Dorit Schuller

Genetic diversity and molecular characterization of
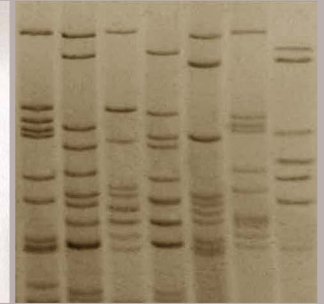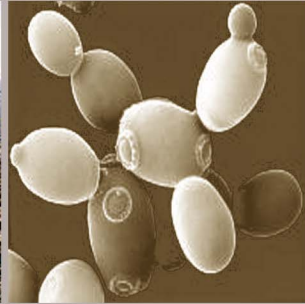*Saccharomyces cerevisiae* strains from winemaking environments

Universidade do Minho
Escola de Ciências

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Genetic diversity and molecular characterization of
Saccharomyces cerevisiae **strains from winemaking environments**

Doutoramento em Ciências

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Professora Doutora Margarida Casal

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É autorizada a reprodução desta tese.

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Abstract

The principal aim of the present work is to assess the genetic diversity of fermenting *Saccharomyces cerevisiae* strains found in vineyards belonging to the Vinho Verde Region in order to create a strain collection representing the region's biodiversity wealth as a basis for future strain selection and improvement programs.

Validation of molecular techniques for accurate genotyping is an indispensable pre-requisite for biogeographical surveys. Molecular typing methods (microsatellite analysis, restriction fragment length polymorphism of mitochondrial DNA (mtDNA RFLP), an optimized PCR-based interdelta method and electrophoretic karyotyping) revealed to be substantially equivalent concerning their discriminatory power among 23 commercial *S. cerevisiae* strains. A large-scale biogeographical survey was devised during the 2001-2003 harvest seasons, comprising three farms, with wineries close to the vineyards, belonging to different sub-regions of the Vinho Verde Region located in northwest Portugal. From 90 grape samples collected, 54 spontaneous fermentations were achieved. The 1620 *S. cerevisiae* isolates obtained were analyzed by mtDNA RFLP. A high biodiversity was evident by the obtention of 297 different profiles, whereas only 17 profiles showed a wider temporal and geographical distribution, characterized by a general pattern of sporadic presence, absence and reappearance. A representative strain of each profile was further analyzed in six microsatellite loci. Accumulation of small allele-frequency differences across six loci in groups of strains allowed the identification of populational structures. Correlation of genetic differentiation with the distance between sampling points suggested a pattern of isolation-by-distance, where genetic divergence increases with the size of a vineyard.

Tracking commercial yeast strains that are used by the wineries since five to ten years revealed to be the adequate experimental model for the environmental risk

evaluation associated with the use of genetically modified yeast strains in the wine industry. The presence of commercial yeast strains was evaluated among the 1620 isolates of the present survey. The results were analyzed including data from an identical study performed in three vineyards of the Languedoc Region (south France). Among the 3780 yeast strains identified after spontaneous fermentation, 296 had a genetic profile identical to that of commercial yeast strains. In samples taken at distances from wineries higher than 100 m, less than 2% of the fermentative microflora had a genetic profile identical to that of commercial yeast. In samples taken at very close proximity to the winery and to water rills, the proportion of commercial yeasts increased to 10-43%. The vast majority (94%) of commercial yeasts were found at a distance of between 10 and 200 m from the winery. Commercial strains, despite their intensive annual utilization, do not seem to implant in vineyards, and do not predominate over the indigenous flora, being their presence characterized by natural fluctuations of periodical appearance/dissappearance as autochthonous strains. The data show that dissemination of commercial yeast in the vineyard is limited to short distances and periods of times and largely favoured by the presence of water runoff. Among the 101 recovered natural isolates of strain Zymaflore VL1, three isolates were characterized by loss of heterozygosity (microsatellite analysis), but revealed the same DNA content as the original commercial strain.

Wines produced in the Vinho Verde Region are characterized by a high volatile acidity. Gene *JEN1* encodes for a monocarboxylate permease in *S. cerevisiae*, which is subject to glucose repression. As a first approach aiming at the obtention of a genetically modified yeast able to remove acetic acid from grape must, a strain expressing *JEN1* in the presence of glucose was achieved.

Resumo

O presente trabalho teve como principal objectivo a avaliação da diversidade genética de estirpes fermentativas de *Saccharomyces cerevisiae* na Região dos Vinhos Verdes no sentido de estabelecer uma colecção de leveduras, que representa a biodiversidade da região, como recurso para futuros programas de selecção e melhoramento de estirpes enológicas.

A validação de métodos moleculares para genotipagem é um pré-requisito essencial para estudos biogeográficos. Neste sentido, foi realizada a análise de 6 *loci* de microsatélites, dos perfis de restrição de DNA mitocondrial (mtDNA RFLP), cariotipagem e de um método optimizado baseado na amplificação de sequências interdelta num conjunto de 23 estirpes comerciais de *S. cerevisiae*. Os métodos revelaram idêntico poder de discriminação, tendo-se obtido 21 perfis distintos, embora a cariotipagem tenha permitido distinguir uma das 3 estirpes que não foram diferenciadas pelos outros métodos.

Tendo em linha de conta a principal directriz enunciada para este trabalho de tese, foi realizado um estudo biogeográfico em larga escala, em três quintas pertencentes a sub-regiões da Região Demarcada dos Vinhos Verdes de 2001 a 2003, com a adega localizada nas proximidades da vinha. Recolheram-se 90 amostras de uva, 54 das quais iniciaram a fermentação espontânea, permitindo a obtenção de 1620 isolados de *S. cerevisiae*. A elevada biodiversidade de estirpes fermentativas foi demonstrada pela obtenção de 297 perfis de mtDNA RFLP, dos quais apenas 17 apresentaram maior distribuição geográfica e temporal. Uma estirpe representativa de cada um dos 297 perfis de mtDNA RFLP foi analisada em 6 *loci* de microsatélites. A acumulação de pequenas diferenças nas frequências alélicas no conjunto dos 6 *loci* permitiu a identificação de estruturas populacionais em grupos de estirpes das diferentes vinhas. A correlação entre a distância dos pontos de

amostragem com a diferenciação genética sugere um padrão de isolamento por distância em função do tamanho da vinha.

O rastreio de estirpes comerciais utilizadas consecutivamente nos últimos 5 a 10 anos nas adegas acima mencionadas, constituiu um modelo experimental adequado para a avaliação de riscos ambientais associados ao uso de leveduras geneticamente modificadas na produção de vinhos. A presença de estirpes comerciais foi pesquisada nos 1620 isolados do presente trabalho. Os resultados obtidos foram analisados incluindo 2160 isolados provenientes de um estudo paralelo realizado em três vinhas da Região Languedoc (Sul de França). De entre os 3780 isolados, 296 apresentaram um perfil genético idêntico às estirpes comerciais utilizadas. Verificou-se uma incidência reduzida de leveduras comerciais (< 2 %) na flora fermentativa de uvas colhidas a uma distância superior a 100 m da adega. A partir de amostras mais próximas da adega e de riachos, a proporção de leveduras comerciais situava-se entre 10-43%, sendo que a maior parte (94%) foi encontrada em locais distanciados 10 a 200 m da adega. O conjunto de dados permitiu concluir que leveduras comerciais, apesar da sua abundante utilização anual, não permanecem nas vinhas e possuem um perfil de aparecimento/desaparecimento semelhante ao da flora indígena, não dominando a flora indígena. A sua disseminação está associada a distancias curtas por tempo limitado, sendo a água um factor importante. Três dos 101 isolados da estirpe Zymaflore VL1, recuperados de vinhas em proximidade da adega, apresentaram perda de heterozigosidade para 6 *loci* de microsatélites, tendo no entanto o mesmo teor de DNA que a estirpe comercial original.

Vinhos produzidos na região dos Vinhos Verdes são caracterizados por uma elevada acidez volátil. Em *S. cerevisiae* o gene *JEN1* que codifica para um sistema de transporte de ácidos monocarboxílicos está sujeito a repressão catabólica pela

glucose. Como primeira abordagem para a obtenção de uma levedura geneticamente modificada, capaz de remover com eficácia ácido acético em mostos de uva, foi obtida uma estirpe que apresenta actividade para este sistema de transporte na presença de glucose.

PUBLICATIONS

Papers published in peer-reviewed journals

Schuller, D., Valero, E., Dequin, S. and Casal, M. 2004. Survey of molecular methods for the typing of wine yeast strains. FEMS Microbiol. Lett. 231, 19-26.

Soares-Silva, I., Schuller, D., Andrade, R. P., Baltazar, F., Cassio, F. and Casal, M. 2003. Functional expression of the lactate permease Jen1p of *Saccharomyces cerevisiae* in *Pichia pastoris*. Biochem. J. 376, 781-787.

Papers to be published in peer-reviewed journals

Schuller, D., Alves, H., Dequin, S. and Casal, M.
Ecological survey of *Saccharomyces cerevisiae* strains from vineyards in the Vinho Verde Region of Portugal

Valero, E., Schuller, D., Cambon, B., Casal., M. and Dequin, S.
Assessment of environmental impact of commercial wine yeast in vineyard ecosystems of different wine producing regions

Schuller, D., Sampaio, P., Pais, C. and Casal, M.
Genetic structure of vineyard-associated *Saccharomyces cerevisiae* populations revealed by microsatellite analysis

Genetic instability of a commercial *Saccharomyces cerevisiae* strain

Other publications

Schuller, D., Valero, E., Cambon, B., Casal., M. and Dequin, S. 2003. Commercial yeast strains do not disseminate significantly in winery ecosystems of wine producing regions in France and Portugal. Proceedings of the " Actes de 83ème Assemblée Générale de l'O.I.V".

Publications in conference proceedings

Exploring genetic tools for the over-expression of the lactate permease Jen1p of *Saccharomyces cerevisiae*: constitutive expression in *S. cerevisiae* and heterologous expression in *Pichia pastoris*. 2003. Yeast 20, S246-S246.

CONTENTS

_____ **_Chapter_** _1_

General Introduction

Saccharomyces cerevisiae is mankind's oldest domesticated organism and the world's premier commercial microorganism for biotechnological applications. During the last decades, science and food technology have contributed at an accelerated rate to the introduction of new products to satisfy nutritional, socio-economic and quality requirements. With the emergence of modern molecular genetics, the industrial importance of *S. cerevisiae* continuously extended beyond traditional fermentation. The demand for suitable genetically modified (GM) *S. cerevisiae* strains for the biofuel, bakery and beverage industries or for the production of high value biotechnological products (e.g. enzymes, pharmaceutical products) will certainly play a crucial role in the future.

The inoculation of selected pure yeast cultures into must is an oenological practice established since the 70's, in order to produce wine with desirable organoleptical characteristics and to guarantee the homogeneity of successive vintages. Nowadays, most of the European wine production relies on the use of such commercial starter yeasts that were selected mainly due to their good fermentation performance. Extensive biogeographical surveys over years and the evaluation of the fermentative flora of a given viticultural region of are the point of departure for further strain selection and improvement programs.

However, the natural availability of yeast strains possessing an ideal combination of oenological characteristics is improbable. In the years following the publication of the *S. cerevisiae* genome sequence (Goffeau *et al.*, 1996), new genetic tools turned the construction of genetically modified wine yeast (GMY) strains possible. Currently, numerous research laboratories worldwide have obtained engineered strains, capable of improving for example processing efficiency, fermentation performance and wine's sensory quality. Their performance under oenological conditions has also

been extensively evaluated. A future introduction of genetically modified wine yeast (GMY) also requires, in agreement with current legislation, a detailed safety and environmental impact evaluation. In accordance with current legislation requirements, strains obtained by self-cloning, based on the use of host-derived genetic material, are most likely to receive approval. Searching for specific strains in winemaking environments, harboring desirable oenological traits, may serve in future as a natural gene pool for the construction of such strains, conferring the exploration of strain diversity a new dimension.

During the last years, interesting evidence was provided concerning the evolutionary processes that shaped the genome of *S. cerevisiae*, hypothesizing an intimate linkage of the evolution of the yeast's genome to winemaking and elucidating also the mechanisms that led to yeast adaptation under specific microenvironments. The high genetic variability among wine yeast strains comparatively to "stable" laboratory strains has been characterized, pointing towards the existence of substantial differences. This emphasizes the necessity for further systematic exploitation of indigenous fermentative strain's diversity and a detailed characterization of their genetic constitution in order to contribute towards the understanding of strains with specific phenotypes.

Scope of this thesis

The principal aim of the present work was to assess the genetic diversity of fermenting *S. cerevisiae* strains found in vineyards belonging to the Vinho Verde Region in order to constitute a strain collection representing the region's biodiversity wealth as a basis for future strain selection and improvement programs.

In the general introduction corresponding to chapter 1, an brief bibliographic revision is made concerning *S. cerevisiae* winery strains, beginning with a description of desirable traits, analyzing the genetic constructions underlying some of the currently available GMY strains, summarizing data concerning the occurrence and survival of genetically modified microorganisms (GMMs), and in particular GMY in natural and confined environments, followed by the description of recent research obtained by global expression analysis that shed more light into the cellular functioning under specific conditions and the forces driving evolution of the *S. cerevisiae* genome. The second part, more generalized, relates to the use of genetically modified microorganisms, making reference, whenever possible, to GMY usage in the wine industry.

Validation of molecular techniques for accurate genotyping is an indispensable pre-requisite for biogeographical surveys. In the second chapter, using a collection of 23 commercial strains, some of the most relevant methods used for the delimitation of *S. cerevisiae* strains are compared, namely: interdelta sequence typing, electrophoretic karyotyping, mitochondrial DNA restriction fragment length polymorphism (mtDNA RFLP) and microsatellite analysis.

The natural fermentative yeast flora has been characterized in many wine-producing regions worldwide. Chapter 3 contains the results of the first extensive ecological survey of fermentative *S. cerevisiae* strains from the Vinho Verde Region, located in

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northwest Portugal. The strains were isolated during 3 years in 3 different sub-regions and enabled the establishment of a strain collection that will be the basis for further strain selection and improvement programs.

From an ecological point of view, commercial strains are non-indigenous, mostly *S. cerevisiae* strains that are used without any containment and are regularly introduced in large amounts in the ecosystem around the winery, together with liquid and solid wine-making residues. Considering these strains an adequate model for a GMY, tracking their dissemination in two different geographical regions (Vinho Verde Region and Languedoc, France) constitutes a solid basis for the risk assessment of GMY usage in the wine production chain. The corresponding data are presented in Chapter 4, being the data from the Vinho Verde Region obtained within the present thesis.

Genotyping *S. cerevisiae* strains by means of recently described microsatellites as genetic markers is associated with a high allelic polymorphism, representing a major source of genetic variation that is important for detecting diversity at both the individual and population levels. Data shown in Chapter 5 is the first large-scale approach demonstrating subtle population structures of indigenous *S. cerevisiae* strains by microsatellite typing.

Wine yeast strains are characterized by a very high genetic instability. As shown in Chapter 6, 101 isolates of the commercial strain Zymaflore VL1 (Lallemand), recovered from the vineyard surrounding three wineries were analyzed by the methods described in Chapter 2, in order to evaluate their genetic variability. This approach allowed also evaluating the usefulness of the different typing techniques to assess variability within isolates belonging to the same strain, complementing the results of Chapter 2.

Wines produced in the Vinho Verde Region are characterized by their sparkling, fruity aroma being the volatile acidity frequently too high. In *S. cerevisiae*, two monocarboxylate symporters have been described: one is shared by acetate, propionate and formate, while the other transports lactate, pyruvate, acetate and propionate, being the latter dependent on the expression of *JEN1*. In order to assess the involvement of *JEN1* in the metabolism of acetic acid, a constitutively *JEN1* expressing strain was constructed and evaluated, as shown in Chapter 7. All data related to the expression of Jen1p in *S. cerevisiae* were obtained within the present thesis.

Finalizing, in Chapter 8, a brief general discussion with concluding remarks is provided, as well as perspectives for future research.

Selection of commercial wine yeast strains

Clonal selection of wild *Saccharomyces* strains isolated from natural environments belonging to the viticultural areas of interest is always the starting point for a wine yeast selection program. Selected yeast starters are nowadays widely used since they possess very good fermentative and oenological capabilities, contributing to both standardization of fermentation process and wine quality. Currently, about 150 different wine yeast strains, mainly *S. cerevisiae*, are commercially available. Considering the current trend towards the production of high quality wines with distinctive and very characteristic properties, the wine-makers demand "special yeasts for special traits" still remains to be satisfied (Mannazzu *et al.*, 2002; Pretorius, 2000; Romano *et al.*, 2003b).

Definition of the appropriate selection strategy should always depend on the traits that a wine strain is supposed to harbor and the number of strains to be screened. The numerous compounds synthesized can vary greatly not only between *S. cerevisiae* strains, but also within different yeast species. As summarized in Table 1.1, numerous oenological characteristics were proposed to be evaluated. Technologically relevant data can be obtained by monitoring the fermentation progress, and quantitative traits are determined by chemical analysis at the end of fermentation.

Finding wine yeast strains possessing an ideal combination of oenological characteristics is highly improbable and therefore strain selection was extended to non-*Saccharomyces* yeasts, e.g. *Candida, Kloeckera, Debaryomyces, Hanseniaspora, Hansenula, Pichia, Metschnikowia, Schizosaccharomyces, Saccharomycodes or Rhodotorula*. Although non-*Saccharomyces* species lack competitiveness in oenological conditions mainly because they are not vigorously

fermenting and possess a lower stress resistance when compared to *S. cerevisiae,* the use of mixed starter cultures or sequential fermentation (e.g. *C. cantarellii/S. cerevisiae*) for directing fermentations towards enhanced glycerol and reduced acetic acid production has been successfully used (Toro and Vazquez, 2002). Non-*Saccharomyces* yeasts such as *Torulaspora delbrucekii* and *Candida stellata* are considered to be positive contributors to the overall organoleptic wine characteristics, while apiculate yeasts such as *Kloeckera apiculata* have a negative influence on wine quality due to pronounced acetic acid and ethyl acetate formation associated with low ethanol production (Ciani and Maccarelli, 1998).

Countless references report the beneficial and detrimental influence of non-*Saccharomyces* yeasts on the volatile composition of musts from varying grape varieties (e.g. Ciani and Maccarelli, 1998; Clemente-Jimenez *et al.*, 2004; Granchi *et al.*, 2002; Mingorance-Cazorla *et al.*, 2003; Plata *et al.*, 2003; Romano *et al.*, 2003c), and considerable differences regarding these compounds were also found among commercial or autochthonous *S. cerevisiae* strains (Patel and Shibamoto, 2003; Romano *et al.*, 2003a; Steger and Lambrechts, 2000).

Non-*Saccharomyces* strains produce and secrete several enzymes e.g. pectinase (increases juice extraction, improves clarification and facilitates wine filtration), ß-glycosidase (hydrolyses non-volatile glycosidic aromatic precursors from the grape) protease (improves clarification process), esterase (contributes to aroma compound formation) or lipase (degrade lipids from grape or yeast autolytic reactions), interacting with grape-derived precursor compounds, contributing thus to reveal the varietal aroma and improve the winemaking process (Esteve-Zarzoso *et al.*, 1998 and references therein, Fernandez *et al.*, 2000; Fleet and Heard, 1993; Otero *et al.*, 2003).

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Table 1.1   *Oenological characteristics considered in the selection of* Saccharomyces cerevisiae *wine strains.*
(Brandolini et al.*, 2002; Caridi* et al.*, 2002; Esteve-Zarzoso* et al.*, 2000; Guerra* et al.*, 1999; Maifreni* et al.*, 1999; Mannazzu* et al.*, 2002; Martinez-Rodriguez* et al.*, 2001; Mendes-Ferreira* et al.*, 2002; Perez-Coello* et al.*, 1999; Rainieri and Pretorius, 2000; Regodon* et al.*, 1997; Romano* et al.*, 1998; Steger and Lambrechts, 2000)*

Oenological characteristics	Comment
Fermentation vigor	Maximum amount of ethanol (%, v/v) produced at the end of the fermentation Desirable: good ethanol production
Fermentation rate	Grams of CO_2 produced during the first 48 hours of fermentation Desirable: prompt fermentation initiation
Mode of growth in liquid medium	Dispersed or flocculent growth, sedimentation speed Desirable: dispersed yeast growth during, but sedimentation at the end of fermentation
Foam production	Height of foam produced during fermentation Undesirable: increased foam production
Optimum fermentation temperature	Thermotolerance and cryotolerance is related to oenological properties Optimum fermentation temperature ranges between 25 and 28ºC
Fermentation purity (FP)	Grams of acetic acid produced/100 ml of ethanol produced Desirable: low FP, considered as performance indicator for wine strains
Volatile acidity, acetic acid production	Selected strains should not release more than 100 – 400 mg l^{-1} during fermentation Undesirable : increased volatile acidity/acetic acid production
Malic acid degradation or production	Whether degradation of production is desirable depends on the characteristics of the must. Malic acid degradation varies between 0-20% depending on the *S. cerevisiae* strain
Glycerol production	Desirable major fermentation by-product (5-8 g l^{-1}) contributing to wine sweetness, body and fullness
Acetaldehyde production	Desirable metabolite in sherry, dessert and port wines being an important character for selection of strains to be applied in wine ageing
Esters, higher alcohols and volatile compounds	Desirable metabolites, markedly influence wine flavor and depend on the presence of precursors related to both grape cultivar and grape maturity. Limited amounts contribute positively to global sensorial characteristics
SO_2 tolerance and production	Antioxidant and antimicrobial agent Desirable: high fermentation vigor and rate in the presence of SO_2 concentrations usually applied in winemaking Undesirable: excessive SO_2 production
H_2S production	Determined as the strains colony color on a bismuth containing indicator medium, e.g. BIGGY Agar H_2S is detrimental to wine quality, considered as off-flavor with very low threshold value (50-80 µg/l)
Stress resistance	Tolerance to combined acid/osmotic stress
Copper resistance	High copper concentrations may cause stuck fermentations Desirable: high copper resistance and the ability to reduce the copper content

S. cerevisiae is not a significant producer of enzymes with relevance in wine production, being mainly ß-glycosidase production reported for this species (Restuccia *et al.*, 2002; Rodriguez *et al.*, 2004). Non-*Saccharomyces* yeasts are commercially available, for example immobilized *Schizosaccharomyces pombe* cells (ProMalic, commercialized by PROENOL) for the deacidification of must by malic acid consumption (Silva *et al.*, 2003).

Genetic engineering of wine yeast strains

Due to the demanding nature of modern winemaking practice, there is a continuously growing quest for specialized *S. cerevisiae* strains possessing a wide range of optimized or novel oenological properties. Genetic improvement of industrial strains by classical genetics (e.g. mutagenesis or protoplast fusion) was followed in the last 20 years by the use of recombinant DNA technologies. The publication of the complete *S. cerevisiae* genome (Goffeau *et al.*, 1996), together with a growing arsenal of recombinant DNA technologies led to major advances in the fields of molecular genetics, physiology and biotechnology, and made the construction of specialized commercial strains possible, mainly by heterologous gene expression or by altered gene dosage (overexpression or deletion).

In 1988, Gist-Brocade obtained a baker's strain where the genes coding for maltose permease and maltase were substituted with a more efficient set of genes from another strain. Since no non-*Saccharomyces* DNA was present, the UK authorities granted consent in 1989. A few years later, a recombinant brewer's strain, obtained in 1993 by Brewing Research International was equally approved. This *S. cerevisiae* strain contained an amylase gene from *Saccharomyces diastaticus* together with a gene for copper resistance. Because of the unwillingness of the industries to face a

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negative consumer reaction none of the strains has gone into commercial production (Moseley, 1999). For the same reasons, no application for the industrial use of genetically modified wine strains has been submitted, although many strains were obtained in numerous laboratories during the last years, as summarized in Table 1.2, in consequence of the increased demand for diversity and innovation within the fermented beverage industry.

The most important targets for strain improvement relate to the improved production technology and quality, such as enhancement of fermentation performance, higher ethanol tolerance, better sugar utilization and nitrogen assimilation, enhanced organoleptical properties through altered sensorial characteristics as summarized by several reviewers (Blondin and Dequin, 1998; Dequin, 2001; Dequin *et al.*, 2003; Pretorius, 2000; Pretorius and Bauer, 2002; Pretorius *et al.*, 2003).

In general, all genetic material used for the construction of microorganisms used for food fermentation should be derived from the host species (self-cloning) or GRAS organisms with a history of safe food use, while the use of DNA sequences from species taxonomically closely related to pathogenic species should be avoided. Heterologous gene expression was used in most cases, being the genes of interest isolated for example from *Lactobacillus casei* (*LDH*), *Lactobacillus plantarum* (*pdc*), *Bacillus subtilis* (*padc*), *Pediococcus acidilactici* (*pedA*), *Schizosaccharomyces pombe* (*mae1* and *mae2*), hybrid poplar (*4CL216*), grapevine (*vst1*), *Aspergillus* sp. (*egl1*, *abfB*, *xlnA*, *rhaA*) or *Fusarium solani* (*pelA*), being others, such as *ATF1*, *GPD1* or *PGU1* derived from *S. cerevisiae* (Table 1.2).

Table 1.2   *Targets for* S. cerevisiae *strain improvement (adapted from Pretorius, 2000; Pretorius et al., 2003), indicating, whenever possible, examples of the strategies used for genetic modifications.*

Improvement	Metabolism / protein(s)	Gene(s)	P	T	Pla	M	Chr	Reference
Sensory quality								
Background flavor complexity and intensity	Aroma-liberating enzymes							
	Endoglucanase	egl1	ACT	-	2µ	CYH2	-	(Pérez-González et al., 1993)
	Arabinofuranosidase	abfB	ACT	-	2µ	CYH2	-	(Sanchez-Torres et al., 1996)
	Endoxylanase	xlnA	ACT	-	2µ	CYH2	-	(Ganga et al., 1999)
	Rhamnosidase	rhaA	GPD	PGK		TRP	-	(Manzanares et al., 2003)
	Acidity adjustment							
	Malate permease	mae1	PGK1	PGK1	2µ	SMR1-140	+	(Volschenk et al., 2001)
	Malic enzyme	mae2						
	Malolactic enzyme	mleS	PGK1	PGK1	2µ	URA3		(Volschenk et al., 1997)
	Acetaldehyde dehydrogenase	ALD6 (deletion)				kanMX4		(Remize et al., 2000)
	Lactate dehydrogenase	LDH	ADH1	ADH1	2µ	G418 (Tn903)	-	(Dequin et al., 1999)
	Glycerol production	G3P dehydrogenase	GPD1	ADH1	ADH1	2µ	ble (Tn5)	-	(Michnick et al., 1997; Remize et al., 1999)
	Volatile phenol formation	Phenolic acid decarboxylase	pdc and padc	PGK1	PGK1	2µ	URA3		(Smit et al., 2003)
	Acetate ester production	Alcohol acetyltransferase	ATF1	PGK1	PGK1	2µ	LEU2	+	(Lilly et al., 2000)
	Hydrogen sulphide production	Sulphite reductase	MET10 (site-directed mutagenesis)						(Sutherland et al., 2003)
Safety and health aspects	Resveratrol production							
	ß-glucosidase	bglN	ACT	ACT	2µ	CYH2	-	(Gonzalez-Candelas et al., 2000)
	Resveratrol synthase	4CL216	ADH2	ADH2	2µ	URA3	-	(Becker et al., 2003)
	Coenzyme-A ligase	vst1	ENO2	ENO2	2µ	LEU2	-	
	Ethyl carbamate elimination	Blocking urea secretion	CAR1 (deletion)						(Pretorius et al., 2003)
Spoilage microorganism control	Production of antimicrobial enzymes							
	Pediocin	pedA	ADH1	ADH1	2µ	URA3	-	(Schoeman et al., 1999)
	Chitinase	CTS1-2	PGK1	PGK1	2µ		-	(Carstens et al., 2003)
	Leucocin	lcaB	ADH1	ADH1	2µ	URA3	-	(du Toit and Pretorius, 2000)
	Glucose oxidase	gox	PGH1	PGK1		URA3	+	(Malherbe et al., 2003)

P: promoter; T: terminator; Pla: Plasmid; M: Marker; Chr: Chromosomal integration

Table 1.2   (cont.)

Improvement	Metabolism / protein(s)	Gene(s)	Construction P	T	Pla	M	Chr	Reference
Fermentation performance Achieving a complete conversion of sugar to alcohol and CO_2 without the development of off-flavors	Stress tolerance — Trehalose	TPS1, TPS2, ATH1						(Pretorius et al., 2003)
	Glycogen	GSY1, GSY2						
	Sterols	SUT1, SUT2						
	Sugar uptake and assimilation — Hexose transporters	HXT1-18						(Pretorius et al., 2003)
	Hexose kinases	HXK1, HXK2						
	Nitrogen assimilation — Proline oxidase	PUT1						(Pretorius et al., 2003)
	Pyrroline-5-carboxylate dehydrogenase	PUT2						
	PUT1 and PUT2 repressor	ure2						(Salmon and Barre, 1998)
	Ethanol tolerance — Sterol accumulation	SUT1, SUT2,						(Pretorius et al., 2003)
	Membrane ATPase activity	PMA1, PMA2						
	Resistance to agrochemicals — Copper chelatin	CUP1						(Pretorius et al., 2003)
Processing efficiency Fining and clarification	Removal of filter-clogging polysaccharides — Endopolygalacturonase	PGU1	PGK1	PGK1	LEU2		-	(Vilanova et al., 2000)
	Pectate Lyase	pelA	ACT	-	CYH	2µ	-	(Gonzalez-Candelas et al., 1995)
	Flocculation timing — Flocculin	FLO1, FLO11	HSP30					(Pretorius et al., 2003)

P: promoter; T: terminator; Pla: Plasmid; M: Marker; Chr: Chromosomal integration

In most cases strong promoters and terminators were used, derived from glycolytic enzymes that are constitutively expressed under fermentative conditions (*ADH1*, *ADH2*, *PGK*) but also from the actin gene (*ACT*). Industrial yeasts usually do not have auxotrophic markers (*LEU2*, *URA2*), therefore the yeast-derived cycloheximide resistance gene *CYH2* or heterologous drug-resistance markers were used such as *ble* (Tn5) or *G418* (Tn903), conferring resistance to phleomycine and geneticine, respectively. Engineering industrial strains with multi-copy shuttle vectors bearing *Escherichia coli* ampiciline resistance and yeast drug-resistance markers is not recommended, since the possibility of DNA transfer to gut microflora is considered remote but existent.

Nevertheless, for wine yeast strains this should not be relevant since cells are removed at the end of fermentation. Plasmid-encoded genes should be preferably integrated, since the elements inserted have to be stable in the newly constructed organism, but such approaches were used in few cases (Lilly *et al.*, 2000; Malherbe *et al.*, 2003; Volschenk *et al.*, 2001). One-step gene disruption with auxotrophic markers as performed for the *GPD* gene (Michnick *et al.*, 1997) results in a self-cloning strain, as previously defined (ILSI, 1999), a much less problematic approach in terms of acceptability evaluation.

Secretion of extracellular proteins, for example the *pedA* - encoding pediocin or *gox*-encoding glucose oxidase, were usually directed by the mating pheromone α factor's secretion signal (*MFa1$_s$*) (Malherbe *et al.*, 2003; Schoeman *et al.*, 1999).

The introduced modifications should not change essential characteristics of the host in the fermentation process. For most genetic modifications it could be shown that apart from the introduced metabolic change, no significant differences were found between wines produced with commercial strain and the corresponding modified

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strain regarding their oenological characteristics. Contrarily, enhanced glycerol production due to modulated *GPD* expression led to a decreased ethanol yield (1%, v/v) and by-product accumulation such as pyruvate, acetate, acetoin and 2,3-butanediol in consequence of carbon flux redirection (Michnick *et al.*, 1997). Deletion of *ALD6* led to reduced acetic acid production (-40-70%) and re-routed the carbon flux towards glycerol, succinate and butanediol (Remize *et al.*, 2000). It was also shown that grape must acidification due to enhanced *LDH* expression and consequent L(+) lactic production depends on the *S. cerevisiae* genetic background and also on the grape variety used for must preparation (Dequin *et al.*, 1999). Wines containing 1.8-2.0% less alcohol were obtained from glucose-oxidase overexpressing strains, since this enzyme produced also D-glucono-δ-lactone and gluconic acid from glucose (Malherbe *et al.*, 2003).

Wines produced by GMY should be, in general, considered as substantially equivalent to "traditional" wines. Compounds like glycerol, acetate ester, malic or lactic acid are natural wine substances, and their content would be merely adjusted or optimized in the sense of enhanced organoleptical characteristics. The expected concentration is very likely to lie within the range that can be found in different wine styles. Besides, facilitated and more economic technological process such as the use of a *S. cerevisiae* strain expressing pectolytic enzymes will have no impact on the composition or properties of the final product since the addition of commercial enzymes is an habitual oenological practice. Anyway, a careful evaluation based on a case-by-case study is indispensable.

A two-step gene replacement was used for the construction of a recombinant sake yeast strain free of bacterial and drug-resistant marker sequences. A point mutation (Gly1250Ser) in the yeast fatty acid synthetase *FAS2* confers cerulenin resistance

and is associated with a higher production of the apple-like flavor component ethyl caproate in Japanese sake. A novel counter-selection marker was used, that consisted of a galactose-inducible overexpression promoter and the *GIN11* growth inhibitory sequence (*GALp-GIN11*). Cells that retain the marker do not grow on galactose because of the growth inhibitory effect mediated by *GIN11* overexpression. A plasmid containing the mutated *FAS2* gene, a drug resistance marker and the counter-selectable marker was integrated into the wild-type *FAS2* locus, and the loss of plasmid sequences from the integrants was done by growth on galactose, which is permissive for the loss of *GAL*p-*GIN*11. Counter-selected strains contained either the wild type or the mutated *FAS2* allele, but not the plasmid sequences, and the resulting difference between the described mutant and the corresponding wild type strain is a single base (Akada *et al.*, 1999; Aritomi *et al.*, 2004). This sake yeast strain was approved as self-cloning yeast by the Japanese Government and does not need to be treated as GMY (Akada, 2002). The mentioned type of counter-selections can also be used for multiple chromosomal gene introductions, as required for engineering of metabolic pathways. Other strategies, for example site-directed mutagenesis of the sulfite-reductase *MET10* gene were used to develop wine yeast with lowered ability to produce hydrogen sufide (Sutherland *et al.*, 2003). The allele *LEU4-1* confers resistance to 5,5,5-trifluoro-DL-leucine and the corresponding strains produce twice the amount of isoamyl-alcohol in laboratory-scale fermentations as the respective parental strains (Bendoni *et al.*, 1999). However, a major limitation of self-cloning is that only oenological traits commonly found in *S. cerevisiae* can be modified.

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Monitoring genetically modified yeasts and other microorganisms in simulated and natural environments

The future use of genetically modified microorganisms will be dependent on the ability to assess potential or theoretical risks associated with their introduction into natural ecosystems. The behavior of genetically modified yeast strains (GMY) within microbial populations of a confined wine cellar and greenhouse vineyard has been evaluated, to our knowledge, in only one study. From the commercial strain VIN13 different genetically modified strains were constructed, containing heterologous genes expressing α-amylase (*LKA1*), endo-β-1,4-glucanase (*end1*), xylanase (*XYN4*) or pectate lyase (*peh1*) under the control of strong promoters and terminators and using the *kanMX* or *SMR*-410 resistance markers. After initial characterization of the autochthonous yeast flora of the newly established greenhouse vineyard, the vines of four blocks (each consisting of 20 vines) were sprayed with yeast suspensions containing 2.5 x 10^6 CFU/ml according to a previously defined scheme. Despite of the high initial cellular concentrations, only few *S. cerevisiae* strains were isolated during the weekly monitoring of yeast populations on grapes, leaves, stems and soil. Results showed that (i) no significant difference between the occurrence of the modified strains compared to the parental commercial strains was evident, even for GM strains that were supposed to have a selective advantage over the parental strains (secreting glucanases and pectinases) showing that the mentioned modifications did not confer any fitness advantage (ii) the overall yeast populations on the sprayed blocks were very similar to the untreated control vines, leading to the conclusion that neither commercial strains nor GMY affect the ecological balance of vineyard-associated flora in a confined system, (iii) no significant differences among the strains were detected concerning their

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fermentation performance during spontaneous micro-vinifications (Bauer *et al.,* 2003).

Risk-assessment of a *S. cerevisiae* strain encoding human coagulation factor XIIIa (rhFXIIIa) did not affect the strain's survival in soil/water or waste water suspensions compared to the parental strain (Fujimura *et al.,* 1994). Taking into account the results on dissemination of commercial yeast strains in wine regions located north Portugal and south France (chapter 4 of the present work) and the above mentioned data, it is desirable to complete biosafety assessment by a small-scale "field release", i.e. experimental (non-confined) wine production using GMY strains. This seems important since it was shown that data obtained from greenhouse environments can be poor predictors of microbial behavior in an open environment (Selbitschka *et al.,* 2003).

Horizontal DNA transfer can occur between yeast species belonging to the *sensu stricto* complex, generating viable hybrids with both parental chromosomal sets (Marinoni *et al.,* 1999). Natural transformation of baker's yeast with plasmid DNA was observed under non-artificial starvation conditions when non-growing cells metabolize sugars without additional nutrients. This was proposed to be an evolutionary mechanism contributing to genetic diversity, being a plausible scenario in natural environments (Nevoigt *et al.,* 2000). At present, studies are underway to evaluate the likelihood of both horizontal and vertical gene transfer among modified commercial wine yeast strains under wine production conditions (Bauer *et al.,* 2003).

Another issue, equally important for the safety assessment of GMY use in wine production, is the evaluation of the potential release and stability of recombinant DNA and the corresponding protein(s) during alcoholic fermentation and wine aging on yeast lees. Autolysis of yeast cells is characterized by a loss of membrane

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permeability, hydrolysis of cellular macromolecules such as DNA and proteins, followed by leakage of the breakdown products in the extracellular environment and occurs after yeast cells have completed their life cycle and entered the death phase. Autolysis experiments were performed in laboratory culture media and showed that incubation at 40ºC during 10-14 days at pH 4.0-7.0 led to degradation of 55% of total DNA, associated with leakage of mainly deoxiriboncleotides and a fewer amount of polynucleotides into the extracellular environment (Zhao and Fleet, 2003).

The exploitation of genetically modified bacterial inoculants for industrial ecology applications has received much attention in the last years. Numerous small-scale field release studies have been performed, briefly summarized as follows.

Plant-growth promoting rhizobacteria are used as inoculants for biofertilization, phytostimulation and biocontrol. Biocontrolling *Pseudomonas putida* strains can colonize the roots of crop plants and produce antifungal metabolites such as phenazine-1-carboxylic acid (PCA) that would be an alternative to the application of chemical fungicides. Perturbations resulting from inoculations with such *Pseudomonas* strains were mainly small, the natural variability of microbial communities seems to surpass the effects of GMMs and the recombinant strain caused changes that were, in general, not significantly different from those caused by the unmodified wild-type strain, while neither metabolic activity of microbial soil populations nor plant height or plant yield were affected (Bakker *et al.*, 2002; De Leij *et al.*, 1995a; De Leij *et al.*, 1995b; Glandorf *et al.*, 2001; Mahaffee and Kloepper, 1997; Viebahn *et al.*, 2003). Compared to the parental strain, the genetic modification did not play an additional role in suppression of *Fusarium* populations that are considered to be non-target organisms playing an important role in functioning of the soil ecosystem (Leeflang *et al.*, 2002). Understanding the mechanisms by which

populations disperse and persist is central to predict the environmental fate of deliberately released bacteria. A genetically modified *Pseudomonas fluorescens* strain was shown to be dispersed between sugar beet leafs by the phytophagous cabbage moth (*Mamestra brassicae*), where the bacteria established viable populations (Lilley *et al.*, 1997), but limited dispersal of the GMM from sugar beet to other plant species was recorded (Thompson *et al.*, 1995).

Biofertilization with highly effective nitrogen-fixing rhizobia is a common practice in agricultural production. The survival of modified *Rhizobium leguminosarum* strains was similar to those of the indigenous population (Hirsch, 1996). The same was reported for *Sinorhizobium meliloti* strains, while the survival of the engineered strains depends on the introduced modification. Inoculating soils with two acid phosphatase negative mutants, unable to use organic phosphorous, led to a weakened survival of the modified strains compared to the corresponding wild-type strain (Da and Deng, 2003). A luciferase (*luc*) gene tagged *S. meliloti* strain was significantly outcompeted by indigenous populations (Miethling and Tebbe, 2004; Selbitschka *et al.*, 2003). Genetically modified *S. meliloti* strains persisted in a site for at least six years after release, despite the absence of the host plant, and horizontal gene transfer and microevolution of a GM plasmid between *S. meliloti* strains was also observed (Morrissey *et al.*, 2002).

Global analysis of gene expression in wine yeast

S. cerevisiae was the first eukaryotic genome sequenced, and will probably become the first organism whose transcriptome, proteome and metabolome complexities will be unlocked. Since many physiological traits are consequences of complicated multigene regulation, understanding the way genes are expressed during wine

fermentation will contribute to the knowledge about the genetic make-up of commercial yeast strains and influence wine strain improvement by genetic engineering. The same approaches are the most appropriate to show that the introduced changes are not associated with adverse or unexpected side-effects such as the production of toxic substances.

Global gene expression after a short-term ethanol stress (30 minutes) was associated with up-regulation of 3.1% and down-regulation of 3.2% of the yeast genes (factor 3 in both cases). Cellular adaptation mechanisms involved, besides the stress gene family, energy metabolism regulation, ionic homeostasis, heat protection, trehalose synthesis and antioxidant defense (Alexandre *et al.*, 2001). Gene expression during alcoholic fermentation in a synthetic must, carried out by a wine yeast strain, is characterized by a tightly controlled and coordinated regulation, depending on the changes in nutritional, environmental and physiological conditions. Major changes in gene expression affected more than 2000 genes, and entry in the stationary phase was associated with a major transcriptional reprogramming. Initial stresses such as high osmotic pressure and acidity did not trigger stress response, but during entry into the stationary phase yeast cells integrated distinct stress signals (ethanol, osmotic, acid, nutrient depletion) in a unique stress response (Rossignol *et al.*, 2003). Further DNA array analysis of *S. cerevisiae* commercial strains refer to the effect of diammonium phosphate (DAP) addition, a common practice to prevent nitrogen-related fermentation problems (Marks *et al.*, 2003), transcriptional response to high (40%, w/v) sugar stress (Erasmus *et al.*, 2003) or differing nitrogen conditions (Backhus *et al.*, 2001).

Comparative gene expression analysis between industrial and non-industrial strains led to the identification of genes contributing to strain fitness in industrial

environments, and it was shown that differences between the wine yeast strain T73 and the laboratory strain S288C reside in 40 genes, and are associated with small changes in promoter regions or variations in gene copy number (Hauser *et al.*, 2001). Comparison of the two popular laboratory strains S288C and CEN.PK113-7D revealed also divergent hybridization patterns in 288 genes, due to differential amplification, gene absence or sequence polymorphisms. Seventeen genes were found to be absent in CEN.PK113-7D and eight genes did not show hybridization signals due to significant differences at the DNA level compared to S288C (Daran-Lapujade *et al.*, 2003).

Recent findings showed that residues inside one of the earliest known wine jars from Egypt contained ribosomal DNA from *S. cerevisiae*, indicating that this yeast was responsible for wine fermentation by at least 3150 B.C. (Cavalieri *et al.*, 2003). Selection for millennia of wine-making may have created unique and interesting oenological traits, but they are not widely distributed, nor can be found in combination in one strain. Specific strains may therefore serve as a natural gene pool for yeast improvement programs. Linking the observed phenotypes with global-expression analysis can provide further information that might be useful for the construction of self-cloning yeast strains. Genes could be uncoupled from their regulatory controls and induced only under fermentation-specific conditions. Such *S. cerevisiae* strains could be for example strains possessing ß-glycosidase activity (Rodriguez *et al.*, 2004) or the capability to reduce copper content in the must by excessive intracellular accumulation (Brandolini *et al.*, 2002), strains with absent sulphite reductase activity (Mendes-Ferreira *et al.*, 2002; Spiropoulos *et al.*, 2000), or strains producing low amounts of acetic acid (Romano *et al.*, 2003a).

Genome evolution and genetic variability in S. cerevisiae

It has been claimed that the genome of *S. cerevisiae* is most probably consequence of a genome duplication that supposedly took place 100 million years ago (Wolfe and Schields, 1997), followed by massive gene loss. Gene duplication can occur as duplication of the entire genome, but also as duplication of single chromosomes (aneuploidy), short chromosomal segments or single genes. Whole genome duplication is believed to have played a major role in biological evolution by creating sets of paralogous genes, which allow functional specialization of the duplicated gene copies upon sequence divergence. *Kluyveromyces lactis* is a species closely related to *S. cerevisiae*, without duplicated genome. By comparison of the gene order of both species it was conclusively shown that a defined chromosomal region in *K. lactis* corresponds to two regions in *S. cerevisiae*, as expected for whole-genome duplication. Four hundred fifty seven duplicate gene pairs were identified, and nearly 90% of the duplicated genes were lost in small deletions. Accelerated evolution, in comparison to *K. lactis*, was verified for 76 gene pairs, but affected only one of the paralogues, leading to the conclusion that the slowly evolving paralogue had probably retained the ancestral gene function, while the evolving paralogue might have acquired a new function (Kellis *et al.*, 2004). The sequencing and genome annotation of the filamentous fungus *Ashbya gossypii* came to the same conclusions (Dietrich *et al.*, 2004). The duplication of chromosomes or chromosomal segments occurs probably in order to balance a deletion with a compensatory copy of a close homologue of the deleted gene, as shown by analyzing 300 deletion strains and their isogenic parental wild-type strains using competitive hybridization of genomic DNA (Hughes *et al.*, 2000).

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One model for genome evolution of wine strains, called "genome renewal" has been proposed by Mortimer *et al.* (2000; 1994). It is based on the finding that most winery strains are diploid, on the fact that 70% of strains revealed to be homozygous for the homothallism gene (*HO/HO*) and on the existence of an inverse correlation between the degree of heterozygosity and spore viability that can range from a few percent to 100%. Genome renewal is also based on the assumption that heterozygote strains arise due to continuous accumulation of genetic damage. After sporulation, the haploid meiotic products will represent many distinct combinations of heterozygosities. Haploid strains are able to perform mating type switching due to the *HO* gene and create homozygous strains by "self-diploidization". The fittest of these new diploids would outgrow and replace the parental strain or sibs with lesser fitness.

In contrast to laboratory strains, wine yeast strains do not maintain genetic uniformity since they possess a remarkable capacity to undergo structural chromosomal reorganizations, resulting in variations regarding both the number and size of chromosomes (Longo and Vezinhet, 1993). These karotype variations in natural and industrial yeast strains are associated with chromosomal translocations due to recombination between homologous sequences interspersed in the yeast genome, such as Ty elements (Casaregola *et al.*, 1998; Codon *et al.*, 1998; Rachidi *et al.*, 1999; Umezu *et al.*, 2002), ribosomal genes (Nadal *et al.*, 1999), or other regions of homology (Neuvéglise *et al.*, 2000; Pérez-Ortin *et al.*, 2002). Such recombination events may confer decisive advantages such as increased sulphite resistance, evolved after microhomology-mediated crossing over between chromosomes VIII and XVI, being considered as a mechanism of adaptive evolution (Puig *et al.*, 2000). Ty-transposon tagging allowed identifying adaptive mutations that increase fitness in laboratory populations of *S. cerevisiae* that underwent 1000 generations. Ty mutations in two loci, one inactivating *FAR3* and one upstream of *CYR1* were

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identified in evolving populations, having small but significantly positive fitness effects (Blanc and Adams, 2003).

Karyotype instability is associated with a rearrangement rate of about 10^{-2} chromosomal changes per generation during vegetative growth. The analysis of several chromosome I variants showed that they differed mainly in their subtelomeric regions that contain genes related to components of the cell wall and membrane (*FLO* family), sugar transporters (*HXT* family) and other genes involved in the assimilation of nutrients (*GAL*, *MAL*, *PHO* genes). It was hypothesized that subtelomeric location of genes, important for biotechnological processes, was probably selected through thousands of years of human biotechnology practice and is beneficial to industrial yeasts since rapid changes allow the acquisition of selective advantages in the sense to improve industrial fitness (Carro *et al.*, 2003). These results were recently confirmed by an oligonucleotide array-based approach for the evaluation of genetic variability among *S. cerevisiae* strains. Single-base pair changes between two 25 bp sequences, especially in the central zone, can disrupt hybridization. Therefore, the design of oligonucleotide arrays with large numbers of such probes can be used to discover approximate locations of allelic differences ("single-feature-polymorphisms") between two strains. Using 14 different yeast strains, it was shown that genome variability is biased towards subtelomeric regions at the ends of chromosomes and is more likely to be found in genes related to fermentation and transport. This kind of approach will be fundamental for future genome evolution and population genetic studies in yeast (Winzeler *et al.*, 2003).

Evolutionary and population geneticists are interested to know the genetic basis of evolutionary adaptation and whether initially identical populations maintained in identical environments will adapt by similar genetic mechanisms. Microarrays were

used to examine the changes in gene regulation that had evolved in three glucose-limited (0.08%, w/v) chemostats populations over 250-450 generations. Two-fold transcription differences between the average transcript level of the evolved strains and the parental strain occurred for 184 genes. Eighty eight named genes were identified, being involved in glycolysis, tricarboxylic acid cycle, oxidative phosphorylation and metabolite transport. Characteristic transcriptional re-programming in the evolved cells appeared to be the same in the three strains, directed towards maximizing ATP production per mole of glucose, enabling the strains to improve the efficiency with which they use glucose. Evolved strains seemed to have reduced pyruvate metabolism into ethanol in favor of respiration. The mechanistic basis for the changes in the evolved strains remains to be clarified, but it was hypothesized that a handful of major changes may led to significant changes in expression of large gene numbers (Ferea *et al.*, 1999). It would be interesting to determine the kind of changes yeast cells undergo when they are maintained during 200-300 generations in wine fermentative conditions. Gaining insight into these mechanisms of adaptive evolution could provide further understanding of the genetic constitution of wine yeast strains.

Adaptation was also studied in a homothallic strain of *S. cerevisiae* from a vineyard in Tuscany. Comparing two haploid derivatives that differ in several morphological and biochemical traits, 6% of the genome (378 genes) showed difference in transcript abundance concerning, for example, genes involved in amino acid biosynthesis and transport of sulfur and ammonia. It was suggested that the observed differences are due to variation in a few regulatory loci that either act on hundreds of loci or initiate cascades of transcriptional control. These studies showed that natural vineyard populations of *S. cerevisiae* can harbor alleles that cause massive alterations in the global patterns of gene expression (Cavalieri *et al.*, 2000).

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Similar results were obtained for global gene expression comparison among four natural *S. cerevisiae* isolates from Tuscan vineyards, but the role that differential gene expression may play in adaptation to new or changing environments still remains to be determined (Townsend *et al.*, 2003).

There is a great potential for understanding molecular population genetics and evolution by the study of gene expression in yeast strains isolated from natural environments. In order to elucidate the response of wild yeast isolates to microclimatic-dependent adaptive genetic changes due to environmental oxidative stress, *S. cerevisiae* strains were collected at different locations from the "Evolution Canyon" in Israel. This canyon provides different microclimates in close physical proximity being the south- and north-facing slopes mainly distinguished by temperature differences and light intensity (the south-facing slope receives a 2-8 times higher irradiation). Response to oxidative stress among the isolated strains, expressed in terms of survival rate in the presence of H_2O_2, was significantly correlated with the micro-climatic niches from where they derived, irrespective of close spatial proximity. Global transcript profiling of 8 strains after a 30 min H_2O_2 treatment showed that strains with a high oxidative stress resistance express only a subset of the common stress response associated with up-regulation of coding regions for cell wall biosynthesis, amino acid biosynthesis, ribosomal proteins, translation factors and fatty acid/lipid biosynthesis (Miyazaki *et al.*, 2003). These results confirm the hypothesis of adaptive evolution and fixation of genes in niche populations.

Using genetically modified microorganisms in food products – general aspects

The principle of substantial equivalence

In May 1997 the European Regulation EC258/97 on novel foods and novel food ingredients (EC, 1997b) came into force and includes within its scope foods and food ingredients (i) containing or consisting of genetically modified organisms (GMO) e.g. genetically modified herbicide tolerant (GMHT) crops, genetically modified starter cultures for food fermentations such as yoghurt containing modified lactic acid bacteria (category (a) Article 1 (2)) or (ii) produced by genetically modified organisms (GMOs), whereas these are not present in the food, e.g. sugar from GM sugar beet or tomato ketchup from GM tomatoes such as FlavrSavr® (category (b) Article 1 (2)). Labeling was mandatory for ingredients derived from GM technology, if the foreign DNA or protein could be evidenced, whereas their absence due to specific production processes did not require labeling. According to this concept, the safety of a food derived from a genetically modified organism to be introduced into the food chain is evaluated by comparing it with the most similar food which has a history of safe use. Labeling refers also to foods containing viable GMMs that must undergo a safety assessment before approval can be given for marketing. This means that, if a food derived from a GM is substantially equivalent, it is "as safe as" the corresponding conventional food item and should be treated as such. A detailed comparison with its "conventional counterpart", the most similar existing food or food component is required, and identified differences are the subject for further toxicological, analytical and nutritional investigations. Detailed knowledge of both the overall characteristics and genetic background of the organisms, the source of the transferred gene(s) and the function of the genes that have been modified is essential for this evaluation. The final outcome of a food product is based on

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processes that are controlled by many different genes, being the function of many genes still poorly understood. Therefore, powerful methods for the identification and characterization of unintended effects on a genomic, proteomic and metabolomic scale are currently evaluated for their routine use (Corpillo *et al.*, 2004; Kuiper and Kleter, 2003; Kuiper *et al.*, 2002).

Safety assessment of genetically modified microorganisms

Based on the comparative "substantial equivalence" approach, the International Life Science Institute (ILSI, a nonprofit worldwide foundation affiliated with the World Health Organization) published a guideline on the safety evaluation (safety assessment of food by equivalence and similarity targeting, SAFEST) of novel foods (Jonas *et al.*, 1996) that was later extended to GMMs (ILSI, 1999). A comparison between the GMM and its conventional, food grade conventional counterpart, (the "comparator", preferably the direct parent or near-isogenic line) is used as a basis for defining three categories for GM-derived products. This classification refers to a GMM considered as belonging to category (a) as defined in Article 1 (2) of the Novel Food Regulation namely "foods and food ingredients containing or consisting of genetically modified organisms within the meaning of Directive 90/220/EEC".

Category 1

- No foreign DNA is included in GMMs belonging to this class, and there are also no changes concerning gene expression. In view of the fact that genetic modification is usually undertaken to achieve a specific phenotypical change, only few examples of self-cloning, such as increasing (e.g. through the use of endogenous promoters) or suppressing (e.g. using antisense or

truncated sequences derived from the host strain) existing gene expression. Products obtained in this way are considered substantially equivalent to foods already available, "as safe as" the counterpart and need no further safety assessment.

Category 2

- If the modification is intended to change gene expression, the novel food is considered substantially equivalent to a traditional counterpart, except for the well-defined difference(s) and the safety assessment focuses on these difference(s). Considering that the viable GMM is an organism already established as safe (GRAS, generally regarded as safe), the emphasis of the evaluation lies on the nature and consequences of the genetic change.

Category 3

- Substantial equivalence of the novel food cannot be demonstrated, either because the differences cannot be defined, or because there is no appropriate counterpart to compare with. Being a safe traditional reference food not available, extensive nutritional and safety evaluation of the food is required.

The safety evaluation of viable GMMs in food raises a number of issues that are not relevant to the safety assessment of foods containing non-viable GMMs e.g. gene transfer, colonization and pathogenicity. Although the recently published UK evaluation report about the impacts of genetically modified herbicide tolerant plants

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(www.number-10.gov.uk/su/gm/index.htm). concluded that trans-kingdom transfer of DNA from GM plants to bacteria is "unlikely to occur because of a series of well-established barriers", low-frequency gene transfer from GM soya to the microflora of the small bowel was recently shown (Netherwood *et al.*, 2004). Safety assessment procedures for GMM use must consider the possibility of interactions between microorganisms of the native gut flora and GMMs.

According to the consensus guidelines published by the ILSI, (ILSI, 1999) the following (herein summarized) information should be provided for food-use safety assessment of a GMM:

Host microorganism

- Complete taxonomic profile;

- Data regarding previous food use of the host and considering following possibilities: (i) the host is deliberately added to perform a particular purpose in safe food products; (ii) the host is not intentionally added, but traditionally found in a viable state in safe food products; (iii) the host is not traditionally found in a viable state in safe food products, being GMMs derived from it not acceptable for food use without a full safety evaluation, including also considerations on the effects on the human immune system;

- Production of any harmful substances such as toxins or allergens: It should be shown that the host does not possess silent genes present in genetically related strains or species that are coding for gene products with harmful potential such as proteins with characteristics of known food allergens or pathogenicity factors e.g. toxins.

Inserted genetic material

- Source of the genetic material inserted;
- Characterization of the inserted genetic material, including key trait gene, marker gene, regulatory and non-coding sequences.

Vector

- Procedures involved in the genetic modification such as source and previous vector use, selection method and sequence of genetic elements used to construct the vector and insert the desired functionality into the GMM. No detailed vector information is required if it can be shown that all DNA other than trait DNA has been discarded from the genetically modified strain.

Genetically modified microorganism

Substantial equivalence should always remain the principal characteristic when a genetically modified microorganism is compared to its comparator. The following information is considered essential to estimate the likelihood of unintended effects from the genetic modification such as (i) changed levels of existing gene products or metabolites due to the presence of foreign gene products (ii) insertional effects of the modification activating (or inactivating) existing genes (iii) transcription of vector sequences.

- Sequence data on any introduced genetic material and flanking regions;
- Relative genetic stability with respect to the intended modification and characterization of DNA rearrangement effects at the sequence level;

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- Consideration of the impact of introduced genes and associated regulatory sequences on the resident gut microflora and evaluation whether the potential for horizontal gene transfer has been increased. Genes that confer a selective advantage such as antibiotic resistance marker genes are of particular concern and should be avoided in GMMs in food;

- Assessment of the modification's consequences on the cell physiology;

- Evaluation of any unintended effects and their potential hazard. Introduced genes may pose a risk of potential allergenicity although microorganisms *per se* used in food are not associated with high risk for food allergy.

In order to facilitate the safety assessment of viable GMMs intended for use and permanence in food, the consensus guidelines contains decision trees for use with GMMs with no foreign DNA (self-cloning, category 1) or GMMs containing foreign DNA (heterologous systems, category 2). The schemes lead through a decision-tree-like set of questions and will assist in deciding whether the data available to the applicant are sufficient or if further information has to be sought and reappraised. Basically the same indications can be found in the Commission Recommendation 97/618/EC (EC, 1997a) concerning the scientific aspects and the presentation of information necessary to support applications for the placing on the market of novel foods and the preparation of initial assessment reports. The suggested use of a case-by-case approach ensures that novel risks are adequately addressed.

Authorisation and labeling of genetically modified organisms for food use

The Novel Food Regulation has been recently amended by three new regulations concerning genetically modified organisms including derived foods and feeds: EC1829/2003 (EC, 2003a), 1830/2003 (EC, 2003b) and 65/2004 (EC, 2004). They define the procedures for authorization, labeling and traceability and were implemented in Europe by 18 April 2004.

Regulation 1829/2003 describes the information to be provided by an applicant seeking authorization to place a product on the market. He has to show that the referred food must not (i) have adverse effects on human and animal health and the environment, (ii) mislead the consumer and (iii) differ from the food which it is intended to replace to such an extent that its normal consumption would be nutritionally disadvantageous for the consumer. Such products must undergo a safety assessment before being placed on the market, including a technical dossier with detailed information concerning results obtained from research and developmental releases in order to evaluate the GMOs impact on human health and environment. This is defined in Annex III of Directive 2001/18/EC (EC, 2001) on the deliberate release into the environment of genetically modified organisms for placing on the market or for any other purpose, that repealed the former Council Directive 90/220/EC (EC, 1990). Since placing on the market includes deliberate release into the environment, an environmental risk assessment in accordance with Annex II of Directive 2001/18/EC has to be carried out (EC, 2002). A summary of the required information is shown in annexes I and II of the present work. The product then goes through the approval procedure between the European Food Safety Agency (EFSA) in Brussels, the European Commission and member states. Labeling is mandatory, even if the recombinant DNA or the corresponding protein cannot be detected in the final product. Foods containing GMOs have to be labeled "genetically modified" or

"produced from genetically modified (name of the ingredient)". Labeling is not required for foods containing traces of GMOs, which are adventitious and technically unavoidable, in a proportion lower than the threshold of 0.9% of the food ingredients (relation between recombinant and non-recombinant ingredient). Whereas the Novel Food Regulation was based on the principle of evidence, in the sense of mandatory labeling for food products containing more than 1% GMOs, Regulation EC1829/2003 is supported by the principle of application, making the declaration of GMO use during the production of food compulsory, but declaration does not rely on the detection of recombinant DNA or protein in the final product.

According to **Regulations Nº 1830/2003** (EC, 2003b) and **65/2004** (EC, 2004), GMOs and products derived from GMOs must be traceable during all stages of their placing on the market through the production and distribution chain in order to facilitate withdrawal of products when necessary and to facilitate the implementation of risk management measures.

USA regulations do not currently require mandatory labeling and segregation of genetically modified crops and products. No special labeling is required for "bioengineered foods" the term used by FDA for those derived by GM technology, "as they are not considered to differ from other foods in any meaningful or uniform way or, as a class, to present any different or greater safety concern than foods developed by traditional plant breeding" (Federal Register of May 29, 1992 57 FR 22984). Evaluation and approval before marketing is only required when the introduced gene encodes a product that had never been a component of any other food such as a new sweetening agent for example. The labeling requirements that apply to foods in general therefore also apply to foods using biotechnology. A label must "reveal all material facts" about a food, for example if a bioengineered food is

significantly different from its traditional counterpart, has a significantly different nutritional property or if a potential allergen is present.

Methods for the detection of GM-derived DNA or protein

In "experimental" wines produced by genetically modified yeast (GMY), no data are so far available about the occurrence and concentration of recombinant cells, DNA and protein. It can be estimated that the number of recombinant cells per bottle would be rather low (1-10 cells), since they are removed by filtration or inactivated by thermal treatment. This implies the use of highly sensitive techniques for tracing recombinant DNA during the wine production chain and in final products. Taking into account the recent European Regulations Nº 1829/2003 and 1830/2003, it is clear that reliable and accurate analytical methods are necessary for food containing GMO or produced from GMO. During the past years, both protein- and DNA-based methods have been developed and applied mostly for detection of transgenic soy and maize and their derivatives, as summarized in Table 1.3.

Table 1.3   *Summary of methods for specific detection of recombinant DNA products in GM foods (from: Ahmed, 2002).*

Parameter	Protein-based			DNA-based			
	Western blot	ELISA	Lateral flow strip	Southern blot	Qualitative PCR	QC-PCR	Real-time PCR
Ease of use	Difficult	Moderate	Simple	Difficult	Difficult	Difficult	Difficult
Needs special equipment	Yes	Yes	No	Yes	Yes	Yes	Yes
Sensitivity	High	High	High	Moderate	Very high	High	High
Duration	2 d	30-90 min	10 min	6 h	1.5 d	2 d	1 d
Cost/sample (US$)	150	5	2	150	250	350	450
Gives quantitative results	No	Yes	No	No	No	Yes	Yes
Suitable for field tests	No	Yes	Yes	No	No	No	No
Employed in	Academic labs	Test facility	Field testing	Academic labs	Test facility	Test facility	Test facility

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For protein-based detection, specific monoclonal and polyclonal antibodies have been developed mainly for immunochemical detection, Western blot analysis and ELISA (enzyme-linked immunosorbent assays). The immunochromatographic assays, also known as lateral flow strip tests, Reveal®CP4 and Reveal®Cry9C detect EPSPS (5-enol-pyruvyl-shikimate-3-phosphate synthase) derived from *Agrobacterium* sp. strain CP4 which confers resistance to the herbicide glyphosate in soybeans and corn, and *Bacillus thuringiensis* Cry proteins that confers protection against insects in corn plants, seeds and grains, respectively. Both kits are commercialized by Neogen (www.neogen.com) and detect GMO presence in 5-20 minutes at a low price, with high sensitivity (< 0.125% mass fraction of GMO) being a reliable field test for controlling the distribution of biotechnology-derived products (Ahmed, 2002; Auer, 2003; Brett *et al.*, 1999; Rogan *et al.*, 1999; Stave, 1999; van Duijn *et al.*, 1999; van Duijn *et al.*, 2002).

PCR-based methods are also applied for detection of GMOs by amplification of genetic elements present in most currently available GMOs in Europe such as the cauliflower mosaic virus (CaMV) 35S promoter, the nopalin synthase (NOS) terminator, or the kanamycin-resistance marker gene (*npt*II). Detection limits range between 20 pg and 10 ng target DNA, corresponding to 0.0001 – 1% mass fraction of GMO. Qualitative PCR results can be confirmed by (i) restriction endonuclease digestion (ii) hybridization with a target-specific DNA probe (iii) PCR product sequencing and (iv) nested PCR (Ahmed, 2002; Auer, 2003; ILSI, 1998; ILSI, 2001; Meyer, 1999; van Duijn *et al.*, 1999; van Duijn *et al.*, 2002). Quantitative-competitive PCR (QC-PCR) relies on parallel amplification of the transgene and of an endogenous reference gene that provides a control for both the lack of inhibition and amplificability of the target DNA in the sample. Quantification is possible by comparing PCR product concentrations from amplifications with varying proportions

of target DNA:standard DNA. This approach was successfully tested in collaborative studies involving 12 European control laboratories, and allowed the detection of 0.1% GMO DNA (Hübner *et al.,* 1999; Lüthy, 1999). A hybrid method consisting of multiplex quantitative PCR coupled to subsequent DNA array technology (MQDA-PCR) was able to test a variety of food and feed products for seven different maize constructs simultaneously at levels as low as 0.1% GM (Rudi *et al.,* 2003). Real-time PCR technologies are highly sensitive and suitable for precise DNA quantification at low thresholds, measuring the production of DNA amplicons during the log-linear phase of PCR amplification. (Ronning *et al.,* 2003; Vaitilingom *et al.,* 1999). PCR products quantitation by means of enzyme linked immunoabsorbent assays (PCR-ELISA) were recently described as a highly sensitive and cheap alternative to real-time PCR (Liu *et al.,* 2004; Petit *et al.,* 2003).

Recently, an interesting solution for identification and tracing of GM crops was suggested, consisting of a noncoding DNA sequence to be incorporated adjacent to the transgene providing an unique "molecular bar code" identification tag, that would not produce a protein or change the organism's fitness and could be read by PCR amplification and sequencing (Breithaupt, 2003; Marillonnet, 2003).

While raw foods can readily be identified as GMOs, detection is more difficult when they are processed: complex processed foodstuffs contain degraded DNA and substances that interfere even with the PCR reaction. Inter-laboratory assessment of procedures was essential and gave rise to international standards development (e.g. DIN, ISO, EN) concerning sampling (DIN, 2003), DNA extraction (DIN, 2002b), DNA-based GMO detection (DIN, 2002a) and protein-based GMO detection (DIN, 2002c).

Technological evolution in GMO design, modifications of government regulations and adoption of risk-assessment guidelines will continue to drive the development of

analytical techniques that will be in the future applied to genetically modified organisms. New profiling methods using transcriptomics, proteomics and metabolomics were proposed as the most adequate non-targeted approaches to detect secondary effects (Kuiper and Kleter, 2003) and proteome analysis demonstrated "substantial equivalence" between a genetically modified virus-resistant tomato and the unmodified hybrids (Corpillo *et al.*, 2004).

Consumer's perceptions and attitudes

Many people question the need to use GM technology in food production, being much of the debate intimately linked to political aspects of globalization and trade. As in any other sector, market economies in agriculture require continuous innovation and reduction of production costs and therefore commercializing GM crops has been perceived as benefiting primarily agro-businesses. One of the most extensive (in terms of the number of people surveyed) public opinion analysis conducted in Europe is the Eurobarometer survey, that has been monitoring changes in attitude towards biotechnology in different European member states since the early 1990s. The last survey conducted in 2001 (Anonymous, 2001) questioning 16 000 Europeans showed a generalized positive view of science and technology, but scientific advance is not regarded as an universal panacea for all problems. Almost all (95%) respondents indicated the consumer's lack of choice about consuming genetically modified food (GMF) as main reason for their negative attitude and 60% expressed the view that GMOs had the potential to have negative effects on the environment. In general, women and older people perceived greater risk. Respondents from northern Europe were more concerned about risks compared to those in southern countries. In previous Eurobarometer surveys (Anonymous, 1997;

Anonymous, 2000), a clear hesitation to accept food products involving GM technology was also apparent. In the USA, where public is much less concerned than in Europe, 53% of the respondents of a survey about acceptability of GMF believe that genetic engineering would improve their quality of life, while 30% were convinced that the new technology "would make things worse" (Priest, 2000). However, the overall American average is much more positive compared to the general European opinion (Kondo and Johansen, 2002).

In view of the fact that many scientific concepts are unknown to the public, the consumer's risk perception and attitudes to risk differ significantly from those defended by scientific risk experts, turning discussions about transgenic technologies complex, increasing at the same time distrust and negativity towards biotechnology in general, and GMO in particular. The fears by the critics of GM technology include alterations in nutritional quality of foods, potential toxicity, possible antibiotic resistance from GM crops, potential allergenicity and carcinogenicity from consuming GM foods, environmental pollution, unintentional gene transfer to wild plants, possible creation of new viruses and toxins, limited access to seeds due to patenting of GM food plants, threat to crop genetic diversity, religious, cultural and ethical concerns, as well as fear from the unknown (Uzogara, 2000). As shown in Figure 1.1, consumer's concern about genetic modification depended on many factors, being minor modifications to food products associated with minor concern, whereas the need for them and the advantages they offer were also rated low.

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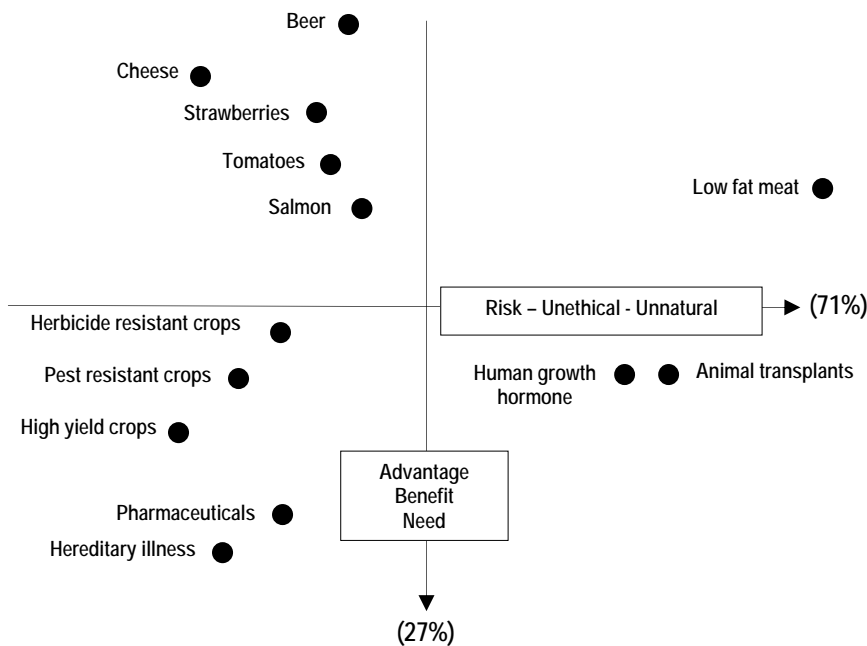
Figure 1.1: Public perceptions of risk versus benefit of genetically modified foods (from Frewer, 2003).

For GM applications in food, benefits were perceived to be marginal, abstract or only on the producer's side. This was verified especially for genetically modified beer, followed by tomatoes, strawberries and salmon. Being beer a traditional lifestyle and convenience beverage like wine, it can be estimated that wine produced by gene technology use would share a comparable consumer opinion. Any modification involving humans and animals was associated with high levels of ethical concern, whereas medical applications such as pharmaceuticals and applications relevant to hereditary disease were perceived to be the most important and necessary (Frewer, 2003; Frewer *et al.*, 1997).

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_____ *Chapter 2*

Survey of molecular methods for the typing of wine yeasts

Survey of molecular methods for the typing of wine yeast strains

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Abstract

A survey of the genetic polymorphisms produced by distinct methods was performed in 23 commercial winery yeast strains. Microsatellite typing, using six different loci, an optimized interdelta sequence analysis and restriction fragment length polymorphism of mitochondrial DNA generated by the enzyme *Hin*fI had the same discriminatory power: among the 23 commercial yeast strains, 21 distinct patterns were obtained. Karyotype analysis gave 22 patterns, thereby allowing the discrimination of one of the three strains that were not distinguished by the other methods. Due to the equivalence of the results obtained in this survey, any of the methods can be applied at the industrial scale.
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Keywords: Molecular typing; Karyotyping; Interdelta sequence; Mitochondrial DNA restriction fragment length polymorphism; Microsatellite; Commercial yeast

1. Introduction

Wine production by the use of selected *Saccharomyces cerevisiae* strains, commercially available as active dry yeast, is widely accepted, being an extensively applied enological practice nowadays. The use of techniques that make it possible to distinguish the inoculated strain from the remaining yeast flora present in the grape must is regarded with great practical interest [1]. In recent years, several methodologies of typing based on DNA polymorphisms have been developed which allowed discrimination among closely related yeast strains.

Chromosome separation by pulsed field electrophoresis [2] revealed considerable variability in the chromosomal constitution of commercial yeast strains [3], and turned out to be a useful method for yeast strain identification [4,5]. As chromosome karyotyping may be too complex, laborious and time-consuming for the analysis of numerous yeast isolates, several other molecular methods of typing have been developed for this purpose.

Restriction fragment length polymorphism (RFLP) analysis of mitochondrial DNA (mtDNA) [5,6] was simplified [7,8] to render it a fast and easy method. Digestion of mtDNA with restriction enzymes like *Hin*fI or *Rsa*I is associated to a high polymorphism, and was also used to study the authenticity of commercial wine yeast strains [9].

The *S. cerevisiae* genome contains repetitive DNA sequences, such as the δ sequences that are frequently associated with the Ty1 transposon [10,11]. The number and the location of these elements have a certain intraspecific variability and were used as genetic fingerprints to identify *S. cerevisiae* strains [11]. Polymerase chain reaction (PCR) profile analysis of δ sequences has a good discriminating power for analyzing commercial strains [12]. On the other hand, it seems to be a minor discriminatory method when used to identify indigenous strains in a given viticultural region [13]. More recently, an extensive BLAST search allowed the optimization of the pair of primers used for interdelta analysis, resulting in highly polymorphic patterns. This improved PCR typing had a similar discriminatory power to pulsed field electrophoresis karyotyping [14].

In the last few years, fingerprinting of microsatellite or simple sequence repeat loci, which are short (1–10 nucleotides) DNA tandem repeats dispersed throughout the genome and with a high degree of variability, has been revealed to be very useful to discriminate *S. cerevisiae*

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strains [15–19]. Searching the genomic DNA database of *S. cerevisiae*, six microsatellite loci were selected that generated 44 genotypes (with a total of 57 alleles) from 51 strains originating from a spontaneous fermentation [20]. This method is fast, allowing multiplex PCR reactions, precise and reproducible, and therefore very powerful.

In the present paper four different genetic fingerprinting techniques (karyotype analysis, δ sequence typing, mtDNA restriction analysis and microsatellite genotyping) were used for the detailed genotyping of 23 commercial wine yeast strains. The analysis of the polymorphisms produced by each of the methods allowed a detailed comparison of the advantages and disadvantages of each method showing the utility and efficiency of these modern approaches for fingerprinting relatively large sets of winery yeast strains.

2. Materials and methods

2.1. Yeast strains

Twenty-three commercially available *S. cerevisiae* wine strains were used in this study. Their geographic origin is indicated in Table 1.

Active dried wine yeast strains were rehydrated and maintained in frozen stocks (glycerol, 30% v/v) at −80°C or, for short-term storage, on YPD agar medium (yeast extract, 1% w/v, peptone, 2% w/v and glucose, 2% w/v).

Table 1
Commercial *S. cerevisiae* strains used in the present study

Strain	Origin
1	Portugal
2	Sangiovese, Italy
3	Bordelais, France
4	Rhône, France
5	Languedoc, France
6	Stellenbosch, South Africa
7	Rhône, France
8	Rhône, France
9	Valencia, Spain
10	Champagne, France
11	Loire, France
12	Champagne, France
13	Gironde, France
14	Languedoc, France
15	Gironde, France
16	Bordelais, France
17	Gironde, France
18	Portugal
19	Portugal
20	Germany
21	Not known
22	Pfalz, Germany
23	Baden-Württemberg, Germany

2.2. DNA isolation

Yeast cells were cultivated in 5 ml of YPD medium (24 h, 28°C, 160 rpm) and DNA isolation was performed using a previously described method [7]. The progress of cell lysis was dependent on the strain and could last between 1 and 3 h. DNA was quantified and used for δ sequence typing, mitochondrial RFLP and microsatellite analysis.

2.3. Delta sequence typing

Amplification reactions were performed on a Bio-Rad iCycler thermal cycler, using the primers δ1 (5′-CAA-AATTCACCTATATCT-3′) and δ2 (5′-GTGGATTTT-TATTCCAAC-3′) (primer pair A [11] or δ12 (5′-TCAA-CAATGGAATCCCAAC-3′) and δ2 (primer pair B)[14]. 15 μl reaction mixture was prepared with 60 ng of DNA, 0.5 U Taq polymerase (MBI Fermentas), Taq buffer (10 mM Tris–HCl, 50 mM KCl, 0.08% Nonidet P-40), 25 pmol of each primer, 0.4 mM of each dNTP and 3 mM MgCl$_2$. After initial denaturation (95°C for 2 min), the reaction mixture was cycled 35 times using the following program: 95°C for 30 s, 43.2°C for 1 min, 72°C for 1 min followed by a final extension at 72°C during 10 min. The amplification products were separated by electrophoresis on a 1.5% (w/v) agarose gel containing ethidium bromide, visualized and photographed.

2.4. Chromosomal polymorphisms

Yeast chromosomal DNA was prepared in plugs as previously described [3], washed in TE buffer (1 mM EDTA, 10 mM Tris–HCl, pH 8.0) at 50°C for 30 min and then washed again three times in the same buffer at room temperature for 30 min. The plugs were loaded in a 1% (w/v) agarose (Seakem® Gold) gel and electrophoresis was performed using a TAFE (transverse alternating field electrophoresis) system (Geneline, Beckman) under the following conditions: constant voltage of 250 V for 6 h run time with 35 s pulse time, followed by 20 h at 275 V with 55 s pulse time at constant temperature (14°C). The electrophoresis buffer consisted of 10 mM Tris base, 0.5 mM EDTA free acid and 4 mM acetic acid. After staining the gel with ethidium bromide, bands were visualized and photographed.

2.5. Mitochondrial DNA restriction patterns

The reactions were performed overnight at 37°C and prepared for a final volume of 20 μl as follows: 17 μl of total DNA (60–120 μg), isolated as described, 0.5 μl of the restriction endonucleases *Hin*fI or *Rsa*I (10 U μl^{-1}, MBI Fermentas), 2 μl of the appropriate 10× buffer and 0.5 μl of RNase (10 mg ml^{-1}) (MBI Fermentas). The DNA fragments were separated on a 1.5% (w/v) agarose gel containing ethidium bromide, visualized and photographed.

2.6. Microsatellite amplification

The six trinucleotide microsatellite loci described as ScAAT1, ScAAT2, ScAAT3, ScAAT4, ScAAT5 and ScAAT6 [20] were amplified in two multiplex reactions using 20 ng of template DNA, 0.5 U Taq polymerase (MBI Fermentas), the corresponding Taq buffer (10 mM Tris–HCl, 50 mM KCl, 0.08% Nonidet P-40), 0.2 mM of each dNTP and 2 mM $MgCl_2$. Multiplex reaction A contained 0.05 pmol of each ScAAT1 and ScAAT6 primer pairs as well as 0.03 pmol of ScAAT4 primer pair. Multiplex reaction B contained 0.05 pmol of ScAAT2, 0.1 pmol of ScAAT3 and 0.075 pmol of ScAAT5 primer pairs. One oligonucleotide of each pair was labeled with fluorescent dye (MWG Biotech). In both cases, the total reaction volume was 6.0 µl, and cycling was performed as described [20] in a Bio-Rad iCycler thermal cycler. PCR reactions were diluted (1:5 for multiplex A and 1:20 for multiplex B), and 2-µl aliquots were mixed with 14 µl of formamide and 0.3 µl of a red DNA size standard (Genescan-500 ROX, Applied Biosystems). Samples were then denatured at 94°C for 5 min and separated by capillary electrophoresis (15 kV, 60°C, 24 min and 27 min for multiplex reactions A and B respectively) in an ABI Prism 310 DNA sequencer (Applied Biosystems) and analyzed using the corresponding Genescan software.

2.7. Reproducibility

All typings were performed at least in duplicate. The reproducibility of the described techniques was also assessed by comparing the results obtained by the analysis of DNA from two independent extractions for five randomly chosen yeast strains.

3. Results

3.1. Delta sequence typing

PCR amplification of δ sequence interspersed regions using primer pair A or B showed a distinct degree of pattern heterogeneity as shown in Fig. 1. For primer pair A, a total of 10 distinct patterns were obtained, and most of them shared three common bands around 500, 750 and 970 bp. These three bands are characteristic for pattern δ_{A3}, the pattern found in 10 of the 23 strains analyzed. Further patterns are characterized by the appearance of an additional band in close proximity to one of the three main bands (e.g., patterns δ_{A1}, δ_{A6}, δ_{A7}), by the absence of some of the three main bands (e.g., patterns δ_{A2}, δ_{A4}), or by the appearance of other extra bands (e.g., pattern δ_{A10}). For primer pair B, almost all patterns appear to have several bands in common of about 400–500 bp, and the presence of many other intense bands of different sizes produced a very high polymorphism compared to primer pair A, allowing the assignment of 21 different patterns among the 23 strains. The group of 10 strains showing the identical pattern δ_{A3} could be distinguished from each other using primer pair B that generated 10
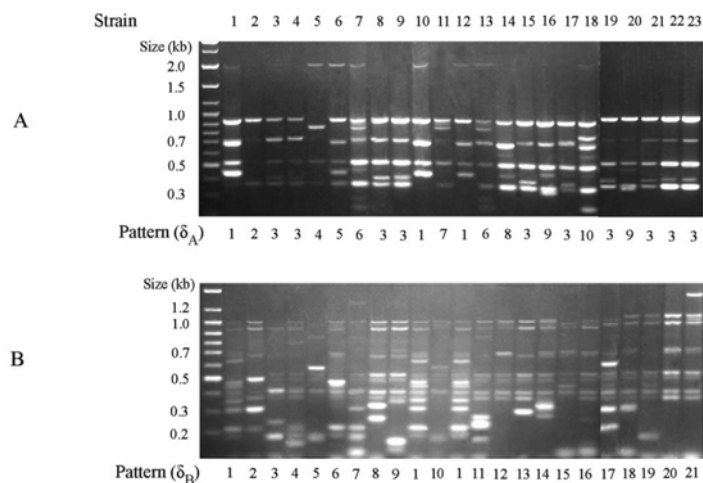

Fig. 1. PCR amplification fragments of δ sequence interspersed regions using primer pair A (A) or B (B). The numbers in the upper part of the figure correspond to the strains used. (δ_A) 1–10 and (δ_B) 1–21 refer to the pattern classification.
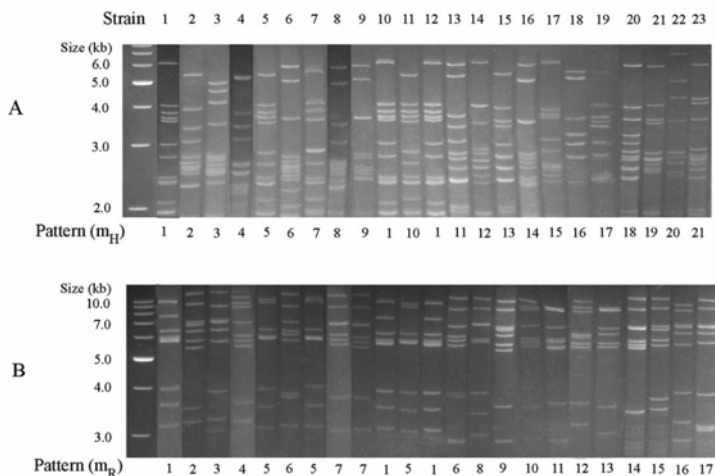
Fig. 2. Mitochondrial DNA restriction patterns of the 23 commercial strains analyzed in this work. The patterns (m_H and m_R) were obtained by digestion with *Hin*fI (A) or *Rsa*I (B). The numbers in the upper part of the figure correspond to the strains used. (m_H) 1–17 and (m_R) 1–21 refer to the pattern classification.

different patterns. Interestingly, strains 1, 10 and 12 show a very characteristic pattern (δ_{B1}), with five bands sized between 300 and 500 bp. These three strains also showed a unique pattern (δ_{A1}) when PCR amplification was performed with primer pair A (Fig. 1), indicating that they are identical or genetically very closely related.

Several faint bands, probably associated with unspecific amplification due to the low annealing temperature and to the high MgCl₂ concentration (3.0 mM), were not always amplified in replicates, but they were not decisive for the assignment of a pattern, as sufficient polymorphisms were obtained from the intense bands.

3.2. RFLP of mitochondrial DNA

The analysis of the genetic variability of 23 *S. cerevisiae* wine strains by means of mtDNA restriction analysis showed a very high level of polymorphisms (Fig. 2). Digestion with *Rsa*I was less discriminating than *Hin*fI, generating 17 and 21 distinct patterns, respectively. Strains 5, 7 and 11 shared pattern m_{R5}, while pattern m_{R7} was shared by strains 8 and 9. The average size of fragments obtained by *Hin*fI digestion was between 2.5 and 6 kb, whereas bigger fragments (mainly between 6 and 10 kb) were obtained by *Rsa*I digestion. Again, with the exception of strains 1, 10 and 12, unique patterns were found with the restriction enzyme *Hin*fI. Fig. 4 shows the identical mtDNA restriction patterns of these three strains using *Hin*fI or *Rsa*I.

3.3. Analysis of chromosomal patterns

As shown in Fig. 3, the pulsed field electrophoretic karyotypes of the 23 strains analyzed showed 22 different chromosomal patterns. In the range below 600 kb, where the resolution is better, the greatest variability was found, both in the position and in the number of bands, which varied from five to 10. There was also considerable variability in the region of approximately 900 kb, where for most strains one or two bands were observed in different positions.

The patterns of strains 10 and 12 (K10) were again identical whereas in strain 1 differences in the zones of about 600 kb (chromosomes XVI–XIII) and 900 kb (chromosomes V–VIII) were observed. A lower weak band was lost and another higher weak band appeared in the zone of around 600 kb. In addition, a band of smaller size in strain 1 replaced a weak band present in the region about 900 kb. Except for these two bands, the pattern of strain 1 is identical to that of strains 10 and 12, indicating that these strains are genetically very closely related.

3.4. Microsatellite analysis

The results obtained for the analysis of the six microsatellite loci ScAAT1–ScAAT6 are summarized in Table 2. Unique patterns were found for 20 strains, while an identical pattern was found for strains 1, 10 and 12. The number of alleles found for each locus varies between three

48
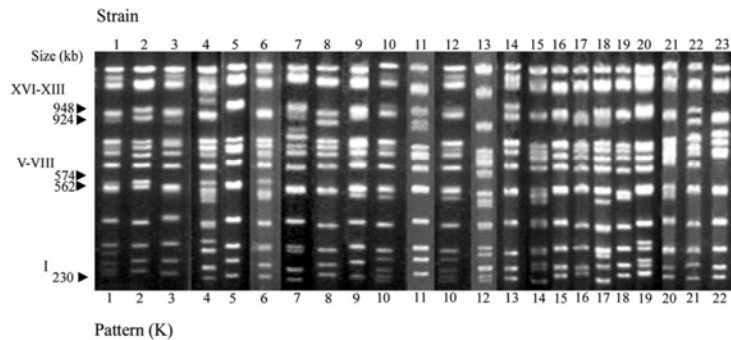
Fig. 3. Electrophoretic karyotype patterns of the 23 commercial strains analyzed. The numbers in the upper part of the figure correspond to the strains used. (K) 1–22 refers to the pattern classification. Numbers on the left give the sizes of chromosomes XVI–XIII, V–VIII and I of the reference strain S288C.

and 15, the loci ScAAT1 and ScAAT3 being characterized by the highest polymorphism. The number of genotypes varied between four and eighteen for each locus separately analyzed (Table 2).

4. Discussion

In the present study, different methods have been applied to genetically differentiate 23 commercial wine starter yeast strains. As summarized in Table 3, depending on the technique used, distinct levels of discrimination were obtained, varying from 10 to 22 different patterns.

The power of discrimination of *S. cerevisiae* strains by PCR-based interdelta typing depended on the primer pairs used. Amplification with the initially described [11] primer pair δ1-δ2 (primer pair A) resulted in 10 different patterns, whereas the substitution of primer δ1 by primer δ12 (primer pair B) resulted in a two-fold increase in the number of patterns obtained (Table 3). The optimized primer pair B, found by an extensive BLAST search, raised the detection of polymorphisms and allowed the unequivocal differentiation of 53 industrial, laboratory and wild-type yeast strains [14]. Delta sequence typing with the standard primer (pair A) has been reported to be very useful and easy to perform for the typing of commercial strains. However, for the delimitation of genetically closely related indigenous yeast strains, this method has a low discrimination power and therefore should be combined with other typing methods like mtDNA or karyotype analysis [13,21]. In the present study, the interdelta typing of the 23 industrial strains with optimized primer pair B had almost the

Fig. 4. Analysis by δ sequence typing, mtDNA RFLP and pulsed field electrophoresis of strains number 1, 10 and 12. The three strains present identical patterns, except the slight differences in strain 1 indicated by arrows.

Table 2
Allelic diversity of the 23 *S. cerevisiae* commercial starter strains

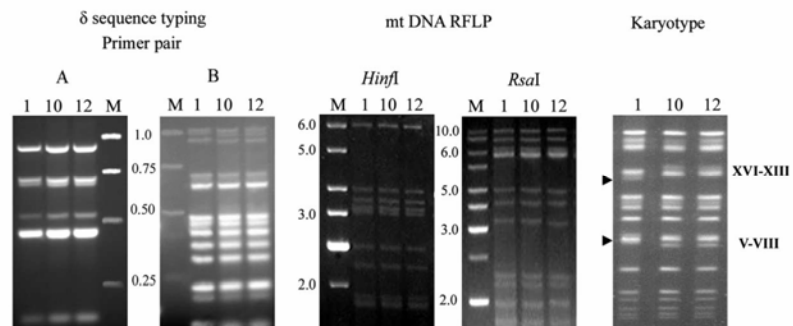| Strain	Microsatellite					
	ScAAT1	ScAAT2	ScAAT3	ScAAT4	ScAAT5	ScAAT6
1	189, 237	375	250, 346	302	219, 222	250, 256
2	201	378	247	329	216	256
3	204, 222	372, 378	259, 265	317, 329	216, 219	256, 259
4	165	384	262, 304	302, 329	216, 219	256, 259
5	246	378	262	329	216	259
6	189, 228	375, 378	250, 262	302, 329	216, 222	256
7	222	369, 384	247	302, 329	216	256
8	195	378	241	332	219	256
9	195, 216	375, 381	256	329	216	256
10	189, 237	375	250, 346	302	219, 222	250, 256
11	195	375	256	329	222	256, 259
12	189, 237	375	250, 346	302	219, 222	250, 256
13	216, 219	372, 378	247, 265	329	216, 219	256, 259
14	174	387	247	338	222	259
15	204, 219	372, 381	265	329	219, 222	256, 259
16	195	378	265	329	222	256
17	201	378	247	329	222	256
18	171, 201	375, 378	259, 268	329	219	256
19	204	369	259, 271	329	219	259
20	192	378	247, 271	329	216	256, 259
21	207	378	262	329, 332	216	256
22	219	381	259	329	219	256
23	189	381	247	290	219	256
Number of alleles	15	7	11	6	3	3
Number of genotypes	18	11	14	8	6	4

same level of discrimination as pulsed field karyotyping. These results are consistent with the ones previously described [14].

As shown in Table 3, the 21 patterns generated by mitochondrial DNA restriction with *Hin*fI match exactly the patterns obtained by PCR typing using primer pair B, microsatellite typing, as well as pulsed field karyotyping (with the exception of strain 1). Additionally, in the present study, digestion with *Hin*fI allowed a much better resolution than with *Rsa*I.

Both mtDNA restriction analysis and electrophoretic karyotyping have been used in numerous studies related to the yeast ecology of spontaneous fermentations, biogeography and biodiversity [22–27]. It was shown that both methods had a very similar resolving power at the strain level. Nevertheless, the results obtained using the improved interdelta typing method are very promising, indicating its equivalence to mtDNA RFLP, karyotyping and microsatellite analysis.

Using interdelta amplification, mtDNA RFLP and microsatellite typing, strains 1, 10 and 12 generated the same patterns (Table 3). The chromosomal patterns of strains 10 and 12 are identical, and were very similar to that of strain 1. Strain 1 differs from the other two strains due to changes in the position of two weak bands in the zones of about 600 and 900 kb. Two pairs of chromosomes, XVI/XIII and VIII/V, very close in size, are found in these regions. Interestingly, a reciprocal translocation between

chromosomes VIII and XVI, generating two new chromosomes VIII[XVI] and XVI[VIII], has been described as occurring frequently in wine yeast strains [28]. This rearrangement, found in wine yeast strains, is involved in their adaptive evolution, since the translocation results in higher expression of *SSU1*, thus enabling the cells to resist higher sulfite concentrations [29]. Indeed, wine yeast strains exhibit either normal chromosome VIII (of about 560 kb), chromosome VIII[XVI] (of about 920 kb), or both [29,30]. Both are actually present in strains 10 and 12 (results not shown) and the opposite variations in the size of bands in strain 1 may indicate different rearrangement events related to these two chromosomes. All these lines of evidence strongly suggest strains 10 and 12 are genetically related to strain 1.

Strain 'families' having the same mtDNA restriction profile and δ sequence PCR product patterns, differing only by faint variations of chromosomal band position or the presence of doublets, have been described [23]. Differently sized chromosomes can be explained by structural reorganizations, leading to structural heterozygosis [30]. Such chromosomal rearrangements have been described in wine yeast genomes during vegetative growth [31] or during wine fermentation [32].

The discrimination obtained by combining the allele sizes from the six microsatellite loci was very high. The combination of the results from loci ScAAT1 and ScAAT3 generated the highest polymorphism (18 and 14 geno-

Table 3
Summary of the results obtained by all typing methods used

Strain	Pattern					
	δ sequence		mtDNA RFLP		Microsatellite ScAAT1–6	Karyotype
	δ_A	δ_B	m_R	m_H		
1	1	1	1	1	1	1
2	2	2	2	2	2	2
3	3	3	3	3	3	3
4	3	4	4	4	4	4
5	4	5	5	5	5	5
6	5	6	6	6	6	6
7	6	7	5	7	7	7
8	3	8	7	8	8	8
9	3	9	7	9	9	9
10	1	1	1	1	1	10
11	7	10	5	10	10	11
12	1	1	1	1	1	10
13	6	11	6	11	11	12
14	8	12	8	12	12	13
15	3	13	9	13	13	14
16	9	14	10	14	14	15
17	3	15	11	15	15	16
18	10	16	12	16	16	17
19	3	17	13	17	17	18
20	9	18	14	18	18	19
21	3	19	15	19	19	20
22	3	20	16	20	20	21
23	3	21	17	21	21	22

For each method a different number was assigned to distinct patterns.

types), and was sufficient for the unequivocal characterization of the present population of 23 strains. However, for studies aiming at the characterization of strains that are genetically more closely related, it may be necessary to include data obtained for the other four loci.

In summary, our results show that microsatellite typing and the optimized interdelta analysis have similar discriminatory power compared with both mtDNA restriction analysis and karyotyping. None of the typing methods was able to discriminate between two *S. cerevisiae* commercial strains (10 and 12). At least two hypotheses can be raised to explain this result: the strains are identical, although having different commercial designations, or the techniques used are not sufficiently accurate to discriminate between them. Concerning the first hypothesis, there are references reporting equivalent situations in commercial yeast strains [9]. The common geographical origin of these two strains supports this hypothesis.

The improved PCR amplification of δ sequences described by Legras et al. [14] is a very convenient method that does not require high equipment investment and can replace other methods advantageously. However, some critical aspects of δ sequence typing have to be mentioned, as the PCR banding patterns depend on the quantity of template DNA [9]. Occasionally, we also found weakly amplified bands that can make the interpretation of the results difficult (not shown).

Mitochondrial DNA restriction analysis could be a good technique to differentiate yeast strains from the same ecosystem. This technique is also easy to use once the conditions have been carefully standardized and the reproducibility is better than that of δ sequences analysis.

Karyotyping was shown to be very efficient in discriminating between strains genetically closely related as we confirmed in this study, and is still the method of choice for the detection of chromosome rearrangements. Nevertheless, this technique is time-consuming and complicated for use in industry.

The detection of microsatellite polymorphisms is a promising and powerful tool, providing accurate and unequivocal results expressed as base pair number (or as a number of repeats). This technique is the most appropriate for large-scale studies like determination of genetic proximity (phylogenetic studies) and biogeographical distribution of indigenous *Saccharomyces* strains and/or species by means of numerical analysis. It requires higher equipment investment and skilled human resources which can be seen as the only disadvantages of this technique.

In conclusion, due to the verified equivalence of the results, any of these methods could be applied for industrial applications, such as quality assurance during dry yeast production, implantation studies or tracing of contamination routes. For standard control during the fermentation process PCR amplification of δ sequences and mtDNA restriction analysis are the most appropriate methods. The choice of the most convenient technique should depend on the resources available and the objective of the work.

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Ecological survey of Saccharomyces cerevisiae *strains from vineyards in the Vinho Verde Region of Portugal*

Ecological survey of Saccharomyces cerevisiae *strains from vineyards in the Vinho Verde Region of Portugal*

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Abstract

One thousand six hundred and twenty yeast isolates were obtained from 54 spontaneous fermentations performed from grapes collected in 18 sampling sites of three vineyards (Vinho Verde Wine Region in northwest Portugal) during the 2001-2003 harvest seasons. All isolates were analyzed by mitochondrial DNA restriction fragment length polymorphism (mtDNA RFLP) and a pattern profile was verified for each isolate, resulting in a total of 297 different profiles, all revealed to belong to the species *Saccharomyces. cerevisiae*.

The strains corresponding to seventeen profiles showed a wider temporal and geographical distribution, being characterized by a generalized pattern of sporadic presence, absence and reappearance. One strain (ACP10) showed a more regional distribution with a perennial behavior. In different fermentations ACP10 was either dominant or not, showing that the final outcome of fermentation was dependent on the specific composition of the yeast community in the must.

Only 24% of grape samples collected before harvest initiated a spontaneous fermentation, compared to 71% of the samples collected after harvest, in a time frame of about 2 weeks. The associated strains were also much more diversified: 267 patterns among 1260 isolates compared to 30 patterns among 360 isolates in the post- and pre- harvest samples respectively.

The present study aims at the development of strategies for the preservation of biodiversity and genetic resources as a basis for further strain development.

Introduction

Traditionally, wine fermentation is carried out in a spontaneous way by indigenous yeast either present on the grapes when harvested or introduced from the equipment and cellar during the vinification process. All recent research agrees that the predominant species on healthy grapes are apiculate yeasts like *Hanseniaspora uvarum* (and its anamorph form *Kloeckera apiculata*) and oxidative species such as *Candida*, *Pichia*, *Kluyveromyces* and *Rhodotorula* (Fleet and Heard, 1993). Contrarily, fermentative species of the genus *Saccharomyces*, predominantly *Saccharomyces cerevisiae*, occur in extremely low number on healthy undamaged berries or in soils (Frezier and Dubourdieu, 1992; Martini *et al.*, 1996; Parish and Carroll, 1985), while damaged grapes are believed to be an important source of *S. cerevisiae* (Mortimer and Polsinelli, 1999). The prevalence of strains belonging to this species is well documented among the wineries resident flora (Beltran *et al.*, 2002; Constanti *et al.*, 1997; Longo *et al.*, 1991; Sabate *et al.*, 2002; Vaughan-Martini and Martini, 1995). The grape's yeast flora depends on a large variety of factors such as climatic conditions including temperature and rainfalls, geographic localization of the vineyard (Longo *et al.*, 1991; Parish and Carroll, 1985), antifungal applications (Monteil *et al.*, 1986), grape variety and the vineyard's age (Martini *et al.*, 1980; Pretorius *et al.*, 1999; Rosini, 1982), as well as the soil type (Farris *et al.*, 1990). Several ecological surveys, using molecular methods of identification, report a large diversity of genetic patterns among the enological fermentative flora. *S. cerevisiae* strains seem to be widely distributed in a given viticultural region (Lopes *et al.*, 2002; van der Westhuizen *et al.*, 2000a; van der Westhuizen *et al.*, 2000b; Versavaud *et al.*, 1995), can be found in consecutive years (Torija *et al.*, 2001; Vezinhet *et al.*,

1992) and there are also strains predominant in the fermenting flora (Frezier and Dubourdieu, 1992; Sabate *et al.*, 1998), hypothesizing the occurrence of specific native strains that can be associated with a *terroir*.

Selected yeast starters are nowadays widely used since they possess very good fermentative and oenological capabilities, contributing to both standardization of fermentation process and wine quality. In the years following the publication of the *S. cerevisiae* genome sequence (Goffeau *et al.*, 1996), enough evidence was provided showing substantial genetic differences among wine yeast strains (Carro *et al.*, 2003; Perez-Ortin *et al.*, 2002a; Perez-Ortin *et al.*, 2002b). Therefore, exploring the biodiversity of indigenous fermentative strains can be an important contribution towards the understanding and selection of strains with specific phenotypes.

The genetic diversity of *S. cerevisiae* strains has been analyzed by several methods such as karyotyping by pulse field gel electrophoresis (Blondin and Vezinhet, 1988), mitochondrial DNA restriction analysis (mtDNA RFLP) (Fernandez-Espinar *et al.*, 2000; Lopez *et al.*, 2001; Querol *et al.*, 1992a; Querol *et al.*, 1992b), fingerprinting based on repetitive delta sequences (Legras and Karst, 2003; Ness *et al.*, 1993) and microsatellite genotyping (Gallego *et al.*, 1998; Hennequin *et al.*, 2001; Pérez *et al.*, 2001). Schuller *et al.* (2004) have recently shown that microsatellite typing, using 6 different loci (Pérez et al., 2001), an optimized interdelta sequence analysis (Legras and Karst, 2003) and RFLP of mitochondrial DNA generated by the enzyme *Hin*fI had the same discriminatory power. In the present work mtDNA RFLP analysis using *Hin*fI was applied as genetic marker for the distinction of *S. cerevisiae* strains.

The aim of the present work was to assess the biodiversity of the fermenting flora found in vineyards belonging to the Vinho Verde Region in order to define strategies

for future wine strain selection programs. Another goal was the establishment of a strain collection contributing to the preservation of *S. cerevisiae* genetic resources.

Materials and methods

Sampling

The sampling plan included a total of 18 sites in three vineyards surrounding a winery, located in northwest Portugal (Região Demarcada dos Vinhos Verdes). In each vineyard, six sampling points were defined according to vineyard geography, and the distance between winery and the sampling sites varied between 20 to 400 m, as shown in Figure 3.1. Two sampling campaigns were performed before (early stage) and after (late stage) harvest, in a time frame of about 2 weeks, in order to assess the diversity among fermentative yeast communities during the last stage of grape maturation and harvest. This experiment was repeated in three consecutive years (2001-2003). Samples were not always collected from the same rootstock, but from the same area (± 1-2 m). The grapevine varieties sampled were Loureiro (vineyard A), Alvarinho (vineyard P) and Avesso (vineyard C), being all white grapes used in the Vinho Verde Region.

Fermentation and strain isolation

From each sampling point, approximately 2 kg of grapes were aseptically collected and the extracted grape juice was fermented at 20ºC in small volumes (500 ml), with mechanical agitation (20 rpm). Fermentation progress was monitored by daily weight determinations.
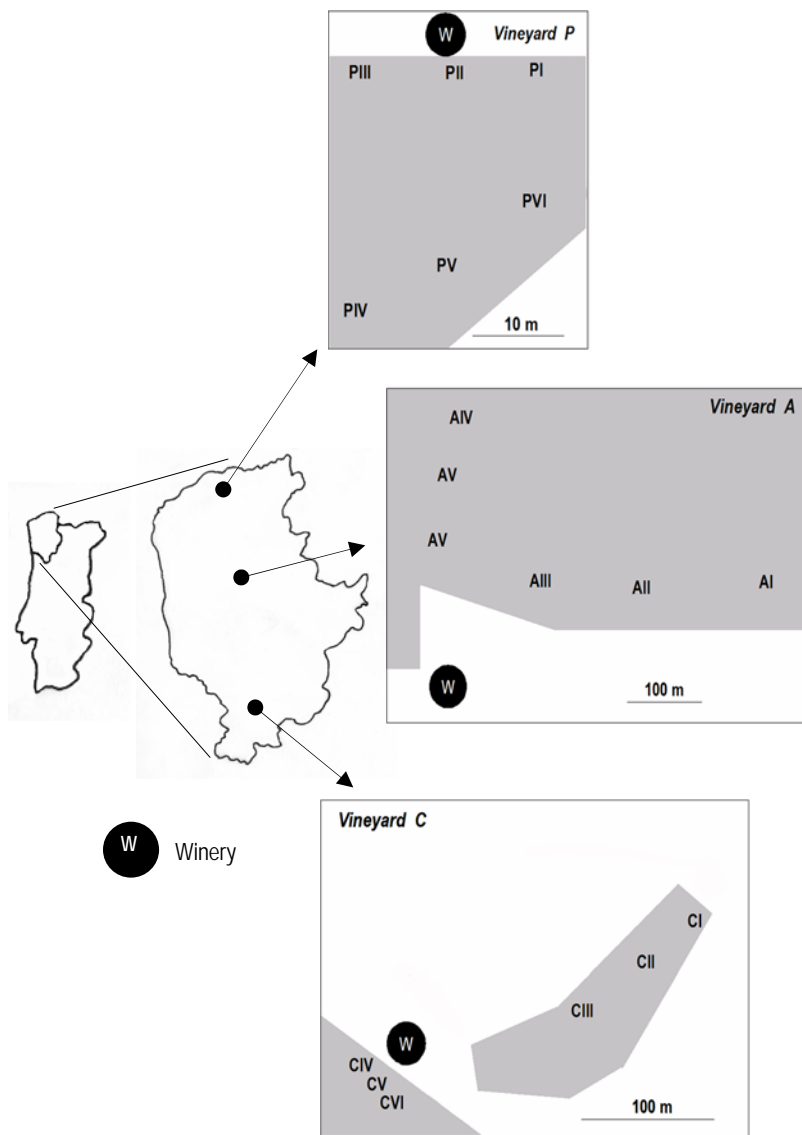
*Figure 3.1   Geographic location of the three vineyards A, C and P in the Vinho Verde Wine Region
with indication of the wineries and the corresponding sampling sites PI-PVI, AI-AVI and
CI-CVI.*

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When must weight was reduced by 70 g/l, corresponding to the consumption of about 2/3 of the sugar content, diluted samples (10^{-4} and 10^{-5}) were spread on YPD plates (yeast extract, 1% w/v, peptone, 1% w/v, glucose 2% w/v, agar 2%, w/v), and 30 randomly chosen colonies were collected after incubation (2 days, 28ºC). The isolates obtained from 54 fermentations throughout this work were stored in glycerol (30%, v/v) at -80ºC.

DNA isolation

Yeast cells were cultivated in 1 ml YPD medium (36 h, 28ºC, 160 rpm) and DNA isolation was performed as described (Lopez *et al.*, 2001) with a modified cell lysis procedure, using 25 U of Zymolase (SIGMA). Cell lysis was dependent on the strain and lasted between 20 minutes and 1 hour (37°C). DNA was used for mitochondrial RFLP.

Mitochondrial DNA RFLP

Restriction reactions were preformed as described (Schuller *et al.*, 2004). The attributed designations for observed distinct patterns were A1-A93, C1-C62 and P1-P135, corresponding to isolates from vineyard A, C and P respectively. Pattern designation ACP10 refers to a strain common to all vineyards and C69P77 and C42P80 were assigned to strains common to vineyard C and P. Pattern profiles that are identical to commercial starter yeasts used by the wineries are designated S1-S6. One representative strain of each of the 297 patterns was withdrawn and tested for growth in a medium containing lysine as sole nitrogen source (Barnett *et al.*, 1990).

Results

In the present work, three vineyards, situated in the Vinho Verde Wine Region, in northwest Portugal, were sampled during the 2001-2003 harvest seasons (Figure 3.1). In order to obtain a more detailed picture of fermenting yeast temporal distribution, two sampling campaigns were performed, one before and another after the harvest, in a time frame of about two weeks. A total of 108 grape samples have been planned (six sampling points x two sampling campaigns x three vineyards x three years), from which 54 started a spontaneous fermentation, 36 were not able to start fermentation after 30 days of incubation, whereas 18 samples were not collected due to unfavorable weather conditions and a bad sanitation state of the grapes in 2002. From the 54 fermentations 1620 yeast isolates were obtained. All the isolates were analyzed by their mtDNA RFLP (*Hinf*I) and a pattern profile was attributed to each isolate, resulting in a total of 297 different profiles.

The total yeast count (cfu in YPD medium) ranged between 1.0×10^6 and 8.0×10^7, corresponding to values generally described for grape must fermentations. All isolates belonged to the species *S. cerevisiae* due to their inability to grow in a medium containing lysine as sole nitrogen source and by their capacity to amplify several *S. cerevisiae* specific microsatellite loci (Schuller *et al.*, in preparation).

The results of mtDNA RFLP for the 1620 isolates are summarized in Table 3.1. Among the total 450 isolates collected in vineyard A, 93 corresponded to unique patterns whereas in C and P a total 450 and 690 strains were isolated, corresponding to 62 and 135 unique patterns, respectively.

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Table 3.1 *MtDNA RFLP analysis of 1620 yeast isolates from fermented must prepared with grapes collected in vineyards A, C and P of the Vinho Verde Region, indicated in Figure 3.1, during the harvest of 2001, 2002 and 2003. E - early sampling stage; L - late sampling stage; NF - no spontaneous fermentation; NC - not collected.*

Vineyard	Year	Stage	Site	Number of isolates	Number of distinct patterns	Number of unique patterns	Common patterns
Vineyard A	2001	E	AI – AVI	NF	-	-	-
		L	AI AIV AVI	NF	-	-	-
			AII		2		
			AIII	90	8	9	A06
			AV		1		
	2002	E	AI – AVI	NF	-	-	-
		L	AI		16		ACP10 A06 A11
			AII		2		ACP10
			AIII	180	9	34	A11 A13
			AIV		6		A06 A13
			AV		9		A13
			AVI		1		-
	2003	E	AI – AVI	NC	-	-	-
		L	AI		3		ACP10 S3
			AII		1		ACP10 S3
			AIII	180	9	46	A06
			AIV		12		-
			AV		19		-
			AVI		2		-
Vineyard C	2001	E	CI CII CIII	NF	-	-	-
			CIV		6		S1 S2 S3 S4 S5
			CV	90	4	2	S4 S5
			CVI		1		S1
		L	CI	NF	-	-	-
			CII		20		S5
			CIII		4		S1 S4 S5
			CIV	150	2	24	S3 S5
			CV		4		S1 S3 S4 S5
			CVI		8		S1 S2 S4 S5
	2002	E	CI CII CIII	NC	-	-	-
			CIV	30	1	1	-
			CV CVI	NF	-	-	-
		L	CI – CVI	NC	-	-	-
	2003	E	CI CIII CIV CV CVI	NF	-	-	-
			CII	30	3	3	-
		L	CI		8		S3 S4 C69P77 C63
			CII		3		C63
			CIII		1		ACP10
			CIV	180	18	32	S1 C42P80
			CV		9		-
			CVI		2		S4

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Table 3.1   *Cont.*

		Site	Number of isolates	Number of distinct patterns	Number of unique patterns	Common patterns
Vineyard P	2001		PI	60	2	2	P136
		E	PII		2		P136
			PIII PIV PV PVI	NF	-	-	-
			PI		6		S3 S5 S6 P136
		L	PII		17		S3 S6 ACP10 P03 P136
			PIII	180	8	62	-
			PIV		21		P03 P24 P50 P136
			PV		15		S3 P24
			PVI		13		S3 S6 P136
	2002	E	PI PII PIII	NF	-	-	-
			PIV PV PVI	NC	-	-	-
		L	PIV	NF	-	-	-
			PI		5		-
			PII		4		ACP10 S6 P136
			PIII	150	1	12	ACP10
			PV		10		S3 S6 P50 P136
			PVI		1		ACP10
	2003	E	PIV PV	NF	-	-	-
			PI		10		-
			PII	120	1	12	-
			PIII		1		P136
			PVI		2		ACP10
		L	PI		15		ACP10 S3 C69P77
			PII		1		-
			PIII	180	9	47	P136
			PIV		18		S3
			PV		5		C42P80
			PVI		5		-

For 11 common patterns (Figure 3.2) found in more than one fermentations (Table 3.1), and also for six commercial starter yeast strains (S1-S6), a wider geographical and temporal distribution was verified. Patterns S1 to S6 corresponded to commercial starter yeasts that had been used in the wineries for the last few years. Perennial strains were associated with more sites of a single vineyard (patterns A06 and S6, P136, P50), but showed also a wider distribution across multiple sampling

sites in two or three vineyards (patterns S3, S4, and ACP10). Patterns S1, S2, C63, A11, A13, P03 and P24 were found only in one year but across several sampling sites of a single vineyard, while strain S5 had a wider distribution across several sampling sites of vineyard C and P. Patterns C42P80 and C69P77 appeared only in a single sampling site during 2003 of both vineyards C and P. Pattern ACP10 is the only "regional" isolate with a wider geographical distribution, whereas A06, A11, A13, C63, P03, P27, P50 and P136 can be considered as "vineyard-strains" due to their occurrence in multiple sampling sites and/or years.
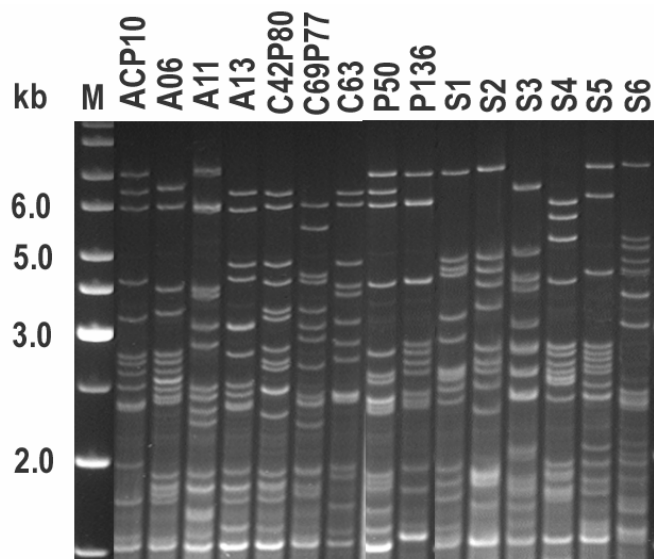

Figure 3.2   Examples of common mitochondrial DNA RFLP (HinfI) patterns, as listed in Table 3.1, found in yeast strains isolated from spontaneous fermentations of must collected as described in Materials and methods.

The wet weather in the summer 2002 resulted in severe fungal infestations and heavy applications of chemical sprays, being probably the reason for the merely 12 unique patterns among the 150 strains collected in the late sampling stage in 2002 in vineyard P. In 2003, this relation was again more similar to the one found in 2001 (47 and 62 unique patterns among each 180 isolates from the late sampling stages of vineyard P).

As shown in Figure 3.3, onset of spontaneous fermentation was verified in almost all grape samples collected in the late sampling campaign. This was rarely the case for most of the samples collected some days before the harvest. Must prepared from grapes collected in the early sampling stage in vineyard A, never started to ferment spontaneously. An accidental agrichemical over-dosage occurred in 2001, resulting in delayed spontaneous fermentation onset for three of the four post-harvest samples (II, III and VI). In the following two years, fermentation profiles were similar to samples from C and P, suggesting the recovery of the intervenient flora.

Fermentation started after six to twelve days being generally accomplished by one to twenty strains. Spontaneous fermentations were performed by one or more predominating strains accompanied by no, few or many "secondary" strains, or by a very heterogeneous yeast community with no prevalent strain(s). This is in agreement with other studies reporting the presence of one or two predominating strains constituting more than 50% of total biomass, and a varying number of "secondary" strains (Constanti *et al.*, 1997; Lopes *et al.*, 2002; Querol *et al.*, 1992a; Querol *et al.*, 1992c; Schütz and Gafner, 1993; Versavaud *et al.*, 1995), or presence of many distinct strains with no prevalence (Pramateftaki *et al.*, 2000; Sabate *et al.*, 1998). The occurrence of both situations has also been reported (Khan *et al.*, 2000; van der Westhuizen *et al.*, 2000a; van der Westhuizen *et al.*, 2000b).
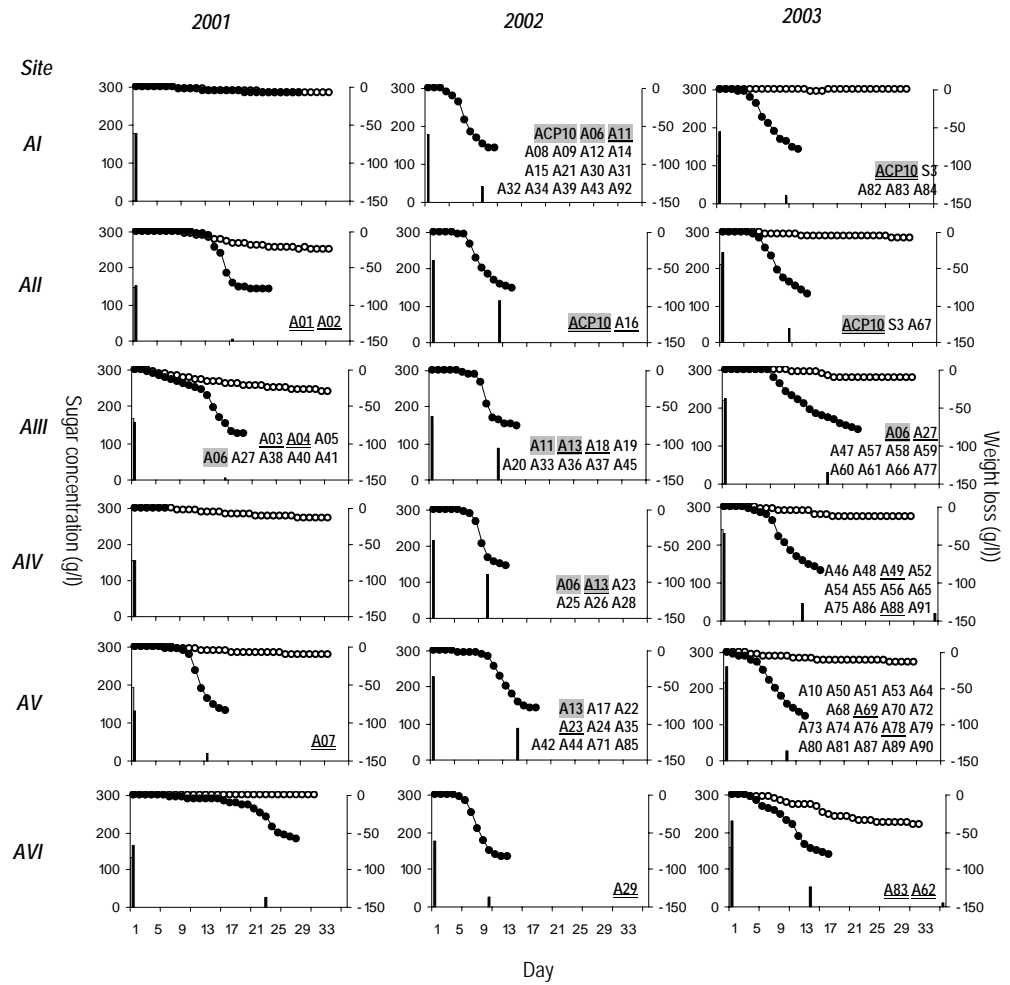
Figure 3.3   *Fermentation profile (lines) and sugar content (bars) of must samples collected in the early (open circles and bars) and late (closed circles and bars) sampling campaigns from which yeast strains analyzed in this work were isolated. In each plot, mtDNA RFLP pattern designations of the yeast isolates are inserted. Predominating strains are double (≥ 50%) or simple (20-50%) underlined. Pattern designations from post-harvest fermentations are bold. Common patterns are in highlighted in grey squares.*
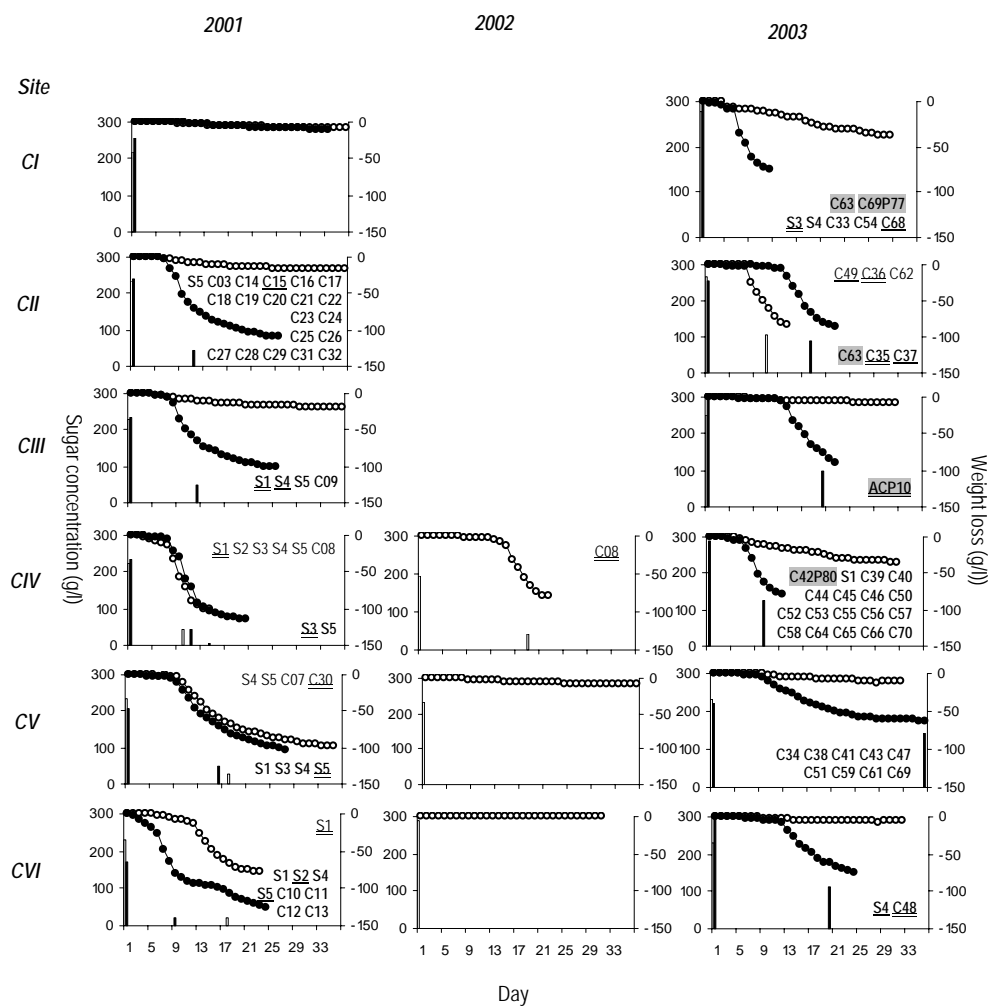
Figure 3.3   *Cont.*
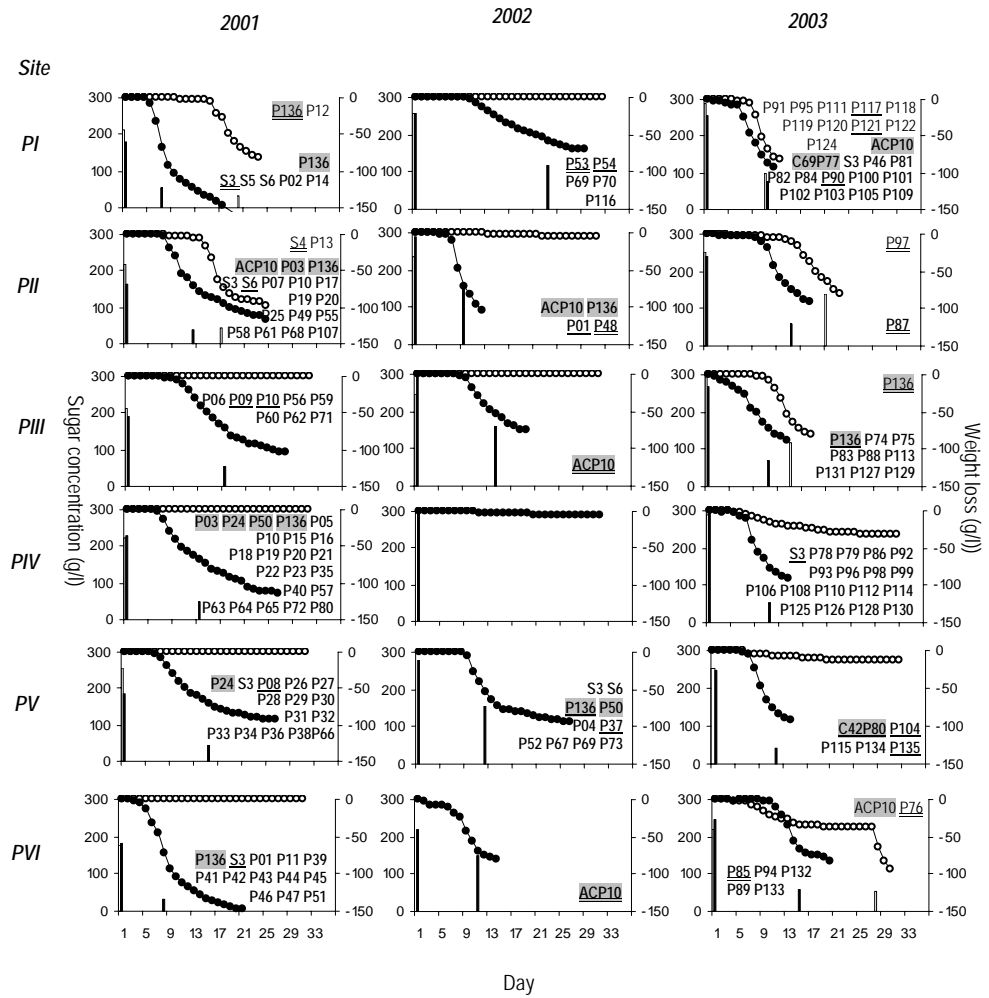
Figure 3.3 Cont.

Apparently no correlation between the number of strains involved in a fermentation and sampling site, year or vineyard was found. The wider distributed strain (ACP10) was dominant in five fermentations (PVI-2002, AI-2003, AII-2003, CIII-2003, PIII-2002) contributing to 77-100% (23 to 30 strains) of the total yeast flora, but was of minor importance in six fermentations (AI-2002, AII-2002, PII-2001, PII-2002, PI-2003, PVI-2003), accounting for only 3-10% (one to three strains), and being accompanied by one to sixteen different strains. The distribution of this strain is not associated with the capability to predominate in fermentation, and competition with accompanying strains seems to play the key role.

Vineyard-specific patterns of samples collected in the early stage did not appear after two weeks at the same site (P, 2001 and 2003, C, 2001) with the exception of the more generalized patterns S1, S2, S3, S4, S5, ACP10 and P136, speaking in favor of a very diversified *S. cerevisiae* flora.

Being the question about origin of wine yeasts still controversial (Martini, 2003; Martini *et al.*, 1996; Mortimer and Polsinelli, 1999; Vaughan-Martini and Martini, 1995), our results clearly indicate that *S. cerevisiae* occurs in vineyard ecosystems belonging to the Vinho Verde Region in sufficient high numbers to conduct a spontaneous fermentation from musts prepared with approximately two kg of grapes. However, some remarks have to be made concerning our experimental approach. Grape must creates selective and very stressful conditions for yeast, totally distinct from the environmental influences in nature. It is therefore clear that our data refer only to *S. cerevisiae* strains capable to survive the conditions imposed by fermentation, under our experimental circumstances, giving therefore a distorted picture (underestimation) of the kind of strains really occurring in vine. As the detection limit of our experimental approach is 3.3% (one strain in 30 isolates), rare

strains, although capable to survive fermentation, might also have not been detected. Searching for *S. cerevisiae* in 18 sites, in two campaigns and over three years using a direct-plating method from single grape berries, as described (Martini *et al.*, 1996) would be highly labor-intensive. Therefore we regard our approach as an acceptable compromise, allowing good estimation of population composition, but preventing a precise description in terms of relative strain abundance in nature.

Discussion

Biogeographical large-scale surveys and studies on the genetic diversity of *S. cerevisiae* strains isolated from spontaneous fermentations have documented the dynamic nature of these populations. In the present study, 297 different genetic patterns have been found among 1620 isolates obtained from 54 small scale fermentations performed with grapes from three vineyards located in the Vinho Verde Region, during a three years period. The overwhelming majority of the patterns were unique, demonstrating an enormous biodiversity of *S. cerevisiae* strains in the Vinho Verde Region. Considering the ratio between the number of isolates and the number of patterns as an approximate biodiversity estimative, our results showed similar values to previously published surveys on genetic diversity of autochthonous oenological *S. cerevisiae* strains in other regions with viticulture traditions such as Bordeaux (Frezier and Dubourdieu, 1992), Charentes (Versavaud *et al.*, 1995; Versavaud *et al.*, 1993), Campagne and Loire Valley (Vezinhet *et al.*, 1992), in France; El Penedèz (Esteve-Zarzoso *et al.*, 2000), Tarragona (Constanti *et al.*, 1997), Priorato (Sabate *et al.*, 1998; Torija *et al.*, 2001) and La Rioja (Gutierrez *et al.*, 1999) in Spain; Germany and Switzerland (Schütz and Gafner, 1993); Tuscany, Sicily (Cavalieri *et al.*, 1998) and Collio (Comi *et al.*, 2000) in Italy; Amyndeon and Santorini (Pramateftaki *et al.*, 2000) in Greece; Western Cape (Khan *et al.*, 2000; van

der Westhuizen *et al.*, 2000a; van der Westhuizen *et al.*, 2000b) in South Africa; Patagonia (Lopes *et al.*, 2002) in Argentina.

The vast majority of the strains did not display a perennial behavior, being the flora of each year characterized by the appearance of many new patterns. This might be attributed to the sampling of only 12 x 2 kg of grapes per vineyard and year, being not enough to grasp the entire biodiversity wealth of a given area. Another reason for the appearance of new patterns could be attributed to recombination and evolutionary forces, but it seems unlikely that such changes occur from one year to another to justify the presence of many distinct patterns in consecutive years. Mitochondrial DNA RFLP patterns are stable when *S. cerevisiae* cells undergo about five to seven divisions during alcoholic fermentation (Schuller *et. al.*, in preparation).

Among all patterns only ACP10 showed a wide regional distribution with a perennial behavior, being a preliminary evidence for a strain representing a "*terroir*" as described (Versavaud *et al.*, 1995; Vezinhet *et al.*, 1992). However, the wider distribution of a strain is not necessarily correlated with a better technological fitness. This makes sense from an ecological point of view, since the selective forces that act in a vineyard are completely different from those that yeast may find in a fermenting grape must. Further physiological characterization under wine making conditions is required to evaluate the potentialities of this strain. The appearance of these strains did not obey to a generalized pattern, but rather to sporadic presence, absence and reappearance, due to natural population fluctuations. The perennial appearance of pattern ACP10 is a consequence of its prevalence in the local microflora. In different fermentations, ACP10 was dominant or not, showing that the final outcome of fermentation was dependent on the specific composition of the yeast community in the must, that is influenced by many factors such as the killer effect which depends

strongly on the ratio of killer to sensitive cells at the beginning of the fermentation (Heard and Fleet, 1986).

Grape variety of vine A was Loureiro, being Alvarinho and Avesso the cultivars of vineyard P and C, respectively, indicating that the grape variety could contribute to the finding of so many distinctive patterns. Traditional wine-making practices are very similar in A, C and P, and differences in climatic influences seem to be of minor influence since the three vineyards are geographically close. However, one can not exclude microclimatic influences, not recorded in the present study.

A first sampling campaign was performed some days before the harvest; a second was carried out a few days after the end of harvest. This was accomplished in a time frame of about two weeks, in order to obtain a more detailed picture of the temporal distribution of fermenting yeast populations during the harvest. As grapes mature to full ripeness, yeasts become more abundant. The last stage of the grape maturation can favor fermentative yeast proliferation on grape surfaces, due to the decrease of grape skin integrity and must leakage from the berries. Insects are the probable source of yeast on damaged grapes. Yeast colonization of grapes can reach values of about 10^5-10^6 cfu/berry (Fleet, 2002). Before vintage, only 5% of the grapes harbor yeasts, being this number much higher (60%) during vintage (Rosini *et al.*, 1982). As expected, only 11 of 45 pre-harvest samples (24%) were able to ferment spontaneously compared to 34 of 48 post-harvest samples (71%). The associated strains were also much more diversified in the late sampling campaign (267 patterns among 1260 isolates) compared to the early stage (30 patterns among 360 isolates). With only one exception (pattern P136), autochthonous strain patterns from the early sampling stage did not appear in the late sampling stage, speaking in favor of a succession of *S. cerevisiae* strains. Alternatively, differences can be attributed to the

fact that different grape bunches were harvested, that may have, although in close proximity to each other, a distinct flora. It seems unlikely that the enormous increase in strain variability at harvest time is due to a spreading of winery-resident flora with harvesting equipment.

The present work is the first large-scale approach about the vineyard-associated strains from the Vinho Verde Region in Portugal, being a useful approach to obtain a deeper insight into ecology and biogeography of *S. cerevisiae* strains, even among geographically close regions. We consider these studies indispensable for the developing of strategies aiming at the preservation of biodiversity and genetic resources as a basis for further strain development.

Acknowledgements

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Chapter 4

Assessment of environmental impact of commercial wine yeast in vineyard ecosystems of different wine producing regions

Assessment of environmental impact of commercial wine yeast in vineyard ecosystems of different wine producing regions

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Running title: Dissemination of commercial wine yeast

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Abstract

In the present study we used commercial wine yeast currently used in wineries as a model to assess the potential environmental risks associated with the utilisation of genetically modified wine yeast strains in the wine industry. To evaluate the dynamics of industrial yeast strains in the vineyard, a large-scale sampling plan was devised over a period of three years, in six different vineyards that have used the same starter yeast for at least five years. Among the 3780 yeast strains identified after spontaneous fermentation, 296 had a genetic profile identical to that of commercial yeast strains. In four of the six vineyards, where the samples were taken at distances from wineries higher than 100 m, only 0-2% of the fermentative microflora had a genetic profile identical to that of commercial yeast. In the other two vineyards, where the samples were taken at very close proximity to the winery and to water rills, the proportion of commercial yeasts increased to 10-43%. The majority (94%) of commercial yeasts were found at a distance of between 10 and 200 m from the winery. Commercial strains were not found in the same site from one year to another, which indicate that their implantation in the vineyard does not occur. Instead these strains are subject to natural fluctuations of periodical appearance/ disappearance as autochthonous strains.

The data show that dissemination of commercial yeast in the vineyard is limited to short distances and periods of times and is largely favoured by the presence of water runoff. If some of these strains are able to remain in the vineyard, they don't become implanted systematically in the ecosystem and are not able to dominate the natural microflora.

Introduction

The predominant yeast species used in the production of wine is *Saccharomyces cerevisiae*, universally known as "wine yeast". Under selective conditions of grape must fermentation, yeasts efficiently compete with other microorganisms present in musts, such as moulds and lactic and acetic acid bacteria. A succession of various yeast species – the apiculate yeasts *Hanseniaspora uvarum* (= *Kloekera apiculata*) and other yeasts of the genera *Metschnikowia*, *Candida* or *Pichia* - are found in the early stages of fermentation. As the concentration in ethanol increases, these species are rapidly outgrown by *S. cerevisiae* which invariably dominates the latter stages of the process.

Since the beginning of the 1980's, the use of active dried *S. cerevisiae* yeast starters has been extensively generalised. Today, the majority of wine production is based on the use of active dried yeast, which ensures rapid and reliable fermentations, and reduces the risk of sluggish or stuck fermentations and of microbial contaminations. Most commercial wine yeast strains available today have been selected in the vineyard for enological traits such as fermentation performance, ethanol tolerance, absence of off-flavors and production of desirable metabolites. These and other technological developments have contributed to an improvement in the quality of wine, and have enhanced the ability of winemakers to control the fermentation process and achieve specific outcomes.

As a result of modern winemaking practices and diversification of wine products, there is an increasing quest for specialised wine yeast strains. During the last two decades a considerable knowledge of *S. cerevisiae* genetics and physiology has been generated as well as numerous genetics tools. Recombinant DNA technologies have been successfully applied to wine yeast, generating specialized wine yeast

strains which have been engineered for specific traits, such as improved fermentation performance and process efficiency, wine sensory quality and health benefits for consumers (Blondin and Dequin, 1998; Butzke and Bisson, 1996; Dequin, 2001; Hansen and Kielland Brandt, 1996; Pretorius, 2000; Pretorius and Bauer, 2002).

While genetically engineered strains could bring significant benefits to both winemakers and consumers, they have not yet been used into the wine industry. From the perspective of a future introduction of this technology, a sound evaluation of the safety and the potential environmental impact of genetically modified wine yeast is absolutely required.

In this context, industrial yeasts used as fermentation starters are a good study model for assessing the potential environmental impact of genetically modified wine yeasts. Commercial yeasts are classically used in winemaking without any special containment and are annually released in large quantities, together with liquid and solid wine-making residues, in the environment around the winery. The behaviour of these yeasts in the ecosystem of the vineyard is totally unknown as well as their potential impact on the natural microflora. In particular, it is not known if commercial strains are able to survive in nature and to become members of the vineyard microflora. These questions have to be addressed since the corresponding genetically modified wine yeasts may be able to compete and influence the fermentations of the following year, specially those performed according to traditional practices which rely on spontaneous fermentations. There is very little available data that could contribute to the evaluation of the importance of starter yeast dissemination and permanence in the vineyard (Frezier and Dubourdieu, 1992; Vezinhet *et al.* 1992; Guillamón *et al.*, 1996). Recently, a large-scale biogeographical

study in South African vineyards was carried out over 4 years. In five areas situated in the Coastal Region vineyards of the Western Cape, 13 samples were collected and commercial yeasts were recovered from three samples (van der Westhuizen *et al.*, 2000a and 2000b). These studies have made it necessary to carry out this type of study on a larger scale, with the aim of increasing the statistic significance of the results obtained.

In order to provide a consistent assessment of potential environmental risks associated with the use of genetically engineered winery yeast strains, a large-scale study was established to assess their fate in the natural environment in different geographical localizations of France and Portugal, using commercially available yeast strains as a model. The present study aims to evaluate the industrial starter yeasts' ability to spread and survive in nature, becoming part of the must microflora.

Materials and Methods

Sampling plan and commercial wine yeast

Grapes were harvested during three consecutive years (2001-2003) in six vineyards, three of which were located in south France and three in northwest Portugal, as shown in Figure 4.1. In France, the wineries were located in the Languedoc-Roussillon Region, around the Mediterranean city of Montpellier, and the vineyards were situated at a distance of between 30 and 80 km. In Portugal, the three wineries were located in the north, centre and south of the Região Demarcada dos Vinhos Verdes, the distance between each being between 40 and 80 km. In each vineyard, six sampling points were defined according to the predominating wind direction at a distance of between 20 to 1000 m from the winery, as shown in Figure 4.1.

In order to evaluate the permanence over years of commercial yeast, a first sampling campaign was performed before the winery started wine production with the use of commercial yeast strains (pre-harvest samples). In a second post-harvest sampling campaign, the grapes were collected after the onset of wine production, in order to evaluate the immediate commercial yeast dissemination from the winery. With the present experimental design, 72 grape samples were planned for each year.

The wineries selected have used one or more commercial yeast strains consecutively in at least the last five years. Tables 4.1 and 4.2 show the commercial yeasts used in each winery during the studied period (2001-2003) and their geographic origin respectively.

Sample collection and yeast isolation

From each sampling point, approximately 2 kg of grapes were collected aseptically and placed directly into sterile plastic bags that were transported to the laboratory in cool bags. At the laboratory, grapes were crushed by hand in the plastic bags, which were opened and 180 ml of juice poured into 250 ml sterile fermentators. The fermentators were placed in a temperature-controlled room at 20°C with mechanical agitation. Daily weight determinations allowed the monitoring of the fermentation progress.

The yeast flora was analysed when the must weight was reduced by 70 g/l, corresponding to the consumption of about 2/3 of the sugar content. Must samples were diluted and spread on plates with YEPD medium (yeast extract, 1% w/v, peptone, 1% w/v, glucose, 2% w/v, agar, 2% w/v), and after 2 days of incubation 30 randomly selected colonies were collected from each spontaneous fermentation.
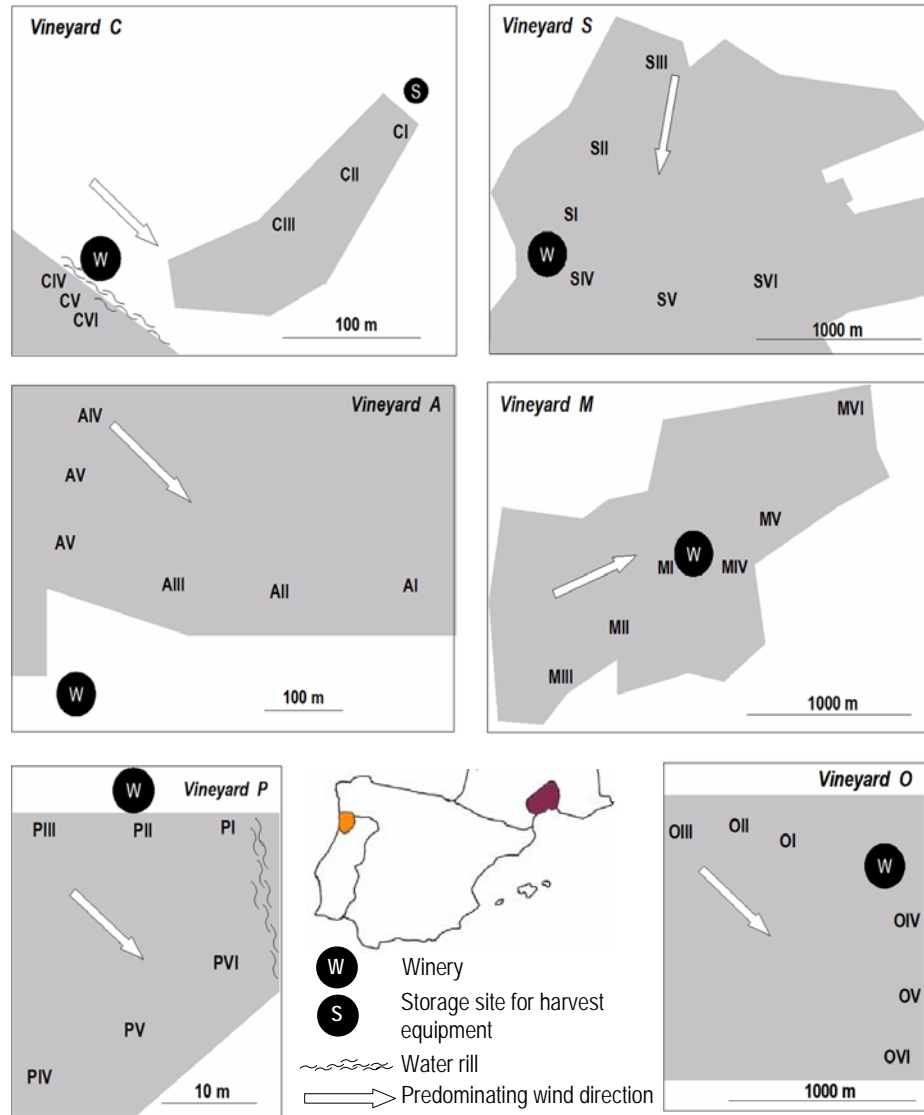
Figure 4.1   *Geographic localization of the vineyards belonging to the Languedoc (S, M, O) and Vinho Verde (A, C, P) Wine Regions and indication of the sampling sites in each of the six vineyards. In each site, 2 samples (pre- and post- harvest campaign) were collected. The factors that may have influence in the dissemination are indicated in the Figure.*

Table 4.1   *Commercial yeast strains used in each winery in the last years. The strains shown in bold were used for at least the last 5 years prior to the study.*

Year	Winery S	Winery M	Winery O	Winery C	Winery P	Winery A
2001	**K1M ICV-INRA** **ICV D254** Enolevure K34 Lalvin QA23 ICV D47	**K1M ICV-INRA** **ICV D254** ICV D80 Uvaline BL Lalvin BM45 Maurivin AWRI2	**K1M ICV-INRA** **Zymaflore VL3** **Maurivin PDM** ICV D254 ICV D47 Uvaline arôme Vitilevure-Chardonnay Anchor VIN 13	**ZymafloreVL1** **ZymafloreVL3** **Zymaflore F10** Zymaflore F15 Uvaferm L2056 Lalvin CY 3079 Uvaferm ALB Uvaferm 228	**Zymaflore VL1** Lalvin EC 1118	**Zymaflore VL1**
2002	**K1M ICV-INRA** **ICV D254** Lalvin QA23	**K1M ICV-INRA** ICV D80 Uvaline BL Lalvin BM45 Maurivin AWRI2 Uvaline CVR	**K1M ICV-INRA** **Maurivin PDM** ICV D47 Anchor VIN 13 Zymaflore VL3 Anchor NT 116 Vitilevure-Sauvignon	**ZymafloreVL1** **ZymafloreVL3** **Zymaflore F10** Zymaflore F15 Uvaferm ALB Uvaferm 228 Uvaferm CS2	**Zymaflore VL1** Lalvin EC 1118 Levuline BRG Fermichamp	**Zymaflore VL1** Lalvin EC 1118
2003	**K1M ICV-INRA** ICV D254 Enolevure K34	**K1M ICV-INRA** Uvaline BL Lalvin BM45 Anchor NT 45 Anchor NT 50 ICV D80 Uvaline CVR Enolevure K34 Maurivin PDM	**K1M ICV-INRA** **Zymaflore VL3** **Maurivin PDM** Vitilevure-Chardonnay Vitilevure-Sauvignon	**ZymafloreVL1** **ZymafloreVL3** **Zymaflore F10** Zymaflore F15 Zymaflore VL2 Uvaferm ALB	**Zymaflore VL1** Fermafine Fermafruit IOC 18-2007 Lalvin CY 3079	**Zymaflore VL1** Lalvin CY 3079

Table 4.2   *Geographic origins of commercial yeast strains used in the studied wineries.*

Strains	Origin
Anchor NT 45	South Africa
Anchor NT 50	South Africa
Anchor NT 116	South Africa
Anchor VIN 13	Stellenbosch, South Africa
Enolevure K34	Valencia, Espagne
Fermafine	Not Known
Fermafruit	Not Known
Fermichamp	Alsace, France
ICV D47	Rhône, France
ICV D80	Rhône, France
ICV D254	Languedoc, France
IOC 18-2007	Not Known
K1M ICV-INRA	Languedoc, France
Lalvin BM 45	Sangiovese, Italy
Lalvin Cy 3079	Bourgogne, France
Lalvin EC1118	Champagne, France
Lalvin QA 23	Portugal
Levuline BRG	Not Known
Maurivin AWR12	Bordelais, France
Maurivin PDM	Champagne, France
Uvaferm 228	France
Uvaferm ALB	Not Known
Uvaferm CS2	Alsace, France
Uvaferm L 2056	Rhône, France
Uvaline arôme	Loire, France
Uvaline BL	Champagne, France
Uvaline CVR	Not Known
Vitilevure Chardonnay	Languedoc, France
Vitilevure Sauvignon	Sauvignon, France
Zymaflore F10	Bordelais, France
Zymaflore F15	Gironde, France
Zymaflore VL1	Guironde, France
Zymaflore VL2	Burgundy, France
Zymaflore VL3	Gironde, France

Growth in medium containing L-lysine

To rapidly discriminate between *Saccharomyces* and non-*Saccharomyces* yeast, every isolate was evaluated according to its ability to grow in L-lysine (Barnett *et al.*, 1990). All isolates that were not able to grow on the YNB medium with L-lysine as the sole nitrogen source but grew on the control medium YNB with ammonium sulphate were considered as *Saccharomyces* and selected for molecular identification.

Molecular identification

Yeast cells were cultivated in 1 ml YPD medium (36 h, 28ºC, 160 rpm) and DNA isolation was performed as described (López *et al.*, 2001) with a modified cell lysis procedure, using 25 U of Zymolase (SIGMA). Cell lysis was dependent on the strain and lasted between 20 minutes and 1 hour (37°C). DNA was used for mitochondrial DNA RFLP (mtDNA RFLP) analysis and microsatellite typing. The following methods were used for the molecular characterisation:

Chromosomal polymorphisms were studied by pulsed field gel electrophoresis. Yeast chromosomal DNA was prepared in plugs as previously described (Blondin and Vezhinet, 1988). The TAFE (transverse alternating field electrophoresis) system (Geneline, Beckman) was used. The gels were run for 26 h: 6 h at 250 V with 35 s pulse time followed by 20 h at 275 V with 55 s pulsed time, at a constant temperature (14°C).

Mitochondrial DNA restriction profiles were analyzed as previously described (Querol *et al.*, 1992). Digestions (*Hin*fI) were performed overnight at 37ºC in a final volume of 20 μl as previously described (Schuller *et al.*, 2004).

Microsatellite analysis was performed using six loci (ScAAT1-ScAAT6) previously described by Pérez *et al.* (2001) that were amplified in two multiplex reactions (Bio-Rad iCycler thermal cycler). The samples were denatured and separated by capillary electrophoresis in an ABI Prism 310 DNA sequencer (Applied Biosystems) and analysed using Genescan software, the complete method is described by Schuller *et al.* (2004).

Results

Sampling sites and Saccharomyces *isolations*

A large sampling plan was followed: a total of 198 samples were collected during three consecutive campaigns (2001-2003), 108 of which were taken in France and 90 in Portugal. It is to be noted, as can be observed in Figure 4.1, that due to geographical constraints, the samples in Portugal were collected much closer to the winery than in France. In the French wineries (S, M and O), the sample sites were located at a distance of between 100 and 1000 m from the winery, whereas in the Portuguese wineries (A, C and P) half of the sampling sites were located at a distance of less than 70 m from the winery and none was located further than 400 m.

Table 4.3 shows the global data in each country broken down into years. Of the 198 samples, 126 musts (64%) produced spontaneous fermentations, 20% and 44% in must from pre-harvest and post-harvest campaigns, respectively. The percentages of spontaneous fermentations were similar in both countries, 66% in France and 60% in Portugal. A total of 3780 colonies were isolated from these fermentations (2160 and 1620 in France and Portugal, respectively).

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Table 4.3   *Global data over the three years studied. Numerical distribution by countries and years.*

	2001		2002		2003		Total
	France	Portugal	France	Portugal	France	Portugal	
Samples	36	36	36	18	36	36	198
Spontaneous fermentations	24	19	33	12	15	23	126
Number of isolates	720	570	990	360	450	690	3780
Non-Saccharomyces strains	314	0	870	0	241	0	1425
Saccharomyces strains	406	570	120	360	209	690	2355

Discrimination between *Saccharomyces* and *non-Saccharomyces* isolated in Languedoc was performed using a selective medium with L-lysine as the sole nitrogen source (Barnett *et al.*, 1990). According to this method only 2 species of the *Saccharomyces* genus (*S. kluyveri* and *S. unisporus*) not habitually found in enological environments are capable of growing with L-lysine. From this we can consider the yeasts isolated after fermentation that can grow in L-lysine as not belonging to the *Saccharomyces* genus. To confirm this hypothesis isolates from the fastest fermentations that grew in a L-lysine medium were identified by PCR-RFLP analysis of the rDNA ITS region (Granchi *et al.*, 1999). The results confirmed that they were non-*Saccharomyces* yeast strains, belonging mainly to the *Kloeckera* genus (data not shown). All isolates not able to grow on the L-lysine medium were therefore selected for molecular identification. In Portugal, all isolates were assigned in different groups according their mtDNA RFLP pattern. One representative strain from each group was randomly withdrawn, and all strains were unable to grow on the L-lysine medium.

Important differences were observed regarding the proportion of *Saccharomyces sp.* found in the isolates after fermentation (weight reduction 70 g/L). In Portugal all the isolates after fermentation were shown to have a *Saccharomyces* type profile, while in France a large proportion of non-*Saccharomyces* was found (Table 4.3, Figure 4.2). The non-*Saccharomyces* represented 66% of the total yeasts isolated in France over the three years. It should be noted that in 2002 88% of the yeasts isolated in France were non-*Saccharomyces*. This year was atypical, characterised by heavy rainfall, before and during the harvest (50% above normal). These conditions not only produced musts with lower sugar content and slower fermentations (data not shown) but also made it necessary to increase the antifungal treatment of the vines (about 30% more than in the other two years of the study).

In Portugal, heavy rainfall and very bad sanitation conditions of the grapes prevented many samples from being collected in 2002, nevertheless the yeasts isolated from the collected samples were all *Saccharomyces*, as in the other two years.

Frequency of commercial wine yeast in each vineyard

The global composition of the yeast population isolated after fermentation from the six wineries over the three years studied, in pre- and post-harvest campaigns is shown in Figure 4.2.

Identification of *S. cerevisiae* strains was performed by different molecular typing methods depending on the specific resources of each laboratory. Chromosomal pattern analysis of 735 *Saccharomyces* isolates from France (wineries S, M and O) was performed, and compared with that of the 19 commercial yeasts used in the

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three wineries. In Portugal, all 1620 isolates were analysed by mtDNA RFLP (*Hin*f*I*),

Legend:

- Zymaflore VL1
- K1M ICV-INRA
- Zymaflore VL3
- Zymaflore F10
- Zymaflore F15
- Lalvin CY 3079
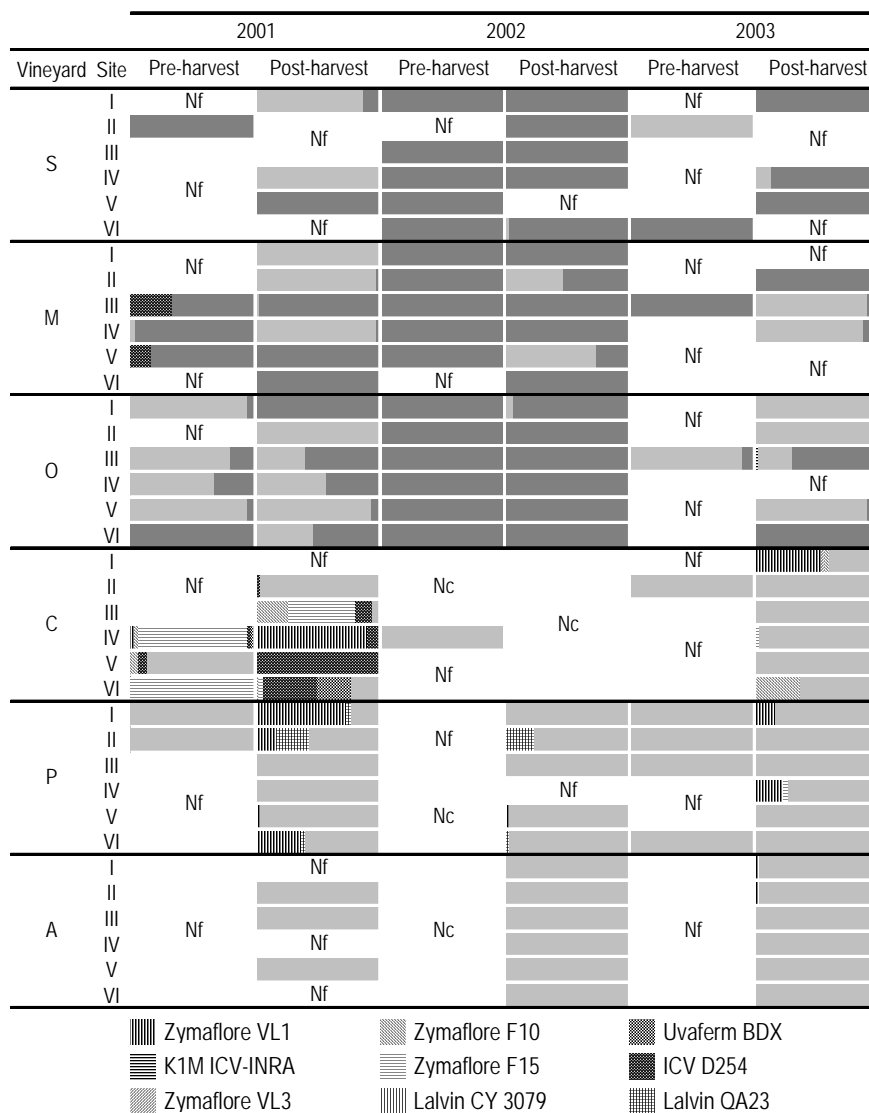- Uvaferm BDX
- ICV D254
- Lalvin QA23

Figure 4.2   Global composition of the yeast populations isolated in each site from the six wineries during the pre- and post- harvest sampling campaigns over three years. The motifs show the commercial yeast, light grey indicating other Saccharomyces *strains and dark grey the non-*Saccharomyces *strains. Nf – No fermentation; Nc – Not collected.*

and their patterns compared to those of a commercial yeasts strain collection, including all strains used by the three wineries. At least one representative isolate of each strain group showing mtDNA RFLP patterns identical to commercial strains was further confirmed by microsatellite analysis of the loci described in the Material and Methods section. In order to evaluate the discriminatory power of these three methods a previous survey of the genetic polymorphisms generated by distinct methods was performed in a total of 23 commercial yeast strains used in the wineries of the 2 countries. The results showed that the discriminatory power of microsatellite typing using these six different loci and that of mtDNA RFLP patterns generated by the enzyme *HinfI* was the same and similar to that of karyotype analysis. Among the 23 commercial yeasts strains analysed, 21 different patterns were obtained using the first two methods and 22 using the last (Schuller *et al.*, 2004). Owing to the verified equivalence of the discriminatory power of these methods any of them can be used for this study and the results obtained can be comparable.

In vineyard S, only 47 % of the isolates collected in 2001 were *Saccharomyces sp.*, the percentage was lower still in the following year, 1% in 2002 and 23% in 2003. The marked strain K1M ICV-INRA was the industrial yeast most commonly used in this winery over the last 15 years, followed by Enolevure K34 and ICV D47, used over the last 10 years (Table 4.1). Over the study period these yeasts were used in large quantities (30% - 40%), nevertheless none of the isolates had an identical genetic pattern to any of the commercial strains used in the winery.

In vineyard M, the marked strain K1M ICV-INRA was the most commonly used industrial yeast in this winery over the last 10 years, followed by ICV D254 used for the last five years (Table 4.1). One hundred ninety four *Saccharomyces sp.* were found in a total of 720 isolates, representing 39% in 2001 and 11% and 44% in 2002

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and 2003 respectively. The karyotype of 15 isolates was identical to the commercial strain ICV D254 that was initially isolated in south France (Table 4.2). They were found in the pre-harvest samples (sites MIII and MV) collected in 2001, and represented 17% and 33% of the fermentative flora in each site respectively. Both samples were collected from opposite directions in relation to the winery, at distances of 500 m (site MV) and 1000 m (site MIII). This fact could indicate previous dissemination, but it cannot be confirmed since the commercial yeast strain ICV D254 was isolated from the same region (Table 4.2).

In vineyard O, as in the other two wineries, the most commonly used industrial yeasts are K1M ICV-INRA (90-95%), together with Zymaflore VL3 and Maurivin PDM, used for over five years. It was in this winery that the largest quantity of *Saccharomyces sp.* were collected in France (324 from a total of 870 isolates), representing 61% of the initial flora collected during 2001, decreasing to 10% in 2002 and increasing again to a similar percentage in 2003 (68%). The karyotype of only one isolate was identical to the commercial strain K1M ICV-INRA; it was found in a sample collected from a distance of 1000 m from the winery during the post harvest campaign of 2003. This isolate was tested, together with others with a chromosomal pattern close to that of K1M ICV-INRA, to assess its resistance to erythromycine and diuron, since this strain possesses a mutation of mitochondrial DNA conferring resistance to these antibiotics. The results confirmed that only this isolate is the marked strain K1M ICV-INRA (results not shown). No commercial yeast strains were detected during the sampling years 2001 and 2002.

In vineyard C, three sampling sites (CIV, CV and CVI) were in close proximity to the winery (20 – 40 m), and none were situated at more than 200 m, as shown in Figure 1. Strains Zymaflore VL1 and F10 were predominantly and continuously used during

more than 10 years, along with several sporadic minority strains (Table 4.1). In 2001, Zymaflore VL1, F10 and F15 were found in sites closer to the winery (sites CIV, CV and CVI), contributing between 7% and 100% of the fermenting flora in both the pre- and post-harvest campaigns. Due to the ground inclination, water runoff flows from the winery to the vine may contribute to the frequent occurrence of commercial strains in these sites (Figure 4.1). From the post-harvest campaign of site CIII, located further, strains Zymaflore F10 and F15 were recovered, but not strain VL1. Strain ICV D254, used from 1998 to 2000, was found in 7 of 8 fermentations, principally in post-harvest fermentations from sites CV and CVI and strain Uvaferm BDX, used in 1998 and 2000, was found to a lesser extent in sites CVI (post-harvest) and CIV (pre-harvest). However, they were not found in the following years. In 2002, due to heavy rain falls and a very bad sanitation condition of the grapes, many samples could not be collected and no commercial strains were recovered. In the following year, samples collected from the closer sites CIV, CV and CVI showed no spontaneous fermentation (pre-harvest) and a smaller quantity of strains F15 and F10 was found in the post-harvest samples, strain VL1 being completely absent. From grapes collected after harvest in site CI, (250 m from the winery) strains VL1, VL3 and F10 were recovered. A small building nearby (20 m) that is used for storage of harvest transport equipment and commercial yeasts may explain strain occurrence at this more distant site. However, dissemination by insects, wind and dust should also be considered.

In vineyard P, located in the north of the Vinho Verde Region, all sampling sites were relatively close to the vinery (10-70 m) due to the small dimensions of the vineyard. Strain Zymaflore VL1 was detected in all three years in post-harvest fermentations only (2001- sites PI, PII, PV and PVI; 2002 – site PV; 2003 – site PI and PIV), while none of the sporadically used strains was found with the exception of 1 isolate Lalvin

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Cy 3079 (post-harvest 2003). It is also noteworthy that site PI of this winery, where the highest number of VL1 strains was recovered, is located close to a rill that transports runoff water from the winery, emphasizing the importance of water as a vehicle for yeast strain dissemination. Furthermore, the dumping site of macerated grape skins is adjacent to site PI, constituting a fermenting sugary substrate harbouring large amounts of yeast that are distributed throughout the vine. Several isolates with a genetic pattern identical to the strain Lalvin QA23 were found in 2001 and 2002 (post-harvest only). Given that this strain was initially selected from the Vinho Verde Region, and given the lack of records concerning its use in former years, it was not possible to decide whether these isolates correspond to the natural yeast flora or to dissemination from the winery.

In vineyard A, samples were collected at greater distances than in the other 2 wineries, ranging from 150-400 m from the winery. None of the 270 strains isolated in the 2001 and 2002 campaigns had the genetic patterns of the commercial yeast strains that have been used in the last five years (Zymaflore VL1 and Lalvin EC 1118), and in 2003 only 1 strain with a VL1-profile was found in each of 2 distant sites (ca. 400 m).

Geographic distribution of recovered commercial yeast strains

Table 4.4 shows the distribution and frequency of commercial yeasts in each vineyard. It should be noted that in the vineyards where the sampling sites were placed at a greater distance from the winery, vineyard A in Portugal and the three French vineyards (S, M, O), the occurrence of commercial yeast was very low, representing between 0% and 2% of fermentative flora. In France, the genetic profile of 16 strains out of 735 *Saccharomyces* isolates (2%) was identical to that of

commercial yeasts. These strains corresponded to 0.8% of the microflora isolated after fermentation. With only one exception, these strains (15 isolates) had an identical profile to that of the autochthonous strain ICV D254 and were found in the same site (winery M), in pre-harvest samples taken in 2001 at the halfway or furthest points from the winery. No commercial yeasts were found from winery S and one colony, isolated in 2003 in winery O (site OIII), had the same profile as K1M ICV-INRA, used in the three French wineries for the last 5-15 years. It is noteworthy that this yeast, that has been used extensively for a considerable length of time, has never been found in the vineyard, except in this case. The same situation occurs in the Portuguese winery A, only 2 isolates (sites AI and AII) with the same profile as the extensively used commercial yeast, Zymaflore VL1, used for five years, was found.

Table 4.4   Commercial yeast strains recovered in each vineyard over the three years studied.

Vineyards	S	M	O	C	P	A	Total
Nº of isolates	570	720	870	480	690	450	3780
Commercial yeast strains	0	15*	1	206	54+18*	2	296
% Commercial strains / nº of isolates	0	2	0.1	43	10	0.5	7.8

*Commercial yeasts initially isolated in the same region

The results were very different in the Portuguese wineries C and P, for which a high number of commercial strains were recovered after fermentation, representing 43 and 10% of the fermentative flora respectively. Indeed, the large majority (94%) of the commercial strains isolated within the six vineyards was recovered from these 2 vineyards only and 70% from the sole vineyard C. The major difference between these 2 vineyards and the 4 other ones is that the sample sites in the 2 former were

placed in close proximity to the winery (Figure 4.1). It can be observed from Figures 4.2 and 4.1 that the majority of the commercial strains in these 2 vineyards were recovered from sites closest to the winery, CIV, CV and CVI in vineyard C and PI, PII and PVI in vineyard P.

An overview of the dissemination of commercial strains in relation to their distance from the winery is shown in Figure 4.3. Ninety four percent of commercial strains were found in a radius of around 10-200 m from the winery and a large majority (78%) was recovered in sites at very close proximity (10-50 m) to the wineries (vineyards C and P), where rills with runoff water were present. Commercial yeasts in pre-harvest campaigns only were collected in sites very close to the winery C (10-50 m). The strain found in the greatest quantity (87%) in the pre-harvest campaign was Zymaflore F15. A higher quantity of commercial strains were recovered in the post-harvest samples, strain VL1 represented 49% of commercial strains recovered after harvest, and derived from sites close to the place where macerated grape skin is deposited or water runoff occurs, never further than 10-20 m from the winery (Figure 4.1 and 4.2). A lower percentage of other predominant strains Zymaflore F10 and F15, formerly used minority strains Uvaferm BDX and ICV D254 and the autochthonous strain Lalvin QA23 was found at sites closer to the winery (10-50 m). Zymaflore F15, F10 and ICV D254 were also found at about 100 m from the winery. The occurrence of several isolates found at 200 m (site I, winery C) can be attributed to the presence (20 m) of a small building for storage of commercial yeast and harvest transport equipment. Two samples taken in France at a distance of 400 and 1000 m contained yeasts with an identical karyotype to that of indigenous strain ICV D254.
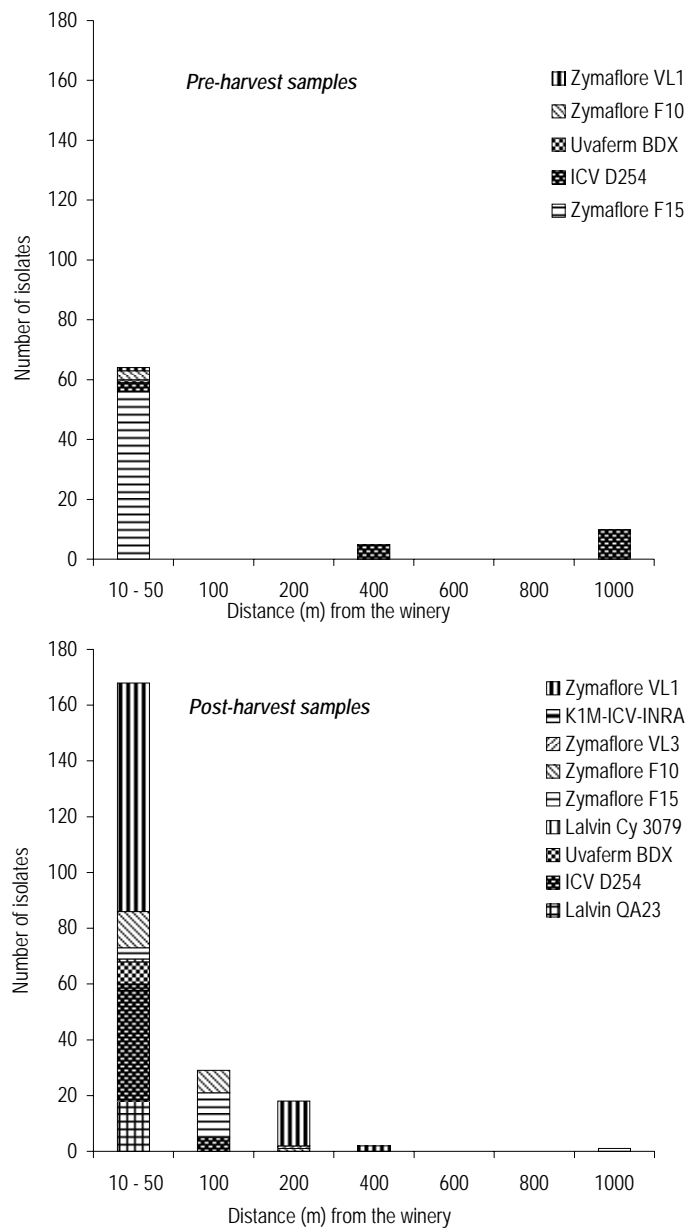
Figure 4.3   *Overall (three years) distribution of commercial yeast strains according to the distance from the wineries in pre-harvest and post-harvest campaigns.*

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Evolution of fermentative flora over the three years

The evolution of the total flora isolated after fermentation in the different wineries of France and Portugal during the three years studied is shown in Figure 4.4. In France we observed that different proportions of *Saccharomyces* and non-*Saccharomyces* were recovered in the different vineyards in the three years. The proportion did not only vary in each vineyard and campaign in any given year, but also in the same site and campaign from year to year. While, for example, in the pre-harvest campaign in vineyard S only non-*Saccharomyces* were recovered in 2001 and 2002, a similar proportion of *Saccharomyces* and non-*Saccharomyces* was recovered in 2003.

As regards commercial yeasts, from a total of 296 recovered over the three years in the six vineyards, 76% were found in 2001, in pre and post-harvest samples collected in vineyard D and post-harvest samples collected in vineyard E.
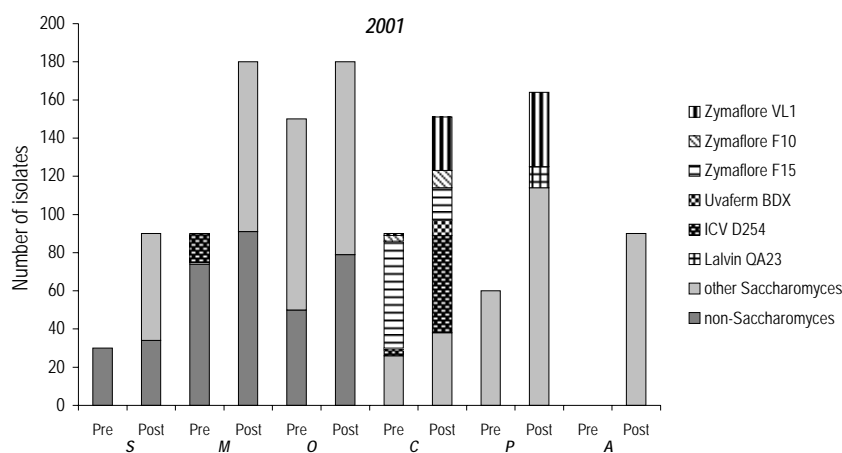

Figure 4.4   *Evolution of the total microflora from each wineries (S, M, O, C, P, A) during the three year's survey in pre- and post-sampling campaigns (Pre and Post).*
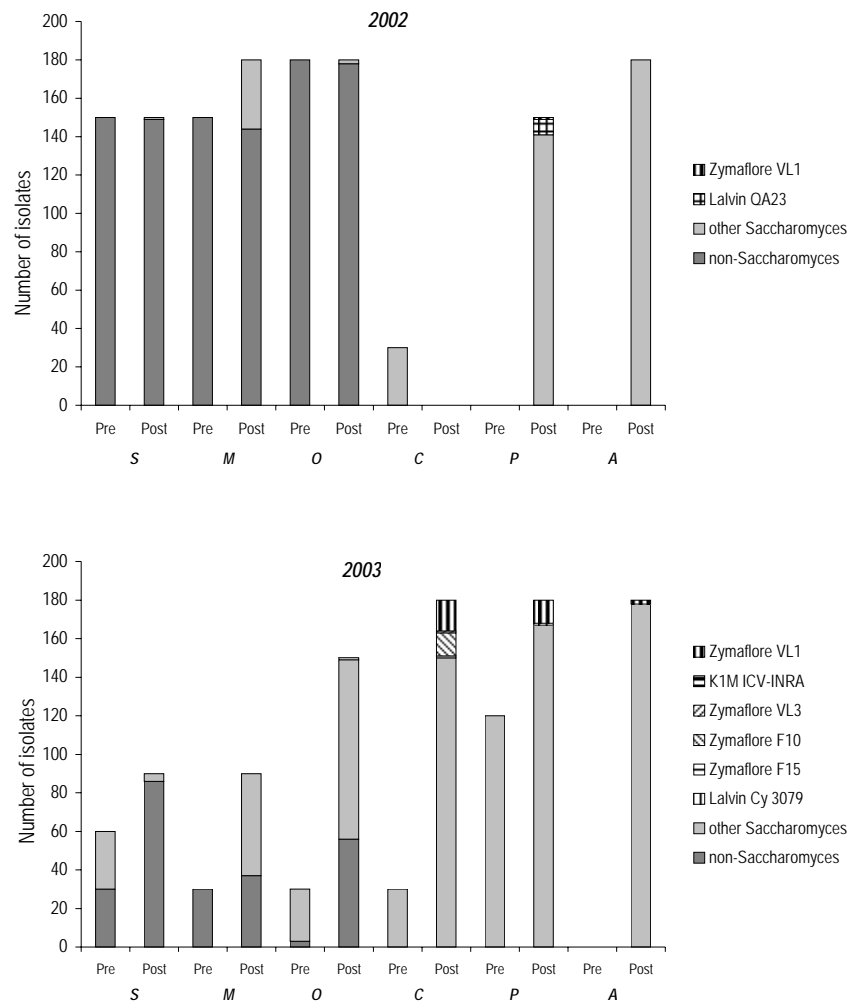
Figure 4.4   Cont.

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In the following two years commercial yeasts were detected only in certain post-harvest but not in pre-harvest samples. As can be observed in Figure 4.4, five different commercial yeast strains were found in the pre-harvest campaign of winery C in 2001, predominantly used strains VL1, F10 and F15 and, in much smaller quantities, previously used strains Uvaferm BDX (1998 and 2000) and ICV D254 (1998–2000), showing the permanence of the latter in the vineyard from one year to another. However, given that these 2 strains appeared in 2001 only, this permanence is limited. The commercial yeasts collected in each site, nevertheless, were different. The highest number of Zymaflore VL1 isolates was obtained from grapes collected after harvest at the site CIV, whereas in samples collected before harvest, VL1 and F10 occurred rarely, contrarily to the abundant strain F15 in the pre-harvest campaign in 2001 sites CIV and CVI (Figure 4.2). This data may suggest a better ability of strain Zymaflore F15 to remain in the vineyard, nevertheless, no isolate of strain F15 was found in 2002 and only one was found in 2003 (Figures 4.2 and 4.4). In this winery no samples were collected in the post-harvest campaign in 2002, and a lower quantity of commercial yeasts was found in 2003. In addition the presence of one isolate of Zymaflore VL3, not present in 2001, was detected. In the post-harvest campaign of 2001 in winery P, 2 commercial yeast strains were isolated, Zymaflore VL1 and the autochthonous yeast Lalvin QA23, this last strain was the only commercial yeast found in the same winery in 2002, but it was not present in 2003. Contrarily, Zymaflore VL1 was not found in this winery in 2002, but was present in 2003, although in lower proportions. The situation observed in Portuguese winery A, as described previously, was similar to that in French wineries, no commercial yeasts were detected in 2001 and 2002, and only 2 isolates of Zymaflora VL1 were found in 2003. In winery M, autochthonous strain ICV D254 was found in the pre-harvest campaign in 2001 and was not found in the following years. Only one isolate of K1M

ICV-INRA was found only in winery O in 2003. As a whole, the evolution of the microflora over the three years studied showed that the same strains were not found in the same sites from one year to another, which indicate that a permanent implantation of commercial yeasts in the vineyard does not occur.

Discussion

To study the importance of dissemination of commercial yeast in the vineyard, a large number of samples (198) was collected from six vineyards in France and Portugal. A similar percentage of must (about 60%) produced spontaneous fermentations in the two countries, this being higher, as expected, in musts from the post-harvest campaign. The first classification in *Saccharomyces* and non-*Saccharomyces* showed, in contrast, significant differences. While a 100% of isolates over the three years belonged to the *Saccharomyces* genus in Portugal, only 44% of the total yeast isolated in France was classified as belonging to the *Saccharomyces* genus. These data confirm previous reports indicating that *S. cerevisiae* is not present in large numbers in vineyards (Fleet and Heard, 1993; Mortimer and Polsinelli, 1999; Pretorious *et al.*, 2000). The percentage of *Saccharomyces* isolated was much lower (12%) in 2002. This year was atypical, characterised by heavy rainfall, before and during the harvest, which made it necessary to increase the antifungal treatment of the vines. As in this study, van der Westhuizen *et al.* (2000b), observed that weather conditions resulting in severe fungal infestations and heavy application of chemical sprays dramatically reduced the number of *S. cerevisiae* strains. These results are also in agreement with the findings of Longo *et al.* (1991) and Ganga and Martínez (2003), which showed a larger proportion of non-*Saccharomyces* yeasts in years of heavy rainfall. Angulo *et al.* (1993) observed that

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the initial yeast flora of the fermentation may be altered by the use of fungicides in the vineyard, reducing the number of fermentative yeasts in the first stages of fermentation. These same factors, climatic and phytosanitary treatment and the proximity of the sample sites to the wineries, may be the reason why only *Saccharomyces* strains were isolated in Portugal. In French vineyards, distribution of *Saccharomyces* and non-*Saccharomyces* strains was different in both sites in the same year and over the years in a particular campaign and vineyard. This was observed previously by van der Westhuizen *et al.*, (2000a, 2000b) and may indicate that external factors had affected the natural *Saccharomyces* population, as stated above, or it may simply be due to the fact that naturally occurring *Saccharomyces* are normally distributed in a rather haphazard manner (Török *et al.*, 1996). Interestingly, in French wineries in 2002, 29 of the 33 spontaneous fermentations were exclusively carried out by non-*Saccharomyces* strains.

The analysis of genetic profiles of 2355 *Saccharomyces* from 3780 isolates resulted in the identification of 296 commercial yeasts, representing 7.8% of the fermentative flora. It should be noted that in this study, fermentation is used as an enrichment tool for *Saccharomyces* strains and that the present results do not allow conclusions about the number of strains occurring on the surface of the grape, that is in fact very low, only reflecting those strains that could possibly have some enological use. The majority (94%) of these commercial yeasts was collected from Portuguese vineyards C and P where sample sites were placed at close proximity to the winery. The presence of water rills in these sites indicates that dissemination is probably largely favoured by liquid effluents. Commercial yeasts were recovered from pre-harvest campaigns only in winery C, in 2001. In the following two years commercial yeasts were found in post-harvest campaigns but not in any pre-harvest samples and we were not able to systematically find the same strains from one year to another in the

same vineyards. Therefore, if some of these strains are able to remain in the ecosystem, as the presence of commercial yeasts in pre-harvest samples taken in 2001 in Portugal suggests, they are not capable of dominating the natural flora of the vineyard.

In the vineyards where the sample sites were at more than 100 m from the winery, the three French vineyards and the Portuguese vineyard A, the frequency of commercial yeasts was very low, 0.7% of fermentative microflora (18 isolates). Fifteen of these 18 isolates were obtained before harvest in France and had an identical genetic profile to that of commercial yeast ICV D254. These findings could be an indication of previous dissemination, but this hypothesis cannot be confirmed since the commercial yeast strain ICV D254 was initially selected from the Languedoc region (Table 4.2) where this study was carried out. Strain ICV D254 notwithstanding, given that it is not conclusive, in these four vineyards dissemination was verified only by the presence of three isolates (0.1% of fermentative flora), 1 of K1M ICV-INRA and 2 of Zymaflore VL1. It could be considered that their presence is due to immediate dissemination, probably mediated by insects or another occasional dissemination vector. It is, in any case, evident that the presence of the most widely used commercial yeast in French wineries (K1M ICV-INRA) and in Portuguese wineries (Zymaflore VL1) for the last five to ten years, is occasional and does not ever dominate the microflora of any of these four vineyards. These results, in accordance with those previously obtained in South African vineyards (van der Westhuizen *et al.*, 2000a and 2000b), indicate a very poor level of dissemination/implantation of commercial yeast in the vineyard ecosystem.

The present study shows that dissemination of commercial yeasts in the vineyard is limited to short distances and periods of time. More than 90% of commercial yeasts

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were found at a radius of between 10 and 200 m from the winery and did not become implanted in the ecosystem in a systematic way. Dispersion of commercial strains seems to be mainly mediated by water runoff and the deposition of macerated grape skins as solid wastes. This situation occurred during the habitual functioning of a winery, where commercial strains are used without any containment. Avoiding grape-skin deposition and canalisation of water-runoff are low-cost measures able to significantly reduce the number of commercial strains around the winery.

Given that they are used in large quantities, commercial strains tend to out-compete autochthonous strains inside the winery (Beltran *et al.*, 2002). However, they do not seem to "implant" in the vineyard, but rather underlie natural fluctuations of periodical appearance/disappearance just as autochthonous strains do, and their higher technological aptitude is not associated with a greater permanence in nature. Moreover, vine-associated autochthonous *Saccharomyces* biodiversity is not affected by long-term use of commercial yeasts (data not shown).

Assuming that genetically modified yeast (GMY) derive from commercial strains, and that genetic changes do not lead to increased fitness in natural environments, our data suggests that future GMY use would not be associated with extensive spreading, permanence and dominance in natural environments close to the site of their usage. Future approaches, evaluating horizontal gene transfer or the strains capacity to recombine with wild indigenous strains will complement data of this study.

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Genetic structure of vineyard-associated Saccharomyces cerevisiae *populations revealed by microsatellite analysis*

Genetic structure of vineyard-associated Saccharomyces cerevisiae *populations revealed by microsatellite analysis*

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Abstract

The analysis of six polymorphic microsatellite loci was performed in 361 *Saccharomyces cerevisiae* isolates, that derive from a previous screening (using mtDNA RFLP) of 1620 isolates obtained from spontaneous fermentations of grapes collected in three vineyards of the Vinho Verde Region, in northwest Portugal, during the 2001 – 2003 harvest seasons. Among the 93 alleles obtained, 52 new alleles were identified. For all loci analyzed, observed heterozygosity was three to four times lower than the expected value, probably due to a strong populational substructuring. Populational structures were identified based on the accumulation of small allele-frequency differences across six loci in groups of strains. Genetic differentiation in the same vineyard in consecutive years was of the same order of magnitude as the differences verified among sampling sites within each vineyard. Correlation of genetic differentiation with the distance between sampling points suggested a pattern of isolation-by-distance, where genetic divergence in a vineyard increased with size.

The present work is the first large-scale approach showing that microsatellite typing reveals a very fine population resolution of indigenous *S. cerevisiae* strains isolated from vineyards. These studies are indispensable for the development of strategies aiming at the characterization of genetic resources as a basis for preservation of biodiversity and further strain development.

Introduction

Commercial *Saccharomyces cerevisiae* wine strains, available as active dry yeast, are obtained by clonal selection of autochthonous strains isolated from natural environments associated with the wine production areas of interest. Such strains are capable to efficiently ferment grape musts and produce desirable metabolites (e.g. glycerol, organic acids and higher alcohols) associated with reduced off-flavors development (mainly H_2S, acetic acid or phenolic compounds). Globally, they enhance the wine's sensorial characteristics and confer typical attributes to specific wine styles (Briones *et al.*, 1995; Regodon *et al.*, 1997).

The development of genetically engineered *S. cerevisiae* strains with improved fermentation capacities or novel oenological properties, able to increase the wholesomeness and sensory quality of wine was suggested as a new approach to satisfy winemaker's demand of "special yeasts for special traits" (Pretorius, 2000; Pretorius and Bauer, 2002). Taking into account that the use of such strains in the near future is not to be expected, mainly due to a very critical consumer attitude concerning genetically modified organisms, we believe that exploring genetic variability of indigenous fermentative strains is an important step towards the selection and understanding of strains with specific phenotypes.

The genetic diversity of autochthonous *S. cerevisiae* strains from wine-producing regions has been analyzed by molecular methods such as karyotyping by pulse field gel electrophoresis (Blondin and Vezinhet, 1988), mitochondrial DNA restriction analysis (mtDNA RFLP) (Querol *et al.*, 1992) or fingerprinting based on repetitive delta sequences (Legras and Karst, 2003; Ness *et al.*, 1993). Schuller *et al.* (2004) have shown that the discriminatory power of six microsatellite loci (Pérez *et al.*, 2001) is identical to mtDNA RFLP (using enzyme *Hinf*I) and an optimized interdelta

sequence method (Legras and Karst, 2003) using a set of 23 commercial *S. cerevisiae* strains. Accurate and easy genotyping of *S. cerevisiae* strains using recently described microsatellites as genetic markers (Gallego *et al.*, 1998; Hennequin *et al.*, 2001; Pérez *et al.*, 2001) is associated with a high allelic diversity representing a major source of genetic variation that is important for detecting diversity at both the individual and population levels. Therefore, this method is most informative to demonstrate subtle population structures.

The aim of the present work was to assess genetic variability among vineyard-associated *S. cerevisiae* populations from the Vinho Verde Region using microsatellite markers. Besides, it was assessed whether such markers were able to reveal population substructures in order to define strategies for future wine strain selection programmes.

Materials and methods

Sampling

The sampling plan included a total of 18 sites in three vineyards surrounding a winery, located in northwest Portugal (Região Demarcada dos Vinhos Verdes). In each vineyard, six sampling points were defined, located at ten to 400 m from each other, according to the vineyard geography, as shown in Figure 3.1 (chapter 3). Two sampling campaigns were performed before (early stage) and after (late stage) harvest, in a time frame of about two weeks, in order to assess the diversity among fermentative yeast communities during the last stage of grape maturation and harvest. This experiment was repeated in three consecutive years (2001-2003), whereas samples were not always collected from the same rootstock, but from the same area (± 1-2 m). The grapevine varieties sampled were Loureiro (vineyard A),

Alvarinho (vineyard P) and Avesso (vineyard C), being all white grapes cultivated in the Vinho Verde Region.

Fermentation and strain isolation

From each sampling point, approximately 2 kg of grapes were aseptically collected and the extracted grape juice was fermented at 20ºC in small volumes (500 ml), with mechanical agitation (20 rpm). Fermentation progress was monitored by daily weight determinations. When must weight was reduced by 70 g/l, corresponding to the consumption of about 2/3 of the sugar content, diluted samples (10^{-4} and 10^{-5}) were spread on YPD plates (yeast extract, 1% w/v, peptone, 1% w/v, glucose 2% w/v, agar 2%, w/v), and 30 randomly chosen colonies were collected after incubation (2 days, 28ºC). The isolates obtained throughout this work were stored in glycerol (30%, v/v) at -80ºC.

DNA isolation

Yeast cells were cultivated in 1 ml YPD medium (36 h, 28ºC, 160 rpm) and DNA isolation was performed as described (Lopez *et al.*, 2001) with a modified cell lysis procedure, using 25 U of Zymolase (SIGMA). Cell lysis was dependent on the strain and lasted between 20 minutes and 1 hour (37°C). DNA was used for mitochondrial RFLP and microsatellite analysis.

Mitochondrial DNA restriction patterns

The reactions were performed overnight at 37ºC and prepared for a final volume of 20 µl as previously described (Schuller *et al.*, 2004). To each isolate a pattern designation was attributed (A1-A92, C1-C70 and P1-P135 for isolates from vineyard A, C and P respectively). When isolates from different samples showed identical patterns, one representative strain from each sample was randomly withdrawn,

resulting in a total of 361 isolates that were further studied by microsatellite analysis. All isolates of this smaller collection were unable to grow in a medium containing lysine as sole nitrogen source and therefore considered as presumptive *S. cerevisiae* strains (Barnett *et al.,* 1990).

Microsatellite amplification

The six trinucleotide microsatellite loci described as ScAAT1, ScAAT2, ScAAT3, ScAAT4, ScAAT5 and ScAAT6 (Perez *et al.,* 2001) were amplified and analyzed as described (Schuller *et al.,* 2004).

Computer assisted analysis

Genetic analysis was performed using the program Arlequin 2.000 (Schneider *et al.,* 1997) and included (i) estimation of allelic frequencies (ii) observed heterozygosity compared to expected values obtained by Hardy-Weinberg equilibrium (iii) estimation of Wright's F_{ST} value (Wright, 1978) and (iv) genetic variation attributable to different hierarchical levels of defined genetic structures (AMOVA analysis). An Euclidean distance-based allelic frequencies matrix was clustered by the unweighted pair group method arithmetic mean (UPGMA) using the program NTSYSpc 2.0 (Applied Biostatistics Inc.) to examine whether genetic divergence was correlated with sampling sites. This program was also used for dendrogram drawing and to calculate a cophenetic correlation coefficient (r).

Results

Six sampling sites in each of three vineyards, located in the Vinho Verde Wine Region, were sampled during the 2001-2003 harvest seasons. Two sampling campaigns were performed (before and after the harvest in a time frame of about 2

weeks) in order to obtain a more detailed picture of *S. cerevisiae* temporal distribution. From the resulting 108 grape samples, 54 started a spontaneous fermentation, 36 were not able to start fermentation after 30 days of incubation, and 18 samples were not collected due to a bad sanitation state of the grapes as a consequence of frequent rain falls in 2002. From the 54 fermentations 1620 isolates were obtained, analyzed by mtDNA RFLP (*Hinf*I) and a pattern profile was attributed to each isolate, resulting in a total of 297 different profiles. When the same profile was found in more than one sample, a strain from each sample was randomly withdrawn resulting in a total of 361 isolates, all assumed to be *S. cerevisiae* strains. This was supported by their inability to grow in a medium containing lysine as sole nitrogen source and by their capacity to amplify previously described *S. cerevisiae* specific microsatellite loci (Pérez *et al.*, 2001). Generally, strains showing different mtDNA RFLP patterns had also distinct genotypes as determined by the allelic combinations for loci ScAAT1-ScAAT6.

The distribution of overall and vineyard-specific allelic frequencies for the loci ScAAT1-ScAAT6 is shown in Table 5.1. The six markers revealed a high degree of genetic variability, being ScAAT1 and ScAAT3 the most polymorphic markers with 29 and 19 alleles, respectively. Besides the 41 alleles (51 strains) previously described for ScAAT1-ScAAT6 (Pérez *et al.*, 2001), 52 new alleles were identified in the present study (361 strains). The vast majority of alleles were evenly distributed among *S. cerevisiae* populations belonging to vineyard A, C and P, but differences are notorious for few alleles as shown in Table 5.1 and summarized in Table 5.2. Populations from C and P share the most frequent alleles for markers ScAAT1, ScAAT2 and ScAAT3 (171, 378 and 265 bp respectively), while populations belonging to A had the highest frequencies at 201, 375 and 259 bp. For ScAAT4 and ScAAT6 the most frequent alleles were 329 bp and 256 bp for all populations, and for

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ScAAT5 the allele 219 bp was most frequent in A and C, and 216 bp in P respectively. Distinct unique alleles were found in each of the three populations, but as their frequencies were very low, ranging between 0.01 and 0.03, they might play only a minor role.

A particular situation seems to be the allele 341 bp (ScAAT4), the second most frequent allele for this locus, that appears predominantly in vineyard A with 14 homozygotic strains and two heteozygotic strains (299/341 and 335/341) and in lower frequency in vineyard C and P with seven and two homozygotic strains respectively. Associated with this allele is almost always the 253 bp allele of locus ScAAT6, indicating that the mentioned strains (at least all strains belonging to the 15 isolates from vineyard A) could be genetically related, although their mtDNA RFLP appears very heterogeneous, as shown in Figure 5.1.

Considerable differences among the populations from different vineyards were also verified for the percentage of heterozygosity, ranging from 3 to 29%. Observed heterozygosity (*Ho*) was about three to four times lower than the expected heterozygosity (*He*) for all loci of the three populations, as shown in Table 5.2.

Table 5.1   Alleles of microsatellite loci ScAAT1 – ScAAT6 and their frequencies in S. cerevisiae *in each of the vineyards A, C and P. New alleles, identified in the present study, are shown in bold. Alleles with major differences concerning their frequencies in each vineyard are highlighted in dark squares.*

ScAAT1					ScAAT2					ScAAT3					ScAAT4					ScAAT5					ScAAT6				
Allele (bp)	Overall	A	C	P	Allele (bp)	Overall	A	C	P	Allele (bp)	Overall	A	C	P	Allele (bp)	Overall	A	C	P	Allele (bp)	Overall	A	C	P	Allele (bp)	Overall	A	C	P
156	0.02	0.01	0.03	0.02	339	<0.01	0	0	0.01	229	<0.01	0	0	0.01	287	<0.01	0	0.01	0	213	0.01	0.01	0.01	0	247	<0.01	0	0	0.01
159	0.01	0	0.03	0	345	0.03	0.06	0.02	0	235	0.01	0.03	0	0	293	0.02	0.04	0.01	0.01	216	0.25	0.29	0.31	0.19	250	<0.01	0.05	0	0.03
165	0.01	0	0.03	0.01	348	0.02	0.03	0.03	0.01	238	0.01	0	0	0.02	299	0.03	0.04	0.03	0.01	219	0.32	0.40	0.33	0.29	253	0.06	0.14	0.06	0.01
171	0.27	0.28	0.41	0.27	351	0.03	0.02	0.04	0.02	241	0.01	0.01	0.02	0.01	302	0.03	0.01	0.04	0.03	222	0.35	0.25	0.31	0.43	256	0.75	0.72	0.75	0.76
174	<0.01	0	0.01	0	357	0.14	0.15	0.08	0.14	**244**	0.03	0.06	0.01	0.01	305	0.03	0.02	0.05	0.02	225	0.06	0.05	0.01	0.1	259	0.14	0.07	0.15	0.20
177	0.01	0.03	0.01	0	360	0.03	0.04	0.01	0.02	247	0.14	0.13	0.16	0.13	311	0.02	0.03	0	0.02						262	<0.01	0.04	0.04	0
180	<0.01	0.01	0	0	**363**	<0.01	0	0.01	0	250	0.04	0.12	0.01	0.01	314	0.01	0.02	0	0.01						265	0.02	0	0.01	0.01
186	0.01	0.02	0	0	**366**	<0.01	0	0.01	0	253	<0.01	0	0	0.01	317	<0.01	0	0	0.01						271	<0.01	0.01	0	0
189	0.01	0.01	0	0.01	369	0.06	0.13	0.09	0.01	256	0.02	0.02	0.03	0.01	323	<0.01	0.02	0	0						277	<0.01	0	0	0.01
192	0.01	0	0	0.01	372	0.05	0.01	0.04	0.08	**259**	0.15	0.23	0.11	0.11	326	<0.01	0.02	0	0										
195	0.04	0.01	0.03	0.08	375	0.24	0.30	0.14	0.22	262	0.18	0.11	0.14	0.22	329	0.64	0.55	0.61	0.73										
198	0.16	0.10	0.07	0.22	378	0.28	0.12	0.28	0.37	**265**	0.21	0.03	0.38	0.26	332	0.03	0.04	0.04	0.04										
201	0.22	0.30	0.16	0.19	381	0.10	0.12	0.15	0.01	268	0.06	0.1	0.05	0.03	335	0.03	0.04	0.02	0.03										
204	0.05	0.03	0.06	0.07	384	0.03	0	0.07	0.02	271	0.11	0.13	0.06	0.11	**338**	<0.01	0	0.01	0										
207	0.02	0	0.03	0.02						274	0.01	0	0	0.02	**341**	0.08	0.16	0.1	0.01										
213	0.01	0.02	0.02	0.01						277	<0.01	0.01	0.01	0.01	347	0.04	0.03	0.03	0.06										
216	0.03	0.06	0.04	0.02						280	<0.01	0	0	0.01	350	0.02	0	0.03	0.02										
219	0.02	0.04	0.01	0.02						**301**	<0.01	0	0.01	0															
222	0.03	0.04	0.06	0						346	<0.01	0.01	0	0															
225	0.01	0.02	0.01	0																									
228	<0.01	0	0.01	0.03																									
237	<0.01	0	0	0.03																									
240	<0.01	0.01	0	0																									
249	<0.01	0	0.01	0.01																									
261	<0.01	0	0.01	0																									
267	<0.01	0.01	0	0																									
273	<0.01	0.01	0	0																									
279	<0.01	0.01	0.01	0																									
300	0.01	0.01	0	0.01																									

Table 5.2   *Numbers of distinct alleles, most common and unique alleles, percentage of heterozygous allele combinations, observed (Ho) and expected (He) heterozygosity as indication of the levels of genetic variation for* S. cerevisiae *populations from vineyards A, C and P.*

Locus		Vineyard A	Vineyard C	Vineyard P
ScAAT1	Number of alleles	20	20	17
	Most frequent allele	201	171	171
	Unique allele(s)	180, 186	261	192,237
29 alleles	Heterozygosity (%)	28	18	24
(156-300 bp)	*Ho*	0.287	0.186	0.236
	He	0.831	0.839	0.832
ScAAT2	Number of alleles	10	13	11
	Most frequent allele	375	378	378
	Unique allele(s)	-	363, 366	-
14 alleles	Heterozygosity (%)	17	28	20
(339-384 bp)	*Ho*	0.191	0.286	0.200
	He	0.836	0.866	0.785
ScAAT3	Number of alleles	13	12	15
	Most frequent allele	259	265	265
	Unique allele(s)	235, 346	301	229, 238, 253, 274
19 alleles	Heterozygosity (%)	20	15	29
(229-346 bp)	*Ho*	0.212	0.157	0.286
	He	0.881	0.807	0.840
ScAAT4	Number of alleles	13	12	13
	Most frequent allele	329	329	329
	Unique allele(s)	323, 326	287, 338	317
17 alleles	Heterozygosity (%)	10	11	16
(287-350 bp)	*Ho*	0.106	0.114	0.157
	He	0.672	0.619	0.468
ScAAT5	Number of alleles	5	5	4
	Most frequent allele	219	219	222
	Unique allele(s)	-	-	-
5 alleles	Heterozygosity (%)	16	23	20
(213-225 bp)	*Ho*	0.170	0.229	0.200
	He	0.713	0.708	0.700
ScAAT6	Number of alleles	6	5	7
	Most frequent allele	256	256	256
	Unique allele(s)	271	-	247, 277
9 alleles	Heterozygosity (%)	3	13	13
(247-277 bp)	*Ho*	0.042	0.142	0.136
	He	0.463	0.427	0.393

Figure 5.1   *Mitochondrial DNA RFLP (Hinfl) of strains from vineyard A displaying the allele combination 341 (ScAAT4) and 253 (ScAAT6).*
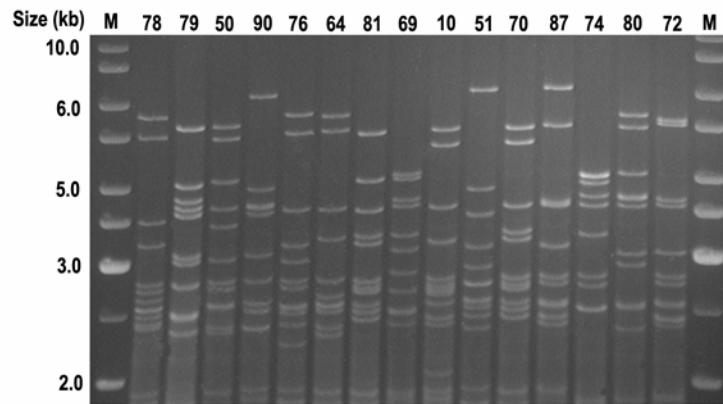
As shown in Table 5.3, the number of different strains among the 30 isolates of each fermentation sample showed a lower (one to ten strains) or higher (11 - 21 strains) biodiversity in 44 and 10 fermentations, respectively. Only 17 genotypes showed a wider temporal and geographical distribution, being the corresponding strains characterized by a generalized pattern of sporadic presence, absence and reappearance across sampling sites, vineyards or years, indicating a very high biodiversity. One genotype ([b]) showed a more regional distribution with a perennial behavior.

Table 5.3   *Number of genotypes found in different samples as defined by microsatellite analysis. The same superscript letters (a-q) represent identical genotypes in different samples. Samples that were not collected or did not initiate spontaneous fermentation are marked (- and * respectively).*

Sampling site	2001		2002		2003	
	Early	*Late*	*Early*	*Late*	*Early*	*Late*
AI	*		-	16 [abc]	*	3 [bn]
AII	*	2	-	2 [b]	*	1 [bn]
AIII	*	8 [a]	-	9 [cd]	*	9 [n]
AIV	*		-	6 [ad]	*	12
AV	*		-	9 [d]	*	19
AVI	*		-	1	*	2
CI	*	*	*	-	*	8 [ejno]
CII	*	20 [p]	*	-	1	3 [e]
CIII	*	4 [lop]	*	-	*	1 [b]
CIV	6 [lmnop]	2 [np]	1	-	*	18 [kl]
CV	4 [op]	4 [lnop]	*	-	*	9
CVI	1 [l]	8 [lmop]	*	-	*	2 [o]
PI	10	6 [fnpq]	*	5	10	15 [bjn]
PII	1	17 [bfgnq]	*	4 [bfq]	1	1
PIII	1 [f]	8	*	1 [b]	1 [f]	9 [f]
PIV	*	21 [fghi]	-	*	*	18 [n]
PV	*	5 [hn]	-	10 [finq]	*	5 [k]
PVI	2 [b]	13 [fnq]	-	1 [b]	2 [b]	5

The pattern and degree of temporal and spatial divergence in the nuclear microsatellites ScAAT1 to ScAAT6 among subpopulations was estimated by F_{ST} determination over all loci by AMOVA analysis, as shown in Table 5.4. The contribution of variation within the populations defined was always very high, ranging from 81 to 93%, as might be expected from a set of highly polymorphic loci. Differences among major groups constitute only up to 7% of variation. Populations from C (2002) are not included, given that just fermentation CIV with a single genetic pattern was obtained. Statistically significant genetic variation was found at every level of analysis (among vineyards, among year-classes).

Table 5.4   *AMOVA analysis, F_{ST} values and distribution of variance components (%) among groups (AG), among populations within groups (APWG), and within populations (WP) based on microsatellite data.*

Source of variation			Variation (%)			F_{ST}	P (r < o)
			AG	APWG	WP		
Among vineyards	2001	A/C/P	2.93	6.53	90.54	0.09	< 0.00001
	2002		6.79	11.27	81.93	0.18	0.001
	2003		1.53	13.85	84.62	0.15	< 0.00001
	2001	A/P	4.32	6.87	88.80	0.11	< 0.00001
	2002		6.79	11.27	81.93	0.18	< 0.00001
	2003		3.08	12.91	84.01	0.16	< 0.00001
	2001	A/C	5.99	8.26	85.94	0.14	0.05
	2003		0.97	17.59	81.44	0.19	< 0.00001
	2001	P/C	2.29	5.57	92.14	0.08	< 0.004
	2003		-0.17	11.75	88.42	0.12	< 0.00001
Among years	2001/2002	A	-2.06	14.61	87.45	0.12	0.014
		P	0.34	6.68	92.98	0.07	0.027
	2002/2003	A	2.19	15.77	82.04	0.18	< 0.00001
		P	0.19	9.54	90.26	0.10	0.037
	2001/2003	A	-2.58	20.01	82.57	0.17	< 0.00001
		C	3.80	10.47	85.73	0.14	< 0.00001
		P	0.21	7.25	92.55	0.07	< 0.00001
Among sampling sites	2001+2002+2003	A	-0.43	17.56	82.87	0.17	< 0.00001
		C	-8.93	22.45	86.48	0.13	< 0.00001
		P	-1.28	8.96	92.31	0.08	< 0.00001

The *S. cerevisiae* populations from A, C and P were significantly different (P$_{(random\ value<\ observed\ value)}$ < 0.001) in three consecutive years, and F_{ST} values range between 0.09 and 0.18, corresponding to a moderate (0.05 – 0.15) to great (0.15 – 0.25) genetic differentiation (Wright, 1978). When populations from different vineyards were pair wise associated (A/C, A/P and P/C) F_{ST} values of the same order of magnitude were found in consecutive years, being higher for A/C and A/P (0.14-0.19 and 0.11-0.18) when compared to P/C (0.08-0.12). Populations within a vineyard varied in consecutive years, being more variable in A (F_{ST} = 0.12 – 0.18) than in P (F_{ST} = 0.07 – 0.12). When samples were pooled across year-classes within the sampling sites of each vinery, the highest F_{ST} value was again obtained for A (0.17)

compared to C (0.14) and P (0.08). Due to a low number of isolates obtained from the early sampling stage, no conclusive results were drawn for the pair wise population comparison from different sampling stages.

Relationships among the populations belonging to six sampling points in three wineries was determined by a cluster analysis (UPGMA) based on a Euclidean distance dissimilarity matrix of allelic frequencies (Figure 5.2).
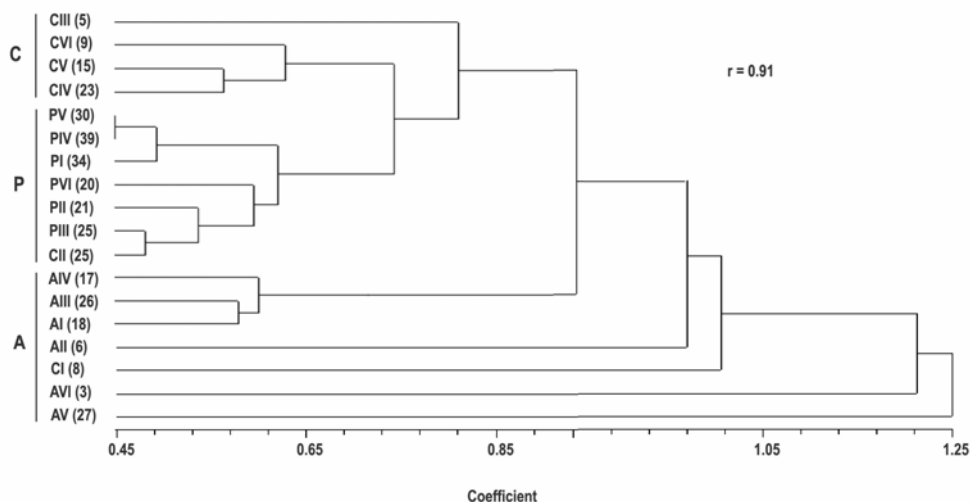

Figure 5.2   *UPGMA phenogram based on Euclidean distance of allelic frequencies from strains found at each sampling site over 3 years. Numbers in parenthesis indicate the number of strains corresponding to unique patterns.*

The cophenetic correlation factor r was 0.91, indicating that the genetic relationships were not distorted by hierarchic clustering. A similar genetic structure was obtained with the neighbor joining algorithm (not shown), being the value for r significantly lower (0.74). At a dissimilarity distance of about 0.60 – 0.65, populations were grouped in three defined clusters, comprising three sampling sites of C, six sampling sites of P (in addition one site of C), and three sites of A, showing the existence of a populational substructure, characteristic for each vineyard. Populations within groups

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C and P are in general more closely related, as indicated by the dissimilarity distance between them. *S. cerevisiae* populations belonging to vineyard A are much more heterogeneous and also more distinct from C and P, which is in accordance with previously shown data (Table 4). Population from CII lies within the cluster P, indicating that genetic differences do not delimit specific populations with fixed geographic boundaries. This is in agreement with the existence of strains with a wider geographic distribution, as previously shown (Table 5.3). Further exceptions from the vineyard - specific population structure were found for sampling sites CI, CIII, CII, and AVI, possibly due to the low number of strains and consequent lack of rigor in the quantification of allelic frequencies. Sampling site V in vineyard A is also located outside the A-cluster and showed the most divergent allelic frequencies from all populations, although a sufficient number of strains (27) were analyzed. Allele 341 (ScAAT4) in strains collected during 2003 in site V may be the main reason for this observation.

Generally, populations from sampling points in vineyard P seem to be more similar to each other, in accordance with data presented in Table 5.4. The most similar populations were from sampling sites IV and V with about 10-15 m distance (see Figure 3.1, chapter 3), but between sites V and VI, located at the same distance, much more differentiated populations were found. In the present study, genetic distances and geographical localization of the populations did not correlate, since strains with most similar genotypes resided in most distant vineyards C - P (~ 100 km). The opposite situation was verified for the closer vineyards A - C (~ 60 km) and A - P (~ 40 km).

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Discussion

Vineyard–associated *S. cerevisiae* populations have never been extensively characterized by microsatellite markers. The initial screening of 1620 isolates by mtDNA RFLP and subsequent microsatellite analysis of 361 strains revealed to be the appropriate strategy for the present large-scale approach, since both methods are equivalent concerning their capacity to discriminate commercial wine yeast strains (Schuller *et al.*, 2004).

For all loci, observed heterozygosity was three to four times lower than the estimated value. Heterozygous genotypes reduction relative to that expected under random mating can be consequence of population substructure. Wine strains of *S. cerevisiae* are usually prototrophic homothallic diploids, mostly homozygous for the homothallism gene (HO/HO) and have high spore viability contrary to strains with heterozygosities that show decreased spore viabilities with increasing number of heterozygous loci, associated with reduced strain fitness. A mechanism called "genome renewal" (Mortimer *et al.*, 1994) has been proposed for natural wine yeast strains that undergo mating among their progeny cells and thereby change a multiple heterozygote into completely homozygous diploids, leading to gradual replacement of heterozygous diploids. Heterozygous deficiencies can also be explained by the presence of null alleles that arise when mutations prevent primers from binding, so that many of the apparent homozygotes can be, in reality, heterozygotes between a visible and a null allele. Both high degrees of homozygosity and appearance of rare alleles point to the existence of genetically isolated subpopulations of yeast strains with distinct genetic constitution.

The dendrogram shown in Figure 5.2 and Amova analysis (Table 5.4) clearly agree in the distinction of the more similar populations belonging to vineyard P and C

compared to A. Allelic frequencies based clustering of at least 10 distinct genotypes lead to the expected result concerning populational structures, showing that ecologically meaningful conclusions require an adequate sample size. As most alleles are widespread, certainly due to the relatively close location of the vineyards, genetic differences among *S. cerevisiae* populations derived mainly from gradations in allele frequencies rather than from distinctive "diagnostic" genotypes. Only the accumulation of small allele-frequency differences across six loci allowed the identification of a population structure.

Several commercial yeast strains have been used for the last years in the wineries that are located within the vineyards and were recovered in the present study. The structure of the dendrogram excluding commercial strains (not shown) is similar to the presented, indicating that the closer genetic proximity of populations from C and P is due to autochthonous strains and that the rate of gene flow caused by continuous use of starter yeasts was not sufficient to genetically homogenize local indigenous strains.

In the present study, 52 new alleles were identified besides the 41 alleles previously described for ScAAT1-ScAAT6 (Pérez *et al.*, 2001). The extension of the current approach to strains isolated from other viticultural regions is desirable, since a preliminary comparison revealed major differences in both allelic combinations and frequencies (our unpublished data).

The occurrence and survival of *S. cerevisiae* in vineyards depends on numerous factors like climatic influence such as rainfall, temperature (Longo *et al.*, 1991; Parish and Carroll, 1985) or viticultural practices like agrichemical applications, grape variety or maturation stage (Pretorius *et al.*, 1999; Rosini, 1982). In the present case, the three geographically close vineyards share climate similarities, but one can

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not exclude microclimatic influences, not recorded in the present study. Geographical distance was not correlated with genetic proximity, since the most distant (100 km) vineyards P and C had most similar populations. This is coincident with data of previous studies (Torija *et al.*, 2001; Versavaud *et al.*, 1995), but it was also shown that this correlation exists among *S. cerevisiae* strains from different Spanish wine regions, being red wine strains significantly grouped according to their geographic origin, independently of the wine type and the grapevine cultivar, and white wine strains according to ecological factors such as wine type of grapevine cultivars (Guillamon *et al.*, 1996). The three sampled sub-regions share similar viticultural practices, being Loureiro the grape variety of vineyard A, Alvarinho and Avesso the cultivars of vineyard P and C respectively. Correlation between grape variety and global genetic constitution of associated strains seems possible but needs further confirmation.

Genetic differentiation (the acquisition of allele frequencies that differ among subpopulations) may result from natural selection favouring different genotypes in different subpopulations, but it may also result from random processes in the transmission of alleles from one generation to the next or from chance differences in allele frequency among the initial founders of the subpopulations. The distinction between little (F_{ST} = 0-0.05), moderate (F_{ST} = 0.05-0.15), great (F_{ST} = 0.15-0.25) and very great (F_{ST} > 0.25) genetic differentiation has been suggested (Wright, 1978), but the identification of causes underlying a particular F_{ST} value can be difficult. AMOVA analysis revealed to be useful for the detection of inter-populational genetic variations among populations that exhibit a high amount of intra-populational variability. Genetic differentiation among populations grouped according to sampling year or site, being the highest value recorded for vineyard A, followed by C and P. Differences in the same vineyard in consecutive years are of the same order of

magnitude as the differences verified among the 3 vineyards, demonstrating the importance of sampling in consecutive years in order to get a realistic picture of yeast population distribution. Values of genetic differentiation are correlated with the distance between sampling points and consequently the size of the vineyards. *S. cerevisiae* strains may become more distinctive in a larger vineyard that constitutes a bigger "evolutionary playground", hypothesizing that local populations may evolve due to multi-factorial influences being the size of the vineyard one of them. Genetic heterogeneity in a vine could follow a pattern of isolation-by-distance, where genetic divergence increases with vineyard size. However, the forces causing a global shift in a vineyard's *S.cerevisiae* population still remain to be clarified.

The present work is to our knowledge the first large-scale approach about the usefulness of microsatellite typing in an ecological survey of indigenous *S. cerevisiae* strains isolated from vineyards. Microsatellite typing with loci ScAAT1-ScAAT6, followed by statistical analysis permitted a very fine population screen, and is therefore the appropriate method to obtain deeper insight in ecology and biogeography of *S. cerevisiae* strains, even among geographically close regions. These studies are indispensable for developing strategies aiming at the preservation of biodiversity and genetic resources as a basis for further strain development.

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Chapter 6

Genetic instability of a commercial Saccharomyces cerevisiae *strain*

Introduction

Wild strains of *Saccharomyces cerevisiae*, isolated from wine, cellars or vineyards are predominantly diploid, homothallic and mostly homozygous (65%), with low (Bakalinsky and Snow, 1990; Barre *et al.*, 1992; Guijo *et al.*, 1997) to intermediate (30%) sporulation capacity (Mortimer, 2000). Aneuploid strains, with approximately diploid DNA contents, have been described (Codon *et al.*, 1997; Nadal *et al.*, 1999; Puig *et al.*, 2000) and meiosis seems not to be a common occurrence in their life cycle (Bakalinsky and Snow, 1990; Barre *et al.*, 1992). Such wine yeast strains present essentially an asexual life cycle and are characterized by high karyotype instability which is believed to be a potential source of genetic variability (Bidenne *et al.*, 1992; Carro *et al.*, 2003; Longo and Vezinhet, 1993; Nadal *et al.*, 1999). Haploid laboratory strains do not undergo by far such extensive changes (Longo and Vezinhet, 1993). Gross mitotic chromosomal rearrangements, such as large regions fusion between homologous and non-homologous chromosomes occur in wine yeast with frequencies around 10^{-5} (Puig *et al.*, 2000). Baker's yeast have a higher chromosomal variability than wine yeasts (Codon *et al.*, 1997).

In chromosome I, several membrane-associated genes are located in subtelomeric regions, and it was hypothesized that subtelomeric plasticity may allow rapid adaptive changes of the yeast strain to specific substrates (Carro *et al.*, 2003). The *SSU1-R* allele, generated by reciprocal translocation between chromosomes VIII and XVI, confers sulfite resistance to yeast cells and was described as first case of adaptive evolution, occurring probably as a consequence of the use for millennia of sulfite as a preservative in wine production (Goto-Yamamoto *et al.*, 1998; Perez-Ortin *et al.*, 2002).

S cerevisiae strains contain between two and 30 copies of at least five retrotransposons (Ty1-Ty5), being the copy number of each highly variable depending on the strain examined. Their internal DNA (5.3 – 5.7 kb) is related to retroviruses and is surrounded by ~350 bp long terminal repeats (LTRs), being retrotransposons Ty1 and Ty2 flanked by delta sequences. Recombination events expel the central sequence at one LTR, leaving a single LTR behind, explaining the dispersed presence of many copies of LTRs throughout the genome. PCR-amplification of segments between repetitive delta sequence were successfully used for the development of PCR-based *S. cerevisiae* strain typing (Lavallée *et al.*, 1994; Legras and Karst, 2003; Ness *et al.*, 1993). Multiple Ty elements mediating reciprocal recombinations (chromosome I/III or III/VII) was shown by fine-mapping of the junctions, demonstrating their crucial involvement in karyotype alterations in natural and industrial strains (Carro *et al.*, 2003; Rachidi *et al.*, 1999; Umezu *et al.*, 2002), together with insertions/transpositions of Y´elements (Neuveglise *et al.*, 2000). Additionally, ribosomal DNA repeats may also contribute to chromosomal sizes changes (Nadal *et al.*, 1999). Small but positive fitness increment due to Ty-promoted genome rearrangements leading to inactivation of *FAR3* and *CYR1* were verified in laboratory populations (Blanc and Adams, 2003).

The mitochondrial genome of *S. cerevisiae* consists of 85.8 kilobases (kb), has a low gene density, a very low GC content (17-18%) and extensive intergenic regions, being composed of long adenosine-thymidine (A+T) stretches and short guanosine-cytidine (G+C) clusters that make up 62% of the genome (Foury *et al.*, 1998). Although the *S. cerevisiae* mitochondrial DNA (mtDNA) molecule is very recombinogenic, the wild-type configuration is preferentially inherited (Piskur, 1994). MtDNA restriction fragment length polymorphism (RFLP) analysis using enzymes like *Hinf*I or *Rsa*I is associated with a high polymorphism, and is a widely used

genetic marker for the distinction of *S. cerevisiae* wine strains (Fernandez-Espinar *et al.*, 2000; Lopez *et al.*, 2001; Querol *et al.*, 1992a; Querol *et al.*, 1992b).

Microsatellites were described as simple sequence repeats (SSR), usually less than 10 bp motif repeats, with a substantial level of polymorphism. In *S. cerevisiae*, microsatellites were described as abundant and highly polymorphic in length (Field and Wills, 1998; Richard *et al.*, 1999), and were used as reproducible and portable typing method (Gallego *et al.*, 1998; Hennequin *et al.*, 2001; Pérez *et al.*, 2001a; Pérez *et al.*, 2001b; Schuller *et al.*, 2004).

The objective of the present work was to assess the usefulness of four genetic fingerprinting methods (interdelta sequence typing, mtDNA RFLP, chromosomal karyotyping and microsatellite analysis), to detect a commercial yeast strain (Zymaflore VL1, Lallemand) and to evaluate whether the permanence of this strain in natural grapevine environments induced genetic changes.

Materials and Methods

Fermentation and strain isolation

The natural isolates of the *S. cerevisiae* strain Zymaflore VL1 were obtained from grapes collected before and after the harvest of the years 2001 – 2003 in different sampling sites close to 3 wineries located in the Vinho Verde Wine Region (northwest Portugal), that predominantly used this yeast for the last five to ten years. Spontaneous fermentations occurred with 500 ml grape juice (obtained from about 2 kg of aseptically smashed grapes) at 20ºC with mechanical agitation (20 rpm). When must weight was reduced by 70 g/l, corresponding to the consumption of about 2/3 of the sugar content, diluted samples (10^{-4} and 10^{-5}) were spread on plates containing

YPD medium (yeast extract, 1% w/v, peptone, 1% w/v, glucose, 2% w/v, agar, 2% w/v) and 30 randomly chosen colonies were collected after incubation (2 days, 28ºC). Among the fermentative flora, derived from 16 fermentations (8 in 2001, 2 in 2002, 6 in 2003), 101 isolates were obtained showing the mtDNA RFLP of the starter strain Zymaflore VL1 (Lallemand). In another fermentation, 500 ml grape must were inoculated with the original, commercially available Zymaflore VL1 strain (0.2 g, following the manufacturers instructions) and fermentations as well as strain isolation (30 isolates) was performed as described above. All 131 isolates used throughout this work were kept in frozen stocks (glycerol, 30 %, v/v) at -80 ºC.

DNA isolation

Yeast cells were cultivated in 5 ml of YPD medium (24 h, 28ºC, 160 rpm) and DNA isolation was performed using a previously described method (Lopez *et al.*, 2001). The progress of cell lysis was dependent on the strain and could last between 1 to 3 hours. DNA was quantified and used for interdelta sequence typing, mitochondrial RFLP and microsatellite analysis.

Microsatellite amplification

The six trinucleotide microsatellite loci described as ScAAT1, ScAAT2, ScAAT3, ScAAT4, ScAAT5 and ScAAT6 (Perez *et al.*, 2001) were amplified and analyzed as described (Schuller *et al.*, 2004).

Delta sequence typing

Amplification reactions were performed on a BioRad iCycler thermal cycler, using the primers δ1 (5′-CAAAATTCACCTATATCT-3′) and δ2 (5′-GTGGATTTTTATTCCAAC-

3′) (primer pair A) (Ness *et al.*, 1993) or δ12 (5′-TCAACAATGGAATCCCAAC-3′) and δ2 (primer pair B) (Legras and Karst, 2003) as described (Schuller *et al.*, 2004).

Chromosomal polymorphisms

Yeast chromosomal DNA was prepared in plugs as previously described (Bidenne *et al.*, 1992). Pulse gield gel electrophoresis was perforemed using the TAFE (transverse alternating field electrophoresis) system (Geneline, Beckman). The gels were run for 26 h: 6 h at 250 V with 35 s pulse time followed by 20 h at 275 V with 55 s pulsed time, at a constant temperature (14°C).

Mitochondrial DNA restriction patterns

The reactions were performed overnight at 37ºC and prepared for a final volume of 20 µl as previously described (Schuller *et al.*, 2004).

Flow cytometry

Yeast cells were cultivated in 50 ml of YPD medium (24 h, 28ºC, 160 rpm), prepared as described (Fortuna *et al.*, 2000) and analyzed in a Partec PAS flow cytometer equipped with an argon-ion laser emitting a 488 nm beam at 15 mW.

Reproducibility

Interdelta typing and microsatellite analysis was repeated for the isolates showing different banding patterns and allelic distributions respectively, using DNA from independent extractions.

Results

In the present work, 131 isolates were analyzed by mtDNA RFLP (*Hinf*I) and revealed a unique and stable banding pattern (range of 1.8 to 5.5 kb), for all isolates, as shown in Figure 6.1. They were selected for further analysis by the other typing methods.
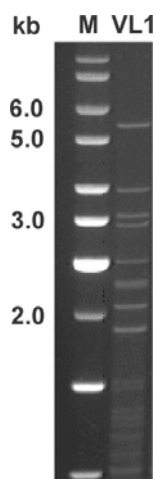

Figure 6.1   *Mitochondrial DNA RFLP (*Hinf*I) of* S. cerevisiae *Zymaflore VL1 (Lallemand).*

Microsatellite analysis using six different loci was also performed. As shown in Table 6.1, 89 isolates recovered from winemaking environments shared the characteristic Zymaflore VL1 allelic distribution (pattern M1), being heterozygous for loci ScAAT1, ScAAT2, ScAAT5 and ScAAT6, and homozygous for loci ScAAT3 and ScAAT4. Three natural isolates, corresponding to patterns M2, M3 and M4 were characterized by complete loss of heterozygosity (LOH), whereas pattern M4 showed a trinucleotide increment from 381 bp to 384 bp in locus ScAAT2. These three isolates were found in different fermentations, from grapes collected in 2002 and 2003 in distinct sampling sites of the same vineyard. One of them, corresponding to pattern M2, was chosen for further analysis by PFGE and showed a chromosomal

constitution similar to the expected pattern for a haploid derivative, characterized by loss of structural heteromorphism for example for chromosomes III and VI (Figure 6.2, pattern KD). Preliminary flow cytometric analysis showed that strains with patterns M2, M3 and M4 had the same DNA content as strain Zymaflore VL1 (not shown).

Table 6.1   *Microsatellite analysis of* S. cerevisiae *Zymaflore VL1 isolates. Patterns M1 – M8 were found among isolates derived from natural environments (N) and from the original commercial (C) strain.*

Pattern	Alleles (bp) of microsatellite loci										Number of isolates	
	ScAAT1		ScAAT2		ScAAT3	ScAAT4	ScAAT5		ScAAT6		C	N
M1	204	219	372	381	265	329	219	222	256	259	29	89
M2		219	372		265	329		222	256			1
M3	204			381	265	329	219		256			1
M4	204			384	265	329	219		256			1
M5	204	219		381	265	329	219	222	256	259		1
M6	204	219	372		265	329	219	222	256	259	1	1
M7	204	219	372	381	265	329		222	256	259		2
M8	204	219	372	381	265	329	219	222		259		5

Patterns M7 and M8, characterized by the absence of alleles 219 and 256 (ScAAT5 and ScAAT6) could be result of microsatellite expansion due to the hypothesized "replication-slippage" model, giving raise to alleles 222 and 259 respectively. This model assumes that during DNA synthesis, the nascent strand dissociates and realigns out of register. When DNA synthesis continues, the repeat number of the microsatellite is altered at the nascent strand (Schlötterer, 2000). Disappearance of alleles 372 and 381 (ScAAT2) may be associated with other mechanisms. Pattern

M6 was detected in one isolate among the population derived from the vineyard (N) and from commercial strains (C), while patterns M5, M7 and M8 were only detected in the isolates recovered from vineyards. Pattern M8 corresponds to the absence of allele 256 (ScAAT6) and was the most frequent variation, but the isolates could be clonal since four of them derived from the same fermentation.

As shown in Table 6.2, interdelta sequence amplification patterns with primer pair B generated a more polymorphic banding pattern when compared to primer pair A, which is in accordance with previous findings for commercial *S.cerevisiae* strains (Legras and Karst, 2003; Schuller *et al.*, 2004). All variants, except DB9 were characteristic for only one of the populations. While patterns DA1 and DB1 are characteristic for strain Zymaflore VL1, variant amplification patterns DA2 and DB2-DB15 were defined by additional bands for primer pair A and B respectively. No correlations were apparently found between microsatellite typing patterns and interdelta sequence amplification patterns. Among the 118 strains with VL1-characteristic microsatellite M1 pattern, 1 (0.85 %) and 17 (15%) strains gave distinct patterns when analyzed by interdelta PCR amplification with primer pair A and B respectively.

Chromosomal polymorphisms were analyzed in 42 of the 101 isolates derived from natural environments. The most abundant pattern K1, shown in Figure 6.2, was considered to be characteristic of strain Zymaflore VL1. Major changes of chromosomal patterns were evident by the absence of one band in the presumable region of chromosomes VI (K2) and III (K4). Minor chromosomal changes, in the same chromosomal regions, were assigned to patterns K3 (Chr. VI), K5 (Chr. III) and K6 (both Chr. III and VI) characterized by double bands closer or more distant than in pattern K1.

Table 6.2   *Patterns for interdelta sequence amplification using primer pair A or B among isolates derived from natural environments (N) and from the original commercial (C) strain* S. cerevisiae *Zymaflore VL1.*

Primer pair	Pattern	Additional bands (bp)	Number of isolates from population	
			C	N
A	DA1	-	30	100
	DA2	120		1
B	DB1	-	26	88
	DB2	180		1
	DB3	170, 580	1	
	DB4	200, 700	1	
	DB5	220		1
	DB6	270		3
	DB7	270, 430		1
	DB8	300		1
	DB9	250	1	1
	DB10	440, 800		1
	DB11	560		1
	DB12	710		1
	DB13	850	1	
	DB14	330		1
	DB15	180, 470		1

One strain (pattern K7) is characterized by changes in chromosomal regions III and V-VIII. When the correspondence between karyotype patterns and the other molecular markers was analyzed, most of the VL1-characteristic pattern K1 matched the corresponding microsatellite and interdelta patterns M1 and DB1 (13 and 12 isolates, respectively), while 2 strains with pattern M7 and M8 corresponded to patterns K3 and K5, respectively. Variant interdelta amplifications patterns DB6 and DB10 were found for two strains belonging to pattern K2 and K5.
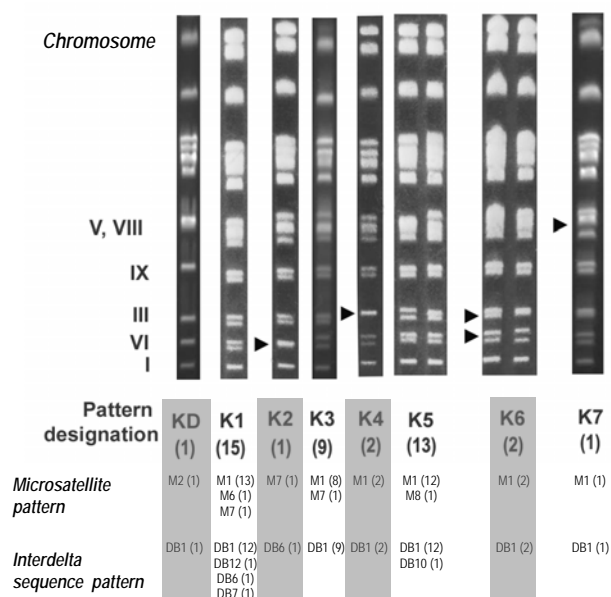
Figure 6.2   *Electrophoretic karyotyping patterns KD, K1-K7 and correspondence to patterns obtained by microsatellite (M1-M8) and interdelta sequence analysis (primer pair B, DB1-DB15). The assignment of different bands to chromosomes is merely indicative and was estimated by similarity between banding patterns of the present strains and laboratory strain S288C.*

Figure 6.3 shows examples of each pattern class DB1-DB15 and DA1-DA2 and shows also, for all strains belonging to each of the interdelta method patterns, the corresponding patterns and numbers of strains obtained by microsatellite analysis and electrophoretic karyotyping. One hundred and two strains showing the VL1-characteristic microsatellite pattern M1 and all variant patterns (with exception of 1 strain, pattern M7) showed the VL1-characteristic interdelta sequence pattern DB1. A similar situation was verified for most of the karyotype variants. Variant DB patterns

were found for three strains with the VL1- characteristic karyotype pattern K1, one strain with pattern K5 and one with pattern K6.
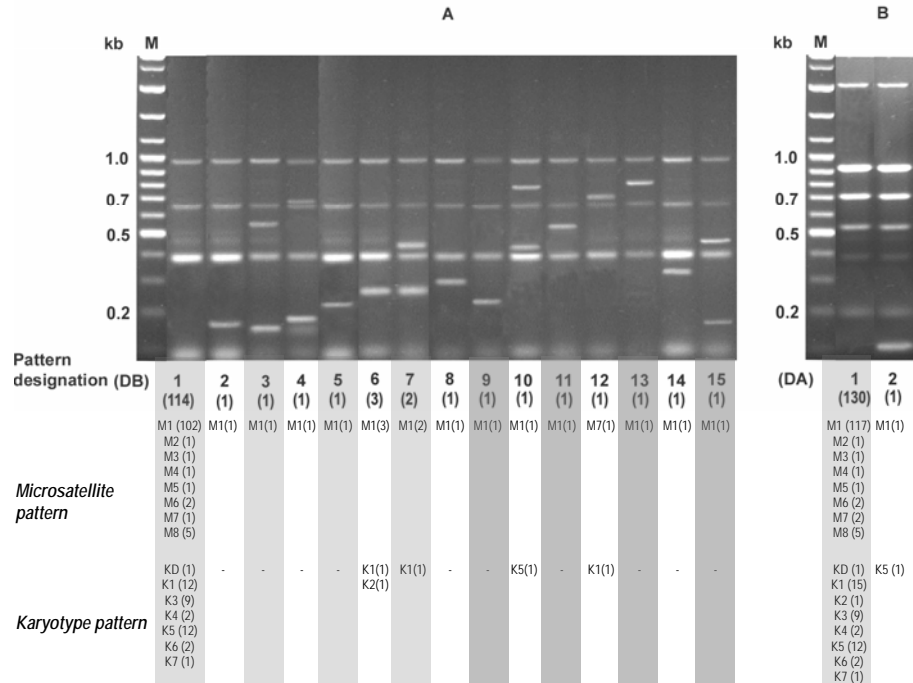

Figure 6.3   *Patterns obtained by interdelta sequence analysis with (A) primer pair B and (B) primer pair A and correspondence to the patterns obtained by microsatellite analysis and electrophoretic karyotyping of strain* S. cerevisiae *Zymaflore VL1. Numbers in brackets indicate the number of isolates.*

Globally, no population-specific changes could be identified since the isolates obtained after fermentations performed with grapes collected close to the wineries where strain VL1 has been used showed the same kind of variations compared to populations obtained from the original commercial strain.

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Discussion

In the present study, 101 isolates of the *S. cerevisiae* strain Zymaflore VL1 (Lallemand) recovered from vines close to the winery were identified by mtDNA RFLP. Evaluation of polymorphisms was achieved by the use of distinct methods (PFGE, interdelta sequence typing and microsatellite analysis) and compared to 30 isolates of the corresponding original commercialized strain.

Microsatellite allelic polymorphisms were found among isolates of the strain VL1. Some of the isolates may be clonal since they derived from the same fermentation sample (4 of 5 isolates M7 and both isolates M6). Microsatellite patterns M1-M3 are characterized by loss of heterozygosity for loci ScAAT1, ScAAT2, ScAAT5 and ScAAT6, localized on chromosomes XIII, II, XVI and IX, respectively. The DNA content of these strains is identical to the parental strain VL1, as determined by flow cytometric analysis (not shown). Considering that these strains had the identical mtDNA RFLP profile like strain VL1, the occurrence of sporulation and subsequent "self-diploidization", such as the previously described "genome renewal" (Mortimer, 2000; Mortimer *et al.*, 1994) could be an explanation of our findings. In this case, chromosomal heteromorphism of strain Zymaflore VL1 would not impart sporulation. Since it was hypothesized that sporulation does not occur in must fermentation (Puig *et al.*, 2000), and considering that the strains were derived from 3 different samples, it seems possible that these changes are associated with the yeast's permanence in natural environments, but this hypothesis needs further confirmations. It has been reported that loci ScAAT1 and ScAAT3 were the most polymorphic (Pérez *et al.*, 2001b; Schuller *et al.*, 2004), but no variant alleles were found in the present study. Microevolutionary trinucleotide expansions were found for loci ScAAT5 and ScAAT6.

Mitochondrial DNA RFLP revealed to be a very stable marker since identical restriction patterns were found for all 131 isolates, but different results were obtained for chromosomal profiles and interdelta sequences amplification.

Transposable elements such as delta elements, either associated with Ty1 and Ty2 retrotransposons or distributed in a random manner throughout the genome of *S. cerevisiae,* indicative of past Ty insertions, are contributing to the variability found within isolates of strain VL1. Although karyotype variability may be Ty-mediated, no correlation between the patterns obtained from both markers was apparent. Variation in delta sequence chromosomal positions may not be associated with gross chromosomal rearrangements, which in turn are mediated also by other repetitive DNA sequences, as mentioned before.

Our data confirmed that natural isolates of strain Zymaflore VL1 show considerable chromosomal DNA polymorphisms (Bidenne *et al.,* 1992; Longo and Vezinhet, 1993), most evident for the smaller chromosomes III and VI. Such rearrangements, abundantly described in *S. cerevisiae*, are considered to be involved in adaptive evolution (Dunham *et al.,* 2002; Infante *et al.,* 2003; Pérez-Ortin *et al.,* 2002). Minor chromosome size polymorphisms were also observed in *Cryptococcus neoformans* and described as rapid microevolutionary changes as result of adaptation to laboratory conditions (Franzot *et al.,* 1998). Whether the karyotype changes observed in the present study may be relevant for the yeast's survival in nature still needs further investigation.

In global terms, besides loss of heterozygosity, no differences in the kind of genetic changes were found among strains recovered from nature and the original commercial strains, as determined by interdelta sequence analysis and amplification of microsatellite loci ScAAT1-6. Yeast cells undergo about 70 generations for both

dry yeast production and cellular multiplication during must fermentations (5 to 7 divisions) (Longo and Vezinhet, 1993), being a reason for the amount of genetic variability among original commercial isolates. However, the higher number of variants found among the natural isolates may be also attributed to the higher number of isolates analyzed compared with the original commercial population.

Among the methods used mitochondrial DNA RFLP showed no polymorphism, since an identical pattern was obtained for all 131 isolates. Electrophoretic chromosome karyotyping is still the method of choice for evaluation of chromosomal rearrangements. The usefulness of delta sequence typing for strain delimitation using primer pair A or B should be carefully evaluated. The use of primer pair A is associated with a low resolution among strains when compared to primer pair B (Legras and Karst, 2003; Schuller *et al.*, 2004). In the present study it was shown that higher pattern stability among isolates belonging to the same strain is obtained for the first primer pair, while the opposite is verified for primer pair B, which can lead to misidentifications.

To summarize, our data show that commercial yeast strains present a considerable genetic instability that can be assessed by interdelta sequence typing, microsatellite analysis and electrophoretic karyotyping. Loss of heterozygosity occured only among isolates belonging to the population recovered from natural environments.

Chapter 7

Functional expression of the lactate permease Jen1p of
Saccharomyces cerevisiae *in* Pichia pastoris

Functional expression of the lactate permease Jen1p of *Saccharomyces cerevisiae* in *Pichia pastoris*

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In *Saccharomyces cerevisiae* the activity for the lactate–proton symporter is dependent on *JEN1* gene expression. *Pichia pastoris* was transformed with an integrative plasmid containing the *JEN1* gene. After 24 h of methanol induction, Northern and Western blotting analyses indicated the expression of *JEN1* in the transformants. Lactate permease activity was obtained in *P. pastoris* cells with a V_{max} of 2.1 nmol $\cdot$ s^{-1} $\cdot$ mg of dry weight^{-1}. Reconstitution of the lactate permease activity was achieved by fusing plasma membranes of *P. pastoris* methanol-induced cells with *Escherichia coli* liposomes containing cytochrome *c* oxidase, as proton-motive force. These assays in reconstituted heterologous *P. pastoris* membrane vesicles demonstrate that *S. cerevisiae* Jen1p is a functional lactate transporter. Moreover, a *S. cerevisiae* strain deleted in the *JEN1* gene was transformed with a centromeric plasmid containing *JEN1* under the control

of the glyceraldehyde-3-phosphate dehydrogenase constitutive promotor. Constitutive *JEN1* expression and lactic acid uptake were observed in cells grown on either glucose and/or acetic acid. The highest V_{max} (0.84 nmol $\cdot$ s^{-1} $\cdot$ mg of dry weight^{-1}) was obtained in acetic acid-grown cells. Thus overexpression of the *S. cerevisiae JEN1* gene in both *S. cerevisiae* and *P. pastoris* cells resulted in increased activity of lactate transport when compared with the data previously reported in lactic acid-grown cells of native *S. cerevisiae* strains. Jen1p is the only *S. cerevisiae* secondary porter characterized so far by heterologous expression in *P. pastoris* at both the cell and the membrane-vesicle levels.

Key words: functional reconstitution, heterologous expression, *JEN1*, lactate transporter, *Pichia pastoris*, yeast.

INTRODUCTION

Lipophilic weak carboxylic acids are used as food preservatives. The undissociated acid accumulates into microbial cells by simple diffusion until equilibrium of concentrations both sides of the plasma membrane [1]. The acid dissociates in the cytoplasm, resulting in toxic accumulation of protons and anions. This phenomenon depends on the microbial strain, the external pH, the external concentration of the acid molecule and its physical characteristics such as pK_a and lipid-buffer partition value.

Monocarboxylic acids are normal end products of the alcoholic fermentation carried out by *Saccharomyces cerevisiae*. Lactic acid, pyruvic acid or acetic acid can be used as sole carbon and energy sources by *S. cerevisiae*. However, glucose-grown cells are not able to metabolize these acids [2]. At concentrations occurring in must fermentation, acetic acid induces cell death [3,4] and inhibits metabolic fermentation/respiration activities [5].

The ABC transporters Yor1p [6] and Pdr12p [7,8] have been reported to contribute to tolerance to monocarboxylic acids in *S. cerevisiae*, possibly by directly extruding the toxic anion through the plasma membrane. Expression of the major facilitator *AZR1* is required for adaptation to acetic acid and to low-molecular-mass organic acids [9]. Two monocarboxylate–proton symporters have been described in *S. cerevisiae*: one is shared by acetate, propionate and formate, while the other transports lactate, pyruvate, acetate and propionate [2,10,11]. The first system is constitutively expressed in cells growing on non-fermentable carbon sources, whereas the lactate transporter is specifically induced by lactate. Both systems are totally repressed by glucose.

In *S. cerevisiae* it was demonstrated that the activity for the lactate–proton symporter is dependent on the expression of *JEN1*

[12]. *JEN1* is the only *S. cerevisiae* member of the sialate–proton symporters subfamily [TC (Transport Commission) no. 2.A.1.12; http://tcdb.ucsd.edu/tcdb/background.php] belonging to the major facilitator superfamily [13]. However, members of other phylogenic subfamilies can be expected to transport monocarboxylic acids such as the five MCP monocarboxylate porters, the FNT acetate–H$^+$ symporter YHL008c or even the *SSU1* putative transporter of unknown mechanism [13].

Jen1p is rapidly and irreversibly inactivated upon the addition of glucose to induced cells [14]. Some of the factors involved in proper localization and turnover of the Jen1 protein were revealed by expression of the JEN1–green fluorescent protein fusion protein in a set of strains with mutations in specific steps of the secretory and endocytic pathways [15]. However, none of the above data discriminate the possibilities of whether Jen1p has regulatory (or sensor) or transport function.

The purpose of the present study was to demonstrate non-ambiguously that Jen1p is a monocarboxylate–proton symporter. Therefore the *JEN1* gene was cloned in *Pichia pastoris* to produce significant amounts of active protein allowing heterologous reconstitution of lactate transport activity in membrane vesicles. The *JEN1* gene was also overexpressed in *S. cerevisiae* (at a lower efficiency however) to characterize the kinetic properties of Jen1p at the cell level.

MATERIALS AND METHODS

Strains and growth conditions

Yeast strains are described in Table 1. Cultures were maintained on YPD. Minimal medium contained Difco yeast nitrogen base

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Table 1   Yeast strains

Strain	Relevant genotype	Source or reference
S. cerevisiae		
W303-1A	a ade2 leu2 his3 trp1 ura3	[31]
BLC203	W303-1A jen1Δ::HIS3	[12]
L19	W303-1A jen1Δ::HIS (p416GPD)	This work
L23	W303-1A jen1Δ::HIS (pDS1)	This work
L79	W303-1A jen1Δ::HIS (p426GPD)	This work
L81	W303-1A jen1Δ::HIS (pDS2)	This work
P. pastoris		
X-33	Wild-type	Invitrogen
BLC536	X-33 (pZPARS)	This work
BLC537	X-33 (pZ-JEN1)	This work
BLC532	X-33 (pPICZB)	This work
BLC549	X-33 (pB-JEN1)-I	This work
BLC550	X-33 (pB-JEN1)-II	This work
BLC551	X-33 (pB-JEN1)-III	This work
BLC552	X-33 (pB-JEN1)-IV	This work
KM71H	arg4 aox1Δ::SARG4	Invitrogen
BLC538	KM71H (pZPARS)	This work
BLC539	KM71H (pZ-JEN1)	This work
BLC534	KM71H (pPICZB)	This work
BLC553	KM71H (pB-JEN1)-I	This work
BLC554	KM71H (pB-JEN1)-II	This work
BLC555	KM71H (pB-JEN1)-III	This work
BLC556	KM71H (pB-JEN1)-IV	This work

(YNB) adjusted to the indicated pH with HCl or NaOH and supplemented with the adequate requirements for prototrophic growth. For growth of *S. cerevisiae*, YNB media were supplemented with different concentrations of glucose and/or acetic acid, as indicated in the text. For growth of *P. pastoris*, specific media were utilized as follows: YPDS medium, glucose (2.0 %, w/v), yeast extract (1.0 %, w/v), peptone (1.0 %, w/v) and sorbitol (1.0 M); MGY medium, YNB (1.34 %, w/v), biotin (4.0 × 10^{-5} %, w/v) and glycerol (1.0 %, w/v); MM medium, YNB (1.34 %, w/v), biotin (4.0 × 10^{-5} %, w/v) and methanol (0.5 %, v/v); MD medium, YNB (1.34 %, w/v), biotin (4.0 × 10^{-5} %, w/v) and glucose (2.0 %, w/v). Agar (2.0 %, w/v) was added for solid media. Liquid *S. cerevisiae* cultures were grown at 28 °C and 180 rev./min and *P. pastoris* cultures were grown at 30 °C and 250 rev./min. The media were supplemented with zeocin (25–100 μg/ml) and ampicilin (100 μg/ml) whenever necessary. The *E. coli* XL1-Blue strain was used for plasmid propagation and amplification according to [16]. Consumption of glucose and acetic acid was determined using a HPLC system (Gilson), equipped with a Merck Polysher OA KC Column (catalogue no. 51270), maintained at 50 °C. The mobile phase was H_2SO_4 (0.025 M, in ultra-pure water), and the flow rate was 0.5 ml · min^{-1}.

DNA manipulation and cloning techniques

DNA cloning and manipulation were performed according to standard protocols [16]. The yeast shuttle vectors p416GPD (*CEN6/ARSH4*) and p426GPD (2μ) were kindly provided by Dr Dominik Mumberg (Institut für Molekularbiologie und Tumorforschung, Philipps-Universität Marburg, Germany) [17]. The gene *JEN1* was amplified from *S. cerevisiae* W303-1A genomic DNA by PCR using primers J7 (5′-CCGGAATTCGTT-ACATAGAGAAGCGAACACG-3′) and J8 (5′-CGCGGATC-CAGTTTCAAAAGTTTTTCCTCAAAG-3′; MWG Biotech) and Platinum *Taq* high-fidelity DNA polymerase (Gibco, catalogue

no. 11304-011). The primers introduced a *Bam*HI (J8) and an *Eco*RI (J7) restriction site at the 5′ and 3′ ends of *JEN1* respectively. The amplified fragment was digested with both enzymes and cloned into the cloning array of the plasmids p416GPD and p426GPD, originating plasmids pDS1 and pDS2 respectively, which were used to transform *S. cerevisiae* (Table 1).

The *P. pastoris* pPICZB plasmid was purchased from Invitrogen. *JEN1* was amplified by PCR from the *S. cerevisiae* W303-1A genome, using the primers JB-EcoRI (5′-CCGGAA-TTCGAAAATATGTCGTCGTCAATTACAG-3′) and JB-XbaI (5′-GCTCTAGAACGGTCTCAATATGCTCC-3′). *JEN1* was also cloned in the replicative expression vector pZPARS [18]. For this purpose, *JEN1* was amplified by PCR from the *S. cerevisiae* W303-1A genome, using the primers JP-SalI (5′-ACGCGT-CGACGTCGTCGTCAATTACAGATGAGAAAATATCTGG-3′) and JP-NotI (5′-ATAAGAATGCGGCCGCATTAAACGGTC-TCAATATGCTCCTCATATGTC-3′). The PCR products were digested and cloned in the expression vector using standard procedures, originating plasmids pB-JEN1 and pZ-JEN1 respectively. The four plasmids were used to transform *P. pastoris*, both X-33 and KM71H strains (Table 1).

RT-PCR (reverse transcriptase PCR) reactions were performed with primers JF (5′-TGACATGGCAGAATTGGAAC-3′) and JR (5′-GGAATTTCTCATGGCCAACA-3′), based on the sequence of *JEN1*, and PF (5′-AAAGATATGGTCATCATCAGAAGAA-3′) and PR (5′-CAAACTTGGATGCTTGGTAGACA-3′) as a reference based on the sequence of *PDA*. The Ready To-Go™ RT-PCR Beads from Amersham Biosciences (catalogue no. 27-9556-01) were used, in combination with 300 ng of DNase-treated RNA and oligo(T)$_{12-18}$ as first-strand primer.

Selection of *P. pastoris* recombinant strains

The integrative vectors were digested with the restriction enzyme *Sal*I (Roche), for integration in the *AOX1* locus. *P. pastoris* X-33 and KM71H cells were transformed by electroporation, the transformants were selected in YPDS medium supplemented with zeocin and each transformant was purified to ensure pure clonal isolates. A direct PCR screening of the *P. pastoris* clones was performed as described by Linder et al. [19] and in accordance to the guidelines provided by the EasySelect™ *Pichia* Expression Kit Instruction Manual (Invitrogen) using the 3′AOX1 (5′-GC-AAATGGCATTCTGACATCC-3′) and 5′AOX1 (5′-GACTGG-TTCCAATTGACAAGC-3′) primers (where AOX is alcohol dehydrogenase). Another PCR reaction was performed to amplify of an internal fragment of the *S. cerevisiae* gene using specific primers Jinv-1 (5′-GAAAGTGGCCGTACATTAC-3′) and JC (5′-GATACCCCAGACACCAAAGAC-3′). Considering that all *P. pastoris* KM71H transformants have a Mut^s phenotype, X-33 integration transformants were tested for their Mut$^+$ or Mut^s phenotype in MM medium. All the transformant strains analysed presented a Mut$^+$ phenotype.

P. pastoris growth conditions for recombinant protein expression

Cells previously grown in solid MD medium for 48 h were inoculated in 100 ml of MGY medium in a 1 litre flask and grown in a shaking incubator until the culture reached $D_{600} = 2.0–6.0$. The cells were harvested and resuspended in 200 ml of MM medium to a D_{600} of 0.5 in a 2 l flask. Methanol (100 %, v/v) was added to the culture to a final concentration of 0.5 % (v/v) every 24 h to maintain induction. Cell-suspension samples of 15 ml were collected over time and analysed for recombinant protein expression.
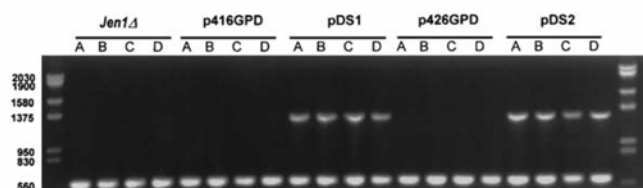
Figure 1   Detection of *JEN1* expression by RT-PCR in cells of *S. cerevisiae jen1*Δ not transformed or transformed with the referred plasmids

Cells were collected during the first exponential growth phase. Letters indicate different culture media: A (YNB; glucose, 2.0 %, w/v, pH 4,0); B (YNB; glucose, 2.0 %, w/v, acetic acid, 0.25 %, v/v, pH 4.0); C (YNB; glucose, 0.1 %, w/v, and acetic acid, 0.25 %, v/v, pH 4.0); D (YNB; acetic acid, 0.25 %, v/v, pH 4.0).

Hybridization analysis

Samples of $10\,\mu g$ of genomic DNA from each *P. pastoris* transformant strain were blotted on to a positively charged nylon membrane (GeneScreenPlus Hybridization Transfer Membrane; NEN™ Life Sciences Products, Boston, MA, U.S.A.) using a PR648 slot-blot filtration manifold (Hoefer Scientific Instruments, San Francisco, CA, U.S.A.). Genomic DNA preparation and slot-blot experimentation were performed according to Ausubel et al. [20]. Total cellular mRNA was prepared from yeast cells, $20\,\mu g$ were electrophoresed on 1.5 % (w/v) agarose Mops/ formaldehyde gels, blotted on to a positively charged nylon membrane and the membrane was hybridized for *JEN1* [12]. Densitometer scanning was performed using the Integrated Density Analysis program from EagleSight® software, version 3.2 (Stratagene, La Jolla, CA, U.S.A.).

Preparation of plasma membranes and yeast cell extracts

Plasma membranes were prepared from *P. pastoris* as described by Van Leeuwen et al. [21]. The purified membranes were resuspended in 10 mM Tris/HCl, 1 mM EDTA, pH 7.4 (about 3 mg of protein/ml) and stored in liquid nitrogen. Total yeast extracts were obtained by the method of Volland et al. [22].

SDS/PAGE and Western blotting

Protein samples were separated by SDS/PAGE (10 % gels) and transferred to a PVDF membrane (Hybond™-P, Amersham Biosciences). The proteins were probed with chicken polyclonal antibody raised against a 13-amino-acid peptide of the N-terminal region (EVYNPDHEKLYHN) of Jen1p. Primary antibody was detected with a horseradish-peroxidase-conjugated anti-chicken IgG secondary antibody (Sigma) detected by enhanced chemiluminescence (ECL®; Amersham Biosciences).

Measurement of transport activity

Cells were harvested, washed twice with ice-cold deionized water and resuspended to a final concentration of about 25–35 mg of dry weight per ml. Uptake rates of labelled monocarboxylic acids were estimated as described previously [15]. The substrates were D,L-[^{14}C]lactic acid (sodium salt, Amersham Biosciences; 4000 d.p.m./nmol), pH 5.0, and [^{14}C]acetic acid (sodium salt; Amersham Biosciences; 3000 d.p.m./nmol), pH 5.0. A computer-assisted non-linear regression analysis program (GraphPad Software, San Diego, CA, U.S.A.) was used to determine the best-fitting transport kinetics to the experimental data and to estimate

the kinetic parameters. All the experiments were performed in triplicate, and the data represent mean values.

Preparation of hybrid plasma membrane vesicles and measurement of labelled lactic acid accumulation

Fusion of proteoliposomes containing cytochrome oxidase with plasma membrane vesicles was performed by the freeze–thaw–sonication procedure as described previously [23] using a 1:20 protein/phospholipid ratio. At time zero, radioactively labelled D,L-lactic acid ($158\,\mu M$) was added to $200\,\mu l$ of hybrid vesicles and energization was started by addition of 15 mM ascorbate, 0.015 mM cytochrome *c* and 0.15 mM TMPD (*N,N,N',N'*-tetramethyl-*p*-phenylenediamine). Aliquots of $15\,\mu l$ were withdrawn at appropriate intervals and diluted with 2.0 ml of ice-cold 100 mM LiCl. The mixtures were filtered through nitrocellulose filters (pore size. $0.45\,\mu m$; Macherey-Nagel) and washed with 2.0 ml of 100 mM LiCl. The filters were introduced into vials and radioactivity was measured as described above. Experiments were carried out at 26 °C.

RESULTS

Constitutive expression of *JEN1* in *S. cerevisiae*

The *S. cerevisiae* strain carrying a genomic deleted *jen1*Δ allele was transformed with either of the plasmid p416GPD and p426GPD (native plasmids) and with the corresponding plasmid containing a copy of *JEN1* under the control of the GPD (glyceraldehyde-3-phosphate dehydrogenase) constitutive promotor (pDS1 and pDS2 respectively; Table 1). *JEN1* transcription was analysed by RT-PCR. Figure 1 shows the detection of *JEN1* transcripts, prepared from exponentially growing cells in different single- and mixed-substrate culture media. As expected, no mRNA signal was found in strains carrying the native plasmids. In contrast, and independently of the carbon source, *JEN1* expression was detected in the strains transformed with the plasmids bearing *JEN1*.

As was previously shown [12,14] in the *S. cerevisiae* strain W303-1A, *JEN1* expression is absent in glucose when under the control of its own promoter. Additionally, in lactic acid-grown cells, a rapid decline of *JEN1* mRNA is observed upon the addition of glucose, the transcripts being completely absent 10 min after the pulse of glucose [14]. These results show that, despite the mechanisms of repression and degradation previously reported, the cloning under a strong promoter allows constitutive expression of the *JEN1* gene whether the carbon source is glucose, acetic acid or a mixture of both.

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Table 2   Kinetic parameters for the transport of monocarboxylic acids in S. cerevisiae jen1Δ transformed with the indicated plasmids

Transport analyses were performed in cells exponentially growing in the following culture media: G (YNB; glucose, 2.0% w/v, pH 4.0); GA (YNB; glucose, 2.0% w/v, and acetic acid, 0.25%, v/v, pH 4.0); A (YNB; acetic acid, 0.25%, v/v, pH 4.0). Units for V_{max} are nmol·s^{-1}·mg of dry weight^{-1}.

| Plasmid | Culture medium | Kinetic parameter | | | |
| | | Acetic acid | | Lactic acid | |
		K_m (mM)	V_{max}	K_m (mM)	V_{max}
p416GPD	G	No activity	No activity	No activity	No activity
	GA	No activity	No activity	No activity	No activity
	A	1.50 ± 0.50	2.69 ± 0.39	No activity	No activity
pDS1	G	0.68 ± 0.17	0.44 ± 0.04	0.60 ± 0.23	0.50 ± 0.07
	GA	2.71 ± 1.05	1.04 ± 0.26	0.35 ± 0.22	0.63 ± 0.11
	A	1.57 ± 0.62	3.07 ± 0.53	0.64 ± 0.28	0.83 ± 0.11
p426GPD	G	No activity	No activity	No activity	No activity
	GA	No activity	No activity	No activity	No activity
	A	1.67 ± 0.31	1.39 ± 0.68	No activity	No activity
pDS2	G	No activity	No activity	No activity	No activity
	GA	No activity	No activity	No activity	No activity
	A	1.06 ± 0.47	2.86 ± 0.48	Not determined	Not determined

Transport of monocarboxylic acids in S. cerevisiae cells expressing JEN1 constitutively

Initial uptake rates of labelled lactic acid were measured at pH 5.0 in exponentially growing cells cultivated in minimal medium at pH 4.0, containing glucose (2.0%, w/v), acetic acid (0.25%, v/v) or a mixture of both glucose and acetic acid in the concentrations indicated (Table 2). Under the growth conditions used, the JEN1 gene cloned in the centromeric plasmid induces permease activity, whereas when cloned in the multicopy plasmid, the uptake of labelled lactic acid obeys a simple diffusion mechanism. In cells carrying no functional copy of JEN1, the transport of labelled lactic acid also obeyed a simple diffusion mechanism.

Measurements of lactic acid uptake were performed in glucose-grown cells of transformed S. cerevisiae strains, incorporating a metabolizable (glucose, 100 mM) or a non-metabolizable (sorbitol, 100 mM) sugar in the assay buffer. The kinetic parameters obtained were of the same order of magnitude as the ones described in Table 2 in the absence of the sugar. These results indicate that the medium composition and osmotic strength do not affect the mechanism of monocarboxylic acid transport.

Transport of labelled acetic acid at pH 5.0 was also evaluated (Table 2). In acetic acid-grown cells, acetate uptake was observed in the four strains studied. This result confirms that besides JEN1 another monocarboxylate permease is present in acetic acid-grown cells of S. cerevisiae [2,12]. In glucose-grown cells (in either the presence or absence of acetic acid) activity of the acetate permease was only found in cells expressing JEN1 from the centromeric plasmid. This indicates that, above a certain level, JEN1 mRNA is not functionally expressed in S. cerevisiae.

Consumption of acetic acid in the presence of glucose in S. cerevisiae

In order to determine whether the constitutive expression of JEN1 in the presence of glucose is associated with the altered consumption of acetic acid, the supernatants of cultures grown in YNB containing glucose and acetic acid were analysed by HPLC. As can be observed in Figure 2, the consumption of both substrates
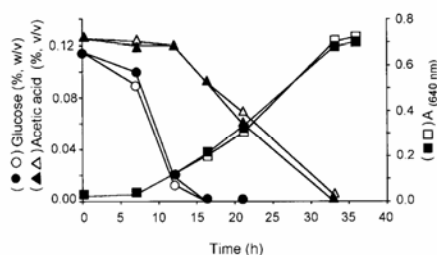
Figure 2   Growth of S. cerevisiae L19 (open symbols) and L23 (closed symbols) strains in YNB medium containing glucose (0.1%, w/v) and acetic acid (0.125%, v/v), pH 4.0

Table 3   Specific growth rate (h^{-1}) of S. cerevisiae jen1Δ transformed with the plasmids p416GPD or pDS1

Cells were cultivated in YNB medium containing the indicated carbon sources, at the initial pH indicated. Data represent results of three independent experiments.

| Culture medium | | | Specific growth rate (h^{-1}) of the transformants | |
Glucose (%, w/v)	Acetic acid (%, v/v)	pH	p416GPD	pDS1
2.0	0.00	4.0	0.22 ± 0.03	0.23 ± 0.02
2.0	0.25	4.0	0.22 ± 0.03	0.23 ± 0.02
2.0	0.30	4.0	0.21 ± 0.03	0.21 ± 0.03
2.0	0.40	4.0	0.18 ± 0.01	0.16 ± 0.02
2.0	0.50	4.0	0.05 ± 0.005	0.05 ± 0.004
2.0	0.60	4.0	0.05 ± 0.005	0.05 ± 0.006
2.0	0.70	4.0	No growth	No growth
0.0	0.25	4.0	0.15 ± 0.01	0.12 ± 0.02
2.0	0	6.0	0.30 ± 0.02	0.26 ± 0.02
2.0	0.25	6.0	0.23 ± 0.03	0.25 ± 0.02
0	0.25	6.0	0.16 ± 0.01	0.13 ± 0.01

was identical in a mixed-substrate medium, containing glucose and acetic acid, at pH 4.0. In cells either expressing JEN1 or not, the consumption of acetic acid was initiated only after glucose exhaustion. As shown in Table 3, glucose- and/or acetic acid-grown cells had very similar growth rates under all the conditions tested, at either pH 4.0 or 6.0. In the media containing glucose and acetic acid, the growth rate decreased and the exponential phase increased (results not shown) with increasing amounts of acetic acid. The plasmids p416GPD and pDS1 were used to transform another S. cerevisiae genetic background (CEN.PK113-13D), and no differences in growth were detected between the strains.

Lactate permease activity in P. pastoris transformants

Jen1p permease activity was evaluated by measuring the initial uptake rates of radioactive lactic acid in different P. pastoris transformants. Cells were grown in mineral medium with gly-cerol (MGY), and incubated further for 72 h in methanol medium (MM). After 24 h induction with methanol, all P. pastoris transformants containing the JEN1 gene presented measurable lactic acid uptake, although with different velocities. After 48 or 72 h of induction, lactate uptake was greatly decreased. The integrative vectors generated higher uptake rates than the replicative vectors (results not shown). Additionally, the P. pastoris
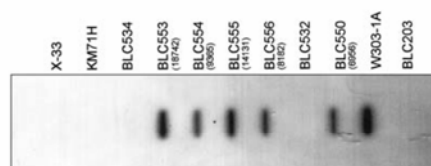
Figure 3 Slot-blot analysis of 10 μg of genomic DNA from *P. pastoris* transformants, using an *JEN1*-specific probe

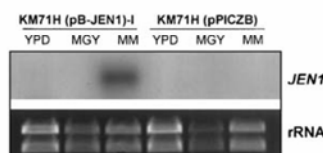Numbers in parentheses refer to the measured spot densities.

Figure 4 Northern blot analysis of *JEN1* expression in *P. pastoris* KM71H recombinant strains

Samples were taken from cells grown in YPD or MGY and from cells transferred to MM medium for 24 h.

KM71H (pB-JEN1) transformant strains analysed exhibited higher lactate-uptake activity than the X-33 (pB-JEN1) recombinant strains. The transformant that displayed the highest level of lactate uptake in all the tested conditions was KM71H (pB-JEN1)-I (strain BLC553; Table 1). In glycerol-containing medium (0 h induction), no measurable lactate-uptake activity could be found for any of the assayed strains (results not shown). In the strains containing the empty vectors (Table 1), no measurable permease activity could be found during the induction time-course tested (results not shown).

Slot-blot analysis of integrated *JEN1*

Semi-quantitative DNA slot-blot analysis was carried out using genomic DNA isolated from the recombinant strains (Figure 3). The *S. cerevisiae JEN1* probe hybridized as expected with the parental *S. cerevisiae* W303-1A genomic DNA control, and failed to produce any signal in all negative controls, whereas signals were obtained with recombinant *P. pastoris* strains. The transformant BLC553 presented the highest number of genomic *JEN1* insertions, in agreement with the observation of the highest permease activity level in this transformant.

Expression analysis of *JEN1* in *P. pastoris*

No *JEN1* transcripts were detected in *P. pastoris* cells grown in media containing glucose or glycerol. However a 2.2 kb mRNA was detected in the KM71H (pB-JEN1)-I transformant induced with methanol (Figure 4). Jen1p was detected in membrane preparations of the *P. pastoris* KM71H (pB-JEN1)-I transformant, as well as in *S. cerevisiae* lactic acid-grown cells. The size of the protein expressed in *P. pastoris* KM71H (pB-JEN1)-I membranes is larger than the one detected in *S. cerevisiae*, which is expected
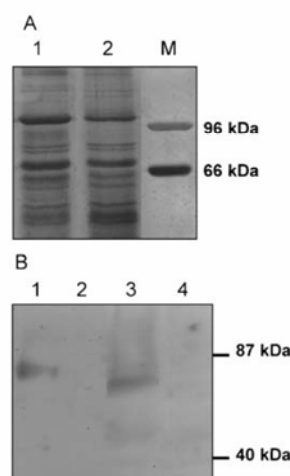

Figure 5 Expression analysis of Jen1p

(**A**) Silver-stained SDS/PAGE of plasma membrane proteins prepared from *P. pastoris* 24 h-methanol-induced cells. Lane 1, *P. pastoris* KM71H (pB-JEN1)-I; lane 2, *P. pastoris* KM71H pPICZB; lane M, SDS/PAGE Standard (Bio-Rad). (**B**) Western blot using a specific Jen1p antibody. Lane 1, plasma membrane proteins from *P. pastoris* KM71H (pB-JEN1)-I 24 h-methanol-induced cells; lane 2, plasma membrane proteins from *P. pastoris* KM71H pPICZB 24 h-methanol-induced cells; lane 3, crude extracts of *S. cerevisiae* W303-1A lactic acid-grown cells; lane 4, crude extracts of *S. cerevisiae* W303-1A glucose-grown cells.

as Jen1p expressed in *P. pastoris* has the hexahistidine and c-Myc tags (Figure 5).

Characterization of the Jen1 permease kinetic parameters

The kinetic parameters of monocarboxylate transport system were determined in the *P. pastoris* recombinant KM71H (pB-JEN1)-I recombinant strain after 24 h of induction in methanol-containing medium, measuring the initial uptake rates as a function of the labelled lactic acid concentration. *P. pastoris* KM71H transformed with the empty vector was used as a control, where labelled lactic acid was transported solely by simple diffusion (Figure 6), with $k_d = 0.040 \pm 0.002 \, \mu l \cdot s^{-1} \cdot mg$ of dry weight^{-1}. The presence of a mediated transport system was indicated by Michaelis–Menten saturation kinetics in the recombinant strain KM71H (pB-JEN1)-I, with $K_m = 0.54 \pm 0.08$ mM D,L-lactic acid and $V_{max} = 2.15 \pm 0.14$ nmol of lactic acid $\cdot$ s^{-1} $\cdot$ mg of dry weight^{-1}.

Lactic acid transport in hybrid vesicles

Plasma membranes from methanol-induced *P. pastoris* KM71H (pB-JEN1)-I recombinant were fused with *E. coli* liposomes containing bovine heart cytochrome *c* oxidase. In control vesicles obtained with *P. pastoris* transformed with the empty vector, the uptake of labelled lactic acid was negligible, before and after energization. In contrast, hybrid vesicles prepared from cells containing *JEN1* accumulated a 6-fold higher lactate concentration inside than outside at pH 6.2 (Figure 7). The essential
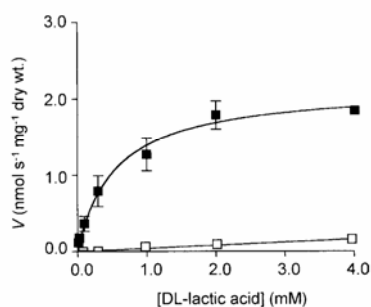
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Figure 6   Initial uptake rates of labelled lactic acid (pH 5.0) measured in *P. pastoris* (pZB-JEN1)-I (■) and pPICZB (□) transformants after 24 h of induction in methanol-containing medium
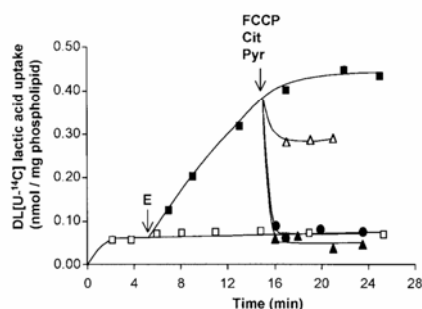

Figure 7   Uptake of radiolabelled lactic acid in hybrid vesicles prepared from *P. pastoris* KM71H (pZB-JEN1)-I methanol-induced cells

The assays were performed with 158 μM radiolabelled lactic acid, at pH 6.2 and 26 °C, either with (■) or without (□) energization. For energization of the system (E), 15 mM ascorbate, 0.015 mM cytochrome *c* and 0.15 mM TMPD were added to the reaction mixture. At the time indicated by the arrow, 10 μM FCCP (▲), 80 mM pyruvic acid (Pyr; ●) and 80 mM citric acid (Cit; △) were added.

contribution of the protonmotive force to the accumulation of lactic acid was shown by the rapid efflux of the accumulated labelled lactic acid upon addition of 10 μM FCCP [carbonyl cyanide *p*-(trifluoromethoxy)phenylhydrazone]. The specificity of transport was investigated by the addition of either unlabelled pyruvic acid (80 mM) or citric acid (80 mM) to the hybrid vesicles after accumulation of the labelled lactic acid. Only pyruvic acid was able to promote the efflux of labelled lactic acid (Figure 7). Such specificity is fully consistent with the results obtained in *S. cerevisiae* whole cells [10].

DISCUSSION

This is the first report of fully functional reconstitution of a *S. cerevisiae* permease in the heterologous host *P. pastoris*. We will therefore comment on some features of the cloning

system used. The *P. pastoris* recombinant strains containing the *JEN1* gene were grown in glycerol and transferred to methanol-containing medium for gene induction by the strong *AOX1* promoter. All recombinant strains presented a maximum initial rate of lactic acid uptake after 24 h of induction. After 48 or 72 h of induction, permease activity was greatly decreased, indicating that continuous overproduction of the membrane protein was deleterious. Independently isolated *P. pastoris* strains transformed with the same expression vector displayed significant differences in the levels of Jen1p activity. Such clonal variation is often observed even within collections of transformants harbouring the same number of expression cassettes [24–26]. By slot-blot hybridization we verified that the clone KM71H (pB-JEN1)-I presenting the highest levels of lactic acid transport also exhibited increased the *JEN1* copy number integrated in the genome. Premature termination of transcription has been described for a number of foreign genes expressed in yeast [27]. Fortunately, this phenomenon was not observed for *JEN1* expression in the KM71H (pB-JEN1)-I transformant. Northern blot analysis in different culture media shows a transcript of similar size to that found for *S. cerevisiae*, corresponding to *JEN1* mRNA (2.2 kb). In the best *P. pastoris* transformant, the kinetic parameters for lactate uptake were found to be $K_m = 0.54 \pm 0.08$ mM lactic acid and $V_{max} = 2.15 \pm 0.14$ nmol of lactic acid $\cdot$ s^{-1} $\cdot$ mg of dry weight^{-1} while in *S. cerevisiae* W303-1A the V_{max} was previously estimated to be 0.40 nmol of lactic acid $\cdot$ s^{-1} $\cdot$ mg of dry weight^{-1} and the $K_m = 0.69$ mM lactic acid [12]. These results represent a 5-fold enrichment of Jen1p in *P. pastoris* transformant cells compared with wild-type *S. cerevisiae*. In contrast, the best constitutive heterologous monocarboxylate overexpression of *JEN1* in the homologous host *S. cerevisiae* had produced only a 2-fold increase in Jen1p V_{max} using the strong GPD promoter while the use of a multicopy vector inhibited growth. Even though it appears that, as in *S. cerevisiae* [28], excessive overexpression of membrane proteins is detrimental to *P. pastoris* cell growth, the amount of heterologous proteins produced in *P. pastoris* was much higher than in *S. cerevisiae* and sufficient for allowing the measurement of lactate transport in reconstituted membrane vesicles. The reconstitution of the activity of lactate permease of *S. cerevisiae* was achieved in hybrid vesicles obtained by fusing plasma membranes from *P. pastoris* KM71H (pB-JEN1)-I with proteoliposomes. The properties of the reconstituted lactate uptake agreed with those of the permease evaluated in *S. cerevisiae* cells. The involvement of the protonmotive force was directly demonstrated in reconstituted *P. pastoris* vesicles by instant release of lactate upon addition of protonophore thus confirming the proton-symport mechanism previously shown in *S. cerevisiae* intact cells. Such *in vitro* measurement of Jen1p-dependent lactate uptake obtained in heterologous membrane vesicles is crucial. Indeed, it is the only measurement that provides non-ambiguous demonstration of Jen1p being a lactate permease. While it is conceivable that in *S. cerevisiae* cells the loss of lactate uptake in *JEN1* deletants and its gain in constitutive overexpression conditions could result from indirect perturbation of regulatory or sensing factors, as has been postulated in the literature [29,30], such regulatory mechanism is very unlikely to occur in heterologously reconstituted membrane vesicles from *P. pastoris*. Indeed, when not transformed, this species does not contain lactate permease activity in the induction conditions used. In conclusion, *JEN1* is a fully functional lactate permease. It is the only functional monocarboxylate transporter gene identified so far in the *S. cerevisiae* genome as Makuc et al. [30] have showed that neither the five members of the monocarboxylate porter subfamily nor the *YHL008c* gene were involved in monocarboxylate transport under all tested conditions.

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_____ *Chapter 8*

General discussion and future perspective

General discussion and future perspective

The principal aim of the present study was to evaluate the biodiversity wealth of fermentative *S. cerevisiae* strains from the Vinho Verde Region in northwest Portugal and the establishment of a yeast strain collection. The work herein presented aimed also at the assessment of molecular methods for *Saccharomyces cerevisiae* strain typing and the evaluation of industrial starter yeasts' ability to spread and survive in nature, in order to provide data contributing to environmental risk assessment associated with the use of genetically engineered winery yeast strains.

Microsatellite analysis (using six different loci), electrophoretic karyotyping, restriction fragment length polymorphism of mitochondrial DNA (mtDNA RFLP), and PCR-based typing based on repetitive delta sequences are major tools in *S. cerevisiae* DNA fingerprinting. No single method seems to emerge as the method of choice, and some methods may perform better than others at different levels of resolution. Each method was evaluated regarding its ability to differentiate 23 commercial *S. cerevisiae* strains. Depending on the technique used, distinct levels of discrimination were obtained, varying from 10 to 22 different genotypic patterns. Twenty one different patterns were obtained by microsatellite analysis, mtDNA RFLP (restriction enzyme *Hinf*I) and interdelta sequence typing using an optimized primer pair. Three strains were not distinguished by each of the methods. Karyotype analysis allowed the discrimination of one of the three strains originating 22 patterns, and is still the method of choice for the detection of chromosome rearrangements between genetically closely related strains.

In the present work, the distinct method's suitability for the typing of 131 isolates of the commercial yeast strain Zymaflore VL1 (Lallemand) was also assessed. MtDNA RFLP revealed to be a very useful marker generating a unique pattern for all isolates.

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The advantage of the interdelta sequence lies in the high polymorphism generated by the differences in the position of delta sequences that are associated to Ty1 and Ty2 elements or are dispersed as solo delta sequences throughout the genome. Interdelta sequence typing using primer pair A generated only 10 distinct patterns among 23 commercial yeast strains, but resulted in a stable and reproductive pattern among the 131 isolates of Zymaflore VL1. In contrary, for primer pair B, a much higher polymorphism was generated at both levels: among strains and also within isolates belonging to the same strain. This is a major drawback of the method, making strain delimitation difficult, since an unknown isolate can not be clearly assigned to a known strain. However, the method's application as quick and easy to perform analysis, for example in the scope of industrial quality assurance, is not invalidated through the present results. For other purposes, this method should be combined with additional analysis.

Genetic changes that isolates of a certain yeast strain such as Zymaflore VL1 undergo can be also evaluated by microsatellite typing and by karyotyping. Minor changes such as microsatellite expansion were observed, but also loss of heterozygosity. However, the assignment of these isolates to strain VL1 was not difficult since the observed change(s) involved mostly one of at least six alleles. Besides, microsatellite typing revealed to be a powerful tool that provides accurate and unequivocal results expressed as base pair number, being the most appropriate approach for large-scale studies like the biogeographical distribution of indigenous *S. cerevisiae* strains by means of numerical analysis, as shown in Chapter five.

Karyotyping was shown to be very efficient in discriminating between genetically closely related strains and is still the method of choice for the detection of genomic reorganizations implying chromosome rearrangements. However, analysis of the

offspring derived from sporulation and self-diploidization of a strain with chromosomal heteromorphism can yield a banding pattern that is likely not to be assigned to the parental strain, leading to misidentification. This would not happen by mtDNA RFLP analysis, and microsatellite typing using several loci should also be able to detect such a situation. Besides, the laborious and time-consuming methodology does not allow a high number of strains to be analyzed simultaneously and is another drawback of this method. Our data show that the methodological approach used in the present thesis for ecological surveys shown in chapters three, four and five is legitimate. The ideal approach for studies similar to those performed in the present thesis would be mtDNA RFLP for a first rapid strain delimitation, followed by electrophorectic karyotyping or microsatellite analysis.

Genetic variations found among 101 isolates of the strain Zymaflore VL1, derived from grapes surrounding wineries where this strain was used, was similar to the changes found in original commercial isolates, indicating that the presence in nature is not associated with genetic changes that can be evaluated by the markers used. This was not unexpected, since our studies also showed (Chapter 4), that the permanence of commercial strains occurs in a limited time frame. Besides, the polymorphisms detected by each marker did not match among the isolates. Among the 101 isolates recovered from natural environments, three are characterized by a loss of heterozygosity for the six microsatellite loci ScAAT1 – ScAAT6, but showed the same DNA content like the original commercial VL1 strain by flow cytometry analysis, hypothesizing the occurrence of sporulation and self-diploidization, in agreement with the previously described mechanism of "genome renewal". Since sporulation has never been observed during fermentation, it is possible that such changes occur during strain's permanence in nature and it would be interesting to search for the reasons which trigger such events. Further comparison of the three

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strains with the heterozygote VL1 strain by oligonucleotide microarrays is one of the aims for future studies. We could expect to find genetic variability upon which selection can act in the sense of adaptive microevolutionary processes that yeast strains may undergo in nature.

One thousand six hundred and twenty isolates of *S. cerevisiae* were obtained from 54 spontaneous fermentations performed with grapes collected in 18 sampling sites of 3 vineyards (Vinho Verde Wine Region, Northeast of Portugal) during the 2001-2003 harvest seasons. Alvarinho, Loureiro and Avesso were the grape varieties in each of the vineyards, and based on mtDNA analysis for strain assignment, 135, 89 and 62 unique patterns were found, respectively. Seventeen strains showed a wider regional and temporal distribution, characterized by a sporadic pattern of appearance, disappearance and reappearance. Six of them corresponded to the commercial yeast strains that were used by the wineries during at least five years before our survey was initiated and one strain showed a wider, regional distribution. Spontaneous fermentations were mostly obtained from grapes collected after the harvest and the fermentative *S. cerevisiae* flora found was very variable.

An identical survey has been completed in three vineyards in the Languedoc Region (France), and non-*Saccharomyces* species such as *Kloeckera apiculata* predominated in numerous spontaneous fermentations. These data indicate that the ecology of grape-associated yeast is complex and may depend on a multitude of factors, being viticultural practices, agrichemical application or climate just a few of them. Since ecologically meaningful data require long term observations, it is desirable to continue these surveys, involving also, if possible, mathematical models.

The present work is the first large-scale approach showing the enormous biodiversity of *S. cerevisiae* strains in the Vinho Verde Region. A strain collection is now

available, constituting an important biotechnological resource for the region's winemakers. Preservation and further exploitation of such strains is of great interest and the 297 strains corresponding to unique mtDNA RFLP patterns were chosen for further characterization. Several phenotypic traits are now being evaluated such as ethanol tolerance, H_2S production, as well as acetic acid and malic acid consumption (not shown in the present thesis). Due to the continuous use of agrichemicals over many years, these strains may also harbor interesting resistance mechanisms that will be subject of further investigations. The results already obtained show a high phenotypic heterogeneity among the strains analyzed and some strains will be further studied in order to evaluate their fermentation performance in synthetic musts and experimental vinifications. A polyphasic approach, integrating all molecular and phenotypic data obtained, is also planned in order to develop tools for yeast strain selection programs.

Using microsatellite typing is a novel approach for revealing populational structures in a large-scale ecological survey of indigenous *S. cerevisiae* strains as shown in the present work. Genetically isolated subpopulations were identified, characterized by a high degree of homozygosity and by the appearance of rare alleles. Clustering of small allele-frequency differences across six loci allowed the identification of a population structure, whereas genetic differentiation was correlated with the distance between sampling points hypothesizing a pattern of isolation-by-distance, where genetic divergence increases with vineyard size. Local populations may evolve due to multi-factorial influences, the size of the vineyard being one of them. The extension of the current approach to strains isolated from other viticultural regions is desirable, and the existence of "diagnostic" genotypes, characteristic for strains of a given viticultural region can be expected. Biopreservation of genetic resources

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should not be a matter of interest involving merely viticultural regions or single countries, but should have rather a broader involvement.

Studying commercial yeast's dissemination in six vineyards belonging to the Vinho Verde and Languedoc wine regions was used as a model for the risk assessment associated with the potential spreading of GMY strains, involving 78 and 120 grape samples and the analysis of 1620 and 2160 strains in Portugal and France, respectively. Despite the regular usage of commercial starter strains in large amounts, their dispersion was generally limited and seems mainly mediated by water runoff and the deposition sites of macerated grape skins. In the vineyards where the samples were located at more than 100 m from the winery, the frequency of commercial yeasts was very low (0-2 % of the fermenting microflora). In sites where the samples were taken at very close proximity to the winery and to water rills, the proportion of commercial yeasts increased to 10-43%. The majority (94%) of commercial yeasts were found at a distance of between 10 and 200 m from the winery. These strains did not become "implanted" in the ecosystem in a systematic way, but rather underlie natural fluctuations of periodical appearance/disappearance just like autochthonous strains.

This is in agreement with data obtained in greenhouse experiments by the Weinforschungsanstalt Geisenheim (Germany) and the Institute for Wine Biotechnology, Stellenbosch University (South Africa), showing that GMY strains have the same survival capability like the non-modified parental strains. Besides, vineyard-associated yeast strains tend to occur as local subpopulations and the population structure of vine-associated autochthonous *S. cerevisiae* biodiversity does not seem to be affected by long-term use of commercial strains.

Globally, and taking into account that (i) the presence of commercial strains was limited in space and time and did not cause significant changes in vineyard-associated *S. cerevisiae* populations (ii) genetic modifications did not confer selective advantages leading to increased fitness in confined environments (data obtained in above mentioned laboratories) and assuming that GMY strains derive from commercial strains, data suggest that GMY use would not be associated to a negative environmental impact. The occurrence of horizontal and vertical gene transfer is currently investigated under laboratory conditions by the Institute for Wine Biotechnology, South Africa. The possible consequences of introduced genetic changes should always be analyzed in the light of recent research showing that genomic plasticity and high genetic variability among strains are very characteristic features of wine yeast strains. We therefore believe that data obtained in the course of the present research, together with results obtained by the colleagues in France, Germany and South Africa provide a solid scientific basis to submit an application for a "deliberate release" trial in the sense of experimental GMY usage in a non-confined winery. Preferentially, a strain should be used that was already evaluated in confined environments and by global gene expression profiling in comparison to the parental strain. GMY tracking should also include runoff water, soils and the winery's interior space, and evaluate potential recombination between GMY strains and autochthonous strains. Besides, more efficient molecular detection methods in environmental samples should be adapted for GMY detection such as fluorescent in situ hybridization (FISH) or temperature- or denaturating gradient gel electrophoresis (TGGE/DGGE).

A *S. cerevisiae* strain constitutively expressing *JEN1* was obtained, capable to transport acetic and lactic acids in the presence of glucose. However, constitutive Jen1p expression alone is not sufficient to mediate the consumption of acetic acid in

the presence of glucose. The molecular basis underlying the transport of monocarboxylic acids in yeast is an important and well-established research line in the Department of Biology and future studies concerning the consumption of monocarboxylic acids will be performed.

The use of genetically modified yeasts in biotechnological applications other than winemaking is a realistic estimation for the near future, since yeast revealed a very useful system for heterologous protein expression and considering also the tremendous advances that are being gained by genome-wide expression profiling. Continuation of the present studies in the outlined directions can contribute towards this goal.

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Annexes

Annex I

Directive 2001/18/EC of the European Parliament and of the Council of 12 March 2001 on the deliberate release into the environment of genetically modified organisms and repealing Council Directive 90/220/EEC

Annex III A
Information required in notifications concerning releases of genetically modified organisms other than higher plants

General information	*Name and address of the notifier* *Name, qualifications and experience of the responsible scientist(s)* *Title of the project*
GMO	*Characteristics of the donor, recipient, and parental organism* scientific name, taxonomy, other names, phenotypic and genetic markers, degree of relatedness between donor and recipient or between parental organisms, description of identification and detection techniques, sensitivity and reliability of detection and identification techniques, description of the geographic distribution and the natural habitat, organisms with which transfer is known to occur under natural conditions, verification of the genetic stability and factors affecting it, pathological ecological and physiological traits, nature of indigenous vectors, history of previous genetic modifications.
Vector	*nature and source,* *sequence of transposons, vectors and other non-coding genetic segments,* *frequency of mobilization of inserted vector and/or genetic transfer capabilities and methods of determination,* *information on the degree to which the vector is limited to the DNA required to perform the intended function.*
Modified organism	*Information relating to the genetic modification:* methods used for the modification, methods used to construct and introduce the insert(s) into the recipient or to delete a sequence, description of the insert and/or vector construction, purity of the insert from any unknown sequence and information on the degree to which the inserted sequence is limited to the DNA required to perform the intended function, methods and criteria used for selection, sequence, functional identity and location of the altered/inserted/deleted nucleic acid segment(s) in question with particular reference to any known harmful sequence.

Information on the final GMO

description of genetic trait(s) or phenotypic characteristics and in particular any new traits and characteristics which may be expressed or no longer expressed,

structure and amount of any vector and/or donor nucleic acid remaining in the final construction of the modified organism,

stability of the organism in terms of genetic traits,

rate and level of expression of the new genetic material – method and sensitivity of measurement,

activity of the expressed protein(s),

description of identification and detection techniques including techniques for the identification and detection of the inserted sequence and vector,

sensitivity and specificity of detection and identification techniques,

history of previous releases or uses of the GMO,

consideration for human, plant and animal health.

Conditions of release and the receiving environment	*Release* description of the proposed deliberate release, including the purpose(s) and the foreseen products, foreseen dates of the release and time planning of the experiment including frequency and duration of the releases, preparation of the site previous to the release, size of the site, method(s) to be used for the release, quantities of GMO to be released, disturbance of the site (type and method of cultivation, mining, irrigation and other activities, worker protection measures, post-release treatment of the site, techniques foreseen for elimination or inactivation of the GMOs at the end of the experiment, Information on and results of previous releases of the GMOs especially at different scales and in different ecosystems. *Environment* geographical location and grid reference of the site(s), physical or biological proximity to humans and other significant biota, proximity to significant biotopes, protected areas or drinking water supplies, climatic characteristics of the region(s) likely to be affected, geographical, geological and pedological characteristics, flora and fauna including crops, livestock and migratory species, description of target and non-target ecosystems likely to be affected, comparison of the natural habitat of the recipient organism with the proposed site(s) of release, any known planned development or changes in land use in the region that could influence the environmental impact of the release
Interactions between the GMOs and the environment	*Survival, multiplication and dissemination* biological features which affect survival, multiplication and dispersal, known or predicted environmental conditions which may affect survival, multiplication and dissemination (wind, water, soil temperature, pH etc.), sensitivity to specific agents *Interaction with the environment* predicted habitat of the GMOs, studies on the behavior and characteristics of the GMOs and their ecological impact carried out in simulated natural environments, such as microcosms, growth rooms, greenhouses, Genetic transfer capability, likelihood of post-release selection leading to the expression of unexpected and/or undesirable traits in the modified organism, measures employed to ensure and to verify genetic stability as well as description of genetic traits which may prevent or minimize dispersal of genetic material, routes of biological dispersal,

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	known or potential modes of interaction with the disseminating agent, including inhalation, ingestion, surface contact, description of ecosystems to which the GMOs could be disseminated, potential for excessive population increase in the environment, competitive advantage of the GMOs in relation to the unmodified recipient or parental organism(s), identification and description of the target organism if applicable, anticipated mechanism and result of interaction between the released GMOs and the target organism(s)if applicable, identification and description of non-target organisms which may be adversely affected by the release of the GMO, and the anticipated mechanisms of any intended adverse interaction, likelihood of post-release shifts in biological interactions or in host range, known or predicted interactions with non-target organisms in the environment, including competitors, preys, hosts, symbionts, predators, parasites and pathogens, known or predicted involvement in biogeochemical processes, other potential interactions with the environment.
Monitoring, control, waste treatment and emergency response plans	*Monitoring techniques* methods for tracing the GMOs and for monitoring their effects, specificity (to identify the GMOs, and to distinguish them from the donor, recipient, or, where appropriate, the parental organisms), sensitivity and reliability of the monitoring techniques, techniques for detecting transfer of the donated genetic material to other organisms, duration and frequency of the monitoring. *Control of the release* methods and procedures to avoid and/or minimize the spread of the GMOs beyond the site of release or the designated area for use, methods and procedures to protect the site from intrusion by unauthorized individuals, methods and procedures to prevent other organisms from entering the site. *Waste treatment* methods and procedures for controlling the GMOs in case of unexpected spread, methods for decontamination of the areas affected, for example eradication of the GMOs, methods for disposal or sanitation of plants, animals, soils etc. that were exposed during or after the spread, plans for protecting human health and the environment in case of the occurrence of an undesirable effect.

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Annex II

Commission Decision of 24 July 2002
Establishing guidance notes supplementing Annex II to Directive 2001/18/EC of the
European Parliament and of the Council on the deliberate release into the environment
of genetically modified organisms and repealing Council Directive 90/220/EEC
(2002/623/EC)

Annex
Guidance notes on the objective, elements, general principles and methodology of the environmental risk
assessment (ERA) referred to in annex II of the Directive 2001/18/EC

General principles:

In accordance with the precautionary principle the ERA should be based on the following general principles:
- Identified characteristics of the GMO and its use which have the potential to cause adverse effects should be compared to those presented by the non-modified organism from which it is derived and its use under corresponding situations.
- The ERA should be carried out in a scientifically sound and transparent manner based on available scientific and technical data.
- The ERA should be carried out on a case by case basis, meaning that the required information may vary depending on the type of the GMOs concerned, their intended use and the potential receiving environment, taking into account, *inter alia*, GMOs already in the environment.
- A general principle for ERA is also that an analysis of the "cumulative long-term effects" relevant to the release and the placing on the market is to be carried out. "Cumulative long-term effects" refers to the accumulated effects of consents on human health and the environment, including flora and fauna, soil fertility, soil degradation of organic material, the feed/food chain, biological diversity, animal health and resistance problems in relation to antibiotics.

Methodology:

Characteristics of GMOs and releases
The following points should be addresses as main steps in the ERA

- Identification of characteristics which may cause adverse effects
disease to humans including allergenic or toxic effects,
disease to animals and plants including toxic, and where appropriate, allergenic effects,
effects on the dynamics of populations of species in the receiving environment and the genetic diversity of each of these populations,
altered susceptibility on therapeutic medical, veterinary, or plant protection treatments, for example by transfer of genes conferring resistance to antibiotics used in human and veterinary medicine,
the potential spread of GMOs in the environment that will depend on
    – Its biological fitness,
    – the conditions of the deliberate release or placing on the market,
    – the likelihood of a deliberate release or placing on the market, or unintentional releases into the environment,

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  – pathways of dispersal of viable material,
  – particular environmental considerations,
  – for plants: the viability of pollen, seeds and vegetative structures,
  – for microorganisms: the viability of spores as survival forms, or the potential of the microorganisms to enter the viable, but not cultivable state.

The transfer of the inserted genetic material to other organisms, or the same organism whether genetically modified or not

  – the reproductive properties of the GMO itself, including the modified sequences,
  – the conditions of release, and particular environmental considerations such as climate,
  – differences in reproduction biology,
  – agricultural practices,
  – the availability of potential crossing partners,
  – transport and pollinating vectors,
  – the availability of hosts for parasites.

Phenotypic and genetic instability

  – if in a transgenic plant line that contains more than one transgene, the subsequent segregation process results in these transgenes being divided up in the progeny, there could be plants with less transgenes but new phenotypes,
  – if attenuated mutants may, due to instability, revert to virulence,
  – if duplication of transgenes leads to silencing,
  – if copy numbers are very high,
  – if re-insertion of transposable elements results in new phenotypes, due to inactivation of the transgene by the insertion of mobile genetic elements,
  – if the level of transgene expression is important.

Interactions with other organisms (other than exchange of genetic material/pollen

  – exposure to humans (such as farmers, consumers),
  – exposure to animals,
  – competition for natural resources,
  – displacement of natural populations of other organisms,
  – delivery of toxic substances,
  – different growth patterns.

Changes in management, including, where applicable, in agricultural practices

  – sowing, planting, growing, harvesting or transporting crops,
  – crop rotation,
  – disease and pest control,
  – resistance management,
  – isolation in land agricultural and aquatic agricultural systems,
  – agricultural practices,
  – management in non-agricultural systems.

- Evaluation of the potential consequences of each potential adverse effect, if it occurs
  The magnitude is to be seen in relation to the baseline and likely to be influenced by:
    – sowing, planting, growing, harvesting or transporting crops,
    – genetic construction,
    – each adverse effect identified,
    – the number of GMOs released (scale),
    – the environment into which the GMO(s) is (are) to be released,
    – the conditions of release, including control measures.
- Evaluation of the likelihood of the occurrence of each individual potential adverse effect
  A major factor in evaluating the likelihood or probability of adverse effects occurring is the characteristics of the environment into which the GMO is intended to be released and the manner of the release.

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- Estimation of the risk posed by each identified characteristic of the GMO
  An estimation on the risk to human health or the environment posed by each identified characteristic of the GMO which has the potential to cause adverse effects should be made as far as possible, given the state of the art, by combining the likelihood of the adverse effect occurring and the magnitude of the consequences, if it occurs.

- Application of management strategies for risks from the deliberate release or marketing of GMO(s)
  Risks should be identified that required to be managed and a corresponding risk management strategy should be defined.

- Determination of the overall risk of the GMO(s)
  An evaluation of the overall risk of the GMO(s) should be made taking into account any risk management strategies which are proposed.