR are shown in Fig. 15.4 (reviewed in Estruch 2000). Two types of transcription factors (Yap1, with 5 homologs in yeast, and Skn7/Pos9) regulate the expression of genes encoding detoxification enzymes. Target gene promoters are either recognized in concert or individually in response to different stresses. Yap1 function is regulated by its nuclear export sequence, which is only recognized by nuclear exporter Crm1 if key cysteine residues are reduced. If oxidized, Yap1 resides in the nucleus and activates transcription of target genes with YRE sequences (Yeast AP1-like Response Elements) in their promoters. In the cytosol, Yap1 is oxidized by a glutathione peroxidase-like enzyme (Gpx3) which serves as a sensor of hydrogen peroxide. Another sensor which acts through Gpx3 in the same pathway is Ybp1 (Fig. 15.4; Veal et al. 2003). Oxidized Yap1 translocates into the nucleus and binds to YRE sequences in its target gene promoters.

Similarly, Skn7 binds to so-called OSRE (for Oxygen Stress Response Element) sequences. Skn7 is a response regulator containing a receiver domain reminiscent of bacterial two component systems. Although it can be activated via the Sln1 branch of the HOG pathway under osmotic stress, activation upon oxidative stress is Sln1 independent (He and Fassler 2005).

OSR pathway activation leads to production of enzymes for detoxification (superoxide dismutase, catalase) and for synthesis of protectants (i.e. glutathione,

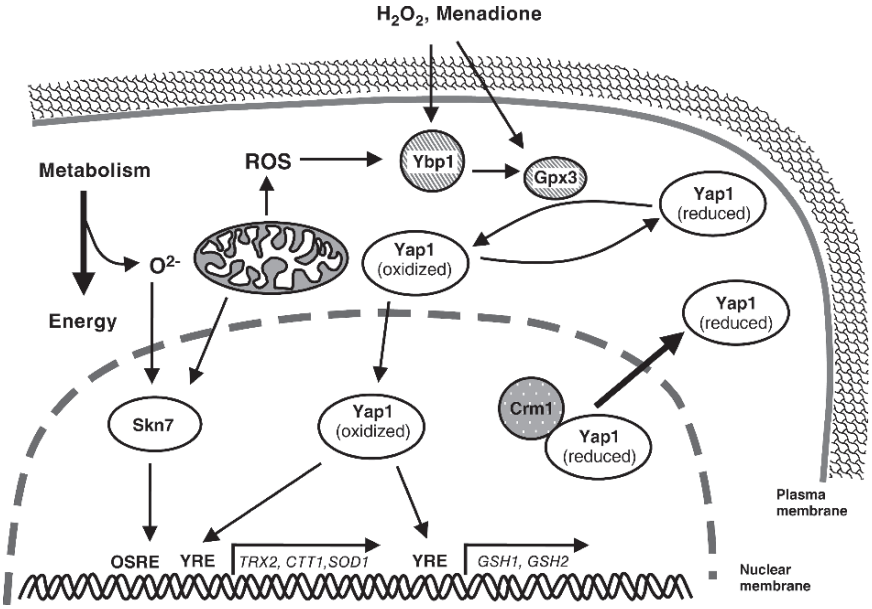

Fig. 15.4 The oxidative stress response (OSR). Reactive oxygen species (ROS) generated by external oxidative stress or by the mitochondrial respiratory chain are converted to a cellular response by two major transcription factors (Yap1, Skn7). Sensor proteins Ybp1 and Gpx2 are depicted. Oxidation of Yap1 favours nuclear localization; the reduced form is exported to the cytosol. *YRE* yeast AP1-like response element; *OSRE* oxygen stress response element. Patterns used to designate protein functions are as explained in legend of Fig. 15.1

thioredoxin, glutaredoxin; see Estruch 2000, for a list of target genes). Decrease in glycolytic flux and increase in pentose phosphate pathway activity leads to the regeneration of NADPH required under oxidative stress.

15.2.5 The General Stress Response (GSR) Pathway

Many different stresses in yeast provoke a similar set of responses. The terms "general stress response" (GSR) or "environmental stress response" (ESR) have been coined for these events. As a physiological result, exposure to one stress condition at sub-lethal doses (e.g. exposure to heat) confers protection not only against higher doses of the same stress, but also against different ones (e.g. ethanol tolerance). This effect is commonly known as "cross protection" (CP).

Control of gene expression in the GSR pathway is mediated by STRE (Stress Response Element) sequences in the respective promoters. Induction also occurs in response to osmotic and oxidative stress, nutrient limitation, heavy metals and DNA damage (Ruis and Schüller 1995; Treger et al. 1998). STRE sequences are recognized by a redundant pair of transcription factors, Msn2 and Msn4, whose subcellular localization depends on cAMP/PKA signalling. The latter also plays a role in sugar sensing as described in Chap. 6 of this volume. Activated protein kinase A (PKA) phosphorylates transcription factors and results in their export to the cytosol. Upon stress (or sugar limitation) the PKA pathway is down-regulated and the de-phosphorylated transcription factors enter the nucleus to activate STRE-dependent gene expression (Estruch 2000).

Signalling to PKA is mediated by the Ras proteins (Fig. 15.5). Under non-stress conditions members of the Hsp70 and Hsp90 chaperone families stabilize the GDP/GTP exchange factor Cdc25, which activates Ras proteins (Ras1,2). Titration of the chaperones by unfolded proteins upon a stress leads to Cdc25 degradation (Wang et al. 2004). This renders Ras inactive and adenylate cyclase activity low. Lower cAMP levels result in interaction of the catalytic PKA subunits (TPK1-3) with their regulatory component Bcy1, keeping the heterotetramer inactive.

Msn2 and Msn4 control expression of genes subject to GSR, but also of genes encoding heat shock proteins, oxidative stress detoxification enzymes and trehalose metabolism. The latter is of particular interest.

The disaccharide trehalose is a major stress protectant (Gancedo and Flores 2004). It has been implicated in protection against dehydration, freezing, heating, and also against toxic compounds such as ethanol, oxygen radicals and heavy metals (Estruch 2000). The reserve carbohydrate glycogen, whose metabolism is controlled in a largely overlapping fashion, also confers increased yeast cell viability in wine fermentations (Perez-Torrado et al. 2002).

Glycogen and trehalose metabolism are summarized in Fig. 15.6 (reviewed in Francois and Parrou 2001). Carbohydrate moieties for anabolism are provided by UDP-glucose. For glycogen, the first glucose molecules are covalently linked to glycogenin (Glg1 or Glg2). Glycogen synthase isozymes (Gsy1, Gsy2) then elongate the chains. They are allosterically activated by glucose-6-phosphate and inactivated by

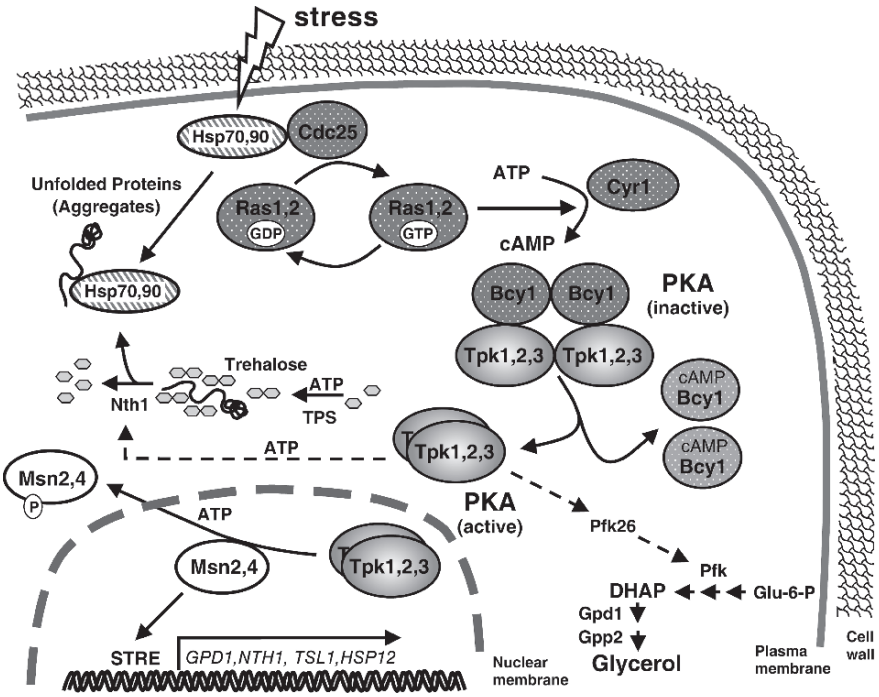

Fig. 15.5 General stress response (GSR). Most environmental stresses activate the cAMP/PKA pathway through Ras1/Ras2. Catalytic subunits of PKA can form an inactive tetrameric complex with the regulatory subunit Bcy1. Dissociation upon cAMP binding leads to partial translocation of active subunits into the nucleus, where a transcriptional response is mediated by Msn2 or Msn4 binding to target promoter elements (*STRE* stress responsive element). Trehalose collaborates with Hsps in the HSR. Glycerol serves as a compatible solute to counteract osmotic stress. Pfk26 phosphofructo-2-kinase (Pf2k). Patterns used to designate protein functions are as explained in legend of Fig. 15.1

PKA dependent phosphorylation. Branching activity is provided by Glc3. For glycogen mobilization, glycogen phosphorylase (Gph1) and a debranching enzyme (Gdb1) liberate glucose-1-phosphate and glucose, respectively. Inversely to glycogen synthase, Gph1 is inhibited by glucose-6-phosphate and activated by a PKA-dependent phosphorylation.

Trehalose is synthesized by a multienzyme complex, composed of two catalytic subunits, Tps1 (trehalose-6-phosphate synthase) and Tps2 (trehalose-6-phosphate phosphatase), and two regulatory subunits (Tps3 and Tsl1). The disaccharide can also be imported from the medium by Agt1. Hydrolysis is catalyzed by either of two trehalases (an acid isoform encoded by *ATH1* and a neutral one encoded by *NTH1*). Consistent with the reported kinetics of trehalose turnover in a laboratory strain (Lillie and Pringle 1980), Nth1 activity is high in log-phase cells, decreases with the diauxic shift and remains low in stationary phase. Similar kinetics for trehalose are observed under vinification conditions, i.e. accumulation after ammonium depletion and degradation in the lag-phase of initial growth (Novo et al. 2003).

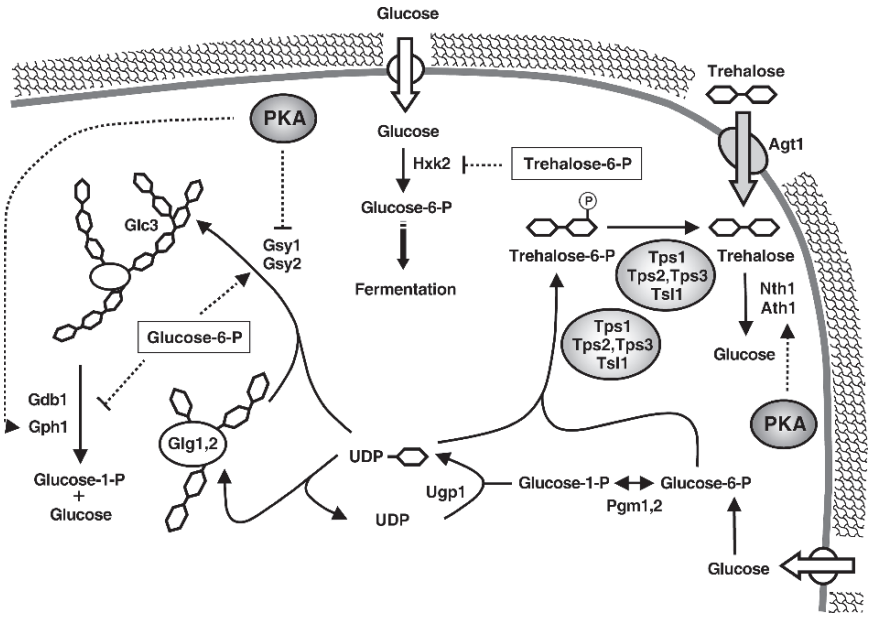

Fig. 15.6 Trehalose and glycogen metabolism in yeast. Enzymes involved in synthesis and degradation of the two carbohydrates are shown. For the sake of clarity, glucose, glucose-6-phosphate and PKA are shown in different locations. Regulatory effects are designated by dotted lines. For further details see text and Francois and Parrou (2001)

During heat shock, trehalose synthesis occurs rapidly, but is degraded soon after. This is consistent with gene expression patterns. Thus, genes for the four biosynthetic subunits are induced under heat stress and repressed by the cAMP/PKA pathway, mediated by STRE sequences in their promoters. Paradoxically, so is *NTH1* expression. Post-translationally, Nth1 is also activated by a PKA-dependent phosphorylation in response to external glucose (Alexandre et al. 2001). A model for the role of trehalose in stress protection may lend physiological significance to these findings (Singer and Lindquist 1998): In early stages, trehalose stabilizes protein structure and prevents aggregation of denatured proteins. Then, heat shock proteins take over this function and trehalose needs to be degraded to avoid interference. This also explains why trehalose confers resistance to various stresses affecting protein folding (heat, cold, and ethanol). This disaccharide has also been shown to stabilize the plasma membrane upon such stresses.

15.2.6 Cross-Pathway Stress Responses

All stresses have some physiological landmarks in common: the responses are transient, i.e. cells first increase production of key proteins and then adapt their physiology, making their presence obsolete. Stress also causes an initial slow-down

in bulk protein synthesis and a growth delay, mediated by cell-cycle control. These features are the result of coordination of different stress responses to respective environmental conditions. Since literature on such "cross-talk" is overwhelming in *S. cerevisiae*, we will only discuss a few examples in this section.

Cell surface is of special interest in providing first contact to adverse conditions (e.g. addition of glucanases in vinification). Although yeast cells activate the CWI program upon such an attack, only components downstream of Pkc1, and not plasma membrane sensors (Wsc1–3; Fig. 15.2), are required. Instead, the response depends on the Sho1-branch of the HOG pathway (Fig. 15.1), but lacks the concomitant glycerol accumulation (Bermejo et al. 2008). This indicates an unanticipated complexity in signal integration between different stress response pathways.

Another example of complex interplay is provided by exposure of yeast cells to toxic levels of acetate (100 mM). Both the HOG and the CWI pathway are activated, although only the former is required to develop resistance. In this case, the Sln1 but not the Sho1 branch of the HOG pathway is essential (Fig. 15.1; Mollapour and Piper 2006). Again, acetate treatment does not result in typical readout of glycerol production.

Response to heat shock also displays significant cross-talk with cell wall construction. Thus, a Cdc37/Hsp90 complex regulates MAPK's in the HOG and the CWI pathway (Fig. 15.3; Hawle et al. 2007). Consequently, impairment of *HSP90* expression (e.g. by a mutant Hsf1 transcription factor) causes destabilization of Mpk1 and reduced Rlm1-mediated transcription in the CWI pathway (Truman et al. 2007).

HSR and GSR are also interdependent: Thus, expression of small heat shock genes (*HSP26, HSP12*) depends on the Hsf1 transcription factor and is induced under various stress conditions. Hsp26 delivers proteins to the Hsp104 complex for refolding or degradation (Fig. 15.3), whereas Hsp12 is located in the cell wall and modulates its texture (Karreman et al. 2007). Ferguson et al. (2005) found that Hsf1 (and thereby *HSP12* expression) is negatively regulated by PKA, but independent of Msn2/Msn4 of the GSR (Fig. 15.5). In contrast, *HSP12* transcription under high pressure depends on these factors (Gibson et al. 2007).

15.3 Physical and Chemical Stress Factors Encountered in Vinification

15.3.1 Responses to Nutrient Limitations

Two major nutrients determine the fate of must fermentations: Sugars and nitrogen source (ammonium, amino acids). Although rarely depleted, limitation of the first triggers cAMP/PKA dependent responses (GSR; Sect. 15.2.5). Nitrogen may also become limiting (i.e. $< 140\,mg\,L^{-1}$) and cause stuck fermentations. As neither limitation causes yeast to enter stationary phase, the transition is postulated to be caused by ethanol concentrations above 2% (Marks et al. 2008).

Stress responses provoked by nutrient limitations are mediated by TOR signalling (for "Target Of Rapamicin"). Two complexes (TORC1 and TORC2) control cell growth (volume) rather than propagation and intervene in different stress responses (reviewed in Jacinto and Lorberg 2008). These include the cAMP/PKA pathway (ribosome biogenesis, trehalose metabolism), interaction of Hog1 and Sch9 (involved in cAMP/PKA-independent glucose signalling; see Chap. 6), cell cycle control (e.g. Rim15), and CWI signalling through Pkc1 activation by a rapamycin-insensitive branch (TOR2 complex).

15.3.2 Ethanol Stress

Conversion of must sugars to ethanol (> 10% v/v), imposes another stress on *S. cerevisiae*. Reported effects are changes in membrane composition and permeability, especially for protons, and protein unfolding (reviewed in Gibson et al. 2007). This triggers typical stress responses (Alexandre et al. 2001): (1) Inhibition of cell cycle and propagation. (2) Induction of GSR, trehalose and glycogen accumulation. (3) Induction of HSR. (4) Measures to counteract loss of plasma membrane potential, e.g. increased activity of the plasma membrane ATPase and expression of the K^+/H^+-exchanger Kha1. (5) Induction of genes encoding vacuolar proteases and their inhibitors. Clearly, ethanol tolerance is a desired trait in wine yeast but has a polygenic basis. It correlates with increased ATPase activity and high levels of oleic acid and ergosterol in the plasma membrane (Aguilera et al. 2006).

Many investigations on ethanol stress response should be taken with caution. Thus, data have been generated by sudden addition of 7–12% ethanol. In contrast, pre-adaptation and cross-protection should occur in must fermentations. Recently, studies on long-term response in must fermentations over a period of 15 days (Zuzuarregui et al. 2006; Marks et al. 2008) led to the following conclusions: (1) In contrast to sudden ethanol addition, glycerol is not accumulated in long-term fermentations. (2) Only a subset of HSR genes induced upon short-term ethanol stress contribute to long-term adaptation. (3) Despite the presence of sugars, genes encoding enzymes of respiration and gluconeogenesis are expressed. (4) Entry into stationary phase is caused by ethanol concentrations of 2%, rather than by nitrogen limitation. (5) A novel fermentation stress response (FSR) pathway is triggered in long-term adaptation (but not in usual laboratory setups, where ethanol does not exceed 2–3% v/v). FSR involves a set of genes which has not been associated with other stresses, including many with as yet unknown functions.

15.3.3 Organic Acids

Besides ethanol, yeast sugar metabolism yields organic acids, of which acetic acid is the most prominent (Chap. 6). Together with proton permeability of the plasma membrane caused by ethanol, cytosolic acidification may result in unfolding of

proteins and apoptotic cell death (Garay-Arroyo et al. 2004). Cells produce vacuolar ATPase as one way to avoid cytosolic acidification. Loss of enzyme function causes sensitivity to oxidative and cell wall stress. Yet, a defect in the CWI pathway does not lack an OSR (Ando et al. 2007). In general, low pH induces expression of genes encoding cell wall proteins (like *CWP1*), enzymes involved in glycerol and trehalose synthesis (e.g. *GPD1, GPP2, TPS1, TSC1*) and in other stress responses (*CTT1, HSP12*; Kapteyn et al. 2001).

15.3.4 Sulphite Resistance

Sulphite is frequently added to musts for microbiological containment, since *S. cerevisiae* is least sensitive to its toxic effects. Resistance has been attributed to continuous selective pressure by use of sulphur compounds over thousands of years. In fact, resistant wine yeast strains contain a specific chromosomal translocation between chromosomes VIII and XVI. The *SSU1* gene (encoding a sulphite excreting pump) thereby is transcribed from a strong promoter and escapes control by the transcription factor Fzf1 (Perez-Ortin et al. 2002).

Sulphite exerts its toxic effects by reacting with cellular compounds, especially with carbonyl groups. Acetaldehyde production can thus serve as a second resistance strategy. Toxic effects are further reduced by sulphite incorporation into methionine and cysteine, consistent with the observation that addition of methionine to the medium results in increased sulphite sensitivity. An adverse effect is observed after deregulation of adenine metabolism (Aranda et al. 2006).

15.3.5 Cold Stress

The molecular response to cold stress (CSR) has received much less attention. Changes in membrane fluidity have been correlated with modulation of gene expression patterns. This largely overlaps with HSR, indicating that the latter generally responds to sudden temperature changes (Aguilera et al. 2007). Yet, components of general and oxidative stress response pathways can also participate in CSR.

15.4 Emerging Issues

A wealth of transcriptome data has been generated for wine yeast. These may not always correlate well with proteome studies (Trabalzini et al. 2003; Shioya et al. 2007). In this context, translational control by so-called P-bodies (complexes of RNA and proteins which serve as mRNA storage and/or degradation entities within the cytoplasm) may explain some of the discrepancies. For example, stationary cells

still react to both oxidative and temperature stresses. Interestingly, this response does not require *de novo* transcription, but specific sets of mRNAs are apparently set free from P-bodies (Aragon et al. 2006). They have also been shown to accumulate in yeast cells upon ethanol stress (Izawa et al. 2007). Such mechanisms are only beginning to be investigated in wine making.

Gross chromosomal rearrangements have also been reported as an adaptation to stress, such as high temperature. They occur predominantly at recombination hot spots (Ty elements, tRNAs, ARS elements and at telomeres) and may lead to local gene amplifications and altered expression of stress-related genes (James et al. 2008). Except for sulphite resistance (Perez-Ortin et al. 2002), how such chromosomal rearrangements can be specifically employed by wine yeast remains elusive.

Finally, with regard to the complex interplay of stress response mechanisms and central carbohydrate metabolism described in this chapter, bioinformatic approaches leading to a systems biology of wine yeast, although still in their infancy, will result in a profound understanding of how signals are integrated by a yeast cell/population within the next decade (Borneman et al. 2007; Pizarro et al. 2007). This knowledge will undoubtedly result in improvements in handling of wine yeast strains and ultimately in even higher quality wines.

15.5 Conclusions

In the process of vinification, yeast is exposed to a variety of different stress conditions, to all of which it has evolved appropriate responses to ensure cell viability. It is to be expected that similar mechanisms will also be operating in non-*Saccharomyces* yeasts. Besides a GSR system commonly employed under adverse growth conditions, specific stresses may also trigger specialized responses. We now have a fairly good overview of the molecular components involved. However, we are only beginning to understand the complex regulatory networks which coordinate different pathways. Especially the observation of cross-pathway protection indicates that a profound knowledge of these processes may have a fundamental impact on applications in wine industry. These will strongly depend on development of systems biology tools.

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Chapter 16
Physical and Chemical Stress Factors in Lactic Acid Bacteria

Jean Guzzo and Nicolas Desroche

16.1 Introduction

Malolactic Fermentation (MLF) takes place after Alcoholic Fermentation (AF) with a delay more or less long according to the wine-making conditions, bacteria concentrate and physical and chemical factors of the wine. This phase consists of an adaptation phase of the cells to stress factors of the wine. It starts as soon as bacterial population reaches a concentrate of 10^6 UFC mL^{-1} and its duration is approximately, 5 days to 3 weeks according to physical and chemical factors (Lonvaud-Funel 1995). Consumption rate of L-malic acid and duration of MLF depend on the biomass present, and the malolactic activity of the lactic acid bacteria. Physical and chemical conditions, notably pH, and availability of sugar, are determining factors for viability and specific activity of *O. oeni* cells (Krieger et al. 1990). Presence of ethanol in high concentrate, sulphites and pH acid, results in this bacterium selection during alcoholic fermentation (Britz and Tracey 1990). Other factors like technological conditions have an influence on survival, bacterial development and malolactic activity.

16.2 Parameters Influencing Malolactic Fermentation and Limiting the Growth of Lactic Acid Bacteria in Wine

16.2.1 Technological Factors

Wine-making methods are the first factors to influence bacterial development and realization of MLF.

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The clarification process is unfavourable for development of lactic acid bacteria. This process eliminates a part of native microflore that is afterwards responsible for spontaneous triggering off of MLF. Moreover, it reduces the quantity of nutriments necessary for development of both fermentations.

Decanting at the end of AF also has an impact on MLF. This process eliminates yeast dregs which autolysis, thus depriving the bacteria of growth factors (mannoproteins, vitamins) which can be liberated during this stage.

Addition of sulphite at the end of AF, in order to limit wine oxidation, is not advised. Due to its antimicrobial activity (Chang et al. 1997), SO_2 induces a decrease in the lactic acid bacterial population and a late starting of MLF.

Finally, usage of stainless steel tanks instead of wooden ones reduces natural developing of the wine during wine-making process.

16.2.2 Microbiological Factors

During the process of wine-making, there is large microbial diversity. *O. oeni* coexists with several other micro-organisms: lactic acid bacteria and essentially yeasts. This bacterium is in competition with the latter for usage of wine nutriments.

During AF, yeasts multiply. Yeasts consume sugar and nitrogenous sources, including amino acids, more quickly than bacteria and liberate toxic metabolites (ethanol, fatty acids, SO_2) for the lactic acid bacteria that are also at the origin of regression of the bacterial population (Alexandre et al. 2004, Guilloux-Benatier et al. 1998). Yeasts can equally produce and liberate substances possessing a bacteriostatic or bactericidal effect which affects MLF (Comitini et al. 2005, Osborne et al. 2006). At the end of AF, yeast autolysis thus liberates growth factors (amino acids, vitamins, mannoproteins) for the lactic acid bacteria (Alexandre et al. 2001, Guilloux-Benatier et al. 2006). These molecules allow a quick growth of the bacteria and a decrease in the latency time between the two fermentations. Yeasts/bacteria interaction is complex, firstly opposing, then synergic, and is still relatively unknown (Alexandre et al. 2004, Guilloux-Benatier et al. 1998).

This phenomenon of competition also exists between different types of lactic acid bacteria. During the wine-making process, opposing effects between different bacterial types entitled *Pediococcus, Lactobacillus, Leuconostoc* and *Oenococcus* can be found (Lonvaud-Funel and Joyeux 1993). These effects are probably due to liberation of components with antimicrobial property such as bacteriocines (Yurdugül and Bozoglu 2002).

As for during the development process of dairy products, phage represents a threat for the MLF process. *O. oeni* strains can be infected by phage (Henick-Kling et al. 1986). These phage attacks appear in wine together with a slowdown of MLF (Henick-Kling 1995). The phages, isolated from wine, are able to induce lytic and lysogene cycles in *O. oeni* (Henick-Kling et al. 1986, Poblet-Icart et al. 1998). Sensitivity of *O. oeni* strains to phage is very variable. Difficulties in MLF due to

phage can therefore result in a need for more time, thus allowing for undesirable bacteria such as *Pediococcus* to develop.

In certain cases, phage attacks can induce a stopping of MLF. Nevertheless, these attacks are limited by the acidity of the wine and less frequent than in the dairy industry.

16.2.3 Physical and Chemical Factors

Wine is a complex environment (sugar, fatty acids, amino acids, organic acids, phenol components, ethanol, SO_2, pH). Its physical and chemical characteristics vary according to numerous conditions: vine variety, climatic conditions, and wine-making conditions. These physical and chemical characteristics do not correspond to the optimum conditions for growth of lactic acid bacteria and particularly *O. oeni* and are, thus, stressful. These parameters therefore have a major impact on the progress of MLF. Principal factors are pH, ethanol content, concentration in sulphites and temperature (Versari et al. 1999).

Acidity. pH: pH is the factor which has most influence on development of lactic acid bacteria in wine. Most lactic acid bacteria are neutrophilic. Generally, the optimum pH growth of lactic acid bacteria is close to neutrality (Hutkins and Nannen 1993). Some families of bacteria such as *Lactobacillus* and *Oenococcus* show more acidophilic behaviour. However, during wine making, the average pH is low (pH between 3.0 and 3.8); the bacterial growth rate is therefore longer. So, the more the pH of wine is low, the more the latency phase between AF and MLF is long. At pH values less than 3.0 bacterial growth is very difficult or impossible according to other physical and chemical factors (Lonvaud-Funel 1995).

Acidity creates major damage at cell level. In fact, the pH modifies the survival of bacteria and can induce a slowdown and/or a stop of their growth. Metabolic changes linked to acid stress are therefore numerous and complex. The main effect of wine pH is to generate a decrease in intracellular pH (pH_{int}). Intracellular pH is a critical factor for controlling cell processes such as enzymatic activity, ATP and RNA synthesis, and DNA replication (Belguendouz et al. 1997, Molina-Gutierrez et al. 2002). Acidity also results in protein denaturation (Molina-Gutierrez et al. 2002).

Variations in pH_{int} influence the enzymatic reactions of the cell. In fact, when pH_{int} becomes too acidic in *O. oeni* cells, enzymatic activities, notably malolactic and ATPase activities, are inhibited (Britz and Tracey 1990, Carrete et al. 2002). Once lower limit of pH_{int} is reached, cell damage is large : enzymatic activities can no longer take place and the cells die (Hutkins and Nannen 1993).

In addition to modifying pH_{int}, extra-cellular pH plays an important role in sugar metabolism (Henick-Kling 1995). A pH level exists for sugar and L-malic acid assimilation. This pH level corresponds to the lower pH from which sugar is used. At pH 3.0, glucose is practically no longer metabolised while L-malic acid is transformed into L-lactic acid and carbon dioxide. Moreover, transport of L-malic acid

is regulated according to the extra-cellular pH. The lower the pH, the higher the diffusion of L-malic acid, in its non-dissociated form is. There is thus a modification in metabolism according to the wine pH.

Acidity can also impair proteins by modifying their ionic interactions thus causing aggregation and denaturation (Cotter and Hill 2003, van de Guchte et al. 2002). DNA structure damage, notably to its topology, was also observed (Drlica 1992). Intracellular acidification is the origin of a DNA rolling decrease and generates DNA depurination and depyrimidation (van de Guchte et al. 2002). These modifications affects the expression of several genes and induces a DNA repair system (Hartke et al. 1996, van de Guchte et al. 2002).

Finally, cytoplasmic membrane is the first target of acidity. Acidity generates large modifications in composition and fluidity of this membrane. At low pH, the barrier role of the membrane is altered, permeases no longer function correctly and molecules can be freely distributed throughout the cell and, exchanges with the extra-cellular environment are then disrupted.

In conclusion, pH is an essential factor in wine; it influences survival and development of lactic acid bacteria by intervening at different levels: modification of the growth rate and pH_{int}, and total membrane change.

Ethanol content. Ethanol, produced by yeast during AF, is considered as one of the main factors which inhibits growth of lactic acid bacteria in wine. The final ethanol content is very variable according to the wine (10–16% v/v). Different types of lactic acid bacteria are more or less tolerant to ethanol. *O. oeni* can tolerate content attaining 14% (v/v) (Alegria et al. 2004, Britz and Tracey 1990). These values are however variable according to the strain studied (Henick-Kling 1995). Resistance to ethanol also varies according to other environmental conditions such as temperature and wine pH (Alegria et al. 2004, Britz and Tracey 1990). Cell tolerance to ethanol diminishes when environmental pH is low and temperature increases. Low concentrate in ethanol (3–5% v/v) can stimulate *O. oeni* growth (Alegria et al. 2004, Britz and Tracey 1990). However, with concentrate higher than 8% (v/v), this metabolite is responsible for the inhibiting, or even bacterial death, of *O. oeni* strains (Capucho and San Romao 1994, Teixeira et al. 2002). It affects the latency phase and growth rate of lactic acid bacteria in the wine. Nevertheless, this inhibiting phenomenon is lessened when ethanol is produced in a progressive manner. In these cases, lactic acid bacteria can adapt itself progressively to ethanol presence. This phenomenon is called “adaptive response” (van de Guchte et al. 2002, Weber and de Bont 1996).

Ethanol toxicity is generally attributed to the fact that this molecule inserts itself into the hydrophobic part of the membrane lipid double layer (Weber and de Bont 1996). Destabilization of the membrane structure then occurs which later affects several cell processes such as DNA replication, enzymatic activities, metabolites transport and peptidoglycane synthesis (Jones 1989, Weber and de Bont 1996). Membrane permeability is modified and it no longer plays its barrier role. Membrane polarity is increased and can favour the passage of other polar molecules through the membrane.

Like acidity, the cell membrane is considered as the first target of ethanol (Garbay and Lonvaud-Funel 1996, Teixeira et al. 2002). Ethanol presence induces

an increase in *O. oeni* membrane fluidity (Chu-Ky et al. 2005, Da Silveira et al. 2002, Da Silveira et al. 2003). The membrane then becomes permeable to many solutions and produces a loss of intracellular material such as co-factors (NAD^+/NADH and AMP) and ions (Da Silveira et al. 2002). The composition of cell membrane is equally dependent on ethanol presence (Teixeira et al. 2002). Cells of *O. oeni* modify composition of fatty acids in its membrane during culture in the presence of ethanol: (1) the proportion of cyclic fatty acids increases (Teixeira et al. 2002) and (2) the membrane protein/phospholipid ratio increases to limit the effect of ethanol on lipids (Da Silveira et al. 2003). Exposure to ethanol can equally induce a dissipation of the membrane electrochemical gradient (Da Silveira et al. 2002, Silveira et al. 2004). An influx of protons can then occur which will affect cell processes dependant on the pH gradient such as ATP synthesis, transportation of amino acids and L-malate. Ethanol also has an impact on enzymatic activities. The malolactic activity is notably modified according to ethanol concentrate. When the concentrate in ethanol is higher than 12% (v/v), malolactic activity is inhibited (Capucho and San Romao 1994).

Ethanol has therefore an important impact on physiology of cells because its presence generates important modifications that are the basis for adaptation of the cells to this stress. Due to these effects on the cell, this component influences MLF development, by notably modifying latency time between AF and the beginning of MLF necessary for the adaptation of the cells.

Temperature. Temperature has an important role in the final quality of wine (Reguant et al. 2005b). It modifies growth speed of all microorganisms (yeasts and bacteria). All bacteria show a growth optimum temperature, as for the pH. Majority of lactic acid bacteria being mesophilic (van de Guchte et al. 2002), its optimum growth is between 25 and 30°C in laboratory culture. In wine the optimum temperature of growth is different from that obtained in a laboratory. The ideal temperature for growth of *O. oeni* in wine and consumption of L-malic acid is between 20 and 25°C, according to the strain tested (Britz and Tracey 1990). This value is modified according to physical and chemical parameters and notably ethanol content. The higher the ethanol content, the more of the optimum growth temperature is decreased.

The average temperature at which MLF is carried out in the cellars is between 18 and 22°C. These conditions are therefore favourable for the growth of *O. oeni*. However, in certain cases temperature is often less than 18°C, bacterial growth is then slower, enzymatic activities are reduced and MLF starts late. At 15°C, MLF is very slow and growth is almost impossible (Britz and Tracey 1990).

Low temperature has an impact on the molecule and notably on transcription, mRNA translation and DNA replication (van de Guchte et al. 2002). In fact, cold temperatures induce formation of secondary structures in the mRNA which slow down translation (Sanders et al. 1999). Temperature changes can equally induce negative DNA rolling and modify topoisomerase activities and DNA gyrase (Abee and Wouters 1999, Drlica 1992).

Finally, as for ethanol and pH, temperature has an important effect on membrane fluidity (Chu-Ky et al. 2005). However, few studies have been carried out in order to measure the impact of low temperatures on *O. oeni* physiology.

Temperature has an important impact on MLF, because it directly influences bacteria growth speed. However, it is an easy factor to control compared to pH and ethanol content.

Sulphites or sulphur dioxide. Sulphur dioxide (SO_2) is another factor which plays an essential role in the growth of *O. oeni* and on the realization of MLF (Reguant et al. 2005a). This component, found in wine with variable concentrate according to the wine-making conditions, has two origins: (1) an exogenous origin and (2) an endogenous origin.

The exogenous SO_2 originates from sulphuring during wine-making. This method consists of adding sulphur anhydrous in salt or gas form to the must. Sulphur dioxide is mainly used for its antioxidant effect and antimicrobial activity. It can be added to grape must when it is being put into the vat, to limit proliferation of lactic acid bacteria and thus avoiding interruption of AF (Chang et al. 1997). It also permits better extraction of anthocyanes.

Endogenous SO_2 originates from yeast metabolism. During AF, yeasts synthesize and naturally liberate molecular SO_2 in wine. The quantity varies according to yeast strain and wine-making conditions (Alexandre et al. 2004, Comitini et al. 2005).

In wine, the SO_2 present is in equilibrium in a free and combined form. Three liberated forms of sulphur dioxide are present: (1) molecular (SO_2), (2) bisulphite (HSO_3^-) and (3) sulphite (SO_3^{2-}). The predominant form varies according to environmental pH (Fig. 16.1). In wine pH, the bisulphite form predominates in equilibrium with the hydrated form (H_2SO_3) which constitutes the active form of free SO_2. This form is responsible for antioxidant activity and antimicrobial effect. It can freely diffuse itself inside the bacteria. SO_2 then reacts with cell

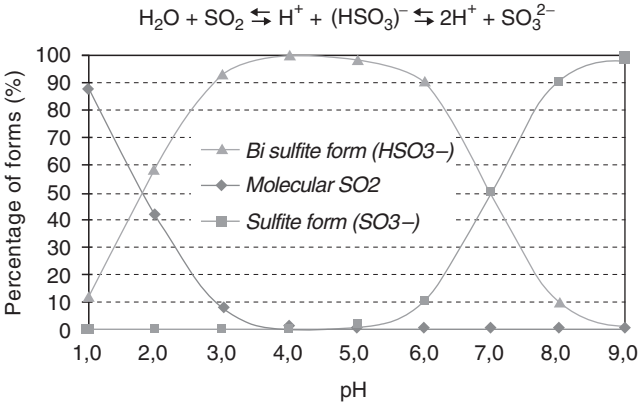

Fig. 16.1 Equilibrium reactions between free sulphur dioxide and proportion of three types for different pH values. Equilibrium equations between the different forms are indicated above the graph

constituents, interferes with protein disulphurous bridges and associates itself with coenzymes and vitamins (Chang et al. 1997). All these reactions induce cell death.

Apart from these free forms, SO_2 can be found in combined forms. Combined SO_2 results from combination of the bisulphite form with other molecules such as sugar, anthocyanes and aldehydes. Antimicrobial activity is weak (SO_2 combined with acetaldehyde) or nonexistent in this form. Temperature and ethanol content modulates antimicrobial effect of SO_2 while modifying the proportion of liberated and combined forms (Britz and Tracey 1990). Moreover, the quantity of molecular SO_2 depends on the quantity of free SO_2, pH and essentially the composition of wine, notably in organic acids and in aldehydes which regulate the balance between the free and combined form (Osborne and Edwards 2007). The Lower the pH in wine, the more SO_2 presents a larger the antimicrobial effect. *O. oeni* is able to develop tolerance to SO_2 (Guzzo et al. 1998). SO_2 has therefore a selective effect on the native microflore of lactic acid bacteria. This parameter allows the selection of *O. oeni*, as for pH and ethanol content. Concentrates of 1–10 mg L^{-1} of free SO_2 and 50–100 mg L^{-1} of combined SO_2 (100–150 mg L^{-1} of total SO_2) are able to inhibit growth of lactic acid bacteria (Reguant et al. 2005a). Tolerance to SO_2 is very variable depending on the *O. oeni* strains (Britz and Tracey 1990).

SO_2 also has an impact on different enzymatic activities. Malolactic activity of cells is sensitive to SO_2 concentration (Henick-Kling et al. 1989, Henick-Kling 1995). Carrete et al. (2002) showed that SO_2 also has a major inhibiting effect on ATPase activity, which reduces by more than 50% for concentrations in total SO_2 of 40 mg L^{-1}. Reduction of this activity induces major loss of viability in cells. In fact, the ATPases, by their implication in the mechanism of MLF and maintaining of pH_{int}, are implied in the survival of *O. oeni*.

Nutritional deficiencies. On top of all these factors, lactic acid bacteria can be confronted with nutritional deficiency. Wine is a poor environment from a nutritional point of view. The nutritional composition of wine can vary. It depends on grape variety, berry maturity, yeast strain and wine-making conditions (Alexandre et al. 2004). Even though wine contains necessary elements for growth, difficulties in development can appear. During AF, yeasts make the environment poorer in carbon (sugar such as glucose and fructose) and nitrogenous sources. Lactic acid bacteria requires carbon and nitrogenous sources as well as remaining mineral elements for their growth.

At the end of AF, residual sugars, essentially fructose, glucose and pentose, are the principal sources of carbon and energy. Their concentration is variable depending on the wine (10–0.1 g L^{-1}). This low concentration is nevertheless sufficient for bacterial growth (Henick-Kling 1995). It is the availability in nitrogenous sources (amino acids, peptides and proteins) that is generally limiting (Alexandre et al. 2004, Remize et al. 2005). Lactic acid bacteria, and particularly *O. oeni*, are auxotroph for a lot of amino acids (Garvie 1967). This deficiency phenomenon is compensated for, at the end of AF by the autolysis of yeast which allows peptides, amino acids and mannoproteins to be freed in large quantities. This phenomenon stimulates growth and malolactic activity of lactic acid bacteria (Alexandre et al. 2004,

Comitini et al. 2005). *O. oeni* is equally able to synthesize and secrete exoprotease which induces yeast lyses and allows for the liberation of nitrogenous nutrients necessary for its growth (Guilloux-Benatier et al. 2000). *O. oeni* is also able to produce proteases and amino peptidases that can hydrolyse macromolecules during autolysis at the end of AF (Farias and Manca de Nadra 2000, Manca de Nadra et al. 1997, Manca de Nadra et al. 1999, Rollan et al. 1998). Protease activities seem to be dependent on stress conditions and nutritional deficiency, with an increase observed during stress conditions (Remize et al. 2005, Rollan et al. 1998).

This deficiency phenomenon can be at the origin of a slowdown of the cell's general metabolism. Deficiency in carbon sources can lead to a decrease in the intracellular ATP pool. A deficiency in phosphate can slow down DNA and RNA synthesis, and nitrogenous deficiency is generally at the origin of decrease in the intracellular protein synthesis (van de Guchte et al. 2002).

Other factors specific to wine. Wine is a particularly complex environment and several other factors have an influence on growth of *O. oeni* and on malolactic activity. Polyphenols and anthocyanes, present in wine, can inhibit bacterial development (Figueiredo et al. 2008). However, the effect of these molecules on malolactic activity is still quite unknown (Reguant et al. 2000, Vivas et al. 1997). As many components are present in wine, some polyphenols and anthocyanes are favourable for bacterial growth and others are unfavourable (Campos et al. 2003). Most of the polyphenols have a neutral effect on the growth of *O. oeni* and their role is very complex (Reguant et al. 2000). Certain of these components such as gallic acid help to stimulate growth (Rozes et al. 2003) and increase malolactic activity, whereas others have an inhibiting effect (Figueiredo et al. 2008, Vivas et al. 1997). However, for all these molecules, little knowledge is available regarding the mechanisms involved in cellular effects.

Other molecules such as fatty acids also have an impact on the growth of *O. oeni*. The decanoic and dodecanoic acids (Lonvaud-Funel et al. 1988), produced by yeast also cause regression of bacterial population. Effects of fatty acids are strongly dependent on concentration and types of fatty acid present. Capucho and San Romao (Capucho and San Romao 1994) showed that the presence of decanoic and dodecanic acids in low concentration (respectively 12.5 and 2.5 mg L-1) stimulates malolactic activity and growth, whereas high concentration of these two components has an inverse effect. Environment pH also plays a role in the toxic strength of fatty acids. At low pH, toxic action of fatty acids increases and these components act in a synergic manner with pH and ethanol (Capucho and San Romao 1994). They then induce inhibition of ATPase activity which is essential for the mechanism of MLF (Carrete et al. 2002). Oleic acid also has an important effect on the growth of *O. oeni*. Assimilation of this component acts as a survival or growth factor, according to the *O. oeni* strain used (Guerrini et al. 2002).

However, role of these molecules remains secondary in comparison with that of physical and chemical parameters of wine. The influence of physical and chemical factors (pH, ethanol content, temperature and sulphite presence) is crucial for realization of MLF.

16.2.4 Common Molecular Responses to Physical and Chemical Stress

The parameters presented previously induce physiological and metabolic damages. Lactic acid bacteria, notably *O. oeni*, must adapt their physiology to environmental changes. Metabolic changes are numerous and complex. Response to different stress can be considered as bimodal with a physiological response that implicates enzymatic systems and an induced response that necessitates changes of genome expression (van de Guchte et al. 2002).

Generally this adaptation phenomenon necessitates a protein synthesis allowing the maintaining of cell activity and integrity of different cell constituents. Among the numerous proteins induced during stress, stress proteins and notably proteins which are induced by thermal shock (HSP for Heat Shock Protein) play an important role. These chaperones are classified in different families according to their molecular mass and activity (Parsell and Lindquist 1993). During different stress, these proteins have several functions: (1) degradation of damaged proteins (proteins not able to return to its initial state), (2) their folding into active conformation (Fig. 16.2).

A wide variety of stress (ethanol, temperature, acid, osmotic, nutritional deficiency, oxidative) induces synthesis of HSP. Certain of these proteins, such as molecular chaperones GroES, DnaK (Kilstrup et al. 1997, Koch et al. 1998) and proteins of the Clp family (Caseinolytic protein), constitute a common response to different

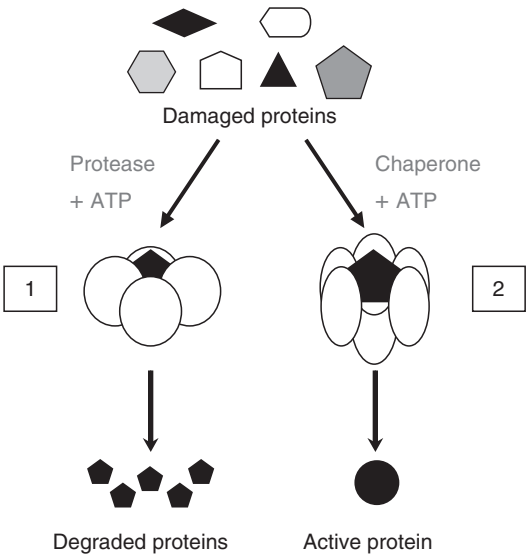

Fig. 16.2 Heat Shock Protein (HSP) simplified action mode according to Abee and Wouters (Abee and Wouters 1999). Proteins damaged by different stress are recognised by the protease (1) either by the chaperone (2) resulting in degradation or restoration of an active protein

stress (Abee and Wouters 1999, Sanders et al. 1999). The universal molecular chaperones GroES and DnaK have a crucial role since these proteins limit aggregation of damaged proteins and allow for folding into active conformation. The Clp family is composed of ATPases (ClpX, ClpC) and protease (ClpP) (Gottesman et al. 1997). The ClpP protease associates itself with a Clp ATPase sub-unit in order to damage those denatured in an irreversible manner during different stress.

Besides intervention of these HSP proteins, other stress proteins more specific to other stress take place in the adaptation and resistance of bacteria to stress. This is notably the case of ASP proteins (Acid Shock Protein), CSP (Cold Shock Protein) and GSP (General Stress Protein) that, respectively, intervene in the adaptation of lactic acid bacteria to acid stress, low temperature and nutritional deficiency (Hartke et al. 1996, Sanders et al. 1999, van de Guchte et al. 2002). The roles of these proteins are different and depend on stress. CSPs, induced by low temperature stress, play a role in the stability of the ARNm and in translation effectiveness. These proteins are implied in the adaptive response of lactic acid bacteria to a drop in temperature (van de Guchte et al. 2002). ASPs intervene in the repairing and deterioration of damaged proteins during acid stress (Abee and Wouters 1999).

The variety and amount of stress proteins induced are variable according to the stress applied and the lactic acid bacteria studied. With *O. oeni*, stress proteins were detected after exposure to different kinds of stress. In fact, ethanol presence in high concentration induces the stress protein synthesis with *O. oeni* (Garbay and Lonvaud-Funel 1996) and notably HSP such as Lo18 protein (Guzzo et al. 1994, Guzzo et al. 1997). Lo18 can act as a lipochaperone on phospholipids (increases the molecular order of phospholipids and regulates membrane fluidity) and as a molecular chaperones on damaged proteins (prevents aggregation of proteins during stress conditions) (Coucheney et al. 2005). A recent study based on monitoring of several stress gene expression in *O. oeni* after direct inoculation in wine, revealed an increase of mRNA rate for stress genes as *hsp18* encoding Lo18 protein, *groEL*, *clpP*, *clpX* and others. These results showed clearly that *O. oeni* can develop an adaptive response in wine (Beltramo et al. 2006).

16.3 Conclusions

In conclusion, we note that wine is an extremely complex environment. Several factors have an effect on the survival and development of bacteria, and notably of *O. oeni*. Furthermore, all these factors interact according to synergic or opposing effects (Britz and Tracey 1990, Versari et al. 1999): pH and SO_2 show opposing effects (Nielsen et al. 1996), low temperature influences tolerance of bacteria to ethanol. Modification of one of these parameters can have serious consequences on the starting and development of MLF. It is also necessary to take account of the fact that the *O. oeni* strains highly differ in their capacities in tolerating different stress and their developing in wine (Britz and Tracey 1990). In wine, *O. oeni* survives and

develops in hostile conditions which form a "multistress" environment for this bacterium. In order to survive and then to develop, this bacterium has therefore to adapt itself during the wine-making process. Multiple adaptation mechanisms are implied for bacterial development in wine.

Intervention of all these technological, microbiological, and physical and chemical factors leaves spontaneous MLF uncertain and difficult to foretell and control.

In order to master this step, several alternatives are currently at the disposal of winemakers and oenologists.

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Chapter 17
Influence of Phenolic Compounds and Tannins on Wine-Related Microorganisms

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17.1 Introduction

Polyphenols represent an important family of compounds found in grapes and wine. To date, more than 8,000 polyphenols have been described and structurally elucidated in plant kingdom. Research on polyphenols mainly started in the early 1930s after the discovery of vitamin P and the "ascorbic acid cofactor" by Rusznyak and Szent-Györgyi (1936). Research on these compounds was intensified especially after the development of more sophisticated analytical methods. The general interest in polyphenols was also intensified after the publication of several epidemiological studies in the early 1990s, which suggested that the negative correlation between coronary diseases and a diet rich in saturated fats in France is mainly due to the relatively high consumption rate of red wine in this country. The polyphenols in red wine have been made responsible for this effect.

To date many polyphenols have been identified in grapevines, grapes, and grape products. They play an important role in the sensorial characteristics of the wine, as they not only contribute to color, but also to tasting sensations, such as bitterness, astringency, and velvety, and hitherto, although to a lesser extend, to the volatile aromatics of a wine.

With regard to the interactions between the polyphenols from grapes and microorganisms, mainly antimicrobial effects have been studied due to the aforementioned belief that polyphenol-rich diets may have an impact on human health. Some researchers have also studied the direct interactions between yeasts and polyphenols.

The following chapters give an overview over the classification, biosynthesis, typical contents of polyphenols in grapes and grape products, and their antimicrobial effects in vitro and in vivo.

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17.2 Classification of the Polyphenols

Generally, polyphenols can be divided in two large groups: flavonoids and non-flavonoids. In the first large group more than 8,000 substances have been identified to-date. Thus, a further classification is necessary, which in this case is limited to the flavonoids occurring in grapes and grape-related products.

The flavonoids of grapes and wines are being subdivided into three groups:

(1) Anthocyanins (anthocyanidin glucosides): malvidin-3-O-glucoside, peonidin-3-O-glucoside, petunidin-3-O-glucoside, cyanidin-3-O-glucoside, delphinidin-3-O-glucoside, and their corresponding acylated forms
(2) Flavonols and flavonol glycosides: kaempferol, quercetin, myricetin
(3) Flavan-3-ols: also often referred to as catechins (catechin, epicatechin, epigallocatechin) and procyanidins or tannins (procyanidins B_1–B_4, T_1–T_6)

The non-flavonoids are also being subdivided into three groups:

(1) Hydroxybenzoic acids: gallic acid, ellagic acid, vanillic acid
(2) Hydroxycinnamic acids: *p*-coumaric acid, caffeic acid, ferulic acid
(3) Stilbenes: resveratrol, resveratrol-glucosides (piceids, see Fig. 17.1), resveratrol polymers (viniferins)

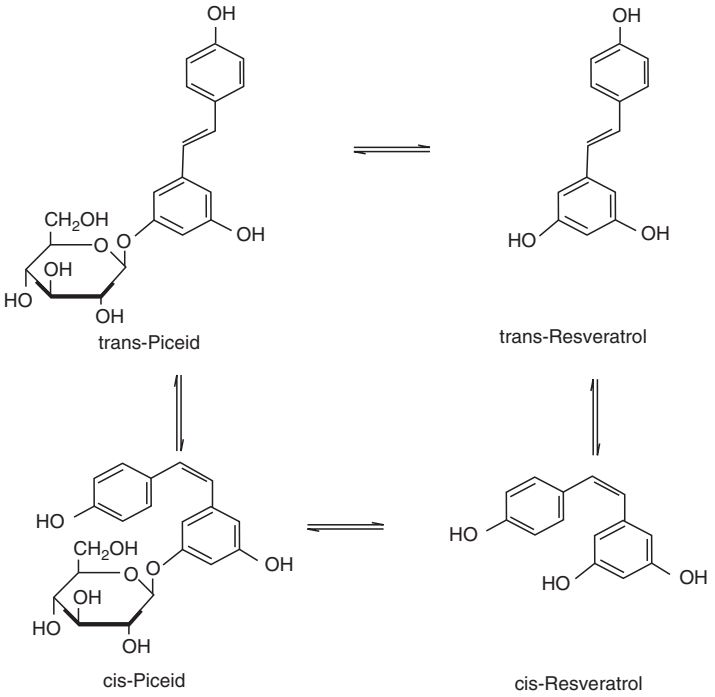

Fig. 17.1 Resveratrol and its glucosides

17.3 Polyphenol Biosynthesis

The precursors of the polyphenols emanate from the carbohydrate metabolism. The biosynthesis of the polyphenols has been investigated in detail (Harborne 1988, Forkmann 1993, Heldt 1996, Winkel-Shirley 2001, Macheix et al. 2005, Jeong et al. 2006, Davies and Schwinn 2006) and can be divided into three partitions:

- *Shikimic acid partition*: synthesis of the amino acids phenylalanine, tyrosine, tryptophan
- *Phenylpropanoid partition*: synthesis of the hydroxycinnamic acids and the precursors of the flavonoids and lignins
- *Flavonoid partition*: flavonoid synthesis

17.3.1 Shikimic Acid Partition

The schematic biosynthesis of the polyphenols is shown in Fig. 17.2. Shikimic acid is formed from phosphoenol pyruvate and erythrose-4-phosphate. The enzyme phenylalanine-ammonium-lyase (PAL) catalyzes the formation of *trans*-cinnamic acid from phenylalanine. The ammonia released during the conversion of both amino acids is probably bound to glutamine synthetase and brought back into the cycle (Heldt 1996).

17.3.2 Phenylpropanoid Partition

The biosynthesis of the phenylpropanes emanates from *trans*-cinnamic acid and, thus, is a subsequent reaction of the shikimic acid partition. The enzyme cinnamic acid-4-hydroxylase (a P450 monooxygenase) hydroxylizes the benzene structure in para-position, which leads to *p*-coumaric acid. Further hydroxylation and methylation leads to the hydroxycinnamic acids caffeic acid, ferulic acid, and sinapic acid. *S*-Adenosyl methionine acts as the methyl donor. Elimination of a C_2 fragment leads to the formation of the benzoic acids, such as salicylic acid.

17.3.3 Flavonoid Partition

The flavonoid biosynthesis starts from the chalcone. The latter is formed from three molecules malonyl-CoA and one molecule *p*-coumaroyl-CoA. The enzyme chalcone synthase (CHS) catalyzes the reaction, which initially leads to the formation of tetrahydroxy chalcone and concurrent elimination of CO_2. This pathway is also called malonate pathway.

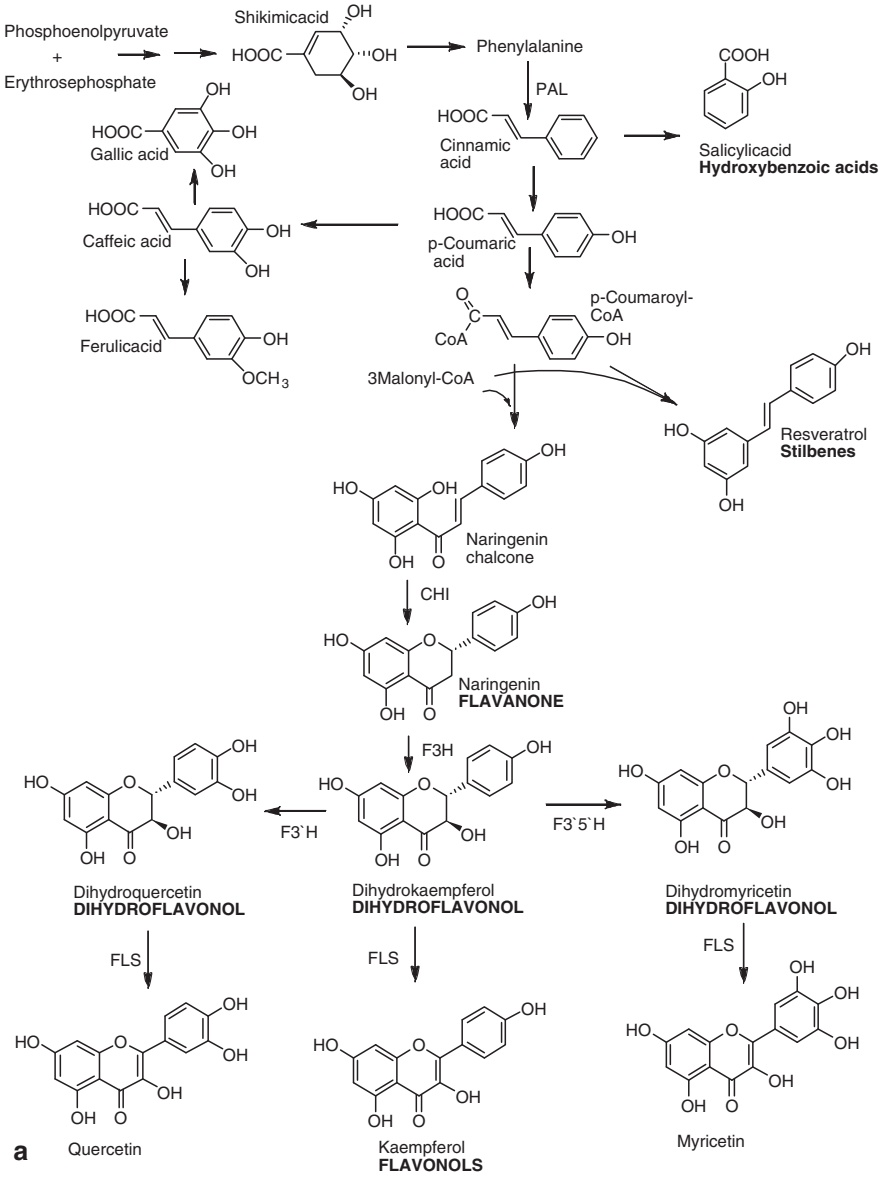

Fig. 17.2 Principle of polyphenol biosynthesis

Some plants, such as grapevine, peanut, pine, and mulberry, also possess a stilbene synthase, which also reacts with three molecules malonyl-CoA and one molecule p-coumaroyl-CoA. Yet, the C9´ atom of the phenylpropanoid is eliminated as CO_2. The formed structure is called resveratrol and belongs to the subclass of the stilbenes. Its effect as an agent against fungal attacks classifies it as a typical phytoalexin.

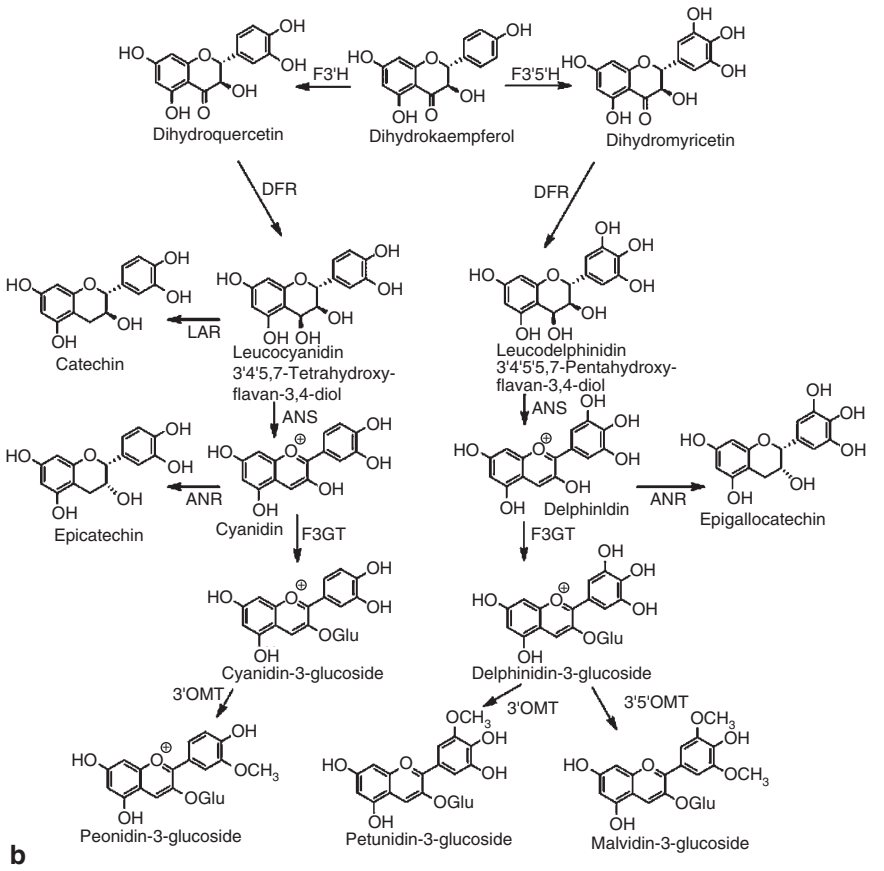

Fig. 17.2 (continued)

The C_6–C_3–C_6 skeleton of tetrahydroxy chalcone is the structural basis for all flavonoids. After ring closure catalyzed through the chalcone isomerase (CHI) naringenin is being formed. The latter is transformed to dihydrokaempferol by the enzyme flavanone 3-hydroxylase (F3H). The enzyme flavonol synthase (FLS) catalyzes the formation of a double bond between the C_2 and the C_3, which leads to the formation of kaempferol (a flavonol).

Other dihydroflavonols can be formed from dihydrokaempferol by the activities of flavonoid 3´-hydroxylase (F3´H) and flavonoid 3´5´-hydroxylase (F3´5´H), leading to dihydroquercetin and dihydromyricetin, respectively (Jeong et al. 2006). Both enzymes belong to the cytochrome P450 family, and it is presumed that the ratio of F3´H and F3´5´H controls the anthocyanin composition of grape skins. The resulting dihydroflavonols are then transformed to quercetin and myricetin by means of flavonol synthase (FLS), and subsequently are glycosylated.

Dihydroflavonol can also be converted to a flavane-3,4-diol through a catalytic reduction by means of dihydroflavonol reductase (DFR). The crystal structure of

Vitis vinifera DFR, heterologously expressed in *Escherichia coli*, was determined recently by Petit et al. (2007).

Flavan-3,4-diols (also called leucoanthocyanidins) are regarded as transient precursors of the anthocyanins, the flavan-3-ols, and the proanthocyanidins (PA), with the latter being polymerized flavan-3-ols. The complete synthesis of this compound is still not yet completely revealed. The interaction of several enzymes is probable (Harborne 1988, Forkmann 1993).

The glucosides of cyanidin, delphinidin, peonidin, petunidin, and malvidin are the characteristic pigments of red grapes and wines. Cyanidin and delphinidin are formed from leucocyanidin and leucodelphinidin, respectively, by anthocyanin synthases (ANS) or, more precisely, leucoanthocyanin dioxygenases (LDOX). These aglycons are glucosylated by means of flavonoid 3-glucosyltransferases (F3GT) using UDP-glucose. The enzymes are therefore UDP-glucose**:**flavonoid 3-O-glucosyltransferases (UF3GT, Jeong et al. 2006). Further modifications, like methylations, occur under the enzymatic control of 3´O- and 3´5´O-methyl transferases 3´OMT, 3´5´OMT, leading to peonidins, petunidins, and malvidins. Numerous enzymes catalyzing flavonoid modifications by hydroxylation, methylation, glycosylation, acylation, and some other reactions have been described (Forkmann 1993, Davies and Schwinn 2006). Pelargonidins are not found in grape wine. It can be assumed that the reaction from dihydrokaempferol to leucopelargonidin is not possible due to high activities of F3´H and F3´5´H, providing a strong biosynthetic drive away from the pelargonidin precursors. Another possible explanation is that the grape DFR does not accept dihydrokaempferol as a substrate, and therefore leucopelargonidin cannot be formed. This situation is met in the case of Petunia, which does not produce pelargonidin (Macheix et al. 2005).

Flavan-3-ols may be formed by two biosynthetic routes, from either leucoanthocyanidins or anthocyanidins (Davies and Schwinn 2006). The reaction type leading to monomeric flavan-3-ols is catalyzed by the NADPH-dependent leucoanthocyanidin reductase (LAR) and is presumably initiated by the separation of the hydroxyl group at the C_4 atom, which leads to a carbocation, which is then reduced to the flavan-3-ol. Thus, the monomeric catechin is formed from leucocyanidin, whereas gallocatechin is formed from leucodelphinidin (Davies and Schwinn 2006, Jeong et al. 2006, Adams 2006). In contrast, epicatechin and epigallocatechin are formed from the anthocyanidins cyanidin and delphinidin (presumably the pseudobase forms), respectively. The corresponding enzyme anthocyanidin reductase (ANR) was first described by Xie et al. (2003).

Bogs et al. (2005) found that the ANR of grape is encoded by a single gene and LAR is encoded by two closely related genes. The ANR gene is expressed in the skin and seeds until the onset of ripening, and the two LAR genes show different pattern of expression in skin and seeds. The expression of ANR and LAR genes is consistent with the accumulation of proanthocyanidins (condensed tannins) in the berry. The recent findings on tannin synthesis support the idea of a close relationship between anthocyanins and proanthocyanidins. Epicatechin is the predominant extension unit in grape hypodermis cells and seeds, suggesting that cyanidin plays a role as an intermediate in tannin biosynthesis in grape berries. In grape skins where also epigallo-

catechin is found as a tannin subunit, cyanidin and delphinidin would be important intermediates in proanthocyanidin biosynthesis (Adams 2006). The polymerization is still a matter of debate. Beside the monomeric flavan-3-ols, derivatives of leucoanthocyanidins seem to play a role, like carbocation products and quinone methide. It is still not known whether the polymerization of PA occurs spontaneously in all tissues or is enzyme catalyzed in some or all cases (Davies and Schwinn 2006).

17.4 Typical Contents of Phenols in Grapes and Grape Products

Polyphenols in grapes and in the respective products vary with season, climatic conditions, soil structure and composition, viticultural practices, and winemaking technology. Especially, the impact of various winemaking procedures on polyphenolic composition has been studied thoroughly.

Some researchers have studied the polyphenolic composition of grape berries. Gómez-Alonso et al. (2007) have published the phenolic compounds in Spanish grape skins and wines of the red grape variety "Cencibel." Table 17.1 summarizes the results.

Trans-coutaric acid is the main hydroxycinnamic acid derivative in the skins. It is well known from literature that this compound acts as a vehicle for the anthocyanins.

Relatively few data are available on the flavonol composition of grapes and their distribution within the grape berry. Tables 17.2 and 17.3 show the flavonol and anthocyanin contents of grape skins and wines of the Cencibel variety.

Downey et al. (2003) have studied the biosynthesis of flavonols in Shiraz and Chardonnay berries. The main flavonols were quercetin-3-glucoside and kaempferol-3-glucoside. Their results show that flavonol biosynthesis in Shiraz starts about 7 weeks before véraison and reaches a maximum 6 weeks after véraison followed by a decline towards harvest date. In Chardonnay biosynthesis starts about 9 weeks

Table 17.1 Molar [%] hydroxycinnamic acid composition of grape skins and respective wines from the Spanish grape variety "Cencibel"

Hydroxycinnamic acid/ester	Grape skins [molar%] (n = 10)	Wines [molar%] (n = 10)
Grape reaction product (GRP)	n.d.	15.65 ± 5.02
cis-Caftaric acid	6.75 ± 2.81	0.64 ± 0.10
trans-Caftaric acid	28.26 ± 2.73	34.28 ± 7.56
Caffeic acid	n.d.	6.25 ± 4.29
cis-Coutaric acid	13.03 ± 1.91	5.91 ± 0.54
trans-Coutaric acid	48.88 ± 4.32	31.17 ± 2.04
p-Coumaric acid	n.d.	2.73 ± 2.46
cis-Fertaric acid	0.81 ± 0.65	0.26 ± 0.20
trans-Fertaric acid	2.27 ± 1.11	2.50 ± 0.59
Ferulic acid	n.d.	0.35 ± 0.34

Table 17.2 Molar [%] flavonol composition of grape skins and respective wines from the Spanish grape variety "Cencibel"

Flavonol	Grape skins [molar%] (n = 10)	Wines [molar%] (n = 10)
Myricetin-3-glucoside	37.37 ± 5.10	38.21 ± 4.69
Quercetin-3-rutinoside	3.35 ± 0.81	n.d.
Quercetin-3-galactoside	4.22 ± 0.76	3.26 ± 0.51
Quercetin-3-glucoside	22.62 ± 3.68	25.37 ± 1.62
Quercetin-3-glucuronide	14.02 ± 1.66	20.52 ± 5.74
Kaempferol-3-rutinoside	2.74 ± 0.58	1.34 ± 0.11
Kaempferol-3-glucoside	4.84 ± 2.66	1.98 ± 1.27
Isorhamnetin-3-glucoside	7.87 ± 2.42	7.25 ± 1.43
Myricetin	1.84 ± 0.38	0.91 ± 0.36
Quercetin	0.53 ± 0.18	0.92 ± 0.35
Kaempferol	0.41 ± 0.25	0.14 ± 0.08
Isorhamnetin	0.18 ± 0.09	0.11 ± 0.06

Table 17.3 Molar [%] anthocyanin composition of grape skins and respective wines from the Spanish grape variety "Cencibel"

Anthocyanin	Grape skins [molar%] (n = 10)	Wines [molar%] (n = 10)
Delphinidin-3-glucoside	15.75 ± 1.84	10.87 ± 1.29
Cyanidin-3-glucoside	2.99 ± 0.69	0.48 ± 0.17
Petunidin-3-glucoside	11.97 ± 0.80	12.41 ± 0.54
Peonidin-3-glucoside	5.77 ± 1.18	4.07 ± 2.06
Malvidin-3-glucoside	37.37 ± 1.85	55.10 ± 3.10

before véraison reaching a peak about 2 weeks after véraison followed by a decline towards harvest date.

Masa and Vilanova (2008) have studied the flavonol composition of Albarín blanco grapes in three consecutive years. According to their results, 30% of the flavonols is made up of dihydroquercetin glycosides, 26.8% is quercetin glycosides, and 10.3% is kaempferol glycosides.

Spanos and Wrolstad (1990) published some data on the polyphenolic composition of white grape juices of the "Thompson seedless" variety. According to their results only very low amounts of polyphenols can be found in these grape juices (Table 17.4).

Some more detailed data was published by Pour Nikfardjam et al. (2000) on the polyphenolic content of commercial and varietal red grape juices from own production (Table 17.5).

As can be seen from Table 17.5, the varietal juices contain much more polyphenols than the commercial samples. This might be due to several reasons: (1) the varietal juices were analyzed shortly after production; (2) other red varieties than in the commercial samples were used; (3) commercial red grape juices may contain also white varieties.

Fuleki and Ricardo-da-Silva (2003) measured the concentrations of catechin and procyanidins in grape juices of North America. According to their results especially high amounts of procyanidins were found in Seyval (13 mg/L) and Niagara (4.4 mg/L), catechin and epicatechin in Elvira (8 and 12 mg/L, respectively), and Chardonnay (4.1 and 4.8 mg/L, respectively). In red grape varieties, Vincent (32 mg/L) had the

Table 17.4 Polyphenolic composition of grape juices from the "Thompson seedless" variety

Polyphenol (mg/L)	Concentration
Caftaric acid	n.d.–14.0
Coutaric acid	n.d.–2.2
Grape reaction product (GRP)	2.4–8.6
Caffeic acid	n.d.–8.6
p-Coumaric acid	0.2–3.3
Ferulic acid	n.d.–0.5
Gallic acid	n.d.–1.6
(−)-Epicatechin	n.d.–2.2
(+)-Catechin	n.d.–7.4
Procyanidin B_1	n.d.–15.4
Procycanidin B_2	n.d.–1.8
Procyanidin B_3	n.d.–4.7
Procyanidin B_4	n.d.–3.5
Procyanidins (Trimers–Tetramers)	n.d.–2.3
Quercetin-glycosides	n.d.–7.5

Table 17.5 Polyphenolic composition of commercial and varietal grape juices from own production

Red grape juice	Commercial juices (n = 27)			Varietal juices (n = 7)		
Phenol (mg/L)	Mean	Min.	Max.	Mean	Min.	Max.
Hydroxycinnamic acids						
Caftaric acid	22.1	7.7	30.3	28.3	19.4	41.0
Coutaric acid	25.1	13.7	67.8	49.8	22.9	105.4
Fertaric acid	1.8	n.d.	5.8	7.5	n.d.	29.9
Ferulic acid	1.4	n.d.	10.0	4.0	n.d.	9.4
Grape reaction product (GRP)	7.9	4.6	13.7	21.1	12.3	30.7
Caffeic acid	4.8	1.6	7.9	9.7	n.d.	22.3
p-Cumaroylglucosyltartrate (*p*-CGT)	1.1	n.d.	n.d.	n.d.	n.d.	n.d.
p-Coumaric acid	0.7	n.d.	n.d.	2.6	n.d.	10.3
Sum	64.9	30.3	120.0	122.9	65.5	226.2
Hydroxybenzoic acids						
3-OH-Benzoic acid	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
4-OH-Benzoic acid	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
Ellagic acid	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
Gallic acid	9.9	1.9	23.4	4.3	1.6	9.6
Protocatechuic acid	10.3	n.d.	24.2	3.2	0.0	8.7
Sinapic acid	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
Syringic acid	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
Tyrosol	9.2	2.3	17.9	11.7	4.8	20.2
Sum	29.4	6.5	55.9	19.2	10.5	29.8
Flavonols						
Avicularin	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
Hyperoside	7.4	n.d.	20.2	4.2	n.d.	8.5

(continued)

Table 17.5 (continued)

Red grape juice	Commercial juices (n = 27)			Varietal juices (n = 7)		
Isoquercitrin	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
Quercetin	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
Rutin	15.5	n.d.	42.6	38.2	24.0	47.6
Sum	22.9	n.d.	62.0	42.4	32.5	55.7
Flavan-3-ols						
Catechin	13.8	n.d.	7.6	2.4	0.0	5.4
Epicatechin	8.8	0.4	15.7	32.4	7.9	68.5
Sum	22.6	1.5	23.3	34.8	11.8	68.5
Proanthocyanidins						
Procyanidin B_2	14.9	0.3	19.1	12.9	0.6	39.9
Anthocyanins						
Delphinidin-3-glucoside	2.9	n.d.	4.3	33.8	10.3	98.4
Cyanidin-3-glucoside	1.6	n.d.	3.6	21.2	2.4	48.2
Petunidin-3-glucoside	2.8	n.d.	4.4	33.6	12.3	92.3
Peonidin-3-glucoside	2.4	n.d.	4.5	113.2	16.2	355.4
Malvidin-3-glucoside	8.3	n.d.	17.5	213.9	45.0	527.0
Peonidin-3-acetylglucoside	1.3	n.d.	2.2	22.3	n.d.	51.8
Malvidin-3-acetylglucoside	1.5	n.d.	4.2	46.7	n.d.	154.2
Peonidin-3-coumaroylglucoside	1.2	n.d.	2.0	12.1	n.d.	42.9
Malvidin-3-coumaroylglucoside	1.4	n.d.	3.2	55.2	n.d.	195.8
Sum	23.5	n.d.	41.9	552.2	137.4	1550.4
Stilbenes						
cis-Piceid	1.6	n.d.	6.0	1.8	n.d.	7.3
cis-Resveratrol	0.0	n.d.	0.5	n.d.	n.d.	n.d.
trans-Piceid	1.0	0.5	3.2	1.2	n.d.	4.8
trans-Resveratrol	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
Sum	2.6	0.5	9.2	3.0	n.d.	12.1
Sum (HPLC)	115.7	43.0	176.1	664.5	202.8	1729.3

highest contents on procyanidins, and Vincent, Maréchal Foch, and Baco noir were highest in catechin (18, 30, and 6 mg/L, respectively).

Table 17.6 summarizes the polyphenolic composition of white wines (Vrhovsek et al. 1997, Pena-Neira et al. 2000, Carando et al. 1999, Teissedre et al. 1996).

A recently published study (Pour Nikfardjam et al. 2007) reported on the polyphenolic content of German white wines (Table 17.7). According to their results, tyrosol is the most abundant polyphenol in German white wines of the Bacchus, Müller-Thurgau, Riesling, and Silvaner varieties. In Red Traminer, *p*-coumaric acid was the main polyphenol, and in Rieslaner 3-hydroxybenzoic acid was dominant.

Table 17.8 shows the composition of colorless polyphenols of red wines (Pour Nikfardjam et al. 2006a, Makris et al. 2006, Woraratphoka et al. 2007).

Table 17.6 Polyphenolic composition of white wines

Polyphenol (mg/L)	Riesling	Riesling	White wine	White wine
Origin	Germany	Germany	Canada	Various countries
Caftaric acid	30.2 (12.1–75.6)	n.d.	n.d.	25.1 (14.2–32.6)
Grp	1.3 (0.2–6.0)	n.d.	n.d.	12.5 (4.0–32.2)
p-CGT	0.5 (0.1–1.2)	n.d.	n.d.	n.d.
Coutaric acid	2.5 (0.9–5.1)	n.d.	n.d.	4.4 (1.2–6.9)
Fertaric acid	2.3 (1.1–3.4)	n.d.	n.d.	2.9 (1.8–3.4)
Caffeic acid	2.3 (0.5–4.8)	n.d.	(1.51–5.20)	1.9 (0.8–3.3)
p-Coumaric acid	1.7 (0.9–2.8)	2.0	(1.57–3.21)	1.8 (1.0–2.8)
Ferulic acid	1.7 (0.9–4.5)	n.d.	(tr-4.42)	0.6 (0.4–0.9)
Protocatechuic acid	n.d.	n.d.	n.d.	0.06
Tyrosol	25.2 (6.3–53.8)	n.d.	n.d.	1.7
Gallic acid	n.d.	n.d.	2.8	0.19
Ellagic acid	n.d.	n.d.	n.d.	0.09
Sinapic acid	n.d.	n.d.	n.d.	0.1
(+)-Catechin	0.9 (tr-2.5)	3.7	(3.80–4.20)	9.8/34.9
Procyanidin B_2	4.9 (tr-9.5)	n.d.	n.d.	n.d.
(−)-Epicatechin	6.1 (1.0–11.8)	1.2	(1.70–3.80)	5.3/21.2
(−)-Epicatechin-3-*O*-gallate	12.3 (0.4–47.2)	n.d.	n.d.	n.d.

The data already show that the origin of the wines has a distinctive effect on its polyphenolic composition. Beside climatic conditions other factors, such as winemaking style and technology, also play a very important role. Especially, fining agents are known to reduce the polyphenol content in wines.

Wang and Huang (2004) studied the flavonol content of wines by means of HPLC and capillary electrophoresis. Their results show that on a quantity basis the most important flavonol is quercetin followed by kaempferol, myricetin, and baicalein (5,6,7-trihydroxyflavone).

Amico et al. (2004) analyzed the flavonol content of Nerello mascalese grape pomace using HPLC-MS-ESI. According to their results, quercetin-glucoside was the main flavonol in grape pomace (170 mg/kg) followed by quercetin-glucuronide (130 mg/kg), isorhamnetin-glucoside (63.8 mg/kg), myricetin-glucoside (21.3 mg/kg), and quercetin (15.3 mg/kg).

Table 17.9 shows the anthocyanin composition of the wines already mentioned in Table 17.8 (Pour Nikfardjam et al. 2006a, Makris et al. 2006, Woraratphoka et al. 2007). Unfortunately, the wines from Thailand were not analyzed on their anthocyanin content.

The anthocyanin content and its composition are mainly dependent on the variety. Some authors have studied the feasibility of using the anthocyanin composition for the determination of the cultivar. It has been shown that the anthocyanin composition remains relatively constant irrespective of the winemaking technology used (Eder et al. 1994, Holbach et al. 1997).

Table 17.7 Polyphenolic composition (mg/L) of German white wines

Cultivar	Bacchus	Müller-Thurgau	Rieslaner	Riesling	Silvaner	Traminer
Number of Samples	$n = 14$	$n = 75$	$n = 7$	$n = 17$	$n = 68$	$n = 10$
Vintage	1997	1989–1998	1992–1996	1996–1998	1993–1998	1998
Gallic acid	0.8 (n.q.–2.9)	1.3 (n.q.–10.1)	0.6 (n.q.–1.7)	n.q.	1.6 (n.q.–14.8)	1.4 (n.q.–2.8)
Protocatechuic acid	4.5 (1.7–11.0)	2.1 (n.q.–7.7)	2.4 (0.7–3.7)	n.q.	3.5 (n.q.–11.0)	3.0 (1.8–4.4)
Tyrosol	13.9 (9.9–22.0)	19.9 (n.q.–39.2)	16.1 (10.8–19.6)	18.0 (n.q.–42.2)	17.4 (3.5–26.7)	16.0 (7.5–24.1)
3-Hydroxybenzoic acid	n.d.	0.2 (n.q.– 12.0)	23.1 (n.q.–36.8)	n.q.	0.9 (n.q.–38.3)	n.d.
Caftaric acid	1.4 (n.q.–3.6)	4.0 (n.q.–19.9)	19.9 (17.2–28.4)	13.7 (0.5–25.9)	8.3 (n.q.–40.2)	2.8 (n.q.–21.1)
Catechin	n.d.	0.3 (n.q.–7.0)	6.0 (n.q.–10.9)	n.q.	1.1 (n.q.–26.3)	n.d.
GRP	2.3 (n.q.–12.4)	3.7 (n.q.–18.6)	8.1 (6.6–12.8)	1.7 (n.q.–5.1)	4.1 (n.q.–18.0)	4.0 (1.5–8.4)
Procyanidin B_2	n.d.	1.7 (n.q.–39.5)	8.1 (n.q.–16.3)	n.q.	0.3 (n.q.–6.7)	n.d.
p-CGT	8.2 (n.q.–16.7)	4.3 (n.q.–23.0)	2.1 (n.q.–3.9)	0.5 (n.q.–1.7)	10.6 (n.q.–61.4)	7.5 (n.q.–24.2)
Syringic acid	n.d.	n.d.	0.6 (n.q.–2.1)	n.q.	n.d.	n.d.
Caffeic acid	1.9 (n.q.–10.0)	0.7 (n.q.–2.5)	2.4 (1.1–2.9)	1.6 (n.q.–3.9)	2.0 (n.q.–13.5)	8.4 (2.1–25.9)
Epicatechin	n.d.	0.3 (n.q.–12.3)	3.3 (n.q.–5.7)	0.9 (n.q.–6.4)	0.3 (n.q.–10.3)	n.d.
Coutaric acid	2.5 (n.q.–6.3)	2.0 (n.q.–8.4)	0.6 (n.q.–2.9)	1.7 (0.7–3.0)	2.7 (n.q.–6.9)	1.3 (n.q.–7.5)
Sinapinic acid	n.d.	0.1 (n.q.–3.0)	1.0 (n.q.–5.1)	n.q.	0.2 (n.q.–4.7)	n.d.
Fertaric acid	1.0 (n.q.–2.5)	1.3 (n.q.–2.7)	3.6 (2.5–4.8)	1.1 (n.q.–2.6)	1.6 (n.q.–3.3)	2.6 (n.q.–5.8)
p-Coumaric acid	12.4 (6.2–18.8)	4.4 (n.q.–15.4)	4.5 (2.2–5.7)	1.3 (n.q.–3.7)	8.2 (0.8–33.3)	21.9 (8.5–47.2)
Ferulic acid	1.1 (n.q.–3.5)	0.3 (n.q.–1.7)	3.2 (1.0–4.6)	0.7 (n.q.–4.7)	0.6 (n.q.–5.5)	1.3 (n.q.–3.6)
Ellagic acid	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
Quercetin-3-galactosid	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
Quercetin-3-rutinosid	n.d.	0.1 (n.q.–3.5)	n.d.	n.d.	n.d.	n.d.
Quercetin-3-glucosid	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
trans-Resveratrol	n.d.	0.2 (n.q.–1.5)	n.d.	n.q.	0.1 (n.q.–1.7)	n.d.
Quercetin	n.d.	n.d.	1.4 (n.q.–4.1)	n.q.	n.d.	n.d.
Sum (HPLC)	50 (37–65)	49 (24–97)	111 (55–140)	48 (14–87)	64 (22–124)	70 (29–137)

Table 17.8 Colorless polyphenolic composition of red wines from various countries

Polyphenol (mg/L)	Cabernet Sauvignon (n = 10)	Cabernet Sauvignon (n = 7)	Merlot (n = 10)	Merlot (n = 5)	Pinot noir (n = 4)	Shiraz (n = 3)	Barbera (n = 3)
Origin of wines	Hungary	Greece	Hungary	Greece	Hungary	Thailand	Thailand
Gallic acid	57.8	n.d.	65.9	n.d.	45.2	16.9	19.5
Tyrosol	89.1	n.d.	81.2	n.d.	117.0	n.d.	n.d.
Caftaric acid	53.5	22.8	51.6	24.3	55.5	n.d.	n.d.
Catechin	81.8	40.5	89.1	42.4	103.0	6.4	4.4
Grp	1.1	n.d.	2.5	n.d.	n.d.	n.d.	n.d.
Procyanidin B_2	43.7	14.1	47.5	5.3	33.9	n.d.	n.d.
Caffeic acid	23.1	3.1	18.5	1.4	28.9	10.7	1.3
Epicatechin	102.8	19.4	126.0	18.6	64.6	3.5	2.1
p-Coumaric acid	6.6	0.9	10.2	1.8	8.9	19.4	2.9
Fertaric acid	3.4	n.d.	3.0	n.d.	8.3	n.d.	n.d.
Rutin	13.1	30.9	16.9	20.7	9.7	1.8	n.d.
Ferulic acid	n.d.	n.d.	2.6	n.d.	n.d.	n.d.	n.d.
trans-Resveratrol	2.8	n.d.	3.9	n.d.	3.2	1.9	0.4
Quercetin	5.6	13.7	11.2	29.9	7.5	2.1	0.6

Table 17.9 Anthocyanin composition of red wines from various countries

Anthocyanin (mg/L)	Cabernet Sauvignon (n = 10)	Cabernet Sauvignon (n = 7)	Merlot (n = 10)	Merlot (n = 5)	Pinot noir (n = 4)	Shiraz (n = 3)	Barbera (n = 3)
Origin of wines	Hungary	Greece	Hungary	Greece	Hungary	Thailand	Thailand
Delphinidin-3-glucoside	92.8	14.1	53.7	17.3	43.0	n.q.	n.q.
Cyanidin-3-glucoside	n.d.	0.6	n.d.	1.4	n.d.	n.q.	n.q.
Petunidin-3-glucoside	74.2	22.1	49.6	31.8	39.9	n.q.	n.q.
Peonidin-3-glucoside	43.5	16.2	38.2	18.8	48.1	n.q.	n.q.
Malvidin-3-glucoside	565.0	391.5	276.0	311.5	316.0	n.q.	n.q.

17.5 Resveratrol

The phytoalexin resveratrol is one of the best analyzed compounds in wine. A plethora of papers has been published on resveratrol, its analysis, and its impact on the antioxidative and anticancer effect of moderate wine consumption.

Table 17.10 summarizes the *trans*-resveratrol and resveratrol derivatives contents of various red varieties and origins.

A strong dependence of resveratrol on vintage and variety has been emerged from these studies. Vintage is important because of the climatic influence on Botrytis

Table 17.10 Resveratrol derivatives (mg/L) in red wines from various countries

Origin	*trans*-Piceid	*cis*-Piceid	*trans*-Resveratrol	*cis*-Resveratrol	Literature
Worldwide	0.5–11.3	n.q.	0.4–10.6	0.4–7.5	Burns et al. (2000)
Hungary	3.8–16.4	n.q.	0.1–14.3	n.q.	Márk et al. (2005)
Canada	0.02–0.98	0.02–0.68	0.71–2.5	0.27–0.88	Soleas et al. (1997)
Austria	1.7–3.6	4.2–5.7	1.3–2.4	0.9–1.6	Vrhovsek et al. (1997)
Spain	0.9–4.0	0.3–1.9	0.6–8.0	0.1–2.5	Lamuela-Raventos et al. (1995)
Portugal	n.d.–50.8	n.d.–17.9	n.d.–5.7	n.d.–9.5	Ribeiro de Lima et al. (1999)

cinerea growth. Several studies have shown that Botrytis-free grapes contain less resveratrol than those with a slight infection. In the event of a very strong infection, the resveratrol concentration is drastically reduced. This is due to the activity of a laccase-like enzyme, a stilbene oxidase, which is produced by the fungus as a means against the antifungal effect of resveratrol. The enzyme catalyzes the oxidation of resveratrol to various products. One of those is the resveratrol-*trans*-dehydrodimer, which was first mentioned by Breuil et al. (1998).

Figure 17.3 shows the dependence of the *trans*-resveratrol content of Hungarian red wines from the "Villány" region on vintage. As can be seen especially, the wines from the 2002 vintage had very high resveratrol contents.

Beside vintage the variety has been identified as a very important factor for resveratrol levels. Here a clear trend to varieties of the Pinot family and other varieties high in phenolics can be observed (Figs. 17.4 and 17.5). Very high levels on *trans*-resveratrol could be found in the varieties Kékfrankos (synonym: Blaufränkisch, Lemberger), Merlot, Pinot noir, and Zweigelt.

In white wines much lower concentrations of resveratrol can be found. One reason for this difference is the lower biosynthesis rate. One of the precursors of *trans*-resveratrol is the *p*-coumaroyl-CoA. This substance is also a precursor of the anthocyanins. It is thus comprehensible that in red grapes more *p*-coumaroyl-CoA is being formed, since it is needed for the biosynthesis of the anthocyanins (Conn et al. 2003). This also explains the occurrence of resveratrol in the grape skins. First, in the skins it can act as a defensive agent against fungal attacks. Second, it is self-evident to place the biosynthesis at the location, where the substance is needed. Research has shown a direct negative correlation between resveratrol formation and anthocyanin content in grape skin cells. This has been attributed to the competitive action of the two enzymes chalcone synthase and stilbene synthase. The decrease of ability of grapes to synthesize resveratrol in response to UV-irradiation following the onset of véraison could be a consequence of the concomitant rise of anthocyanin accumulation in these fruits (Jeandet et al. 1995).

Another reason for the much lower resveratrol levels in white wines are caused by the different winemaking procedures. Mash fermentation or mash heating are generally not applied to white grapes, thus not leading to an opportunity to sufficiently extract the resveratrol from the grape skins.

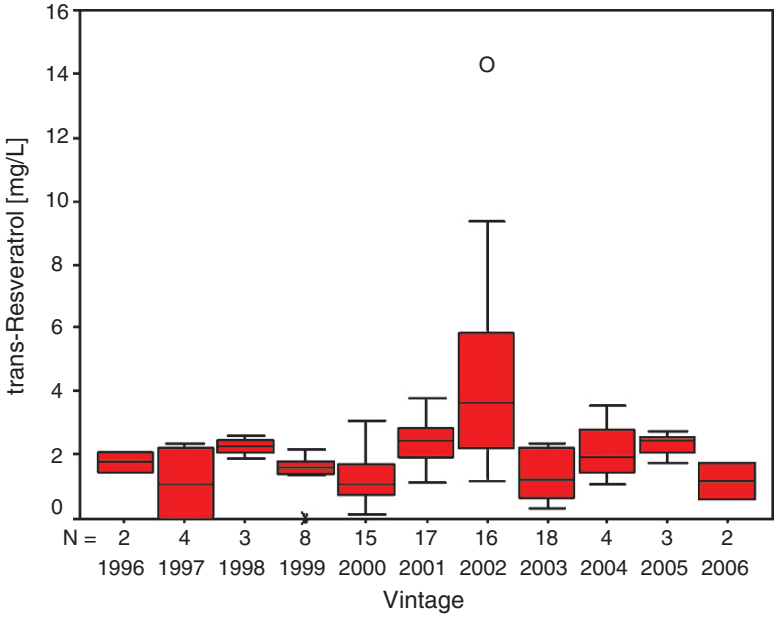

Fig. 17.3 Trans-resveratrol content [mg/L] of Hungarian red wines from the "Villány" region according to vintage

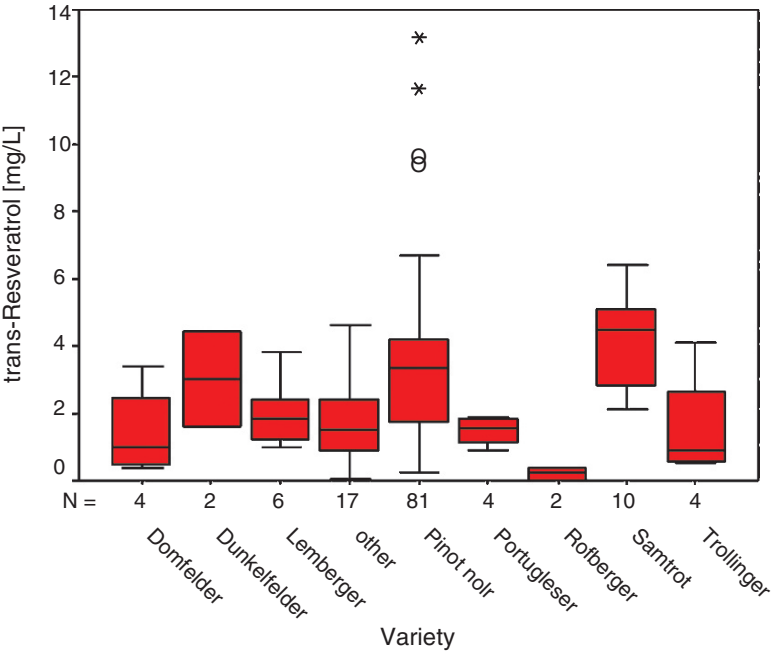

Fig. 17.4 Trans-resveratrol content [mg/L] of German red wines

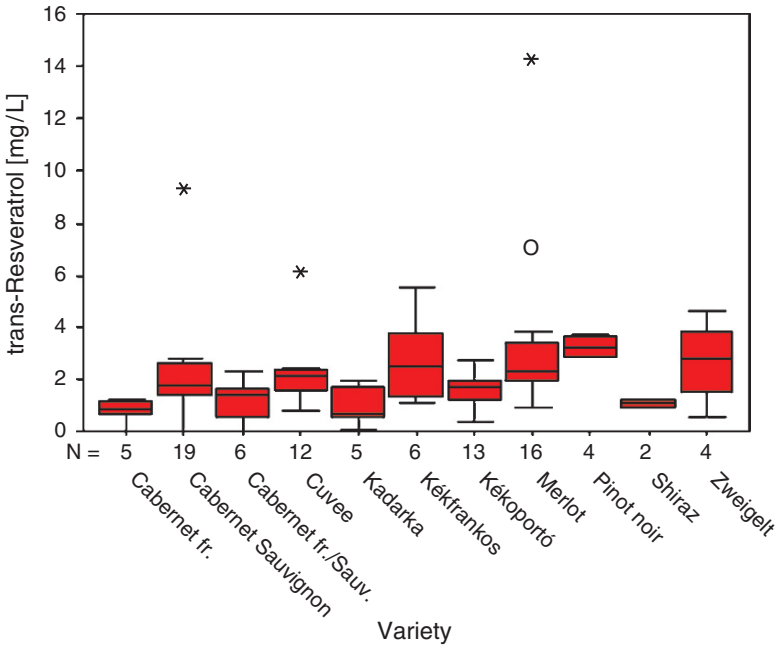

Fig. 17.5 Trans-resveratrol content [mg/L] of Hungarian red wines

Still there are white wines, which, due to their different winemaking style, show resveratrol levels, which are comparable to those of red wines. These wines are for instance the wines from the Tokaj region in Hungary. Especially, those Tokaj wines with a high amount of botrytized berries (Tokaji Aszú) show high resveratrol levels. Obviously, the concentrating effect of the berry shrinking caused by Botrytis cinerea and the extraction of these berries in the high alcoholic base wine lead to these high resveratrol levels.

Another typical product of the Tokaj region is the so-called Forditás (twist). Here the already extracted Aszú berries are extracted again with the aid of a high alcoholic base wine. The result is a wine rich in extract and polyphenols. Folin values for these wines can reach 1,700 mg/L and an antioxidative capacity (TEAC value) of 10.8 mmol/L. These are values which otherwise are only reached by red wines (Pour Nikfardjam et al. 2006b).

Resveratrol derivatives can also be found in grape juices. Because of the missing fermentation step, most of the derivatives are present in their glucoside form (piceid). Partly, resveratrol levels are reached that are comparable or even higher than in wines. Generally, the *cis*-isomers are more common in grape juices (see Fig. 17.1). Table 17.11 shows an overview over the resveratrol levels in commercially available German grape juices and variety-pure grape juices (Pour Nikfardjam et al. 2000).

The high resveratrol levels of some juices are partly due to the variety influence. Hitherto, the effect of the production technique has to be taken into account. Especially during grape juice production, the length of the mash enzymation has a decisive influence on the resveratrol levels.

Table 17.11 Resveratrols in commercial and variety-pure grape juices

Grape juice	*trans*-Piceid (mg/L)	*cis*-Piceid (mg/L)	*trans*-Resveratrol (mg/L)	*cis*-Resveratrol (mg/L)
Red (commercial)	0.53–7.34	0.08–5.66	n.q.–1.09	n.d.–0.23
Red (variety-pure)	n.d.–4.8	n.d.–8.7	n.d.–0.5	n.d.–0.5
White (commercial)	n.d.–0.48	n.d.–0.34	n.d.–0.19	n.q.
White (variety-pure)	n.d.–0.2	n.d.–1.0	n.d.	n.d.

17.6 Antimicrobial Effects of Polyphenols in Grapes

Grapes can suffer from several stress factors, which can be divided into biotic and abiotic stress: herbivory, fungal and viral pathogens, wounding, high light and UV radiation, ozone, extreme temperature, drought, salinity, nutrient deficiency and imbalances, application of herbicides and fungicides. Secondary plant metabolites, like the polyphenols of grapes, are part of a generalized stress response. Leshem and Kuiper (1996) introduced the term *general adaption syndrome*. Plants use a broad repertoire of defense strategies against phytopathogenic fungi and other biotic stressors. These are among other things based on the following:

1. Presence of several polyphenol subclasses (see above) with high chemical reactivity (antioxidant and radical scavenging properties)
2. Crosslinking of microbial enzymes
3. Inhibition of microbial cellulases and pectinases
4. Chelation of metal ions necessary of enzyme activity
5. Formation of a physical barrier
6. Accumulation of phytoalexins after contact with fungal elicitors
7. Formation of flavonoid oxidation products (FOP)

The early activation of the phenylpropanoid metabolism is a strict prerequisite in the expression of disease resistance in plants and is, to a certain extent, dedicated to the reinforcement and chemical modification of plant cell walls (Matern and Grimmig 1993). Cinnamic acids can rapidly be incorporated into the polysaccharide fraction within 24 h following fungal elicitation. The acylation confers increased mechanical rigidity and renders the polysaccharides insensitive to hydrolytic enzyme activities by pathogenic fungi. Elicitation is also known to cause an oxidative outburst by reactive oxygen species (ROS) as a very rapid response. As ROS are toxic to cell membranes and other plant structures, the cells need a kind of self-protection. Hydroxycinnamic acids and esters are known as efficient radical scavengers and can fulfill this important task. Also other elicitor-inducible substances with antifungal properties were described, like the formation of aromatic aldehydes (vanillin, 4-hydroxybenzaldehyde,) produced from cinnamic esters (Matern and Grimmig 1993).

17.6.1 Resveratrols

The grapevine produces a multitude of chemical agents against fungal and bacterial attacks. Phytoalexins are defined as low molecular weight substances, which are synthesized de novo by the plant cell after fungal infection as part of a hypersensitive reaction. This concept excludes those substances that are present in the plant before any infection occurs. The most prominent agent is resveratrol, a stilbene derivative (Fig. 17.1), which occurs as *cis*- and *trans*-form. For steric reasons the *trans* form is the favored structure. In grapes the stilbene monomers occur as glucosides; they are termed piceids or polydatins. A number of derivatives are known in the meanwhile, present in different tissues, like in root tissue. Typical substances are the amurensins, the viniferins, astringin, piceatannol, pterostilben, cyphostemmin, pallidols, and gnetins (Bavaresco et al. 2002, Pezet et al. 2003, Baderschneider and Winterhalter 2000, Huang et al. 2000, Ducrot et al. 1998, Carando et al. 1999)

The biosynthesis of resveratrol is different within the *Vitis* species. There is a significant correlation between their sensitivity against fungal diseases and the biosynthesis of resveratrol. In *V. amurensis* and *V. labrusca*, several authors could prove a significantly higher concentration of resveratrol and its derivatives compared with *V. vinifera*. The biosynthesis is carried out primarily before the véraison. Young, unripe grape berries are highly resistant against Botrytis. After véraison the anthocyanins are formed whereas the biosynthesis of resveratrols declines (Jeandet et al. 1995).

The fungus Botrytis cinerea is able to produce a stilbene oxidase, to be protected against the toxic resveratrol. This enzyme oxidizes resveratrol to higher molecular weight substances. Such a degradation product is resveratrol-*trans*-dehydrodimer (Breuil et al. 1998).

17.6.2 Flavonoids

Resveratrol is a quite efficient protective molecule for the grapevine but not enough to be on the safe side. In most grapes, resveratrol is only 1% or less of the total concentration of polyphenols. Therefore, a broad arsenal of other polyphenolics are found in the grapes, like flavonol glycosides and flavan-3-ols, including the proanthocyanidins. Flavonol glycosides, like quercetins, myricetins, and kaempferols, are only localized in the berry skin, working mainly as protective agents against radiation and UV light, whereas flavan-3-ols are situated in the berry skins and the grape seeds. Flavonols and flavanols possess remarkable antimicrobial properties (Harborne 1988, Treutter 2006).

In spite of the fact that anthocyanins are mainly discussed in relation to their function as attractants or to their protective function against UV radiation and light, they seem also to support plants against microbes. The structures of nearly 540 different anthocyanins have been elucidated, and more than half of these have been reported after 1992 (Anderson and Jordheim 2006). Several dozens of anthocyanin-based structures are found in grapes and red wines.

17.6.3 Formation of Flavonoid Oxidation Products (FOP)

The biological effects of flavonoids are linked to their antioxidative properties and their potential cytotoxicity. They act as scavengers of free radicals, like reactive oxygen species (ROS), and prevent their formation by metal chelation. The behavior of oxidized flavonoids (quinones and related oxidation products) is different and has attracted much attention in plant physiology. Quinones are strong antibiotics, possess tanning properties, and are able to alkylate proteins (Pourcel et al. 2006). For example, kaempferol and quercetin polymers, produced by polyphenol oxidases (PPO), have a stronger ROS scavenger effect than the corresponding monomers.

Three enzymes responsible for flavonoid oxidation are laccase (EC 1.10.3.2), catecholoxidase (EC 1.10.3.1), and peroxidase (EC 1.11.1.7). Laccases are *o*-diphenol- and *p*-diphenol: dioxygen oxidoreductases, belonging to the group of blue copper oxidases. Those multi-copper glycoproteins consist of four histidine-rich copper binding domains. Catechol oxidases are less glycosylated and possess two copper binding domains. Peroxidases (POD) are hemoproteins, which oxidize phenols with concomitant reduction of hydrogen peroxide to water. PODs are able to form ROS, like superoxide anion radicals and hydroxyl radicals. Not all plants possess these three groups of enzymes.

Most PPOs are found in a latent form and must be activated. Catechol oxidases are localized in plastids whereas PODs can have different locations. In healthy, not senescent cells the enzymes and substrates are distributed in different subcellular compartments. This is a kind of self-protection. Anthocyanins, flavonol glycosides, flavan-3-oles, and proanthocyanidins are sequestered in vacuoles. Oxidation can occur only after senescence or plant stress (wounding, elicitation by phytopathogenic microorganisms).

It is assumed that the physiological role of flavonoid oxidation is the protection during seed and plant development and the defense against pathogen attacks. Flavonoids lead to browning of seed hulls and reinforce the barrier against water permeation. There is a positive correlation between oxidation of procyanidins and their crosslink in the cell wall. The autoxidation of quercetins leads to activated oxygen and subsequently to the formation of hydrogen peroxide. This is the substrate for POD, which enhances the autoxidation and induces the formation of 3,4-dihydroxybenzoic acid, a well known antifungal agent. Quinones are toxic against pathogens. Wounding increases the formation of polyphenols and corresponding oxidizing enzymes, and the oxidized tannins can react by covalent binding with pectinases, cellulases, and laccases of fungi, leading to their inhibition (Pourcel et al. 2006).

17.6.4 Salicylic Acid: Systemic Acquired Resistance

Salicylic acid (2-hydroxybenzoic acid; see Fig. 17.2) is formed through elimination of a C_2 fragment from the phenylpropanoids. Following fungal infects or UV irradiation, higher salicylic acid concentrations can be found in plants. Presumably, the influences from outside induce the biosynthesis of defensive and protective agents (Heldt 1996). This is exploited during the induction of the so-called systemic acquired

resistance (Durner et al. 1997). Grapes are sprayed with an aqueous salicylic acid solution. This induces the biosynthesis of phytoalexins and protects the plant and its fruits (Ryals et al. 1996). There is big interest especially in ecologically orientated wineries to use salicylic acid against downy mildew. Several studies have confirmed a dosage dependent fungistatic and fungicidic effect of salicylic acid (Amborabé et al. 2002). Furthermore, salicylic acid is an important mediator with hormone-like character in plants. Kreava et al. (1998) have shown that injection of an aqueous salicylic acid solution into grape berries retards ripening of these berries. Obviously, the plant possesses the potential to delay ripening in certain cases.

To-date only little is known on the incorporation of salicylic acid after wine ingestion in humans. Yet, this seems to be of reasonable importance, given the fact that high consumption of salicylic acid can lead to hypersensibilization (Haeberle 1987). In contrast to this negative impact also positive effects have been reported, such as antithrombotic and blood-diluting effects (Muller and Fugelsang 1994).

One study has analyzed the impact of an exogenous salicylic dosage on the final salicylic acid levels in wines. No elevated salicylic acid levels were found. Generally, the concentrations on this compound were very low. In white musts and wines a mean of 0.01 mg/L was found. In red musts and wines the mean was slightly higher with 0.16 mg/L. In total none of the wines had salicylic levels above 0.43 mg/L (Pour Nikfardjam et al. 1999).

17.7 Antimicrobial Effects of Polyphenols in General

Flavonoids in food and their health effects have been studied thoroughly (Rice-Evans and Packer 1998, Yao et al. 2004). Especially, the berry fruits are rich sources of bioactive compounds, such as flavonoids, phenolics, and organic acids, which have antimicrobial activities against human pathogens. Among different berries and berry phenolics, cranberry, cloudberry, raspberry, strawberry, and bilberry especially possess clear antimicrobial effects against, for example *Salmonella* and *Staphylococcus*. Complex phenolic polymers, like ellagitannins, are strong antibacterial agents present in cloudberry and raspberry. Several mechanisms of action in the growth inhibition of bacteria are involved, such as destabilization of cytoplasmic membrane, permeabilization of plasma membrane, inhibition of extracellular microbial enzymes, direct actions on microbial metabolism, and deprivation of the substrates required for microbial growth. Antimicrobial activity of berries may also be related to antiadherence of bacteria to epithelial cells, which is a prerequisite for colonization and infection of many pathogens. Antimicrobial berry compounds may have important applications in the future as natural antimicrobial agents for food industry as well as for medicine (Puuponen-Pimiä et al. 2005).

Every plant has developed its own strategy to fight against fungal, bacterial, and viral diseases. This is demonstrated with a few examples from different plant sources (Table 17.12). It can be expected that the phenolics of grapes and wines possess similar effects, based on their broad range of these substances and their similar structures.

Table 17.12 Examples for antimicrobial effects of different polyphenols

Product	Polyphenols	Effects and target	Literature
Artichoke	Chlorogenic acid isomers, combined with flavones	Antimicrobial	Zhu et al. (2004)
Finnish berry fruits	Flavonoids, including anthocyanins, hydroxycinnamic acid derivatives	Antimicrobial	Heinonen (2007)
Finnish berry fruits	Flavonoids, phenol carbonic acids	Antimicrobial against pathogenic colon bacteria, mostly gram-negative, *salmonella*, *staphylococcus*	Puuponen-Pimiä et al. (2001, 2005)
Native olive oil	Hydroxytyrosol derivatives Tyrosol, Oleuropein, Decarboxymethyl-Ligstrosid Aglycon, Pinoresorcinol	Antibacterial, *Helicobacter pylori* (partially resistant against antibiotics)	Romero et al. (2007)
Cranberry	Proanthocyanidins	Inhibition of adherence of uropathogenic P-fimbriated *Escherichia. coli*	Foo et al. (2000)
Red wine, Cranberry	Flavonoids, anthocyanins	Anticariogenic, growth inhibition of *Streptococcus mutans*	Thimothie et al. (2007)

17.8 Polyphenols Formation During Winemaking

Some polyphenols are formed when the berries are crushed and fermented. One of these phenols is tyrosol. It is formed through deamination from the amino acid tyrosine. A further hydroxylation leads to hydroxytyrosol. The latter was first analyzed in olive oil of extra vergine quality. Later it was shown that it is ubiquitous in all parts of the olive tree. In 1982, it was discovered in fermented products. Tommaso et al. (1998) analyzed several white wines from Italy and found a mean concentration of 1.8 mg/L.

The Grape Reaction Product (GRP, 2-*S*-glutathionyl-caftaric acid) and the GRP2 (2,5-*S*-Diglutathionyl-caftaric acid) are also only formed after crushing and under oxidative conditions. Caftaric acid is oxidized by tyrosinase to the respective quinone, which than reacts with glutathione (GSH) to GRP. Thus, the GRP can be regarded as an indicator for an enzymatically driven oxidation. Especially, in grape material with a high amount of infected berries, the laccase from Botrytis cinerea can further oxidize GRP and lead, after incorporation of another GSH molecule, to the GRP2. Tyrosinase cannot oxidize GRP or GRP2. The GSH level of the must and wine is of utmost

importance for further browning reactions. As long as enough GSH is present, the quinone formed from caftaric acid reacts with GSH and browning does not occur.

A further large group of phenols which is only formed during winemaking are the degradation products of the anthocyanins and flavan-3-ols. They can react with several wine compounds and form new substances with interesting characteristics. Mainly three compounds or compound groups take part in the reactions:

1. Flavan-3-ols (catechins)
   It is well known that anthocyanins react with flavan-3-ols to form higher molecular compounds. Partly the acetaldehyde, which is formed during fermentation, acts as a bridge between the anthocyanin and the flavan-3-ol.
2. Ellagitannins
   Ellagitannins are extracted from the wood and, thus, mainly occur in wines with Barrique maturation. Ellagic acid is part of the lignin structure of the wood and is hydrolyzed from the wood by enzymatic and chemical processes. It preferably reacts with flavan-3-ols forming new structures.
3. Various wine compounds with low molecular mass
   The anthocyanins can react with various wine compounds. This leads to the formation of the so-called pyranoanthocyanins. Molecules, like pyruvate, vinylphenol, or acetone react with an anthocyanin molecule, forming a new pyran ring. These pyranoanthocyanins have been found in fermenting and aged wines. Other wine compounds can react with the newly formed pyran, leading to highly complex structures, which have been identified in port wines and partly account for their blue color tonality (Mateus et al. 2003).

For more details see Ribéreau-Gayon et al. (2000), Monagas et al. (2005), and Cheynier (2006).

17.9 Interactions of Polyphenols with Wine Yeasts *(Saccharomyces cerevisiae)* and Other Microorganisms Before/During/After Fermentation

The levels of anthocyanins and proanthocyanidins recovered in red wines at the end of fermentation represent 20–40% of their amounts in the corresponding grapes (Cheynier 2006). Principally, the amount of polyphenols is decreased by mashing and pressing, and a large quantity of these secondary grape metabolites remain in the pomace (Hang 1998, Kammerer et al. 2005). The biggest loss is found for white wines which contain only minor concentrations of flavonoids, stilbenes, and hydroxycinnamic acid derivatives. Also, yeasts can decrease a part of these substances by adsorption. It was shown that the *Saccharomyces cerevisiae* strain 9CV could decrease especially *p*-coumaric acid derivatives up to 29%. In the case of anthocyanins, the 3-O-glucosides and the *p*-coumaroylated anthocyanins were depleted by 52.6% and 36.6%, respectively (Morata et al. 2005).

Some research has also shown that the yeast strain has a clear impact on the final polyphenolic composition of the wines. Caridi et al. (2004) have analyzed the effect of two *S. cerevisiae* strains (Sc2659 and Sc1483). According to their results Sc2659 led to deeper color, higher polyphenol content, and higher antioxidative capacity.

Saccharomyces yeasts are sufficiently tolerant of the presence of polyphenols. Therefore, it is no problem to ferment grape musts to wines or fruit juices to fruit wines. Nevertheless, a certain inhibitory effect on fermentation, but also on the inhibition of microbial spoilage and deterioration, can be found for several microorganisms, depending on the concentration and composition of the polyphenols. A remarkable inhibition of fermentation is known for several benzoic acid derivatives. Free benzoic acid is found in different Vaccinium fruits, for example, 0.6–1.3 g/L in lingonberries, which are difficult to ferment. 0.2–0.3 g/L prevent the growth of yeasts (Visti et al. 2003). Inhibitory effects on the fermentation are also found for the gallic acid and ellagic acid derivatives.

Under certain circumstances, the undesired fermentation of fruit juices can be delayed for days and weeks. We found that pasteurized and bottled apple juices made from polyphenol-rich cider apples are stable for a long period after opening the bottles. On the contrary, if we investigated apple juices made from dessert (table) apples with low polyphenol concentrations, the fermentation started within a few days after opening of the bottles (unpublished). It seems that polyphenolics inhibit foreign microorganisms.

The antimicrobial effect of apple, grape, orange, and red beet phenolics was shown for *Alicyclobacillus acidoterrestris*, a thermophilic spoilage bacterium (Wieland et al. 2002). Cloudy juices contained a significantly higher amount of polyphenolics and inhibited the growth of *Alicyclobacillus*, whereas the corresponding clear products, where a part of polyphenolics was removed by clarification, were sensitive against spoilage. The best growth was found in apple juices made from clear apple juice concentrates, due to their low polyphenol concentration. The authors recommend gentle fruit processing and the preservation of these protective substances in the juices. Similar results for *Alicyclobacillus* were found by Brodbeck et al. (2004) for iced tea beverages. Beside the factors temperature (>25°C), pH (<5.8, optimum <4.6), oxygen (>0.1%, strictly aerobic), ascorbic acid, redox potential, and storage time, the growth is dependent on the presence or absence of polyphenolics. Freshly prepared tea contains antimicrobial flavan-3-ols, whereas soluble tea extracts are diminished by 90%. Therefore, the inhibiting effect on microorganisms gets lost. The addition of polyphenolics to iced teas is recommended by the authors to ameliorate the stability.

After fermentation, yeast cell walls are protected by wine polyphenols toward the action of hydrolytic enzymes. Salmon et al. (2003) studied the effect of wine polyphenols on the shape of wine yeast after fermentation. Their results show that yeast cells in the absence of wine polyphenols rapidly reach a flat shape after the end of alcoholic fermentation. With wine polyphenols they keep a spherical and almost intact shape, which might be an indicator for a decelerated yeast cell autolysis evoked through the aforementioned antioxidative protection against hydrolytic enzymes through the wine polyphenols.

Polyphenols interact also with the bacteria of the malolactic fermentation. Some phenolics can influence the growth of bacteria in a positive or a negative way. 50–150 mg/L caffeic acid supported the growth and the degradation of malic acid in Merlot wine, whereas ferulic acid was inhibitory; coumaric acid had even a more negative effect (Krieger-Weber 2007). In the same study, the tannins from grape seeds inhibited the growth of malolactic bacteria.

17.9.1 Increasing the Live Expectancy of Saccharomyces with Resveratrol

Studies, which have been conducted at the Harvard Medical School in the US, have revealed that resveratrol increases the live expectancy of *S. cerevisiae*. The activity of sirtuin 2 (Sir2) is increased. Sirtuins are enzymes (NAD-dependent histone deacetylases), which are important for DNA regeneration. The calorie consumption is mediated by Sir2 (Horwitz et al. 2003). In some organisms, this "calorie restriction" has led to an increased live expectancy (Barger et al. 2003). This could be shown for rats, mice, and *Drosophila melanogaster* (Conti et al. 2006, Rauser et al. 2004). In the studies of Horwitz et al. (2003), the life span of *S. cerevisiae* was extended by 70%.

Studies on other organisms, such as mice, have revealed that resveratrol promotes longevity and improves glucose homeostasis by stimulating the Sir1-mediated deacetylation of the transcriptional coactivator PGC-1α (Koo and Montminy 2006).

17.10 Conclusions

The biosynthesis of polyphenols is an energy-costly process for the grape but necessary for the survival of the fruit. The antimicrobial properties play a major role in this context. Grapes and wines are characterized by a very complex composition of these secondary plant metabolites. This complexity is also due to the fact that the original polyphenols are intensely transformed during the processing into must and wine. A further factor is the subsequent chemical transformation of anthocyanins and colorless polyphenols during wine ageing. Presently, it is essentially nothing known on inhibiting or supporting effects of these products on yeasts and other microorganisms.

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Chapter 18
Microbial Interactions

Leon M.T. Dicks, Svetoslav Todorov, and Akihito Endo

18.1 Introduction

Wine fermentation is, as in many other food fermentations, characterized by complex chemical and microbial interactions. Lactic acid bacteria and yeast are the first to develop after crushing of grapes. Bacterial numbers increase to 10^3 or 10^4 cfu ml^{-1}, but decline to almost undetected levels during alcoholic fermentation (Fleet 2001). *Oenococcus oeni*, *Leuconostoc mesenteroides* subsp. *mesenteroides*, *Lactobacillus plantarum*, *Lactobacillus casei* and *Pediococcus damnosus* (previously *Pediococcus cerevisiae*) are major species present (Davis et al. 1985; Wibowo et al. 1985, 1988). At the end of alcoholic fermentation, growth commences and cell numbers increase to approx. 10^7 cells ml^{-1}. *O. oeni* usually predominates in wines of low pH (<3.5), while *P. damnosus* grows in wines with higher pH resulting in spoilage of wine (Davis et al. 1985; Wibowo et al. 1985, 1988). A number of acetic acid bacteria have been isolated from wine, e.g. *Acetobacter aceti*, *Acetobacter pasteurianus, Gluconobacter oxydans* (Drysdale and Fleet 1988), *Acetobacter (Gluconacetobacter) liquefaciens* and *Acetobacter (Gluconacetobacter) hansenii* (Du Toit and Pretorius 2000). The phylogenetic relatedness of wine lactic acid bacteria and acetic acid bacteria with other species in the same genus is shown in Figs. 18.1 and 18.2, respectively.

Yeast numbers at time of harvest range between 10^4 and 10^6 cfu g^{-1}. The most predominant genera are *Rhodotorula*, *Cryptococcus, Candida, Hanseniaspora* (anamorph *Kloeckera*), *Metschnikowia* and the yeast-like fungus *Aureobasidium pullulans* (Fleet 2003). As alcoholic fermentation commences, growth of species less resistant to ethanol, e.g. *Hanseniaspora*, *Candida*, *Pichia*, *Kluyveromyces*, *Metschnikowia* and *Issatchenkia*, is suppressed and *Saccharomyces cerevisiae* proliferates (Gao and Fleet 1988; Heard and Fleet 1988). However, at low temperatures (15–20°C) the filamentous fungi are less sensitive to ethanol and may increase in numbers equal to that of

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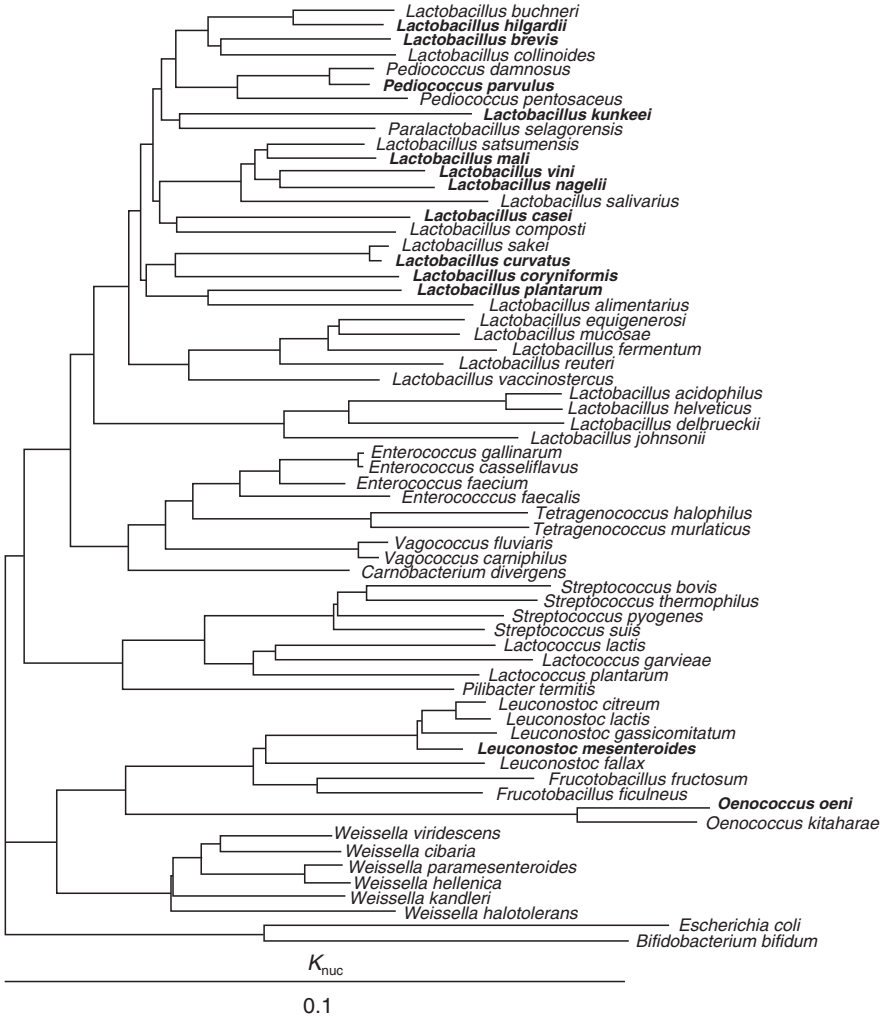

Fig. 18.1 Phylogenetic relatedness of malolactic bacteria with other lactic acid bacteria (malolactic bacteria are in ***bold***)

S. cerevisiae (Heard and Fleet 1988; Erten 2002). This could have an important impact on wine, as *Candida stellata,* for instance, changes glycerol content and alters flavour profile (Ciani and Ferraro 1998; Soden et al. 2000).

A number of filamentous fungi have been isolated from wine, especially when grapes are harvested after heavy rainfall or at the end of a wet season. The most predominant genera are *Botrytis*, *Uncinula*, *Alternaria*, *Plasmopara*, *Aspergillus*, *Penicillium*, *Rhizopus*, *Oidium* and *Cladosporum* (Emmett et al. 1988; Fugelsang

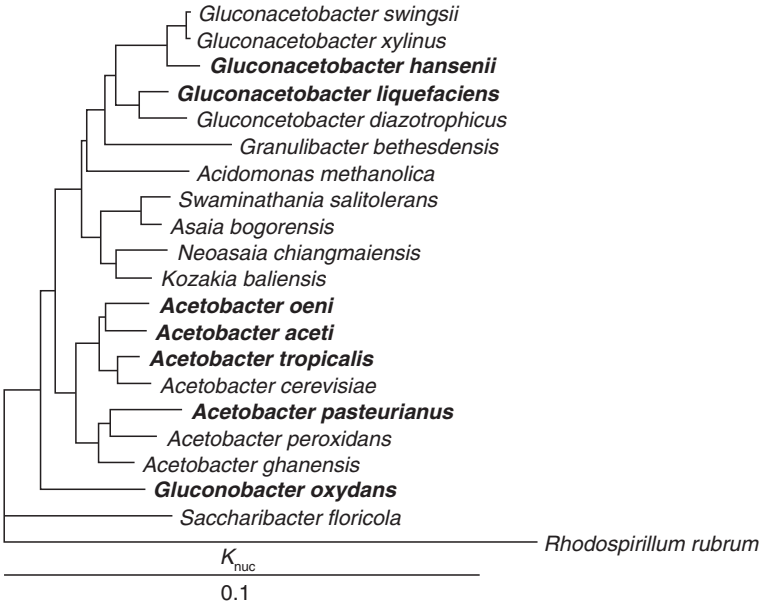

Fig. 18.2 Phylogenetic relatedness of acetic acid bacteria isolated from wine (printed in ***bold***) and other acetic acid bacteria

1997; Fleet 2001). *Botrytis cinerea*, *Aspergillus* spp. and *Penicillium* spp. produce acetic acid that may alter the flavour of wine (Drysdale and Fleet 1988). Mushroom taints (1-octen-3-one) produced by the mildew fungus *Uncinula necator* is often detected during the onset on alcoholic fermentation, but disappears as *S. cerevisiae* converts the compound to 3-octanone (Darriet et al. 2002; Stummer et al. 2003). Mycotoxins, such as ochratoxin A, is a major concern (Markaki et al. 2001; Tateo and Bononi 2001; Sage et al. 2002; Stander and Steyn 2002; Hocking et al. 2003). Fortunately, yeast cell wall components, particularly 1,3-β-glucans, have the ability to adsorb mycotoxins (Lyons 2002) so that little is left after completion of alcoholic fermentation. Mannoprotein and β-glucans, typically produced by *B. cinerea,* may inhibit the growth of *S. cerevisiae* (Lafon-Lafourcade 1984; Reed and Nagodawithana 1988; Doneche 1993). Some filamentous fungi, on the other hand, may stimulate the growth of *S. cerevisiae* and thus alcoholic fermentation (Reed and Nagodawithana 1988).

The secret to good winemaking is to manage the process in such a way that interactions between yeast, lactic acid bacteria, and yeast and lactic acid bacteria are controlled at all times. This may be difficult, as seen with stuck or sluggish malolactic fermentations and the sudden production of off-flavours. Many underlying factors may be responsible for this, including viticultural practices, such as spraying of the grapes with fungicides, high levels of flavenoides and phenolic compounds in grapes, or flavenoides and phenolic compounds released from oak barrels during maturation (Middleton and Chithan 1993).

At the onset of fermentation, yeast and bacteria are in fierce competition for nutrients. Both groups have developed unique survival strategies to compete against each other and cope with conditions in a rather extreme environment. *S. cerevisiae* has a very active metabolism, tolerates oxygen and relatively high levels of SO_2. During alcoholic fermentation, some strains produce SO_2, short chain fatty acids, peptides, proteins or glycoproteins, such as killer toxins, and lytic enzymes that are used to inhibit the growth of malolactic bacteria (Henick-Kling and Park 1994; Bisson 1999; Bauer and Pretorius 2000). Lactic acid bacteria, on the other hand, are slow growing, fastidious and require adequate levels of carbohydrates, amino acids and vitamins to survive. Like yeast, they prefer acidic environments. It is thus understandable that they have, over the years, developed an arsenal of antimicrobial compounds to compete with faster growing organisms, including yeast. Many lactic acid bacteria, including malolactic species, produce bacteriocins (antimicrobial peptides) and bacteriocin-like substances (BLIS) active against other bacteria. A few lactic acid bacteria have also developed the ability to produce antifungal compounds (Schnürer and Magnusson 2005).

It is clear that organisms forced to co-survive in the same habitat have developed unique forms of "communication". We are only beginning to understand the regulation of enzymatic reactions and competition between microbial cells through quorum sensing. Most of these studies have been done on microorganisms in niches other than wine (Bisson 1999; Fleet 2003).

18.2 Interactions Among Yeast

It is not surprising to observe fierce competition amongst organisms in a rapid changing environment such as wine; from nutrient rich grape must to an environment with high levels of SO_2 and ethanol, and onto a secondary stage (malolactic fermentation) where almost all nutrients have been depleted. Despite this, little is known about the interactions between yeast. Different strains of *S. cerevisiae* produce different end products (e.g. glycerol, acetic acid, hydrogen sulfide) and at variable concentrations (Henschke 1997). Some authors reported inhibition of *S. cerevisiae* by *Metschnikowia pulcherrima*, commonly found in grapes (Nguyen and Panon 1998). Yeast produces short- to medium-chain fatty acids (e.g. hexanoic, octanoic and decanoic acids) during alcoholic fermentation. At certain levels, these may become inhibitory to *S. cerevisiae* (Edwards et al. 1990; Viegas and SaCorreira 1997; Bisson 1999).

S. cerevisiae is more tolerant to low oxygen levels compared to other yeast (Hansen et al. 2001). Non-*Saccharomyces* species, on the other hand, utilize amino acids and vitamins, and may limit the growth of *S. cerevisiae* during early alcoholic fermentation (Bisson 1999; Mortimer 2000). Some yeast, such as *Kluyveromyces apiculata* (*Kloeckera apiculata*) and *M. pulcherrima* are proteolytic (Charoenchai et al. 1997; Dizzy and Bisson 2000) and can generate amino acids to stimulate the growth of *S. cerevisiae*. Killer strains of yeast produce killer toxins (van Vuuren and Jacobs 1992; Shimizu 1993; Musmanno et al. 1999; Guriérrez et al. 2001) and may predominate later

stages of alcoholic fermentation. Killer activity has been reported in *S. cerevisiae, Candida* spp., *Pichia* spp. and *Hanseniaspora* spp. (Fleet and Heard 1993).

18.3 Yeast–Bacteria Interactions

Malolactic fermentation usually occurs 2–3 weeks after completion of alcoholic fermentation (Lonvaud-Funel 1999). With the conversion of L-malic acid to L-lactic acid and CO_2, pH of wine increases, flavour compounds are produced, residual sugars are fermented and the wine becomes microbiologically stable, i.e. microbial growth stops (Fleet 2001). Little is known about the interactions between yeast and bacteria. A number of papers have been published on the production of short chain fatty acids (e.g. hexanoic, octanoic, decanoic), SO_2, peptides and proteins by *S. cerevisiae* (Markides 1993) and their effect on microbial growth (Fornachon 1968; Lonvaud-Funel et al. 1988; Wibowo et al. 1985, 1988; Dick et al. 1992; Markides 1993; Capucho and San Romao 1994; Eglinton and Henschke 1996; Guilloux-Benatier et al. 1998). A few studies have shown that yeast may stimulate the growth of *O. oeni* and thereby malolactic fermentation. This is, however, ascribed to cell lysis towards the end of alcoholic fermentation (Fornachon 1968; Guilloux-Benatier et al. 1998; Patynowski et al. 2002).

O. oeni is inhibited by high SO_2-producing yeast, but not by low SO_2-producing strains (Osborne and Edwards 2006). It is, however, difficult to draw a clear correlation between SO_2 produced by yeast and bacterial inhibition, as mechanisms other than SO_2 may be involved (King and Beelman 1986; Eglinton and Henschke 1996; Caridi and Corte 1997; Larsen et al. 2003). Dick and co-workers (1992) isolated two antibacterial cationic proteins from *S. cerevisiae* that inhibited the growth of malolactic bacteria. Comitini et al. (2005) reported the inhibition of malolactic bacteria by a heat and protease sensitive compound isolated from *S. cerevisiae*. In both studies the proteinaceous compounds were only partially characterized and their molecular weights were not determined.

In rare cases *Bacillus* and *Clostridium* spp. have been isolated from wine. The source of contamination is most probably contaminated water or bottles that have not been adequately sterilized (Sponholz 1993; Fugelsang 1997; Fleet 1998; Du Toit and Pretorius 2000). Since these organisms are not typically found in wine, their interaction with yeast has not been studied.

18.4 Bacterial Interactions

Like most other lactic acid bacteria, strains isolated from wine produce H_2O_2, diacetyl, low molecular organic antimicrobial products and bacteriocins (Riberau-Gayon et al. 1998; Bauer et al. 2002, 2003; Nel et al. 2002). Obligately homofermentative species produce two moles lactic acid per mol glucose fermented. Diffusion of the

lipophilic, undissociated form of lactic acid across the cytoplasmic membrane leads to a collapse of the electrochemical proton gradient and consequent inactivation of substrate transport systems (Kasjket 1987).

Hydrogen peroxide, produced by LAB in the presence of oxygen, oxidizes sulfhydryl groups, leading to the denaturing of a number of enzymes (Byczkowski and Gessner 1988). Peroxidation of membrane lipids increases membrane permeability (Byczkowski and Gessner 1988). H_2O_2 may also act as precursor for production of bactericidal free radicals such as superoxide (O_2^-) and hydroxyl (OH^*) radicals that damage DNA (Byczkowski and Gessner 1988).

Diacetyl, produced by lactic acid bacteria from the fermentation of citrate, reacts with arginine, making it metabolically unavailable (Jay 2000). Diacetyl levels of 344 μg ml^{-1} inhibited the growth of *Listeria*, *Salmonella*, *Yersinia*, *E. coli* and *Aeromonas* (Jay 2000). Accumulation of diacetyl, acetoin and acetic acid in wine varies according to the rate of malolactic fermentation. With active malolactic fermentation, acetic acid production from a given amount of citric acid is relatively high and levels of diacetyl and acetoin produced low. As malolactic fermentation (and cell growth) slows down, cells secrete less acetic acid and more diacetyl and acetoin. Similar results were recorded for laboratory strains of *Leuconostoc mesenteroides* (Lonvaud-Funel 1999).

Carbon dioxide is mainly produced by heterofermentative LAB. The precise mechanism for its antimicrobial action is still unknown. CO_2 may play a role in creating an anaerobic environment which inhibits enzymatic decarboxylations. The accumulation of CO_2 in the lipid bilayer of cell membranes may lead to permeability dysfunction (Lindgren and Dobrogosz 1990). Studies conducted on Gram-negative psychrotrophic bacteria have shown that growth is inhibited by CO_2. As little as 10% CO_2 could decrease viable cell numbers by 50% (Wagner and Moberg 1989). Levels of 20–50% CO_2 have strong antifungal activity (Lindgren and Dobrogosz 1990).

Bacteriocins produced by a variety of lactic acid bacteria have been reported (reviewed by De Vuyst and Vandamme 1994), but only a limited number of papers have been published on bacteriocins produced by malolactic bacteria (Lonvaud-Funel and Joyeux 1993; Strasser de Saad and Manca de Nadra 1993).

Bacteriocins are ribosomally synthesized and are usually active against genetically related species (Jack et al. 1995). They are grouped into four classes, based on their structure (Klaenhammer 1993). Class I bacteriocins are small lantibiotics (<5 kDa), containing amino acids lanthionine, α-methyllanthionine, dehydroalanine and dehydrobutyrine. Class I peptides are further divided into type A, i.e. elongated peptides with a net positive charge (amphipathic peptides such as nisin) and type B, i.e. globular peptides, e.g. mersacidin and actagardine (Brotz et al. 1998; McAuliffe et al. 2001). Class II bacteriocins are small (<10 kDa), heat-stable, non-lanthionine containing peptides and are sub-divided into three groups, *viz.* class IIa characterized by a conserved N-terminal amino acid sequence and being active against *Listeria* spp., class IIb for which two peptides are needed (Nisen-Meyer et al. 1992), and class IIc that includes all other bacteriocins not classified into any of the two groups (Chen and Hoover 2003). Best known examples of class II bacteriocins are pediocin PA-1 (class IIa) produced by *Pediococcus acidilactici* and pediocin-like peptides. All pediocin-

like bacteriocins have a consensus amino acid sequence motif in the N-terminal part of the mature peptide. Members of pediocin-like peptides show high degree of homology (40–60%) when the corresponding amino acid sequence is aligned. In particular, the cationic N-terminal domain contains the homology region YGNGVXCXXXXCXV or "pediocin box" with two cystein residues forming a disulphide bridge (Eijsink et al. 1998). Bacteriocins classified as class III are large (>30 kDa), heat-labile proteins. Complex bacteriocins with carbohydrate or lipid moieties are classified as class IV. More recently, a new classification system was proposed by Cotter et al. (2005a) where only two classes of bacteriocins are proposed, e.g. lanthionine-containing bacteriocins (lantibiotics) in class I and non-lanthionine containing bacteriocins into class II. The lanthionine-containing bacteriocins are similarly classified into elongated amiphilic cationic bacteriocins and globular lantibiotics. The peptides are further divided into 11 sub-groups based on their unmodified peptide sequences (Cotter et al. 2005b).

The mode of activity of most bacteriocins is the formation of pores and destabilization of the cell membrane. Model membrane studies with nisin A have shown that lipid II acts as a docking station (Wiedemann et al. 2001). After binding, nisin wedges itself into the cell membrane to form short-lived pores which disturb the integrity of the cytoplasmic membrane and causes efflux of ions and other cell components (Driessen et al. 1995). At high concentrations of nisin, pore formation may occur in the absence of lipid II, provided the cell membrane contains at least 50% negatively charged phospholipids (Wiedemann et al. 2001). Under these conditions, the positively charged C terminus of nisin is important for initial binding and antimicrobial activity. Mersacidine and the antibiotic vancomycin also bind to lipid II, but to a different part of the molecule (Breukink and De Kruijff 1999).

The following are examples of antimicrobial peptides produced by lactic acid bacteria isolated from wine and their possible application as natural antibacterial compounds in winemaking.

The effect of nisin on *O. oeni* and wine related bacteria was studied by Rojo-Bezares et al. (2007). The following MIC_{50} values have been reported for nisin in the presence of potassium metabisulphite and ethanol: 0.024 µg ml^{-1} for oenococci, 12.5 µg ml^{-1} for lactobacilli, pediococci and leuconostocs, 200 µg ml^{-1} for acetic acid bacteria and ≥ 400 µg ml^{-1} for yeast. Synergistic effects on bacterial growth inhibition were observed, and potassium metabisulphite MIC_{50} values decreased from one to three orders of dilution, when combined with sub-inhibitory concentrations of nisin in the growth media. Significant differences in nisin sensitivity were observed between Gram-positive and Gram-negative bacteria, and between *O. oeni* and other lactic acid bacteria. Optimal combinations of nisin and metabisulphite can thus control growth of spoilage bacteria in wine and may also lead to lower levels of addition of SO_2 to wine (Rojo-Bezares et al. 2007).

Lonvaud-Funel and Joyeux (1993) reported growth inhibition between *P. pentosaceu*s, *L. plantarum* and *O. oeni* isolated from wine. Pediocin N5p, produced by a strain of *P. pentosaceus* isolated from Argentinean wines, inhibited *Pediococcus* spp., *O. oeni* and *Lactobacillus* spp. (Strasser de Saad and Manca de

Nadra 1993). The strain produced 4,000 U (units) pediocin N5p per ml in grape juice medium, with optimal production at pH of 7.5 and 30°C. Activity of peptide remained unchanged at pH 2.0–5.0, and at 4°C and 30°C, but was inactivated at pH 10.0. When submitted to 30 min at 80°C, 100°C and 115°C, maximal stability was observed at pH 2.0. Ethanol concentrations of 10% (v/v) had no effect on activity at low pH or in the presence of 40–80 mg l^{-1} SO_2 (Strasser de Saad et al. 1995).

Pediocin PD-1, produced by *P. damnosus* NCFB 1832 differs from other pediocin-like bacteriocins in that it is not active against pediococci and has a unique sensitivity pattern to proteolytic enzymes (Green et al. 1997). Compared to other pediocin-like bacteriocins, pediocin PD-1 is not very active against *Listeria monocytogenes*. The peptide is hydrophobic, resistant to heat (10 min at 121°C), remains active after 30 min of incubation between pH 2.0 and 10.0 (Green et al. 1997) and is not affected by 15% (v/v) ethanol and 100 ppm SO_2, or a combination thereof (Green et al. 1997). The peptide is active against a range of Gram-positive bacteria, including members of the genera *Clostridium, Bacillus, Staphylococcus, Enterococcus, Propionibacterium, Lactobacillus, Leuconostoc* and *Oenococcus* (Nel et al. 2002). Pediocin PD-1 effectively removed established biofilms of *O. oeni* which formed on stainless steel surfaces in Chardonnay must and out competed nisin in the same experiment (Bauer et al. 2002, 2003; Nel et al. 2002).

Navarro et al. (2000) tested 42 strains of *Lactobacillus*, *Leuconostoc*, *Pediococcus* and *Lactococcus*, isolated from Rioja red wines, for antimicrobial activity against *O. oeni* and *S. cerevisiae*. Only nine strains, all classified as *L. plantarum*, showed antimicrobial activity. *L. plantarum* J-51 had the broadest spectrum of activity, inhibiting the growth of 31 strains. Bacteriocin J-51 is stable between pH 3 and 9 and withstands 100°C for 60 min. The gene encoding bacteriocin J-51 has a 366-bp fragment with 95% homology to the *pln* locus of *L. plantarum* C-11. The deduced precursor peptide differed from that of planraricin C-11 by a single mutation (Gly7 to Ser7) in the leader peptide.

Nisin and pediocin PA-1 are expressed in *Lactococcus lactis* (Horn et al. 1999). Schoeman et al. (1999) were the first to express the genes encoding pediocin PA-1 in *S. cerevisiae*. This was followed by the expression of plantaricin (bacteriocin) 423 in the same host (Van Reenen et al. 2002).

In a purified form bacteriocins are colorless, tasteless and odorless and will not influence the organoleptic quality or bouquet of the wine. Extensive tests on a number of bacteriocins have proven that they are not toxic and are safe for human consumption. Since most bacteriocins have a narrow spectrum of antibacterial activity, they have little effect on the normal population of intestinal bio-microflora (De Vuyst and Vandamme 1994, Verellen et al. 1998). Furthermore, recent studies (Bauer et al. 2002, 2003; Nel et al. 2002) have shown that bacteriocins may be used to control biofilm formation in stainless steel tanks, suggesting that they are natural and bio-safe sanitizers. If technology can be developed to produce bacteriocins cost-effectively, they may replace, or at least lower, the use of sulphur dioxide, detergents and sanitizers.

Bacterial population regulate their behavior and expression of genes through production of low-molecular-mass signalling molecules (Whitehead et al. 2001). These quorum sensing molecules are produced throughout growth and, at a specific level, they stimulate or inhibit gene expression to change the behaviour of the whole population. In the case of Gram-negative bacteria, *N*-acyl-homoserine lactones act as signalling molecules that regulate biofilm formation, production of exo-polymers and extracellular hydrolytic enzymes. In Gram-positive bacteria, amino acids and small peptides have similar functions. Yeasts, on the other hand, use biocarbonate (Ohkuni et al. 1998), acetaldehyde (Richard et al. 1996) and ammonia (Palkova et al. 1997) as signaling molecules. Farnesol prevents the transformation of *Candida albicans* from yeast to hyphal stage and biofilm formation (Hornby et al. 2001; Ramage et al. 2002). An excellent example of quorum sensing between bacteria and yeast is the production of a compound by *Pseudomonas aeruginosa* that kills the growth of *C. albicans* (Hogan and Kolter 2002). Similar forms of communication could be present between malolactic bacteria and yeast in wine.

18.5 Conclusions

The secret to good winemaking is to manage the fermentation processes, i.e. complex microbial interactions occurring between yeast, malolactic bacteria and between yeast and malolactic bacteria. Although a number of antimicrobial and growth stimulating compounds have been identified for yeast, filamentous fungi and bacteria, more research is needed at the molecular level to fully understand microbial interactions occurring at each of the phases during wine making. Little is known about gene expression and stress signals produced by yeast and malolactic bacteria. The role of signal molecules in quorum sensing therefore needs to be studied.

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Part V
Molecular Biology and Regulation

Chapter 19
Genomics of *Oenococcus oeni* and Other Lactic Acid Bacteria

Angela M. Marcobal and David A. Mills

19.1 Introduction

Lactic acid bacteria (LAB) is a diverse group of low GC Gram-positive bacteria that produce lactic acid as a major end product. LAB act in concert with other microbes in the creation of some of mankind's favorite fermented products, one of them being the production of wine. In the case of wine production, the main microbial workhorse is the alcohol-producing yeast *Saccharomyces cerevisiae*, which consumes majority of grape sugars and produces significant levels of ethanol. Along with fermentative yeast, LAB are a common presence in wine production having both beneficial and detrimental attributes. Numerous species of LAB have been identified on grape surfaces or in fresh musts, consisting mostly of members of the genera *Oenococcus*, *Lactobacilli*, *Leuconostoc*, *Lactococcus* and *Pediococcus* (Osborne and Edwards 2005). However, once the main alcoholic fermentation is carried out and the level of ethanol increases, a more restricted group of oenococcal, lactobacilli and pediococcal species are generally witnessed.

Perhaps the most useful role that LAB performs in the production of wine is the conversion of L-malate to L-lactate. This process is known as the malolactic fermentation (MLF) (Davis et al. 1985) in which malate is decarboxylated to lactate and CO_2 released. The development of MLF is often considered a good winemaking practice since it consumes available substrates and thereby increases microbiological stability of the wine. MLF also improves organoleptic complexity of wine through production of lactate and other flavor compounds such as diacetyl (Kunkee 1991; Lonvaud-Funel 1995).

Recently genome sequences of *O. oeni* and many other wine-related LAB have been determined (Bolotin et al. 2001; Chaillou et al. 2005; Kleerebezem et al. 2003; Makarova et al. 2006). Of the specific LAB isolates sequenced to date, only *O. oeni* PSU-1 originated from wine. Consequently, this review mainly focuses on aspects of *O. oeni* evolution as well as select topics on carbon and nitrogen metabolism in relation to growth of *O. oeni* in wine.

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H. König et al. (eds.), *Biology of Microorganisms on Grapes, in Must and in Wine*,

19.2 Comparative Genomics of Wine-Related LAB

At the time of this writing, complete or high-quality draft genome sequences for 23 food or beverage-related LAB species are publicly available (http://www.ncbi.nlm.nih.gov/), providing a tremendous resource for researchers worldwide. Of these, nine sequences are from LAB species that are generally found in wines or musts (Table 19.1). Several groups have compared and contrasted these genomes, and their subgroups, revealing the shared and unique features of the whole clade (Canchaya et al. 2006; Lorca et al. 2007; Makarova et al. 2006; Makarova and Koonin 2007; Siezen et al. 2004). Interestingly, phylogenetic trees constructed on the basis of concatenated ribosomal proteins or RNA polymerase subunits mostly match what had previously been determined on the basis of rRNA sequence analysis (Makarova et al. 2006). However, several differences are seen. *Lactobacillus casei* is clearly shown to be more closely associated with the *L. delbrueckii* group and distinct from *Pediococcus*, thereby contrasting previous phylogenies (Schleifer and Ludwig 1995). In addition, the genus *Lactobacillus* appears to be paraphyletic with respect to the *Pediococcus-Leuconostoc* groupings. Confirming a previous observation by Yang and Woese (Yang and Woese 1989), the *Leuconostoc* branch exhibits an accelerated evolution by comparison to *Pediococcus*, with the genera *Oenococcus* evolving faster than *Leuconostoc*.

A most intriguing aspect of comparative genomics is the ability to reconstruct the evolution of the LAB clade by denoting genes gained and lost since the split from a *Bacillales* ancestor (Makarova et al. 2006; Makarova and Koonin 2007). Figure 19.1 details the reconstruction of gene content evolution in *Lactobacillales*. Overall, there is a general pattern of gene loss that is much greater than the level of genes gained. Notable aspects are early loss of genes involved in biosynthesis of cofactors as well as loss of oxidase-related genes and catalases, suggesting an ancient transition to anaerobic (or microaerophilic), nutritionally rich environments similar to that found in animal intestinal tracts. Of those genes gained in the LAB

Table 19.1 General features of sequenced genomes of lactic acid bacteria found in wine

	Sequence status[a]	NCBI Accesion No.	GC%	Size (nt)	No. of genes	rRNA operons
O. oeni PSU-1	Complete	NC_008528	37.8	1,780,517	1,741	2
O. oeni ATCCBAA-1163	Draft	AAUU01000000	37.9	1,753,447	1,678	1
Lb. brevis ATCC367	Complete	NC_008497	46.0	2,340,228	2,299	5
Lb. plantarum WCFS1	Complete	NC_004567	44.4	3,348,625	3,144	5
Lb. casei ATCC334	Complete	NC_00852	46.5	2,924,325	2,846	5
Lb. sakei subsp. *sakei* 23 K	Complete	NC_007576	41.2	1,884,661	1,963	7
P. pentosaceus ATCC2574	Complete	NC_008525	37.3	1,832,387	1,826	5
L. lactis subsp. *lactis* IL1403	Complete	NC_002662	35.3	2,365,589	2,424	6
Lc. mesenteroides ATCC8293	Complete	NC_008496	37.6	2,075,763	2,088	4

[a]Data obtained from JGI (http://www.jbi.doe.gov)

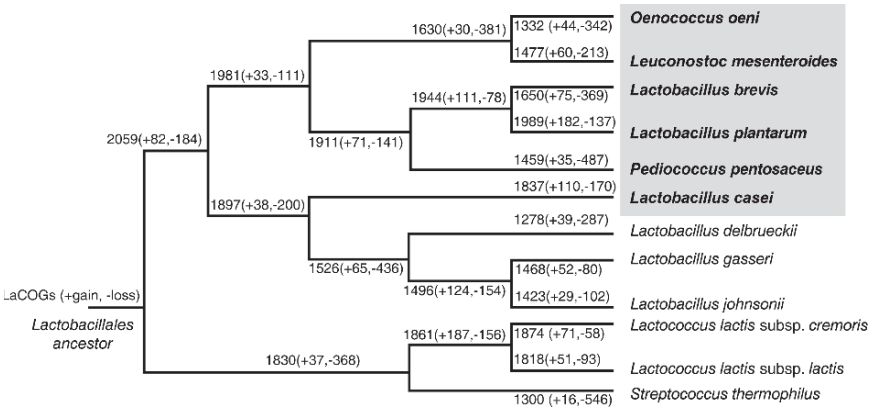

Fig. 19.1 Reconstruction of gene content evolution in *Lactobacillales*. The number of shared genes (LAB-specific Clusters of Orthologous Genes; LaCOGs), including those gained or lost, are depicted at each branch. *Bold* and *shaded* species are commonly found in wine. The figure is based on a previous figure from Makarova and Koonin (2007)

clade, most common are those involved in sugar metabolism, likely arising from horizontal gene transfer as species adapted to distinct niches.

19.3 The Genomic Content of *O. oeni* PSU-1

19.3.1 Genomic Analysis and Evolution

Using pulse field gel electrophoresis, Tenreiro and coworkers (Ze-Ze et al. 1998, 2000) generated a detailed physical map of the genome of two *O. oeni* strains, PSU-1 and GM, estimating the genome size at ~1.857 and ~1.932 Kb respectively. Both genomes are structurally similar; the differences between them are believed to be point mutations and/or insertion/deletion events (Ze-Ze et al. 2000). The actual sequenced genome of *O. oeni* PSU-1 contains 1,780,517 nt with a G + C content of 38% (Makarova et al. 2006). Like other members of the *Lactobacillales*, *O. oeni* PSU-1 exhibits significant lineage specific gene loss in the divergence from the nearest *Leuconostoc* ancestor (Table 19.2; Makarova et al. 2006). Numerous genes related to different biosynthesis pathways that are present in the nearest ancestor are lacking in *O. oeni*. This includes genes involved in the biosynthesis of cofactors such as glutathione, riboflavin or thiamine. PSU-1 also lacks genes related to metabolism of xylose, sucrose, as well as some components of the mannose phosphotransferase system. This latter observation is consistent with previous studies which concluded that *O. oeni* is unable to metabolize these carbohydrates (Beelman et al. 1977; Garvie 1986). Furthermore, genes involved in the synthesis of several amino acids, such as arginine, leucine, valine, tryptophan, methionine,

Table 19.2 Examples of gene loss in *O. oeni* compared to the common ancestor of *O. oeni* and *Lc. mesenteroides*

Functional group	Gene	Protein description	COG number
Synthesis of cofactors	*ribC*	Riboflavin synthase α-chain	COG0307
	ribD	Pyrimidine reductase, riboflavin biosynthesis	COG1985
	ribH	Riboflavin synthase β-chain	COG0054
	thiD	Hydroxymethylpyrimidine/ phosphomethylpyrimidine kinase	COG0351
	thiE	Thiamine monophosphate synthase	COG0352
	thiF	Dinucleotide-utilizing enzymes involved in molybdopterin and thiamine biosynthesis family 2	COG0476
	thiM	Hydroxyethylthiazole kinase, sugar kinase family	COG2145
	thiJ	4-Methyl-5(β-hydroxyethyl)-thizaole monophsophate	COG0693
Carbohydrate metabolism	*xylA*	Xylose isomerase	COG2115
	xylB	Xylulose kinase	COG3507
	sacA	Sucrose-6P hydrolase	COG1621
	manY	Phosphotransferase system, mannose/fructose/N-acetylgalactosamine-specific component IIC	COG3715
	manZ	Phosphotransferase system, mannose/fructose/N-acetylgalactosamine-specific component IIC	COG3716
Amino acid metabolism	*argB*	Acetylglutamate kinase	COG0548
	argC	Acetylglutamate semialdehyde dehydrogenase	COG0002
	argF	Ornithine carbomayltransferase	COG0078
	hisA	Phosphoribosylformimino-5-aminoimidazole carboxamine ribonucleotide (ProFAR) isomerase	COG0106
	hisB	Imidazoleglycerol-phosphate dehydratase	COG0131
	hisD	Histidinol dehydrogenase	COG0141
	hisF	Imidazoleglycerol-P synthase	COG0107
	hisG	ATP phosphoribosyltransferase	COG0040
	hisZ	ATP phosphoribosyltransferase involved in histidine biosynthesis	COG3705
Oxidative stress response	*gshAB*	Glutathione biosynthesis bifunctional protein	COG1171
Replication, repair, recombination	*mutS*	Mismatch repair ATPase (MutS family)	COG0249
	mutL	DNA mismatch repair enzyme (predicted ATPase)	COG0323
	mutY	A/G-specific DNA glycosidase	COG1194
	radC	DNA repair protein	COG2003
	recQ	Superfamily II DNA helicase	COG0514
	recT	Recombinational DNA repair protein	COG3723
	mutT	NTP pyphosphohydrolase	COG0494

glutamate, isoleucine, or histidine are missing in PSU-1. Since arginine and isoleucine are necessary for growth of *O. oeni* (Garvie 1967; Remize et al. 2006), the high content of these amino acids in wine/must might explain the lack of the genes involved in their biosynthesis.

Yang and Woese (1989) previously designated *O. oeni* as a rapidly evolving organism on the basis of 16S rRNA-based phylogeny and unusual alterations in normally conserved 16S rRNA positions. Phylogenetic trees constructed from concatenated ribosomal proteins and RNA polymerase subunits confirmed that *O. oeni* evolves faster than other *Leuconostoc* (Makarova and Koonin 2007). One possible explanation for the accelerated evolution is the fact that *O. oeni* PSU-1 has lost *mutS* and *mutL,* genes involved in mismatch repair (MMR) (Table 19.2). Other genes related to DNA maintenance are also missing including genes involving DNA repair (*radC* and *recT*), DNA helicase (*recQ*), glycosidase (*mutY*) and the NTP pyrophosphohydrolase (*mutT*).

Recently Marcobal et al. (2008) demonstrated that spontaneous mutation rates for *O. oeni* and the newly discovered *O. kitaharae* are significantly higher than those from neighboring taxa *Pediococcus pentosaceus* and *Leuconostoc mesenteroides*, which possess *mutS* and *mutL*. Since MMR systems more effectively repair transition mutations by comparison to transversions, the absence of *mutSL* results in an increase in transition substitutions (Choy and Fowler 1985). Comparison of allelic nucleotide substitutions in the two *O. oeni* genome sequences (PSU-1 and BAA-1163) showed the ratio of transitions to transversions is significantly higher than in other paired genomes from the *Bacillales* (Marcobal et al. 2008). These data confirm that the mutational spectrum in *O. oeni* is clearly different, and the elevation of transition nucleotide substitutions is consistent with the lack of MMR in the species.

19.3.2 Select Topics Related to Major Metabolic Pathways in O. oeni

Carbohydrate metabolism: The main sugar sources in musts and wines are glucose and fructose. In the initial characterization of *O. oeni* PSU-1 Beelman and coworkers (Beelman et al. 1977) demonstrated growth in glucose, fructose, ribose, trehalose and cellobiose as well as production of mannitol from fructose. In addition, growth was initially observed in sucrose, lactose, maltose and raffinose; however when tested 2 years later the strain had lost these capabilities. Given the high mutation rate of the species (Marcobal et al. 2008), it is likely that these functions were lost by spontaneous mutation or, perhaps, were originally encoded on plasmids and subsequently lost. While the more current version of PSU-1 does not house any plasmids (Tenreiro et al. 1994) many other *O. oeni* do contain such elements.

Once transported into the cell *O. oeni* metabolizes hexoses and pentoses via the phosphoketolase pathway yielding D-lactic acid, ethanol and CO_2. Genes involved in the phosphoketolase pathway in the PSU-1 genome are shown in Fig. 19.2.

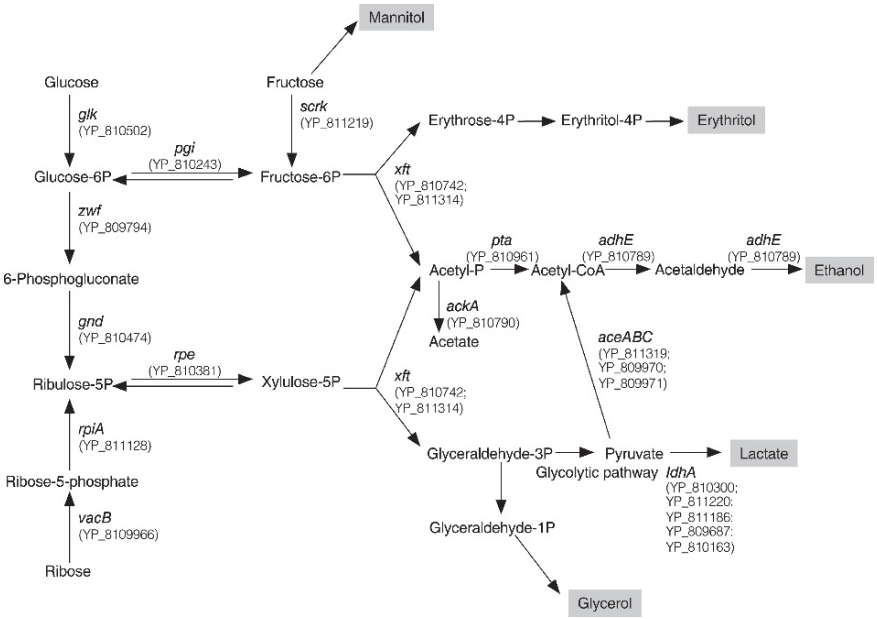

Fig. 19.2 Metabolism of glucose by the phophoketolase pathway by *O. oeni* PSU-1. *Abbreviations*: *aceABC* pyruvate dehydrogenase; *adhE* acetaldehyde/ethanol dehydrogenase; *glk* glucokinase; *gnd* 6-phosphogluconate-dehydrogenase; *ldhA* lactate dehydrogenase; *pta* phosphotransacetylase; *pgi* phophoglucose-isomerase; *rpe* ribulose-5-phophate-3-epimerase; *rpiA* ribose-5-phosphate-isomerase; *scrK* fructokinase; *vacB* ribokinase; *xft* phosphoketolase; *zwf* glucose-6-phosphate-dehydrogenase. NCBI accesion numbers are indicated in brackets

Interestingly PSU-1 appears to have lost the gene encoding mannitol dehydrogenase (COG0246) which converts fructose to mannitol even though this conversion was witnessed in the original isolate (Beelman et al. 1977).

Malic acid metabolism: Malic acid is the large fraction of organic acids found in grape juice and wine; its consumption results in an increase in the wine pH, with consequent change in the organoleptic characteristic of wine (Saguir and Manca de Nadra 2002). The acid flavor of the malate is reduced by conversion to a monocarboxylic lactate, thus softening the mouthfeel of wines that have gone through MLF. This conversion is realized through the action of a malate permease (TC#2.A.69) which transports malate into the cell and a malolactic enzyme which decarboxylates it to lactate. Cox and Henick-Kling (Cox and Henick-Kling 1989) first demonstrated that wine-related LAB obtain chemiosmotic energy from this process and are thus able to generate additional ATP. Genes contained within *mle* locus possess a relatively conserved structure compared to nearly all sequenced LAB (Fig. 19.3), with the malolactic enzyme (*mleA*) and permease (*mleP*) transcribed in the same orientation and a *lysR* regulator (*mleR*) transcribed in the opposite direction upstream of *mleA*. Interestingly, *O. oeni* PSU-1 has four other permeases in the *mleP* transporter class (TC#2.A.69) elsewhere on the genome – more than any other sequenced LAB (Lorca

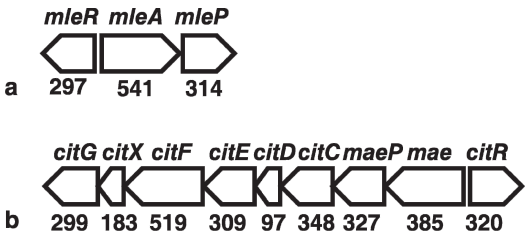

Fig. 19.3 Genetic organization of genes involved in (**a**) malate and (**b**) citrate metabolism in *O. oeni* PSU-1. *Abbreviations*: *mleA* malolactic enzyme; *mleP* malate permease; *mleR* lysr-type regulatory protein; *citC* citrate ligase; *citD* citrate lyase γ subunit; *citE* citrate lyase β subunit; *cit F* citrate lyase α subunit; *citg* phosphoribosyl-dephospho-coa synthetase; *citR* citrate lyase regulator; *citX* phosphoribosyl-dephospho-coA transferase; *mae* malate oxidoreductase; *maeP* predicted permease

et al. 2007). While the exact substrate specificities for these additional transporters are yet to be determined, an expansion of permeases in this class could suggest an increased ability to transport malate in *O. oeni*. At present the role of the *mleR* regulator is unclear in *O. oeni;* however the same gene in *L. lactis* is critical for MLF capacity (Renault et al. 1989).

Citric acid metabolism: The consumption of citrate by *O. oeni* has been the subject of numerous studies (Bartowsky and Henschke 2004; Lonvaud-Funel 1999). Citrate can not be used by *O. oeni* as a sole carbon source, but the co-metabolism with sugars stimulates growth and contributes to flavor development by production of diacetyl (Salou et al. 1994), a buttery flavor desired in some wine styles. Genome analysis of *O. oeni* PSU-1 reveals genes necessary for catabolism of citrate to pyruvate. PSU-1 encodes a single citrate lyase cluster of nine genes, many of which are shared by several other sequenced LAB strains (Fig. 19.3; Mills et al. 2005). Interestingly, the citrate transporter, *maeP*, is in the same transporter class (TC#2.A.69) as the malate permease discussed above. Others have shown that citrate transporters (CitP) can transport malate as well (Marty-Teysset et al. 1995). *O. oeni* PSU-1 also contains genes encoding the complete butandiol pathway including two different α-acetolactate synthases- one anabolic (*ilvB*) and another catabolic (*alsS*) – of which the latter is part of an operon with an acetolactate decarboxylase (*alsD*). Different authors have demonstrated that this latter operon is constitutively expressed (Garmyn et al. 1996; Ramos et al. 1995).

Nitrogen metabolism: Nitrogen composition of wine changes along the process of vinification. During alcoholic fermentation, amino acids are both consumed and released by the yeast (Alexandre et al. 2001; Guilloux-Benatier et al. 2006). In addition, autolysis of yeast results in release of amino acids, peptides and proteins thus generating available nitrogen compounds for LAB growth (Alexandre et al. 2001; Guilloux-Benatier and Chassagne 2003; Martinez-Rodriguez et al. 2001). The *O. oeni* PSU-1 genome contains numerous genes with predicted specificity for peptide and amino acid transport including: six members of the amino acid-polyamine-organocation family (TC#2.A.3), a major facilitator subfamily transporter

(TC#2.A.1), a member of the branched chain amino acid:cation symporter (LIVCS) family (TC#2.A.26) and nine ABC-type transporters (TC#3.A.1) with amino acid or peptide specificies (Lorca et al. 2007). The PSU-1 genome also encodes genes involved in biosynthesis of alanine, aspartate, asparagine, cysteine, glutamine, lysine, methionine and threonine but lacks genes involved in synthesis of arginine, histidine, leucine, isoleucine, valine, proline, serine and tryptophan (Makarova et al. 2006; Mills et al. 2005).

19.4 Conclusions

Recent access to whole genome sequence provides a wealth of information for wine-related LAB and, in particular, *O. oeni*. Notably, whole genome comparisons have enabled phylogenetic reconstruction of the whole LAB group thereby allowing in depth analysis of the species evolution – an analysis which will continue to drive discovery for some time to come. Access to *O. oeni* genome sequence also enables prediction of key pathways involved in central carbon and nitrogen metabolism and facilitates "omics" research strategies aimed at understanding implementation of this important starter culture in wine processing. This, in turn, will foster modeling of *O. oeni* growth and metabolism in order to predict optimum strategies for efficiently carrying out the MLF with a desired flavor outcome. The advent of genomic biology for wine-related LAB represents a paradigm shift that sets the stage for a more comprehensive understanding of their complex interactions within wine and the winery environment.

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Chapter 20
Genome of *Saccharomyces cerevisiae* and Related Yeasts

Bruno Blondin, Sylvie Dequin, Amparo Querol, and Jean-Luc Legras

20.1 Introduction

Many different yeast species can take part in spontaneous alcoholic fermentations, but *Saccharomyces cerevisiae* is the main species involved which is commonly used as a starter for alcoholic wine fermentation, because of its optimal technological and sensory properties. *Saccharomyces cerevisiae* wine yeasts are thought to have been selected over a long period of time, through the unwitting use of this species, by man, for alcoholic fermentation. These yeasts are particularly well adapted to harsh conditions prevailing in grape musts and wines (strong acidity, high sugar or alcohol concentration, presence of sulfites, etc.), which have shaped their genome (Pretorius 2000; Querol et al. 2003). Identification of the molecular basis of specific adaptation to wine environment is therefore a key element to wine yeast genome research. The availability of the first yeast (laboratory) genome sequence (Goffeau et al. 1996) and the development of genome-wide analysis tools (De Risi et al. 1997) have greatly facilitate studies of wine yeast genome architecture over the last ten years. This has in turn opened up new possibilities for more detailed investigations of divergence between wine and laboratory yeasts. It will also be challenging to uncover the genetic basis of extensive phenotypic diversity of wine yeasts, which remains poorly understood. The identification, of variation contributing to the properties of wine yeast, among the huge number of genomic differences, should make it easier to exploit yeast biodiversity, improve selection procedures and define molecular targets for genetic improvement of wine yeasts.

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20.2 *Saccharomyces* Wine Yeasts

The taxonomy of *Saccharomyces* wine yeasts has undergone multiple changes, due to successive reorganizations of the classification of this group (Pretorius 2000; Rainieri et al. 2003). *Saccharomyces* wine yeasts are all now considered to belong to the genus *Saccharomyces*. This taxon initially included four species — *S. cerevisiae*, *S. bayanus*, *S. paradoxus*, *S. pastorianus* — but has since been enriched by the addition of another three species: *S. kudriavzevii, S. mikatae* and *S. cariocanus* (Vaughan-Martini and Martini 1998; Naumov et al. 2000a; Rainieri et al. 2003). Most wine yeasts used for alcoholic fermentation are now recognized as *Saccharomyces cerevisiae*. However, *Saccharomyces bayanus* may also mediate alcoholic fermentation, particularly in low-temperature conditions, as they are cryotolerant (Naumov et al. 2000b). Strains of *S. paradoxus* have been isolated from vineyards, but their potential contribution to wine fermentation is unknown (Redzepovic et al. 2002).

Another level of complexity within the genus *Saccharomyces* group arises from the ability of strains of different species to mate and form hybrids (de Barros Lopes et al. 2002). In recent years, the molecular characterization of *Saccharomyces* wine strains has revealed some of these strains to be hybrids (Masneuf et al. 1998). This situation extends the range of phenotypic diversity for wine yeasts and has potentially significant industrial implications. Recent advances in molecular knowledge for such hybrid genomes are discussed in this chapter (see Sect. 20.6).

20.3 Genetic Properties of *S. Cerevisiae* Wine Strains

The genome structure of *S. cerevisiae* is intimately linked to its genetic properties, which reciprocally influence the life style and genome characteristics of this yeast. *S. cerevisiae* strains are mostly diploid in natural conditions and display vegetative reproduction through multi-polar budding. Under specific nutritional conditions cells may sporulate to form four haploid spores of different mating types, a or α. One peculiarity of *S. cerevisiae* wine strains is that many are homothallic, and descendants of these haploid spores mate with their own progeny to form a diploid. Homothallism is frequent in wine yeasts, with about 70% of strains known to be homothallic (Mortimer 2000), but the ecological significance of this property remains unclear. Mortimer et al. (1994) suggested that homothallism may provide required conditions for a process he called “genome renewal”. According to this model, yeasts accumulate mutations during the vegetative stages of their life, rendering them heterozygous for various traits. Upon sporulation and the self-mating of homothallic spores, homozygous diploids are generated. This process makes it possible to eliminate recessive mutations deleterious for the strain or to ensure that recessive mutations increasing strain fitness are expressed. Genome renewal is therefore likely to play a role in adaptation of yeasts to stressful wine environment. Mortimer (2000) proposed that about one third of wine *S. cerevisiae* cells were

homozygous. Conversely, high levels of heterozygosity was deduced from various approaches and was shown to lead to massive differences in gene expression among segregants (Bradbury et al. 2005; Cavalieri et al. 2000).

Little is known about the sexual activity of yeasts in wine environments. The frequency at which yeasts sporulate and mate in such environments is unknown. The ability of wine yeasts to sporulate is highly heterogeneous and varies from 0 to 100% on laboratory media (Johnston et al. 2000). The external crossing rate of *S. cerevisiae* was recently estimated indirectly, through an analysis of the whole-genome sequences of three strains and the sister species *S. paradoxus* (Ruderfer et al. 2006). It was concluded that outcrossing was rare, occurring once every 50,000 divisions, corresponding to once every 17–137 years assuming that one to eight divisions occur per day.

20.4 *Saccharomyces Cerevisiae* Genome – Genome Variation

The genome sequence of a laboratory strain of *S. cerevisiae* was released in 1996 (Goffeau et al. 1996). The yeast genome is quite small, at only 12 Mb. It is highly packed, with about 6,000 genes distributed over 16 chromosomes. *S. cerevisiae* also has two small cytoplasmic genomes: mitochondrial DNA and killer dsRNA. We will first focus here on the chromosomal genome and its variation in wine strains.

20.4.1 The Ploidy of Wine Yeast

Early genetic studies on wine yeasts indicated that most strains were diploid though some were polyploid or aneuploid (Cummings and Fogel 1978; Thornton and Eschenbruch 1976; Takahashi 1978; Bakalinsky and Snow 1990). Various genetic data and DNA analyses have suggested that aneuploidy was common in flor yeasts (Martinez et al. 1995; Guijo et al. 1997). More accurate information about yeast aneuploidy was recently obtained through CGH analysis, with Infante et al. (2003) confirming that flor yeasts are aneuploid for several chromosomes. Unexpectedly, a similar karyotyping of commercial "fermentation" strains revealed no whole-chromosome aneuploidy (Dunn et al. 2005). This discrepancy suggests that the aberrant segregation of genetic markers is frequent in wine strains and that the abundance of aneuploidy may have been overestimated from genetic analysis. An estimation of the DNA content of a large set of commercial "fermentation" strains recently showed that most of these strains had a DNA content close to 2 n (Bradbury et al. 2005). Moreover, Legras et al. (2007) recently reported that 88% of the *S. cerevisiae* strains had allele patterns consistent with a diploid state. Unlike other industrial yeasts (baker's yeast and brewing yeast strains), which have ploidy levels exceeding 2 n, most of the *Saccharomyces cerevisiae* strains used in wine-making

seem to be diploid. Flor yeasts have features closer to other industrial yeasts, with more common aneuploidy and polyploidy certainly in relation to the occupancy of a specific ecological niche.

20.4.2 Chromosomal Rearrangements

The existence of gross chromosomal rearrangements — translocations, deletions and amplifications of chromosomal regions — was rapidly suspected based on the high level of chromosome polymorphism found in wine yeasts. Analysis of wine yeast chromosomes by pulsed-field gel electrophoresis (PFGE) demonstrates major chromosome length polymorphism between wine yeast strains (Vezinhet et al. 1990; Yamamoto et al. 1991). Such variations in chromosome size clearly resulted from gross chromosomal rearrangements (GCR). Indeed, characterization of the chromosome structures of various wine strains has revealed the existence of translocations or deletions (Bidenne et al. 1992; Carro et al. 2003). Recombination between repeated Ty sequences interspersed throughout the genome is shown to be a major cause of chromosomal translocation (Rachidi et al. 1999; Codon et al. 1998). Other types of repeated sequences —, tRNA and telomeric Y' sequences - may also serve as substrates for ectopic recombinations leading to chromosomal rearrangements (Carro et al. 2003).

A broad view of the genomic constitution of wine yeasts has been provided by the CGH analysis of commercial strains reported by Dunn et al. (2005). This analysis reveals the existence of a set of deleted or amplified genes common to wine and other industrial yeasts, mostly corresponding to repeated genes (*Ty1, CUP, ASP3, ENA*). Some gene copy-number changes are specific to wine yeasts and have been identified as a possible wine yeast signature (Dunn et al. 2005). The differences between wine strains are moderate and mostly concern genes encoding membrane transporters. The genes amplified in wine yeasts are mostly located at the ends of chromosomes and belong to various functional categories, although several of these genes are involved in cell detoxication (Dunn et al. 2005). These data confirm the plasticity of subtelomeric regions in yeasts and their role in adaptation to industrial environments (Louis 1995).

One of the main conclusions of the CGH analysis is that wine yeasts are closely related. Indeed, "fermentation" strains do not contain the extensive amplifications of chromosomal regions observed in flor strains (Infante et al. 2003). It has been suggested that flor yeasts must deal with high acetaldehyde concentrations during wine aging, potentially inducing double strand breaks, the processing of which may favor GCR (Infante et al. 2003). The differences between the environments of fermentation and flor yeasts may therefore support different evolutionary processes. CGH analysis is subject to certain limitations, which must be taken into account. This approach cannot detect reciprocal translocations or account for the existence of genes other than those already identified in the sequenced laboratory strain.

The effects on yeast fitness of most of these rearrangements remain unclear. In flor yeasts, the amplified regions were shown to contain genes potentially useful for strain adaptation, such as *ADH*, which encodes alcohol dehydrogenase. This enzyme can detoxify the medium by removing acetaldehyde. In addition, a key gene for velum formation, *FLO11/MUC1,* encoding a cell wall mucin, is found amplified and has an altered expression due to a promoter modification (Infante et al. 2003; Fidalgo et al. 2006). The best studied case of contribution to adaptation is that of a translocation between chromosome VIII and XVI, which has a direct impact on sulfite resistance (Perez-Ortin et al. 2002). The *SSU1* gene encodes a plasma membrane protein that is thought to extrude sulfite anions and confers sulfite resistance (Park and Bakalinsky 2000). *SSU1* is located on chromosome XVI in the laboratory strain, but an allele conferring higher levels of sulfite resistance is found associated with a translocation onto chromosome VIII (Goto et al. 1998) (see Fig. 20.1). A survey of the translocation distribution shows that it is widespread in wine yeasts. This translocation is the only clear example identified to date in wine yeast in which a chromosomal rearrangement has been shown to be involved in adaptation to the wine environment and to be selected in response to a technological practice — extensive sulfite use.

The chromosomal structures of wine yeasts display significant levels of instability. Several authors have reported chromosome instability during vegetative growth, based on PFGE analysis (Longo and Vezinhet 1993; Puig et al. 2000; Carro and Piña. 2001). The effect of these rearrangements on strain performance remains unclear although no differences in fermentation properties are found between different structural variants (Longo and Vezinhet 1993). During laboratory storage or industrial propagation, wine yeasts may undergo minor chromosomal changes, as suggested by slight variations observed between strains from different manufacturers (Dunn et al. 2005).

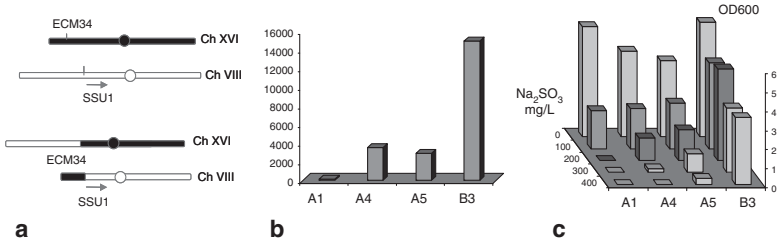

Fig. 20.1 Illustration of chromosomal translocation and its effect on *SSU1* expression and the sulfite resistance of wine yeast. (**a**) Schematic representation of the translocated chromosomes VIII and XVI at the *SSU1* locus. (**b**) Expression level of *SSU1* (arbitrary units) in four strains carrying no Ch VIII–XVI translocation (strain A1), one translocation (A4, A5) or two translocations (B3). (**c**) Growth of the four strains with varying amount of sulfite

20.4.3 Microsatellites and Snps

Microsatellites are direct repetitions of two- to six-base nucleotides displaying variation in the number of repeats. Microsatellite expansion and contraction lead to polymorphic sites, which can be used for strain typing. Highly polymorphic loci have been identified, and microsatellite typing has been shown to be an efficient tool for discriminating between wine strains (Perez et al. 2001; Legras et al. 2005). Microsatellite-based approaches have proven highly relevant for population genetic studies of wine yeasts (Legras et al. 2007). DNA sequence variation corresponding to single nucleotide polymorphisms (SNPs) or insertion/deletions (indels) has been inferred from gene sequence comparisons and oligonucleotide array hybridizations (Winzeler et al. 2003). DNA sequence analysis of selected loci (multilocus sequence typing, (MLST)) has been used to asses the genealogical relationships between wild and industrial *S. cerevisiae* strains in population-based studies (Fay and Benavides 2005). Overall DNA divergence between strains was estimated at about 0.42% (bp per 100 bp) for industrial strains and at 0.14% for wild strains isolated from grapes, suggesting lower diversity in the wild strains. In addition, the complete genomes sequencing of various *S. cerevisiae* strains, including some vineyard isolates, has either been completed or is underway, and this should provide more accurate data concerning DNA sequence variation (see Sect. 20.5).

In a few cases, sequence polymorphisms have been shown to influence phenotypes of industrial relevance. Using a QTL approach Marullo et al. (2007) identified a single nucleotide polymorphism which affects phenolic off-flavour formation by wine yeast. Guillaume et al. (2007) have shown that mutations in an hexose carrier were responsible of an enhanced capacity to ferment fructose. Single nucleotide polymorphisms may also alter gene expression and account for most expression variation between strains. Difference in gene expression between strains have been reported including under conditions of alcoholic fermentation (Zuzuarregui et al. 2006). Expression variation are thought to contribute to phenotypic diversity (Landry et al. 2006). Indeed wine *S. cerevisiae* display strong expression difference compared to laboratory strains. In such a comparison we observed that many cellular functions were concerned including carbon metabolism and stress response (Blondin, unpublished data, see Fig. 20.2). Little is known about the polymorphisms responsible of such expression variations. The search for eQTL has proven to be a relevant approaches to link genetic polymorphism to regulatory variation as demonstrated by Yvert et al. (2003).

20.4.4 Mitochondrial Genome

S. cerevisiae has a small (75 kb), circular mitochondrial DNA genome. The mitochondial genome encodes a small set of proteins involved principally in respiration (Chen et al. 2000). Mitochondrial DNA is not essential for *S. cerevisiae* survival. The mitochondrial genome has often been overlooked in yeasts used for industrial

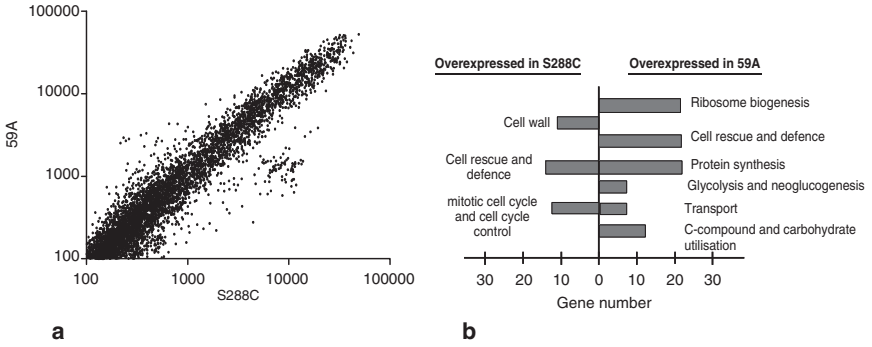

Fig. 20.2 Compares gene expression between a wine-derived haploid clone (59A) and a standard laboratory strain (S288C) under conditions of alcoholic fermentation (50% of fermentation progress). (**a**) scatter plot of whole genome expression. (**b**) MIPS functional categories over represented in the set of genes over expressed in 59A and S288C strains. More than 350 genes are found differentially expressed between the two strains (Ambroset, personal communication)

fermentations during which respiration is not required. However, there is evidence to suggest a complex interplay between mitochondria and other cellular functions (Chen et al. 2000). It was observed that the ethanol resistance of yeasts depended on mitochondria and that the ethanol tolerance of a laboratory strain could be enhanced by introducing mitochondria from a flor yeast (Jiménez and Benitez 1988; Ibeas and Jimenez 1997). Mitochondrial DNA has been shown to display a strong polymorphism in wine yeasts, and particularly in flor yeasts, in which it is thought to reflect strain adaptation. Indeed, this polymorphism has been exploited as a target for wine yeast typing (Vezinhet et al. 1990; Martinez et al. 1995).

20.5 Lessons from Yeast Sequencing

In 1996, the budding yeast *S. cerevisiae* became the first eukaryotic organism to have its genome completely sequenced (Goffeau et al. 1996). The strain sequenced, S288C, is a commonly used laboratory strain that was obtained in the 1950s by mating a strain isolated from a rotten fig (EM93) with a commercial strain (Mortimer and Johnston 1986). While experimental conditions may have left a significant footprint on the evolution of S288C (Gu et al. 2005), since 1996, the S288C genome sequence has been the only reference sequence available for *S. cerevisiae*. Today the genomes of several other yeast strains have been sequenced, including that of RM11–1a, a haploid derivative of a natural vineyard isolate, (http://www.broad.mit.edu/annotation/genome/saccharomyces_cerevisiae/Home.html), the clinical isolate YJM789 (Wei et al. 2007), and the diploid, heterozygous wine yeast strain EC1118 widely used as starter

in the wine industry (http://www.cns.fr/externe/English/Projets/Projet_RA/organisme_RA.html). The sequence divergence between these strains and the reference strain has been estimated at 0.5–1%, similar to that between humans and chimpanzees.

The sequence of YJM789 has been extensively compared with that of S288C (Wei et al. 2007). Some chromosomal rearrangement events have been identified in the genome of YJM789 relative to S288c. Several ORFs with sequences are entirely absent from S288C; on the other hand, several genes that are missing compared to the genome of S288C have also been identified. A divergence level of 0.6% was observed between YJM789 and S288C (Wei et al. 2007). Sequence information also allowed to correct or clarify some previous findings. In particular, several genes predicted to be absent by CGH approaches were in fact present but highly divergent. *PDR5* is such a polymorphic gene, which contains more than 250 SNPs compared to the S288C allele. The rate of substitution polymorphisms with respect to the reference genome was unequally distributed between and across the chromosomes. Many orthologs between YJM789 and S288c contain nucleotide polymorphism affecting the sequence or length of the gene product. For example, the *AMN1* gene, which is responsible for yielding non-clumpy cells has an inactivated mis-sensed polymorphism in S288C (Yvert et al. 2003) whereas the corresponding gene in YJM789 and in RM11–1a, which are more clumpy than S288C, lacks this. Similarly, *FLO8*, a transcription factor involved in pseudohyphal formation has an inativating amber mutation in S288C but not in the pseudohyphae forming YJM789 strain.

Recently, analysis of the EC1118 genome (Dequin et al., unpublished data) has revealed a sequence divergence relative to S288C or RM11 of 0.5 or 0.25% respectively. It has been estimated that there is less than 0.1% heterozygosity between EC1118 haplotypes, a value in the range of the variation observed between human beings (0.1–0.01%). About 50,000 SNPs and only a moderate number of indels (5,000) with respect to the S288c genome have been identified in the genome of EC1118, suggesting that, as observed for YJM789, SNPs might be a primary cause of heritable phenotypic variation between strains. The genome of EC1118 has, in addition, remarkable peculiarities. Interestingly, the genome of this strain contained entirely new regions carrying several genes involved in metabolic and transport functions The study of the distribution of these fragments within the *S. cerevisiae* species, as well as their potential contribution to the adaptation of yeast to wine environment is under way.

Several large-scale initiatives aiming at sequencing genomes of many *S. cerevisiae* isolates have been recently conducted. The *Saccharomyces* Genome Resequencing Project (SGRP) has completed the sequencing of 37 S. *cerevisiae* haploids strains with a coverage of between 1× and 3× http://www.sanger.ac.uk/Teams/Team71/durbin/sgrp/index.shtml. A similar project is ongoing at the Genome Sequencing Center, Washington University in Saint-Louis (USA). The amount of genomic information generated through these initiatives will undoubtedly facilitate the identification of sequence changes potentially involved in the adaptation of strains to different environments.

20.6 Hybridization Between Species of the Genus *Saccharomyces*

Wine fermentations were initially developed by primitive agricultural societies, which unwittingly selected *Saccharomyces* wine strains based on their ability to ferment substrates with high sugar contents, and to adapt to fermentation conditions predominant in each region. Thus, yeast strains from the Mediterranean region tolerate high sugar and alcohol contents and high fermentation temperatures, whereas, strains from areas of oceanic or continental climate are better adapted to lower sugar and alcohol contents, and lower fermentation temperatures.

Saccharomyces genus yeasts have a very interesting mechanism for adapting to industrial processes: the generation of inter-specific hybrids. Inter-specific hybridization generates new combinations of genes of potential adaptive value, conferring, on hybrids, selective advantages over their parental species under fluctuating or intermediate environmental conditions (Masneuf et al. 1998; Greig et al. 2002).

20.6.1 Hybrids Between S. bayanus and S. cerevisiae

The best described examples of natural hybrid yeasts are those of lager brewing yeasts from the taxon *S. pastorianus* (syn. *S. carlsbergensis*). These yeasts are partial allotetraploids, generated by a hybridization event between *S. cerevisiae* and a *S. bayanus*-related yeast (Hansen and Kielland-Brandt 1994). *S. bayanus* x *S. cerevisiae* hybrids have also been found in wine fermentations (Masneuf et al. 1998; González et al. 2006) but have been analyzed in less detail than their counterparts in lager-making.

The transfer of chromosomes from lager strains to a laboratory *S. cerevisiae* strain and the subsequent analysis of meiotic crossing over and restriction analysis of cloned genes (reviewed in Kodama et al. 2005), show that lager yeasts contain three types of chromosomes: (1) homologous chromosomes (*S. cerevisiae*-like type); (2) homeologous chromosomes (*S. bayanus*-like type); and (3) mosaic, recombinant chromosomes composed of homologous and homeologous segments. The presence of mosaic chromosomes in lager hybrids was recently confirmed by comparative genome hybridization (CGH) (Bond et al. 2004; Kodama et al. 2005) and genome sequencing (Kodama et al. 2005).

The parental species *Saccharomyces bayanus* is a controversial taxon, containing diverse strains with different genetic and physiological traits (Rainieri et al. 2003). The type strain of this species, originally isolated from spoiled beer, was recently suggested to be a hybrid between *S. cerevisiae* and *S. bayanus* (Nguyen et al. 2000), based primarily on the presence of subtelomeric repeated sequences and genes (Nguyen and Gaillardin 2005). The suggested hybrid nature of this species led to the proposal that *S. uvarum*, a former taxon included in *S. bayanus*, be reinstated as a distinct species (Pulvirenti et al. 2000; Nguyen and Gaillardin 2005).

However, the presence of certain introgressive subtelomeric sequences is not necessarily indicative of a hybrid genome (Naumova et al. 2005).

20.6.2 Hybrids between S. cerevisiae and S. kudriavzevii

In two recent studies, new hybrids resulting from hybridization between *S. cerevisiae* and *S. kudriavzevii* were described in wine (González et al. 2006; Bradbury et al. 2005; Lopandi 2007) and brewing (González et al. 2007) from three different Belgian Trappist beers, and from English, German and New Zealand ale beers.

The hybrid nature of some of these wine strains was unknown when they were originally identified by Schütz and Gafner (1994) as the predominant strains during spontaneous wine fermentations in the Zurich region of Switzerland; some were selected as commercial strains adapted to fermentation in cold areas of Central Europe (strains W27 and W46 from Lallemand Inc.). New hybrids also appear to predominate in some Austrian wine regions (Lopandi 2007), with others corresponding to commercial wine yeast strains selected for their interesting enological properties (Bradbury et al. 2005). González et al. (2006) also found a *S. uvarum* x *S. cerevisiae* x *S. kudriavzevii* hybrid strain in Switzerland. The sequencing and restriction analysis of gene regions on different chromosomes and comparative genome hybridization analysis with DNA chips showed that *Saccharomyces kudriavzevii* hybrids may also display differences in aneuploidy and chimeric chromosomes due to recombination between 'homeologous' chromosomes from different parental strains (see Fig. 20.3), (Belloch et al.- manuscript in preparation).

20.6.3 Interesting Properties of Hybrids

In subsequent studies, commercial *S. cerevisiae* x *S. kudriavzevii* hybrids (W27 and W46 from Lallemand Inc.) and commercial *S. uvarum* x *S. cerevisiae* hybrid (S6U, also from Lallemand Inc.) were enologically and physiologically characterized (González et al. 2006; Belloch et al. 2008). Their properties were compared with those of reference strains of three parental species: *S. uvarum*, *S. cerevisiae*, and the type strain of *S. kudriavzevii* IFO 1802- one of the two strains available for this species. This study concluded that these hybrids outperformed the parental species for wine-making purposes in several key aspects: (1) they produced significantly smaller amounts of acetic acid, a compound that reduces the quality of wines; (2) they produced larger amounts of glycerol, a favorable compound; and (3) they produced and released more flavors and aromas (González et al. 2007). These hybrid strains retained ethanol tolerance and ability to grow in media with a high sugar content of the *S. cerevisiae* parent, but clearly grew better at low temperatures, a trait inherited from the non *S. cerevisiae* parent (Belloch et al. 2008).

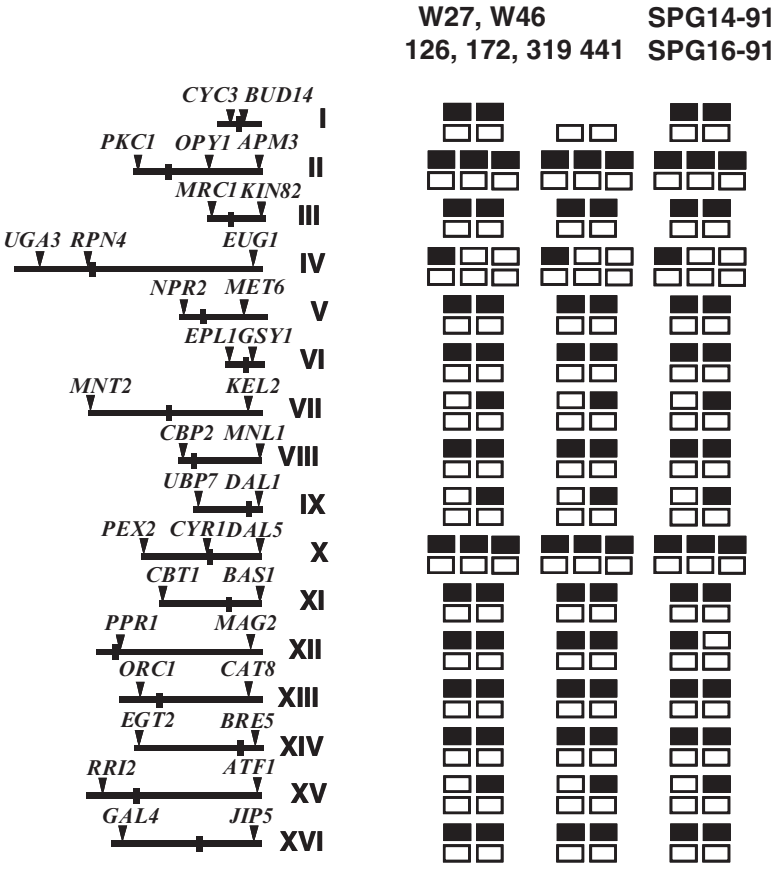

Fig. 20.3 Genotypes of the *S. cerevisiae* x *S. kudriavzevii* wine hybrids. Each square corresponds to a copy of each gene region according to its chromosome location indicated at the left. White and black squares represent alleles of *S. cerevisiae* and *S. kudriavzevii* origins, respectively. The presence or absence of alleles coming from each parent species is determined by restriction analysis of the 35 gene regions amplified by PCR with general primers. Adapted from González et al. (2008)

This characteristic is considered of great value due to the current trend for wineries to carry out wine fermentation at low temperatures to obtain fruity and aromatic wines. However, many selected *S. cerevisiae* strains are not well adapted to such low-temperature conditions, which may lead to inadequate yeast growth and slow or stuck fermentations.

Studies of natural hybrids present in fermentation processes have opened up a new strategy for the development of novel industrial yeasts – the generation of artificial hybrids. Producing hybrids between *S. cerevisiae* wine strains and strains of other species could be a useful strategy for obtaining yeasts with novel characteristics of enological interest.

20.7 Wine Yeast Population Diversity and History

The recent MLST study of Fay and Benavides (2005) and the Multi locus microsatellite study of Legras et al. (2007) have unveiled the impact of domestication on yeast genetic structure. The structure of these yeast populations is highly related to the technological origins of the strains, so their denomination "bread", "beer", "wine", "sake" and "palm wine yeast" is highly appropriate (Fig. 20.1). In addition, the wide distribution of some yeasts (beer, bread, cheese etc.) suggest migration with human activities. This process has been particularly spectacular for wine yeasts, with 95% of wine yeast strains isolated around the world belonging to the same cluster (Legras et al. 2007; Fig. 20.1). Geographical isolation explains 28% of this genetic structure and this also implies that *S. cerevisiae* has been domesticated in different areas around the world: Asia and Mesopotamia for wine, Africa for palm wine, and, probably, South America for *pulqu*e and *chicha* (Barrio 2008 personal communication) (Fig. 20.4).

Fst genetic distances calculated between different groups of strains characterized by MLMT (Legras et al. 2007) provide additional information. For wine, the basal position of a Lebanese group of strains suggests a migration from Mesopotamia, accompanying vine domestication consistent with known patterns of vine migration (Arroyo-Garcia et al. 2006). However, more strikingly, the substructure within the wine yeast cluster is also consistent with historical knowledge. The two main branches of the tree suggest two migration routes. As expected, a clustering of Mediterranean vineyards was observed, corresponding to well documented intensive exchanges around the Mediterranean Sea. The second branch suggests a second migration route along the Danube Valley. The position of French vineyard groups also suggests migration along the Rhone Valley towards Burgundy and Alsace or Nantes, and could be explained the transfer of vines: Ugni blanc, the main grape variety used in Cognac, originated from Italy and Muscadet (Nantes) and was imported from Burgundy during the fifteenth century (Viala and Vermorel 1901).

The estimates of the timing of wine yeast divergence of Fay and Benavides, (2005) and Legras et al. 2007 (2,700–27,000 and 4,500–32,000 years from the present, respectively) highlight the contemporary nature of wine yeast migrations. The most probable estimate suggests that the advent of wine making, corresponding to the oldest archaeological site displaying remains of wine technology, occurred around 10,000 years from now (Legras et al. 2007).

The extent of yeast domestication, as proposed by Martini (1993) or Fay and Benavides (2005), also requires consideration. The drive towards continuous growth of flor yeasts on the surface of wine during the sherry wine process is clearly an example of human control. These yeast strains possess highly specific traits, including gene amplification and mutation (Infante et al. 2003; Fidalgo et al. 2006).

However, for other types of wine strains, the continuous control of yeast populations by humans has not been clearly demonstrated, particularly as some debate remains concerning the origin of wine yeasts (Constanti et al. 1997; Le Jeune et al.

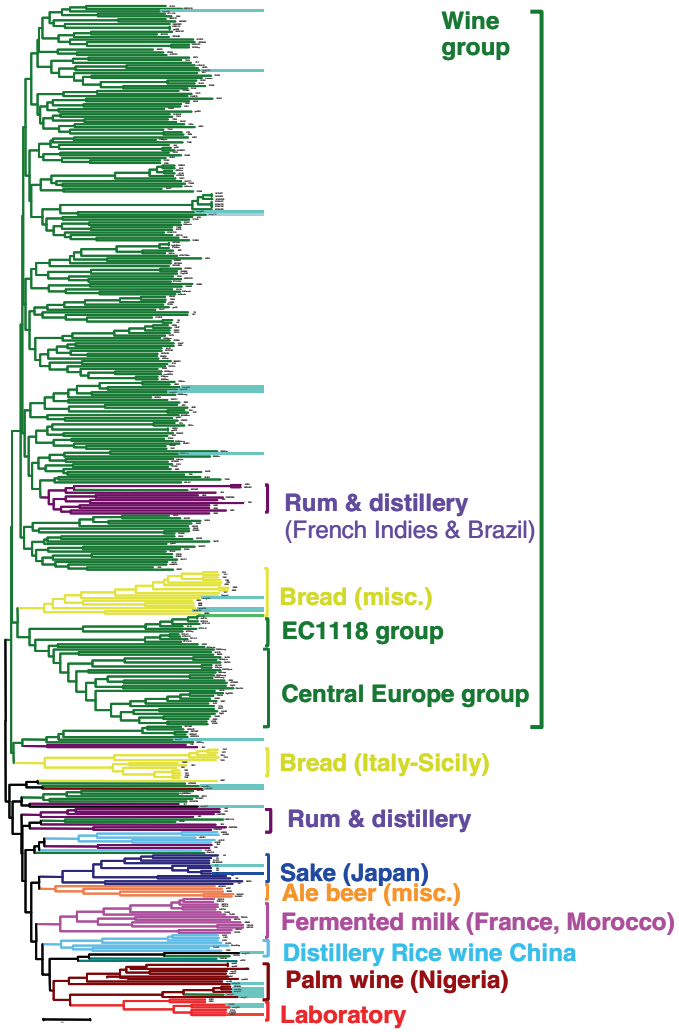

Fig. 20.4 Neighbor-joining tree showing clustering of 393 yeast strains isolated from different sources, modified from Legras et al. (2007). The tree is constructed from the Dc chord distance between strains based on the polymorphism at 12 loci and is rooted according to midpoint method. Branches are coloured according to substrate from which strains have been isolated. Sequences stains are highlighted – S288C in red, EC1118 in bright green, sake K7 in blue and strains from Sanger institute in light blue. Color code: Wine *dark green*; Cider *light green*; Bread *yellow*; Beer *orange*; fermented milk *pink*; Sake from Japan *dark blue*; Chinese, Rice wine and distillery from Vietnam and Thailand *light blue*; sorghum beer or palm wine from Africa *brown*; Oak tree *blue-green* from America; distillery from South America and rum from French Indies *purple*; Laboratory strains *red*

2006). However, the specific karyotypic signature observed in micro-array CGH (Dunn et al. 2005) and the evolution of the *SSU1* gene conferring SO_2 resistance (Perez-Ortin et al. 2002) demonstrate the adaptation of yeast to vine and winery environments.

Thus, man have made a major contribution to the expansion and evolution of this yeast species, selecting *Saccharomyces cerevisiae* stains or hybrids and spreading strains adapted to human technology. Conversely, modern yeast genomes contain footprints of human history.

20.8 Conclusions and Future Prospects

Characterization of wine yeast genome has highlighted differences between wine and laboratory strains and revealed high level of genomic diversity among wine strains. However, it has also become clear that genomic data obtained with the S288C strain, represented a relevant blueprint for more detailed exploration of the architecture of wine yeast genome. The main limitation is the existence of hybrid strains/genomes, the analysis of which requires information for other genomes. It also remains unclear whether wine yeasts harbor genes absent from standard laboratory yeast. The knowledge accumulated for wine yeast genomes indicates that the genomic diversity of wine yeasts results from exploitation of the many possible mechanisms underlying genome plasticity in yeast (Barrio et al. 2006). The evolution and adaptation of *S. cerevisiae* to stressful wine environment has clearly involved combinations of different mechanisms acting at different genome scales, with large or local effects.

Knowledge of wine yeast genome is clearly entering a new phase, given the availability of new genome scanning technologies, such as DNA chip-based analysis or massive sequencing techniques. This will probably soon lead to massive influx of data, shedding new light on specific features of wine yeast genome. The first complete genome of a commercial diploid strain will soon be released (see Sect. 20.5). We can foresee a future in which whole-genome sequence data or similar information (e.g. whole-genome SNP distribution) will be available for many wine strains. Use of this information may become the gold standard for genetic work with wine yeasts and for addressing the genetic basis of phenotypic diversity.

Bridging the gap between genome variation and phenotypic diversity will undoubtedly be a major challenge for wine yeast geneticists. Knowledge concerning the precise role of each of the 5,700 yeast genes in wine alcoholic fermentation will clearly be useful. Most of the available information concerning *S. cerevisiae* genes was obtained under laboratory conditions, without taking into account the specific physiological situation faced by yeasts in wine-making. Various functional genomic approaches have been applied to wine yeast to specify the role of genes playing a key role in alcoholic fermentation conditions, such as the *ALD* or *HXT* genes (Saint-Prix et al. 2004; Luyten et al. 2002). Genome-wide approaches have been used to investigate gene expression patterns under enologically relevant situa-

tions (Backhus et al. 2001; Rossignol et al. 2003; Beltran et al. 2006; Zuzuarregui et al. 2006). Such global approach will help to specify key targets of wine yeast adaptation. The understanding of genetic bases of yeast phenotypic diversity will require a combination of approaches. Methods of quantitative genetic and QTL analysis have proved efficient to investigate the genetic basis of wine yeast traits (Marullo et al. 2007). Such approaches should help identification of polymorphisms affecting technological or sensorial properties of yeasts which will be a key objective of wine yeast genome mining.

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Chapter 21
The Genome of Acetic Acid Bacteria

Armin Ehrenreich

21.1 Introduction

Acetic acid bacteria are Gram-negative, rod-shaped, and acido-tolerant to acidophilic organisms with a strictly aerobic type of metabolism. These fastidious organisms thrive in complex, nutrient-rich environments of high osmolarity such as honey bees, on the surface of fruits, in nectar, plant saps, fruit juices, cider, must, wine, sake, beer or other sweet or alcoholic beverages (Lambert et al. 1981; De Ley et al. 1984; Gupta et al. 2001). Some genera can grow in highly concentrated sugar solutions of up to 30% (w/v) glucose and at pH values as low as 2.5. The most prominent physiologic feature of acetic acid bacteria is that they derive their energy from incomplete oxidations of a multitude of substrates, mainly alcohols, sugars and other polyols. The most important of these oxidations with respect to oenology are the oxidation of ethanol to acetate and that of glucose to gluconic acid. As most of their oxidation products are acids, and as acetic acid bacteria rapidly convert large amounts of substrate, they quickly acidify their environment during growth. This inhibits growth of many other bacteria and gives them a selective advantage as they can tolerate low pH values. Another oenologically important aspect is their tendency to form biofilms. A remarkable example of these biofilms is the Mother of Vinegar. This is a massive gelatinous or leathery layer that develops on the surface of nutrient rich, unstirred alcoholic solutions such as wine during prolonged aerobic incubation that transforms wine to vinegar by oxidizing ethanol to acetic acid. The matrix of this biofilm consists of cellulose fibrilli that are synthesized by *Gluconacetobacter xylinum* (Wong et al. 1990; Ross et al. 1991). Besides cellulose fibrillae, a number of other exopolysaccharides are formed by acetic acid bacteria, such as levans or acidic polysaccharide acetan. Some strains of Gluconobacter show viscous growth in beer due to formation of dextrans.

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even degrade sugars to acetic acid but instead, preserve the carbon skeleton of their substrates and perform only a few stereo- and regioselective oxidation steps with dehydrogenases located in the cell membrane with their active site facing towards the periplasm. This architecture has the advantage that the substrates and products need not be transported across the cell membrane as they can enter or leave the periplasmic space by diffusion. A multitude of such dehydrogenase activities located in the cytoplasma membrane of acetic acid bacteria have been described in literature. Purified enzymes are either flavoproteins containing covalently bound FAD or quinoproteins containing pyrroloquinoline quinone (abbreviated PQQ) as cofactor (Matsushita et al. 1994; Adachi et al. 2003; Davidson 2004). Although a large number of membrane-bound dehydrogenases are characteristic of acetic acid bacteria, these enzymes are not confined to this group of organisms. Several pseudomonads and even *E. coli* also contain a PQQ-dependent glucose dehydrogenase in their cytoplasmic membrane (Toyama et al. 2004). But in contrast to acetic acid bacteria, *E. coli* is only able to synthesize the apoenzyme and needs to take up the PQQ from the environment to form an active enzyme (Neijssel 1987).

Acetic acid bacteria carry out oxidation of ethanol to acetate in two consecutive steps using membrane-bound quinoproteins – ethanol dehydrogenase and acetaldehyde dehydrogenase. This ethanol oxidation occurs in all acetic acid bacteria except the genus *Asaia*. Besides ethanol, a large number of alcohols, sugars and other polyols are oxidized to their corresponding acids or ketones in one or few steps by the action of various membrane-bound dehydrogenases. For example, glycerol is oxidized to dihydroxyacetone by the quinoprotein polyol dehydrogenase or D-sorbitol is oxidized to L-sorbose by a D-sorbitol dehydrogenase (Deppenmeier et al. 2002; Adachi et al. 2003). The occurrence of different dehydrogenases is species or even strain-specific and their classification based on existing literature is difficult, because at least some of them have a broad substrate spectrum. In most studies enzymes have been purified and characterized only according to their activities, while the corresponding genes have not been cloned and sequenced. As subunit composition and molecular weights are often similar, the number of different enzymes that are responsible for observed activities is not known. For example alcohol- and aldehyde dehydrogenase of acetic acid bacteria oxidize many straight and branched chain alcohols and aldehydes to their corresponding carboxylic acids (Sievers and Swings 2005) or the polyol dehydrogenase is able to oxidize glycerol, D-arabitol, D-sorbitol and many other sugar alcohols (Matsushita et al. 2003).

Many oxidations carried out by membrane-dependent enzymes can be described empirically according to the Bertrand-Hudson rule, which states that polyols with *cis*-arranged secondary hydroxyl groups in D-configuration to the adjacent primary alcohol (D-*erythro* configuration) group are oxidized to corresponding ketoses.

As compared to complete oxidations, these incomplete oxidations extract only few electrons per mole of substrate. Therefore acetic acid bacteria have to convert large amounts of substrates to produce little biomass. This fact makes these organisms interesting for industrial applications because acetic acid bacteria couple distinct stereo- and regioselective oxidation reactions of many substrates to reduction of oxygen and only little substrate is lost for biomass formation (Deppenmeier et al.

2002). From this perspective they can be seen as living oxidative catalysts. This is the reason for employment of acetic acid bacteria in many biotechnological processes such as the production of vitamin C, gluconic acid, dihydroxyacetone and many others (Macauley et al. 2001).

In addition to the set of membrane-bound dehydrogenases catalyzing irreversible oxidations, a second set of dehydrogenases, using NAD(P) as cofactor, is located in the cytoplasm. These soluble enzymes convert similar or even same substrates as their membrane-bound counterparts in reversible reactions. For example, a soluble, NADP dependent alcohol dehydrogenase, a glucose dehydrogenase and a gluconat dehydrogenase have been isolated and characterized (Deppenmeier et al. 2002). The physiological role of this second set of dehydrogenases in metabolism is not yet clear but it is assumed that soluble, NAD(P)-dependent enzymes contribute only little to the overall oxidation of the substrates. For example, a membrane-bound quinoprotein glucose dehydrogenase has been shown to be 30 times more active than a NADP-dependent soluble glucose dehydrogenase (Pronk et al. 1989).

21.4 Overview on the Genome of *Gluconobacter oxydans* 621H

Despite their great relevance for biotechnology, only one genome of an acetic acid bacterium has currently been published. Additionally the unpublished genome sequence of *Gluconacetobacter diazotrophicus* PAL5 is available in genome databases. The published genome from *G. oxydans* strain 621H allows reconstruction of the unique metabolism of this ketogenic acetic acid bacterium that is adapted to growth in concentrated sugar solutions (Prust et al. 2005).

The complete genome has a size of 2.9 Mb and consists of a circular chromosome of 2.7 Mb and 5 plasmids of 163.1-, 26.6-, 14.6-, 13.2- and 2.7-kb size. It contains 2,601 predicted protein-encoding open reading frames, 55 tRNA genes and 4 copies of rRNA operons (Prust et al. 2005). 89.9% of the DNA codes for proteins or stable RNAs.

A prominent genetic feature is the high number of insertion sequences and transposases found in the genome: a total of 82 insertion sequences and 98 transposases together with two genome regions potentially representing inserted prophages could be recognized. According to the classification of Mahillon and Chandler, ten copies of insertion sequences can be attributed to the family IS12528 and eight copies to IS1032 (Mahillon and Chandler 1998). Although several of the insertion sequences seem to be defective, the functional copies may be responsible for the marked genetic and, as a consequence, physiological instability that has been observed with several acetic acid bacteria (Kondo and Horinouchi 1997a, c).

The 5 plasmids that are present in the strain *G. oxydans* 621H are cryptic. The genome sequence has not helped much in deciphering their role as around 70% of the open reading frames located on them, code for hypothetical proteins of currently unknown function based on sequence homologies. Among other things, open reading frames with suggested functions code for putative proteins of plasmid replication, a DNA helicase II (*umuD*), a restriction and modification system,

21.2 Acetic Acid Bacteria in Oenology

Production of vinegar from wine was documented as early as 4000 B.C. (Deppenmeier et al. 2002). The English word 'vinegar' derives from the old French 'vin aigre', meaning 'sour wine'. A diluted sour wine with modest acetic acid concentration has been used at all times as a drink in Mediterranean countries on account of their warm climate. The sensory threshold of acetic acid in wine is around 700 mg L^{-1}; concentrations larger than 1.2–1.3 g L^{-1} are generally regarded as unpleasant (Dittrich 1987). Though a certain level of acetic acid is required in some wines to develop a more complex, 'desirable' taste. Acetic acid bacteria are also important for the development of a characteristic aroma in several other foodstuffs such as Kombucha. The characteristic sweet and sour flavor results from ethanol, acetate and gluconic acid produced by yeasts, acetic acid bacteria and lactic acid bacteria (Chen and Liu 2000; Greenwalt et al. 2000).

Formation of stronger vinegar taste is seen as a major and irreversible wine fault and acetic acid bacteria are the major source for this spoilage.

The acetic acid is either formed in the must or later if oxygen is available (Bartowsky and Henschke 2008). As acetic acid bacteria can be ubiquitous on the surface of grapes, they are inoculated in high numbers in the must where they can proliferate. They are responsible for rapid acidification during the initial stages of fermentation (Drysdale and Fleet 1988). Under prolonged sticky weather in autumn, acetic acid bacteria can even form acetic acid at the grape by infecting the sap through tiny injuries. These injuries can be caused by insects or by *Botrytis* infections. As fungus breaks through the skin of the grape, tiny droplets of sap can leak from the berry providing substrate for the bacteria (Barbe et al. 2001).

If acetic acid bacteria develop in the must, increased amounts of acetic acid, gluconic acid, keto-gluconic acids as well as dihydroxyacetone are formed, although the latter is usually reduced again during fermentation by yeasts (Dittrich 1987). The produced carbonyl compounds account for an increased SO_2 binding capacity of the must (Barbe et al. 2001).

As the must contains high concentrations of sugar and comparatively little ethanol, these are especially favorable conditions for members of the 'suboxydans' group of acetic acid bacteria. In contrast to must, wine contains only small amounts of sugar and high concentrations of ethanol. This results in the prevalence of acetic acid bacteria of the 'peroxydans' group whenever wine is stored with exposure to air (Joyeux et al. 1984; Bartowsky et al. 2003).

Their obligate oxygen dependence alleviates control of acetic acid bacteria. As preparation of red wine usually requires more intense mechanical processing of the mash as compared to white wines, more oxygen is available to the acetic acid bacteria. This manifests itself in higher concentrations of acetic acid in many red wines. The concentration of acetic acid in wines is commonly measured in the form of volatile acidity. The accepted critical value of volatile acidity is 1.6 g L^{-1} for red wines as opposed to 1.2 g L^{-1} for white wines (Dittrich 1987).

Ethylacetate is another important wine fault that has been attributed to acetic acid bacteria, although wine spoilage yeasts or lactic acid bacteria seem to be the more

important elicitors (Rojas et al. 2003). Transcription of an esterase which seems to be responsible for ethylacetate formation in *Acetobacter pasteurianus* is induced by ethanol (Kashima et al. 1999). Ethylacetate does not contribute to volatile acidity and is formed by esterification of acetate with ethanol. Wines with a high level of acetic acid are therefore more likely to suffer from ethylacetate formation. While at low concentrations ethylacetate contributes to richness and sweetness of wine, above a sensory threshold of 150–200 mg L^{-1} it produces a 'nail polish remover' aroma. In vinegars the esters weaken the strong smell of acetic acid (Kashima et al. 1999).

Besides causing off-flavors in wine and other alcoholic beverages such as beer, acetic acid bacteria are generally regarded as harmless and are not pathogenic to humans or animals (Gupta et al. 2001). They have only sporadically been ascribed to induce bacterial rot of apples, pears or other fruits. These infections are accompanied by different shades of browning. Sometimes they also cause spoilage of canned pineapples. After heating in the canning process, the diseased tissue turns pink to brown due to the presence of 2,5-diketogluconic acid (Cho et al. 1980).

21.3 Overview on Acetic Acid Bacteria

Traditionally, acetic acid bacteria have been taxonomically divided into the 'suboxydans'- and the 'peroxydans' group (Leisinger 1965). These two groups are physiologically defined with respect to their further utilization of the acetate formed from oxidation of ethanol: Members of the 'suboxydans' group, represented by the genus *Gluconobacter*, show strong ketogenesis from polyols and prefer habitats rich in sugar (De Ley et al. 1984; Gupta et al. 2001). They are not capable of complete acetate oxidation to CO_2 because they lack a full TCA cycle as well as the enzymes needed for a glyoxylic acid shunt. While *Gluconobacter* is able to oxidize glucose in the must with a high yield of gluconic acid, members of this genus are not very active in forming acetic acid or ethylacetate.

In contrast to the 'suboxydans' group, members of the 'peroxydans' group prefer alcohol-enriched niches and are capable of slowly oxidizing acetate or lactate completely to CO_2 after depletion of primary carbon sources (De Ley et al. 1984). While *Gluconobacter* can be found during the early stages of cider manufacture, *Acetobacter aceti* and *Acetobacter pasteurianus* as members of the 'peroxydans' group are usually isolated in later stages (Passmore and Carr 1975). Despite their ability to oxidize acetate completely, also termed 'over-oxidation', organisms of the 'peroxydans' group seem to be mainly responsible for vinegar formation. The capability for over-oxidation requires a complete TCA cycle as well as a glyoxylic acid shunt.

Traditionally all acetic acid bacteria of the 'peroxydans' group are attributed to the genus *Acetobacter* (Leisinger 1965). Later, the genus has been split into the genera *Acetobacter* and *Gluconacetobacter*. Many new genera have also been added to this group over time (Yamada and Yukphan 2008).

While incomplete oxidation is a common phenomenon in many microorganisms, acetic acid bacteria are highly specialized in this kind of metabolism. They do not

a heavy metal resistance system, a conjugation system as well as for a C_4-dicarboxylate transporter and two alcohol dehydrogenases of unknown substrate specificity (Prust et al. 2005). The occurrence of numerous plasmids in *Gluconobacter* or other acetic acid bacteria has already been reported in several studies. Some of the plasmids contain identified dehydrogenase- or antibiotic resistance genes (Verma et al. 1994; Krahulec et al. 2003), but most of them are cryptic plasmids with no obvious function (Valla et al. 1987; Beppu 1993; Fomenkov et al. 1995; Trcek et al. 2000).

21.5 The Membrane Bound and the Soluble Dehydrogenases

Using bioinformatic tools, more than 75 genes coding for potential oxidoreductases, mainly with unknown substrate specificity, were identified on the genome of *G. oxydans*. Fifteen of them are predicted to be located in the cytoplasmic membrane. This illustrates that a large amount of oxidative potential of this organism is still unknown (Prust 2004).

The genes of three already biochemically characterized membrane-bound quinoprotein dehydrogenases have been identified: The quinohemoprotein alcohol dehydrogenase complex, the major polyol dehydrogenase and the glucose dehydrogenase. In addition there are four genes with high homology to quinoprotein dehydrogenases that were not previously known. One is predicted to be a periplasmic enzyme while other three can be assumed, due to sequence homology, to be located in the cytoplasmic membrane. Their C terminus, containing the PQQ binding domain faces towards the periplasmic space (Prust 2004). One of these enzymes has recently been suggested to be a quinoprotein *myo*-inositol dehydrogenase, as a deletion mutant is no longer able to grow on *myo*-inositol as sole energy source (Hölscher and Görisch 2007).

The quinohemoprotein alcohol dehydrogenase complex present in the genome is exclusively known from acetic acid bacteria (Reid and Fewson 1994; Toyama et al. 2004) and is composed of three subunits in *G. oxydans* as well as in *Acetobacter pasteurianus* (Kondo and Horinouchi 1997b). The larger subunit is the catalytically active quinohemoprotein that binds the PQQ cofactor, contains a heme *c* and requires Ca^{2+} (Anthony 1996). The second largest subunit, coded adjacent to the large subunit, is the triheme cytochrome *c* that transfers the electrons from the quinoprotein to the ubiquinone pool in the membrane. Therefore it connects ethanol oxidation to the electron transport chain. The smallest subunit, encoded in a distant genome location, is of uncertain function. It has been suggested, that it might be a kind of chaperone that helps to keep the complex in correct conformation (Kondo et al. 1995). The subunits of purified enzyme complexes have molecular masses of 83, 51 and 15 kDa in *G. oxydans* as deduced from the genome sequence (Prust 2004) and 78, 48 and 20 kDa in *A. pasteurianus* as estimated by SDS–PAGE.

Acetic acid bacteria form acetic acid from ethanol in two consecutive steps. The acetaldehyde resulting from the alcohol dehydogenase complex is oxidized to acetate by aldehyde dehydrogenase. The aldehyde dehydrogenase complex of

G. oxydans 621H is composed of three subunits of 83-, 52- and 17-kDa size while the subunits of the aldehyde dehydrogenase complex from *A. polyoxygenes*, an industrial vinegar producer, have molecular weights of 78, 45 and 14 kDa (Tamaki et al. 1989; Matsushita et al. 1994). Although these enzyme systems which carry out the formation of acetic acid in vinegar fermentation, are present in *Gluconobacter,* they are more active in members of the genera *Acetobacter* and *Gluconacetobacter*. These genera seem to play a much more important role in vinegar formation than *Gluconobacter* that is more specialized in the direct oxidation of sugars and other polyols (Matsushita et al. 1994).

The major polyol dehydrogenase is a quinoprotein that oxidizes several polyols such as D-mannitol, D-sorbitol, glycerol, *meso*-erythritol, D-arabitol and also D-gluconic acid. It is composed of a large 79.6 kDa SldA and a small 13.7 kDa SldB subunit. The SldA subunit contains the active site with the PQQ-binding domain. SldB seems to be involved in processing or stabilizing SldA (Shinjoh et al. 2002b; Hoshino et al. 2003).

The quinoprotein glucose dehydrogenase catalyzes the oxidation of D-glucose to D-gluconolactone that spontaneously hydrolyzes to D-gluconic acid. The enzyme consists of a single subunit of 87 kDa and has an amino acid sequence that is 56% identical to the well-studied enzyme from *E. coli* (Yamada et al. 2003, Prust 2004).

A second group of membrane-bound dehydrogenases has flavin in the form of covalently bound FAD as cofactor. Examples are the gluconate-2-dehydrogenase (Elfari et al. 2005) and the sorbitol dehydrogenase (Adachi et al. 2003). *Gluconobacter* contains two types of membrane-bound sorbitol dehydrogenases (Toyama et al. 2005). Beside the major polyol dehydrogenase that has also been described as a PQQ containing sorbitol dehydrogenase (Shinjoh et al. 2002b), there is another enzyme with FAD and heme *c* as prosthetic groups coded by the *sldSLC* genes. It is composed of three subunits of 61.5, 52 and 22 kDa size. Both enzymes produce L-sorbose from D-sorbitol. In older studies, the product from D-sorbitol oxidation was described as D-fructose but a more recent study does not support this finding, at least for the thermotolerant strain *G. frateurii* THD32 (Toyama et al. 2005).

Besides the membrane-bound dehydrogenases there is an alternative set of soluble NAD(P) dependent enzymes in the cytoplasm for metabolizing sugars, sugar derivatives, polyols and alcohols in reversible reactions (Adachi et al. 2001; Prust et al. 2005). There are several soluble enzymes that catalyze oxidations of polyols or reduction of ketosugars (Klasen et al. 1995). Other soluble enzymes can oxidize ethanol, acetaldehyde or glucose. Like their membrane-bound counterparts, at least some of those enzymes seem to act on a broad substrate spectrum (Schweiger et al. 2007). The resulting products are phosphorylated and can be channeled into the intermediary metabolism. For example a NADPH-dependent sorbose reductase converts L-sorbose to D-sorbitol (Shibata et al. 2000; Shinjoh et al. 2002a; Soemphol et al. 2007). D-Sorbitol can then be oxidized by a sorbitol dehydrogenase to D-fructose (Parmentier et al. 2003) which in turn can be channeled into the pentose phosphate pathway. The physiological role of multitude of soluble enzymes is not yet clear. They might be responsible for assimilating a wide variety of sugars, alcohols or

their oxidation products and channel them into the intermediary metabolism, mainly the pentose phosphate cycle, in order to supply biosynthetic precursors. Alternatively it has been proposed that the soluble dehydrogenases might be involved in substrate degradation to provide energy for the maintenance of cells during stationary growth phase (Deppenmeier and Ehrenreich 2008).

21.6 Structure of the Respiratory Chain

All acetic acid bacteria, with the exception of *Acidomonas methanolica* when grown on methanol, are oxidase negative (Matsushita et al. 1992; Sievers and Swings 2005). This means that a cytochrome c oxidase is missing. Biochemical investigations identified ubiquinol oxidases of the cytochrome *o* type in *Gluconobacter*. A second, cyanide-insensitive alternative ubiquinol oxidase has also been detected. In summary, biochemical studies predicted a respiratory chain in *Gluconobacter* that consists of cytochrome *c* from the membrane bound dehydrogenases, ubiquinone and ubiquinol oxidase. In contrast *Acetobacter* and *Gluconacetobacter* have terminal ubiquinol oxidases of the cytochrome a_1, cytochrome *d* or cytochrome *o* type (Matsushita et al. 1994). *A. aceti* expresses cytochrome a_1 in shaking cultures and cytochrome *d* in static cultures (Matsushita et al. 1994).

Gluconobacter, *Gluconacetobacter* as well as *Acidomonas, Asaia,* and *Kozakia* mainly contain ubiquinones of the Q-10 type whereas *Acetobacter* uses ubiquinones of the Q-9 type (Yamada et al. 1997). The terminal oxidases are of the cytochrome a_1, cytochrome *d* or the cytochrome *o* type (Matsushita et al. 1994).

Data from the genome sequence derive a detailed picture of the components of the respiratory chain in *G. oxydans* 621H that is also summarized in Fig. 21.2. Numerous membrane-bound dehydrogenases transfer electrons through their cytochrome *c*-containing subunits or domains to ubiquinol. The organism also contains a non-proton-translocating NADH:ubiquinone oxidoreductase that allows feeding of electrons from NADH into the ubiquinol pool. In contrast to a proton-translocating NADH:ubiquinone oxidoreductase (complex I), this enzyme generates no proton gradient during its redox reaction. Two operons are identified that code for ubiquinol oxidases: bo_3 type and *bd* type. The bo_3 type enzyme has been studied biochemically and shown to generate a proton gradient (Matsushita et al. 1987). The *bd* type ubiquinol oxidase probably represents the observed cyanid-insensitive oxidoreductase that is produced at low pH values (Matsushita et al. 1989). There is also a ubiquinol:cytochrome *c* oxidoreductase (complex III) encoded in the genome. But its function is not yet clear as a cytochrome *c* oxidase that would be needed to reoxidize this enzyme is missing.

No genes are found in the genome of *G. oxydans* 621H that can be involved in any anaerobic respiration. This is in agreement with the obligate aerobic growth physiology of the organism. The proton motive force that is generated by the respiratory chain is used by an F_1F_0-type ATPase to generate ATP. The short electron transport chain seems to have a rather limited ability to conserve energy by proton

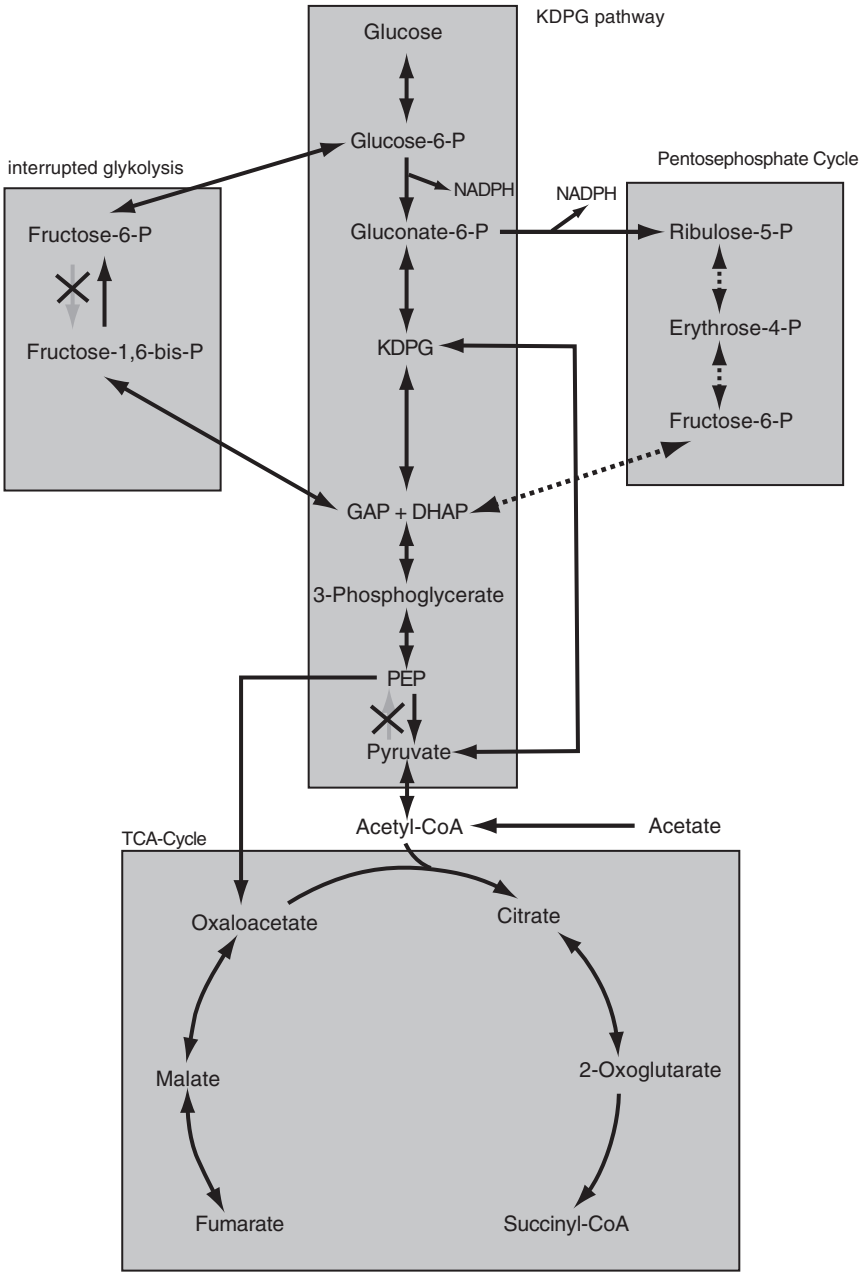

Fig. 21.1 Overview of central metabolism of *Gluconobacter oxydans* as it can be deduced from the genome sequence. An Entner–Doudoroff pathway is present as well as the pentosephosphate pathway. There is no glycolysis due to a missing phosphofructokinase. Also a gluconeogenesis from pyruvate is not possible because no PEP forming enzyme has been identified. The TCA cycle is not complete in this "suboxidizer" and can therefore only be used for providing biosynthetic precursors because the thiokinase and the succinate-dehydrogenase are absent. Beside the shown reactions, a multitude of soluble dehydrogenases are present that can channel many substrates as carbon sources into various points of the central metabolism

translocation during its redox reactions. But it can be assumed that the energy balance is better when considering that many oxidation products are acids. Additional protons are thus formed at the outer side of the cell membrane while proton consumption by oxygen reduction occurs at the inner side. Overall the respiratory system of Gluconobacter seems to be more adapted to fast oxidation of large amounts of substrate than efficient energy conservation.

21.7 The Central Metabolism

Classical biochemical investigations suggest that the pentosephosphate pathway is the most important route for carbon breakdown in acetic acid bacteria (Leisinger 1965). As a phosphofructokinase is missing, no glycolysis is present (Attwood et al. 1991). Cellulose-forming *Gluconacetobacter* strains have been found to posses a more active Entner-Doudoroff pathway than pentosephosphate pathway (White and Wang 1964a, b). The Entner-Doudoroff pathway was also demonstrated in *Gluconobacter* (Leisinger 1965; Kersters and De Ley 1968). All these points have been affirmed by the genome sequence of *G. oxydans* 621H. There is indeed no gene for a phosphofructokinase, and the complete sets of enzymes for the pentose phosphate cycle as well as for the Entner-Doudoroff pathway are present in the genome. Further investigations will have to clarify the physiological relevance of each of the two pathways for conversion of hexoses to pyruvate. On the basis of genome sequence it is supposed that phosphoglycerate kinase and pyruvate kinase are the only substrate-level phosphorylation reaction possible in *Gluconobacter* as an acetate kinase is missing.

Pyruvate is converted through the pyruvate dehydrogenase to acetyl-CoA, which can be fed into the incomplete TCA cycle. *G. oxydans* also contains a pyruvate decarboxylase that degrades pyruvate to acetaldehyde, which in turn can be further oxidized to acetate by a soluble acetaldehyde dehydrogenase. The significance of this reaction for overall acetate generation is not known.

From biochemical investigations it is known that *Acetobacter* and *Gluconacetobacter* have a complete TCA cycle and that a glyoxylate bypass is used during growth on ethanol containing media (Stouthamer et al. 1963). In contrast, *Gluconobacter* has an incomplete TCA cycle and no glyoxylate bypass. The genome sequence confirms that the succinate thiokinase as well as the succinate dehydrogenase are missing. As the TCA cycle is not closed, a putative PEP carboxylase supplies oxaloacetate as acceptor for acetyl-CoA (Prust 2004). The lack of a complete TCA cycle has profound consequences for the biology of this organism, because a complete oxidation of substrates degraded via acetyl-CoA is not possible. It has been proposed that complete oxidation can be done via repeated passage of the pentose phosphate cycle especially during stationary growth phase (Prust 2004). Overall it seems that the main physiologic role of the central metabolism as summarized in Fig. 21.1, is not substrate degradation but generation of building blocks for anabolism. In this context it is interesting that the *G. oxydans*

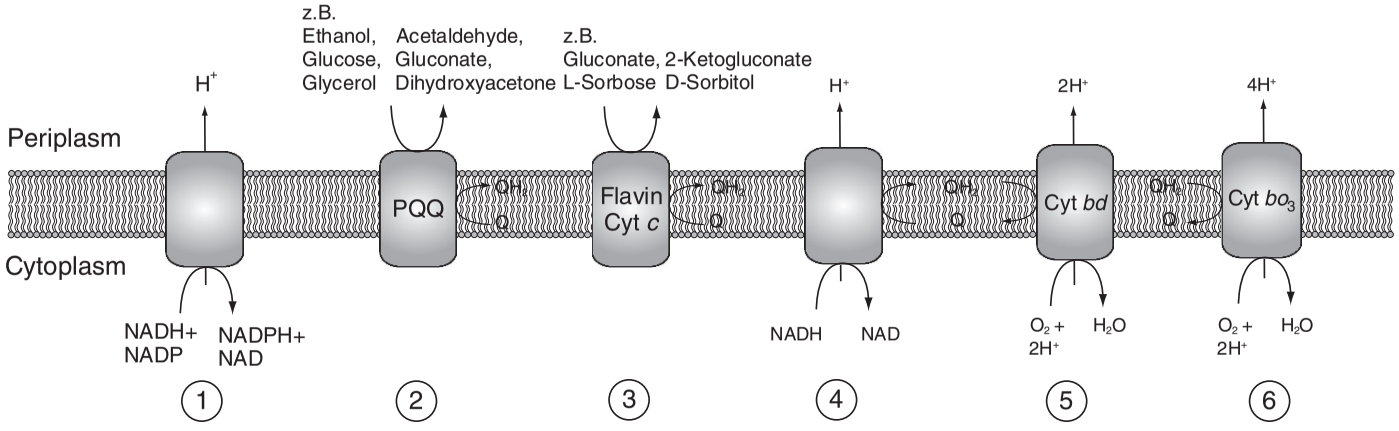

Fig. 21.2 Electron transport in acetic acid bacteria. Various PQQ and flavin dependent dehydrogenases (*circles 2* and *3*) with their active site facing toward the periplasm are present in the cell membrane. They transfer the electrons from substrate oxidation to quinone. A type I NADH dehydrogenase that is unable to translocate protons is also present (*circle 4*) to feed electrons into the quinone pool. The quinone is reoxidized either by a cytochrome *bd* or cytochrome bo_3 (*circles 5* and *6*) containing quinone oxidases. *Gluconobacter* also has a membrane-bound transhydrogenase to transfer electrons between the NAD and NADP pools (*circle 1*)

621H genome sequence reveals the presence of an operon with three genes coding for a membrane bound transhydrogenase. This enzyme couples the equilibrium between the NADH and NADPH pools to the proton motive force. In *E. coli* the membrane-bound transhydrogenase functions in the direction of generating NADPH from NADH (Sauer et al. 2004). Accordingly its physiological function might be to supply *Gluconobacter* with the NADPH needed to reduce assimilated products of direct oxidation in order to channel them into intermediary metabolism. It has also been suggested that transhydrogenase might be involved in the creation of a proton gradient from NADPH generated from substrate oxidations by the soluble dehydrogenases (Prust et al. 2005).

The gluconeogenetic abilities of metabolism are limited. While gluconeogenetic hexose or pentose formation is possible from trioses, there is no ORF coding for a phosphoenolpyruvate synthase, pyruvate orthophosphate dikinase or any other phosphoenolpyruvate synthesizing enzyme. This means that *G. oxydans* cannot produce hexoses from pyruvate or acetate which is in accordance with the observation that *Gluconobacter* is not able to grow on lactate (De Ley 1963). It can be deduced from the genome sequence, that the organism takes up polyols, sugars and oxidized sugar derivatives, phosphorylates these substrates by specific kinases and uses the dehydrogenases and isomerases to channel them into the intermediary metabolism that provides the building blocks for biosynthesis (Deppenmeier and Ehrenreich 2008). This would also account for the presence of the Entner–Doudoroff pathway, as this pathway conserves less energy via substrate level phosphorylation than glycolysis but converts gluconate in only two reaction steps into the important biosynthetic precursor pyruvate.

Opposed to its restricted central metabolism, *G. oxydans* 621H is able to synthesize all amino acids, nucleotides and many vitamins de novo (Prust 2004). Nevertheless all *Gluconobacter* strains do require growth factors. Panthotenic acid, niacin, thiamin and *p*-aminobenzoic acid are required by 96, 40, 8 and 4% of the strains respectively (Gosselé et al. 1980). Nitrogen is assimilated at the level of ammonium that is taken up by specific transporters and incorporated into the metabolism via the glutamine synthetase and the glutamat synthase reactions while a NAD(P) specific glutamate dehydrogenase is missing (Prust et al. 2005). No genes are found in the genome of *G. oxydans* 621H for the reduction of N_2 or NO_3^-. But there are other acetic acid bacteria known such as *Gluconacetobacter diazotrophicus*, *Gluconacetobacter johannae* and *Gluconacetobacter azotocaptans* that are capable of fixing molecular nitrogen (Sievers and Swings 2005).

According to the *G. oxydans* 621H genome sequence sulfur is taken up as sulfate by a permease and reduced via a phosphoadenosine-phosphosulfate synthase (PAPS) system. The serin-O-acetyltransferase and cystein synthase reactions then incorporate the sulfur in the amino acid cysteine (Deppenmeier and Ehrenreich 2008).

One of the most important cofactors synthesized by *G. oxydans* is PQQ, as it is essential for the synthesis of the quinoproteins. The biosynthesis of PQQ that is coded by the *pqqABCDE* operon has been studied in *Methylobacterium extorquens* and *Klebsiella pneumoniae*. All but one of the essential genes have been identified

in the genome of *G. oxydans*. The missing gene is an additional *pqqF* gene that has low sequence similarity between species (Goodwin and Anthony 1998). A good candidate has been recently identified by transposon mutagenesis (Hölscher and Görisch 2006).

21.8 Conclusion and Outlook

Acetic acid bacteria are truly fascinating organisms with unusual biochemistry. While consequences of their metabolism are mostly detrimental in oenology, especially the formation of an excess of acids from sugars in the must or from ethanol in wine during storage, their metabolism in the right dosage also gives a balanced aroma to the wine. Although acetic acid bacteria have been studied since Pasteur's times, their physiology and biochemistry still hold many unanswered questions. The genome sequence of *G. oxydans* 621H permits a detailed look at metabolic pathways and enzymology of a representative of the 'suboxydans' group of acetic acid bacteria, providing a solid fundament for further investigations and demonstrating the enormous power of genome sequencing for microbial research. It should be complemented in the future by more publicly available genome sequences of other acetic acid bacteria, especially from representatives of the 'peroxydans' group.

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Chapter 22
Systems Biology as a Platform for Wine Yeast Strain Development

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22.1 Introduction

Over a period of several millennia, *Saccharomyces cerevisiae* yeasts have been selected for their fermentative capacities, robustness and the sensorial attributes that they impart to wine. This has led to the generation of large collections of *S. cerevisiae* strains that can be inoculated into grape juice to deliver reliable fermentation performance as well as a diversity of predetermined flavors and wine styles. In addition, *S. cerevisiae* is one of the most intensively studied eukaryotes, providing a platform for cutting-edge research into fundamental biological systems. By combining these two discrete areas, fundamental knowledge can be leveraged by industry to improve both production efficiencies and wine quality, while fundamental research can benefit from the focused research directions and financial support of industry.

22.2 Not All Yeasts are Created Equal

The *S. cerevisiae* species is comprised of hundreds of phenotypically diverse strains which are generally thought to have arisen due to human intervention during multiple rounds of wild yeast 'domestication' (Querol et al. 2003). The short generation time of *S. cerevisiae*, combined with clonal selection and passaging has driven the rapid evolution of genetically diverse strains which have specialized functions related to their use. Laboratory strains have been selected for ease of use, consistent phenotypes, stable genotypes and the ability to grow quickly and reproduce in nutrient-rich, laboratory growth media. In contrast, industrial strains are

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generally exposed to very different selection pressures, mainly driven by production efficiencies, with particular focus on fermentative capacity. For example, yeast strains used in winemaking must not only be able to survive stresses of high sugar and low pH, as found in grape juice, they must also be able to rapidly ferment sugars in this medium, while tolerating increasing levels of ethanol (Bauer and Pretorius 2000).

The divergent outcomes of these selection pressures are, of course, most evident when comparing laboratory and industrial yeasts. For example, as opposed to winemaking strains, laboratory strains such as S288C are able to ferment only in the most hospitable of grape musts. This is a clear and major difference in the two yeast types, most likely reflecting the adaptation of laboratory strains to a relatively stress-free environment. Differences between industrial strains can be more subtle, but are nevertheless significant, even amongst those used for the same process. Several studies which investigated the effect that different yeast strains have on winemaking have made it clear that the particular strain of wine yeast used can dramatically impact the sensory properties of wine, even when identical grapes are used (Table 22.1) (Lambrechts and Pretorius 2000; Swiegers et al. 2005).

22.3 Understanding Strain Diversity

One of the major benefits of fundamental yeast research to winemaking is the access to powerful molecular and systems biology tools that have been developed for detailed studies of yeast biology. These technologies allow for the investigation of yeast at a whole-cell level using ‘omic approaches, and provide the means to accurately characterize both intra- and inter-specific diversity which differentiate yeast strains and species.

Table 22.1 Aroma compounds that display yeast-strain dependent levels in wine

Compound(s)	Aroma	Reference
Acetic acid	Vinegar, pungent	Delfini and Cervetti (1991), Eglinton and Henschke (1999)
Higher alcohols	Marzipan, floral, rose	Giudici et al. (1990), Rankine and Pocock (1969)
Esters	Banana, pear, apple, pineapple, violets	Soles et al. (1982)
Acetaldehyde	Sour, green apple	Longo et al. (1992), Romano et al. (1994)
Volatile phenols	Smoky, vanilla, clove-like	Van Wyk and Rogers (2000)
Hydrogen sulfide	Rotten eggs	Rauhut et al. (1996), Mendes-Ferreira et al. (2002)
Volatile thiols	Passion-fruit, cat urine, box tree	Howell et al. (2004)

22.3.1 Genomics

Historically, techniques utilising amplified fragment length polymorphisms (AFLP), restriction fragment length polymorphisms (RFLP) and chromosome length polymorphisms (CLP) (Van der Westhuizen and Pretorius 1992; de Barros Lopes et al. 1999; Fernandez-Espinar et al. 2000) have been used to differentiate between strains of *S. cerevisiae* and also track the genomic content of intra- and inter-specific hybrids. However, these techniques do not assay the entire genome such that important variation can often go undetected. Genomic technologies, including genome sequencing and comparative genome hybridization (CGH), which more thoroughly assay genome differences between strains, have therefore taken over from these other technologies for understanding yeast strain diversity (Bisson et al. 2007).

The genomic sequencing of laboratory strain S228C was a scientific *tour de force*, requiring efforts of 75 laboratories over 8 years (Goffeau et al. 1996). This data provided unparalleled insight not only into the structure of the yeast genome, but of eukaryotic genomes in general as it was the first available eukaryotic genome sequence. However, due to high resource cost required for traditional genome sequencing, the 10 years following the release of the S288C genome saw only two more strains of *S. cerevisiae* (RM11-1a and YJM789) sequenced to a near-complete state (Wei et al. 2007; http://www.broad.mit.edu/annotation/genome/saccharomyces_cerevisiae/Home.html), although low coverage (generally 1- to 2-fold sequencing coverage) is available for at least 36 other strains (http://www.sanger.ac.uk/Teams/Team71/durbin/sgrp/).

While, these sequencing efforts are small in number, they have unearthed a huge amount of genetic diversity, particularly at the level of single nucleotide polymorphisms (SNPs). As an example, there are approximately 60,000 SNPs between S288C and YJM789 (Wei et al. 2007). Interestingly, this level of nucleotide variation (0.6–0.7%) is much higher than that observed between any two humans (<0.1%), and approaches the divergence recorded between humans and chimpanzees (1%; Mikkelsen et al. 2005).

To overcome limitations imposed by the cost and speed of current DNA sequencing, researchers have turned to CGH analysis to perform whole-genome comparisons between large numbers of strains. CGH uses DNA micro-array technology to detect alterations in DNA content (deletions and duplications) between two genomes where it provides cost-effective and rapid mapping of yeast strain variation (Lashkari et al. 1997; Primig et al. 2000; Steinmetz et al. 2002; Infante et al. 2003; Winzeler et al. 2003; Dunn et al. 2005; Gresham et al. 2006; Pope et al. 2007; Schacherer et al. 2007). As an example of the application of CGH, Dunn et al. (2005) used CGH to map variation in four commercial wine strains and uncovered a ‘commercial wine yeast signature’, comprising a set of genomic alterations common to the four wine strains tested. In addition to common genomic alterations, there were numerous differences that were strain specific and even one case of a difference between two isolates of the same strain. High levels of genomic variation are therefore present between wine yeasts, and furthermore, extensive variation exists even when strains are isolated from similar geographic locations (in this case France), or even when different isolates of the same strain are investigated.

While CGH currently represents the most rapid and economical method for detailed characterization of yeast strains at the whole-genome level, it is limited as it requires prior knowledge of the genome and provides no indication of positional rearrangements or the presence of novel DNA sequences. Most importantly though, this method cannot reliably and unambiguously detect SNPs, and this is especially problematic given that such a large amount of this type of variation was detected by the limited *S. cerevisiae* genome sequencing that has been performed to date. SNPs are therefore likely to be responsible for much of the phenotypic variation observed in wine yeast. This has left researchers with the dilemma of using CGH and missing out important polymorphic differences, or trying to secure the large resource base necessary to perform traditional DNA sequencing. This is now changing however, as next generation DNA sequencing technology and particularly massively parallel sequencing, has matured to a point where it is possible to sequence several yeast genomes in less than a week (Table 22.2) and for orders of magnitude lower costs than traditional Sanger sequencing technologies (Bennett 2004; Margulies et al. 2005; Shendure et al. 2005). As an example of this, Borneman et al. (2008) recently applied parallel pyrosequencing (Margulies et al. 2005) to rapidly produce the first genome sequence of an *S. cerevisiae* wine strain. Interestingly, genomic comparisons showed that this strain differed from both S288C and YJM789 by about 0.6% at the nucleotide level. This is very similar to the nucleotide divergence recorded between YJM789 and S288C (Wei et al. 2007) showing that the strains represent three approximately equidistant lineages. By addressing these limitations of traditional DNA sequencing, namely high cost and slow turn-around, it will be routine practice to sequence diverse yeast strains, providing the ultimate picture of yeast diversity and a wealth of knowledge on how differences at the DNA level can translate into phenotypic variation.

22.3.2 Transcriptomics, Proteomics and Metabolomics

While genomics can detect all differences present in the DNA from a collection of yeast strains, many nucleotide differences may represent ‘silent’ mutations and will ultimately have no phenotypic consequence. Transcriptomics, proteomics and metabolomics aim to address this by identifying phenotypic variation which can then be related back to causative genomic differences.

Of these three techniques, it is metabolomics which is most likely to be relevant for characterizing the consequences of yeast variation in winemaking process. Wine represents the combined metabolomes of grape, yeast and bacteria that are

Table 22.2 Next-generation commercial sequencing technologies

Company	Technology	Read length (bp)	Output from one run (days)	Yeast genome equivalents
Roche	Pyro-sequencing	~250	>100 Mbp (3)	~10
Illumina	Sequencing by synthesis	~35	~1.3 Gbp (3)	~100
ABI	Ligation-mediated sequencing	~35	~2.5 Gbp (8)	~200

present in the fermentation. Drinking wine is therefore drinking a combined metabolome, and human olfaction and taste receptors represent very sensitive and efficient detectors of many of the metabolites being consumed. Thus, any differences between wines comprised of different metabolomes will be reflected in the sensory qualities of the product. By integrating genomic and metabolomic data with consumer testing, it should be possible to identify genomic variations that produce metabolomic varation that translates into discernable wine qualities. This knowledge can then provide the basis for future strain development.

22.3.3 Systems Biology

Systems biology is an emerging discipline which promises to revolutionize the study of yeast growth and metabolism (reviewed in Mustacchi et al. 2006; Oliver 2006; Borneman et al. 2007; Pizarro et al. 2007). Systems approaches aim to encompass data provided by genomics, transcriptomics, proteomics and metabolomics under a mathematical framework. By harnessing the predictive nature of this mathematical base, systems biology provides researchers with the ability to model the behavior of complex systems, such as whole yeast cells; preliminary work is already modelling some facets of the yeast cell (Colman-Lerner et al. 2005; Klipp et al. 2005; Westergaard et al. 2006; Castrillo et al. 2007).

22.4 Strain Development

Strain development in *S. cerevisiae* can be accomplished through a variety of techniques which are broadly grouped as either genetically modified (GM) or non-GM approaches (Pretorius 2000). Both approaches can benefit from systems-based techniques which can allow for identification of beneficial existing variation, but can also identify areas of cellular metabolism where new, unexplored types of variation can prove beneficial.

22.4.1 Non-genetic Engineering Approaches

Non-GM techniques for strain development rely on existing variation, or variation that can occur in a population through mutation (reviewed in Chambers et al. 2008). The main techniques involve either mating or adaptive evolution.

22.4.1.1 Mating-Based Techniques

While many industrial strains of *S. cerevisiae*, such as wine yeasts, display low rates of sporulation and fertility (Mortimer et al. 1994; Codon et al. 1995), it is usually possible to cross these strains to combine diverse phenotypes. In some

cases, intra-specific mating cannot be accomplished by conventional means, but techniques such as rare mating and protoplast fusion can be used to produce viable intra-specific hybrids (Pomper et al. 1954; Arima and Takano 1979; Mukai et al. 2001; de Barros Lopes et al. 2002). In addition, these procedures can be used to extend mating outside of the *S. cerevisiae* species boundary to produce inter-specific hybrid strains (Gunge and Nakatomi 1972; Seki et al. 1985; Farahnak et al. 1986; Pina et al. 1986; Lucca et al. 2002; Pasha et al. 2007). This harnesses even more genetic variation, producing hybrid strains that contain genetic contributions from both *S. cerevisiae* and non-*S. cerevisiae* species.

In a wine context, there are several examples of both natural and laboratory-derived inter-specific hybrids which are in use today. The commercial wine yeast S6U is a naturally derived hybrid strain formed between *S. cerevisiae* and *S. bayanus* (Masneuf et al. 1998) while natural hybrids of *S. cerevisiae* and *S. kudriavzevii*; and even triple *S. cerevisiae*, *S. kudriavzevii* and *S. bayanus* hybrids have been documented (Bradbury et al. 2006; Gonzalez et al. 2006; Lopandic et al. 2007). Laboratory-derived inter-specific hybrids are now being used for winemaking with a *S. cerevisiae*, *S. kudriavzevii* hybrid (AWRI 1503), the product of a rare mating between a diploid wine strain of *S. cerevisiae* and a haploid strain of *S. kudriavzevii*, being used with great success in many of the world's leading wine-producing regions. This highlights the benefits of introducing novel genetic variation from outside of *S. cerevisiae*.

In the context of non-GM strain development strategies, systems approaches allow more precision in breeding, through the identification of genetic variation which produces strain-specific desirable characters. A micro-array approach has recently been developed to identify regions of the *S. cerevisiae* genome that are responsible for phenotypic differences between strains (Steinmetz et al. 2002). For this method, micro-arrays are used to map nucleotide variation in two genetically diverse strains that have a measurable difference in a desirable phenotype. These parental strains are crossed and F1 progeny are scored for the presence (or level) of the phenotype. Micro-arrays are then used to track the genetic contributions (through identification of the parental nucleotide variants) made by each parent to a pool of F1 progeny displaying the desirable phenotype and a pool of progeny which lack the phenotype. Statistical algorithms are applied to identify genomic regions (quantitative trait loci, QTLs) that are over-represented in the progeny displaying the phenotype (Fig. 22.1). These areas are predicted to contain genetic loci that contribute to the desirable character. This technique has recently been used to map loci responsible for intra-specific differences in acetic acid production and ethanol robustness – two phenotypes that are very important to the wine industry (Hu et al. 2007; Marullo et al. 2007), demonstrating the power of the method for identifying genetic basis of industrially important phenotypes.

In addition to identifying important variant loci, the presence of specific nucleotide polymorphisms can be detected using high throughput genotyping strategies (reviewed in Syvanen 2005). As large numbers of recombinant progeny can be rapidly screened, a successive backcrossing strategy can be applied with desirable variation being introduced into new strains while limiting the carry-over of collateral

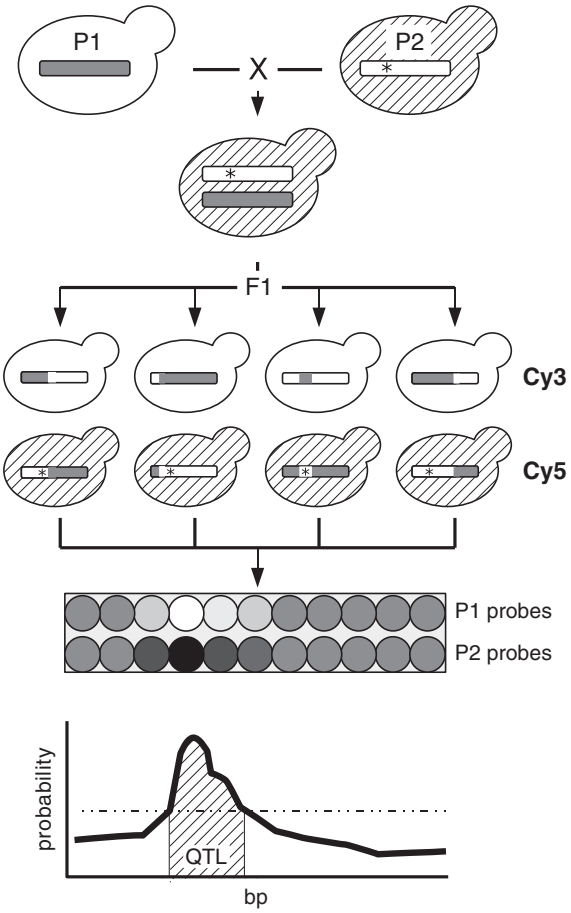

Fig. 22.1 High resolution, micro-array-based QTL mapping. Two strains, P1 and P2, which differ in a phenotype of interest (*white cell*, no phenotype; *hatched cell*, desirable phenotype), are screened by micro-array analysis for genomic loci which show strain specific hybridization patterns (P1 probes and P2 probes). Strain P2 contains a single locus (*) which contributes to this desirable phenotype. These strains are crossed and the heterozygous diploid is sporulated. F1 progeny are pooled into two groups depending on their phenotype (*white*, no phenotype; *hatched*, desirable phenotype). Genomic DNA is isolated from the two groups and labeled with either Cy3 (no phenotype) or Cy5 (desirable phenotype). These two samples are then competitively hybridized to a micro-array (*light grey box*) comprised of the parental strain-specific probes identified previously. For any region which is segregating randomly in the F1 progeny, the Cy5 and Cy3 signals on the micro-array should be present in an approximately 1:1 ratio (*grey*). Regions which are linked to the desirable phenotype will show ratios of >1 (*black*) in P2-specific probes and <1 (*white*) in P1 specific probes. Statistical analysis of the probe signals then identifies QTL regions that are associated with phenotype of interest by searching for regions of the chromosome in which multiple flanking probes show a concerted movement away from the expected 1:1 ratio of Cy3 and Cy5 signals

DNA variation (Fig. 22.2). Even for complex traits, which result from the cooperative action of multiple genetic loci, high throughput screening can be used to simultaneously track multiple genetic loci in large numbers of progeny. Using this approach, sufficient recombinant progeny can be tested, such that a strain with the desired combination of cooperative loci (which can appear at very low frequency, depending on the number of genes) can be isolated in the new strain background.

22.4.1.2 Adaptive Evolution Strategies

In adaptive evolution, strains are either successively passaged or grown in continuous culture under conditions which progressively select for a trait of interest (Chambers et al. 2008). As strains are grown over large numbers of generations, multiple mutations, which act cooperatively to enhance the selected phenotype, can accumulate over time. Adaptive evolution is therefore an ideal technique for isolating phenotypes that have a multi-genic basis.

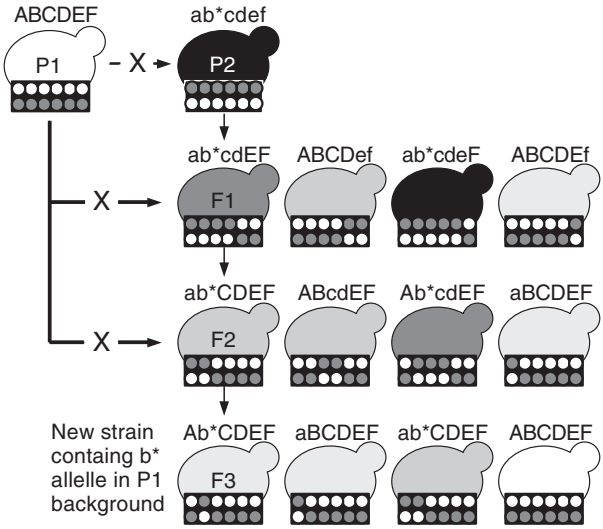

Fig. 22.2 A backcrossing strategy to transfer desirable alleles into new strains. Strain P2 contains a desirable characteristic which results from a DNA difference in the b locus (b*) and which would be beneficial in the P1 strain background. Micro-arrays (black rectangles) are used to identify genomic differences between the two strains (compare patterns of *white circles*). These two strains are then crossed and the same micro-array platform is used to determine the genomic contribution of each parent to each of the F1 progeny. The strain which contains the largest contribution by parent P1, while retaining the b* allele, is selected (strain F1) and crossed back to P1. The screening process is then repeated through multiple generations (F2, F3, etc.) until a strain is isolated that contains all the alleles of the P1 parent, with the exception of the b* allele. This strain (F3) essentially represents the original P1 parental strain with the exception of the desirable b* allele

Adaptive evolution has been used extensively to increase output of industrial strains and is especially suited to developing strains with improved use of specific nutrients such as glucose (Paquin and Adams 1983; Zeyl 2005) or, in more extreme situations, pentose sugars such as xylose (which are normally very poorly utilized by *S. cerevisiae*) (Sonderegger and Sauer 2003; Sonderegger et al. 2004; Attfield and Bell 2006) and to isolate strains which are resistant to stresses imposed during fermentation by metabolites such as acetic acid (Aarnio et al. 1991) or ethanol (Brown and Oliver 1982; Jimenez and Benitez 1988). The latter study is especially significant for the wine industry as it was performed in a wine strain and reflects a phenotype which is especially important for minimizing risk of stuck or sluggish fermentations, which can occur when using strains that are not particularly robust.

Systems techniques can contribute to development of selection regimes and help analyze new strains produced by adaptive evolution (Fig. 22.3). Adaptive evolution requires genetic variation, and the identification of 'useful' variation that contributes to desirable phenotypes remains one of the greatest challenges to this field. Different forms of CGH have been successfully applied to the identification of such variation (Dunham et al. 2002; Gresham et al. 2006). This technique is still not widely applied, but advances in the methodology and improvements in resolution should eventually make this a method of choice for identifying the approximate location of DNA variation among strains. However, while the use of high-resolution CGH may provide the short-term solution for characterizing strains, the incorporation of new-generation sequencing will undoubtedly become the standard approach for strain analysis, making these other techniques all but obsolete.

In addition to genomic contributions to non-GM strain development, metabolomics can also be applied to identify biochemical pathways that are altered as a result of adaptive evolution (Allen et al. 2003; Hua et al. 2006; Cakir et al. 2007). When combined with data from genomic experiments, complementary data sets will enable discovery of industrially relevant genetic variation and lead to a clear understanding of how this variation shapes the phenotype.

22.4.2 Genetic Engineering Approaches

Unlike non-GM approaches, GM techniques allow genetic variation from any source to be incorporated into a yeast strain, using a variety of techniques. These techniques can utilize extra-chromosomal, plasmid vectors or integrate DNA into the yeast genome.

22.4.2.1 Extra-Chromosomal Addition of DNA

There are two main types of plasmid used in yeast molecular biology, which differ slightly in their copy number and segregation properties. Two-micrometer plasmids function in a manner similar to bacterial plasmids, being present

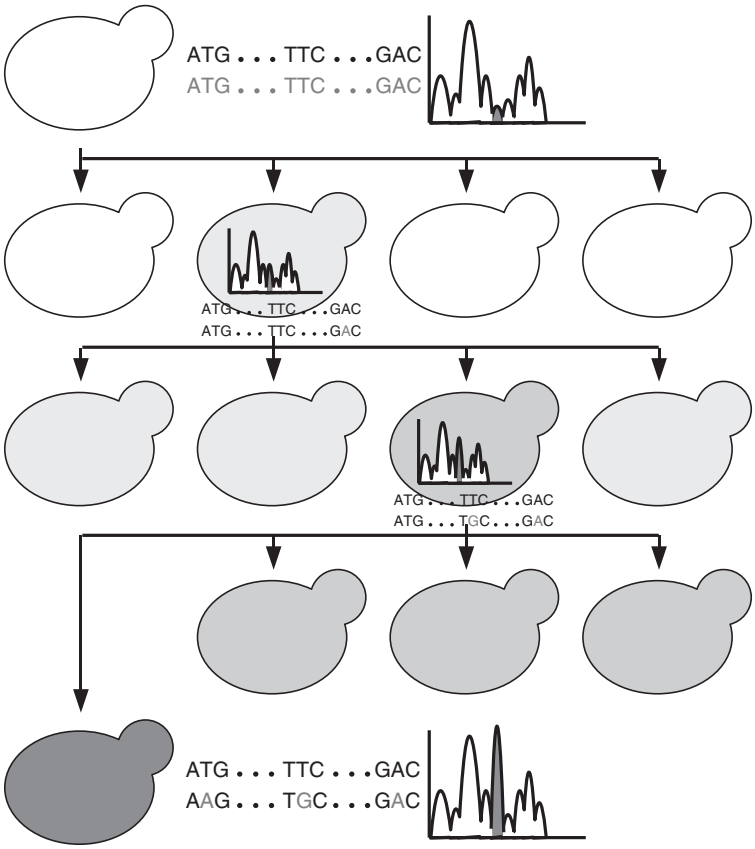

Fig. 22.3 Systems analysis of adaptively-evolved strains. A parental strain (*white cell*) is identified for development. The genome sequence of this strain is known (right of cell), as is the metabolomic profile of wine produced by this strain (far right of cell). After the first round of adaptive evolution, the best performing strain (*light grey*) is selected and subjected to a second round of adaptive evolution. This process is reiterated until suitably performing strain (*dark grey cell*) is isolated. The mutations which arose during adaptive evolution to produce the desired phenotype can then be determined at the genomic level (mismatched *grey* nucleotides) and differences in metabolomic profiles can be used to determine the impact of the mutation(s) on cellular metabolism (compare *grey peaks* in chromatograms)

in high copy and segregating randomly during cell division, while CEN plasmids, which contain a yeast centromeric region, act more like mini chromosomes and are present in low copy number. Both types of plasmid are very easy to introduce into yeast by transformation, but as they are extra-chromosomal, they are unstable in the absence of selection and this is especially true for the 2-μm variety (Fig. 22.4a).

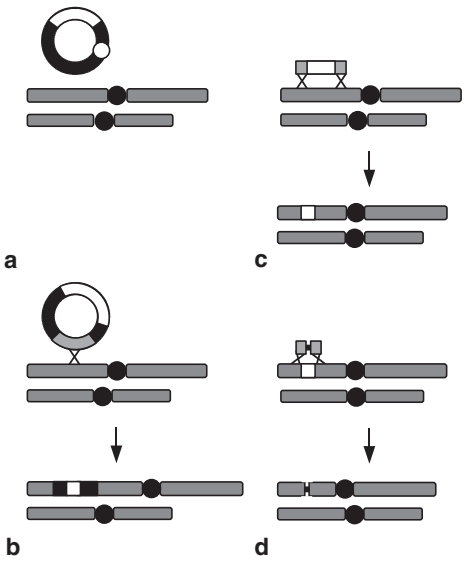

Fig. 22.4 Genetic modification techniques in *Saccharomyces cerevisiae*. (**a**) Extra-chromosomal plasmids. DNA cassette to be introduced (*white bar*) is inserted into a plasmid backbone (*black ring*). The plasmid backbone contains sequences which allow for the plasmid (and the inserted DNA cassette) to replicate autonomously in yeast (*white circle*). (**b**) Integrating plasmids. These plasmids cannot replicate autonomously in yeast and must be integrated into one of the chromosomes to be maintained. Chromosomal integration is achieved by including a region of DNA (*grey bar*) which is homologous to the area of the genome where integration is to take place. A single homologous recombination event (X) integrates the plasmid into the genome. (**c**) Integration of linear DNA. DNA cassette is flanked by two regions which are homologous to targeted sites in the genome. Two recombination events are required to integrate the DNA cassette into the chromosome. (**d**) Storici cassette removal (Storici and Resnick 2003). In many situations, it is desirable to remove all of part of a DNA cassette that has been chromosomally integrated as this often contains marker sequences such as antibiotic resistance genes. Here, a linear piece of DNA which contains only the flanking DNA regions is transformed into yeast to remove the cassette inserted in (**c**). Two homologous recombination events remove the cassette, while leaving any required alterations to the genome (*small black bar*)

22.4.2.2 Chromosomal Integration of DNA

There are two main procedures for integrating extraneous DNA into chromosomes of *S. cerevisiae*. In the first, DNA is incorporated at specific chromosomal sites through recombination between a region present on a circular piece of DNA (generally a plasmid which cannot replicate in yeast) and a second, homologous genomic region (Fig. 22.4b). A single recombination event integrates the entire plasmid into this locus, producing two copies of the region duplicated in tandem used in the homologous recombination. While this procedure allows specific fragments of DNA to be inserted into precise positions of the genome, it also requires integration of accessory DNA such as the plasmid backbone and selectable markers.

In the second technique, linear, rather than circular DNA is integrated into chromosomes. However, unlike the previous technique, the integration of linear DNA requires two recombination events between two separate homologous regions (Fig. 22.4c). While this requirement reduces the efficiency of integration, the homologous recombination machinery of *S. cerevisiae* nonetheless ensures its success, even when very small (35 bp) regions of homology are used (Wach et al. 1994). Also, as two regions of homology are used, it is possible to incorporate both new DNA as well as delete existing genomic DNA by specifying the incorporation of the marker sequences between two disjunct sections of the genome. Additional benefits can also be achieved with this technique when specific selectable markers and a second transformation step are used, as it is possible to remove all marker sequences and accessory DNA required for the first transformation step (Storici and Resnick 2003). This so-called 'delitto perfecto' technique allows gene deletions, integration of foreign DNA or even single nucleotide alterations to be made without any 'unwanted' sequences, such as antibiotic resistance genes, being left in the genome; a key criticism of many current GM techniques (Fig. 22.4d).

22.4.3 Industrial GM Strains

Outside of the pharmaceutical industry where GM strains are used for production of a number of peptide drugs including insulin, and vaccines such as that directed against hepatitis, there has been very little use of GM techniques in the development of industrial yeast strains that have subsequently been used in production (McAleer et al. 1984; Kjeldsen 2000; Verstrepen et al. 2006). This is especially true in the wine industry, where many countries have effectively banned the use of GM yeast strains for wine production.

Recently, however, two GM wine yeast strains have been granted GRAS (generally regarded as safe) status by the Food and Drug Administration in the USA – ECMo01 and ML01 (GRAS Notice numbers GRN 000120 and GRN 000175). In ECMo01, an additional copy of the *S. cerevisiae DUR1,2* gene, altered for increased expression, was inserted into a wine yeast strain to lower the levels of urea present during fermentation (Fig. 22.5). In alcoholic fermentations, urea

Fig. 22.5 (continued) *ADH1* promoter and terminator on a non-integrative plasmid. High levels of *GPD1/2* divert carbon from ethanol and produce higher levels of glycerol and acetic acid. High levels of acetic acid are undesirable in wine, so the *ALD6* gene is deleted from the genome by homologous recombination (by replacement with the KanMX selectable marker). Loss of *ALD6* stops the accumulation of acetic acid resulting in increased glycerol production. Another carbon diversion strategy involves using *PGK1* sequences to express the *GOX1* gene of *Aspergillus niger*, with the cassette integrated by homologous recombination (Malherbe et al. 2003). *GOX1* causes carbon to be diverted from ethanol to gluconic acid which is non-fermentable

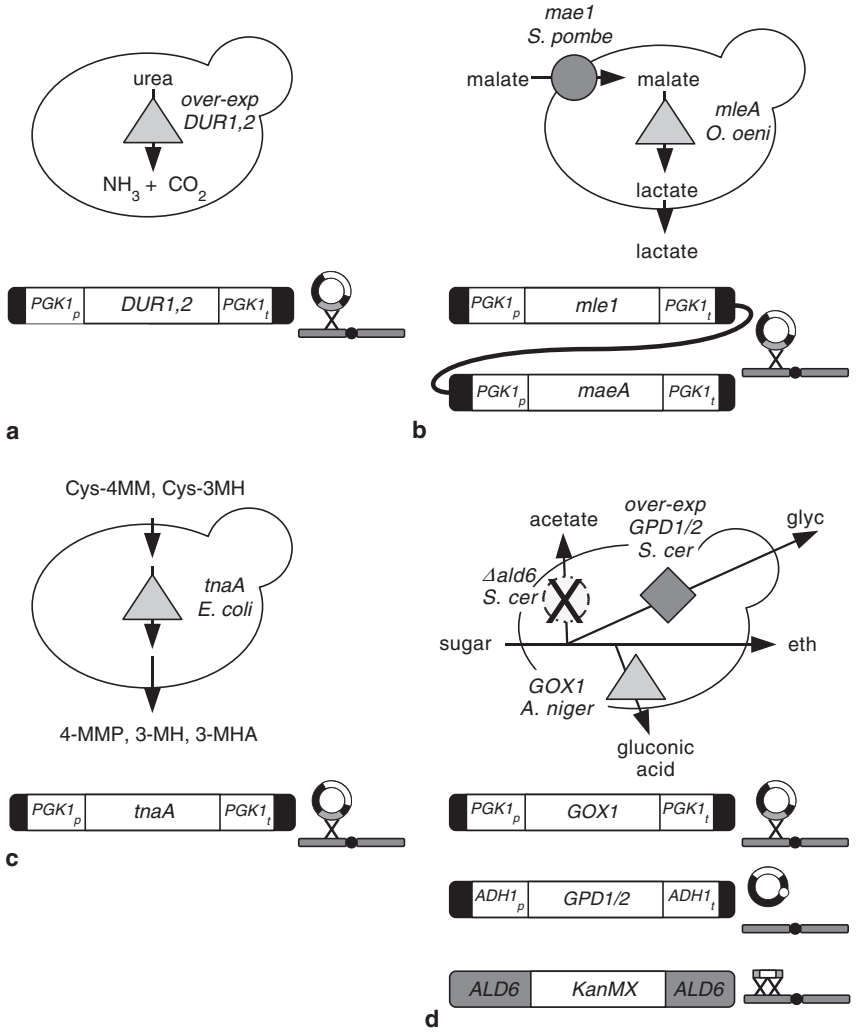

Fig. 22.5 Schematic representations of GM yeast strains. (**a**) Strain ECMo01 (Coulon et al. 2006). The *DUR1,2* gene of *Saccharomyces cerevisiae* is over expressed by replacing the native promoter and terminator sequences with those of the *S. cerevisiae PGK1* gene ($PGK1_p$ and $PGK1_t$). The gene cassette is chromosomally integrated by a single recombination event. *DUR1,2* converts urea into ammonia and carbon dioxide reducing the potential formation of ethyl carbamate. (**b**) Strain ML01 (Husnik and van Vuuren 2006; Husnik et al. 2006). The *mae1* gene of *Schizosaccharomyces pombe* and the *mleA* gene of *Oenococcus oeni* are expressed using *PGK1* sequences. This dual cassette is chromosomally integrated by a single recombination event. Mae1 transports malate into the cell where MleA completes the malolactic process by converting the malate into lactate. (**c**) A flavor-active GM strain (Swiegers et al. 2007). The *Escherichia coli tnaA* gene is expressed using the *S. cerevisiae PGK1* sequences and chromosomally integrated by a single recombination event. The *tnaA* gene encodes a cysteine-β-lyase which liberates the aromatic, passion fruit thiols, 4-mercapto-4-methylpentan-2-one (4-MMP) and 3-mercaptohexan-1-ol (3-MH) from their non-volatile, odorless precursors Cys-4MMP and Cys-3MH. (**d**) Construction strategies for a low-ethanol producing yeast (Remize et al. 1999; Eglinton et al. 2002). Either the *GPD1* or *GPD2* gene of *S. cerevisiae* is over-expressed by placing it under the control of the

reacts with ethanol to form ethyl carbamate, a potential carcinogen. By lowering the levels of yeast-generated urea, the possibility of wine ferments containing unacceptable levels of this metabolite is substantially reduced (Coulon et al. 2006). In the case of ML01, two foreign genes, the *mae1* malate permease of *Schizosaccharomyces pombe* and the *mleA* malolactic gene of *Oenococcus oeni*, were integrated into the genome of a common wine yeast strain (Fig. 22.5b). These two genes enable the ML01 wine yeast to degrade malic acid during the primary fermentation, a process which is normally performed by malolactic bacteria after completion of ethanolic fermentation (Husnik and van Vuuren 2006; Husnik et al. 2006).

In addition to these production strains there has been a great deal of GM-based wine yeast strain engineering performed in research environments (reviewed in Pretorius 2000; Schuller and Casal 2005; Pretorius and Høj 2005; Pretorius 2006; Verstrepen et al. 2006). Like ECMo01 and Ml01, some of these, involve only one or two genetic modifications (Fig. 22.5c) (Lilly et al. 2006; Swiegers et al. 2007). However, many desirable phenotypes require rerouting of central metabolism, and this can produce complex side effects. In such cases, it is likely that multiple cooperative and compensatory genetic modifications will be required to redirect metabolism with only desirable outcomes (Fig. 22.5d) (Remize et al. 1999; Eglinton et al. 2002; Malherbe et al. 2003; Blank et al. 2005; Cambon et al. 2006; Heux et al. 2006). Systems biology, and especially metabolic modeling, is now being applied to reduce the possibility of metabolic side effects and strategically select genes or enzyme activities to be incorporated into yeast strains to produce desirable metabolic alterations (Famili et al. 2003; Forster et al. 2003; Bro et al. 2006). As an example of the power of this approach, Bro et al. (2006) used a metabolic model of *S. cerevisiae* central metabolism to screen around 3,500 genetic alterations (gene deletions, amplifications and addition of foreign enzyme activities) in silico to identify 56 reactions that could increase ethanol production prior to constructing any prototype strains. This strategy greatly increases the speed at which suitable prototype strains can be developed, as the laboratory-based work is the real bottleneck in generating new industrial yeasts.

Systems biology is therefore likely to have its greatest impact on current GM approaches to strain development in the area of in silico prediction. Unlike non-GM techniques, the precise nature of GM methods mean that the new variation is known and its impact on the phenotype can be predicted prior to the strain being created. Thus, techniques such as CGH and genome sequencing are not required to characterize the strain, once it is produced. However, allowing researchers to computationally screen for desirable genetic alterations from the thousands of available possibilities is crucial to optimizing modern GM strain development. For winemaking, the combination of metabolic modeling and systems-level knowledge on how genomic modifications affect gene activity will provide the greatest benefit. Such data will allow researchers to rationally design genomic alterations which will drive metabolic activity through specific biochemical pathways.

22.5 Conclusion

Holistic understanding of yeast genetics and physiology that comes with systems-based approaches will facilitate development of better informed classical and GM strategies for strain improvement programs. In the case of GM strategies, systems biology will also allow for large-scale computational evaluation of genetically engineered strains, prior to their physical construction. This will vastly speed up strain development while simultaneously expanding the range of potential alterations to be considered.

The future of yeast strain development is therefore very bright. It is built on the promise of accelerated growth in the knowledge and understanding of complex hierarchies of systems that comprise a living cell, and how interactions between these levels bring life to a complex milieu of bio-chemicals. Systems biology also has the potential to enable development of strains tailored, for example, to suit specific grape juices or to meet diverse and expanding consumer preferences. This will allow winemakers to consistently produce a premium product and give consumers confidence that wine they are purchasing will meet or exceed their expectations of quality and value.

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Chapter 23
Plasmids from Wine-Related Lactic Acid Bacteria

Juan M. Mesas and M. Teresa Alegre

23.1 Introduction

Lactic acid bacteria (LAB) are important in wine-making for several reasons. First, they are responsible for malolactic fermentation (MLF) that provokes deacidification of wine by transformation of malic acid into lactic acid. Second, they can spoil wine as a consequence of metabolism of various substrates, leading to so-called "diseases of wine." Third, some strains can produce undesirable molecules that can be considered as toxic products, such as the biogenic amines and the precursors of ethyl carbamate. Finally, wine-related LAB are more recently reported to be a source of enzymes of interest in wine-making. For reviews of LAB in wine-making, see Gasson and de Vos (1994), Lonvaud-Funel (1999), Matthews et al. (2004), and Swiegers et al. (2005).

In view of the above, the genetic manipulation of wine-related LAB to improve their beneficial effects and minimise their negative effects is one of the main objectives of research on wine-making LAB (Pretorius and Høj 2005).

A large number of LAB isolated from different sources contain one or more plasmids: some of these are cryptic and have been used to develop cloning vectors (Shareck et al. 2004), while others have been studied for the traits they encode, including bacteriocin production, sugar catabolism, heat stress response, antibiotic resistance, bacteriophage resistance, metal ion resistance and polysaccharide biosynthesis (Salminen and von Wright 1998; Gasson and Shearman 2003). Parallel to these findings on plasmids from LAB, wine-making researchers have investigated the presence of plasmids in LAB from grapes, must and wine, their putative roles, and their possible use as cloning vectors enabling manipulation of wine-related LAB. The LAB associated with grapes, must and wine belong to the genera *Lactobacillus* (*Lb.*), *Leuconostoc* (*L.*), *Oenococcus* (*O.*) and *Pediococcus* (*P.*)

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H. König et al. (eds.), *Biology of Microorganisms on Grapes, in Must and in Wine*,

(Chap. 1). The present chapter aims to review findings on plasmids from LAB strains of these four genera which have been isolated from wine-related sources.

23.2 Lactobacillus

Lactobacillus is a diverse genus of homo- and hetero-fermentative LAB whose species are widespread in a variety of natural habitats including the gastrointestinal tract of man and animals, wine and other alcoholic beverages, and fermented vegetables. Many species of the genus *Lactobacillus* are important in the industrial preparation of fermented milk, meat and vegetable products.

Plasmids are present in most, but not all, *Lactobacillus* species. According to Wang and Lee (1997) at least 38% of the species of *Lactobacillus* contain plasmids that vary widely in size (from 1.2 to more than 150 kb), in number (from 1 to more than 10 plasmids in a single strain) and in mode of replication (generally theta replication for large plasmids and rolling-circle [RC] replication for small plasmids). Although most of the plasmids of *Lactobacillus* remain cryptic (Shareck et al. 2004), some functions have been found to be plasmid-linked. According to Wang and Lee (1997) such functions can be grouped into four classes: (1) hydrolysis of proteins; (2) metabolism of carbohydrates, amino acid, and citrate; (3) production of bacteriocins, exopolysaccharides, and pigments; and (4) resistance to antibiotics, heavy metals, and phages.

A large number of vectors based on native plasmids of *Lactobacillus* strains have been developed to transform and/or to conjugate species of *Lactobacillus* and other gram-positive bacteria, and a number of vectors derived from plasmids of non-lactobacilli strains can replicate in *Lactobacillus* species (Wang and Lee 1997; Shareck et al. 2004). Nevertheless, very few such plasmids have been reported from strains of *Lactobacillus* species isolated from wine-related sources.

De las Rivas et al. (2004) described the complete nucleotide sequence of pPB1, a small cryptic plasmid (2,899 bp) isolated from *Lb. plantarum* BIFI-38 (a wine-related strain). This plasmid replicates via an RC mechanism and is composed of two modules, a replication module that shows 94.5% identity to an analogous region of the *L. lactis* plasmid pCI411 (Coffey et al. 1994), and a mobilisation module that shows 97.5% identity to *Lb. plantarum* plasmid pLB4 (Bates and Gilbert 1989). These findings suggest that pPB1 originated by modular exchange of large DNA fragments between two plasmids. Evolution based on accumulation of modular units is well established in RC plasmids (Francia et al. 2004).

In some cases, functions undesirable for wine-making have been associated with the presence of plasmids in *Lactobacillus*. For example, Lucas et al. (2005) reported a strain of *Lb. hilgardii* isolated from wine that contains several plasmids, one of which (pHDC, 80 kb), encodes histamine production, and is probably also harboured by some histamine-producing strains of *Tetragenococcus muriaticus* and *O. oeni*. More recently, Suzuki et al. (2005, 2006) have reported three plasmids

involved in resistance to hop compounds of three LAB isolated from spoiled beers. These plasmids are pRH45 (15,136 bp) from *Lb. brevis*, pRH20690 (13,022 bp) from *Lb. lindneri*, and pRH478 (14,567 bp) from *P. damnosus*. The very similar characteristics and high percentage of nucleotide-sequence identity between these three plasmids suggest a common origin. In addition, a *P. pentosaceus* plasmid (10.1 kb) named pRS5 – isolated from the same wine strain from which pRS4 (Alegre et al. 2005) was isolated, and currently under study by our group – shows a region with high nucleotide-sequence identity (unpublished results) with the three plasmids reported by Suzuki's group. This suggests a close relationship between some plasmids of LAB present in beer and wine.

23.3 Leuconostoc

The genus *Leuconostoc* comprises a diverse group of heterofermentative LAB of considerable industrial importance, traditionally used in various food and wine fermentations. Few species of *Leuconostoc* have been documented to be related with wine: *L. oenos*, now reclassified as *O. oeni* (Dicks et al. 1995), and *L. mesenteroides* are the most frequent, if not the only, wine-related species of this genus. In general – and with the exception of *L. oenos*, which has been always isolated from wine-related sources – the species of the genus *Leuconostoc* including *L. mesenteroides* have been isolated from other sources, including fermented vegetable, milk and meat products (Dellaglio et al. 1995). There have been few studies reporting the presence of plasmids in species of *Leuconostoc*: those concerning *L. oenos* (*O. oeni*) will be discussed in Sect. 23.4, and the rest here.

O'Sullivan and Daly (1982) were the first to report the presence of plasmids in *Leuconostoc*. They found at least one plasmid, ranging in size from 2.5 to 40 MDa, in every one of the ten strains analysed. Later, Orberg and Sandine (1984) documented plasmids from 1 to 76 MDa in *L. lactis*, *L. paramesenteroides* and in several subspecies of *L. mesenteroides*, while Dellaglio et al. (1984) reported the presence of plasmids from 1.2 to 3.5 MDa in 15 strains of *L. paramesenteroides*. In 1988 Cavin et al. reported a mutant derived from strain *L. mesenteroides* ssp. *mesenteroides* 19D unable to metabolise citrate and lacking a plasmid of 14.6 MDa.

Although some *Leuconostoc* plasmids have been reported to be associated with metabolic functions such as lactose utilisation and citrate permease activity (O'Sullivan and Daly 1982; David et al. 1992), and bacteriocin production and immunity (Hastings et al. 1991), most of the plasmids of *Leuconostoc* remain cryptic.

To date, two types of plasmid replicated by the RC mechanism have been characterised in *Leuconostoc*, pCI411 and pFR18. pCI411 from *L. lactis* (2,926 bp) has a replication origin of the pE194-type, and can be introduced into *Leuconostoc*, *Lactococcus*, *Streptococcus*, *Lactobacillus* and *Bacillus* (Coffey et al. 1994). pFR18 (1,828 bp) from *L. mesenteroides* ssp. *mesenteroides* has similar characteristics of the pT181 family of plasmids (Biet et al. 1999), and its derivatives are able to transform *Lb. sakei* and several species of *Leuconostoc*.

A new cryptic plasmid, pTXL1 (2,665 bp) from *L. mesenteroides* ssp. *mesenteroides*, the first small theta-replicating plasmid described in *Leuconostoc*, was noted by Biet et al. (2002). Derivatives of this plasmid replicate in several LAB.

Finally, pIH01, a small plasmid (1,822 bp) from *L. citreum*, has been characterised by Park et al. (2005) as a new member of the pT181 family. A pIH01 derivative carrying the erythromycin resistance gene (*ermC*) from pE194 has been able to transform *Leuconostoc* strains, *Lb. plantarum*, and *Lactococcus lactis*.

23.4 Oenococcus oeni

This LAB, formerly called *L. oenos*, is a heterofermentative coccus which is usually present on grapes, in must and in wine and is the major agent of the MLF (Van Vuuren and Dicks 1993; Lonvaud-Funel 1995; Versari et al. 1999). According to Lonvaud-Funel (1999) *O. oeni* could be the sole LAB that is genuinely beneficial in wine-making. Due to the important role of *O. oeni* in the process of wine-making, the presence of plasmids in this LAB and their putative roles have been investigated for the last 20 years; there have also been various attempts to develop cloning vectors and transformation protocols for *O. oeni* based on these plasmids.

23.4.1 Plasmids

The earliest reported attempt to find plasmids in *O. oeni* appears to have been that of Sgorbati et al. (1985), who examined extra chromosomal DNA in 52 strains belonging to the heterolactic bacterial species most commonly found in alcoholic beverages (wine, cider, beer, etc.). More than 50% of the strains, belonging to *L. oenos* species and *Lactobacillus* genus, carried plasmids. Two years later the same group (Sgorbati et al. 1987) analyzed 35 new strains of *L. oenos*, finding plasmids ranging from 3.3 to 39 MDa in 11 of these strains. The plasmids were characterised by restriction analysis and one of them, pBL34 from strain *L. oenos* Lco 34, was associated with resistance to the pesticides aldrin, bromophos-methyl and heptachlor.

Using new methods of DNA extraction, Janse et al. (1987) isolated 11 plasmids, ranging from 2.4 to 4.6 kb, from 8 of a total of 42 strains of *L. oenos*. Five of these strains contained only one plasmid and the other three contained two plasmids each. According to these authors, the relatively small size of these plasmids, their low frequency of appearance and their low copy number together suggest that *L. oenos* carries little genetic information in these extrachromosomal elements.

In 1988 Cavin et al. investigated the presence of extrachromosomal DNA in 22 strains of *Leuconostoc*, 8 of them of *L. oenos*. They found that only 2 of the *L. oenos* strains carried plasmids, strain 8413 from the Institute of Oenology of Bordeaux which carried a plasmid of 3.5 MDa later called pLo13 (Fremaux et al. 1993), and strain ATCC 23279 which carried a plasmid of 4.1 MDa. These authors

concluded that the frequency of appearance of plasmids is low in *L. oenos* by comparison with other *Leuconostoc*.

In an attempt to differentiate strains of *L. oenos* from New Zealand isolates, Kelly et al. (1993) found three new strains harbouring unreported plasmids of 4, 22 and 33 kb, with unknown metabolic roles.

The first plasmid of *L. oenos* to be analyzed at the molecular level (Table 23.1) was pLo13 (Fremaux et al. 1993), a cryptic plasmid of 3,948 bp present in strains 8413 and HB156, both from French wines. Sequence analysis indicated that pLo13 replicates by the RC mechanism. Later, Prévost et al. (1995) reported the isolation and partial characterisation of 6 cryptic plasmids present in 6 of 15 strains of *L. oenos* isolated from wines and ciders. Every one of the six strains harboured a single plasmid, with three different plasmids altogether, pUBLO1 (3.9 kb), pUBLO5 (4.3 kb) and pUBLO6 (26 kb); pUBLO1 is the previously reported pLo13.

Zúñiga et al. (1996a) reported the nucleotide sequence of p4028, a plasmid of 4,410 bp present in *L. oenos* CECT 4028 isolated from wine. Sequence analysis revealed 5 ORFs grouped in two clusters separated by a short non-coding sequence. No relationships between pLo13 and p4028 were found.

The nucleotide sequence of pOg32, a cryptic plasmid of 2,544 bp stably maintained in several strains of *L. oenos* isolated from Portuguese wines, was reported by Brito et al. (1996), who described some similarities between pOg32 and pLo13, such as the presence of three major ORFs and the use of an RC replication mechanism.

Following the change of denomination of *L. oenos* to *O. oeni*, studies on wine-related heterofermentative cocci have tended to centre specifically on *O. oeni* rather

Table 23.1 Plasmids of *Oenococcus oeni* with complete nucleotide sequence in databases

Plasmid	Size (pb)	Source	Accession number	Characteristics[a]	Reference
pLo13[b]	3,948	French wine, IOB[c]	M95954	RC, Cryptic	Fremaux et al. (1993)
p4028[b]	4,410	Spanish wine, CECT[d]	Z29976	Theta, ATPase	Zúñiga et al. (1996a)
pOg32[b]	2,544	Portuguese wine	X86402	RC, Cryptic	Brito et al. (1996)
pRS1	2,523	Spanish wine	AJ006467	RC, Cryptic	Alegre et al. (1999)
pRS2	2,544	Spanish wine	AJ310613	RC, Cryptic	Mesas et al. (2001)
pRS3	3,948	Spanish wine	AJ310614	RC, Cryptic	Mesas et al. (2001)
pOM1	3,926	Japan	AB208028	(RC), Cryptic	–

[a]*RC* replication by rolling-circle mechanism; *Theta* replication by theta mechanism; () putative replication mechanism; *Cryptic* unknown function; *ATPase* DNA-dependent ATPase

[b]From strains previously designated as *L. oenos*

[c]*IOB* Institute of Oenology of Bordeaux

[d]*CECT* Spanish Type Culture Collection

than on the genus *Leuconostoc*. Zavaleta et al. (1997) studied the genetic diversity of different strains of *O. oeni* finding that 26% of the strains have extrachromosomal DNA, a higher percentage than the 16 and 8% found by Janse et al. (1987) and Fremaux et al. (1993) respectively. Brito and Paveia (1999) used a large-scale isolation technique to screen 30 strains of *O. oeni* for extrachromosomal DNA, finding large plasmids (ca. 40 kb) in 18 strains and small plasmids (2.5–4.5 kb) in 6 strains. Some of the small plasmids corresponded with, or were similar to, other plasmids already reported, such as pOg32, pLo13 and p4028. This study suggested that large plasmids may be frequent in *O. oeni*, but were difficult to detect due to their low copy number and problems of isolating them.

In 1999 our group published the nucleotide sequence of pRS1 (2,523 bp), a cryptic plasmid of a strain of *O. oeni* isolated from Spanish wines (Alegre et al. 1999). This plasmid shows high homology with pOg32 and, like pLo13 and pOg32, it contains 3 ORFs coding for a replication-initiation protein (Rep), a plasmid recombination enzyme (Pre), and an unknown protein. These features prompted us to postulate the existence of a family of small cryptic plasmids in *O. oeni* that is widespread among strains isolated in diverse countries. Supporting our hypothesis, the nucleotide sequence (3,926 bp) of a plasmid (pOM1) of an *O. oeni* strain from Japan that is highly homologous to pLo13 has recently been deposited in the databases. Our group also reported the nucleotide sequences of two other RC plasmids stably maintained in a single strain of *O. oeni*, its natural host (Mesas et al. 2001). One of these plasmids, pRS2 (2,544 bp; Fig. 23.1), shows high homology with pOg32 and pRS1, while the other, pRS3 (3,948 bp), is practically identical to pLo13. This finding suggests that the family of small RC plasmids of *O. oeni* can be split into two subfamilies that can coexist in a single strain of *O. oeni*. Attempts to identify possible roles of these plasmids using cured strains (Mesas et al. 2004) have been unsuccessful. Recently Walling et al. (2005) detected strains of *O. oeni* containing a putative *dps* gene by using the same primers that correlate this gene with plasmid pF8801 of *P. damnosus*. This finding raises the possibility of searching for this gene among small plasmids of *O. oeni* without known functions.

23.4.2 Development of Cloning Vectors and Transformation Systems for O. oeni

Despite increasing knowledge of the genetics of *O. oeni*, no useful procedures for its genetic manipulation have been developed. Dicks (1994) reported the transformation by electroporation of *L. oenos* using a protocol in which competent cells were prepared in the presence of lysozyme; however, it has proved difficult to reproduce these results and no new reports based on this protocol have been reported. Several other groups of researchers, including our own group, have dedicated a lot of effort to developing cloning vectors based on small cryptic plasmids of *O. oeni*, and trying to develop an efficient transformation system for *O. oeni*, but so far without success. In our laboratory we have tried to electrotransform

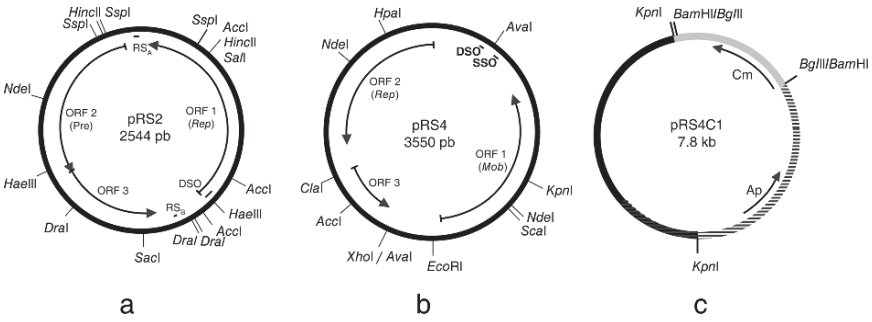

Fig. 23.1 Genetic map of small RC-cryptic plasmids from wine related strains: (**a**) pRS2 as representative of the family of small cryptic plasmids of *O. oeni*; (**b**) pRS4 a plasmid from *P. pentosaceus* with similar characteristics to the family of pRS2; (**c**) pRS4C1 a shuttle cloning vector derived from pRS4. *ORF* Open reading frames; *DSO* double-strand origin; *RS* recombination sequences; *SSO* single-strand origin; *Pre* recombination enzyme; *Rep* replication protein; *Mob* mobilization protein; *Ap* ampicillin resistance marker; *Cm* chloramphenicol-resistance marker

O. oeni using well-established electroporation protocols developed for other LAB (see Kim et al. 1992; Berthier et al. 1996; Caldwell et al. 1996; Alegre et al. 2004; Rodríguez et al. 2007) with gram-positive vectors like pCU1 (Augustin et al. 1992), pBT2 (Brückner 1997) and pRS4C1 (Alegre et al. 2004; Fig. 23.1), as well as derivative plasmids of pRS1, pRS2 and pRS3 (Table 23.1); again, however, we have not had any success.

The introduction and expression of plasmids in *O. oeni* appears to give better results than those obtained by electroporation using conjugation. Zúñiga et al. (1996b, 2003) were able to develop reproducible methods to transform *O. oeni* by conjugation using conjugative transposons and plasmids. They transferred transposons Tn916 and Tn925 from *Enterococcus faecalis*, and plasmids pIP501 and pVA797 from *Lactococcus lactis,* into *O. oeni* by conjugation. However these conjugative plasmids showed structural instability. Later, Beltramo et al. (2004) developed pGID052, a new plasmid that can be successfully transferred by mobilisation from *Lactococcus lactis* to *O. oeni*. This low-copy-number plasmid seems to be structurally and segregationally stable and, to date, is the only realistic candidate vector for future genetic manipulation of *O. oeni*.

23.5 Pediococcus

Pediococci are homofermentative LAB that are commonly found in nature in fermenting plant materials (Giacomini et al. 2000), as well as in beer, cider and wine (Fernández et al. 1995; Gindreau et al. 2001). Several pediococcal strains are important in the fermentation of vegetables, soy milk and meat, and in flavour

development of Cheddar cheese (Fleming and McFeeters 1981; Smith and Palumbo 1983; Thomas et al. 1985). In addition to their ability to produce fermented foods, there are pediococcal strains that produce bacteriocins active against gram-positive pathogenic and food-spoilage bacteria (Daeschel and Klaenhammer 1985; González and Kunka 1987; Hoover et al. 1988; Motlagh et al. 1994). Many pediococcal strains harbour plasmids that encode a variety of traits, while others, being cryptic, have been used to develop cloning vectors (Shareck et al. 2004). To date, genetic studies on pediococcal plasmids have yielded considerable knowledge; however, only few studies on plasmids from wine-related strains of *Pediococcus* have been reported.

23.5.1 Plasmids

One of the first indications of the presence of plasmids in *Pediococcus* was reported by González and Kunka (1983), who detected plasmids from 4.7 to 30 MDa in strains of *P. pentosaceus* and *P. acidilactici*, though no information was provided about their metabolic function. Later, curing studies with novobiocin indicated that production of a bacteriocin-like substance by *P. cerevisiae* FBB63 might be linked to a 10.5-MDa plasmid (Graham and McKay 1985). Daeschel and Klaenhammer (1985) reported that the production of a bacteriocin named pediocin A was associated with a plasmid present in two strains of *P. pentosaceus* isolated from cucumber fermentations. Both bacteriocin immunity and bacteriocin production were encoded by the plasmid. González and Kunka (1986) found that the abilities to ferment raffinose, melibiose and sucrose by three strains of *P. pentosaceus* were encoded by plasmids. The same two authors also reported in 1987 the association of sucrose fermentation and production of pediocin PA-1 with plasmids pSRQ10 and pSRQ11 respectively in *P. acidilactici*, and speculated on the natural role of such plasmid-linked properties that may give to the strains containing these plasmids a selective advantage in nature.

Other authors (Hoover et al. 1988; Halami et al. 2000) have reported linkage of pediococcal sugar utilisation, and bacteriocin production and immunity, to plasmids, but there have been no published reports relating such functions with plasmids of strains of pediococci isolated from wine. However, because some strains of *Pediococcus* isolated from wine seem to produce bacteriocins (Strasser and Manca 1993), it seems likely that some functions of wine-related pediococci may be plasmid-linked.

Some lactic acid bacteria can induce viscosity in wine, beer and cider by production of exopolisaccharides (EPS); these LAB are designated ropy strains. In a study of the presence of plasmids in a ropy strain of *Pediococcus* isolated from Basque Country ciders, Fernández et al. (1995) found six plasmids, one of them related with the ropy character and resistance to oleandomycin. These results are in line with those of Lonvaud-Funel et al. (1993), who observed that non-ropy derivatives of ropy strains of *Pediococcus* had lost some of their

plasmids. Plasmid pF8801 was detected in ropy strains of *P. damnosus* (Lonvaud-Funel et al. 1993) and partially sequenced in order to develop primers specific for the detection by PCR amplification of other ropy strains. The complete nucleotide sequence of this plasmid (Walling et al. 2005) revealed genes for maintenance (*rep*) and transfer (*mob*), and a putative glucosyltransferase gene named *dps*.

In addition to pF8801, only a few plasmids of *Pediococcus* have been completely sequenced (Table 23.2). The first reported complete nucleotide sequences of plasmids of *Pediococcus* were those of pMSB 74 from *P. acidilactici* (Motlagh et al. 1994) and of pUCL287 from *P. halophilus* (Benachour et al. 1997), later reclassified as *Tetragenococcus halophila*. Because plasmid pUCL287 was shown to be a theta-replicating plasmid unrelated with previously well-characterised theta-type replicons, a new family of theta-type replicons represented by pUCL287 was set up. Plasmid pMD136 of *P. pentosaceus* (Giacomini et al. 2000) is another completely sequenced pediococcal plasmid that has been reported to use theta replication; however, while pMD136 showed homology with the theta-type replicons of *Lactococcus lactis*, pSMB 74 was suggested to be a member of the pUCL287 replicon family.

The nucleotide sequence of cryptic plasmid pRS4 (Fig. 23.1) from a strain of *P. pentosaceus* isolated from wine was reported by our group (Alegre et al. 2005). This is the first RC-replicating plasmid of a *Pediococcus* strain that has been completely sequenced and used as a cloning vector for LAB (Fig. 23.1). A second plasmid, named pRS5, which replicates via the theta mechanism, and isolated from the same strain, is currently being studied (see Sect. 23.2).

Table 23.2 Plasmids of *Pediococcus* with complete nucleotide sequence in databases

Plasmid	Size (bp)	Species	Source	Accession number	Characteristics[a]	Reference
pSMB 74	8,877	*P. acidilactici*	–	U02482	Theta, Pediocin AcH	Motlagh et al. (1994)
pUCL287	8,738	*T. halophila*[b]	–	X75607	Theta, Cryptic	Benachour et al. (1997)
pMD136	19,515	*P. pentosaceus*	Cucumber	AF033858	Theta, Pediocin A	Giacomini et al. (2000)
pRS4	3,550	*P. pentosaceus*	Spanish wine	AJ68953	RC, Cryptic	Alegre et al. (2005)
pF8801	5,558	*P. damnosus*	French wine	AF196967	(RC), *dps*	Walling et al. (2005)
pRH478	14,567	*P. damnosus*	Japanese beer	AB218963	(Theta), *hor* A	Suzuki et al. (2006)

[a]*Theta* replication by theta mechanism; *RC* replication by rolling-circle mechanism; () putative replication mechanism; *Cryptic* unknown function; *Pediocin AcH* contains *pap*-gene cluster of the Pediocin AcH; *Pediocin A* contains regulatory genes involved in Pediocin A activity; *dps* glucan synthase gene; *hor* A hop-resistance gene

[b]Previously called *P. halophilus*

23.5.2 Plasmid Transfers into Pediococcus

A number of reports show that some broad-host-range plasmids can be transferred by conjugation and/or electroporation into *Pediococcus* species (González and Kunka 1983; Kim et al. 1992; Benachour et al. 1996; Caldwell et al. 1996; Rodríguez et al. 2007). However, of these only one – pRS4 – has been isolated from a wine-related strain of *Pediococcus* (Alegre et al. 2004). In addition, pRS4 derivatives seem to be useful vectors for food-related LAB, since they replicate in species of *Lactobacillus* and *Pediococcus* but not in *Enterococcus faecalis* (Mesas et al. 2006).

23.6 Conclusion

Plasmids are frequently present in LAB species, but only a few plasmids have been reported from wine-related LAB strains. Some genera like *Lactobacillus* and *Pediococcus* seem to be plasmid-rich, while *Leuconostoc* and *Oenococcus* are not. In general, large plasmids replicate via the theta mechanism and contain genes coding for different traits, while small plasmids usually replicate by the RC mechanism and are cryptic. The stable maintenance of such small cryptic RC plasmids in their hosts, under an apparent absence of selective pressure, can be explained – as in the case of the RC plasmids of *Bacillus* (Guglielmetti et al. 2006) – by considering that they promote recombination and consequently enhance the ecological adaptability of their hosts.

The presence of plasmids in *O. oeni*, the most important LAB in wine-making, has attracted the attention of several groups of researchers. As a consequence of these studies, it can be concluded that small plasmids are less frequent in *O. oeni* than in other LAB. A few small cryptic plasmids are widespread among strains of this species (Fremaux et al. 1993; Brito et al. 1996; Zúñiga et al. 1996a; Alegre et al. 1999; Mesas et al. 2001), and there have been some attempts to use these plasmids as tools for genetic manipulation of LAB, but without successful results to date. The low degree of conservation of *rep* and *pre* sequences in the small RC plasmids of *O. oeni* can be attributed to the fast rate of evolution of *O. oeni* compared with other gram-positive bacteria (Yang and Woese 1989; Brito et al. 1996; Makarova and Koonin 2007). Large plasmids, sometimes with known roles, are also more frequently present in *O. oeni*, than would be expected a priori (Sgorbati et al. 1987; Brito and Paveia 1999), suggesting that a lot of genetic information from *O. oeni* may be contained in large plasmids.

Future work on wine-related-LAB plasmids will require new vectors and transformation systems, notably for *O. oeni*; however, the availability of LAB genome sequences (Makarova and Koonin 2007) together with the development of other genetic tools will undoubtedly facilitate the development of strains of LAB with improved wine-making properties.

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Chapter 24
Rapid Detection and Identification with Molecular Methods

Jürgen Fröhlich, Helmut König, and Harald Claus

24.1 Introduction

The only absolute criterion of purity for a bacterial culture is that it has been derived from the progeny of a single cell. Failure to apply this criterion may lead to much effort in proving the purity of a culture. All strains upon which research is to be based should therefore be rigorously purified before starting to investigate the properties of individual organisms (Johnstone 1969). Ecologically oriented wine microbiologists are especially faced with the problem of how to obtain a pure culture of certain microbial strains from their densely populated natural habitats. The used methods comprise thereby a range from simple devices up to very complex machines. Most approaches to identify and enumerate microbes in wine use enrichment techniques (Fugelsang and Edwards 2007). Such indirect methods do not enumerate the original cell number in the sample, but their progeny, as enriched in a specific medium. Fugelsang and Edwards (2007) describe both general and selective growth media for plating yeasts and bacteria from wine. Unfortunately, plating and enrichment procedures are time consuming as colonies for some wine-related microbes take up to a week or more to appear on a Petri dish. Additionally, once colonies appear on a plate, the identification of the microbes requires further testing. Moreover, sublethally injured or viable but nonculturable cells, common in wine, may fail to grow on plates but are metabolically active. As a rule, culture-based techniques typically underestimate the size and diversity of a population (Kell et al. 1998; Millet and Lonvaud-Funel 2000). For monitoring the succession of a microbiota, cultivation-free molecular biological approaches were applied which give a more realistic view of a population. These spatiotemporal "snapshots" are often presented in the form of gel electrophoretic pattern of PCR amplicons or

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pictures of fluorescence in situ hybridization (FISH) which allow a simultaneous visualization of the main role players within a population on species level (Amann et al. 1995; Mills et al. 2002; Hirschhäuser et al. 2005; Röder et al. 2007a, b). Unfortunately, there are no cultivable-free techniques available that could represent and monitor populations on strain level. Up to now, all methods that fit the strain level are culture dependent. With focus on a single cell, micromanipulation techniques are alternative methods to traditional cultivation approaches and a useful tool when complex habitats are investigated without cultivation (Fröhlich 2002; Fröhlich and König 1998; 1999a, b; 2000; Fröhlich et al. 2002). After the isolation of a single cell, different methods for identification on species or strain level can be applied, which are described below.

24.2 Micromanipulation Techniques

24.2.1 Historical Perspective

Since the beginning of the twentieth century, several attempts have been made to improve the management of single prokaryotic and eukaryotic cells by using micromanipulator techniques. Thereby, a suspension of an adjusted concentration of microorganisms was aspirated into a simple capillary tube, so that a single cell was transferred statistically in a defined volume (Harbeck and Rothenberg 1995).

Moreover, a survey of the chief methods devised for single organism cultures was presented by Johnstone (1969, 1973). These include the block cut method for the selection of an isolated organism on a lightly inoculated nutrient gel, formation of droplets with micropipettes, which are searched for those containing single organisms, and isolation by carrying the selected organisms across the sterile gel surface with a microneedle. Because of technical problems and disadvantages, these methods were not adopted for routine isolation.

Other attempts to improve the management of single microbial cells by using micromanipulator techniques have been described in the literature. Either microneedles or microcapillaries were used for the separation of single bacterial cells (Skerman 1968; Bakoss 1970; Johnstone 1973; Thomsen et al. 2004). The techniques suggested more than 30 years ago were based on the state of the art at that time. They were faced with several technical disadvantages, which hampered routine usage of the isolation techniques for a broad spectrum of prokaryotes in a microbiological laboratory. The magnification was limited and a transfer of single cells was hardly possible (Skerman 1968). It was designed for use with low power objectives (e.g., 10×) with a working distance of 7 mm or more. The instrument consists of a lens collar and magnetic tool carrier. The lens collar was clamped onto the objective and it contained two steel slides which permitted the magnet tool carrier to slide along freely. Knobs or microloops were the most useful tools for the isolation of cells from colonies on solid agar plates. By several operations, cells

were floated across the surface of solid media by lateral movement of the Petri dish and they were well separated from the original population. Attempts to lift single organisms in a loop for transfer were rarely successful. So far, this method has been applied for the isolation of large filamentous bacteria (Bradford et al. 1996) and cyanobacteria (Bowyer and Skerman 1968). Bakoss (1970) cloned single cells of leptospires with a micropipette connected to a syringe via a thin polyethylene tube, which was fastened to the holding clip of a micromanipulator. He used a syringe as a simple pneumatic system. The disadvantage of this micromanipulator technique was that it was laborious. A mechanical micromanipulator with a microneedle was also used (Sherman 1973) to separate the four spores from a cluster in a yeast ascus. This technique was also applied for this purpose in our institute and it is also suitable for the separation of larger bacterial cells (>3 μm) by moving them onto an agar surface. Coccoid bacteria from the "corn cob" of human dental plaques were successfully isolated by Mouton et al. (1977) with microneedles designed to be a double-angulated microhook as described by Johnstone (1973). Single selected spores of *Bacillus cereus* adhering to the glass point of capillary tubes were selectively removed from Petri dishes (Hamilton 1978). Micromanipulation was also successfully applied for the isolation of *Pedomicrobium* cultures from water samples (Sly and Arunpairojana 1987). Luttermann et al. (1998) described a micromanipulation method for transferring micro-objects such as bacteria from agar plates with microcapillary tubes. An angulated capillary tube (angle of 90°) is positioned between the condenser and the objective. The agar plate with the selected bacteria is moved below the opening of the capillary tube with the microscope stage. The aspirated bacterium is placed on the surface of a solid medium or in liquid media in microtiter plates.

24.2.2 Modern Equipment

Since the 1960s, the technical equipment of micromanipulators has been greatly improved. A long distance objective (Zeiss, Oberkochen, Germany) with a magnification of hundred-fold is now available. This allows manipulation at a magnification of a thousand-fold and more with an inverse microscope. The capillary tools can be positioned quickly and precisely. The available pneumatic or hydraulic systems are very accurate pressure devices.

For the isolation of microbial cells, a commercial micromanipulator (Eppendorf, model 5171) equipped with a pressure device (Eppendorf model 5246 plus or CellTram Oil) and mounted onto an inverse phase contrast microscope (Axiovert 25; objective CP "Achromat" 100×/1.25 Oil Ph2; Zeiss) is used (Bactotip method) (Fröhlich 2002; Fröhlich and König 1998a, b; 1999a, b; 2000; Fröhlich et al. 2002; Prüss et al. 1999; Fig. 24.1). The magnification is adjusted from 400× to 1,000×. The micromanipulator is used according to manufacturer's instructions (micromanipulator 5171: Operating Manual; CellTram Oil: Operating Manual; Transjector 5246: Operating Manual; Eppendorf, Hamburg, Germany). The diameter of the opening

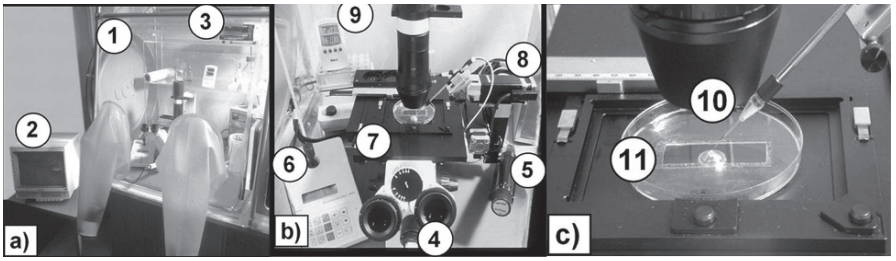

Fig. 24.1 Working station for the manipulation of single cells with a COY chamber (**a**) for the aerobic and anaerobic isolation. The isolation of single cells is performed using an inverse microscope and a micromanipulator device (**b**). The spreaded cells are aspirated by the application of a Bactotip (**c**). Technical specifications: COY chamber (1), monitor (2), O_2/H_2 electrode (3), camera (4), CellTram Oil (5), joystick (6), inverse microscope (7), micromanipulator (8), thermometer/hygrometer (9), Bactotip (10), cover slip with spreaded bacteria (11)

of the capillary tip can be adjusted to the size of the bacterial cell of interest. For the isolation of bacteria, a sterile capillary tube ("Bactotip"; Fig. 24.2a, b) is used, which preferably possesses a beveled tip (angle 45°) usually with an opening of about 5–10 µm at the anterior end. The sterile Bactotips are produced by Eppendorf (Hamburg) or can be manufactured with a capillary puller (Saur, Reutlingen, Germany) and a microgrinder (Saur, Reutlingen) using capillary tubes type GB 100 TF-8P (Science Products GmbH, Hofheim, Germany). The posterior end of the Bactotip is sealed with a droplet of sterile oil. If desired, the inner surface of the tip can be siliconized with dichlorodimethylsilane (Fluka Chemie AG, Buchs, Switzerland). This is advisable if the bacteria tend to adhere to glass surfaces. Our experiments show that desiccation and oxygen stress (Krämer 1997) for the isolation of anaerobic and aerobic microorganisms can be avoided by using a glove box with a N_2/H_2 (95:5; v/v) atmosphere (COY chamber, Toepfer Lab Systems, Göppingen, Germany). The relative humidity in the chamber is adjusted from 95 to 100%. The microscope bulb is replaced by an optical fiber device (Schott, Mainz, Germany) which reduces the IR radiation. The microscope is equipped with a CCD camera (Type AVTBC12CE, Zeiss) and a monitor (Type PM 95 B, Zeiss).

24.3 Isolation Techniques

24.3.1 Bactotip Method

This technique (cf. Fig. 24.2a) is used when single cells are cultured in liquid media or genes are going to be amplified by single-cell PCR. Cultures or complex mixtures of prokaryotic or eukaryotic strains are diluted in 1–10 mL phosphate-buffered saline

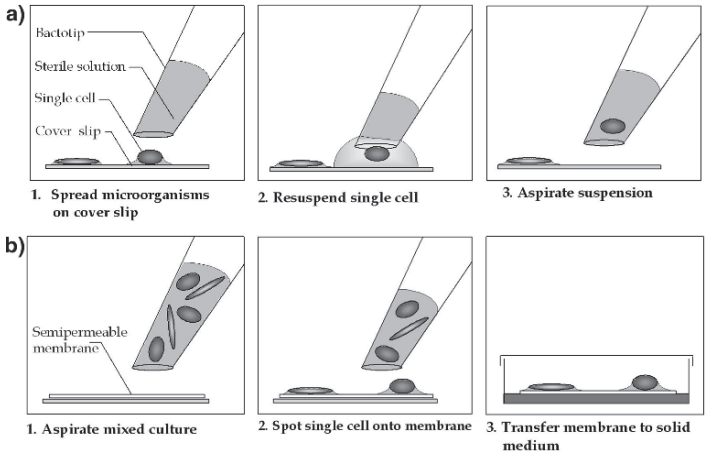

Fig. 24.2 Bactotip (**a**) and Membrane (**b**) methods for the isolation of single cells

(1× PBS). An aliquot of the suspension (10 µL) is spread as a thin film on a sterile microscopic cover slip (24 × 60 mm). A small volume of buffer or medium (ca. 0.1–0.2µL) is aspirated into the capillary tube. When the opening of the Bactotip is brought close to the surface of a distinct microorganism, a droplet flows out of the tip and moistens the selected cell. The cell is suspended in the droplet after detaching from the glass surface and aspirated into the Bactotip. About ten single microbial cells are successively removed from the microscopic slide within 30 min by aspirating them together with the droplet into the Bactotip. The withdrawn single cells can be transferred in Eppendorf reaction tubes or Hungate tubes (anaerobes) containing 0.3 mL of the corresponding liquid medium. The tubes are incubated at, e.g., 37°C for 10–72 h (Fröhlich and König 1999a, 2000; cf. Prescott et al. 2002).

24.3.2 Membrane Method

In contrast to the Bactotip method, an appropriate dilution (ca. 0.1 µL) of a mixed culture is sucked into the capillary tube (cf. Fig. 24.2b; Fröhlich et al. 2002). The tip is brought close to the surface of a semipermeable membrane (dialysis hose; Roth) and single cells are spotted under visual control on the membrane in a distance of 5–10 mm to each other. Subsequently, the membrane will be removed with sterile tweezers and transferred onto a solid medium. Nutrients diffuse through the membrane and enable individual cells to grow up to colonies. In contrast to the Bactotip method, more cells can be isolated in a little while. The use of a dialysis membrane has the advantage of a very smooth surface compared with

the application of agar layers, so that very small microorganisms can be separated without limitation of the visual control.

24.3.3 Efficiency of the Cloning Procedure

The efficiency rate of the cultivation from freshly grown laboratory cultures was between 30% (*Escherichia coli*) and 70% (*Staphylococcus aureus*) (Fröhlich and König 1999a), and the isolation of lactic acid bacteria (LAB) could be performed with similar rate. The *Oenococcus oeni* strains B70 and B139^T could be isolated with an efficiency between 63 and 67%. Similar results could be obtained with the species *Lactobacillus brevis* (66%) and *Pediococcus damnosus* (70%). The fastidious anaerobe *Bifidobacterium bifidum* could be micromanipulated anaerobically in the anaerobic chamber with a yield of 30% (Fröhlich et al. 2002). Thereby, the selection of the isolation method did not have any influence. The single cells grew up to a visible density or a visible colony in 10–72 h. A single cell could also be transferred onto solid media in Petri dishes as proved with *B. cereus*. The colonies become visible after incubation overnight at 37°C. Furthermore, single cells (e.g., *B. cereus*) were directly grown in the Bactotip.

Spreading of the bacterial suspension onto a microscopic slide after an appropriate dilution of the original culture was a prerequisite for the rapid isolation of the single cells, while the isolation of a single bacterial cell directly out of a droplet containing a suspension of a mixed microbial population was not successful. The cells should be transferred to the culture medium within 30 min after spreading. The application of the Bactotip method allows the transfer of single prokaryotic cells to different culture vessels such as Eppendorf reaction tubes, Hungate tubes, onto the surface of solid media in Petri dishes or to subject the isolates to single-cell PCR. Cells were also directly grown in the Bactotip. The advantage of the Bactotip method compared with conventional isolation methods can be seen in the ability to pick out a single prokaryotic cell under direct visual control and to grow pure cultures of distinct aerobic and anaerobic cells directly out of a mixed natural or laboratory population in a relatively short time.

24.4 Laser Micromanipulation Systems

24.4.1 Optical Tweezers

Ashkin et al. (1987) described the use of infrared laser beams (1,064 nm) for trapping and manipulation of biological specimens such as the single cells of *Escherichia coli* and *Saccharomyces cerevisiae*. This method was improved and

successfully applied for the isolation of hyperthermophilic bacteria and archaea (Huber 1999; Huber et al. 1995).

A neodymium laser is focused by a microscope objective. The movement of the microscope stage is computer controlled. A rectangular glass capillary with a predetermined breaking point is used as separation chamber (inside dimensions 0.1 × 1 mm, length 10 cm), which is filled with fresh medium (90%) and the mixed microbial population (10%). A single selected cell is fixed with the laser beam and is separated from the mixed culture by moving the microscope stage. The capillary is broken at the predetermined breaking point and the single cell is transferred to the culture medium. The culture efficiency after an incubation time of up to 5 days was 20–100%. The isolation of dead cells could be prevented by application of fluorescent dyes staining viable cells for example with bis-(1,3-dibutylbarbituric acid) trimethine oxonol (Beck and Huber 1997). Photo damage can be reduced to background level under anaerobic conditions (Neumann et al. 1999). This method is a promising tool for the isolation of microorganisms, which cannot be obtained in pure culture by conventional methods.

24.4.2 Laser Microdissection

Schütze et al. (1998) described a laser pressure catapulting method (LPC), which uses a laser (Robot-Micro-Beam) for the microdissection and transfer of single cells. This method has been successfully applied for the isolation of single cells from human tissues. The specimens are spread on a sheath of a 1.35-µm thin polyethylene membrane. With the high photonic energy of a focused nitrogen laser, a selected single cell is precisely circumscribed and the selected cell together with a small surrounding strip of the polyethylene membrane is cut out. The round polyethylene slip with the selected cell still adheres to the polyethylene membrane. The laser is then focused below the microdissected target cell and the microdissected sample is catapulted into the oil-dampened cap of a common Eppendorf reaction tube positioned above the sample with a laser shot of increased energy. The cells are subjected to single-cell nested PCR. In principle, this method can be applied to cells of any size, but an application for the isolation of viable prokaryotes has not been published so far.

24.5 Molecular Biological Techniques

24.5.1 Resolution of Molecular Biological Methods

The identification and classification of microorganisms are of great importance in microbial ecology. Many different molecular biological methods are presently being applied for microbial identification and classification. Each of these

methods permits a certain level of phylogenetic classification from higher levels to the genus-, species-, subspecies-, and strain-specific level (Fig. 24.3). Moreover, each method has its advantages and disadvantages with regard to convenience of application, reproducibility, equipment, and level of resolution. Despite of a greater genome in size, the comparison of eukaryotic and prokaryotic microorganisms reveals that classical phylogenetic marker like small subunit (SSU) rDNA is less sensitive in resolution for eukaryotic cells. For yeast, only the species level could be reached (gray-colored columns, Fig. 24.3) or for fungi like *Botrytis* sp. ITS analysis or SSU rDNA matches only the genus level or phylogenetic levels below (dark gray-colored columns ITS (dotted column), Fig. 24.3; Hirschhäuser and Fröhlich 2007). Bacterial species are considered to be groups of strains that are characterized by a certain degree of phenotypic consistency, by a significant degree of DNA hybridization (>70%) and over 97% of SSU ribosomal RNA (rRNA) gene sequence identity (Stackebrandt et al. 2002). Although 16S rRNA gene sequences and DNA–DNA hybridization continue to be considered as molecular criteria for species delineation, it is anticipated that much additional taxonomic information and therefore a more adapted natural species concept can be extracted from complete genome sequences. On the base of comparison of whole genome sequencing, modern phylogenetic marker beyond the unsatisfactory rRNA approach was compiled by Coenye and coworkers (2005).

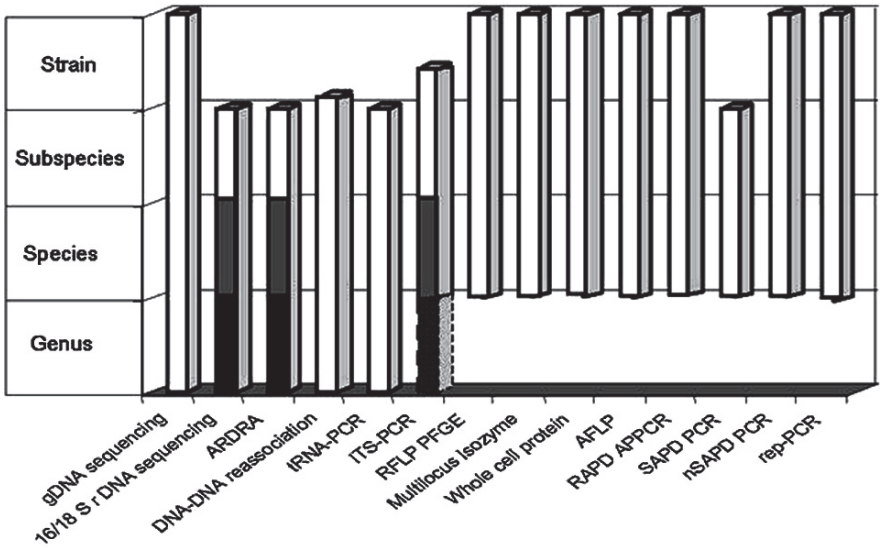

Fig. 24.3 Taxonomic resolution of some approaches for assessing taxonomic relationships. Taxonomic categories from domain to family were not figured. However, sequencing of genomic DNA (gDNA) or SSU rDNA fit also these phylogenetic levels

24.5.2 Molecular Biological Techniques

For identification on species level, the rRNA approach could be applied. After isolation of a clone from an axenic culture or manipulated cell, the rDNA could be amplified by a standard PCR technique following methods like cloning and sequencing. Also, rDNA-derived techniques like FISH or T/DGGE could be used.

DNA-based typing methods, in which a DNA banding pattern is generated, include the separation of macrorestriction fragments by pulsed-field gel electrophoresis (PFGE) and various PCR-based methods like rep-PCR, BOX-PCR, nSAPD-PCR, RAPD-PCR, and AFLP fingerprinting (Van Belkum et al. 2001; Tenover et al. 1995; Gurtler and Mayall 2001; Fröhlich and Pfannebecker 2007; Pfannebecker and Fröhlich 2008). The banding patterns obtained by these methods can be altered in various ways. Chromosomal rearrangements including large insertions, deletions, or mobile genetic elements like transposons or retrotransposons can have an enormous effect on banding patterns generated by restriction enzyme analysis-based methods like PFGE, as well as on patterns obtained with several PCR-based methods. The loss or gain of restriction sites or primer-binding sites can also result in modified patterns. Methylation of the restriction sites also hampers the cleavage by restriction enzymes. Subsequently, a part of the methods is described more precisely. Further assembled methods for acetic acid bacteria and yeast could be consulted in Sects. 2.3, 2.4, and 3.3.2.

24.5.2.1 Decontamination and DNA Enrichment Techniques for Low Contents of Genomic DNA (gDNA)

In the past, PCR of single cells was difficult to perform because the reaction often failed or amplification techniques like nested PCR are very sensitive to contaminated DNA. Nowadays, standard PCR approaches can be decontaminated using psoralen (Jinno et al. 1990) or genomic DNA of a single cell can be amplified in an isothermal PCR approach (Notomi et al. 2000; Hayashi et al. 2007). Moreover, after application of FISH, single cells with a bright fluorescent signal were isolated using a micromanipulator and the genome of the single isolated cells served as a template for multiple displacement amplification (MDA) using the Phi29 DNA polymerase (Kvist et al. 2007).

24.5.2.2 Amplified Ribosomal rDNA Restriction Analysis (ARDRA)

On the surfaces of grapes, in musts and wines, LAB are found in a great variety. The occurrence of wine-spoiling LAB and yeasts during fermentation highlights the close relationship between the wine microbiota and the quality of the wine. For these reasons, the analytical technique ARDRA allows a fast and reliable identifica-

tion of wine microorganisms. The analyses launch with the amplification, directly from colony, of 16S/18S rDNA and later digestion with one of the following restriction enzymes *Bfa*I, *Mse*I, and *Alu*I. A sequential use of the three enzymes is proposed to simplify LAB wine identification, first *Mse*I, then *Bfa*I, and finally *Alu*I digestion. The discriminated isolates belonged to the species *Lactobacillus brevis*, *L. collinoides*, *L. coryniformis*, *L. hilgardii*, *L. mali*, *L. paracasei*, *Leuconostoc mesenteroides*, *Oenococcus oeni*, *Pediococcus parvulus*, and *P. pentosaceus* (Rodas et al. 2003).

24.5.2.3 Nested Specifically Amplified Polymorphic DNA PCR (nSAPD-PCR)

The nSAPD-PCR was developed as a versatile method for identification and discrimination of strains and genotypes from various organisms from bacteria to humans and was also successfully applied to distinguish between strains of LAB like *Oenococcus oeni*, *Pediococcus parvulus*, *Lactobacillus hilgardii*, *Leuconostoc mesenteroides*; yeasts like *Saccharomyces cerevisiae*, *Dekkera bruxellensis*, *Candida* sp.; and fungi like *Botrytis cinerea*, *Sclerotinia minor*, *Sclerotinia sclerotiorum*, *Monilinia fructigena* (Fröhlich and Pfannebecker 2007). The method is based on the principle of the RAPD-PCR. Unlike the RAPD-PCR, the nSAPD-PCR uses specific primers including the *Not*I recognition site and additional nucleotides. The whole primer set consists of 20 primers (first PCR: 4 primers, nested PCR: 16 primers). In contrast to the RAPD-PCR, the nSAPD-PCR primers are not restricted to a small group of species. The nSAPD-PCR is a method which improves the strain discrimination power of RAPD-PCR in combination with a high reproducibility. This could be achieved by using a prolonged ramp in the first PCR (SAPD-PCR) which supports the annealing step. The nested PCR without a ramp and the usage of an enhancer solution improves the specificity. After electrophoretic separation, reliable DNA fingerprints were generated for cluster or descent analysis. As a rule, the first PCR discriminates in dependence from the investigated species on the species or subspecies level, while the nested SAPD-PCR is able to resolve strains (Fig. 24.3).

24.5.2.4 Sequence Characterized Amplified Region PCR (SCAR-PCR)

A further method which could be applied directly after (n)SAPD-PCR is the sequence characterized amplified region PCR. A species- or strain-specific band of a pattern was cut off the gel, reamplified by PCR, and subsequently cloned (Nakano et al. 2004). Finally, the insert was sequenced and specific primers flanking the amplified segment were generated (Fröhlich and Pfannebecker 2007).

24.5.2.5 Fluorescence In Situ Hybridization (FISH)

A rapid method for the identification and enumeration of LAB and yeasts from wine is the fluorescence in situ hybridization (Amann et al. 1995). This technique uses fluorescently labeled oligonucleotide probes targeting the rRNA of a species. Probes were used to identify species in different wines, making it evident that direct identification and quantification from natural samples without culturing are also possible. The results show that FISH is a promising technique for the rapid identification of LAB and yeasts, allowing positive identification within a few hours for common wine species *Oenococcus oeni*, *Pediococcus damnosus*, *P. parvulus*, *P. pentosaceus*, *Lactobacillus plantarum*, *Lb. casei/paracasei*, *Lb. brevis*, *Lb. hilgardii*, and most *Leuconostoc* species among others (Blasco et al. 2003; Hirschhäuser et al. 2005; Röder et al. 2007a, b). Xufre et al. (2006) developed 26S rRNA gene probes targeting the D1–D2 region for identification of numerous wine-related yeast including *Saccharomyces cerevisiae*, *Candida stellata*, *Hanseniaspora uvarum*, *H. guilliermondii*, *Kluyveromyces thermotolerans*, *K. marxianus*, *Torulaspora delbrueckii*, *Pichia membranaefaciens*, and *Pi. anomala*. Moreover, Röder et al. (2007a, b) demonstrated that there are further target sides downstream the common used D1–D2 regions of the large subunit (LSU) rRNA of yeasts. Though, low signal intensity due to poor probe hybridization efficiency is one of the major drawbacks of rRNA-targeted in situ hybridization. To overcome problems with a poor signal-background ratio or confusion with autofluorescent wine constituents, several attempts were performed to increase the yield in fluorescence. Stender et al. (2001) used peptide nucleic acid probes to identify the spoilage yeast *D. bruxellensis* because PNA–rRNA hybrids are very stabile under stringent hybridization conditions. The usage of unlabeled helper probes (Fuchs et al. 2000) or side probes, a set of partially complementary fluorescently labeled probes, also supports the microscopic analysis (Hirschhäuser et al. 2005; Röder et al. 2007a, b). It could also be shown that the nature of the labeled fluorescent dye was also involved in hybridization. Differences in quantum yield could be measured if dyes like carbocyanine 3 (CY3), carboxyfluorescein (FAM), or carboxytetramethylrhodamine (TAMRA) were used. In comparison to FAM and TAMRA, CY3 is the most used label in FISH, since it has a high absorption coefficient and a high quantum yield, shows little bleaching and is pH insensitive (Fuchs et al. 2001). On the other hand, FAM shows a fluorophor-dependent quenching at various target sites by electronic interaction with guanosine. This quenching phenomenon led to the development of so-called smart probes. They, like molecular beacon, are self-complementary at the 5′ and 3′ endings but instead of a fluorescence resonance energy transfer (FRET) dye system, the overlapping fluorochrom is quenched via charge transfer by the cumulative guanosine nucleotides. If the probe is involved in hybridization with the requested DNA target, the dye nucleotide interaction will be finished and finally emission occurs. Another approach for improving FISH is the use of fluorescent DNA oligonucleotides

modified to contain locked nucleic acid (LNA) residues. This increases the thermal stability of hybrids formed with RNA. The LNA-based probes detect specific RNAs in fixed yeast cells with an efficiency far better than conventional DNA oligonucleotide probes of the same sequence (Thomsen et al. 2005; Kubota et al. 2006).

24.5.2.6 PCR Temperature/Denaturing Gradient Gel Electrophoresis (PCR-T/DGGE)

PCR–DGGE and PCR–TGGE of rRNA gene fractions were applied to differentiate individual wine yeast isolates (Manzano et al. 2004, 2005) or to monitor the succession of the yeast microbiota during fermentation (Mills et al. 2002). The study of Mills and coworkers revealed that PCR–DGGE signals for several non-*Saccharomyces* yeast populations could persist into the fermentation and long after these yeasts could be identified on culture media. Furthermore, these methods were used to investigate the LAB microbiota on grape surfaces that were enriched by means of different media. Spano et al. (2007) used the PCR–DGGE for monitoring *Lactobacillus plantarum* and *Oenococcus oeni* in red wine. They reported that the PCR–DGGE method, based on the *rpoB* gene as molecular marker, is a reproducible and suitable tool to monitor spoilage microorganisms during wine fermentation. PCR–DGGE was also used to examine the bacteria that developed in enrichment cultures from grapes. Species of the genera *Lactobacillus*, *Enterococcus*, *Lactococcus*, and *Weissella* were detected in enrichments by plating and PCR–DGGE (Bae et al. 2006). The complexity and diversity of the wine microbial consortium on grape berries, in must during fermentation and in wine during aging, was investigated by Renouf et al. (2007). On grapes, 52 different yeast species and 40 bacteria could be identified. The diversity was dramatically reduced during winemaking then during aging. Nevertheless, the routine usage of these techniques for identification purposes is technically problematic because each visible band of a gel must be previously identified by control strains. Also, it was observed that different DNA amplicons could be "felted" during PCR amplification and band comigration while gel electrophoresis occurs (Gafan and Spratt 2005).

24.5.2.7 Real-Time PCR/Quantitative PCR (qPCR)

Real-time PCR is a PCR-based method using fluorescently labeled probes and DNA-intercalating fluorescence stain. The method permits a quantification of DNA during the amplification. For indirect cell counting standardization with housekeeping genes or reference samples with known DNA content were used. A few grapes- and wine-related microbes could be identified and monitored so far. Hierro et al. (2007) applied the real-time PCR for the rapid quantification of *Saccharomyces* sp. and *Hanseniaspora* sp. during fermentation. They designed specific primers for the region

spanning the internal transcribed spacer 2 (ITS2) and the 5.8S rRNA gene or universal yeast primers were designed from the variable D1/D2 domains of the 26S rRNA gene (Hierro et al. 2006; Phister et al. 2007). The qPCR assay for enumeration of *Hanseniaspora* sp. in must and wine can detect 10 cells per mL. The approach is linear over four orders of magnitude and is not influenced by high concentrations of contaminating *S. cerevisiae* DNA. Also, *D. bruxellensis*-infected wines were investigated by using the same gene region. The assay was linear over a range of cell concentrations (6 log units) and could detect as little as 1 cell per mL in wine (Phister and Mills 2003). Rawsthorne and Phister (2006) analyzed *Zygosaccharomyces bailii* a major food and beverage spoilage organism in grape juice and wine. They could detect 22 cells per mL from grape juice. The assay was equally efficient in wine, detecting 6 cells per mL and provides a rapid and accurate method to establish the levels of the total *Z. bailii* population which consists of both viable and nonviable cells. The correlation was high between qPCR and total cell count as determined by fluorescent microscopy. For the detection of spoiling pediococci, also a real-time PCR approach could be established. The detection limit in wine was 40 cells per mL for ropy *P. damnosus* (Delaherche et al. 2004).

24.5.2.8 Pulsed-Field Gel Electrophoresis (PFGE) for Strain-Specific Differentiation

Placing DNA samples in a solid matrix (most commonly agarose or polyacrylamide) and forcing the molecules to migrate through the gel under a static electric field is the basis of conventional gel electrophoresis. The separation of molecules of different sizes predominantly depends on the sieving properties of the gel matrix. In conventional agarose, all DNA molecules larger than 20 kb will show essentially the same mobility in a static electric field and, thus, will not be separated from each other.

PFGE avoids molecular sieving (and its limitations) by using a completely different separation mechanism that maintains size-dependent electrophoretic mobilities of large DNA molecules. In the absence of external forces, DNA molecules exist in a relaxed form. Under the influence of an electric field, the DNA samples elongate, align with the field, and migrate toward the anode by a process termed "reptation." After the removal of an electric field, the elongated DNA molecules relax back to their original state. When a second electric field is applied in a different angle to the first field, the DNA must change conformation and reorient before it can migrate in the direction of the second field. The time required for this reorientation has been found to be very sensitive to the length of the molecule. Smaller molecules that rapidly reorient migrate most of the time along the electric field. Larger DNA molecules take more time to realign after the field is switched than smaller ones, because of the physical barrier of the agarose matrix. Hence, molecules of increasing size must spend a large portion of each switching cycle reorienting before they can migrate through the gel. As long as the alternating fields are equal with respect to length and voltage, the DNA will migrate in straight path down the gel that reflects the sum of the many zigzag steps actually taken.

PFGE allows separation of large DNA molecules (up to 12 Mb) due to molecular reorientation produced by periodic changes in the electric field (Herschleb et al.

2007). The change frequency of electric fields is referred as switch interval, switch of time, or time pulse. Duration of alternative electric fields establishes the DNA dimensions range that are possible to resolve in PFGE. Pulses can go from some seconds to resolve molecules with some kb to more than 1 h for molecules larger than 5 Mb.

The opposing electric fields create two distinct directions for the DNA molecules as they travel through the gel. The reorientation angle or rotor angle is the difference between these two paths. For most purposes, a fixed angle of 120° is sufficient; however, adjusting the reorientation angle can improve separation of small and large DNA molecules in a mixture.

Besides pulse time and reorientation angle, PFGE resolution is remarkably sensitive to changes in all electrophoretic parameters such as agarose concentration and quality (Kirkpatrick et al. 1993), buffer composition, and temperature. Selection of the gradient voltage is also very important since any change in this parameter drastically changes the dimension of resolved molecules.

Modifications of the original method introduced by Schwartz and Cantor (1984) are field inversion gel electrophoresis (FIGE), contour-clamped homogeneous electric fields (CHEF), or transversal alternating field electrophoresis (TAFE). Rotating field electrophoresis (RFE) is an improvement of the original method, allowing continuous variations of all relevant PFGE parameters, as well as two-dimensional separations and conventional electrophoresis. Straight-line separations of DNA fragments in the 0.1–6,000 kb range are accomplished by varying pulsed time and orientation of the electrodes (Ziegler et al. 1987).

Conventional methods of DNA extraction use forces that lead to breakage of DNA molecules, reducing their dimensions. With adequate protocols, it is possible to obtain DNA molecules of 500 kb in solution. For larger molecules, it is necessary to protect them from both mechanical shearing and nucleolytic degradation during the entire isolation process. Individual cells are embedded in agarose, which protects the DNA against breakage while allowing the free flow of solutions necessary for lysis and digestions (Herschleb et al. 2007).

The development of methods to prepare and analyze large DNA molecules contributed to the development of PFGE as a powerful instrument in molecular biology. It is used to evaluate genome dimensions, to construct physical genome maps, cloning of large DNA inserts, separation of large plasmids, and to analyze genomes of eukaryotic cells.

PFGE analysis of large genomic DNA fragments obtained by digestion with rare cutting enzymes (macrorestriction analysis) and of whole chromosomes (karyotyping) are techniques with high reproducibility and discriminative power, which are widely used for epidemiological studies with clinical bacteria (Ribot et al. 2001; Andrei and Zervos 2006) and yeasts (Bellis et al. 1987; Jang et al. 2005; Lukácsi et al. 2006).

The strain specificity of macrorestriction profiles obtained by PFGE makes this technique also useful to track biotechnological-relevant bacteria in natural environments (Claus et al. 1992, 1995) and food (Huys et al. 2006). For vinology, it might become a powerful tool for monitoring the fate of starter cultures and analyzing microbial wine communities. Tables 24.1 and 24.2 comprise PFGE approaches to differentiate wine-relevant bacteria and yeasts on the strain level.

Table 24.1 Use of macrorestriction profiles to characterize wine-relevant lactic acid bacteria

Species	References
Oenococcus oeni	Lamoureux et al. (1993)
	Daniel et al. (1993)
	Kelly et al. (1993)
	Tenreiro et al. (1994)
	Zapparoli et al. (2000)
	Sato et al. (2001)
	Guerrini et al. (2003)
	Malacrinò et al. (2003)
	Lechiancole et al. (2006)
	Larisika et al. (2008)
Leuconostoc sp.	Tenreiro et al. (1994)
Pediococcus sp.	Luchansky et al. (1992)
	Barros et al. (2001)
	Simpson et al. (2002, 2006)
Lactobacillus sp.	Zapparoli et al. (1998)
	Rodas et al. (2005)

Table 24.2 Use of karyotyping to characterize wine-relevant fungi

Species	References
Saccharomyces sp.	Giudici et al. (1998)
	Vaughan-Martini et al. (1993)
	Versavaud et al. (1995)
	Nadal et al. (1996)
	Guijo et al. (1997)
	Petersen et al. (1999)
	Naumov et al. (2001)
	Mitterdorfer et al. (2002)
	Naumova et al. (2003)
	Špirek et al. (2003)
	Martinez et al. (2004)
	Cocolin et al. (2004)
	Glover et al. (2005)
	Puverenti et al. (2005)
	Valero et al. (2005, 2007)
	Divol et al. (2006)
	Ribeiro et al. (2006)
	Le Jeune et al. (2007)
Botrytis cinerea	Vallejo et al. (1996)

The duration of the PFGE procedure (4 days from sample preparation to result) might be regarded as the main drawback of the method, especially when there is a demand of rapid reactions toward spoiling microorganisms to control vinification processes. In future, this hindrance might be overcome by new technical developments which could shorten PFGE runs (Birren and Lai 1994; Wagner and Lai 1994) and short protocols for sample preparation (Ribot et al. 2001; Simpson et al. 2006).

24.6 Conclusions

Modern techniques for separation of microbial cells followed by DNA amplification and application of molecular biological tools permit even the investigation of a single cell without cultivation. Many of the technical problems regarding the separation of a single cell have been solved. For cultivation, the remaining problems to be solved are now less of technical nature than choosing a suitable medium composition for growing cells of, e.g., unknown systematic affiliation.

Different diagnostic techniques support the identification and characterization on variable phylogenetic levels for culturable or nonculturable microorganisms. Techniques like FISH, DGGE, qPCR, fingerprint approaches, and others are suitable for simultaneous identification or cell counting or monitoring of wine-related microorganisms. Nevertheless, the technical progress in the development of genetic tools and devices cannot hide the fact that there are unsolved problems in investigation of cell-to-cell communication, cofermentation, and genetical drift in focus of environmental changes and changes of the gene regulation and physiology of a certain strain.

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Chapter 25
Maintenance of Wine-Associated Microorganisms

Helmut König and Beate Berkelmann-Löhnertz

25.1 Introduction

A great variety of microorganisms growing on grapes, in must or in wine have been isolated, which also have an influence on wine quality. They belong to acid tolerant microorganisms such as lactic acid bacteria, acetic acid bacteria and yeasts. On grapes also molds can be found (Table 25.1). The most important species for conversion of must into wine are the yeast *Saccharomyces cerevisiae* and the lactic acid bacterium *Oenococcus oeni*, which perform alcoholic and malolactic fermentation, respectively. Both species are used as starter cultures. A variety of techniques and media are available for the enrichment, culture and preservation of these microorganisms (Kirsop and Doyle 1991; Atlas and Parks 1993). For selected species culture and preservation procedures are described (Tables 25.1–25.3).

25.2 Bacteria

25.2.1 Genera of Acetic Acid Bacteria

Acetic acid bacteria (AAB) are acid and ethanol-tolerant aerobic bacteria, which oxidize ethanol to acetic acid. Species of the genus *Acetobacter* can also completely oxidize acetate to CO_2 in the presence of oxygen. Therefore, AAB are common wine spoilage microorganisms, because higher concentrations of acetic acid ($> 1\ g\ L^{-1}$) cause an off-flavor in wine (volatile acidity). Species of the three genera *Acetobacter*, *Gluconacetobacter* and *Gluconobacter* have been found on grapes, in must and wine (Table 25.2). They are grown at 25–30°C.

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Culture media for acetic acid bacteria	
YPM medium (DSMZ 2008)	
Yeast extract	5.0 g
Peptone	3.0 g
Mannitol	25.0 g
Agar	12.0 g
Destilled water	1,000.0 mL
No pH adjustment	
$CaCO_3$ (30 g) may be added to the medium	

Preservation. Agar cultures of the bacteria can be kept at 4°C for 1 or 2 months. Lyophilized cells can be kept alive for 10 years. Methods are described by Kirsop and Doyle (1991).

Table 25.1 Recommended media for cultivation of fungi from grapes

Group	Genus	Culture media	Species (examples)
Ascomycota	1. *Acremonium* Link	DRBC	*A.* spp.
	2. *Arthrinium* Kunze	DRBC	*A.* spp.
	3. *Aspergillus* Fr.: Fr.	CZ (CBS), MEA, AFPA, DRBC	*A. aculeatus* Iizuka; *A. alliaceus* Thom & Church; *A. auricomus* Saito; *A. candidus* Link; *A. carbonarius* Bainier; *A. carneus* Blochwitz; *A. clavatus* Desm.; *A. flavipes* Thom & Church; *A. flavus* Link; *A. fumigatus* Fresen; *A. japonicus* Saito; *A. niger* aggregate; *A. ochraceus* K. Wilh.; *A. ostianus* Wehmer; *A. parasiticus*; *A. terreus* Thom; *A. terreus var. africanus* Raper & Fennell; *A. ustus* Thom & Church; *A. versicolor* Tirab; *A. wentii* Wehmer
	4. *Aureobasidium* Viala & G. Boyer	DRBC	*A.* spp.
	5. *Beauveria*	DRBC	*B. bassiana* Vuill.
	6. *Botrytis* P. Micheli	OA, DRBC	*B. cinerea (Sclerotinia fuckeliana; causal agent of grey mold)*
	7. *Chaetomium* Kunze	DRBC	*C.* spp.
	8. *Chrysonilia* Arx	DRBC	*C.* spp.
	9. *Curvularia* Boedijn	DRBC	*C.* spp.
	10. *Dendryphiella*	DRBC	*D.* spp.
	11. *Drechslera* S. Ito	DRBC	*D.* spp.

(continued)

Table 25.1 (continued)

12. *Emericella* Berk.	DRBC	*E.* spp.
13. *Epicoccum* Link	DRBC	*E. nigrum*
14. *Eurotium* Link: Fr.	DRBC	*E. amstelodami* L. Mangin; *E. chevalieri* L. Mangin
15. *Fusarium* Link	DRBC	*F.* spp.
16. *Geotrichum* Link	DRBC	*G.* spp.
17. *Gliocladium* Corda	DRBC	*G. spp.*
18. *Guignardia*	OA (*Guignardia*)	*Guignardia bidwellii (causal agent of black rot)*
19. *Histoplasma* Darling	DRBC	*H.* spp.
20. *Neurospora*	DRBC	*N. tetrasperma* Shear & Dodge
21. *Nigrospora* Zimm.	DRBC	*N.* spp.
22. *Oidium*	Obligate biotrophic! Cultivation only on potted vines.	*O. tuckeri (Erysiphe necator, causal agent of powdery mildew)*
23. *Penicillium* Link	CZ (CBS), MEA, DRBC	*P. aurantiogriseum* Dierckx; *P. bilaiae* Chalabuda; *P. brevicompactum* Dierckx; *P. canescens* Sopp; *P. chrysogenum* Thom; *P. citrinum* Thom; *P. corylophilum* Dierckx; *P. crustosum* Thom; *P. echinulatum* Fassatiova; *P. expansum* Thom; *P. fellutanum* Biourge; *P. funiculosum* Thom; *P. glabrum/spinulosum;* *P. griseofulvum* Dierckx; *P. implicatum* Biourge; *P. janczewskii* K.M. Zalessky; *P. miczynskii* Zaleski; *P. minioluteum* Dierckx; *P. novae-zeelandiae* J.F.M. Beyma; *P. olsonii* Bainier & Sartory; *P. oxalicum* Currie & Thom; *P. pinophilum* Hedgcock; *P. purpurogenum* Stoll; *P. raistrickii* G. Sm.; *P. restrictum* J.C. Gilman & E.V. Abbott; *P. roqueforti* Thom; *P. rugulosum* Thom; *P. sclerotiorum* van Beyma; *P. simplicissimum* Thom; *P. solitum* Westling; *P. thomii* Maire; *P. variabile* Sopp; *P. verruculosum* Peyronel; *P. waksmanii* Zaleski

(continued)

Table 25.1 (continued)

Group	Genus	Culture media	Species (examples)
	24. *Periconia* Tode ex Fr.	DRBC	*P*. spp.
	25. *Pestalotiopsis* Steyeart	DRBC	*P*. spp.
	26. *Phoma* Sacc.	DRBC	*P*. spp.
	27. *Pithomyces*	DRBC	*P. chartarum* Ellis
	28. *Scytalidium* Pesante	DRBC	*S*. spp.
	29. *Trichothecium*	MEA	*Trichothecium roseum* Link
	30. *Truncatella* Steyeart	DRBC	*T*. spp.
	31. *Ulocladium* Preuss	DRBC	*U*. spp.
Deuteromycotina	32. *Alternaria* Nees: Fr.	MEA, PCA (CBS), DRBC	*Alternaria alternata*
	33. *Cladosporium* Link	MEA, DRBC	*Cladosporium herbarum*
	34. *Monilia*	OA	*Monilia fructigena*
	35. *Paecilomyces* Bainier	MEA, OA, DRBC	*Paecilomyces variotii*
	36. *Stemphylium* Wallr.	DRBC	*S*. spp.
	37. *Trichoderma* Pers.	OA, MEA	*T*. spp.
Oomycota	38. *Plasmopara*	Obligate biotrophic! Cultivation only on potted vines.	*P. viticola* ("*Peronospora*" causal agent of downy mildew)
Zygomycota	39. *Cunninghamella* Matr.	DRBC	*C*. spp.
	40. *Mucor* P. Micheli: Fr	MEA 4% (CBS), DRBC	*M. mucedo*, *M. hiemalis, M. piriformis*
	41. *Rhizopus* Ehrenb.	MEA	*R. stolonifer*
	42. *Syncephalastrum*	DRBC	*S. racemosum* J. Schröt.

AFPA = *Aspergillus flavus/A. parasiticus* selective medium; CZ (CBS) = Czapek agar (formula used at CBS); MEA = malt extract agar; MEA 4% (CBS) = mout extract agar 4% (formula used at CBS); OA = oatmeal agar; PCA (CBS) = potato carrot agar (formula used at CBS); PDA = potato dextrose agar. CBS = Centraalbureau voor Schimmelcultures, The Netherlands. DRBC = Dichloran Rose-Bengal Chloramphenicol Agar (Oxoid) (cf. Serra et al. 2005)

Table 25.2 Recommended media for cultivation of bacteria from grapes, must and wine

Group	Genus	Culture media	Species (examples)
Gram-positive bacteria			
Lactic acid bacteria	1. *Lactobacillus*	MRS, SL	*Lb. brevis*, *Lb. buchneri*, *Lb. casei*, *Lb. curvatus*, *Lb. delbrueckii*, *Lb. diolivorans*, *Lb. fermentum*, *Lb. fructivorans*, *Lb. hilgardii*, *Lb. jensenii*, *Lb. kunkeei*, *Lb. mali*, *Lb. nagelii*, *Lb. paracasei*, *Lb. plantarum*, *Lb. vini*
	2. *Leuconostoc*	MRS	*Lc. mesenteroides*
	3. *Oenococcus*	MRS, TJ	*O. oeni*
	4. *Pediococcus*	MRS	*P. damnosus, P. parvulus, P. pentosaceus; P. inopinatus*
	5. *Weissella*	MRS	*W. paramesenteroides*
Proteobacteria			
Acetic acid bacteria	1. *Acetobacter*	YPM	*A. aceti, A. cerevisiae*, *A. pasteurianus*
	2. *Gluconacetobacter*	YPM	*G. hansenii; G. liquefaciens*
	2. *Gluconobacter*	YPM	*G. oxydans*

Table 25.3 Recommended media for cultivation of yeasts from grapes, must and wine

Genus	Culture media[a]	Species[b] (examples)
1. *Brettanomyces*	GPYA + $CaCO_3$, 25°C	*B. anomala*; *B. bruxellensis* (teleomorphic form: *Dekkera*)
2. *Candida*	GPYA, 25°C	*C. agrestis*, anamorphic (synomyn of *Saturnispora zaruensis*); *C. albicans; C. apicola; C. boidinii; C. cantarellii; C. catenulata*; *C. colliculosa* (synonym of *Torulaspora delbrueckii*); *C. diversa; C. famata* (synonym of *Debaryomyces hansenii); C. glabrata*; *C. incommunis; C. inconspicua; C. intermedia*; *C. krusei*; *C. montana*; *C. norvegica*; *C. parapsilosis*; *C. pelliculosa* (synonym of *Pichia anomala*); *C. pulcherrima* (synonym of *Metschnikowia pulcherrima*); *C. rugosa*; *C. sake; C. solani*; *C. stellata; C. tenuis*; *C. tropicalis*; *C. vanderwaltii; C. veronae* (synonym of *Pichia mexicana*); *Candida valida* (synonym of *Pichia membranifaciens*); *C. versatilis*; *C. vinaria*; *C. vini* (synonym of *Kregervanrija fluxuum*); *C. zeylanoides*
3. *Citeromyces*	GPYA, 25°C	*C. matritensis*

(continued)

Table 25.3 (continued)

Genus	Culture media[a]	Species[b] (examples)
4. *Cryptococcus*	PDA, 25°C	*C. albidus*; *C. humicola*; *C. laurentii*; *C. luteolus*
5. *Debaryomyces*	GPYA, 25°C	*D. carsonii*; *D. etchellsii*; *D. hansenii*; *D. polymorphus*
6. *Dekkera* (anamorphic form: *Brettanomyces*)	GPYA + $CaCO_3$, 25°C	*D. anomala*; *D. bruxellensis*
7. *Dipodascus*	GPYA, 25°C	*D. ingens* (synonym of *Magnusiomyces ingens*)
8. *Endomyces*	GPYA, 25°C	*E. fibuliger* (synonym of *Saccharomycopsis fibuligera*)
9. *Endomycopsella*	GPYA, 25°C	*E. vini* (synonym of *Saccharomycopsis vini*)
10. *Filobasidiella*	PDA, 25°C	*F. neoformans*
11. *Filobasidium*	PDA, 25°C	*F. capsuligenum*
12. *Geotrichum*	EMSA, 24°C	*G. fermentans*
13. *Guehomyces*	PDA, 25°C	*G. pullulans*
14. *Hanseniaspora*	GPYA, 25°C	*H. guilliermondii*; *H. occidentalis; H. osmophila; H. uvarum*; *H. valbyensis*; *H. vineae*
15. *Hasegawaea*	GPYA; 25°C	*H. japonica* (synonym of *Schizosaccharomyces japonicus*)
16. *Hyphopichia*	MYA, 25°C	*H. burtonii* (synonym of *Pichia burtonii*)
17. *Issatchenkia*	GPYA, 25°C	*I. terricola*
18. *Kazachstania*	GPYA, 25°C	*K. exigua; K. transvaalensis; K. unispora*
19. *Kloeckera*	GPYA, 25°C	*K. apiculata* (synonym of *Hanseniaspora uvarum*); *K. corticis*
20. *Kluyveromyces*	GPYA, 25°C	*K. marxianus; K. thermotolerans* (synonym of *Lachancea thermotolerans*)
21. *Kregervanrija*	GPYA, 25°C	*K. fluxuum*
22. *Lachancea*	GPYA, 25°C	*L. kluyveri*; *L. thermotolerans*
23. *Leucosporidium*	PDA, 20°C	*L. scottii*
24. *Lipomyces*	GPYA, 25°C	*L. starkeyi*
25. *Lodderomyces*	GPYA, 25°C	*L. elongisporus*
26. *Magnusiomyces*	GPYA, 25°C	*M. ingens*
27. *Metschnikowia*	GPYA, 25°C	*M. pulcherrima*; *M. reukaufii*
28. *Nadsonia*	GPYA, 25°C	*N. fulvescens*
29. *Octosporomyces*	GPYA, 30°C	*O. octosporus* (synonym of *Schizosaccharomyces octosporus*)
30. *Pachytichospora*	GPYA, 25°C	*P. transvaalensis* (synonym of *Kazachstania transvaalensis*)
31. *Pichia*	GPYA, 25°C	*P. anomala* (asexual form: *Candida pelliculosa*); *P. burtonii; P. canadensis*; *P. carsonii (*synonym of *Debaryomyces carsonii)*; *P. etchellsii* (synonym of *Debaryomyces etchellsii*); *P. farinosa*; *P. fermentans*; *P. guilliermondii*; *P. jadinii*; *P. membranifaciens*; *P. silvicola*; *P. subpelliculosa*

(continued)

Table 25.3 (continued)

32. *Rhodotorula*	PDA, 25°C	*R. acuta* (synonym of *Sterigmatomyces elviae*); *R. aurantiaca; R. bogoriensis; R. glutinis; R. minuta; R. mucilaginosa*
33. *Saccharomyces*	GPYA, 25°C	*S. cerevisiae; S. bayanus; S. exiguus* (synonym of *Kazachstania exigua*); *S. kluyveri* (synonym of *Lachancea kluyveri*); *S. unisporus* (synonym of *Kazachstania unispora*)
34. *Saccharomycodes*	GPYA, 25°C	*S. ludwigii*
35. *Saccharomycopsis*	GPYA, 25°C	*S. fibuligera; S. vini*
36. *Saturnispora*	GPYA, 25°C	*S. zaruensis*
37. *Schizosaccharomyces*	GPYA; 25°C	*S. pombe; S. japonicus*; *S. versatilis; S. octosporus*
38. *Sporidiobolus*	PDA, 25°C	*S. pararoseus*; *S. salmonicolor*
39. *Sporobolomyces*	PDA, 25°C	*S. roseus*
40. *Sterigmatomyces*	PDA, 25°C	*S. elviae*
41. *Torulaspora*	GPYA, 25°C	*T. delbrueckii; T. globosa*
42. *Trichosporon*	PDA, 25°C	*T. beigelii* (synonym of *Trichosporon cutaneum*); *T. pullulans* (synonym of *Guehomyces pullulans*); *T. cutaneum*
43. *Torulopsis*	GPYA, 25°C	*T. versatilis* (synonym of *Candida versatilis*)
44. *Wickerhamiella*	GPYA, 25°C	*W. domercqiae*
45. *Williopsis*	GPYA, 25°C	*W. californica*; *W. saturnus*
46. *Yarrowia*	GPYA, 25°C	*Y. lipolytica*
47. *Zygoascus*	GPYA, 25°C	Z. *hellenicus*
48. *Zygosaccharomyces*	GPYA, 25°C	*Z. bailii*; *Z. bisporus; Z. florentinus* (synonym of *Zygotorulaspora florentinus*); *Z. rouxii*
49. *Zygotorulaspora*	GPYA, 25°C	*Z. florentinus*

All genera belong to the Ascomycota.

[a]cf. also Barnett et al. (1990); Robert et al. (2008)

[b]Index fungorum (2008); Robert et al. (2008)

25.2.2 Genus Lactobacillus

Species of the genus *Lactobacillus* have complex nutritional requirements for amino acids, peptides, nucleic derivatives, vitamins, salts, fatty acids or fatty acid esters and fermentable carbohydrates. Nutritional requirements are generally characteristic for species or strains. Pantothenic acid and nicotinic acid are required by most species and thiamine by heterofermentative species. Some may require folic acid, riboflavin, pyridoxal phosphate, p-aminobenzoic acid, biotin and B12. Nutritional requirements are the result of minor defects in the chromosome and they are met when the media contain fermentable carbohydrates, peptone, meat and yeast extract. Supplementation with tomato juice, manganese, acetate and oleic esters are even essential for some species (Kandler and Weiss 1986). These compounds are included in the MRS-medium (de Man et al. 1960).

Culture media	
MRS-medium (de Man et al. 1960)	
Casein peptone	10.0 g
Meat extract	10.0 g
Yeast extract	5.0 g
Glucose	20.0 g
K_2HPO_4	5.0 g
Diammonium citrate	2.0 g
Sodium acetate	5.0 g
$MgSO_4 \times 7\ H_2O$	0.2 g
Tween 80	1.0 g
Agar	15.0 g
Distilled water	1,000.0 mL
pH	6.2–6.4
Sterilization	121°C, 15 min
SL-medium (Rogosa et al. 1951)	
Casein peptone	10.0 g
Yeast extract	5.0 g
Glucose	20.0 g
K_2HPO_4	6.0 g
Diammonium citrate	2.0 g
Sodium acetate $\times 2\ H_2O$	25.0 g
$MgSO_4 \times 7\ H_2O$	0.5 g
$MnSO_4 \times 4\ H_2O$	0.2 g
$Fe_2SO_4 \times 7\ H_2O$	0.04 g
Tween 80	1.0 g
Agar	15.0 g
Distilled water	1,000.0 mL
Sterilization	121°C, 15 min

Lactobacilli grow best in slightly acidic media with an initial pH between 4.5 and 6.5. Growth ceases below 3.5. Most species are aerotolerant, but grow better under microaerophilic or anaerobic conditions. 5% CO_2 stimulates growth. Surface growth on solid media is enhanced by anaerobiosis of reduced oxygen pressure and 5–10% CO_2. The growth temperature ranges from 2 up to 53°C. Optimal growth is usually at mesophilic temperatures between 30 and 40°C. The optimal pH usually is 5.5–6.2. Growth is often inhibited above pH 7.0. Most strains exhibit proteolytic acitivity.

Non-selective MRS-medium (de Man et al. 1960) can be applied, when lactobacilli form a predominant flora. A more selective medium is the acetate medium (SL-medium) (Rogosa et al. 1951), required when lactobacilli are part of a complex microbial flora. Growth of yeasts may be prevented by the addition of cycloheximide (100 mg L^{-1}). Manganese is required as cofactor for enzyme activity (e.g. lactate dehydrogenase, malolactic enzyme, RNA polymerase, xylose isomerase, NADH oxidase, superoxide dismutase) (Caspritz and Radler 1983; Archibald 1986; De Angelis and Gobbetti 1999).

Agar is dissolved separately by steaming in 500 mL distilled water. All other ingredients are dissolved without heating in 500 mL water. The pH is adjusted to 5.4 with glacial acetic acid. This solution is added to melted agar and boiled for 5 min.

Preservation. For short term preservation, cultures are preferably inoculated into MRS-medium stabs after colonies become visible. The cultures are stored at 4°C to 7°C for 1 month or at −20°C for several months. For long term preservation, the cells of the late growth phase are collected by centrifugation, resuspended in skim milk or horse serum containing 7.5% glucose and lyophilized. Ampules are sealed under vacuum and stored at 5–8°C. Strains can be kept for 10–20 years at −76°C or in liquid nitrogen over 30 years. Freezing in glass capillary tubes is convenient (Kirsop and Doyle 1991).

Some special growth requirements of selected species (Kandler and Weiss 1986):

Lactobacillus brevis. Calcium pantothenate, niacin, thiamine, folic acid are required for growth, while riboflavin, pyridoxal and vitamin B_{12} are not.

Lactobacillus buchneri. As described for *L. brevis*.

Lactobacillus casei. Riboflavin, folic acid, calcium pantothenate and niacin are required for growth. Pyridoxal or pyridoxamine is essential or stimulatory. Thiamine, vitamin B_{12} and thymidine are not needed.

Lactobacillus curvatus. Some strains grow at 2–4°C.

Lactobacillus delbrueckii. Pantothenic acid and niacin are required for growth. Some strains require riboflavin, folic acid, vitamin B_{12}, and thymidine. However, thiamine, pyridoxine, biotin and p-amino benzoic acid are not required.

Lactobacillus diolivorans. Fermentative growth on 1.2-propanediol producing 1-propanol and propionic acid.

Lactobacillus fermentum. Calcium pantothenate, niacin, thiamine are required for growth, while riboflavin, pyridoxal and folic acid are not. Stimulants are tomato and orange juice, extracts of green beans, beetroots, bulb, cabbage and spinach.

Lactobacillus fructivorans. Enhanced growth takes place in the absence of O_2. Fructose is reduced to mannitol. Acidophilic, pH optimum 5.0–5.5, no growth happens at an initial pH above 6.0. Mevalonic acid, tomato juice and/or ethanol are required for growth.

Lactobacillus hilgardii. Optimal growth occurs at pH 4.5–5.5. Growth takes place in the presence of 15–18% ethanol.

Lactobacillus kunkeei. Citrate and malate are utilized in the presence of glucose. Mannitol is produced from fructose.

Lactobacillus paracasei. Growth at 10 and 40°C, some strains at 5 and 45°C.

Lactobacillus plantarum. Calcium pantothenate and niacin are required for growth. Thiamine, pyridoxal, pyridoxamine, folic acid, vitamin B_{12}, riboflavin, thymidine and deoxyribosides are not required.

Lactobacillus vini. Citric and malic acids are utilized.

25.2.3 Genus Leuconostoc

Leuconostocs can be isolated on media containing thallous acetate and crystal violet (Cavett et al. 1965). Rich media with complex growth factors and amino acids are required (Dellaglio et al. 1995). Optimal growth occurs at pH 6–7. Growth is stimulated by addition of 0.05% cysteine. Growth on agar plates is stimulated in the presence of a gas mixture of 19.8% CO_2, 11.4% H_2 and nitrogen.

Glucose is used by all species, but fructose is preferred except for *L. mesenteroides* subs. *cremoris*. All species require nicotinic acid, thiamine, biotine and pantothenic acid, while cobalamine and p-aminobenzoic acid are not required. Growth occurs between 5 and 30°C with an optimum between 20 and 30°C (Garvie 1986a).

Leuconostoc mesenteroides subsp. *mesenteroides*

Growth occurs between 10 and 37°C with an optimum between 20 and 30°C. *Leuconostoc mesenteroides* requires up to eight amino acids, some strains more than eight amino acids. Glutamic acid and valine is required by all. *L. mesenteroides* subsp. *mesenteroides* requires only glutamic acid and valine. None require alanine.

Culture media. MRS-medium (see Sect. 25.2.2).

Preservation. Stock cultures can be prepared from all species in the late growth phase by lyophilization in horse serum containing 7.5% glucose. Once dried, cultures can be kept under vacuum at 10°C.

25.2.4 Genus Oenococcus

Until 2006, the genus *Oenococcus* contained only one species, *O. oeni* (formerly *Leuconostoc oenos*; Dicks et al. 1995), which was isolated from must. A second species, *O. kitaharae* (Endo and Okada 2006) was isolated from a composting destilled shochu residue.

O. oeni is more acid and ethanol tolerant than other lactic acid bacteria. It can grow at pH 3.0 and 10 vol% ethanol (van Vuuren and Dicks 1993; Versari et al. 1999). *O. oenos* can be enriched from must and wine on tomato juice agar with an initial pH below 3.5 and 6 vol% ethanol containing cycloheximide to prevent growth of yeast (Kunkee 1967) for 8 days.

This more defined medium can replace the tomato juice medium for culturing some *Oenococcus* strains (Theobald et al. 2005, 2007b, 2008).

Culture media	
Tomato juice medium (TJ)	
Basal medium:	
Peptone from meat	5.0 g
Yeast extract	5.0 g
Tryptone from casein	2.0 g
Glucose	5.0 g
Fructose	5.0 g
Citric acid	3.0 g
Tween 80	1.0 g
Magnesium sulfate × 7 H_2O	0.5 g
Distilled water	1,000.0 mL
pH 6.0	
75% basal medium +25% tomato juice (Garvie and Mabbitt 1967) (pH 6.0) (v/v)	
20 min 121°C	
MAC medium (Theobald et al. 2008)	
Peptone from meat	5.0 g
Tryptone from casein	20.0 g
Glucose	5.0 g
Fructose	5.0 g
Arabinose	1.5 g
Citric acid	3.0 g
Tween 80	1.0 g
Manganese sulfate × H_2O	1.9 g
Magnesium sulfate × 7 H_2O	0.5 g
Cysteine	1.5 g
Vitamin solution	10.0 mL
Amino acid solution	100.0 mL
Distilled water	1,000.0 mL
pH 6 .0	

Amino acid solution. DL-alanine (2 g L^{-1}), L-arginine × HCl (2 g L^{-1}), L-aspartic acid (3 g L^{-1}), L-glutamate (3 g L^{-1}), glycine (2 g L^{-1}), L-histidine × HCl (2 g L^{-1}), L-leucine (2 g L^{-1}), L-lysine × HCl (2 g L^{-1}), L-proline (2 g L^{-1}), DL-aminobutyric acid (1 g L^{-1}), L-asparagine (1 g L^{-1}), L-cysteine (1 g L^{-1}), L-isoleucine (1 g L^{-1}), L-methionine (1 g L^{-1}), L-phenylalanine (1 g L^{-1}), L-serine (1 g L^{-1}), L-threonine (1 g L^{-1}), L-tryptophan (1 g L^{-1}), L-tyrosine (1 g L^{-1}), L-valin (1 g L^{-1}). The amino acids are dissolved in 1 L of distilled water by heating and stored at −18°C.

Vitamin solution. Pyridoxol hydrochloride (100 mg L^{-1}), nicotinic acid (100 mg L^{-1}), calcium D-(+) pantothenic acid (100 mg L^{-1}), riboflavine (100 mg L^{-1}), thiamine (50 mg L^{-1}), folic acid (20 mg L^{-1}), p-amino benzoic acid (10 mg L^{-1}), cyanocobalamine (1 mg L^{-1}), D(+) biotin (1 mg L^{-1}), myo-inosit (1 mg L^{-1}).

Riboflavine is dissolved in distilled water by heating, folic acid in 50 mL distilled water by adding some drops 1 M NaOH, biotin in a solution of 0.2 g KH_2PO_4 and 0.2 g K_2HPO_4 in 10 mL. The other vitamins are dissolved in 600 mL distilled water. The solution is combined and filled up to 1 L.

Oenococcus oeni. Guanine, adenine, xanthine, uracil, riboflavin, folic acid, nicotinic acid and thiamine are essential. Stimulants are pyridoxol, biotin, yeast preparations, juice, manganese, cystein, arabinose and epigallocatechin gallate (Theobald et al. 2005, 2007a, b). A natural source of arabinose is araban and arabinogalactan. Arabans occur as side groups of pectins and in soft fruits (e.g. grapes) and sugar beets.

Inosite, cobalamin, 4-amino benzoic acid are not necessary. *Oenococcus* strains need up to 16 amino acids. Glutamic acid and valine are not required, but all strains need α-amino butyric acid. Asparagine and lysine (Weiler and Radler 1972) and carotinoids (β-carotin) do not stimulate growth. Polyphenols such as epigallocatechin-3-gallate can act as stimulators (400–500 mg L^{-1}) or inhibitors (>543 mg L^{-1}) on growth (Theobald et al. 2007a). In the case of some strains tomato juice can be completely replaced by 34 mM manganese (Theobald et al. 2005).

Oenococcus oeni can be used as starter culture for decreasing the concentration of malic acid, which reduces the acidity of wine.

Preservation. Stock cultures can be kept on tomato juice agar stabs for 1 month at room temperature. They can be stored for several years in glass capillary tubes at −76°C in fresh tomato juice medium supplemented with glycerol (10%) (Kirsop and Doyle 1991).

25.2.5 Genus Pediococcus

All species need nicotinic acid, pantothenic acid and biotin, while none requires thiamine, p-aminobenzoic acid and cobalamine. In the case of *P. damnosus* and *P. parvulus* 5 days may be required for growth. *P. pentosaceus* grows more rapidly. It grows well aerobically on agar plates. Growth can be improved in an atmosphere of H_2 and 10% CO_2. All species grow at 30°C but optimum temperatures range from 25 to 40°C. Actidione can be used to suppress yeast growth (Garvie 1986b).

Pediococci grow best in rich media. Most strains need amino acids such as alanine, aspartic acid, glutamic acid, arginine, histidine, isoleucine, phenylalanine, proline, threonine, tyrosine, valine, tryptophan, cystine, glycine and leucine and some strains need lysine, methionine and serine (Simpson and Tachuchi 1995). Many strains are stimulated by peptides.

No single medium or incubation condition can be used for isolation and growth of all species. Pediococci can be isolated in the presence of lactobacilli by using MRS medium in which glucose has been replaced by 1% mannose, cellobiose or salicin (Back 1978). Yeast and gram-negative bacteria can be inhibited by the addition of cycloheximide, crystal violet, 2-phenylethanol, sorbic acid and acetic acid (thallous acetate). Many gram-positive species – except strains from *Pediococcus*, *Leuconostoc* and some lactobacilli – are inhibited by vancomycin (Simpson et al. 1988).

Culture media. In general the MRS medium (de Man et al. 1960; see Sect. 25.2.2), YPG medium (Garvie 1978) and TGE medium (Biswas et al. 1991) are sufficient.

Pediococcus damnosus. Cells grow within 2–3 days at 22°C. Addition of cysteine improves growth. On agar surfaces, colonies grow better under anaerobic conditions. The final pH is 4.0. The optimal pH is 5.5. Cells grow at pH 4.2, but not at

pH 8.5. The maximum pH for growth is 6.5–7.0. The pH of the MRS medium is adjusted to 5.2. Growths occur in the range of 8–30°C. Hop bitter acid can be used for the isolation of *Pediococcus damnosus*.

Pediococcus parvulus. Cultures are improved by addition of cysteine. Some strains require asparagine but no folinic acid. Upper pH limit is between 7.0 and 7.5, the pH optimum is at about 6.5. Optimum growth temperature is 30°C; maximum temperature is between 37 and 39°C. Colonies are obtained at 30°C within 48 h. Strains grow in the presence of 8% NaCl. They are unable to use pentoses.

Pediococcus pentosaceus. Colonies are visible after 24 h at 30°C. Folinic acid is required by some strains. Growth is obtained at pH 4.5 and 8.0. The final pH in MRS broth is 4.0, the optimum is between 6.0 and 6.5. The optimal temperature is between 28 and 32°C, the maximum temperature is 39–45°C. Growth occurs in the presence of 10% NaCl.

Preservation. Pediococci can be kept on agar slopes at 4°C for about 3 months. They can be preserved by lyophilization (Garvie 1986b). Storage is improved by the addition of calcium carbonate (1%) to the culture medium. Cells from the late exponential phase should be suspended in horse serum containing 7.5% glucose. Cells can also be stored in growth medium and glycerol (1:1) (Weiss 1991). They can be lyophilized in the presence of horse serum and 7.5% glucose. Cells of the late growth phase should be used. Dried cultures will survive at 10°C under vacuum. Cultures can be preserved for 3–4 month in skim milk supplied with glucose (1.0%), yeast extract (0.3%) and calcium carbonate (1.0%).

25.3 Yeast

More than 100 yeast species have been isolated from grapes, must and wine. The most important for wine making is *Saccharomyces cerevisiae*, which converts glucose and fructose to ethanol and CO_2. Wine-related yeasts can be grown on one of four solid media.

Preservation. Yeast can be kept on agar slopes at 4°C for several months. They can be stored in growth medium supplemented with glycerol (10%) at –76°C, or liquid nitrogen for several years. The maintenance of yeast (subculturing, drying and freezing in liquid nitrogen) was described by Kirsop (1991).

25.4 Fungi

Different fungi can grow on grapes. They can be grown in the following culture media (Table 25.2). Ingredients are dissolved in 1 L distilled water and sterilised by autoclaving at 121°C for 15 min, unless stated otherwise.

Addition of 1 mL trace elements solution per litre is recommended for avoiding atypical colony growth and colour.

Culture media for yeasts	
Emmons' modified Sabouraud's agar (EMSA) (ATTC 2008)	
Sabouraud dextrose broth (BD 238220)	30.0 g
Agar	20.0 g
pH	6.8–7.0
GPYA (glucose-peptone-yeast extract agar) (CBS 2008)	
Glucose	40.0 g
Peptone	5.0 g
Yeast extract	5.0 g
Agar	15.0 g
Distilled water	1,000 mL
Sterilize 15 min at 110°C (0.5 atm)	
MYA (malt-yeast agar) (CBS 2008)	
Yeast extract	3.0 g
Malt extract	3.0 g
Glucose	10.0 g
Bacto peptone	5.0 g
Agar	15.0 g
Distilled water	1,000 mL
PDA (potato-dextrose agar) (CBS 2008)	
Potato extract	0.231
Dextrose	20.0 g
Agar	15.0 g
Distilled water	0.77 L
pH = 6.6	

Aspergillus flavus/A. parasiticus selective medium (AFPA; Oxoid)	
Peptone	10.0 g
Yeast extract	20.0 g
Ferric Ammonium Citrate	0.5 g
Dichloran	0.002 g
Chloramphenicol	0.1 g
Agar	15.0 g
Distilled water	1,000 mL
Final pH ± 6.3	
Note: dichloran and chloramphenicol can be added before sterilisation	
Czapek agar (CZ; CBS)	
Saccharose	30.0 g
$NaNO_3$	3.0 g
K_2HPO_4	1.0 g
KCl	0.5 g
$MgSO_4 \times 7\ H_2O$	0.5 g
$FeSO_4 \times 7\ H_2O$	0.01 g
Agar	15.0 g
Distilled water	1,000 mL
pH = 6.0–6.5	

Malt extract agar (MEA; Difco, Bacto)	
Malt extract, Difco	30.0
Bacto Agar	15.0
Distilled water	1,000 mL
Final pH 5.5 ± 0.2 at 25°C	

Trace elements-solution: 1 g $ZnSO_4 \times 7\ H_2O$ and 0.5 g $CuSO_4 \times 5\ H_2O$ in 100 mL water.

Malt extract agar 4% (2%) (MEA 4% (2%); formulae used at CBS). Add water to malt extract from the brewery until it contains 10% sugar (measurement with aerometer). Mix 400 mL (200 mL) of this solution with 15 g agar and 600 (800) mL water. Malt agar may also conveniently be prepared with malt syrup (10–40 g L^{-1}) or malt powder (10–20 g L^{-1}).

Oatmeal agar (OA; formula used at CBS). Boil 30 g oat flakes in 1 L water and simmer gently for 2 h. Filter through cloth and fill up to 1 L. Add 15 g agar to 1 L and sterilize by autoclaving at 121°C for 15 min. When using powdered oatmeal, filtering is superfluous. Lupin stems may be placed in slants with oatmeal agar. This is also commercially available.

Oatmeal agar (*Guignardia*)

Add 20 g oat flakes (Bio Hafer Gold "Holo" of *Neuform*) to 500 mL distilled water and stir gently. After 15 min add 10 g Agar and sterilise by autoclaving at 121 °C for 15 min. For 500 mL medium use a 1-L-bottle for autoclaving (Attention! Use a 1-L-bottle for 500mL medium because it foams over easily), Final pH: 6.5.

Potato carrot agar (PCA; formula used at CBS). 40 g carrots and 40 g potatoes are separately washed, pealed, chopped, boiled in 1 L for 5 min and filtered off. It is then sterilized for 60 min at 1 atm overpressure (121°C). 250 ml potato extract, 250 mL carrot extract, 500 mL distilled water, and 15 g agar are taken and sterilized at 121°C for 15 min.

Potato dextrose agar (PDA). 300 g scrubbed and diced potatoes are added to 900 mL water and boiled for 1 h. This is then passed through a fine sieve and sterilized for 60 min at 121°C (1 atm overpressure). 230 mL potato extract, 15 g agar and 20 g dextrose are then mixed, filled up to 1 L and boiled until dissolved. This is also commercially available.

Preservation. The maintenance of filamentous fungi (subculturing, drying and freezing in liquid nitrogen) is described by Smith (1991).

25.5 Conclusions

In Tables 25.1–25.3 most of the microbial species (bacteria, yeast, fungi), which have been isolated from grapes, must and wine are compiled. Relatively few media are required to grow most of them. Since conventional isolation procedures need

several days, they are not very suitable for diagnostic purposes. For isolation and rapid detection of certain species or strains micro-manipulation and molecular methods (fluorescently labeled probes, PCR procedures) have been developed (Fröhlich 2002: Fröhlich and König 1998, 1999, 2000, 2004; Hirschhäuser and Fröhlich 2007; Röder et al. 2007a, b; Pfannebecker and Fröhlich 2008). It is expected that molecular detection methods will be helpful tools to learn more about the diversity and identity of microbial strains on grapes, in must and wine that have not yet been cultured.

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Chapter 26
DNA Arrays

José E. Pérez-Ortín, Marcel·lí del Olmo, and José García-Martínez

26.1 Introduction

The molecular bases of the technological properties of wine yeast strains are still largely unknown. *Saccharomyces cerevisiae* wine yeast strains are able to completely ferment sugar-rich natural musts under conditions that other strains are unable to. It is clearly possible that the impressive adaptation of these strains to the enological environment depends on specific expression profiles of their genomes (Wang et al. 2007). This should be the consequence of genetic differences with regard to other kinds of *S. cerevisiae* strains. Enological yeast strains have been selected for thousands of years for their ability to efficiently ferment grape-juice sugars under rather stressing conditions. where cell survival can depend on the ability to quickly adjust to the changing environment. Among stress conditions cells have to face, are, acidic pH (2.9–3.6), hyperosmotic stress due to high sugar concentration in musts (up to 260 g L^{-1}), low nitrogen content and presence of inhibitors such as sulphite and, later, nitrogen and carbon sources starvation and high ethanol concentration (up to 15% v/v) (reviewed in Attfield 1997 and Pizarro et al. 2007). It is worth mentioning that during the whole wine production process, from the preparation of active dry yeast to biological ageing (if required), the plethora of stress situations to which cells are subjected is even wider. Responses to stress situations include transcriptional and post-transcriptional mechanisms. Molecular responses to stress situations have been studied in detail in laboratory strains (see Estruch 2000 for a review). However, these strains do not exhibit the same properties as industrial strains and, therefore, their responses may be quite different. Comparative analysis of gene expression between industrial and non-industrial strains, and also between different industrial strains, could lead to identification of genes involved in the appropriateness of strains for industrial environments.

The discovery of features that differentiate wine yeast strains from others and their responses to vinification stress situations have been mostly analysed by DNA

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macro- and microarrays. Unexpectedly, these studies have shown that significant differences exist, even when comparing laboratory strains. Thus, in order to identify gene expression patterns of a particular (wine) strain, an individual analysis of that strain is required. Moreover, because non-wine strains are unable to grow under distinctive conditions of wine fermentations, it is impossible to explore expression patterns of a yeast strain under those conditions if it is not an enological one. However, because wine strains have not been studied as model systems for gene expression until very recently, not many studies have been conducted to date. It is therefore valuable to compare, where possible, the expression patterns of wine strains with those from standard laboratory ones.

The fact that "standard" *S. cerevisiae* genome stored in databanks is of a particular derivative (FY1679) of the S288c strain has to be taken into account. This strain is a haploid derived from a natural one cultured for 60 years in quite different conditions to those operating in "natural" environments. Thus, the study of other strains, particularly "natural" ones, could have an additional benefit: to understand the functional clues for many genes and to build a "comprehensive" yeast genome in which all existing alleles of every gene are described and from which an "ideal" yeast strain can be derived.

We review herein the current state of DNA array technology that has been applied to these strains (mostly micro-arrays, but also macro-arrays). This is an update to a previous review (Pérez-Ortín et al. 2002b), but also includes part of published work up to 2002 in order to maintain logic of descriptive reasoning.

26.2 Short Overview of the DNA Array Technology

A DNA array (also commonly known as gene or genome chip, DNA chip, or gene array) is a collection of DNA spots, commonly representing single genes arrayed on a solid surface (glass, plastic, silicon chip or nylon) by the covalent attachment to chemically suitable matrices, or simply by electrostatic binding. The affixed DNA segments are known as probes, and many thousands can be placed in known locations on a single DNA microarray (see Fig. 26.1 for a schematic representation of DNA chip technology).

DNA arrays can be fabricated using variety of technologies, including printing with fine-pointed pins onto either glass slides or nylon membranes, photolithography using pre-made masks, ink-jet printing, or electrochemistry on microelectrode arrays. From the printing surface and the technology used for fabrication and processing, different kinds of DNA arrays can be distinguished:

Spotted microarrays: Here probes are cDNA or small fragments of PCR products that correspond to mRNAs and are spotted onto a glass surface. This type of array is typically hybridised with cDNA from two samples to be compared, and is labelled with two different fluorophores. The two labelled cDNA samples are mixed and hybridised to a single microarray that is then scanned to visualise the

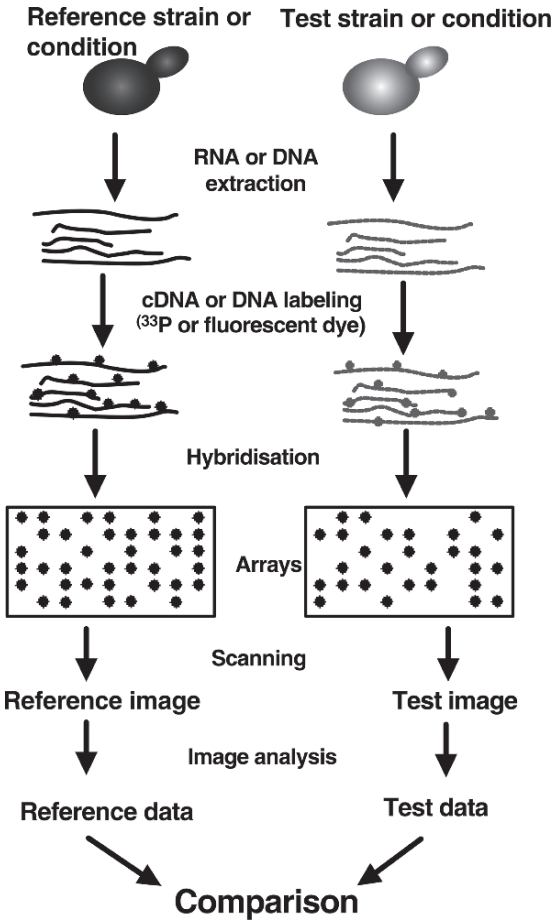

Fig. 26.1 Schematic representation of different steps in the DNA array processing and analysis. Note that in the hybridisation step in macroarrays and in some kinds of oligonucleotide arrays, two independent hybridisations are performed whereas in most glass microarrays, both test and reference labelled samples, are hybridised simultaneously on the same slide

two fluorophores after excitation with a laser beam of a defined wavelength. Relative intensities of each fluorophore may then be used in ratio-based analysis to identify up-regulated and down-regulated genes. Absolute levels of gene expression cannot be determined in the two-colour array, but relative differences in expression among different spots (=genes) can be estimated.

Spotted macroarrays: Similar to the previous method, but in which probes are immobilised onto a positively charged nylon membrane. mRNA is radioactively labelled (usually ^{33}P). Each condition (e.g., wild-type and mutant) is hybridised independently with a stripping step between them, which allows the use of same arrays for different sample replicates.

Oligonucleotide microarrays: The probes are designed to match parts of the sequence of known or predicted mRNAs. Designs are commercially available which cover complete genomes from different companies. These microarrays can provide estimations of absolute value of gene expression. Oligonucleotide arrays can be either produced by piezoelectric deposition with full length oligonucleotides or by in situ synthesis. Long oligonucleotide arrays are composed of 50–60-mers, and are produced by ink-jet printing on a silica substrate. Short oligonucleotide arrays are composed of 25–30-mer and are produced by photolithographic synthesis on a silica substrate or piezoelectric deposition on an acrylamide matrix.

Genotyping microarrays: These are particular types of DNA microarrays used to identify genetic variation in individuals and across populations. Short oligonucleotide arrays can be used to identify single nucleotide polymorphisms (SNPs) which are thought to be responsible for genetic variation.

Tiling arrays: They are a kind of microarray that includes overlapping oligonucleotides designed to blanket entire genome each 5–20 nucleotides without any previous knowledge of coding regions. They can be used either for genotyping or expression studies.

26.3 DNA Array Technology and Wine Yeast Expression Research

26.3.1 Impact of DNA Array Technology on Yeast Gene Expression Research

Genome sequencing initiatives have led to the discovery of many gene sequences but not their function. However, one of the techniques based on genome knowledge, DNA arrays, provides one entry point for functional genomics. The advances in the array-based techniques allow the whole genome to be monitored on a single chip. We can obtain a picture of the interactions between thousands of genes simultaneously compared to traditional methods. The first organisms to benefit from such analysis are the model ones whose genomes have been completely characterized. The yeast *S. cerevisiae* is a popular model for wide-expression studies for several reasons: the sequence of the entire genome was accomplished before other eukaryotes in 1997 (Goffeau et al. 1996, 1997), its genome size is relatively small and considerable genetic information and powerful tools are available for analysis. The leading role of this eukaryotic model has been evident in the development of array technology (Wodicka et al. 1997; De Risi et al. 1997; Hauser et al. 1998). Moreover, it is continuously being improved because this yeast is the working horse for development of different technical improvements (Hughes et al. 2001; García-Martínez et al. 2004; David et al. 2006). Most of these studies may be of some practical application to the biology of industrial yeasts and, therefore, to improvement of winemaking processes.

Logically, most DNA array studies, including all the first ones, were done in laboratory strains of *S. cerevisiae* growing in laboratory conditions. These expression studies, together with the abundant knowledge about yeast genetic regulatory mechanisms, have provided data to link genes and pathways to phenotypes in such a way that components of any metabolic and regulatory pathway can be determined. This will help to elucidate their role in both cellular physiology and the way in which their mechanism works. Thus, initial studies compared expression patterns of one third of yeast genome in different metabolic states (Lashkari et al. 1997). More comprehensive studies characterized genes that are differentially expressed during the shift from fermentation to respiration (DeRisi et al. 1997; ter Linde et al. 1999; Kuhn et al. 2001), during sporulation (Chu et al. 1998) or during the cell cycle (Cho et al. 1998; Spellman et al. 1998). Other major work in this field discerned the function of regulatory proteins, such as transcription factors or subunits of transcription complexes, and studied the consequences of over-expression or examined mutants (DeRisi et al. 2000; Holstege et al. 1998; Kobor et al. 1999; Myers et al. 1999; López and Baker 2000; Zhu et al. 2000 ; Lee et al. 2000; Sudarsanam et al. 2000; Carmel-Harel et al. 2001).

Whole-genome expression profiling can measure the transcriptional gene response to conditions or treatments of interest in a single assay, such as chemical or environmental agents (Jelinsky and Samson 1999; Jelinsky et al. 2000; Gasch et al. 2000; Hughes et al. 2000a; Pizarro et al. 2007). In an experiment that studied varied environmental conditions, the authors actually found that while a large set of genes showed similar response to almost all conditions studied, others were specialized for specific responses (Gasch et al. 2000). This experiment has already served as a model to design approaches to study wine strain responses to general stress responses or to particular stresses that occur in must fermentation. In this sense, Alexandre et al. (2001) identified different genes expressed after ethanol exposure, and concluded that, in addition to previously identified genes, other different mechanisms, such as ionic homeostasis, heat protection and antioxidant defense, respond to ethanol stress. These studies applied to wine strains (see below) will be of great interest because ethanol is a final product of must fermentation and high concentrations are reached.

Sugar fermentation by wine yeast strains is followed by rapid growth and production of ethanol and CO_2, although it can be arrested if sugar or nitrogen is depleted or if growth inhibitors are present (reviewed in Pretorius 2000). A better understanding of not only metabolic changes that accompany the shift from one carbon source to another (DeRisi et al. 1997; ter Linde et al. 1999; Kuhn et al. 2001), but also nutrient-sensitive signaling pathways (Hardwick et al. 1999; Cox et al. 1999; Ogawa et al. 2000) can help to both improve technical conditions in which cellar fermentation takes place and prevent either sluggish or stuck fermentations. DNA array technology is essential for information about these pathways. Although array experiments have mostly used laboratory strains, some interesting studies have been carried out with this technology in recent years in brewing (James et al. 2003), sake (Wu et al. 2006), baker's (Tanaka et al. 2007) and wine (see below) yeasts. Data acquired allows a better understanding of the molecular mechanisms underlying

these processes and also help to identify gene targets or gene expression patterns that allow industrial yeast strains to adapt to their particular conditions. In future, this technology is likely to become even more relevant for these purposes.

26.3.2 Expression Studies of Wine Yeasts

Given the utility of DNA arrays in investigating how interesting genes change their expression throughout a biological process, several attempts were carried out in wine yeasts at the beginning of the twenty-first century (reviewed in Pérez-Ortín et al. 2002b; Pizarro et al. 2007). Once more, the yeast *S. cerevisiae* was the first microorganism in which wild living strains (different from the model strain used for the array probes design) of industrial interest were studied using this powerful technique. As mentionedearlier, vinification is an important industrial process in which yeast strains play the main role, and their efficiency in the process has a capital influence on the final product. Thus, knowledge of genetic features, as well as specific expression profiles in different growth conditions of the wine yeast strain, can help provide us with a more clear understanding of biological process of fermentation at a molecular level, and how the gene expression is regulated in relation to changes in the physical and chemical properties of the growth medium.

Until the development of DNA microarray analysis, some traditional gene expression studies including only small number of genes were conducted with wine yeasts. The first gene expression study in wine yeasts was conducted on a haploid strain, V5 (a non-usual wine strain) by Northern analysis of 19 genes which had been previously described as being expressed in laboratory growth conditions or on molasses during the stationary phase and/or under nitrogen starvation. Nine genes, including members of the *HSP* family, showed a transition-phase induction profile (Riou et al. 1997). A more comprehensive study was conducted on the same haploid wine strain and on a reference strain FY69 (S288c background) by the same group. In this case, 99 genes from chromosome III were studied by Northern blot analysis (Rachidi et al. 2000). A particular wine strain, T73, isolated from Alicante wines (Querol et al. 1992), has been selected in our laboratory for the expression studies of particular sets of genes. A molecular study using Northern blot was conducted (Puig and Pérez-Ortín 2000a, b). The expression patterns of glycolytic genes, and nine other genes that were characterised by DeRisi et al. (1997) as showing a peak of induction at the diauxic shift, were studied. T73 strain (and other commercial wine yeast strains) has also been useful to demonstrate the relevance of the expression of genes involved in the response to osmotic stress (mainly *GPD1*, encoding glycerol-3-phosphate dehydrogenase gene) during the first hours of vinification (Pérez-Torrado et al. 2002, Zuzuárregui et al. 2005). Gene expression analysis has also been carried out along bench-top trials of industrial wine yeast propagation to identify stress responses that might be relevant for the performance of active dry yeasts. After testing expression profiles of a selected set of stress gene markers, induction of stress responsive gene *TRX2* during the batch stage of industrial growth suggest that an

oxidative stress response can occur at the transition from fermentative to respiratory metabolism (Pérez-Torrado et al. 2005).

Partial transcriptomic analysis with commercial wine yeast strains, which differ in their fermentative behaviour, has also helped to understand these differences and obtain clues to understand best adaptation of several strains. Our research groups have carried out several similar analysis. A first study limited to two commercial strains and several well characterised stress-responsive genes (*HSP* family and others) showed that *HSP12* can serve as a molecular marker for stress-resistance in wine yeasts (Ivorra et al. 1999). Later on, analysis of this kind with 14 enological strains demonstrated that it is possible to establish a correlation between stress resistance and fermentative behaviour (Zuzuárregui and del Olmo 2004a). Besides, although each strain shows a unique pattern of gene expression (Carrasco et al. 2001), higher (and in some cases maintained) mRNA levels of many stress genes tested were found in the strains with severe fermentative problems (Zuzuárregui and del Olmo 2004b).

The application of DNA array technology to wine strains has extended the landscape of expression studies. Different genomic studies on wine yeast using DNA array analysis have used various approaches in relation to growth conditions as well as experimental design of the assay. Thus, whereas some experiments use standard laboratory culture media (non-vinification conditions) for growing laboratory strains as well as wine strains (Cavalieri et al. 2000; Hauser et al. 2001), most simulate vinification conditions, to a greater or lesser extent, by growing the wine yeast strain on a synthetic must, thus mimicking their natural environment. The use of standard laboratory conditions enables the comparison of specific metabolic and physiological features of natural isolates or commercial wine yeast strains in relation to laboratory strains which have already been studied in many ways, including DNA arrays, for many years. Thus information obtained with reference strains can be used for in-depth studies or to investigate into pathways and molecular mechanisms that govern winemaking by enological yeast strains. For instance, variation of global gene expression levels in natural isolates has been shown to be unexpectedly high: 6% of the genome showed differences higher than two in a comparison between the progenies of a single wine strain (Cavalieri et al. 2000). This agrees with results obtained when comparing different laboratory reference strains (Primig et al. 2000), and shows that wine strains are highly heterozygotic. As most metabolic differences are segregated as a suite of traits, the authors conclude that this results from a change in a single regulatory gene, such as a transcription factor, from a small number of them. Recently, Wang et al. (2007) have shown that, in fact, 45% of expression differences between wine and laboratory strains are exclusively due to *trans* factors, and that a further 45% are attributable to both *trans* factors and *cis* (structural genes) variation. An example of this behaviour is seen with many amino acid biosynthetic genes. A different case has been observed for the *YHB1* gene (Hauser et al. 2001, see below) and for genes for copper resistance and filigreed phenotypes (Cavalieri et al. 2000) in which changes are not in *trans* but in *cis*.

Although use of laboratory culture conditions facilitates the analysis of yeast, it does not efficiently reproduce the natural environment for wine yeast, i.e.: high sugar concentration, acidic pH and variable nitrogen conditions. The first comprehensive

study into global gene expression during vinification was carried out by Rossignol et al. (2003). This study revealed changes in the expression of more than 2,000 genes during vinifications carried out in a synthetic medium mimicking a natural must, in which growth arrest was caused by nitrogen exhaustion. The results indicated that entry into the stationary phase triggered major transcriptional reprogramming with unique stress response involving hundreds of genes encoding proteins involved in various cellular processes, many of unknown function.

In general, different studies on enological yeast strains have concluded that the genes involved in both amino acid biosynthesis and purine biosynthesis showed high expression levels (Backhus et al. 2001; Cavalieri et al. 2000; Hauser et al. 2001), indicating higher growth rate.

One drawback of sequence-specific hybridisation technology, such as DNA arrays, is that each strain has its own DNA sequence. Up to 37,000 SNPs can be found when comparing laboratory strains (Schacherer et al. 2007); the problem gets more complicated for non-laboratory yeast strains. For instance, recent sequencing of the genome of a pathogenic *S. cerevisiae* strain has shown that several new genes, about 6,000 indels and more than 60,000 SNPs, differentiate that particular strain from the S288c background (Wei et al. 2007). The results from the array study of these strains can be, therefore, less accurate and comprehensive than for the reference, or similar, strain. This problem can be solved by using other genomic methods not based on previously known sequences, such as SAGE (Serial Analysis of Gene Expression) or massive parallel sequencing (MPS) technologies (Wang et al. 2007). A study of wine strain fermentation with SAGE technology has shown that up to 10% of the expressed mRNAs do not correspond to previously annotated ORFs, and that up to 20% of the expressed genes, particularly those expressed in the late stationary phase, do not match known yeast genes (Varela et al. 2005). Therefore, they can represent novel wine yeast-specific genes not present, in DNA arrays.

26.3.3 Expression Responses of Wine Yeasts to Stress Situations During Vinification

Since the analysis carried out by Rossignol et al. (2003), other works have utilized array technology to understand molecular reasons for the adaptation of wine yeasts to the plethora of stress conditions taking place during wine production (Attfield 1997; Pizarro et al. 2007): rehydration, osmotic stress, nitrogen deprivation, cold stress, and increase in ethanol concentration.

Two studies have focused on the response of a commercial wine yeast to rehydration and its adaptation to osmotic stress at the beginning of vinification. In the first study (Rossignol et al. 2006), rehydration was carried out in a glucose complete medium, which allowed identification of phenomena related to the re-establishment of the fermenting stage. The authors found a substantial transcriptional remodelling: while dried yeasts displayed an expression profile typical of respiratory-grown cells starved for nitrogen and carbon, and which had been highly stressed during rehydration,

many genes involved in biosynthetic pathways, in either the transcription or protein synthesis, were co-ordinately induced, while genes subject to glucose repression were down-regulated. Genes involved in general stress response were repressed during rehydration despite high sugar concentration, while acid stress specific genes were induced, probably, in response to organic acid accumulation. In the second analysis (Novo et al. 2007), rehydration was carried out in water in order to separate this process from adaptation to osmotic pressure. This study indicates that after a rehydration period of 30 min, a further hour in water does not introduce any relevant changes into the global gene expression. The incubation of rehydrated cells in the presence of fermentable carbon sources results in the activation of some genes of the fermentation pathway, of the non-oxidative branch of the pentose phosphate pathway, and others related to ribosomal biogenesis and protein synthesis. Another study, focusing on yeast response to high sugar concentration, was carried out by Erasmus et al. (2003). For this analysis, rehydrated wine yeast was inoculated in Riesling grape juice containing 40% (w/v) of equimolar amounts of glucose and fructose, and the global expression was compared with the same must containing 22% sugar. Although sugar concentration in this study is not that usually found in vinification, some results coincided with those described by Rossignol et al. (2003). The glycolytic and pentose phosphate pathway, together with structural genes involved in the formation of acetic acid from acetaldehyde and succinic acid from glutamate, appeared to be up-regulated, while genes involved in the de novo biosynthesis of purines, pyrimidines, histidine and lysine were down-regulated by sugar stress. Besides, important changes in the expression of stress genes were found. These changes affected not only genes involved in the production of compatible osmolyte glycerol (*GPD1*), but also the heat shock proteins *HSP104/12/26/30/42/78/82* and *SSA3/4,* among others.

Nitrogen sources have been accepted as the main growth-limiting factor as its deprivation imposes a nutritional stress on metabolic activities. Thus, under nitrogen restriction for example, fermentation rates in the stationary phase are not sustained, and cease before fermentation is completed (reviewed in Pretorius 2000). This might be due to the reduced cell viability (Bisson 1999). Moreover, different nitrogen sources are classified as "good" or "poor" on the basis of the growth rate they support. Microarray analysis has shown that each source causes specific "signature transcripts" responses on laboratory strain yeast cells grown in a chemostat (Boer et al. 2007). This is of great importance because it is a common practice to supplement poor musts with an external nitrogen source: diammonium phosphate (DAP). Several studies have been carried out recently to understand how yeast cells respond under vinification conditions to nitrogen depletion and to nitrogen re-feeding and how it depends on the specific nitrogen source.

In the first of these studies, yeast cultures were grown in the presence of arginine as the only nitrogen source and two different concentrations of it were used (Backhus et al. 2001). This study determined how the expression of single or multiple genes (involved in the same, or related, metabolic pathway) varies between both experimental conditions. At an elevated nitrogen concentration, usually at the start of fermentation, fermentative activity is exalted and glycolytic enzymes present high expression

levels (a typical fermentative process with anaerobic pathways acting). In low nitrogen conditions, the expression of many ribosomal protein genes is also enhanced and, in spite of still high glucose concentration, some glucose-repressed genes are clearly over-expressed. One suggestion is that the response to low nitrogen conditions can lead to a switch from fermentation to respiration. This is reminiscent of the known "Pasteur effect": inhibition of fermentation by the presence of O_2 at low nitrogen conditions. Although the Pasteur effect has been considered irrelevant to laboratory yeast growth conditions (Lagunas 1986), it might well be relevant now to winemaking conditions. The known consequence of this situation is arrested fermentation. At a gene expression level, this accounts for the practice of adding a supplementary nitrogen source to sluggish or stuck fermentations.

One of the procedures followed in wineries to avoid sluggish or stuck fermentation is the early addition of DAP. Marks et al. (2003) described how 350 genes were affected by the addition of this salt to Riesling must in a late vinification stage when no yeast active growth was found. Genes involved in small molecule transport and urea biosynthesis were down-regulated after salt addition. On the other hand, genes involved in amino acid or purine metabolism, ribosomal proteins and sulphur assimilation were up-regulated, suggesting an active metabolism. These results are in agreement with those found by Rossignol et al. (2003) during the whole alcoholic fermentation, indicating that the TOR pathway plays a critical role in changes in gene expression in response to nitrogen depletion.

A more comprehensive and realistic study of the transcriptional response of *S. cerevisiae* to different nitrogen concentrations during alcoholic fermentation has been recently published (Mendes-Ferreira et al. 2007a, b). In this work, 11 samples corresponding to several time points in control vinifications, nitrogen-limiting fermentations and re-fed (with DAP) fermentations were compared. Approximately 70% of the yeast transcriptome was altered in at least one of the fermentation stages considered, and nitrogen concentration was shown to have a decisive effect on these gene expression changes. This analysis indicates that the early response to nitrogen limitation is characterised, as already described by Backhus et al. (2001), by the induction of genes involved in respiratory metabolism, which is followed by general down-regulation of the genes associated with catabolism. Surprisingly, slight increase of genes encoding ribosomal proteins and those involved in ribosome biogenesis is found during nitrogen starvation. This study identified 36 highly expressed genes under low nitrogen conditions or in the absence of nitrogen in comparison with a nitrogen replete condition. These "signature" genes genes can be useful for predicting nitrogen deficiency, and therefore for diagnosing wine stuck/sluggish fermentations (Mendes-Ferreira et al. 2007b). This analysis also demonstrated that the main transcriptional effect of re-feeding is the increase in the expression of those genes involved in glycolysis, thiamine metabolism and energy pathways (Mendes-Ferreira et al. 2007a), similar to the results found by Marks et al. (2003) after DAP addition. In agreement with the aforementioned chemostat results, another study has revealed that the effect of re-feeding depends on the nitrogen source added: a different gene expression reprogramming has been found when ammonia or amino acids are added to nitrogen limiting vinifications (Jiménez-Martí and del Olmo 2008).

Ammonia addition determines higher expression of genes involved in amino acids biosynthesis, while amino acids addition directly prepares cells for protein biosynthesis.

A global analysis has also been conducted in vinifications carried out at low temperatures. These conditions are often considered to introduce improved sensory qualities into wine. In experiments carried out at 13 and 25°C, Beltrán et al. (2006) have reported a response to cold stress at the initial stage of fermentation followed by an up-regulation of genes concerned with cell cycle, growth control and maintenance at the middle and late stages of the process at 13°C in relation to those at 25°C. Besides, several genes involved in mitochondrial short-chain fatty acid synthesis are also more expressed at the lower temperature. These transcriptomic changes are correlated with higher cell viability, improved ethanol resistance and increasing production of short chain fatty acids and their corresponding esters.

Another important stress condition during vinification is that caused by progressive ethanol production. Although molecular clues of ethanol resistance are not well understood (Pretorius 2000), they are known to be dependent on plasma membrane variations. Alexandre et al. (2001) made a global analysis, in a laboratory strain, of the short-term ethanol stress that we have previously cited. In our laboratory we studied yeast response to sudden ethanol addition. A laboratory strain stops growing when ethanol is added to 7.5%. Growth is reassumed after several hours. At that time, a specific increase in the level of mRNAs of genes encoding cell wall components, hexose transporters and enzymes for carbohydrate metabolism is seen (Antúnez and Pérez-Ortín, unpublished). Despite data available from global analysis of ethanol response in yeast laboratory strains (Alexandre et al., 2001), there are no published papers in which this topic is considered in wine yeasts. Some studies with wine yeast strains not specifically devoted to ethanol stress, however, can provide some insight into the topic. For instance, Backhus et al. (2001) and Rossignol et al. (2003) found changes in the levels of the expression of genes involved in biosynthesis of fatty acids, phospholipids and ergosterol during vinification. The expected higher expression levels for genes encoding these membrane components (*ACS1-2, FAA1-4, OLE1, ERG1-12,20,25, CHO1-2, PSD1-2*,…) were also observed in conditions resembling those of natural fermentation (Backhus et al. 2001). Usually, wine strains are more ethanol-resistant than laboratory ones. A comparative study has not been done with wine yeasts but with sake yeast strains. Using microarray studies to select target genes and ethanol sensitivity data of corresponding knockout strains Hirasawa et al. (2007) have found that tryptophan biosynthesis might confer ethanol resistance to yeast cells. Both tryptophan addition to the culture and overexpression of tryptophan biosynthesis genes conferred ethanol tolerance to yeast strains. For biological ageing of sherry wines, the involvement of *flor* wine yeast that differs from those involved in the previous fermentative stage is essential. During this process, yeast cells are affected by ethanol stress in combination with oxidative and acetaldehyde stress. Global studies have been carried out to compare transcriptome of *flor* yeasts and laboratory strains under conditions mimicking biological ageing of sherry wines (Infante 2002). This analysis has allowed identification of 51 genes which were

more expressed in the *flor* yeast, including several genes related with the adaptation of high ethanol concentration, oxidative stress resistance, glycerol assimilation and the utilisation of reserve carbohydrates. One of these genes, *MUC1/FLO11*, already related with biofilm formation (Reynolds and Fink 2001), has been proposed by these authors as being responsible for velum formation by these yeasts. Indeed, further studies have indicated that this gene is required for the air-liquid interface biofilm (Zara et al. 2005), and is the primary factor in *flor* formation caused by cell surface hydrophobicity in wild-type *flor* yeast (Ishigami et al. 2006).

Overall, stress-response genes in wine yeast strains are expressed at quite a high level, although expression level varies depending on the specific pathway in which the gene product acts. The expression levels for some of these genes have been taken as indices of the physiological status of fermenting cultures (see above). Moreover in the particular case of low-nitrogen conditions, cultures showed higher mRNA levels for genes involved in nitrogen compound recycling, and in translation and oxidative carbon metabolism (Backhus et al. 2001).

26.3.4 Comparative Studies Between Wine and Non-Wine Yeast Strains

In the global transcriptomic studies described so far one strain that is well adapted to vinification process has been considered. Using arrays analyses to understand the reasons for wine yeast adaptation is also an interesting approach to take. In this sense, our group carried out a comparative study between two wine yeasts, one capable of completing vinification and the other that left residual sugar at the end of the process (Zuzuárregui et al. 2006). The results obtained indicated that, together with changes in the expression of several genes that could explain some metabolic peculiarities of these strains, there was a differential expression of genes related to carbohydrate metabolism, transport and metabolism of nitrogen compounds, sterol transport and metabolism, and stimulus response.

One of the main conclusions of that work lies in the strain showing fermentative problems; changes in gene expression found in the early stationary phase correspond to those usually found in non-problematical wine yeasts under conditions of a late stationary phase, indicating a decrease in the fermentation-respiration balance. Although the expression levels of genes involved in the oxidative metabolism remain low (Backhus et al. 2001) in high nitrogen conditions in natural anaerobic fermentation (when fermentation is the main energy-generating process), the transcriptional response to nitrogen limitation for instance, involves induction of the genes involved in oxidative glucose metabolism, despite high glucose concentration available (Mendes-Ferreira et al. 2007a). Further studies would be required to completely understand the glucose metabolism changes during vinification, but global studies like those described can be potentially interesting to obtain some clues.

Several transcriptomic and genomic differences have been found between wine and non-wine yeast strains by means of array studies. For instance, in the T73 wine

yeast strain in relation to oxidative metabolism, *YHB1*, a gene encoding a flavohaemoglobin, whose expression is elevated in aerobic conditions in laboratory strains (Liu et al. 2000), is only slightly expressed in wine yeast (Hauser et al. 2001). A small deletion found in its promoter is thought to be the reason. This event may reflect the physiological features of the wine strain, which has been evolving for billions of generations under almost anaerobic conditions of wine fermentation.

As copper sulphate and other related salts, such as sodium sulphite, potassium bisulphite, and sulphur dioxide, have all been used in controlling mould growth on grapes, or during the winemaking process, to stabilize wine and kill bacteria, some specific genes, whose products can act in a kind of detoxification process, may be overexpressed in wine strains. Thus, genes involved in sulphur (*SUL1-2*) and ammonia (*MEP2*) transport (Cavalieri et al. 2000), or that involved in sulphite resistance (*SSU1*) were found to be over-expressed in wine yeast strains (Hauser et al. 2001). Pérez-Ortín et al. (2002a) investigated in great detail possible mechanisms for the expression regulation of the *SSU1* gene of the T73 wine yeast strain. A rearrangement of the promoter of *SSU1* was detected and led to an up-regulation in its expression. We can conclude that human involvement and traditional vinification protocols led to a selection of wine yeasts which resist these agents. Recently, Aa et al. (2006) analyzed both the population genetic variation and population structure of *S. cerevisiae* by sequencing the coding region of *SSU1* and three other loci (*CDC19*, *PDH1*, *FZF1*) in 27 strains from very different locations in Italy and Pennsylvania, collected from oak forests and vineyards. The phylogenetic reconstruction showed existence of differences between oak strains and wine strains, indicating that differences within *S. cerevisiae* populations are more likely due to ecological than geographic factors. The high sequence polymorphism found in the *SSU1* gene suggests the existence of a diversifying selection on its protein product, thus supporting our previous proposal of a strong selection for this gene during the historical use of sulphur-based fungicides in winemaking. Surprisingly however, the *CUP1* gene, which is related to copper resistance (Karin et al. 1984; Winge et al. 1985), is less expressed in YPD in the T73 wine strain than in the S288c background (Hauser et al. 2001). This is due to a small deletion in the *CUP1* locus region (Pérez-Ortín et al. 2002b).

26.4 Genomic Studies in Wine Yeast Strains

Other than evaluating the changes in the expression levels of many genes, if not all genes of a yeast strain under many different conditions, or comparing these expression levels between different wine strains, DNA array analysis can also be used for a variety of genomic research applications: systematic characterisation of genes discovered by sequencing projects, identification of new transcripts unidentified to date, detection of aneuplodies or partial chromosome deletions, chromosomal rearrangements yielding genome-wide amplifications, identification of interesting Quantitative Trait Loci (QTLs), etc.

DNA arrays promise a deeper understanding of yeast genome plasticity. Allelic variations can be detected in any strain by analyzing the patterns obtained by hybridizing genotyping arrays with total genomic DNA (Winzeler et al. 1998). These arrays are strictly dependent on precise sequence of the target; therefore, point changes in the genes (or in their transcripts) may produce differences in signal intensity, or even no signal at all. Even though the use of oligonucleotide arrays is the only way to analyze allelic differences in detail, it can, however, produce results which are difficult to interpret if yeast strains are being analyzed that are not from the S288c background. For instance, Primig et al. (2000) have shown that SK1 strain has more genetic variation as polymorphisms and deletions (34%) when comparing the S288C standard background with the W303 background (5%).

In similar experiments, but with full-length ORF PCR-product arrays, comparative wide-expression analysis of different yeast strains, and a correlation among profiles, can monitor chromosome aneuploidy or chromosomal segment duplications (Hughes et al. 2000b). Differences in the ploidy are, in part, responsible for different developmental, morphological and physiological characteristics, which are especially important for industrial yeast strains, as already indicated. In order to detect ploidy-regulated genes, isogenic sets from haploid to tetraploid were generated and their mRNA levels were monitored. The relationship between cell size and the ploidy can be explained by the repression of some G1 cyclins, as can the invasive growth by decreased expression of *FLO11* as the ploidy increased (Galitski et al. 1999). Hauser et al. (2001) found important differences between laboratory and wine strains when both expression and genomic hybridisation values for transposon (Ty) ORFs were analyzed. The low expression of these ORF in wine yeast strain seems to be due to the fact that the laboratory strain (S288c genetic background) has more copies of transposable elements (Ty1, Ty2, Ty3 and Ty4) than the wine yeast strain. This factor, also shared by other industrial yeast strains such as brewer's yeast strains (Codón et al. 1998), agrees with the suggestion that a negative selection for transposon accumulation might exist in the Ty elements recently expanded in laboratory strains, but in the more competitive wine fermentations (Jordan and McDonald 1999; Rachidi et al. 1999).

Length polymorphisms in the chromosome from a "cava"-making yeast strain were investigated by means of DNA array technology, but only with a small number of genes from yeast chromosome I (Carro et al. 2003). A DNA "mini-array" containing 14 probes for several ORFs covering the complete length of chromosome I, was hybridized with different isolated and radioactively labelled length variants of that chromosome (belonging to different strains). The results delimited a polymorphic region in the right arm of chromosome I that suffered varying deletions of different cava strains tested.

Using the array-based Comparative Genomic Hybridization (aCGH) technique, Infante et al. (2003) found that two prominent variants of *S. cerevisiae flor* yeast strains differ from one another in the DNA copy number of 116 genomic regions that comprise 38% of the open reading frames (ORFs). They also found that the majority of them correspond to a widespread amplification of genomic fragments. By analyzing different situations found, the authors suggest that amplifications were produced by

gross chromosomal rearrangements (GCRs) mediated by identified hotspots (transposon LTRs, tRNAs, subtelomeric repeated sequences, etc.), helped by bursts of double-strand breaks (DSBs) mainly produced by both acetaldehyde and ethanol. One of the unique properties of *flor* yeast is the production and release of high amounts of acetaldehyde as a consequence of ethanol assimilation. Since some of the genes among those involved in these copy number variations have functions related to specific phenotypes that are characteristic of *flor* yeast strains, one possible suggestion is that this mechanism is responsible for the adaptive evolution of these yeasts. Actually, two changes in *FLO11* (a large deletion in the promoter and another in the coding region) differentiate *flor* yeast strains from other non-floating strains (Fidalgo et al. 2006).

The aCGH technique also allowed Dunn et al. (2005) to analyze four commonly used commercial wine yeast strains. They assayed three independent isolates from each strain, and compared them with laboratory strain S288C. All four wine strains displayed common differences with regard to laboratory strain S288C. Some may be specific to commercial wine yeasts. Slight differences inter- or intra-strain were observed, indicating that they are closely related and quite genetically stable. Among the variations, there are genes that code for transporter proteins (mainly transporter or sugar utilization genes, which vary depending on the particular substrate in that yeast growth). Moreover, genes exist that are involved in drug resistance (or detoxification). The authors not only propose a "commercial wine strain signature", comprising genes whose copy number is altered in all the wine yeast isolates examined in relation to the S288C strain, but also suggest that the differences in the fermentation and organoleptic properties of different strains may arise from a small number of genetic changes.

Varela et al. (2005) used the SAGE technique (Velculescu et al. 1997) to quantify expression profiles of wine yeast under winemaking conditions. Apart from corroborating results from other authors (expression profiles from well characterized ORFs), they also found transcripts corresponding to intergenic regions (NORFs tags, 10%), and others that did not match the published reference yeast genome (NID tags, 22%). Since the authors detected the expression of genes that had not been reported in other studies using SAGE, it can be suggested that winemaking conditions impose a substantially different environment for yeast growth, especially in the late stationary phase when the highest number of NORFs and NIDs are observed. Perhaps the high amount of ethanol achieved in this phase could be responsible for these physiological changes.

26.5 Conclusions

The use of DNA array techniques for wine yeast research has started recently. Despite the fact that other genomic techniques, such as SAGE and MPS, have provided interesting results in relation to wine yeast during fermentation (Varela et al. 2005; Wang et al. 2007), DNA arrays are currently much more feasible and straightforward, and are providing more clues towards an understanding of the biotechnology

process. It is particularly important to know why some yeast strains are able to perform winemaking whereas others are not, why some of them are more resistant to particular stresses, and how the evolution has modelled the genome of this organism. This knowledge will be of great importance in the improvement of current winemaking technologies and accompanying yeast strains.

To date, transcriptomic studies undertaken in the vinification context have only been carried out with *S. cerevisiae* strains. It would be desirable to carry out analysis of this kind in the future with other yeast and bacterial strains which are involved in wine production to a lesser or greater extent (lactic and acetic acid bacteria and yeasts belonging to *Kloeckera* and *Hanseniospora,* among others).

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Chapter 27
Application of Yeast and Bacteria as Starter Cultures

Sibylle Krieger-Weber

27.1 Introduction

Although wine yeasts have been known for a long time, the production of wine has remained more of an art than a science until 40 years ago. The production and use of active dry yeasts (ADY) began in the United States in the mid-1960s and expanded worldwide thereafter (Degre 1993). In inoculated fermentations, selected strains of *Saccharomyces cerevisiae* are usually added to achieve a population of about 10^5–10^6 cells ml^{-1} in the must to ensure a quicker start for fermentation, to out-compete and dominate indigenous yeast strains and provide a wine with distinctive characters.

The history of controlled malolactic fermentation (MLF) is even shorter. Despite the early discovery of Müller-Thurgau in 1891 of lactic acid bacteria (LAB) contributing to the acid reduction in wine, by degrading malic acid to lactic acid and CO_2, commercial starter cultures were only introduced to the markets in the beginning of the 1980s. Most commonly *Oenococcus oeni* (ex *Leuconostoc oenos*) starter cultures are used, but there are also some preparations with lactobacilli reported to give good results (Prahl 1989). Malolactic (ML) starter cultures for easy direct inoculation were only made available in the early 1990s.

27.2 Application of Yeast Starter Cultures

In spontaneous alcoholic fermentations, there is an early and rapid succession of yeast species such as *Hanseniaspora, Kloeckera, Candida stellata*, *Metschnikowia pulcherrima*, *Torulaspora delbrueckii* or *Pichia*, which commonly grow in must (Henschke 1997), but subsequently die, while generally *Saccharomyces cerevisiae* dominate and lead and complete alcoholic fermentation (Fleet and Heard 1993). The critical point of a spontaneous alcoholic fermentation is around 4% vol. alcohol (Dittrich and Grossmann

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2005), when the non-*Saccharomyces* yeast die-off and strains of *Saccharomyces cerevisiae* become dominant. However, dominance of *Saccharomyces* cerevisiae does not guarantee a successful alcoholic fermentation; it also depends on genetic disposition of the dominant strain. There has been much discussion over the years concerning the relative merits of spontaneous alcoholic fermentation versus induced alcoholic fermentations. A definitive resolution of this issue is unlikely, as it partially depends on stylistic preferences, grape variety, grape juice composition and vintage.

27.2.1 Selection of the Yeast Strains for Winemaking

Today worldwide more than 200 different yeast strains are commercially available. These yeast strains were selected for specific properties (Table 27.1), which can be divided in two groups: desirable and undesirable features (Degre 1993), and also technological and qualitative properties as described by Dittrich and Grossmann (2005).

27.2.2 Wine Yeast Characterization

Oxygen and nitrogen requirements: As already reported in Chap. 19, yeast can generally synthesize all amino acids and nitrogenous bases required for their growth from ammonium ions, although yeast growth is accelerated when ready-made building blocks, amino acids, are available in the growth medium. Nitrogen content of must can often be a limiting factor (Amerine et al. 1980); a relationship had been found between the initial concentrations and the maximum fermentation rate (Bely et al. 1991). A value less than approximately 150 mg L^{-1} assimilable nitrogen (YAN) in the must is associated with greater chance of fermentation problems (Henschke and Jiranek 1993). Additions of nitrogen during the stationary phase can be effective, but some authors have demonstrated this effect being strain specific (Jiranek et al. 1991). Julien et al. (2000) proposed a method to quantify nitrogen and oxygen yeast requirements and to study these requirements depending on the yeast strain. Nitrogen requirements were determined during the stationary phase of yeast fermentation. To quantify the effectiveness of nitrogen addition during this phase, constant rate fermentations were performed. Very important differences were observed: some strains needed twice the nitrogen compared to other strains to maintain the same fermentation rate. Based on these results selected yeast strains have been classified as low, medium or high nitrogen demanding. Oxygen is another important factor for yeast metabolism during winemaking since it is required for the synthesis of sterols and fatty acids. Sablayrolles et al. (1996) demonstrated the advantage of combined additions of oxygen and nitrogen to prevent sluggish or stuck alcoholic fermentations. Again different selected yeast strains vary in their oxygen requirement (Julien et al. 2001).

Table 27.1 Selection criteria for yeast strains for commercial use

Desirable	Undesirable
Qualitative properties	*Qualitative properties*
Production of positive fruit aromas and esters	Production of sulfur dioxide
Production of β-glucosidase	Production of hydrogen sulfide
Production of glycerol	Production of S-deriving compounds
Production of manno-proteins	Production of volatile acidity and ethyl acetate
	Production of SO_2-binding compounds (acetaldehyde, pyruvate…)
For special applications:	Formation of ethyl carbamate precursors
Degradation of malic acid	Production of polyphenol oxidase
Formation of lactic acid	Production of biogenic amines
Formation of isoamylacetate	
Fast autolysis	
Technological properties	*Technological properties*
Complete fermentation of sugar	Foaming properties
High tolerance to alcohol	Biofilm formation
Resistance to sulfur dioxide	Activity during fermentation
Minimal lag-phase on rehydration	
Fermentation at low temperatures	
Tolerance to high temperatures	
Fermentation under pressure	
Activity during fermentation	
Killer phenomena	
For special applications:	
Agglomerization properties	
Sedimentation properties	

Temperature and alcohol tolerance: Temperature strongly influences yeast growth. *Saccharomyces cerevisiae* can grow over a range of 0–45°C, and the optimum temperature for alcoholic fermentation ranges between 20 and 30°C (Henick-Kling 1988). Again it is important to look at selected yeasts for their temperature tolerances, which is normally done in a minimal synthetic medium at 10, 12, 15, 20, and 30°C, to select the best yeast strain for specific vinification conditions.

Alcohol tolerance is tested in the same medium. Most selected yeast strains will tolerate up to 14% vol., but for fermentation of high maturity red grape juices the use of yeast with higher alcohol tolerances, 16% vol. and above, is highly recommended.

27.2.3 Choice of the Active Dry Yeast Starter Cultures

In the seventies and eighties, when the first active dry yeast starter cultures were used for winemaking, yeast had been selected mainly for their technological

advantages; nowadays their sensory properties and contribution to overall wine quality are just as important. Thus, the more recently selected strains have more than a simple role of fermenting sugar into ethanol. Although there is a high probability that inoculated *S. cerevisiae* will dominate fermentation (Schütz and Gafner 1993), seeding will not necessarily guarantee 100% dominance of the strain or its exclusive contribution to fermentation. Significant factors that affect this outcome will be the population of the indigenous yeasts already in the juice, choice of yeast strain and its adaptation to specific wine environment. It is crucial to choose a suitable yeast strain, which can grow and express its metabolic activity under given conditions, e.g. a yeast strain with good tolerance to low temperatures for cool white wine fermentations, or a yeast strain, which can tolerate higher temperatures in high pH, high alcohol red wine vinifications. Since it is not always easy to select the right yeast strain for specific wine conditions and sensory contribution, Lallemand has put a "yeast chart" (Table 27.2) to help winemakers conquer the challenge of choosing the right yeast for every fermentation.

The plurality and variation of selected active dry yeast preparations available for winemaking can be even more confusing because producers provide different information on their yeast strains. To help the winemaker with the best choice, the Research Station of Geisenheim has developed a unique data sheet to record most important yeast strain characteristics of selected yeast strains available in the German market. This data was collected in a data base and can be accessed in electronic form on the web page of the research station or under www.hefefinder.de. The system proposes the most suitable yeast strain for a particular wine environment and wine style based on details given in a questionnaire by any person and also proposes a ranking within several yeast strains.

Table 27.2 Quick yeast chart

Criteria for selecting a yeast strain	Rating
Suited for white wine vinification	1–4
Suited for rosé wine vinification	1–4
Suited for red wine vinification	1–4
Suited for restart stuck fermentations	1–4
Sensory effect	Neutral – esters – EVC[a]
Temperature range (°C)	Range does not indicate "optimum temperature range"
Fermentation speed	Slow – moderate – fast
Competitive factor	Sensitive – neutral-active
Alcohol tolerance	Max. alcohol levels tolerated
Relative nitrogen needs[b]	Low – medium – high
H_2S Production (60 ppm N)	Low – medium – high
H_2S Production (170 ppm N)	Low – medium – high

Highest rating (compatibility) = 4, Lowest rating = 1

[a]EVC = Enhances Varietal Character

[b]"relative nitrogen requirement" refers to how much nitrogen one strain requires relative to the other strains on the chart under nitrogen-limiting conditions

27.2.4 Utilization of Active Dry Yeast Starter Cultures

Beside the traditional method of inoculating freshly prepared juice with an amount of actively fermenting juice, two types of yeast starter cultures are used: liquid starter cultures and active dry yeast preparations. Most yeast starter cultures are pure and consist of only one strain of *Saccharomyces cerevisiae*. Liquid yeast preparations have a limited market because of their short shelf-life. Liquid starter cultures are prepared by the winery, or by a commercial supplier (e.g. local wine laboratories or institutes). The main difference between these cultures and active dry yeast cultures is that they are not subjected to drying, and thus have a high population of viable cells, but only for a short time frame. Main applications are for specific juices like fermentation of dry berry selections or ice wines and even in the preparation of sparkling wines.

Active dry yeast starter cultures are grown over different propagation steps with adequate supply of oxygen and nutrients to produce yeast that contain optimal amounts of protein, ergosterol, unsaturated fatty acids, and reserve materials (Monk 1986). The yeast is then dried to conserve it during transport and storage. In addition to the strain, the trehalose content in the cell is one of the most important factors that affects the resistance of yeast to drying and subsequent rehydration. Therefore, there is a strong incentive for yeast producers to stimulate formation of trehalose during production in order to increase resistance of yeast cells to the stresses of dehydration and rehydration" (Degre 1993).

Active dry yeast rehydration: Commercial preparations of dry yeast normally contain less than 8% residual moisture, in most cases even less (6%). Thus, active dry yeast must be rehydrated for revitalization. Rehydration of active dry yeast is very critical, because if it is not done properly it can leak large amounts of cellular components and loose viability and vitality (Henick-Kling 1988). Although yeast rehydration is a straight forward operation and several scientific and technical papers have been published on correct techniques for obtaining healthy membranes and subsequent optimum technological performance, manufacture's instruction vary.

Degre (1993) proposed as a general procedure for vinification:

- Sprinkle 500 g of dry yeast into 5 l of warm water (35–40°C)
- Stir the suspension after 5 min to re-suspend all cells
- Leave yeast cells not more the 30 min in this suspension to avoid use-up of their reserve material
- Add yeast to 20–25 hL must to be fermented, which would correspond to a dosage 25–30 g hL^{-1} (ca. 2–4 × 10^6 cfu ml^{-1})

Some yeast producers recommend manufacturer precise "clean, chlorine free water and 15–30 min holding time before stirring". Other producers prefer to carry out "the soaking of the yeast" in a mixture of juice and water at 35–40°C, because with the addition of juice the yeast cells, which will start budding according to theory during rehydration will have a source of nutrition and can also adapt to the juice/must environment. This procedure may have advantages if the rehydration

exceeds the recommended 30 min. Radler et al. (1985) had, in his most complete studies on wine yeast rehydration, obtained maximum values for cell viability and fermentation activity when rehydration temperatures ranged between 38 and 45°C. Within a time frame of 2 h for rehydration, no change in these activities was observed, but the composition of the rehydration medium had an important influence. It was found that a mixture of grape juice and water, solutions containing sugars, vitamins or salts did have an impact on the metabolic activity of rehydrated yeast. Best activity was achieved when rehydrating in 1% KCl solution. Rehydration in more than 50% juice is not recommended, because of high osmotic pressure, low pH and sometimes high SO_2 levels or fungicide levels.

Active dry yeast rehydration using a yeast rehydration nutrient: Studies of Fornairon-Bonnefond et al. (2002) have shown positive impact of specific sterols during the rehydration phase on the structure of the plasma membrane resulting in better fermentation capacity, particularly under difficult wine conditions. Since the membranes are stressed from the drying and rehydration processes, the yeast needs to mobilize lipid reserves for repair as shown by Beker et al. (1984). More recently Soubeyrand (2005) showed that yeast can also incorporate extra-cellular lipids, including sterols, which is again interesting as these molecules can play an important role in yeast cell vitality in the final stages of alcoholic fermentation (Luparia et al. 2004). In grape musts sterols are present in the form of phytosterols, but their nature differs from the sterols synthesized by the yeast during growth. Because of the differences in the chemical structure, these phytosterols were not sufficient to guarantee yeast integrity during the whole alcoholic fermentation (Luparia et al. 2004). Soubeyrand (2005) has studied the possibility of incorporating specific yeast sterols during rehydration by addition of specific inactive yeast preparations naturally rich in sterols to the rehydration medium. The influence of rehydrating ADY in the presence of micronutrient and /or sterol and unsaturated fatty acid enriched inactivated yeast suspension on yeast viability (Kontkanen 2004) which was remarkable. Higher maximum yeast cell density (Fig. 27.1) and shorter overall fermentation lengths (Fig. 27.2) were observed when using these types of rehydration nutrients especially under high sugar concentrations. The impact obtained on the later performance of the yeast is excellent. The recommended procedure for yeast rehydration using a rehydration nutrient is shown in Table 27.3.

Utilization of multiple strain *Saccharomyces cerevisiae* starter cultures: Spontaneous alcoholic fermentations are generally driven by more than one yeast strain. New molecular biological methods allowed detecting different yeast populations in wild fermentation depending on the phase of the fermentation. The succession of different yeast strains could lead to more complexity in the aroma profile of the final wine in both a positive and a negative sense. Besides the recommendation of achieving complexity in a more controlled manner by preparing separate ferments with different selected yeasts and then blending the ferments mixed cultures of *Saccharomyces cerevisiae* had been developed, in imitation of the variability of a spontaneous alcoholic fermentation. The yeast strains are produced as single cultures and the final mix is done by blending the dried pure cultures. Still the market for mixed *Saccharomyces cerevisiae* starter cultures is small also due to the mixed

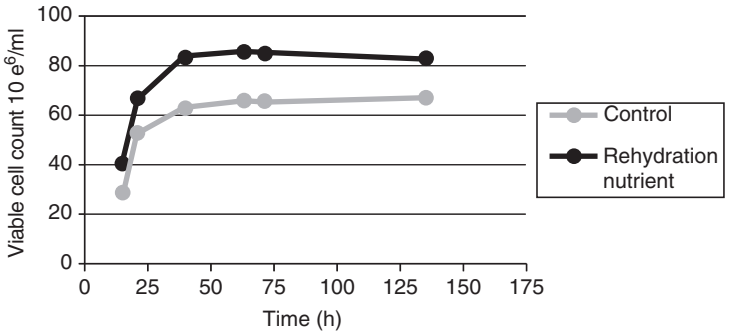

Fig. 27.1 Effect of rehydrating ADY in a micronutrient and sterol enriched inactivated yeast suspension on the cell viability at the end of alcoholic fermentation (potential alcohol 14% vol., fermentation temperature 28°C); *Grey line* control (rehydration without addition): 28×10^6 cfu ml^{-1} (42%); *Dark Line* rehydration in presence of a specific rehydration nutrient: 42×10^6 cfu ml^{-1} (50%)

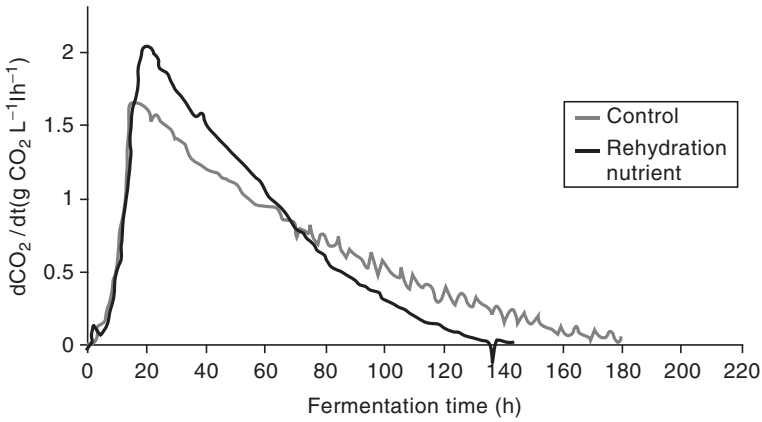

Fig. 27.2 Variation of CO_2 production by *Saccharomyces cerevisiae* strain EC1118 in a Chasan must (240 g l^{-1} sugar and 266 mg l^{-1} FAN) rehydrating ADY in a micronutrient and sterol enriched inactivated yeast suspension compared a standard rehydration in water only. *Grey line* control (rehydration without addition); *Dark line* rehydration in presence of a specific rehydration nutrient. Fermentations carried out in 1.1 l fermenters under isothermal conditions (28°C) with gentle stirring (Sablyrolles 1993). The CO_2 production rate was calculated automatically from the weight loss of the fermenter expressed as a function of fermentation time

success of these inoculations. Due to the variability of the juice/wine matrix, wine conditions may favor one or the other strain and allow dominance by one strain as otherwise it can induce a negative interaction between strains, which will have an impact on the sensory profile of the final wine.

Inoculation of grape must with *Saccharomyces* and Non-*Saccharomyces* strains: In some cases, wine produced with pure yeast mono-cultures lack flavor complexity that may originate from good indigenous fermentations. But wild

Table 27.3 Instructions for optimal yeast rehydration using a rehydration nutrient for the inoculation of 100 hl must

Step	Action
1	Suspend 3 kg (30 g hL^{-1}) of yeast rehydration nutrient in 20 times of its weight in clean water (43°C).
2	Once the temperature of yeast rehydration nutrient solution has dropped to 40°C, add 2.5 kg active dried yeast (25 g hL^{-1}). Stir gently to break up any clumps. Let suspension stand for 15–30 min, the stir gentle again.
3	Over a period of 5 min slowly combine an equal amount of must to be fermented with the yeast suspension. This will help the yeast to adapt to the cooler temperatures in the must and will avoid cold shock caused by a rapid temperature drop exceeding 10°C. This atemperation step may need repeating for very low temperature must. Each atemperation step should last about 5 min.
4	Add the yeast suspension to the bottom of the fermentation tanks as you begin filling the vessels with must.

fermentations require more vigilance and are a gamble, as explained earlier in this chapter, leading to off-flavors or stuck fermentations with high amounts of residual sugar because of the dominance mainly of undesirable non-*Saccharomyces* strains. Only recently wine research has discovered these "exotic" yeast strains and more knowledge is available on their real impact on the sensory profiles of wines (Ciani 1997). Some of these strains like *Pichia fermentans*, *Candida stellata* or *Torulaspora delbrueckii* have been studied for their interesting organoleptic contributions (Clemente-Jimenez et al. 2005; Ciani and Ferraro 1996; Moreno et al. 1991). Although some of these strains could improve the wine bouquet, most of them are not able to complete alcoholic fermentation. For this reason incorporation of a *Saccharomyces cerevisiae* strain with non-*Saccharomyces* strains was studied to overcome these shortcomings. The first blended commercial *Saccharomyces cerevisiae*/non-*Saccharomyces* starter cultures was introduced at the beginning of the twenty-first century with mixed success due to unpredictable interactions between the yeast populations induced by the wine matrix favoring the dominance of one strain over the other. A recent study by Languet et al. (2006) showed good success by reproducing the natural succession of yeast population with sequential inoculation of a non-*Saccharomyces* strain first followed by a good fermenting *Saccharomyces cerevisiae* strain during a later stage of alcoholic fermentation. These sequential inoculations have not only shown better results in terms of intensity but also in terms of sensory complexity.

Utilization of yeast starter cultures in the production of sparkling wine: For the production of sparkling wine or champagne style wines both liquid yeast starter cultures and dried yeast starter cultures are used for secondary in-bottle fermentation. The liquid cultures have to be built up under sterile conditions not only to increase the volume of the inoculums, but also to adapt to difficult conditions in the sparkling base wines. It is also obligatory to acclimatize active dried yeast cultures prior to inoculation for the secondary fermentation, since the direct addition of rehydrated yeast suspension to a medium containing higher levels of alcohol can damage the yeast cell. Again variations of protocols exist; one widely used is described below:

– Rehydration of the ADY according to the yeast producer's instructions, preferably in the presence of a rehydrating nutrient.
– Addition of the yeast suspension to a part of the sparkling base wine (3–10% of the total volume) supplemented with grape juice (up to 50 g l^{-1}) or sugar (between 50–100 g l^{-1}) and ammonium phosphate (0.5–2 g l^{-1}). A variation of the traditional method of starter culture preparation is the use of a mixture of equal parts of base wine, water and tirage liqueur (Wilkinson 1986)
– Acclimatization for 12–20 h (the yeast starts to produce alcohol) at 20–25°C. The suspension must be occasionally stirred for oxygenation to stimulate yeast growth. If conditions are very difficult or the base wine temperatures are very low, acclimatization can be also done at constant lower temperatures
– Again, it is crucial to avoid temperature differences of more than 5°C when transferring the acclimatized yeast solution to the final wine volume

Utilization of yeast starter cultures to restart stuck fermentations: Dr. Paul Monk used to say "The best solution for a stuck fermentation is prevention". Problems occur because of highly clarified must, low temperature fermentations, high temperatures especially in the presence of alcohol, lack of nitrogen, micro-nutrients, sterols, high concentrations of sugar or alcohol, negative interactions with other wine microbes, spray residues. Various factors can have a negative impact on yeast vitality (Dittrich 1977), and as many circumstances can cause stuck alcoholic fermentations as numerous protocols studied and proposed to cure stuck fermentations (Graf and Bannister 1996; Leske and Henschke 1996; Bisson and Butzke 2000; Fischer 2000). All protocols recommend racking off the old yeast and using an alcohol tolerant and vigorous fermenting yeast strain, to restart the stuck fermentation. Most protocols also recommend the addition of SO_2 (30 mg l^{-1}) and/or lysozyme to avoid growth of spoilage bacteria or wild yeast. If potential inhibitory substances are expected to be in the wine, addition of yeast hulls at 25 g hL^{-1} is recommended (Lafon-Lafourcade et al. 1984). After yeast hulls have settled (ca. 48 h) the wine has to be racked or filtered. The preparation of the rescue yeast varies between different yeast producers and research groups. Grossmann has listed in Sect. 7.6 of their wine microbiology book (2005), four different recommendations published in wine literature.

Lallemand recommends:

1. Rehydration of rescue yeast (50 g hL^{-1}) in a rehydration nutrient: Calculation of the appropriate amount of yeast rehydration nutrient at 1.25 times the weight of the yeast to be used. Suspension of the rehydration nutrient in 20 times of its weight of 43°C clean water, gentle mixing, allowing the solution to cool to 40°C. The rescue yeast is sprinkled on the suspension and stirred gently to mix and avoid clumping. The suspension stands for 15–30 min.
2. In the meantime, in another container, preparation of starter mixture with 2.5% of volume of stuck wine and 2.5% of volume of water and a complete yeast nutrient (50 g hL^{-1} wine and water mix). Sugar levels are adjusted to 5 Brix (50 g l^{-1}) with juice, concentrate or sugar. The temperature of the mixture has to be adjusted to 25–30°C.
3. The rehydrated rescue yeast suspension has to be slowly added to this wine/water/sugar mix. The temperature should be maintained at 15–30°C. The sugar

levels are monitored and when the sugar level has dropped by half (approximately 2.5 Brix – ca. 25 g l^{-1} sugar) stuck wine is added to the starter in batches of 20% of the total volume of stuck wine (total of five additions to the starter). Temperature should be maintained between 20 and 25°C. A very critical point is to avoid sugar completion before the addition of the next batch. Only at the last batch of added stuck wine should the sugar be allowed to completely deplete.

More recently the Gafner's group (Sütterlin et al. 2004) proposed the use of *Zygosaccharomyces bailii* to rebalance the glucose-fructose-ratio back to values above 0.1 because of its fructophilic character. Best results were achieved when the *Zygosaccharomyces bailii* strain was inoculated together with a strong fermenting *Saccharomyces cerevisiae* strain since the *Zygosaccharomyces bailii* was loosing viability after the correction of the glucose-fructose-ratio, and the *Saccharomyces* strain took over and fermented the wine to dryness. The first *Zygosaccharomyces bailii* starter culture to restart stuck alcoholic fermentations was introduced to the German market in 2007.

Another innovative approach is the use of immobilized *Saccharomyces cerevisiae* yeast strains selected for its strong fermenting properties and its high tolerance to alcohol. One typical technique of immobilization is encapsulationwhich involves coating microorganisms in a rigid alginate matrix (natural polysaccharide extracted from seaweed). The encapsulation allows substrates and metabolites to diffuse easily throughout the gel matrix without releasing yeast cells into must or wine. They have the advantage of being easily introduced and removed from the media after transformation of sugar into alcohol.

27.3 Application of Bacterial Starter Cultures

For a long time spontaneous acid reduction observed in wine was related to precipitation of tartaric acid only, though in 1891, Müller-Thurgau had already postulated that acid reduction could be due to bacterial activity. In 1913 Müller-Thurgau and Osterwalder, with their epoch-making investigation into lactic acid bacteria in wine, explained bacterial degradation of malic acid to lactic acid and CO_2 according to the formula:

$$C_4H_6O_5 = C_2H_6O_3 + CO_2$$

They called this phenomenon biological deacidification or malolactic fermentation, and *Bacterium gracile* was described as the responsible agent. Since these early findings, research on lactic acid bacteria (LAB) has progressed. The name, *Bacterium gracile*, which was used frequently in the past to recognize the organism which caused the malolactic fermentation, was revised. Findings by Radler (1963) showed that lactic acid bacteria of grape must and wine belong

to the genera *Lactobacillus*, *Leuconostoc*, *Pediococcus* and more recently to *Oenococcus* (Dicks et al. 1995). Different LAB enter into grape juice and wine from the grape berry surface, stems, leaves, soil and winery equipment. However, due to the highly selective environment of different juices and wines, only few types of LAB are able to grow in wine (Wibowo et al. 1985). Studies from several countries indicate that *Oenococcus oeni* is the predominant species conducting malolactic fermentation in wine, even though the LAB composition of grape must at the beginning of the alcoholic fermentation is dominated by *Lactobacillus* strains described in more detail in Chap. 1 of this edition by König and Fröhlich.

Historically, MLF was described as a phenomenon that is capricious and not completely understood, but is of great importance to the final product. In the not-too-distant past, winemakers were content to let nature take its course, and to merely wait for the MLF to occur spontaneously (Rich Morenzoni 2005). This practice was responsible for typical MLF comments such as – "It doesn't go when I want it to", and "I don't like what it does to the wine." Recent research into MLF has helped us to understand better this process of biological wine de-acidification and the limiting factors in wine impacting on the performance of lactic acid bacteria responsible for the biotransformation. "When we encounter a wine that has undergone a spontaneous MLF, it means that a lactic acid bacterium has overcome these hardships and has taken up residence in the wine. However, it does not mean that these bacteria will give us a malolactic fermentation that we can predict, nor will it give us one that has the positive organoleptic and sensory profiles that we want. It only means that a lactic acid bacterium is present in the wine, and that it, not the winemaker, has the ultimate control upon the quality of the finished product" (Rich Morenzoni 2005).

27.3.1 Selection and Characterization of Lactic Acid Bacteria for Winemaking Starter Culture Preparations

As explained previously, relying on indigenous bacterial microflora to complete timely a desirable malolactic fermentation can be precarious, even in low pH musts and wines. Even when desirable malolactic acid bacteria are established in a winery, the onset of the malolactic fermentation may take several months and may occur in some barrels and tanks but not in others. For this reason, induction of the MLF by the use of selected bacterial starter cultures is becoming the preferred option. *Oenococcus oeni* is the MLF organism of choice, but not all strains of this bacterium are good candidates for use as starters. Selecting strains of *Oenococcus oeni*, which are best in terms of performance and most interesting in flavor production, is a multifaceted and challenging task (Bou and Powell 2005). It is imperative to isolate and domesticate only natural

malolactic bacteria (MLB) strains. Wines which employ natural selective pressures of low pH, low cellar temperature, high alcohol and high SO_2 are used to supply isolates of malolactic bacteria. The physiology and genetic profiles of new and interesting strains are determined in the laboratory and in pilot vinifications. One of the first selection criteria for a selected bacteria isolate is its ability to withstand rigors of stress induction during the production process (Bou and Powell 2005) and the freeze-drying step. Besides high resistance to limiting wine conditions as pH, alcohol, SO_2 and temperature, the bacteria are also selected for desirable metabolic activities and absence of undesirable features (Table 27.4).

Table 27.4 Selection criteria for malolactic bacteria strains for winemaking

Desirable	Undesirable
Technological properties	*Technological properties*
Resistance to stress during production	Formation of excessive exo-polysaccharides
Resistance to freezing and freeze-drying	Host of pro-phages
Resistance to low pH	Too high tolerance to SO_2
High tolerance to alcohol	Too fast degradation of malic acid (for red wine application and color stabilization)
High tolerance to SO_2	
Good performance at low temperatures	
Short lag phase	
Fast degradation of malic acid	
Good tolerance of oxygen	
Tolerance of pesticides	
Elevated resistance to Lysozyme	
Production of bacteriocins	
Qualitative properties	*Qualitative properties*
β-glucosidase activity	Production of biogenic amines
Esterase activity	Production of ethyl carbamate
Production of positive fruit aromas	Production of S-deriving compounds
Reducing Vegetative notes	Production of volatile acidity
Rounding the mouthfeel	Production of ethyl lactate
Lowering astringency	Production of mousy taint
Lowering bitterness	Production of volatile phenols
Increasing complexity	Production of geranium off-flavors
Lowering overall SO_2 (degradation of acetaldehyde and keto-compounds)	Production of (excessive) amounts of diacetyl
	Fast degradation of citric acid
Production of acetaldehyde (red wine color stabilization)	Production/degradation of acetaldehyde
	Degradation of citric acid
Production of moderate amounts of diacetyl	
Production of butandiol	
Low affinity to glucose	

27.3.2 Malolactic Starter Culture Preparations

Control of malolactic fermentation, which is an integral part of the winemaking process, was often ignored until ML starter cultures became available. Liquid ML cultures were available and used for decades until the early 1980's when frozen and freeze-dried malolactic bacteria starter cultures were developed. The 1990s saw the development of direct inoculation freeze-dried ML starter cultures and their use has virtually revolutionized the control and predictability of malolactic fermentation in wine (Specht 2005). Table 27.5 summarizes the parameters which are applicable to different types of ML starters used in winemaking.

Most starter cultures available for winemaking benefit from storage under refrigerated and/or frozen conditions in their original, unopened package; the container should not be opened until just before use. In addition, the freeze-dried bacteria should avoid contact with oxygen, excess moisture and high temperature as these conditions are detrimental to survival of the bacteria. In order to obtain the maximum effect from ML bacteria starter cultures always follow bacteria producers' recommendations for handling and storage.

The listing below gives an overview of the most common instructions for ML starter culture preparations:

Frozen ML starter cultures

1. Thaw in room temperature water and not in the refrigerator. Mix 3 l water, 3 l grape juice and 30 g yeast extract. Adjust pH to 4.0 with calcium carbonate or other permitted buffer and mix thoroughly. Add 170 g of thawed culture, seal carboy and mix thoroughly. Hold at 18–24°C for 48 h before inoculation.
2. Directly add the frozen pellets to the wine

Liquid ML starter culture suspensions

Use clean settled juice without added SO_2. If possible heat the juice to 60°C. Adjust sugar level to 180 g L^{-1} with water (if juice is not available substitute with a mix of 50% finished wine (<10 ppm free SO_2 and low total SO_2), 25% water and 25% apple juice). Adjust pH 3.5–3.6 with calcium carbonate. If inoculating wine at pH < 3.2, adjust pH again to 3.4 as an intermediate step.

Add culture and maintain temperature at 22–26°C.

Monitor to 100% malic acid degradation, then expand again as a 10% inoculum at each build-up stage or inoculate.

If finished wine is used to prepare the starter then expand culture by doubling starter volume with wine until it is 5–10% of the amount to be inoculated.

Direct inoculation starter cultures (MBR®)

A special preparation is NOT REQUIRED but may be suspended in clean chlorine free water at 20°C for a maximum of 15 min to help in handling.

Table 27.5 Properties of ML starter cultures (adapted from Specht 2005)

	Type of malolactic bacterial culture				
Property	Frozen	Liquid suspension	Direct inoculation (mbr)	Quick build-up culture (1-step)	Traditional freeze-dried (standard)
Storage tempert. and shelf life	Up to 120 days @ −26°C or up to 1 year @ −29°C in a non-defrosting freezer	Up to 2 days @ room temperature or up to 2 weeks @ 4°C	Up to 18 months @ 4°C or up to 30 months @ −18°C	Up to 18 months @ 4°C or up to 30 months @ −18°C	Up to 18 months @ 4°C or up to 30 months @−18°C
Open container	Once thawed *use immediately* do not re-freeze	*Use immediately*	*Use immediately*	*Use immediately*	*Use immediately*
Time for starter preparation	48 h before inoculation	10-fold expansion in 3–7 days	0–15 min	18–24 h	3–14 days
Nutritional supplements	30 g yeast extract to activation media	~1 g yeast extract per liter growth medium	Proprietary MLB nutrients recommended under more challenging MLF conditions.	Proprietary activator. MLB nutrients recommended under more challenging MLF conditions.	Proprietary MLB nutrients recommended under more challenging MLF conditions.
Usage rates	Red wine ~1 g hL^{-1} White wine ~3–8.5 g hL^{-1}	2–5% inoculation volume or when using finished wine to prepare the starter, then 5–10% inoculation volume.	~1 g hL^{-1}	~0.5 g hL^{-1}	~1 g hL^{-1}

Quick build-up starter cultures (1-STEP® Kit)

Rehydration Phase: Mix and dissolve content of the activator mix in 100 l of potable water at 18 and 25°C. Add content of the bacteria sachet and dissolve carefully by gentle stirring. Wait for 20 min.

Acclimatization phase: Mix the bacteria/activator solution with 100 l of wine, pH > 3.5; Temperature between 20 and 25°C. Wait between 18 and 24 h.

Transfer the activated culture to 1,000 hL of wine.

Traditional freeze-dried STANDARD starter cultures

Rehydrate in 50:50 water: wine mix. Wine should be pH > 3.3 and total SO_2 < 30 mg L^{-1}.

Monitor malic acid drop and when ~2/3 is converted to lactic acid, expand as a 5% inoculum into wine. Make sure pH > 3.3 and alcohol <12.5%.

Monitor malic acid drop and when ~2/3 is converted to lactic acid, expand as a 4% inoculum into wine.

27.3.3 Choice of the Appropriate Malolactic Starter Cultures

There are two basic considerations when selecting a malolactic starter culture:

1. Security – The culture's compatibility with the wine environment
2. Sensory – The desired contribution of different ML strains.

For successful induction of malolactic fermentations, it is critical that the most appropriate preparation of malolactic bacteria is selected for the prevailing wine conditions (Table 27.6).

Since the four main limiting factors (alcohol, pH, temperature and SO_2) have a cumulative stress effect on cultures, Lallemand has developed a table, which allows scoring cumulative "points" of the impact of different wine parameters (Table 27.7).

The resulting "TOTAL" corresponds to the level of difficulty of a wine to start MLF:

- <13 points = favorable
- 13–22 points = not so favorable
- 23–40 points = difficult
- >40 points = extreme

Depending on the *Oenococcus oeni* strain, direct inoculation starter cultures will tolerate in general:

- Alcohol tolerance <15%vol
- PH tolerance >3.1
- Total SO_2 tolerance <60 ppm
- Temperature tolerance >12°C

In addition to the wine conditions described in Tables 27.6 and 27.7, other conditions for alertness when planning selection, preparation and inoculation for MLF include:

- Wines which have struggled to complete alcoholic fermentations are more likely to be deficient in nutrients required to support bacteria during the MLF.
- Nutrient limitation is considered to be one of the major causes of incomplete malolactic fermentations.
- Lower the wine pH below 3.5, higher the bacterial nutrient demand to perform the MLF.
- Bacterial ability to grow and conduct MLF will gradually decrease as wine temperatures fall. Depending upon the wine alcohol content, higher wine

Table 27.6 General characterization of wine conditions for MLF

Wine conditions for MLF	Alcohol (% v/v)	pH	Free SO_2 measurable (mg/l)	Total SO_2 (mg/l)	Temperature (°C)	Problems associated with alcoholic fermentation	Recommended method of MBR® inoculation
Favorable	<13	>3.4	<8	<30	18–22	None	Direct (MBR) – *no acclimatization*
Difficult	13–15	3.1–3.4	8–12	30–40	14–18	Yeast stress	Direct (MBR) or with acclimatization method
Harsh	15–17	2.9–3.1	12–20	40–60	10–14	Sluggish/ Stuck	Direct (MBR) culture with acclimatization procedure
Extreme	>17	<2.9	>20	>60–80	<10		MLF unlikely
Stuck/ partial MLF							MBR acclimatization methods for inoculation of wines with stuck MLF (see Chap. 27.2.4 below)

Table 27.7 Scorecard for determining the ease of malolactic fermentation

	1 point each	2 points each	8 points each	10 points each		Score
Alcohol (% vol)	<13	13–15	15–17	>17	=	
pH	>3.4	3.1–3.4	2.9–3.1	<2.9	=	
Free SO_2 (mg/L)	<8	8–12	12–15	>15	=	
Total SO_2 (mg/L)	<30	30–40	40–60	>60	=	
Temperature (°C)	18–22	14–18 or 18–24	10–14 or 24–29	<10 or >29	=	
Yeast's nutritional needs	Low	Medium	High	Very high	=	
Ease of Alcoholic Fermentation	No problems	Transient yeast stress	Sluggish/ stuck AF	Prolonged yeast contact	=	
Initial level of malic acid (g/L)	2–4	4–5 or 1–2	5–7 or 0.5–1	>7 or <0.5	=	
Maximum AF rate (maximum loss of brix/day)	<2	2–4	4–6	>6	=	

Note: Other, currently less well-known factors that are not considered in this scorecard may include the level of dissolved oxygen, polyphenolic content, lees compacting, pesticide residues, etc.

Total score for the ease of malolactic fermentation: Total

temperatures can also be inhibitory to the development and activity of ML bacteria. A general guideline to avoid inhibitory effects is:

1. Wine Alcohol Content (% v/v) Temperature for MLF should not fluctuate :
   Less than 14.5% 28°C
   Greater than 14.5% 23°C
2. Wine volatile acidity above 0.4 g L^{-1} (as tartaric acid) is likely to inhibit malolactic bacteria
3. Wines stored for more than 3 months on yeast lees are best racked clean before attempts to conduct MLF.

Various acclimatization procedures exist to help overcome limiting wine conditions. Protocol described in Sect. 27.2.4 below has been developed to aid inoculation of MBR bacteria preparations into wines, to cure stuck malolactic fermentations.

27.3.4 Restarting Stuck Malolactic Fermentations

Winemakers are aware that *Oenococcus oeni* bacteria, responsible for malolactic fermentation, are successful only if they adapt to the harsh environment of a fermenting must or finished wine. Direct-addition MLB strains from serious producers that have been selected both for their positive sensory contributions and their ability to perform under the difficult situations are described above. During production, MLB cells undergo a biophysical conditioning that induces the formation of a protective protein. In this physiological state, the cells are harvested and then freeze dried. As a result, they are able to develop a natural resistance to wine conditions and can therefore be added directly to wine without a significant loss of viability.

Sometimes, a stuck MLF can be completed simply by adding a freshly rehydrated direct-addition malolactic bacteria culture. At other times a more extensive adaptation of the MLB is needed to achieve completion. This adaptation can be critical in reducing the effect of an unfavorable wine matrix on the bacteria, favoring successful completion of the MLF. Lallemand Australia has worked in conjunction with their MLF R&D team to develop a MLB acclimatization strategy for finishing wines with stuck malolactic fermentations.

Adaptation Protocol For Handling Stuck Malolactic Fermentations (Specht 2005)

Stage 1

- Pre-treat wine and adjust temperature
- Prepare the stuck-MLF wine by removing any lees, potential inhibitory toxins, and inhibiting spoilage organisms. A small amount of SO_2 and/or lysozyme (or filtration) may be necessary to control undesirable Lactobacillus or Pediococcus bacteria
- Lysozyme is very effective at inhibiting spoilage lactic acid bacteria, especially when the wine is above pH 3.5. If using Lysozyme, be sure that no residual activity remains in the treated wine before inoculation with malolactic bacteria

- In a wine with a stuck MLF suspected of containing substances toxic to malolactic bacteria, a pretreatment with Inactive Yeast Residues (yeast hulls) at 6.25–12.5 g hL^{-1} is recommended. Prepare the yeast hull suspension in water or wine, and then add it to the stuck wine while mixing
- Finally, adjust the temperature of the MLF-stuck wine to 18–22°C (65–72°F)

Stage 2

Acclimatize the bacteria culture in three steps:
Step 1: Prepare medium
Step 2: Rehydrate culture
Step 3: Add rehydrated culture to medium to acclimatize.
Note: The volumes below are based on restarting 10,000 l of stuck-MLF wine.

Step 1: Preparation of the acclimatization medium
Combine:

- 10 l of grape juice (free of SO_2)
- 10 l of water (free of chlorine)
- 20 l of stuck-MLF wine
- After adding all ingredients, adjust the pH to between 3.6 and 4.0
- Adjust temperature to 25–30°C

Step 2: Rehydration of ML bacteria starter culture

- Adjust the temperature of 5 l of tap water (free of chlorine) to 22–25°C. Suspend 1 kg of ML rehydration nutrient into the 5 l of tap water.
- Rehydrate 100 g of direct inoculation malolactic bacteria in the 5 l of tap water/nutrient suspension.

  Allow bacterial suspension to stand for 15 min.

Step 3: Acclimatization of malolactic bacteria

- Mix acclimatization medium (from Step 1) with the rehydrated malolactic bacteria (from Step 2).
- Allow malolactic bacteria to acclimate at 22–25°C for at least 2 h and not more than 4 h.
- After this first acclimatization step, double the volume of acclimatization culture with the stuck wine (e.g., 50 l culture and 50 l wine). If on-site malic acid analysis is not available to monitor MLF progress, it can be assumed that the inoculation culture will be ready in 4–6 h. CO_2 evolution should be evident and/or a slight lactic smell detected. If rapid malic acid analysis is available, 50–70% of the malic acid should be converted before proceeding to Stage 3.

Stage 3

- Add ML nutrient and then acclimatized culture from Stage 2 to the stuck wine.
- Add the nutrient (20 g hL^{-1}) to wine prior to inoculation. The aim is to overcome any nutrient shortages and minimize the risk of residual nutrients in the wine.
- With gentle stirring to avoid excessive aeration, transfer the active acclimatized malolactic culture to 10,000 l of stuck-MLF wine.

- Regular analysis for malic acid (every two weeks) and volatile acidity (weekly) is recommended.

27.3.5 Contribution of the Malolactic Starter Culture to the Sensory Quality of Wine

Reduction of wine acidity and modification of wine flavor due to this secondary bacterial fermentation are often considered to benefit wine quality. The advantage of induction of malolactic fermentation (MLF) by inoculation with selected strains of lactic acid bacteria is twofold. First there is a better control over the time and speed of malic acid conversion and second, a positive influence on wine flavor and quality. Research in recent years has revealed the positive contribution of specific bacteria starters and conditions, including the rate and timing of inoculation for MLF, to the sensory profile of white, red and rosé wines. The metabolic activity of malolactic bacteria (MLB), as well as the kinetics of MLF, will influence the sensory profile of the wine in relation to different winemaking techniques, physical and chemical composition of the wine (pH, alcohol, temperature, citric acid level, SO_2 and aeration) and presence of lees (Lallemand Winemaking Update 01/2007).

MLF reveals varietal aromas: Of all the lactic bacteria active in wine, *Oenococcus oeni* is the one most often responsible for malolactic fermentation. It reduces acidity and modifies the sensory profile of the wine, which has beneficial effect on its quality. For example, the intensity of the floral, fruit, spice and honey notes, are associated with the increase of volatile compounds linked to glycosides and released during MLF. A study done by Ugliano and Moio (2006) validates the role of *O. oeni* in the evolution of the varietal's volatile compounds. Their work shows that the concentration of total glycosides drops significantly during MLF. The hydrolysis of glycosylated aromatic precursors and, consequently, corresponding aromas from the grapes are revealed. The importance of this phenomenon depends both on the bacteria used for MLF and the composition of the wine. In other words, the expression of these varietal aromas, whose importance is considerable to the overall aroma of the wine, depends not only on the potential of the grape varietal, but on the type of malolactic culture as well. This confirms previous observations on the glucosidase activity of MLB. For example, during MLF the glucosidase activity of *O. oeni* releases volatile compounds linked to the aromatic precursors of the grape, including 3-hydroxydamascone, alpha-terpineol, vanillin, methyl vanillate, 4-hydroxybenzoate and tyrosol, from extracts of Chardonnay (Bartowsky and Henschke 2004), as well as linalool, alpha-terpenol, nerol and geraniol extracts of Muscat (Ugliano et al. 2003). These studies suggest that the glycosidic activity of *O. oeni* and subsequent release of the aroma moieties during MLF have the potential to increase the sensory characteristics of the wine.

Diacetyl Management – The Influence of MLB Inoculation Rate and Timing of Addition on the Aroma Profile: Diacetyl is one of the main aromatic compounds produced during malolactic fermentation and is responsible for the

butter and hazelnut notes typical of MLF. Its impact is very important on the profile of the wine, and depending on the desired wine style, it is either sought-after or very undesirable. Indeed, various studies by Martineau and Henick-Kling (1995) and Bartowsky and Henschke (2004) have shown that the production of diacetyl by different *O. oeni* starters could result in completely different aromatic profiles. Each bacteria starter's potential for producing diacetyl is a criterion to consider when choosing a malolactic culture. Beyond diacetyl, the type of starter chosen can also modify other aroma families.

A high level of inoculation with the malolactic cultures not only accelerates the start and speed of the malolactic fermentation, but also results in a low level of diacetyl. In general, it is recommended the wine is inoculated at a population level above 10^6 CFU/mL to reach the critical bacteria population to ensure the rapid initiation of MLF and the regular degradation of the malic acid. Krieger (2005a, b) studied the diacetyl level in a Pinot noir wine where MLF was initiated with different inoculation rates for the malolactic cultures. A low inoculation rate of 2×10^4 CFU ml^{-1} had a prolonged lag phase (14 days) and produced 3.9 mg l^{-1} of diacetyl, whereas a rate of 4×10^6 CFU ml^{-1} immediately initiates the degradation of malic acid and produced 0.8 mg l^{-1} of diacetyl. Inoculation at a rate greater than 2×10^6 CFU ml^{-1} resulted in wines under the diacetyl perception threshold of at nearly 1.5 mg l^{-1} for white and rosé wines.

The timing of inoculation can be just as crucial on the final wines sensory properties. Riesling wines were made using different timing for inoculation with malolactic cultures and carried out in collaboration with DLR Neustadt and Trier (Krieger 2006). These experiments have demonstrated that co-inoculation – the simultaneous inoculation of yeast and bacteria – does not influence the alcoholic fermentation or increase volatile acidity; but it does reduce the overall MLF duration. The co-inoculated Riesling wines did not have buttery or milky aromas associated with MLF, but did have a high intensity of varietal fruit aromas. The diacetyl produced under such reducing conditions during the alcoholic fermentation was immediately transformed into butanediol, which has no odor at this concentration. The same wines inoculated for MLF after alcoholic fermentation had more typical MLF sensory character with dominant notes of butter hazelnut while the fruit was diminished. The control wines with no MLF were more acidic, green and vegetative.

The Sensory Impact of Post MLF Winemaking Techniques: The choice between aging on lees and filtering after malolactic fermentation influences the sensory profile of the wine. The yeast lees can degrade the diacetyl, and bâtonnage can reduce or even eliminate the buttery aroma. The production of diacetyl increases while the wine is in contact with oxygen. Oxygen encourages the oxidation of acetolactate into diacetyl. Nielsen and Richelieu (1999) showed that the accumulation of diacetyl in a semi-aerobic environment could be six times higher than in a completely anaerobic environment. Moreover, the reduction of diacetyl into acetoin and butanediol depends on the redox potential of the wine. A low redox potential is associated with a low level of diacetyl.

27.4 Conclusion

More than 200 strains of active dry wine yeast are available worldwide, offering the wine industry a significant biological diversity. The number of commercially available active dry malolactic starter cultures is still rather limited, but has increased more recently. While active dry yeast starter cultures mostly belong to *Saccharomyces cerevisiae*, starter cultures for the induction of the malolactic fermentation mainly consist of *Oenococcus oeni*. Both yeast and bacteria strains had been selected for their tolerances to limiting wine conditions, their sensory and enological properties to meet creative and security needs of the modern wine industry. It is crucial to know wine parameters and properties of the selected starter cultures to select the right yeast strain, the right bacteria strain and the correct nutrition strategy to match the grapes, fermentation conditions and stylistic goals.

Future demands may also see the application of yeast strains other than *Saccharomyces cerevisiae* or lactic acid bacteria starter cultures other than *Oenococcus oeni*.

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