

# Isolation, selection, and characterization of highly ethanol-tolerant strains of *Oenococcus oeni* from south Catalonia

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**Summary.** Twenty-one strains of *Oenococcus oeni* were isolated during the malolactic fermentation of wines from south Catalonia. Due to their high ethanol tolerance (14 %, or more), these strains may serve as promising starters. The strains were screened by assays in a wine-like medium and by their co-inoculation in wine, resulting in the selection of well-performing strains, subsequently shown not to produce the main biogenic amines and lacking the genes involved in their synthesis. The genetic diversity of the isolates was studied by multilocus sequence typing (MLST), in which seven housekeeping genes were sequenced. Although the concatenated allelic profile of some strains was the same, the profiles obtained by random amplification of polymorphic DNA together with the variable number of tandem repeats at several loci showed that none of the strains were identical. A phylogenetic tree was constructed based on MLST with the seven genes and clearly showed two phylogroups, in accordance with previous studies. The best-performing strains occurred in members of both subgroups, suggesting that the grouping of housekeeping genes is not directly related to adaptation and ethanol tolerance. [Int Microbiol 2013; 16(2):113-123]

**Keywords:** *Oenococcus oeni* · malolactic fermentation · wine production · multilocus sequence typing (MLST) · strain selection

## Introduction

*Oenococcus oeni* is the major species among lactic acid bacteria (LAB) involved in the malolactic fermentation (MLF) of wine [17,35]. MLF, in which L-malic acid is decarboxylated to L-lactic acid, is a crucial step in winemaking as it provides enhanced organoleptic qualities and microbial stabilization of the wine [1,13,19,21]. However, bacterial development and

MLF are not always successful as they are limited under the harsh environmental conditions of wine [32], mainly the presence of ethanol. Ethanol resistance is a unique characteristic of *O. oeni*; however, at concentrations >12 % (v/v), ethanol can affect growth and malolactic activity [6,37]. Moreover, the other typical harsh conditions of wine (few nutrients, phenolics, low pH) restrict cell growth such that MLF is sluggish or even fails [7]. To survive and adapt to this harsh environment, *O. oeni* has developed various strategies, including the production of ATP by consuming organic acids (mainly L-malic, but also citric acid), the synthesis of stress proteins [2], and modifications in the composition of its membranes [30].

Currently, climate change poses a major additional problem for MLF. Over the last 10–30 years, observations in various winemaking regions of the world have provided evidence

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of earlier fruit maturation patterns and, consequently, modified vine development, both of which have been attributed to rising temperatures worldwide [18]. Faster ripening of the grapes leads to a higher sugar content and thus a higher ethanol content of the wines [23,34]. In the prestigious qualified appellation of Priorat, in south Catalonia (north-eastern Spain), wines easily reach an ethanol content of 14 % and sometimes higher [14]. Moreover, the low acidity of these wines together with the above-described stress factors has lowered their L-malic acid content, thus restricting the growth of *O. oeni*.

An understanding of the molecular mechanisms of adaptation of *O. oeni* is crucial to obtaining starter strains that are

better adapted to the harsh conditions that occur in wine during its production [1]. The intraspecific genomic diversity in *O. oeni* is well established and is related to the geographical origin of the isolates [22], and thus to the particular conditions of the wines from these regions. The aim of this study was to use wines produced in south Catalonia to isolate and select strains of *O. oeni* that are able to tolerate high ethanol concentrations, as potential candidates for MLF starter cultures. These strains were also tested for their genetic ability to produce biogenic amines in an attempt to find non-producer strains and thus prevent related health problems in wine consumers. Finally, the selected strains were genetically charac-

**Table 1.** Strains of *Oenococcus oeni* used in this study and the wines from which they were isolated. For each wine, the different RAPD-PCR profiles obtained are signalled alphabetically, and the assigned strain names are in parentheses

Cellar	Wine appellation	Variety	Wine	Profiles (strains)		
C1	DOQ Priorat	Grenache	1P	A (1P1)	B (1P2)	C (1P3)
		Cabernet Sauvignon	2P	C	D (2P2)	E (2P10)
		Cabernet Sauvignon	3P	F (3P1)	G (3P2)	
		Cabernet Sauvignon	4P	F		
		Cabernet Sauvignon	5P	F		
		Carignan	6P	A	F	G
		Grenache	7P	F	G	
C2	DOQ Priorat	Cabernet Sauvignon	8P	H (8P4)	I (8P7)	
		Grenache	9P	I		
		Grenache	10P	J (10P2)	K (10P4)	
		Syrah	11P	H	I	J
		Grenache	12P	I	K	
C3	DOQ Priorat	Carignan	13P	L (13P1)	M (13P5)	
		Cabernet Sauvignon	14P	L		
		Syrah	15P	L		
		Merlot	16P	L		
C4	DOQ Priorat	Grenache	17P	M		
C5	DOQ Priorat	Grenache	18P	N (18P7)		
		Carignan	19P	N	O (19P2)	
C6	DO Tarragona	Grenache	1T	P (1T1)		
		Grenache	2T	P	Q (2T1)	R (2T2)
		Grenache	3T	S (3T1)	T (3T7)	
		Grenache	4T	P		
		Grenache	5T	U (5T8)		

terized by multilocus sequence typing (MLST) in order to determine their phylogenetic relationships.

## Materials and methods

***Oenococcus oeni* strains.** Most strains used in this study were isolated from 24 red wines from south Catalonia (Table 1) of the 2008 vintage. The wines, all of which had an ethanol content of around 14 % (v/v), were taken from six different cellars and two different appellations of origin: DOQ Priorat and DO Tarragona. Other strains used were CH11 (Chr. Hansen, Hoersholm, Denmark), CECT 217<sup>T</sup> (= ATCC 23279<sup>T</sup>, from the Spanish Type Culture Collection, Valencia, Spain), and the sequenced strain PSU-1 (= ATCC BAA-331).

**Isolation and growth conditions.** Wine samples taken during MLF were inoculated (100 µl) on plates of MRS agar [15] supplemented with DL-malic acid (6 g/l), fructose (5 g/l), L-cysteine (0.5 g/l), nystatin (100 mg/l), and sodium azide (25 mg/l). These plates of MRSmf medium (pH 5.0) were incubated at 27 °C in a CO<sub>2</sub> incubator until the colonies had grown. Ten colonies were collected from each plate. Each one was inoculated into MRSmf broth medium and incubated until the end of the exponential phase (*ca.* OD<sub>600</sub> 1.4), usually 7 days.

**Identification and typification of strains.** Cells were incubated with lysozyme (50 mg/ml) for 30 min at 37 °C, after which their genomic DNA was extracted using a High Pure PCR template kit (Roche, Mannheim, Germany) following the manufacturer's instructions. Total DNA concentrations were calculated by measuring absorbance at 260 nm using a Nanodrop 1000 spectrophotometer (Thermo Fisher Scientific, Bremen, Germany). To identify *O. oeni*, species-specific PCR [36], which amplifies a fragment of the malolactic enzyme gene of *O. oeni*, was performed using 2 ng of the DNA.

Isolates identified as *O. oeni* were typed using multiplex random amplification of polymorphic DNA (RAPD)-PCR [26], with two primers, Coc and On2, developed by Cocconcelli et al. [10] and Zapparoli et al. [36], respectively. The amplification products were resolved by electrophoresis in 1.4 % (w/v) agarose gels run at 100 V for 2 h 45 min and stained with ethidium bromide. DNA molecular weight markers II and VI (proportion 1:2) from Roche Diagnostics (Basel, Switzerland) were used for reference purposes.

For a final comparison of the typing profiles of the strains obtained in this study, the RAPD-PCR method with only the Coc primer was used [10], under the same electrophoretic conditions as described above. Strain typing was also verified by the multilocus variable number of tandem repeat (VNTR) method, following the protocol of Claisse and Lonvaud [9].

**Screening of strains by MLF assays in wine-like medium and previous optimization of conditions.** The wine-like medium contained ethanol (10, 12, or 14 %, v/v) added aseptically to the following sterilized base medium: 2 g fructose/l, 2 g tartaric acid/l, 0.5 g citric acid/l, 0.6 g L-malic acid/l, 5 g yeast extract/l, 0.1 g acetic acid/l, and 5 g glycerol/l, adjusted to pH 3.4 with 1 N NaOH. Growth conditions were optimized by testing different inoculants of different sizes (1, 2.5, 5 and 10 %, v/v) and different ethanol concentrations. The optimal conditions, once identified, were then used in all subsequent experiments.

MLF assays were carried out in wine-like medium with every strain and were run in duplicate. Each isolated and typed strain was cultured in tubes containing 5 ml of MRSmf broth medium until an OD<sub>600</sub> of approximately 1.4, equivalent to 10<sup>9</sup> cells per ml, was reached. After centrifugation of 1.25 ml of cultured cells, the pellet was inoculated in 50 ml of wine-like medium and cultured at 20 °C. For comparison, the type culture strain CECT 217<sup>T</sup> was included in these assays. The changes of MLF was followed by analyzing L-malic acid formation using a commercial kit (Roche, Darmstadt, Germany).

The strains were first selected on the basis of their total consumption (%) of L-malate and their malolactic efficiency or consumption rate, calculated as the amount of L-malate (mg/l) consumed per hour of fermentation, during the period in which malolactic activity was detected. These values, for both 12 and 14 % ethanol, were analyzed statistically by grouping the strains in hierarchical clusters by Euclidean distance mapping, using SPSS version 19.0 (SPSS Inc., Chicago, IL, USA).

**Strain selection according to performance in a co-inoculation assay in real wines.** The selected strains of *O. oeni* were grown in MRSmf broth medium at pH 5.0 at 27 °C. Cells of seven strains were collected in the exponential phase (*ca.* OD<sub>600</sub> 1.4) and, after centrifugation, co-inoculated to a final concentration of 2 × 10<sup>6</sup> colony-forming units (CFU)/ml per strain in 500-ml flasks containing two different red wines in which alcoholic fermentation was recently completed (Table 2). The wines were from two wine appellations of south Catalonia: DOQ Priorat and DO Terra Alta (near DO Tarragona). They had a high ethanol content (15.5 % in wine W1 and 13.6 % in wine W2) and a low L-malic acid content. These characteristics

**Table 2.** Main characteristics of the wines (after alcoholic fermentation) used in the co-inoculation study

Wine	W1	W2
Wine appellation	DOQ Priorat	DO Terra Alta
Grape variety	Grenache	Cabernet Sauvignon
Ethanol (% v/v)	15.5	13.6
pH	3.4	3.3
L-Malic acid (g/l)	0.43	1.20
Acetic acid (g/l)	0.52	0.49
Citric acid (mg/l)	65	226

are representative of wines from this area. The incubation temperature was 20 °C. MLF was monitored by analyzing L-malic acid and cell viability in culture samples until L-malic acid had been fully consumed. Acetic acid and citric acid were also analyzed at the end of MLF, using a Boehringer (Mannheim, Germany) enzymatic kit. Thirty colonies from the last sample of each wine were picked, identified, and typed as explained above.

**Quantification of biogenic amines.** Decarboxylase activity was activated according to the method of Bover-Cid and Holzapfel [4], subculturing the bacteria three times in modified MRS broth containing 0.1 g of the precursor amino acids L-histidine HCl, tyrosine di-sodium salt, L-ornithine HCl, and L-lysine HCl/100 ml and supplemented with 0.005 g of pyridoxal-5-phosphate/100 ml. The cultures were grown at 28 °C to the late exponential phase after which duplicate aliquots of 0.1 ml were removed and inoculated into 100 ml of screening decarboxylase medium with and without precursor amino acids (0.2 g/100 ml). These cultures were incubated at 28 °C, centrifuged, and the pellet was discarded. The biogenic amine content of the supernatants was determined by high-performance liquid chromatography (HPLC) with a Agilent 1100 (Agilent Technologies, Böblingen, Germany), following the method of Gómez-Alonso et al. [16].

**Detection of genes that encode for biogenic amines.** The presence of genes for biogenic amines was studied in the selected strains by a specific multiplex PCR method [11,12], designed to detect the four genes involved in the production of histamine (histidine decarboxylase, *hdc*), tyramine (tyrosine decarboxylase, *tdc*), and putrescine, via either ornithine decarboxylase (*odc*) or putrescine transcarbamylase (*ptc*). Gene fragments were PCR-amplified with Taq polymerase (Invitrogen) using 100 ng of bacterial DNA and the following oligonucleotides: HDC3 (5'-GATGGTATTGTTTCK-TATGA-3') and HDC2 (5'-CCCGTGTTCCTTTGTCACCT-3') for *hdc*; TD2 (5'-ACATAGTCAACCATRTTGAA-3') and TD5 (5'-CAAATGAAGAAGA AGTAGG-3') for *tdc*; ODC1 (5'-NCAYAARCAACAAGYNGG-3') and ODC2 (5'-GRTANGGNTNNGCACCTTC-3') for *odc* [12]; and the degenerate primers PTC1 (5'-GGWCAAATTCATYITIGG-3'), and PTC2 (5'-CCRTA CCAWACATGIGTRTA-3') for *ptc*. The amplification program, following that of Coton et al. [12], was 95 °C for 5 min, 35 cycles of 1 min at 95 °C, 52 °C for 1 min, 72 °C for 90 s, with a final extension at 72 °C for 5 min. Besides the selected strains, control positive strains were included: *Lactobacillus brevis* IOEB 9809 (from the Institut d'Oenologie, Bordeaux, France) for *tdc* and *ptc*, *O. oeni* IOEB 9204 for *hdc*, and *Lactobacillus saerimneri* 30A (ATCC 33222) for *hdc* and *odc*.

Aliquots of 18 µl of each PCR sample were analyzed on 1 % (w/v) agarose gels (Invitrogen) in 1× TBE buffer, run at 100 V for 45 min, and the resulting bands then visualized by ethidium bromide staining on a GelDoc2000 (BioRad, Ivry sur Seine, France).

**Multilocus sequence typing (MLST) and bioinformatic analysis.** Bacterial genomic DNA was extracted, amplified and sequenced following the method of Bihère et al. [3] and Bridier et al. [5]. Briefly, PCR was carried out in a 50-µl reaction volume containing a DNAzyme PCR master mix (Finnzymes), 10 ng of template DNA, and 10 pmol of each primer associated with one of the seven target genes. The seven targeted housekeeping genes (*gyrB*, *g6pd*, *pgm*, *dnaE*, *purK*, *rpoB* and *recP*) were amplified using the primers described by Bihère et al. [3]. The PCR program was as follows: 95 °C for 3 min, 30 cycles of 95 °C for 30 s, 55 °C for 30 s, and 72 °C for 30 s, with a final extension at 72 °C for 5 min. To better amplify some loci, the annealing temperature was lowered to 45 °C and the number of cycles increased to 35. To amplify the transposon sequence in the *purK* locus, the elongation time was increased to 1 min. PCR fragment amplification was verified by electrophoresis of the PCR products in 1 % agarose gels containing 10 µl of GelRed (Biotium)/100 ml agarose. The PCR products were then sequenced by Eurofins Medigenomix GmbH.

For each locus, the sequences obtained for the different strains were compared and assigned with allele numbers. The sequences obtained for the seven genes of the MLST were analyzed, edited and compared using Bionumerics 5.1. The phylogenetic tree derived from these sequences and from publicly available data [3,5] was constructed by the neighbor-joining method with a Kimura two-parameter distance model, using MEGA 4 [33].

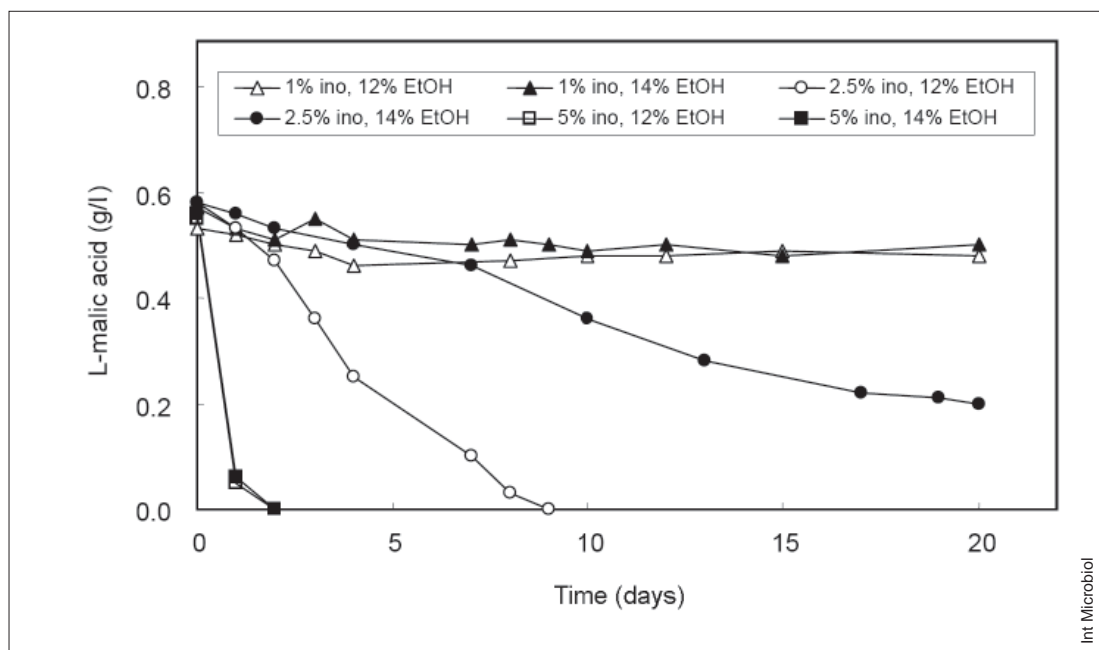
Most of the nucleotide sequences of the MLST loci were the same as reported by Bihère et al. [3] and Bridier et al. [5]. The new sequence types found for strain 8P7 were deposited in GenBank under accession numbers KC923244 (*purK*) and KC923245 (*recP*).

## Results

**Diversity of *Oenococcus oeni* strain profiles in wines.** For all wine samples (Table 1), the LAB populations accounted for 10<sup>4</sup>–10<sup>7</sup> CFU/ml. From these plates, 190 colonies were taken from the DOQ Priorat wines and 50 colonies from the DO Tarragona wines. Almost all of the isolated colonies (99.6 %) were identified as *O. oeni* by species-specific PCR [36], with 21 strain profiles (A to U), differentiated using multiplex RAPD-PCR [26], some of them found in different cellars. From the electrophoresis gels, six different profiles of strains that grew in wines from DO Tarragona (shown in Table 1 as strain profiles P–U) and 15 different profiles of strains that grew in wines from DOQ Priorat (shown in Table 1 as strain profiles A–O) were constructed.

**Optimization of strain screening conditions in wine-like medium.** Among these 21 strains, four (1P2, 10P4, 3T1 and 5T8) were randomly chosen, grown in MRSmf broth medium (reaching 10<sup>9</sup> cells/ml), and inoculated at 1, 2.5, 5, and 10 % (v/v) into wine-like medium containing 10, 12, or 14 % (v/v) ethanol. With 10 % inoculation and 10 % ethanol, MLF was completed in less than 24 h for all strains. The results obtained for one of the strains (5T8) inoculated in different proportions in 12 and 14 % ethanol are shown in Fig. 1. The results for the other strains were similar. A bacterial inoculant of 5 % carried out MLF quickly, while, at the other extreme, an inoculant of 1 % resulted in no MLF after 20 days of incubation. MLF carried out with an inoculant of 2.5 % was, as expected, faster when less ethanol was present. Accordingly, 2.5 % was considered to be the most appropriate inoculant size for screening strains in medium or wine containing 12 % or 14 % ethanol.

**Screening of strains in wine-like medium.** All 21 isolated strains (profiles in Table 1) and the type culture strain CECT 217<sup>T</sup> were tested in wine-like medium under the optimized conditions described above. In the presence of 12 %

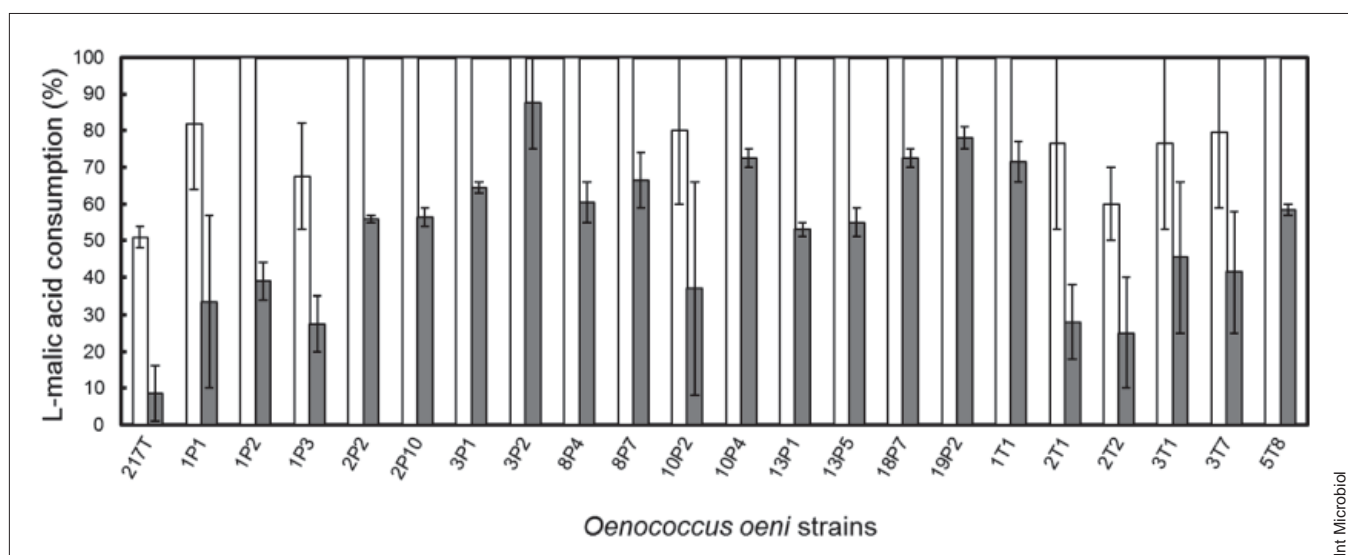


**Fig. 1.** Consumption of L-malic acid in wine-like medium containing ethanol (12 % and 14 %, v/v), by strain 5T8 inoculated in proportions of 1, 2.5, and 5 %.

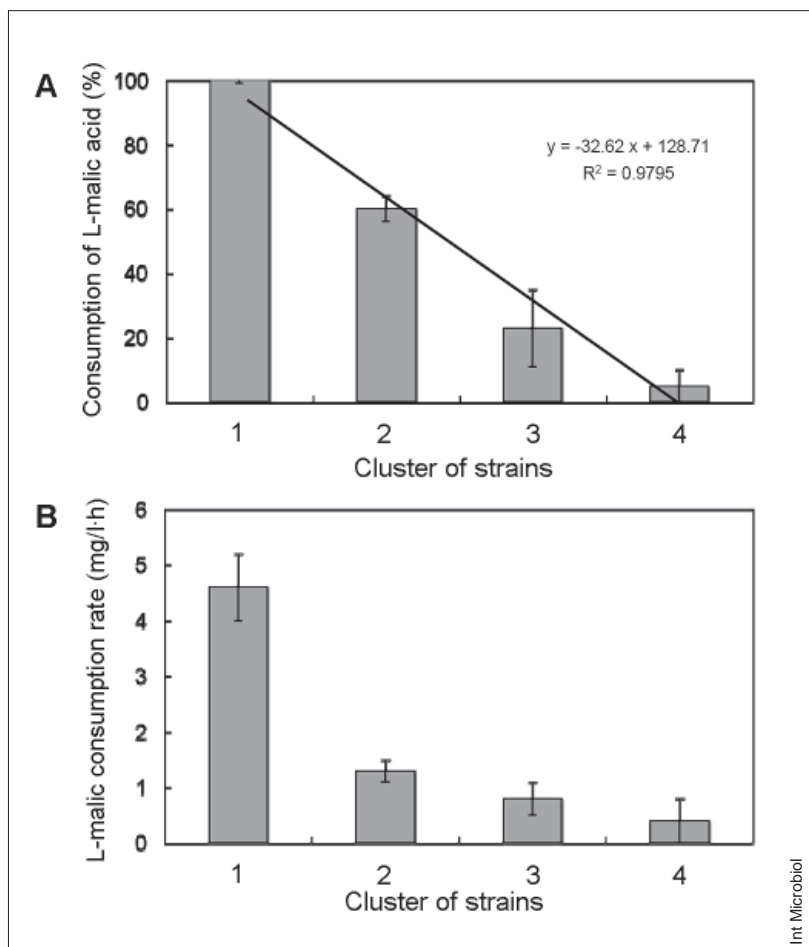
ethanol, most of the L-malic acid was consumed by almost all of the strains (Fig. 2) whereas in the presence of 14 % ethanol, only one strain, 3P2, consumed almost all of the malic acid. Other strains identified as good malic acid consumers were 8P7, 10P4, 18P7, 19P2, and 1T1. Strains that consumed less malic acid were more or less the same in wine-like medium

containing 12 % or 14 % ethanol and included strain CECT 217<sup>T</sup>. As expected, in the presence of 12 % ethanol, the malolactic efficiencies were generally higher, around 6 mg/l per h, and were two to three times higher than those obtained with 14 % ethanol.

These data on L-malic acid consumption were analyzed statistically by grouping the strains in clusters (Fig. 3) and us-



**Fig. 2.** Consumption of L-malic acid (initial 0.6 g/l) by the different strains of *Oenococcus oeni* when growing in wine-like-medium containing 12 % ethanol (white columns) or 14 % (grey columns), at pH 3.4 and 20 °C. Data are the mean ±SD values for duplicate assays.



**Fig. 3.** Consumption of L-malic acid (A), and malolactic efficiency or consumption rate (B), by typed strains of *Oenococcus oeni*, grouped in statistical clusters, when grown in wine-like medium containing 12 or 14 % ethanol.

ing the data from both 12 % and 14 % ethanol. When the strains were grouped in four clusters depending on their consumption of L-malic acid (Fig. 3, top) and their consumption rate (Fig. 3, bottom), the correlation was good. The strains in cluster 1 were 3P2, 8P7, 10P4, 18P7, 19P2 and 1T1, whereas strain CECT 217<sup>T</sup>, among others, belonged to cluster 4.

**Co-inoculation assay in real wines.** In this assay, the selected strains in cluster 1 (3P2, 8P7, 10P4, 18P7, 19P2, and 1T1) were used and co-inoculated. In addition, commercial strain CH11 was added to allow its comparison with these strains and because it is the strain usually used by the cellar of wine W1 to promote MLF. Since W1 and W2 were different, malolactic performance differed as well, but for both wines malic consumption was complete around day 37 (Fig. 4). The population of bacteria in W2 remained stable at  $10^7$  CFU/ml, but in W1 at the end of MLF the population had decreased from  $2.1 \times 10^7$  CFU/ml to  $5 \times 10^4$  CFU/ml.

Citrate consumption was highest (82 %) by cultures in W2 and at the end of MLF was 38 mg/l (Table 2). Citrate levels in

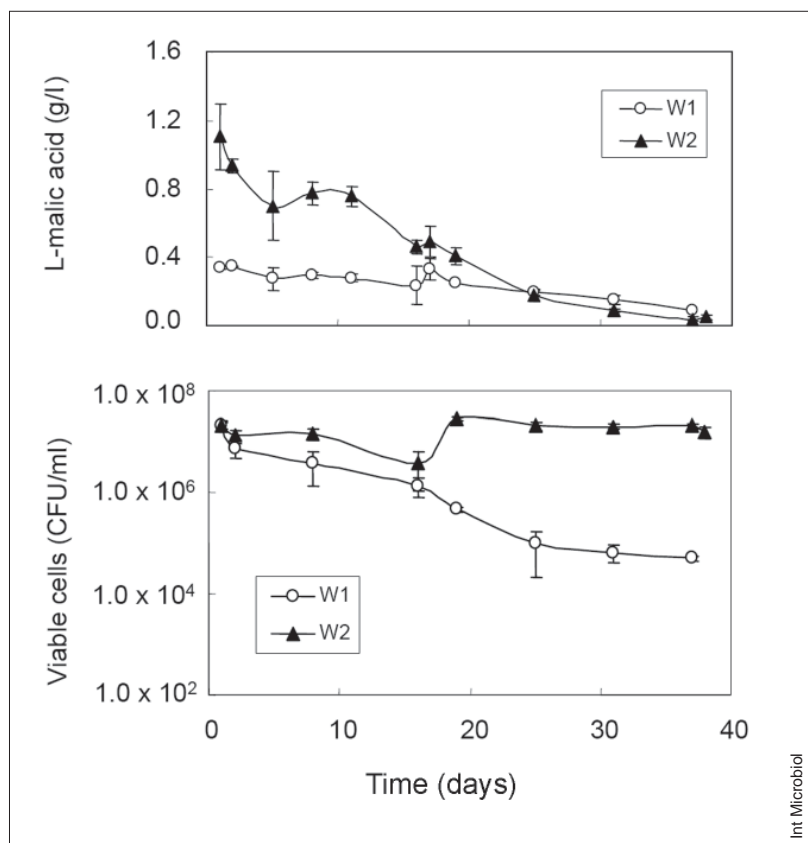
wine W1 did not change significantly throughout the assay. In W2, acetate was not produced during MLF, while in W1 production was 0.1 g acetate/l.

All 30 colonies isolated from each wine at the end of MLF were identified as *O. oeni*. The main RAPD-PCR typing profiles of colonies from W1, in which population viability was low ( $5 \times 10^4$  CFU/ml), were the same as the profile of strain CH11 strain (results not shown), identifying this strain as predominant in W1. For W2, in which population viability was high ( $1.6 \times 10^7$  CFU/ml), three different profiles were obtained, corresponding to strains 3P2 (70 % of total isolates), CH11 (15 %), and 1T1 (15 %).

#### **Absence of biogenic amines and the genes encoding their synthesis in selected strains.**

None of the strains identified as the best performers in the MLF assays (3P2, 8P7, 19P2, 1T1) produced biogenic amines. Electrophoresis of the PCR products of the genes *hdc*, *tdc*, *odc*, and *ptc* showed bands corresponding to genes involved in amine production in the positive control strains (see Materials and meth-





**Fig. 4.** Consumption of L-malic acid (A), and bacterial population (B) in wines W1 and W2 (see Table 2) co-inoculated with seven strains of *Oenococcus oeni* (3P2, 8P7, 10P4, 18P7, 19P2, 1T1 and CH11). Data are the mean  $\pm$ SD values for triplicate assays.

ods): 2300 base pairs (bp) for *hdc*, 1133 bp for *tdc*, 900 bp for *odc*, and 500 bp for *ptc*. However, none of these bands were detected for the strains tested in this study (results not shown).

**Genetic diversity of selected *Oenococcus oeni* strains.** The four strains selected in the microvinifications (3P2, 8P7, 19P2, 1T1) and the two strains that did not perform

well in that experiment (2T2 and CECT 217T) were analyzed by MLST to evaluate their phylogenetic relationships. The seven genes targeted by MLST were successfully amplified and sequenced for all strains. The combination of alleles obtained at each locus defined four distinct allelic profiles or sequence types (STs) (Table 3). The STs of strains CECT 217<sup>T</sup> and 8P7 (designated ST 200 and ST 201, respectively) were

**Table 3.** Alleles of seven housekeeping genes in *Oenococcus oeni* strains determined by multilocus sequence typing (MLST)

Strain	<i>gyrB</i>	<i>g6pd</i>	<i>pgm</i>	<i>dnaE</i>	<i>purK</i>	<i>rpoB</i>	<i>recP</i>	Sequence types*
PSU-1	1	1	1	1	1	1	1	1 same ST as in [3]
1T1	8	11	5	9	8	3	1	82
2T2	8	11	5	9	8	3	1	82 same ST as in [5]
3P2	8	11	5	9	8	3	1	82
8P7	4	1	5	2	30	1	38	201 new ST
19P2	5	1	4	2	2	1	1	11 same ST as in [3]
CECT 217 <sup>T</sup>	4	1	5	2	2	1	1	200 new ST (with known allele but in a new order)

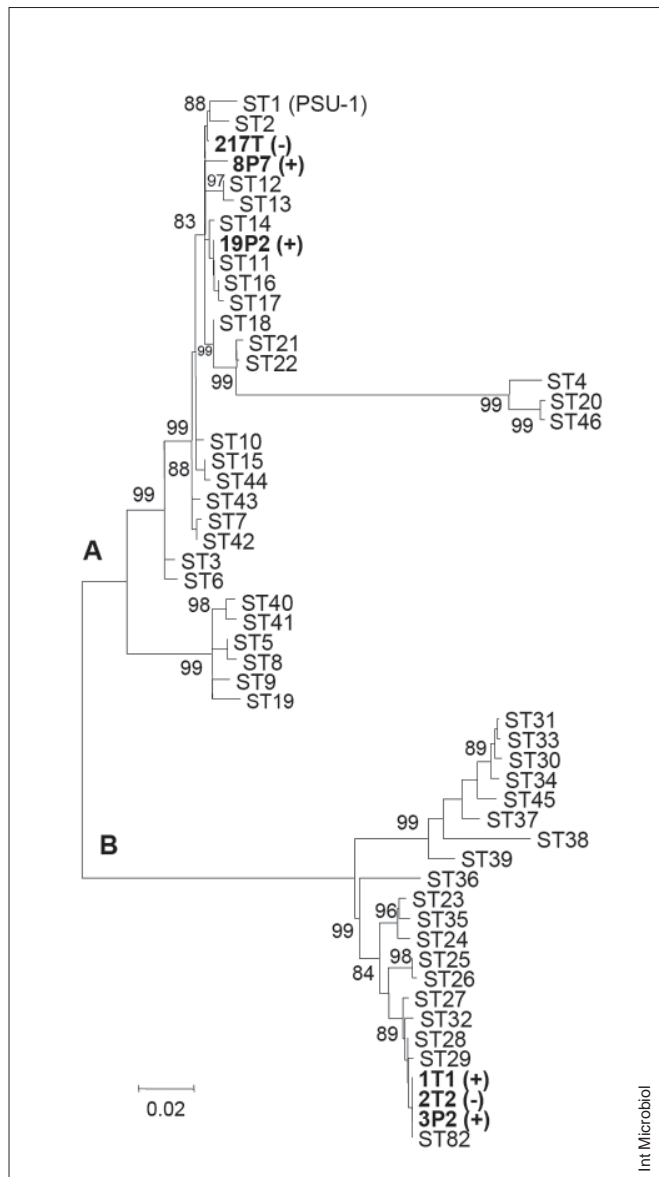
\*Sequence types (STs) were deduced from the allelic profiles obtained for each strain and were attributed according to the findings of Bihère et al. [3] and Bridier et al. [5].

unique, as they had not been detected in previous studies. By contrast, the ST of strain 19P2 (ST 11) was the same as that of a strain reported in [3], and the STs of strains 3P2, 1T1, and 2T2 (ST 82) were the same as that of a strain described in [5].

To evaluate the phylogenetic distribution of the six strains, a 4055-bp sequence was produced for each one by concatenating the sequences of all seven loci analyzed. The concatenated sequences were compared with one another and with 45 similar, previously reported sequences representing the genetic diversity of *O. oeni* [3,5]. A phylogenetic tree derived from the aligned sequences was produced by the neighbor-joining method (Fig. 5). As anticipated from previous studies [3,38], *O. oeni* strains formed two major phylogroups (A and B),

strongly supported by the bootstrap values. The three strains with unique STs were in phylogroup A. Strains CECT 217<sup>T</sup> and 8P7 were closely related to each other but more distantly related to strain 19P2. The three other strains grouped in phylogroup B. To determine whether the strains that grouped with this MLST were different, they were analyzed again by RAPD-PCR using the Coc primer. The results showed that all strains used in this study had different profiles (Fig. 6). This finding was confirmed by the typing method based VNTR at several loci [9], which likewise showed the different profiles of the analyzed strains (data not shown).

Note that the distribution of the six strains in MLST did not correlate with their malolactic performance. In fact, efficient and inefficient strains were equally distributed in the two phylogroups. Moreover, a poorly performing strain (2T2) had the same MLST profile (and therefore the same phylogenetic grouping) as the efficient strains 1T1 and 3P2.

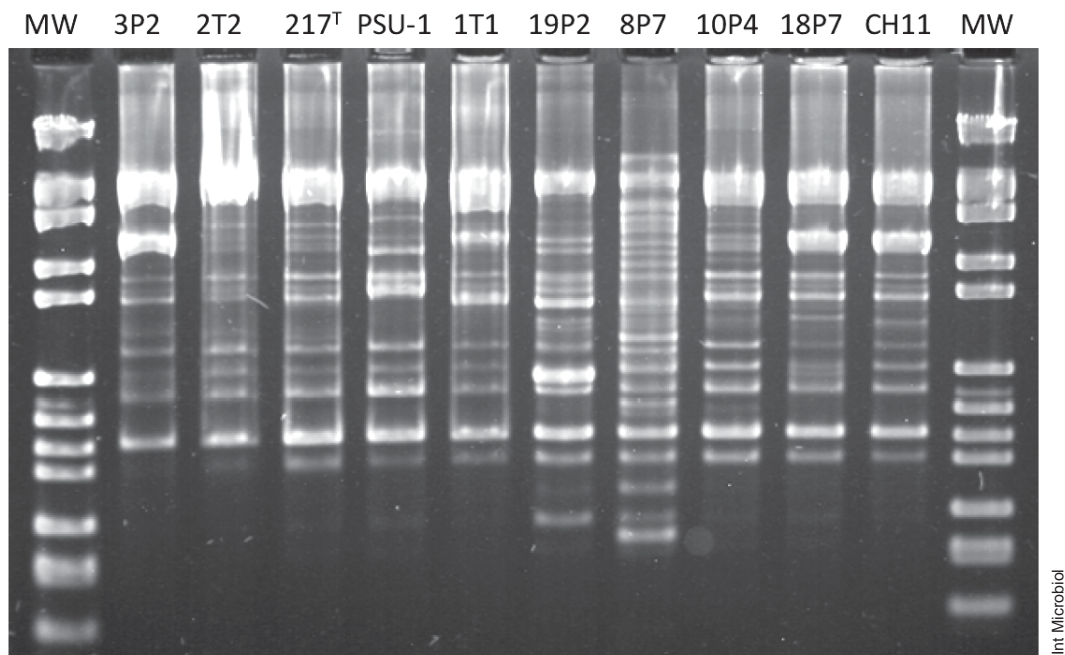


## Discussion

The isolation of 240 LAB colonies from wines with an ethanol content of 14 % and undergoing MLF demonstrates the high ethanol tolerance of the isolates from these wines, suggesting that they are good candidates for use as starters in these kinds of wine. Typing of the isolates yielded the same multiplex RAPD-PCR profiles in several colonies, which allowed us to reduce the number of strains to a total of 21 (Table 1). The same profile, indicative of the same strain, was found in different wines produced in the same cellar. For instance, profile F appeared in five different wines (three Cabernet-Sauvignon, one Carignan, and one Grenache) vinified in cellar C1. This suggests the adaptation of some strains to their environment. By contrast, one only strain, profile M, appeared in wines from cellars C3 and C4, perhaps because these two cellars are located relatively close to each other. Four of the best MLF strains, chosen for their technological properties, and two others, chosen for purposes of comparison, were also typed by MLST. This analysis revealed only four different se-

**Fig. 5.** Phylogenetic distribution of *Oenococcus oeni* strains. The neighbor-joining phylogenetic tree was constructed from the concatenated sequence deduced from the MLST data for each strain. The six strains in this study are indicated in bold and compared with 45 representative strains of the *O. oeni* species characterized in previous studies. The plus and minus symbols in parentheses indicate the efficient and less efficient strains, respectively, as determined by phenotypic characterization. The two major phylogroups (A and B) are indicated.





**Fig. 6.** RAPD-PCR with primer Coc of *Oenococcus oeni* strains used in this study. MW: molecular weight markers.

quence types, but failed to discriminate three strains. This was unusual because it is generally assumed that MLST discriminates bacterial strains better than methods based on banding patterns, such as RAPD or pulse field gel electrophoresis [3]. By applying a typing method based on the VNTR at several loci [9], we found that the strains were indeed different, based on their different profiles (data not shown). An examination of the RAPD-PCR profiles obtained with the Coc primer verified that these strains differed from each other (Fig. 6). Our findings support the use of MLST with these seven loci as a good technique for studying phylogenetic relationships, although, at least for differentiating *O. oeni* strains, it is not as discriminative as RAPD-PCR [22,26] or VNTR methods.

To screen strains in wine-like medium, we optimized the inoculant size and the ethanol content, with the aim of obtaining MLF assay times that were not too short or too long, and which discriminated among strains. After analyzing the results obtained for some strains (Fig. 1), we chose an inoculant size of 2.5 %. Bearing in mind that growth of the inocula in MRSmf broth medium yielded a population size of  $10^9$  per ml (with a 2.5 % inoculant), the initial population in wine-like medium assays was about  $2.5 \times 10^7$  per ml, a reasonable number of viable cells to initiate MLF. This level of inoculation is similar to that reported in previous studies [27,29]. As expected, there were significant differences in MLF effectiveness

depending on the ethanol content, from eight days for 12 % ethanol to more than 20 days for 14 % ethanol.

Screening of the strains in wine-like medium containing 14 % ethanol showed important differences, although the observed trends in malic acid consumption among strains were similar at the two ethanol concentrations. On the basis of both malic acid consumption and its efficiency (consumption of malic acid per unit time), the strains were grouped statistically into four groups, and the six strains of cluster 1 were selected. These six strains were representative of different cellars (1 from C1, 2 from C2, 2 from C5, and 1 from C6), varieties (2 from Cabernet-Sauvignon, 3 from Grenache, and 1 from Carignan) and appellations (5 from DOQ Priorat and 1 from DO Tarragona). L-Malic acid consumption by the best-performing strains in the presence of 12 % and 14 % ethanol (Fig. 3, bottom) was around 6 and 2 mg/l·h, respectively. These efficiencies were higher than those previously obtained in wine-like medium [28,31].

Another step in selecting the best strains was the co-inoculation of the six selected strains and a commercial strain into two wines. This procedure of co-inoculating different strains simulates real conditions in the cellar, where several LAB strains can be found in each wine, one of which usually predominates at the end of the MLF [8]. In our study, two wines were inoculated with a mixture of seven strains that completed

MLF in 37 days (Fig. 4). Nevertheless, in wine W1 the number of viable LAB cells decreased considerably. This wine had a very high ethanol content (15.5 %) and a low L-malic acid content (0.43 g/l), which made it especially difficult for bacteria to grow and to carry out MLF. Despite the relatively small viable population in W1, MLF was completed at the same time as in wine W2. As pointed out by other authors [6], ethanol level has a more important effect on growth than does malolactic acid activity.

The citrate content did not change in W1, whereas in W2 most of the citrate was consumed (from 226 to 38 mg/l); however, there was no increase in the amount of acetate. This quantity of citrate was low (near 1 mmol/l), sufficient to produce just 1 mmol/l acetate (0.06 g/l), which is very low with respect to the initial 0.49 g/l. The difference in citrate consumption between W1 and W2 can be explained by differences in the consumption ability of the predominant strains, with the predominant strain in W1, CH11, as non-citrate consuming.

The isolates of *O. oeni* at the end of MLF in wine W1, which had a very low and decreasing population of bacteria, were mainly typed as the commercial strain CH11, which is known to perform well. In W2, however, in which MLF was higher because of an initially higher malic acid content and a sustainable viable LAB population, the predominant isolates were typed as strain 3P2. This strain performed better than all the others, including the commercial strain CH11, and it was therefore selected for further studies.

One of the considerations in the selection of MLF strains is that they should not produce biogenic amines [20]. Although most *Oenococcus* strains produce few or no amines [24], the optimal strains would be those lacking the genes encoding enzymes involved in amino acid decarboxylation [25]. For this reason, the strains selected in this study were tested for amine production and for the presence of four genes essential to the production of the main amines (histamine, tyramine, and putrescine). The results showed that none of these strains produced these amines nor did they contain the corresponding genes. These strains are therefore good candidates for MLF as their presence in wine does not pose any risk to human health.

The phylogenetic distribution of the six strains selected was determined by comparing their MLST data with those from previously characterized *O. oeni* strains. The six strains were equally distributed in the two phylogroups, A and B (Fig. 5), distinguished by other authors in their analyses of several dozen *O. oeni* strains [3,5]. In the study of Bilhère et al. [3], most of the commercial strains, which are assumed to

be those best adapted for MLF, were grouped in subpopulation A, suggesting that their genotypic traits are related to their phenotypic traits with respect to MLF performance and adaptation to wine. However, our strains could be assigned to both subgroups, with the best-performing strain (3P2) placed in subgroup B. This suggests that phenotypic grouping is not related to the sequences of the housekeeping genes used in the analysis and that other genes are more closely related to adaptation and ethanol tolerance. This becomes even more apparent by considering the low performing strain 2T2, as its MLST profile was exactly the same as the profiles of the efficient strains 1T1 and 3P2. Bridier et al. [5] showed that *O. oeni* strains from a given geographic zone group together to form a well-defined subgroup amongst the strains of phylogroups A or B. This was not the case, however, for the strains described in this study, isolated from cellars in the Priorat and Tarragona DOs. Not only were those strains scattered amongst the two major phylogroups, so were the strains from the same DOQ Priorat.

It is still unclear whether this dispersion means that strains from the DOQ Priorat and DO Tarragona are extremely divergent from a genetic point of view. These findings point to the need to analyze many more strains before this question could be answered. However, our results showed that strains belonging to phylogroups A and B could be encountered in these regions, and that strains within both types differed in their MLF efficiencies. This suggests that strains adapted to given regions should be selected on the basis of geographic origin as well as on their behavior, as determined in winemaking trials, rather than on phylogenetic criteria.

In conclusion, in this study some strains that could carry out MLF in high ethanol wines were selected for their performance in wine-like media and by co-inoculation assay with different strains in real wine. The genetic diversity of these strains was studied by multi-locus sequence typing, and they grouped in two phylogroups, as in previous studies, but this grouping was not related to their ability to adapt and perform an efficient MLF. The absence of genes for biogenic amines in these strains was confirmed, which suggests that they would be good candidates as starter cultures.

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