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Selection and characterization of autochthonous strains of *Oenococcus oeni* for vinification in Priorat (Catalonia, Spain)

Judit Franquès[1], Isabel Araque[1], Mariette El Khoury[2], Patrick M. Lucas[2], Cristina Reguant[1] and Albert Bordons[1*]

[1]Grup de Biotecnologia Enològica, Departament de Bioquímica i Biotecnologia, Facultat d'Enologia, Universitat Rovira i Virgili, c/ Marcel·lí Domingo 1, Campus Sescelades N4, 43005 Tarragona, Catalonia, Spain
[2]Unité d'Œnologie, Institut des Sciences de la Vigne et du Vin, Université de Bordeaux, F-33882 Villenave d'Ornon, France

Abstract

The use of autochthonous strains of *Oenococcus oeni* for inducing malolactic fermentation (MLF) in wines is increasing because they are well adapted to the conditions of a specific area. The main aim of this work was to select *O. oeni* strains from Priorat (Catalonia, Spain) wines that would be able to carry out the MLF while maintaining the characteristics of the wine. Forty-five autochthonous strains were selected based on their degradation of L-malic acid, resistance to low pH and high ethanol, and the absence of biogenic amine genes. The three strains with the best characteristics were inoculated and MLF was carried out successfully with the final wines showing good characteristics. One of the inoculated *O. oeni* strains would be a good candidate to study as possible starter culture. It shows that autochthonous *O. oeni* strains, once selected, have the potential to be used as an inoculum for wines.

Keywords: autochthonous strains, malolactic fermentation, *Oenococcus oeni*, Priorat, wine

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*Corresponding author : albert.bordons@urv.cat

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Introduction

Oenococcus oeni is the major species among lactic acid bacteria (LAB) involved in the malolactic fermentation (MLF) of wine (Wibowo *et al.*, 1985; Henick-Kling, 1993). During MLF, L-malic acid is decarboxylated to L-lactic acid, resulting in wine deacidification. This is a crucial step in red winemaking as it provides enhanced organoleptic qualities and microbial stabilization of the wine (Davis *et al.*, 1988; Lonvaud-Funel, 1999; Liu, 2002; Bartowsky, 2005; Cappello *et al.*, 2017).

MLF can be induced by inoculating commercial starters of *O. oeni*. However, this is not always successful because wine is a very harsh environment for bacterial growth (Coucheney *et al.*, 2005; Spano and Massa, 2006; Ruiz *et al.*, 2010), mainly due to the presence of ethanol (Capucho and San Romão, 1994; Zapparoli *et al.*, 2009). The other limiting conditions of wine (few nutrients, phenolic compounds, low pH) may restrict cell viability in such a way as to make MLF sluggish or even fail (Carreté *et al.*, 2002). In addition, the application of similar commercial bacterial starters across different world regions may lead to a certain product uniformity (Mas *et al.*, 2016). Hence, the application of an autochthonous starter culture, well adapted to the conditions of a specific wine-producing area, has already been suggested (Nielsen *et al.*, 1996; Ruiz *et al.*, 2010). For this reason, several studies have been performed on the characterization of *O. oeni* biodiversity with the aim of selecting putative autochthonous starter cultures (Capozzi *et al.*, 2010; Capozzi *et al.*, 2014; González-Arenzana *et al.*, 2014; Lamontanara *et al.*, 2014).

Climate change poses a major additional problem for MLF. Over the last 10-30 years, evidences of earlier fruit maturation patterns, and consequently modified vine development, have been observed, both of which have been attributed to rising temperatures worldwide (Jones *et al.*, 2005). The faster ripening of the grapes leads to a higher sugar content and therefore a higher ethanol content in the wines (Mira de Orduña, 2010; Webb *et al.*, 2011). In the prestigious qualified appellation of Priorat in southern Catalonia (northeast Spain), wines easily reach an ethanol content of 14%, and sometimes more (De Herralde *et al.*, 2012). The low acidity of these wines together with the earlier fruit maturation patterns diminishes their L-malic acid content, thus restricting the growth of *O. oeni*. Resistance to these harsh conditions (high ethanol and low pH) was the main criterion for strain selection in this work.

The preferences of consumers call for superior wines from a particular region to possess unique qualities and character (terroir wines) that differentiate them from wines of the same variety from other regions (Bisson *et al.*, 2002). Wines perceived as being of high quality can be produced anywhere, even though, according to the concept of terroir, the local environment will influence the composition of the wine produced in a specific growing region (Gilbert *et al.*, 2014; Zarraonaindia *et al.*, 2015). Among other things, this involves the contribution of the indigenous microbiota in shaping the unique quality of the wine (Bartowsky *et al.*, 2015).

A huge diversity of autochthonous *O. oeni* strains performing MLF has been found in wines (Reguant and Bordons, 2003; El Khoury *et al.*, 2017), and this diversity is important within the same location (López *et al.*, 2007; Cappello *et al.*, 2010). Population structure analyses of strains in wines from diverse geographic origins have shown that there are two major genetic groups of *O. oeni* strains, known as A and B (Bilhère *et al.*, 2009; Bridier *et al.*, 2010). For this reason, a minimal genetic characterization of the selected strains using the single nucleotide polymorphism (SNP) method was included as an aim in this work.

Another decisive characteristic for our strain selection was the absence of the ability to produce biogenic amines (BA), which are compounds that are undesirable in wines because they may induce headaches, respiratory distress, hyper-hypotension and various allergenic disorders (Silla-Santos, 1996). Different results have been reported for BA production by *O. oeni* and other LAB and it is of utmost importance to avoid formation of these amines during MLF (Costantini *et al.*, 2006; Landete *et al.*, 2007a).

The main aim of this study was therefore to characterize LAB strains isolated from Priorat wine samples in order to select those with the best characteristics for application as oenological starter cultures. Hence, the selected strains needed to be evaluated by inoculating them into a real Priorat wine in the cellar, checking their imposition and analysing the final wines.

Materials and methods

1. Strains

A total of 45 autochthonous LAB strains (Table 1) isolated from Priorat wines from vintages 2012 and 2013 and described in a previous study (Franquès *et al.*, 2017) were chosen following the criterion of

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having been isolated at least twice in two different wines. They consisted of 41 *O. oeni* strains, two *Lactobacillus plantarum* strains, one *Fructobacillus tropaeoli* strain and one *L. mali* strain. Six of the *O. oeni* strains were found in both 2012 and 2013 vintages.

2. L-malic acid degradation test in a wine-like medium

The strains were grown in MRSm1 (Franquès *et al.*, 2017), which is MRS (De Man *et al.*, 1960) supplemented with L-malic acid (3 g/L), fructose (5 g/L), L-cysteine (0.5 g/L) and tomato juice (100 mL/L) at pH 5, until A_{600nm} = 1.6. The pellet obtained was inoculated (2%) into 50 mL of wine-like medium (WLM) (Bordas *et al.*, 2013), which contained ethanol (12 or 14% v/v) added aseptically to the following sterilized base medium: 2 g/L fructose, 2 g/L tartaric acid, 0.5 g/L citric acid, 2 g/L L-malic acid, 5 g/L yeast extract, 0.1 g/L acetic acid, and 5 g/L glycerol, adjusted to pH 3.4 with 1 N NaOH. Then, it was incubated at 20ºC, in duplicate for each strain. The L-malic acid was measured enzymatically (Miura One, TDI S.A.) and both the L-malic acid consumption and fermentation speed were calculated.

3. Stress resistance test

The strains were precultured in a grape juice medium (GJM) similar to that used for El Khoury *et al.* (2017), which contained per liter 250 mL white grape juice (final sugar content of 50 g/L), 5 g yeast extract, 1 mL Tween 80, and 6% (v/v) ethanol, at pH 4. When the population reached approximately 10^8 CFU/mL, they were inoculated (0.2%) into 10 mL tubes of the same GJM so that a stress resistance test could be carried out in eight conditions combining different pH (2.8, 3, 3.3, 3.6 and 4) and ethanol concentrations (6, 12, 14 and 16%, v/v). The growth of the strains at 20ºC was followed for 3 weeks, checking the OD_{600} every 48 hours with a POLARstar Omega spectrophotometer (BMG Labtech).

4. Biogenic amine gene detection

The detection of the histidine decarboxylase (*hdc*), tyrosine decarboxylase (*tdc*) and ornithine decarboxylase (*odc*) genes was performed by specific PCRs. The DNA extraction was performed according to Ruiz-Barba *et al.* (2005). The *hdc* gene was detected using HDC3 and HDC4 primers (Coton and Coton, 2005), the *tdc* gene using P1-rev and p0303 primers (Landete *et al.*, 2007b), and the *odc* gene using primers 3 and 16 (Marcobal *et al.*, 2005). LAB strains having the BA genes were used as positive controls: *Lactobacillus* sp. 30a (ATCC 33222) for *hdc*, *L. brevis* Enolab 4415 (kindly provided by Sergi Ferrer, University of Valencia) for *tdc*, and *O. oeni* Enolab 4783 (also provided by S. Ferrer) for *odc*.

The *hdc* PCR products were analysed using MultiNA equipment (Microchip Electrophoresis System for DNA/RNA Analysis, Shimadzu) and the MultiNA kit (DNA 100-1500 bp, Shimadzu). SYBR Gold buffer (Invitrogen) diluted 100 times in TE (pH 8) and the molecular marker phiX174-HaeIII digest (Promega) were used. The *tdc* and *odc* PCR products were checked by electrophoresis in 1.2% (w/v) agarose gels with Tris-borate-EDTA buffer (TBE) 0.5x (80V, 1h30) and dyed with ethidium bromide. DNA molecular weight markers 1KB Plus Invitrogen (REF 10787-018) were used for reference purposes.

5. Classification of strains in phylogroups using SNP genotyping

The simple nucleotide polymorphism (SNP) technique was used to analyse *O. oeni* strains and include them in phylogroups A and B. Two other *O. oeni* strains with previously characterized SNPs (Campbell-Sills *et al.*, 2015) were also included in the study: PSU-1 (ATCC-BAA-331) and ATCC-BAA-1163, which belong to groups A and B, respectively. Before genotyping, a real time PCR was performed to compare the DNA samples with each other and check the DNA concentration of each.

SNP methodology was applied following Campbell-Sills *et al.* (2015) and El Khoury *et al.* (2017). Manual curation and selection were performed to select 39 SNPs, which were amplified and sequenced using the Sequenom strategy. The genotyping results of these SNPs for each strain were concatenated into a single sequence of 39 bp. The sequence alignments and phylogroup analysis were performed using MEGA software 6.0.5 (Tamura *et al.*, 2013) with 1000 bootstrap replications on neighbour-joining distance calculation using Kimura 2-parameter.

6. Performance of MLF in industrial wines inoculated with the selected strains

The selected strains (WW strains, from the Wildwine project) were used as starter culture for the inoculation of two industrial wines (one from Grenache and the other from Carignan) at the Ferrer-Bobet winery, located on road T-740, between Falset and Porrera, in Priorat. The alcoholic fermentations (AF) were carried out with autochthonous *S. cerevisiae* strains (CECT13132, CECT13133 and CECT13134) isolated in the same Priorat area (Mas *et al.*, 2015; Padilla *et al.*, 2017). The main analytical

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characteristics at the end of AF are shown in Table 2. For each wine, the strains were grown separately in 1.5 L of MRSm1 medium until OD_{600} = 1.6, corresponding to 10^9 CFU/mL. The total pellet obtained was washed with saline solution and used to inoculate (2%) oak barrels containing 225 L of wine. Other barrels were inoculated with the commercial *O. oeni* CH11 strain (Viniflora® CH11, from Chr. Hansen A/S, Hørsholm, Denmark) as a control, following the manufacturer's indications.

All barrels were kept in the Ferrer-Bobet winery cellar and set aside for MLF at 20ºC. L-malic acid consumption was followed by enzymatic analysis in the laboratory of the same winery. The final MLF samples were collected and cultured in solid MRSm3 (Franquès *et al.*, 2017), which is MRSm1 supplemented with nystatin (100 mg/L) and sodium azide (25 mg/L). In order to confirm the presence of the bacteria inoculated, 30 colonies of each sample were selected and their DNA extracted, then they were typed using the VNTR technique (Claisse and Lonvaud-Funel, 2012) including the modifications made by Franquès *et al.* (2017).

7. Wine chemical analysis

The main chemical characteristics (sugars, ethanol, glycerol, pH, total and volatile acidity, organic acids, nitrogen, sulphur dioxide and phenolics; see details in Table 2) of the final real wines after MLF by the selected strains were analysed following OIV methods (OIV, 2009) by the Catalan Institute of Vineyard and Wine (INCAVI, Vilafranca del Penedès, Catalonia, Spain).

Results and discussion

In a previous study (Franquès *et al.*, 2017), a survey of autochthonous LAB was carried out in the Catalan wine region of Priorat, with 166 strains being identified and typed. Of these, the 45 that were isolated at least twice were chosen to select strains with the greatest potential as malolactic cultures.

1. L-malic acid degradation test in wine-like medium

The 45 strains (41 *O. oeni* strains and four non-*Oenococcus* strains) were characterized by their L-malic acid degradation efficiency and their fermentation speed in WLM at 12% and 14% ethanol (Table 3). A clear difference could be seen between most strains of *O. oeni* and the few strains of other species. Regarding the *O. oeni* strains, three different behaviour groups were observed, with 75% (group E3) being accounted for by strains that consumed 100% of the L-malic acid (2 g/L) in both 12% and 14% (v/v) ethanol fermentations and were the quickest to do so. Some of the most rapid strains reached L-malic acid consumptions of 42 mg/L/h and 28 mg/L/h in the presence of 12% and 14% ethanol, respectively. This speed is equivalent to a consumption of 2 g/L L-malic acid in just four days.

The four non-*Oenococcus* isolates were considered as belonging to group E1 because none of them consumed more than 80% of the L-malic acid in 14% (v/v) ethanol, despite the fact that most of them consumed all the L-malic acid in 12% ethanol. *L. mali* and *F. tropaeoli* were slower than most *O. oeni* strains but quicker than the *L. plantarum* strains in both the 12% and 14% ethanol fermentations. The better performance of *O. oeni* over other species confirms once again its known characteristic of being the predominant LAB of MLF in wine (Wibowo *et al.*, 1985; González-Arenzana *et al.*, 2013).

2. Stress resistance test

Using both the viable culture results and the OD_{600} measurements over three weeks, the studied isolates were classified into five different groups depending on their degree of resistance, with R0 being the least resistant and R4 the most resistant, as shown in Table 1.

Twenty-one strains were unable to grow in the preculture step (pH 4 and 6% ethanol) in GJM. These were tagged as R0 and discarded from the experiment, as they could not grow in the least stressful condition. These 21 strains included 14 *O.oeni* that had been considered part of the more efficient E3 group in the previous experiment. Regarding these differences, it must be borne in mind that the efficiency assay was done in WLM, which is not as restrictive as the GJM medium used in the stress resistance test. Moreover, the inocula for WLM were grown in the rich MRS medium at 2%, whereas the inocula for GJM were grown in GJM at 0.2%. Therefore, the initial population in WLM was higher than in GJM. Finally, it must be taken into account that the goal of the efficiency assay was to measure the degradation of L-malic acid, while that of the resistance test was to see the possible growth under the different stress conditions.

The 12 isolates in group R1 showed good growth at pH 4, 3.6 and 3.3 with 6% ethanol. The growth kinetics of strain 1Pw4, representative of this R1 group, can be seen in Figure 1. In all other conditions, there was no growth even after three weeks of tracking. The four isolates in group R2 showed good growth in 6% ethanol and pH 4, 3.6 and

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Table 1. LAB strains used and characterized in this study.

Strains	Species	Efficiency group [a]	Resistance group [b]	Phylogroup [c]	Gene *odc*	Gene *hdc*
1Pw1 †	*Oenococcus oeni*	E3	R1	B	—	—
1Pw3	”	E2	—	—	—	+
1Pw4	”	E3	R1	B	—	—
1Pw5	”	E1	—	—	—	+
1Pw6	”	E3	—	—	—	—
1Pw7	”	E3	—	—	—	+
1Pw8	”	E3	—	—	—	—
1Pw9	”	E3	R4	A	—	—
1Pw10	”	E2	—	—	—	+
1Pw11	”	E3	—	—	—	+
1Pw12	”	E3	—	—	—	+
1Pw13	”	E3	R4	A	—	—
1Pw14	”	E3	—	—	—	+
1Pw15	”	E3	—	—	—	—
1Pw16 †	”	E3	—	—	—	—
1Pw17 †	”	E1	R3	A	—	—
1Pw18	”	E3	—	—	—	+
1Pw19	”	E1	—	—	+	—
1Pw20	”	E3	R1	B	—	—
1Pw2 †	”	E3	R1	B	—	—
2Pw2	”	E3	R1	B	—	—
2Pw3	”	E3	R1	B	—	—
2Pw5	”	E3	—	—	—	—
2Pw6	”	E3	—	—	—	—
2Pw7 †	”	E3	—	—	—	—
2Pw8	”	E3	—	—	—	—
2Pw9	”	E1	—	—	—	—
2Pw10	”	E2	R2	A	—	—
2Pw11	”	E3	R1	B	—	—
2Pw12	”	E3	R1	B	—	—
2Pw13	”	E3	R2	B	—	—
2Pw14	”	E3	R2	A	—	—
2Pw15	”	E3	R4	A	—	—
2Pw16	”	E3	R3	A	—	—
2Pw17	”	E3	R3	A	—	—
2Pw19 †	”	E3	—	—	—	—
2Pw20	”	E3	R3	A	—	—
2Pw21	”	E3	R2	B	—	—
2Pw22	”	E3	R4	A	—	—
2Pw23	”	E3	R4	A	—	—
2Pw24	”	E3	R4	A	—	—
1Ptr11	*Fructobacillus tropaeoli*	E1	R1	—	—	—
1Pma1	*Lactobacillus mali*	E1	—	—	—	—
1Ppl21	*L. plantarum*	E1	—	—	—	—
1Ppl24	”	E1	R1	—	—	—

[a] Efficiency groups of strains according to Table 3, with E1 being the least efficient and E3 the most efficient.
[b] R1 is the group with the least resistant strains and R4 the most resistant strains. Strains with an empty field belong to group R0, since they were unable to grow with 6% ethanol at pH 4.
[c] Phylogroup of *O. oeni* strains. † These strains were found in both the 2012 and 2013 vintages.

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Table 2. Main analytical characteristics of the wines made at the Ferrer-Bobet winery after MLF with inoculated strains of *O. oeni*. WW: mix of selected strains (1Pw13, 2Pw15 and 2Pw22) from the Wildwine project. CO: commercial *O. oeni* strain CH11. AF: alcoholic fermentation.

Inoculated strains		WW	CO	WW	CO
Wine variety		Grenache	Grenache	Carignan	Carignan
Contents at end of AF	Glucose + fruct	0.25		0.45	
	pH	3.20		3.18	
	Ethanol	14.95±0.11		13.95±0.10	
	Acetic acid	0.31		0.44	
	L-malic acid	0.4		1.0	
Glucose + fructose	g/L	0.16	0.23	0.13	0.21
Glycerol	g/L	6.80	6.80	8.66	8.43
pH		3.32	3.32	3.34	3.33
Tartaric acid	g/L	2.41	2.49	2.51	2.47
Total acidity	g/L	5.7±0.2	5.9±0.2	6.5±0.2	6.5±0.2
Volatile acidity	g/L	0.45±0.07	0.41±0.08	0.55±0.09	0.54± 0.08
L-lactic acid	g/L	0.41	0.21	0.72	0.7
L-malic acid	g/L	< 0.1	0.4	< 0.1	< 0.1
Citric acid	g/L	< 0.05	< 0.05	< 0.05	< 0.05
Alpha-amino nitrogen	mg/L	13.2	55.9	47.7	48.2
Ammoniacal nitrogen	mg/L	7.8	7.9	5.9	6.5
Folin-Ciocalteu Index		62.96	67.71	55.32	53.89
Anthocyanins	mg/L	466	484	555	546
Tannins	g/L	3.71	3.76	2.53	2.50

3.3 conditions. During the third week, the R2 isolates showed little growth in the medium with 12% ethanol and pH 4. The five isolates in group R3 showed active growth in 6% ethanol and pH 4, 3.6 and 3.3 conditions and in 12% ethanol and pH 4. Little growth was also noticed during the third week in the medium with 14% ethanol and pH 4. Finally, there were six isolates in group R4 that showed good growth in 6% ethanol and pH 4, 3.6 and 3.3 conditions and in 12% and 14% ethanol and pH 4 conditions. Little growth was also noticed during the third week in the medium with 6% ethanol and pH 3, as shown in Figure 2 for strain 2Pw22, which is representative of this R4 group.

Of the few non-*Oenococcus* isolates, only the *F. tropaeoli* and one of the *L. plantarum* strains were classified in the R1 resistance group (Table 1). Nevertheless, all these isolates presented very low efficiency when degrading L-malic acid (Table 3).

3. Biogenic amine gene detection

MLF is generally considered to be a crucial factor for BA production, and studies have shown that the main BA generated in this phase are putrescine, histamine and tyramine (Lonvaud-Funel, 2001; Marcobal *et al.*, 2006). Consequently, the isolates were tested for the presence of the corresponding genes.

None of the strains tested contained the *tdc* gene fragment, only one (1Pw19) contained the *odc* gene fragment, and eight (18%) contained the *hdc* gene fragment (Table 1). The strains that contained any of the three BA genes were discarded in the selection. This ensured that those selected would have no risk of producing these amines. Incidentally, all the strains harbouring BA genes were also discarded in the stress resistance test (see above).

4. Classification of *O. oeni* strains in phylogroups using SNP genotyping

SNP genotyping was carried out on 22 *O. oeni* preselected strains, in line with the above results and discarding the least resistant strains (group R0) and those containing BA genes. The 22 strains are those shown in Table 1 with their resistance group (R1, R2, R3 or R4). They could be assigned to the A and B subpopulations described earlier (Bilhère *et al.*, 2009; Borneman *et al.*, 2012; Campbell-Sills *et al.*, 2015; El Khoury *et al.*, 2017). In order to verify this, the known PSU-1 and ATCC-BAA-1163 strains representative of the A and B subpopulations, respectively, were included in the SNP analysis.

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Table 3. L-malic acid consumption (2 g/L) in wine-like medium (WLM) and MLF speed of the predominant strains from different species. Strains are grouped by their efficiency according to both degradation and MLF speed. Assays were done in duplicate with both 12% and 14% (v/v) ethanol.

Species	Efficiency group	No. of strains	% no. strains / total	% L-malic acid consumed		MLF speed (m
				12% Ethanol	14% Ethanol	12% Ethanol
	E3	34	75	100	100	nov-42
O. oeni	E2	3	7	100	80-100	nov-14
	E1	4	9	50-100	50-100	avr-21
L. mali	E1	1	2	100	73	15
F. tropaeoli	E1	1	2	100	66	15
L. plantarum	E1	2	4	36-100	< 50	avr-15

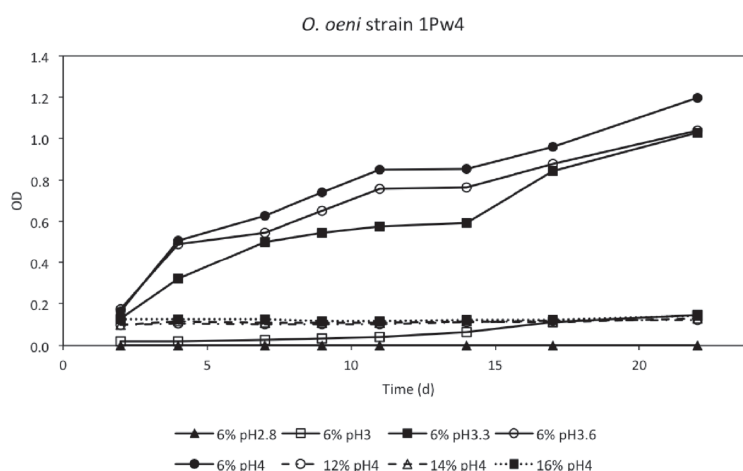

Figure 1. Growth kinetics of *O. oeni* 1Pw4, a representative strain of the R1 resistance group.

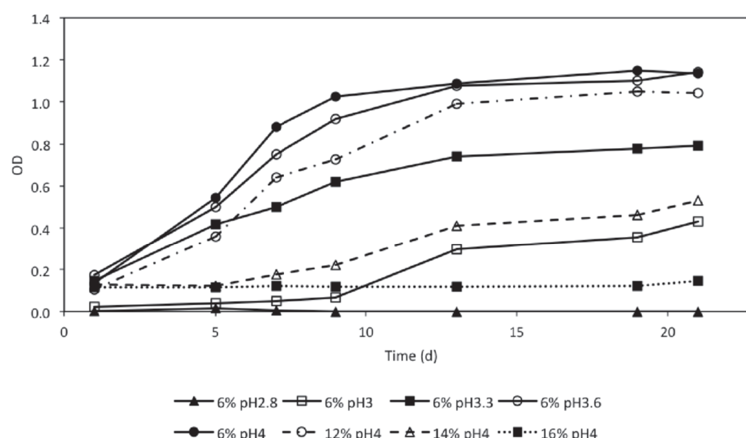

Figure 2. Growth kinetics of *O. oeni* 2Pw22, a representative strain of the R4 resistance group.

A total of 39 SNPs were manually selected following El Khoury *et al.* (2017) and checked for each of the 22 selected strains and for the two "control" strains characterized previously (Bridier *et al.*, 2010). SNP data analysis revealed that all 24 strains possessed SNP combinations corresponding to seven of the predefined sequence types (ST) (El Khoury *et al.*, 2017). Using these data, an unrooted tree was reconstructed by the neighbour-joining method (Figure 3). The result confirmed the assignment of all strains to groups A (upper branch) and B (lower branch) (Figure 3).

As can be seen in Figure 3, 12 of the 22 strains were assigned to phylogroup A and the other 10 to

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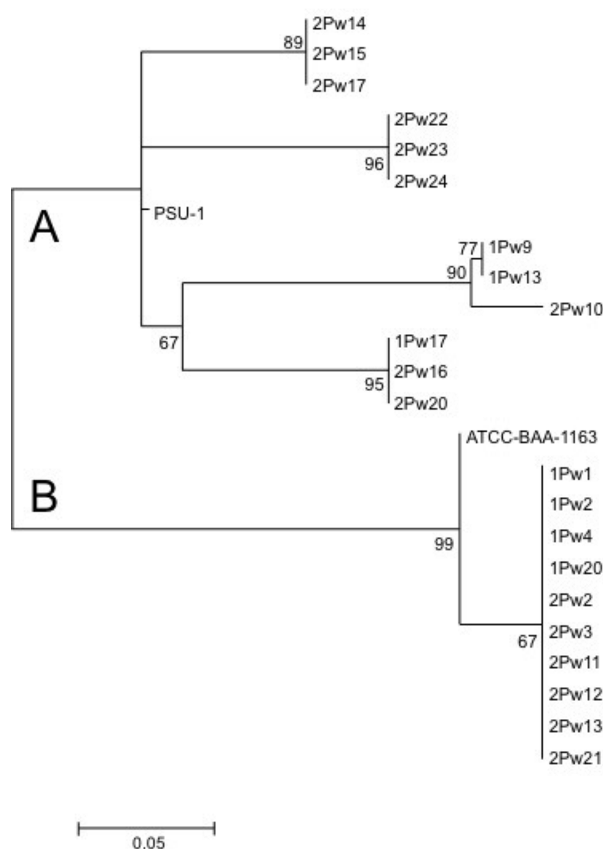

Figure 3. Distribution of preselected *O. oeni* strains in phylogroups. The neighbour-joining tree was constructed using the 39 concatenated sequences of single nucleotide polymorphism (SNP) identified by analysing 24 strains. The number of nodes indicates the bootstrap values (%). The scale bar represents the number of substitutions per site.

group B. The strains with the best characteristics, i.e. the six in group R4 (Table 1), are located in phylogroup A. This agrees with the hypothesis that it is usually the A strains that are best adapted to wine conditions (Campbell-Sills *et al.*, 2015), but we must not forget that some B strains with good malolactic behaviour in MLF were also isolated by our group from other wines of the same region (Bordas *et al.*, 2013).

5. Performance of MLF in industrial wines inoculated with three selected strains of *O. oeni*

Of the initial 45 LAB strains, the best were selected according to the results shown above, i.e. the L-malic acid degradation test, the absence of BA genes and the stress resistance test. The best strains (Table 1) were the six in the resistance group R4 (1Pw9, 1Pw13, 2Pw15, 2Pw22, 2Pw23 and 2Pw24), which were assigned to the best efficiency group E3 and lacked the three BA genes. Bearing in mind that some of these strains presented the same SNP profile (see Figure 3: 1Pw9-1Pw13 and 2Pw22-2Pw23-2Pw24), three were selected, one from each SNP profile: 1Pw13, 2Pw15 and 2Pw22. All were *O. oeni* and had been isolated from different cellars (Franquès *et al.*, 2017). As expected, the strains with the best inoculum characteristics were from *O. oeni*, the species most used for MLF induction (Kunkee, 1984; Solieri *et al.*, 2010).

Barrels from the Ferrer-Bobet cellar with 225 L of Grenache and Carignan wines were inoculated with a mixed pellet of these three selected WW strains (1:1:1), previously grown in MRSm1 medium. As a control, other barrels of the same wines were inoculated with a commercial strain (CH11) of *O. oeni*.

The inoculum prepared with the three WW strains completed MLF in one Grenache (10 d) and one Carignan (77 d) wine. Final viable cell numbers of LAB (in MRSm3) were 10^4 and 10^5 CFU/mL in these Grenache and Carignan wines, respectively. In spite of these low numbers, all colonies were verified to be *O. oeni*. The imposition of two inoculated *O. oeni* strains (1Pw13 and 2Pw22) in those MLFs was confirmed by typing 30 colonies using the

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VNTR technique (Table 4). Another strain (profile "I"), which was not inoculated, was found at a low concentration (4%) in the Carignan wine. This "I" strain had been isolated previously in the same cellar (Franquès *et al.*, 2017). The 2Pw15 strain was not recovered from any wine. The 1Pw13 strain was recovered from every wine and was the predominant strain in every case. The profile of the commercial strain *O. oeni* CH11 was found exclusively in the wines inoculated with this strain.

MLF was successfully carried out in the Grenache and Carignan wines in the cellar. This was despite the high ethanol content, especially for the Grenache wine (14.95%). The duration of MLF with WW strains was shorter in the Grenache than in the Carignan wine. Initial L-malic acid was low in the wines (0.4 and 1 g/L in Grenache and Carignan, respectively), but these concentrations are the ones currently measured in Priorat wines. The better MLF performance in the Grenache than in the Carignan wine despite the harsher conditions (more ethanol and less L-malic acid) must surely be due to the different wine matrix of the two wine varieties, and in the Carignan there is probably some growth-limiting nutrients or other inhibiting substances (Gockowiak and Henschke, 2003).

MLF in the Grenache wines was performed by autochthonous strains and not by the commercial strain. Thus, the WW strains selected and used as inoculum were efficient. It is worth noting that the 1Pw13 strain, which was detected as the predominant strain in both the Grenache and Carignan wines (Table 4), performed the MLF in relatively short times. Moreover, this strain managed MLF without special previous adaptation, since the WW strains were grown in rich MRS medium and then harvested and inoculated directly into the wine. Therefore, it can be suggested that 1Pw13 is a good candidate for a starter culture and also for studying the molecular mechanisms of stress response to wine adaptation.

Table 4. Proportions of *O. oeni* strains found at the end of MLF carried out in the cellar and inoculated with the three selected strains (1Pw13, 2Pw15 and 2Pw22). Proportions were obtained by typing 30 colonies of each sample by the VNTR technique. "I" is a wild (not an inoculated) strain that was previously found in the same cellar.

Wine	Grenache	Carignan
Duration of MLF (d)	10	77
O. oeni strain	Proportions (%)	
1Pw13	73	88
2Pw22	27	8
"I"	—	4

6. Wine chemical analysis

The results of the main analytical characteristics of the final wines after MLF using the three selected strains are shown in Table 2. It can be seen that all the wines had a high ethanol content, nearly 15% in the Grenache.

The initial L-malic acid was low, especially in this Grenache, with only 0.4 g/L. MLF was carried out quickly by WW strains in this wine and after 10 days the L-malic acid was exhausted. However, in the Grenache wine inoculated with the commercial *O. oeni* strain, the L-malic acid content did not decrease after more than two months. Consequently, the L-lactic acid content rose to 0.41 g/L in the Grenache wine with WW strains and only to 0.21 g/L in the one inoculated with the commercial strain. MLF was slower in the Carignan wines, but after 77 days L-malic acid was exhausted in the Carignan wine with WW strains, and two days earlier in the one inoculated with the commercial strain. Since the initial content of L-malic acid was 1 g/L in the Carignan wines, the L-lactic acid content (around 0.7 g/L) was higher than in the Grenache, as expected.

Meanwhile, citric acid was not detected (< 0.05 g/L) in any of the final wines (Table 2). It suggests that it was completely consumed by the same LAB strains, probably in connection with the lower initial concentration of L-malic acid in these wines. *O. oeni* usually degrades citric acid slightly after L-malic acid consumption (Bartowsky and Henschke, 2004), and here the low levels of L-malic acid would have facilitated the rapid consumption of citric acid. Despite this, volatile acidity remained at a reasonable level.

Other differences between the wines inoculated with WW strains and the commercial strain include the final residual sugar contents, which were slightly lower in those inoculated with WW strains (0.16 and 0.13 g/L glucose+fructose in Grenache and Carignan, respectively) than in those inoculated with the commercial strain (0.23 and 0.21 g/L in Grenache and Carignan, respectively). Another difference was that the alpha-amino nitrogen content was much lower in the Grenache wine with WW strains (13.2 g/L) than in the one inoculated with the commercial strain (55.9 g/L). This lower residual sugar content and alpha-amino nitrogen content in the wines fermented with WW strains can be seen as another positive characteristic of these strains. It means that they have an active metabolism under

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these harsh conditions and that these wines would run less risk of being contaminated by other bacteria.

Conclusions

In this study, three *O. oeni* strains were selected from 45 autochthonous LAB strains from Priorat wines based on their efficiency in degrading L-malic acid and most especially their resistance to high ethanol and low pH. The absence of biogenic amine genes in these strains was verified, and the SNP analysis placed them in phylogroup A, the same group in which other good wine-adapted strains have been found. After being inoculated into industrial wines, one of the strains showed good performance when carrying out the MLF. The characteristics of these wines suggest that this strain would be a good candidate for starter culture.

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