J Ind Microbiol Biotechnol (2008) 35:27–33
DOI 10.1007/s10295-007-0262-0

ORIGINAL PAPER

Correlation between indigenous *Oenococcus oeni* strain resistance and the presence of genetic markers

Vincent Renouf · Arnaud Delaherche · Olivier Claisse · Aline Lonvaud-Funel

Received: 21 February 2007 / Accepted: 19 September 2007 / Published online: 18 October 2007
© Society for Industrial Microbiology 2007

Abstract This study reports on monitoring *Oenococcus oeni* intraspecific diversity evolution during winemaking. Three different wines were monitored. The proportion of *O. oeni* species was determined by species-specific PCR and *O. oeni* strains were distinguished by multiplex PCR-RAPD. Each strain was tested by PCR for 16 significant markers revealed by a previous genetic comparison between a strong oenological potential strain and one with poor oenological potential. Population levels and diversity changed according to winemaking stages, oenological practices and the chemical properties of the wine. In all situations, *O. oeni* was the best-adapted species. Within the *O. oeni* group, intraspecific strain diversity decreased and the malolactic fermentation was the result of the most resistant strains with the highest number of markers.

Keywords *Oenococcus oeni* · Intraspecific diversity · Genetic markers

Introduction

Lactic acid bacteria (LAB) play an important role in winemaking mainly through the malolactic fermentation (MLF), which decreases total acidity, improves microbiological stability and enhances organoleptic properties. Winemakers are aware of the importance of a successful MLF. However, MLF sometimes fails, due to very harsh environmental conditions in the wine for bacterial survival and growth, i.e. low pH, high alcohol content, high SO_2 concentrations, and low temperatures [18]. The efficiency of the bacteria depends on their inherent ability to survive and proliferate in wine. The development of new industrial starters for direct inoculation represents a considerable advantage in winemaking technology [9]. These considerations led us to search for a new approach using comparative genomics. This was based on two *Oenococcus oeni* strains: 1491, which has strong oenological potential, and 8413, which has undesirable characteristics. A Khi_2 test confirmed their statistical difference ($P < 0.05$) and revealed 11 genetic markers [5, 6]. According to annotated gene banks, these genetic markers are involved in cadmium transporting P-type ATPase, Dps ferritin, polysaccharide biosynthesis export protein, maltose phosphorylase, transcriptional regulator, hypothetical protein, predicted transcriptional regulators, alcohol–sugar dehydrogenase, copper chaperone, and Arabinose efflux protein MFS. Statistical analysis also revealed five other genes in the oenological *O. oeni* collection, with putative functions intervening in multistress response, transport and structure membrane protein. These five selective genes encode for thioredoxin, glycerol uptake facilitator protein, Arabinose efflux permease, glycotransferase involved in cell wall biogenesis, and a hypothetical protein [7].

We monitored the whole population of lactic acid bacteria and the ratio of *O. oeni* species in three different wines. Indigenous *O. oeni* strains were isolated and used to study intraspecific diversity. The objective was to understand the selection mechanism among indigenous strains during the first stages of winemaking by using the genetic markers previously cited.

V. Renouf (✉) · A. Delaherche · O. Claisse · A. Lonvaud-Funel
UMR Œnologie ISVV Université Victor Segalen Bordeaux 2/ INRA, 351, cours de la Libération, 33405 Talence, France
e-mail: v-renouf@enitab.fr

Materials and methods

Samples

Washings of grapes, fermenting musts, and wines were collected, as described by Renouf et al. [13, 14], from three vineyards in the Bordeaux area. After harvest, we monitored changes in the bacterial population throughout the winemaking process until post-fermentation addition of SO_2 in the three wines (Table 1).

Bacteria isolation and population enumeration

Serial dilution of each sample was used to inoculate duplicate plates of grape juice medium which contained (l^{-1} distilled water): 5 g yeast extract, 500 mL red grape juice and 2 ml tween 80. The pH was adjusted to 4.8 with 5 N KOH. Yeast growth was inhibited by adding 100 mg/L pimaricine. Plates were incubated at 25 °C for 7 days under anaerobic conditions. Colonies were counted on plates with 30–300 colonies. From a wine sample, three replications were made for the population enumeration.

O. oeni identification

In order to assess the ratio of *O. oeni* to the total LAB population on the culture plates, we tested 25% of the colonies on counted plates. Each colony was cultured in liquid medium, with a similar composition to that of the plates, but without agar for 7 days. DNA was extracted from 10 mL liquid culture according to the protocol described by Renouf et al. [13]. The *O. oeni* species-specific PCRs [8] were then performed using 2 ng of the DNA extracted from each colony. Positive amplification indicated that the colony sampled belonged to the *O. oeni* species group. Compilation of PCR responses for each colony gave the percentage of *O. oeni* species among the LAB population. When amplification was negative, the species was identified by PCR-DGGE targeting the *rpoB* gene [15].

The typing of *O. oeni* strains was based on a multiplex RAPD-PCR method using two primers, On2 and Coc, developed by Cocconcelli et al. [4] and Zapparolli et al. [20], respectively, and as used by Reguant and Bordons [12].

Presence of gene markers

The presence of 16 genetic markers was determined for each *O. oeni* colony isolated at different winemaking stages: (1) grape surface at harvest, (2) grape must before alcoholic fermentation (AF), (3) during AF, (4) at devatting (when wine is transferred from one tank to another and separated from the lees), and (5) during MLF.

The oligonucleotide primers used for PCR amplification and sequencing were designed with the ePrimer3 program (Table 2), and positive and negative PCR results for markers test presence were validated by the method of Delaherche et al. [6]. Each 25 µL amplification reaction mixture contained 2 ng DNA template, 2 µL custom-made PCR Master Mix (MP Biomedicals-Qbiogene, France), and 5 pmol of each primer. The reaction mixture was preheated for 5 min at 95 °C and subjected to 30 cycles, each consisting of denaturing (30 s, 95 °C), annealing (30 s, 55 °C), and extension step (30 s, 72 °C), in an iCycler iQ (Bio-Rad, France). In addition with the conventional negative PCR control run without DNA, a positive control with the DNA of the 1491 *O. oeni* strains was used for each PCR test. Amplified products were resolved on 1.5% agarose gel containing ethidium bromide and viewed under a UV-transilluminator.

Table 1 Main characteristics of the wines monitored during industrial winemaking

Wine	A	B	C
Origin	Saint-Emilion	Graves	Médoc
Grape variety	Merlot	Cabernet-Sauvignon	Petit-Verdot
Sulphur dioxide added at harvesting	8 g/hL	7 g/hL	4 g/hL
Initial cold maceration	5 days at 10 °C	No	No
Must sugar concentration (g/L)	254	220	238
Alcoholic fermentation yeast	Indigenous flora	Indigenous flora	Levain 522 Davis
Post AF vatting	4 days	6 days	10 days
Ethanol content at the end of AF (% v/v)	14.65	12.00	13.95
pH before MLF	3.49	3.66	4.12
Free SO_2 before MLF (mg/L of H_2SO_4)	7	10	9
Total SO_2 before MLF (mg/L of H_2SO_4)	41	50	44
TPI before MLF	69.8	75.6	65.2
CI before MLF	1.84	2.02	1.76

CI colour intensity, *TPI* total polyphenol index

Table 2 Markers and sequences of the primers used in PCR detection

Marker	Forward primer (5′→3′)	Reverse primer (5′→3′)	Amplicon length (bp)
Cadmium transporting P-type ATPase-M1	GAAGCTCAAGATACCATCC	CGACTTGTGCACAGATTCC	650
Dps ferritine-M2	TTGGTTAATTCAGCCGTTGT	ATTGATCACGATGTCCCAAC	500
Polysaccharide biosynthesis export protein-M3	CTCGTAAGCATGGTTCTCTC	ATTGGTTTGATGAAAAATGG	565
Maltose phosphorylase-M4	ACGCATGATTCCTCATTATC	GGTCTTTCAAAATACCATCG	600
Transcriptional regulator-M5	TGGCAAACGTCTCAATCAAC	AGCTTACGGCTGATGCTTT	380
Hypothetical protein-M6	TACTGTTCGTCAGCCGATGT	CTCCCGACAAACTGCTAATG	400
Predicted transcriptional regulators-M7	CAATCAAGCCGGAATAGTT	TGACCAGTTCGAATGAATTC	462
Hypothetical protein-M8	ATGACGCCATTCTATATCCA	ATTTGCCTCGATAGTTTCTG	605
Alcohol–sugar dehydrogenase-M9	GGAAACAATTTACGCTTGC	CGGCCTGTTTGATAAAGAA	471
Copper chaperone-M10	CCTCCTACTTAACCTTGACG	AGTCCCACCTCCTGAATAAA	420
Arabinose efflux protein MFS-M11	TGGCTTAATCCCATCAGAAA	CCAAATTGTCCAGAATACCG	600
Thioredoxin-M12	GTTTCTGAAGACCCGCTTA	TGATGCCCCCTTCGTAAT	300
Glycerol uptake facilitator protein-M13	CTAACGCATTCCTGAAGAAC	CCCAACTATATTCCCAGTGA	602
Arabinose efflux permease-M14	TTTTATCTGTCCAAGCAGGT	AATTAGAAGAACGCTGATAGCC	330
Glycosyltransferases involved in cell wall biogenesis-M15	TGTTAACGATACGAAGCGCG	GAATCACTCCATTCCGTCACC	600
Hypothetical protein lp_3433-M16	AAATAACGCAGGCCAATC	CCATGATTCCTGGTTTACTGAG	569

Chemical analysis of the wines

Analyses were carried out using the official methods or those recommended by the International Organization of Viticulture and Wine.

Results and discussion

Despite the influence of various winemaking processes, LAB developed homogeneously during the initial winemaking stages (Fig. 1). At harvest, the average LAB population on the grape surface was 10^2–10^3 CFU/grape. After a transitory growth phase in must to reach approximately 10^4 CFU/mL, the LAB population decreased during AF. In wine A, which had a higher ethanol level, LAB could no longer be counted (< 1 CFU/10 mL) when the AF was completed. Then LAB population subsequently began to grow again, reaching 10^7 CFU/mL, and malic acid consumption was activated.

O. oeni represented a minority of the species on the grape surface. The main species, identified by PCR-DGGE, belonged to the *Lactobacillus* genus, mainly *Lactobacillus plantarum* and *L. casei* [13, 16]. The proportion of *O. oeni* increased throughout AF and it was the only species present during MLF in the three cases studied (Fig. 2). Identification

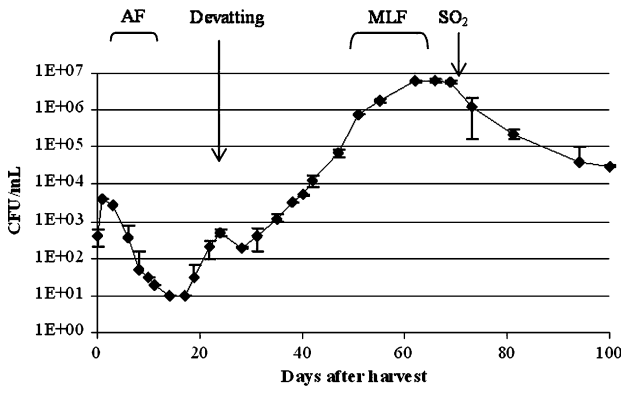

Fig. 1 Changes in the total lactic acid bacteria population during winemaking. The example shown is wine B. The *vertical bars* represent the standard deviation

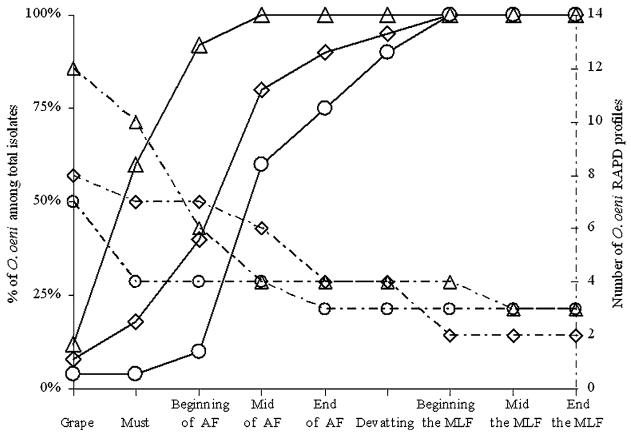

Fig. 2 Changes in the percentage of *Oenococcus oeni* (*solid line*) (*left Y*-axis) and the number of *O. oeni* RAPD profiles (*dotted line*) for the three wines (*open triangle* A; *open circle* B and *open diamond* C) (*right Y*-axis)

Table 3 Presence (+) or absence (−) of the strains and the 16 genetic marker for each strain isolated from the three wines and the frequency of marker detection among all strains

Wines	Strains	Stages of the winemaking					Markers															
		Berries	Must	AF	Devatting	MLF	M1	M2	M3	M4	M5	M6	M7	M8	M9	M10	M11	M12	M13	M14	M15	M16
Wine A	A-XII	+	−	−	−	−	−	−	−	−	−	−	+	−	+	−	−	+	−	−	−	−
	A-XI	+	−	−	−	−	−	−	+	+	−	+	−	−	+	−	−	+	−	+	+	−
	A-X	+	−	−	−	−	+	−	−	−	+	−	−	−	−	+	+	+	+	−	−	+
	A-IX	+	−	−	−	−	+	+	−	+	+	−	−	−	−	+	+	−	+	−	−	+
	A-VIII	+	−	−	−	−	−	−	+	+	−	+	−	−	+	+	−	+	−	+	+	−
	A-VI	+	+	−	−	−	−	−	+	+	−	+	+	−	+	−	−	+	−	+	+	−
	A-V	+	+	−	−	−	+	+	−	−	+	−	−	−	+	+	+	+	+	−	−	−
	A-VII	+	−	−	−	−	−	−	+	+	−	+	+	+	+	+	+	+	−	+	+	−
	A-IV	+	+	+	+	−	+	−	+	−	+	+	+	+	+	+	+	+	+	+	+	+
	A-III	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
	A-II	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
	A-I	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
	Total	12	6	4	4	3	7	5	8	8	7	8	7	5	10	9	8	11	7	8	8	6
							58%	42%	67%	67%	58%	67%	58%	42%	83%	75%	67%	92%	58%	67%	67%	50%
Wine B	B-VIII	+	−	−	−	−	−	−	−	−	−	−	+	−	+	−	−	−	−	−	−	−
	B-VII	+	−	−	−	−	−	−	−	−	−	−	+	−	−	−	−	+	−	−	−	−
	B-VI	+	−	−	−	−	−	−	+	−	−	+	+	+	+	−	−	+	−	+	+	−
	B-V	+	+	−	+	−	−	−	+	−	−	+	+	+	+	−	−	+	−	+	+	+
	B-IV	+	−	+	+	−	+	−	+	−	−	+	+	+	+	+	+	+	−	+	+	+
	B-III	−	+	+	−	+	+	−	+	+	+	+	+	+	+	+	+	−	+	+	+	+
	B-II	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	−
	B-I	+	+	+	+	+	+	−	+	+	+	+	+	+	+	+	+	−	+	+	+	+
	Total	7	4	4	4	3	4	1	6	3	3	6	8	6	7	4	4	5	3	6	6	4
							50%	13%	75%	38%	38%	75%	100%	75%	88%	50%	50%	63%	38%	75%	75%	50%
Wine C	C-VIII	+	−	−	−	−	−	−	−	−	−	−	+	−	−	−	−	−	−	−	−	+
	C-VII	+	+	+	−	−	−	−	+	−	−	+	+	+	+	−	−	+	−	+	+	−
	C-VI	+	+	+	−	−	−	−	+	−	+	+	+	−	−	+	−	+	−	+	+	−
	C-V	+	+	+	−	−	+	−	+	−	−	+	+	−	+	−−	+	+	−	+	+	+
	C-IV	+	+	+	+	−	−	−	+	−	−	+	+	+	+	−	−	+	−	+	+	−−
	C-III	+	+	+	+	−	+	−	+	−	−	+	+	+	+	+	+	+	−	+	+	+
	C-II	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
	C-I	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
	Total	8	7	7	4	2	4	2	7	2	3	7	8	5	6	4	4	7	2	7	7	5
		27	17	15	12	8	50%	25%	88%	25%	38%	88%	100%	63%	75%	50%	50%	88%	25%	88%	88%	63%

Table 3 continued

Wines	Strains	Stages of the winemaking				Markers													
		Berries	Must	AF	Devatting	MLF	M1	M2	M3	M4	M5	M6	M7	M8	M9	M10	M11	M12	M13	M14	M15	M16
Total	Total						15	8	21	13	13	21	23	16	23	17	16	23	12	21	21	15
	Average frequency detection						54%	29%	75%	46%	46%	75%	82%	57%	82%	61%	57%	82%	43%	75%	75%	54%
	Standard deviation of frequency						5%	15%	10%	21%	12%	10%	24%	17%	6%	14%	10%	16%	17%	10%	10%	7%

was then refined up to the strain level, using the multiplex RAPD-PCR [12]. Among the various molecular available methods, the multiplex method combining a short primer (Coc) with a longer primer (On2), specific to a fragment of the malolactic enzyme of *O. oeni* was highly discriminating, rapid and reproducible, making it possible to analyze a large number of strains in a reasonable time, so all the isolates from the three wines were analysed using this method. Like species diversity, strain diversity among *O. oeni* decreased during fermentation. The residual strains in wine during MLF were detected at harvest and resisted the increasingly inhospitable environment as winemaking progressed. Ultimately, during MLF, only two strains were present in wines B and C and three in wine A.

A previous genomic comparison between two strains with different oenological potential revealed 16 significant genetic markers [7]. We used these markers to characterize the resistance of indigenous strains of *O. oeni*. All strains were tested by PCR for the 16 genetic markers. Some strains had relatively few markers (A-XII, B-VIII, C-VIII), while others had all 16 (A-I, A-II, A-III, B-II, C-I, C-II) (Table 3).

The average number of markers per strain increased as winemaking progressed, reaching a maximum during MLF (Fig. 3). Finally, the strains responsible for MLF were those with the highest number of markers. In wines A and C, these strains (A-I, A-II, AIII, C-I and C-II) had all the 16 markers. These strains originated from the grape surface and were present throughout the winemaking process. On the grape surface and in the must, these strains were diluted among others that had fewer markers, but they became dominant at the end of AF and were the only ones during MLF. These observations confirmed the relevance of the genetic markers investigated for assessing the resistance and growth potential of *O. oeni* strains, as well as their capacity to complete MLF in wine.

Some markers may characterize resistance to early stress factors, such as high sugar concentrations and low water activity in fresh must, or low temperatures in initial cold maceration. Others may withstand later stresses, such as those resulting from yeast metabolism, e.g. ethanol and other inhibiting metabolites, and, finally, other markers may contribute to the strains' ability to survive throughout the winemaking process. The first marker (M1) targeted the cadmium-transporting P-type ATPase gene, which was revealed to play a key role in environmental stress response [1]. In *Klebsiella* spp. cadmium toxicity was affected by glucose concentrations [2]. Similar phenomena may have occurred for *O. oeni* in the must. This [19] demonstrated the implication of the metal ions like Cd(II) in bacteria homeostasis and the relationship with the DPS ferritin gene, which was targeted by the second marker (M2). The M12 marker targeted the thioredoxin gene, which was also

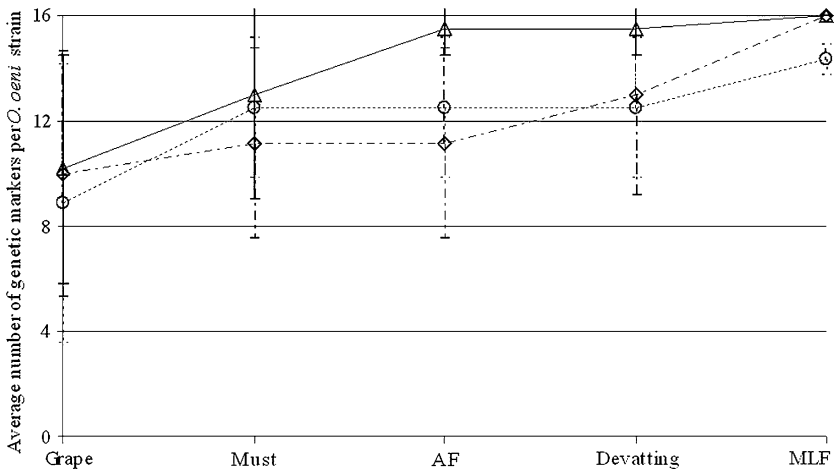

Fig. 3 Change in the average number of genetic markers per *Oenococcus oeni* strain in the three wines at different stages of winemaking (*open triangle* wine A, *open circle*: wine B, *open diamond* wine C). The *error bars* show standard deviation

known to play a major role in response to environmental stresses [21] notably in *O. oeni* [10]. Other markers targeted essential cellular functions viz. the cell wall organization (M15) and the transcription (M5 and M7). The importance of metabolite transport was also underlined by the markers M11, M14 and mainly M13, which was only detected in strains present at all winemaking stages. Glycerol transport has been described as a strategy for preserving cell membrane integrity [17].

Wine A made from the most highly sulphited must at harvest had the lowest pH and the highest alcohol level, and could consequently be considered the most hostile environment. In this wine, the proportion of non-*O. oeni* species and the number of markers per strain increased more quickly than in the other two wines. *O. oeni* was the only species present by mid-AF, and the strains present exhibited most of the markers. This wine resulted from the must with the highest sugar concentration and was also subjected to initial cold maceration. The high sugar concentration and low temperature probably led to osmotic and temperature stresses, which may have accelerated selection of the most resistant strains. In wine C, *O. oeni* strains with numerous markers became dominant after devatting. Strain diversity remained relatively high during AF started with commercial yeast. The yeast strain probably had an impact on the *O. oeni* population via interactions [11]. Long post-AF vatting in wine C may also intervene since during maceration, phenolic compounds were extracted from grape solids and they were known to have a significant impact on bacterial viability [3].

Finally, the genomic approach, based on the presence of 16 significant genetic markers, was very accurate for determining the resistance of indigenous *O. oeni* strains. Some markers were detected less frequently than the others. Genes implied directly in multistress response were the most relevant. Their target by simple methods of PCR could be adapted to the selection of the *O. oeni* industrial starter able to resist to constraints commonly met in the wine as to the description of *O. oeni* intraspecific diversity evolution. Our hypotheses are not exhaustive. That should be tested using other tools, such as proteomic and transcriptomic approaches, to elucidate the adaptation mechanisms of *O. oeni* strains in wine and the relevance of certain physiological changes.

References

1. Banjerdkij P, Vattanaviboon P, Mongkolsuk S (2004) Cadmium-induced adaptive resistance and cross-resistance to zinc in *Xanthomonas campestris*. Curr Microbiol 47:260–262
2. Brynhildsen L, Lundgren BV, Allard B, Rosswall T (1988) Effects of glucose concentrations on cadmium, copper, mercury, and zinc toxicity to a *Klebsiella* sp. Appl Environ Microbiol 54:1689–1693
3. Campos FM, Couto JA, Hogg TA (2003) Influence of phenolic acids on growth and inactivation of *Oenococcus oeni* and *Lactobacillus hilgardii*. J Appl Microbiol 94:167–174; Microbiol Lett 246:111–117
4. Cocconcelli PS, Porro D, Galandini S, Sentini L (1995) Development of RAPD protocol for typing of strains of lactic acid bacteria and enterococci. Lett Appl Microbiol 21:376–379
5. Delaherche A (2006) Adaptation d'*Oenococcus oeni* à l'environnement œnologique approches génomiques comparées, transcriptomiques et protéomiques. Ph D Thesis. Université Bordeaux 2, France
6. Delaherche A, Bon E, Gindreau E, de Daruvar A, Lonvaud-Funel A (2007a) The use of comparative genomic analysis to identify distinct genetic markers of *Oenococcus oeni* during adaptation in wine. Appl Environ Microbiol (in press)
7. Delaherche A, Bon E, Dupré , Lucas M, Arveiller B, De Daruvar A, Lonvaud-Funel A (2007b) Intraspecific diversity of *Oenococcus oeni* strains determined by sequence analysis of target genes. Appl Microbiol Biotechnol 73:394–403
8. Divol B, Tonon T, Morichon S, Gindreau E, Lonvaud-Funel A (2003) Molecular characterization of *Oenococcus oeni* genes encoding proteins involved in arginine transport. J Appl Microbiol 94:738–746
9. Gindreau E, Joyeux A, De Revel G, Claisse O, Lonvaud-Funel A (1997) Evaluation de l'établissement de levains malolactiques au sein de la microflore bactérienne indigène. J Int Sci Vigne Vin 31:197–202

10. Guzzo J, Jobin MP, Delmas F, Fortier LC, Garmyn D, Tourdot-Marechal R, Lee B, Divies C (2000) Regulation of stress response in *Oenococcus oeni* as a function of environmental changes and growth phase. Int J Food Microbiol 55:27–31
11. Lemaresquier H (1987) Inter-relationships between strains of *Saccharomyces cerevisiae* from the Champagne area and lactic acid bacteria. Lett Appl Microbiol 4:91–94
12. Reguant C, Bordons A (2003) Typication of *Oenococcus oeni* strains by multiplex RAPD-PCR and study of population dynamics during malolactic fermentation. J Appl Microbiol 95:244–353
13. Renouf V, Claisse O, Lonvaud-Funel A (2005a) Understanding the microbial ecosystem on the grape berry surface through numeration and identification of yeast and bacteria. Aust J Grape Wine Res 11:316–327
14. Renouf V, Walling E, Coulon J, Lonvaud-Funel A (2006a) Conseils pratiques pour la mise en place d'un suivi microbiologique des vins. Rev Oenol 119:39–42
15. Renouf V, Claisse O, Miot-Sertier C, Lonvaud-Funel A (2006b) Lactic acid bacteria evolution during winemaking: use of the *rpoB* gene as a target for PCR-DGGE analysis. Food Microbiol 23:136–145
16. Renouf V, Claisse O, Lonvaud-Funel A (2007) Inventory and monitoring of wine microbial consortia. Appl Microbiol Biotechnol 75:149–164
17. van der Heide T, Boolman B (2000) Osmoregulated ABC transport system of *Lactococcus lactis* senses water stress via changes in the physical state of membrane. Biochemistry 97:7102–7106
18. Wibowo D, Eschenbruch R, Davis CR, Fleet GH, Lee TH (1985) Occurrence and growth of lactic acid bacteria in wine. A review. Am J Enol Vitic 36:302–313
19. Theil EC (2007) Coordinating responses to iron and oxygen stress with DNA and mRNA promoters: the ferritin story. Biometals (in press)
20. Zapparolli G, Torriani S, Pesente P, Dellaglio F (1998) Design and evaluation of malolactic enzyme gene targeted primers for rapid identification and detection of *Oenoccocus oeni* in wine. Lett Appl Microbiol 27:243–246
21. Zeller T, Klug G (2006) Thioredoxins in bacteria: functions in oxidative stress response and regulation of thioredoxin genes. Naturwissenschaften 93:259–266