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53

Malolactic fermentation: genetics and control

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INTRODUCTION

The control of malolactic bacteria, the lactic acid bacteria (LAB) that carry out malolactic fermentation (MLF), is an important part of the technology of modern commercial wine production. This reaction involves the decarboxylation of L-malic acid naturally occurring in wines to L-lactic acid and CO_2, and makes sharp and acid wines smooth and palatable. There are some advantages to this reaction: 1) a 0.1 to 0.3% reduction in the total acidity of wines (Castino *et al.*, 1975; Fornachon, 1957; Pilone *et al.*, 1966; Rankine, 1977); 2) organoleptic modifications, as a consequence of the formation of flavor compounds from the sugar and organic acid catabolism (Chen *et al.*, 1983; Delfini and Di Stefano, 1984; Fornachon and Lloyd, 1965; Pilone *et al.*, 1966); and 3) microbial stability: wines in which MLF is completed are less susceptible to spoilage (Kunkee, 1967a; Rankine, 1972; Rankine and Bridson, 1971). For these reasons MLF is advisable in some wines. There are different methods for initiating it: 1) stimulation of indigenous LAB development; 2) inoculation with wine already undergoing MLF; 3) passage of the wine over supports of immobilized LAB or enzymes; and 4) inoculation with either laboratory-prepared or commercial strains. These two last methods make it possible to control the time, speed and final characteristics of wines. Many authors have used LAB starters to control the process of MLF (Beelman *et al.*, 1977; Beelman *et al.*, 1980; Descout, 1980; Gallander, 1979; Kunkee, 1967b).

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MLF is not always beneficial and can be responsible for undesirable changes in the sensory properties or color of wine, and may even lead to generation of amines (Davis *et al.*, 1985). In some low acid wines MLF should be completely inhibited to prevent excessive deacidification. Inhibition can be achieved by: maintenance of wine pH at less than 3.2; alcohol concentration above 14%; low storage temperatures; addition of bacterial inhibitors such as SO_2, fumaric acid, or sorbic acid; early racking and clarification; reduction in skin contact and hot-pressing; pasteurization; and sterile filtration (Davis *et al.*, 1985).

In addition to the traditional methods for controlling MLF described above, the emerging capabilities of genetic manipulation of LAB introduce new perspectives related to basic and applied aspects.

MATERIALS AND METHODS

Program selection for malolactic starters

In order to select suitable LAB strains, able to perform MLF in wine, the following criteria were applied: 1) high malolactic activity; 2) tolerance to high concentrations of ethanol; 3) resistance to high levels of SO_2; 4) ability to grow and perform malolactic fermentation at low pH; 5) resistance to low temperatures; 6) inability to produce dextran; 7) production of good organoleptic properties in wine; and 8) no presence of lysogenic phages and resistance to infection.

The rate of L-malic, citric or tartaric acid degradation was measured in synthetic medium C with 10 g/l of the appropriate organic acid (Chalfan *et al.*, 1977). Degradation of fumaric acid (0.5 g/l) was observed in TJ medium (Chalfan *et al.*, 1977). MLF was also monitored in sterile filtered rosé Bobal wine with 12% ethanol, 7.19 g/l of total acidity stated as tartaric acid, 2.15 g/l of L-malic acid, and 50 and 16 mg/l of total and free SO_2 respectively.

To determine the malolactic activity of the strains, ten lots of Bobal wine having the characteristics above described were adjusted to pH values of 3.1, 3.2, 3.3, 3.4, 3.5, 3.6, 3.7, 3.8, 3.9 and 4.0 with NaOH or HCl. In all the other experiments the pH of the wine was set at 3.5. To raise the original ethanol content of the wine (12% v/v), absolute ethanol was added to obtain 13, 14, 15, and 16% in the wine. Potassium metabisulphite was added to attain concentrations of 100, 150 and 200 mg/l of total SO_2 (50 mg/l of SO_2 were originally present in the wine). The final concentrations of free and total SO_2 were verified by the titration method with Iodine (García Barceló, 1976). To assess the influence of temperature, inoculated tubes were placed at 10, 15, 18, 20 and 25°C. The rest of the experiments

were performed at 25°C.

The ability to grow in wine at pH values of 3.5 or lower was tested. The strains were inoculated in sterile wines at different pH values: 3.1, 3.2, 3.3, 3.4 and 3.5. Several methods for evaluating LAB growth were tested: microscopic counting, particle counting (Coulter Counter ZM), spectrophotometric method and viable counts on MLO or MRS solid medium.

To test the ability of LAB to produce dextran from sucrose, ATB medium to which 5% sucrose has been added was inoculated with cultures and incubated in anaerobiosis for 14 days at 28°C (Garvie, 1967). To detect the presence of lysogenic phages, induction was accomplished with Mitomycin C as in Arendt *et al.* (1991).

Preculture media for inoculation in wine

Fourteen preculture media were tested for the growth of a *Leuconostoc oenos* strain (M42) isolated from wine. Shirac wine with a low pH (3.09) and a high L-malic acid content (7 g/l) was inoculated with the M42 strain grown in different preculture media:

1.- Must
2.- Must+0.5% yeast extract
3.- Must+0.5% yeast extract+5 mg/l Tween 80
4.- Must+0.5% yeast extract+5 mg/l Tween 80+2.3% tomato juice
5.- Must+0.5% yeast extract+5 mg/l Tween 80+2.3% tomato juice+10% wine
6.- Must+0.5% yeast extract+5 mg/l Tween 80+2.3% tomato juice+20% wine
7.- Must+0.5% yeast extract+5 mg/l Tween 80+2.3% tomato juice+40% wine
8.- Must+0.5% yeast extract+5 mg/l Tween 80+2.3% tomato juice+5% ethanol
9.- Must+0.5% yeast extract+5 mg/l Tween 80+2.3% tomato juice+10% ethanol
10.-Wine+0.5% yeast extract+5 mg/l Tween 80+2.3% tomato juice+0.5% glucose
11.-MLO
12.-MLO+0.7% malic acid
13.-MLO+0.7% malic acid+10% wine
14.-MLO+0.7% malic acid+5% ethanol

Commercial red must was diluted twofold with water, and the pH of all the media adjusted to 4.5. The wine for inoculation was a Shirac with a pH of 3.09, 7 g/l of malic acid, and 2 g/l of reducing sugars. Cells at mid-logarithmic phase were inoculated in sterile Shirac wine up to a final

concentration of 10^5 cfu/ml. Growth in wine was followed by plate viable counts in MLO medium. Population dynamics and malolactic activity were analyzed throughout.

Quantification of malolactic activity

Approximately 10^{11} cells were harvested from cultures by centrifugation at 10,000 x *g* for 20 minutes at 4°C. The pellets were washed with 10 ml of 0.05 M KH_2PO_4-Na_2HPO_4 buffer (pH 6.0), and finally resuspended in 2 ml of the same buffer. Cells were disrupted with 2 g of glass beads for 10 minutes at 4°C, and the crude extract was centrifuged at 48,000 x *g* for 30 min at 4°C to eliminate the debris. The supernatant fraction was recovered and stored at -20°C until use.

Protein content of the cell-free extracts was measured by the Micro BCA Protein Assay Reagent (Pierce) and the malolactic activities with a CO_2 electrode (Lonvaud and Ribéreau-Gayon, 1973). The reaction mixture was 2.5 ml of 0.2 M sodium-potassium phosphate (pH 6.0), 0.45 ml of 0.05 M L-malic acid, 0.45 ml of 0.05 M NAD^+, and 0.7 ml of 0.02 M $MnCl_2$. Crude extracts were added to a final protein concentration of 0.1-0.2 mg/ml, and water was supplied up to 5 ml total volume. The activities were measured as μmoles CO_2/min/mg protein.

Genetic techniques

The isolation of MLF^- mutants could provide good material for studying the physiology of MLF, as well as recipient cells for cloning the structural gene responsible for this character. Mutagenesis of selected strains was accomplished with UV light, and colonies isolated in MMM medium (Zúñiga *et al.*, personal communication). To clone the MLF gene into cells, several plasmids were tested as cloning vectors (pGK12, pGK13, pVA797, pLO1). Electrotransformation was carried out with a Bio-Rad Gene Pulser using different conditions: voltage, resistance, buffers, cuvettes, competent induction procedures, etc. Conjugation was accomplished on membranes lied on solid media, and different selection conditions were tested. Cryptic plasmids were isolated from *Leuconostoc oenos* with the method of alkaline lysis (Maniatis *et al.*, 1982). Nuclease activity of *L. oenos* was tested following the method of Mayo *et al.* (1991).

Analytical techniques

Metabolite analyses were accomplished with one or several of the following techniques: a) paper chromatography (Pardo and Zúñiga, 1992), b) enzymatic assays (Boehringer-Mannheim, 1984), c) HPLC analysis (Frayne, 1986), or d) CO_2 electrode measurements (Lonvaud and Ribéreau-Gayon, 1973).

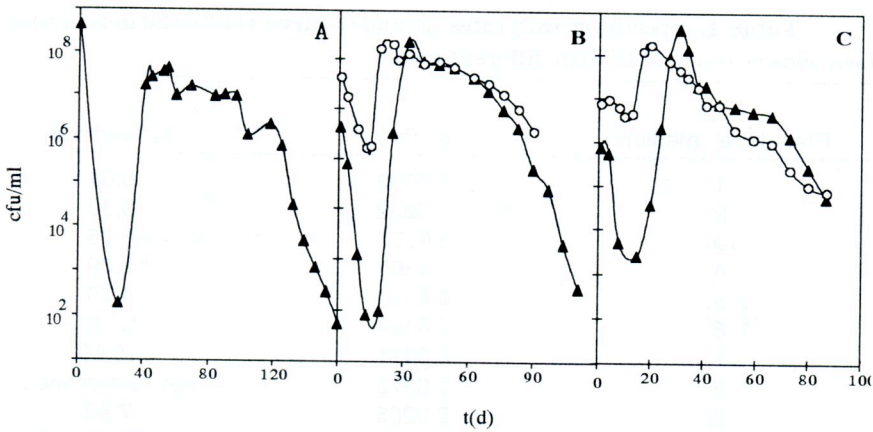

Figure 1.-Growth of *L. oenos* M42 in wine at pH 3.3. Panel A: inoculated with cells from MLO. Panel B: inoculated with wine from panel A. Panel C: inoculated with wine from panel B. Open circles: inoculum of 10%. Closed triangles: inoculum of 1%.

RESULTS

Program selection for malolactic starters

Forty-three LAB strains isolated in the Utiel-Requena region and belonging to the species *Lactobacillus brevis, Lactobacillus confusus, Lactobacillus fructivorans, Lactobacillus hilgardii, Lactobacillus plantarum, Leuconostoc oenos,* and *Leuconostoc paramesenteroides,* as well as eight unidentified strains, were tested using the selection criteria described above (see Material and Methods). Strains such as *L. brevis* and *L. hilgardii* which were less active in the L-malic degradation in synthetic Medium C (Chalfan *et al.*, 1977) were discarded.

L. oenos strains were able to degrade the malic acid by overcoming the negative factors found in wine: low pH, high ethanol concentration, low temperatures, and SO_2 concentrations of 50 mg/l. In addition, they could not degrade tartaric acid, which promote the 'tourné' spoilage, did not produce dextran from sucrose, and were able to grow in wine at low pH. Of the seven strains belonging to *L. oenos* the most interesting were G6, M41, M42, and 172 (Pardo, 1987). Strain G41 seems to carry a lysogenic phage, making it unsuitable as a malolactic starter. Since *L. oenos* has been described as the species responsible for MLF in many viticultural areas, such as Utiel-Requena (Pardo and Zúñiga, 1992), our studies have focused on this organism.

Strains were inoculated in wine to observe their ability to grow in

Table 1.- Specific growth rates (μ) and % survival after 20 min in wine of *Leuconostoc oenos* M42 with different media.

Preculture medium	μ (h^{-1})	% survival
1	0.0093	0.01
2	0.0232	0.30
3	0.0377	0.75
4	0.0350	2.00
5	0.0560	4.70
6	0.0484	12.40
7	0.0411	15.60
8	0.0372	not determined
9	0.0288	7.50
10	0.0118	59.30
11	0.1002	0.00
12	0.0709	0.00
13	0.0753	8.10
14	0.0753	0.03

this environment. A pH interval of 3.1 to 3.5 was chosen because it is crucial for bacterial growth. To study the development of bacteria in the wine we used the counts of viable cells because other methods were not appropriate. Of the selected *L. oenos* strains, three of them were able to grow in wine (G6, M41 and M42). The strain most resistant to the acidic conditions was M42, which grew at pH 3.3; G6 at 3.4 and M41 at 3.5. When bacteria previously grown in synthetic medium (MLO) were inoculated in wine, the viability was reduced from about 10^8 cfu/ml to 10^2 cfu/ml) or less (Figure 1). After a more or less extended period these strains began a logarithmic growth, reaching maximum populations of 10^7-10^8 cfu/ml. These values seem to be characteristic of the wines (Beelman *et al.*, 1982; Beelman *et al.*, 1980; Costello *et al.*, 1985). We thought that the initial mortality could be reduced by successive culture in wine, and we managed to induce the malolactic fermentation in a wine of pH 3.1, using a final cellular concentration as low as 7.5×10^5 cfu/ml.

Preculture media for inoculation in wine

To reduce the initial high mortality of the bacteria inoculated in wine, different preculture media were used (see Materials and Methods).

Growth kinetics of *L. oenos* M42 in some preculture media are shown in Figure 2. The highest specific growth rate for the preculture media was obtained when the M42 strain was grown in MLO medium (Table 1). Addition of L-malic acid, ethanol or wine led to a slowdown of these values. The supplementation of must with yeast extract, Tween 80, or tomato juice increased the growth rates, whereas the presence of wine

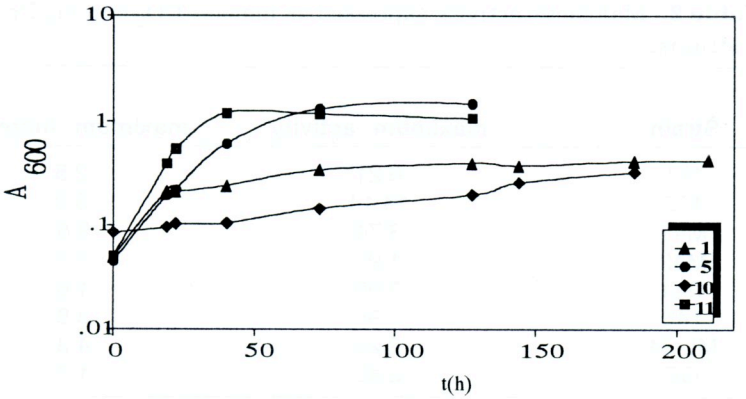

Figure 2.-Growth of *L. oenos* M42 in different preculture media (1, 5, 10, and 11).

or ethanol reduced them.

When active growing cells were inoculated in wine, a week after inoculation we could not detect any viable cells even spreading undiluted samples onto MLO plates. We therefore decided to study the survival of *L. oenos* M42 in wine coming from the different preculture media after shorter periods of time. Samples of wines were taken 20 min after inoculation, and the results are shown in Table 1. Generally, the viability drops dramatically, and survival rates are quite small. The wine kills all the cells when must alone or the synthetic medium MLO (media 1 and 14) is used as preculture. The addition of L-malic acid to the MLO (medium 12) does not increase the viability of the cells, but this can be achieved by the addition of different supplements to the must (media 2-4). However, values rise when wine or ethanol is included in the preculture medium, either for supplemented must or MLO (media 5-9, 13-14). In these cases, values obtained with ethanol are lower than those observed with wine, and an increase in the values yields better results. The best medium is supplemented wine (medium 10), which can provide up to 60% survival in wine after 20 min.

Nevertheless, 60% survival is quite low if we take into account the fact that samples are processed after only 20 min in wine. In an attempt to increase these values, we tested the viability of cells grown in preculture medium 10 to early stationary phase, and 70% survival was achieved. Another important factor is the pH of the preculture, because as the difference between the pHs of the preinoculum and the wine increases, the viability will decrease. When the pH of medium 10 was lowered from 4.5 to 3.5, the survival of exponential cells increased from 60% to 90% after 20 min in the wine (pH 3.1).

Table 2.- Malolactic activity expressed as mmoles CO_2/min/mg Pr of the *L. oenos* strains.

Strain	maximum activity	maximum increase
171	6.21	2.5
172	14.14	9.0
M41	3.74	3.8
M42	1.91	2.2
G41	3.00	1.6
T46	3.95	3.8
ML-34	2.28	4.3
G6	2.42	1.7

The growth phase of a starter in a preculture medium affects its technological properties. To test this, wine supplemented with yeast extract, Tween 80, tomato juice, and glucose, at a pH of 4.5 was used to grow *L. oenos* M42. Cells collected at early stationary phase provided the greatest viability when inoculated in wine. The viability drop can be prevented by subculturing in wines at progressive decreasing pH values. The number of cells required for malolactic fermentation can also be lowered (see before).

Malolactic bacteria may be inoculated at the following stages of vinification: 1) before alcoholic fermentation; 2) during alcoholic fermentation; and 3) after completion of alcoholic fermentation. Inoculation prior to alcoholic fermentation is not recommended when heterolactic bacteria are used because they can attack the sugars present in wine, producing high amounts of acetic and lactic acids that spoil the wine (Davis *et al.*, 1985). To check this assumption we inoculated *L. oenos* strain M42 both before and after alcoholic fermentation. When inoculation was before, MLF was faster (4 instead of 11 days) and acetic acid production was slightly lower (0.24 instead of 0.32 g/l). Sensory analysis revealed that wines inoculated before alcoholic fermentation were preferred by a taste panel. These results argue for an inoculation of the malolactic starter before alcoholic fermentation.

Characterization of the malolactic activity

The proper use of *L. oenos* as starters in wine involves studying the conditions that lead to the highest malolactic activity, such as the influence of the L-malic acid concentration or the growth phase of the cultures.

Growth and malolactic activities of two pattern strains (172 and M42) are presented in Figures 3-4. All the *L. oenos* strains that we have

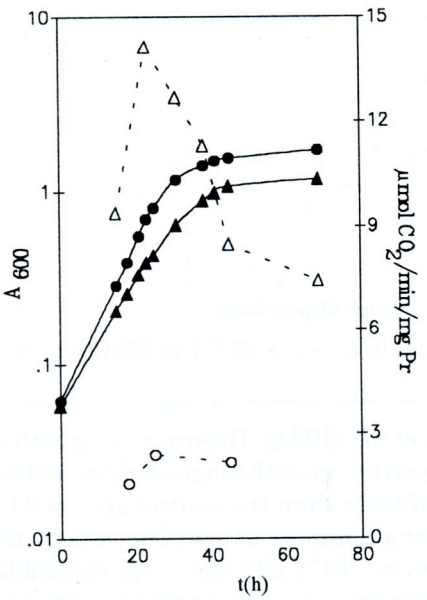

Figure 3.-Growth (closed markers) and malolactic activity (open markers) of cell free extracts of *L. oenos* 172 in MLO medium at pH 4.8. Triangles: 10 g/l DL-malic acid added. Circles: no malic acid added.

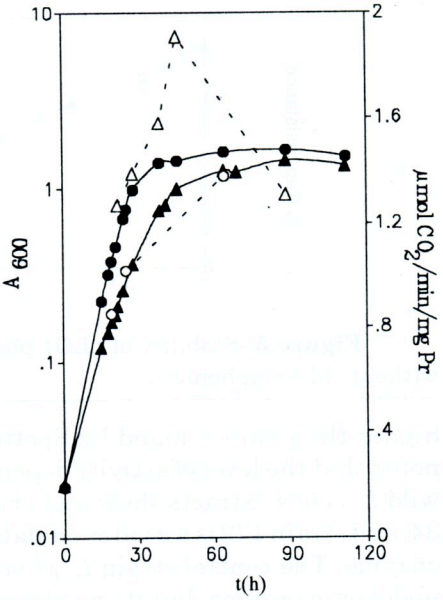

Figure 4.-Growth (closed markers) and malolactic activity (open markers) of cell free extracts of *L. oenos* M42 in MLO medium at pH 4.8. Triangles: 10 g/l DL-malic acid added. Circles: no malic acid added.

analyzed show inducible malolactic enzymes. We have found two different behaviors: a first group (strains 171, 172, and ML-34, Figure 3) had always low levels of malolactic activity during the growth in the absence of malic acid, and a second one (strains M41, M42, G41, and T46, Figure 4) increased this activity during growth both in the presence and absence of malic acid. For all the strains, the maximum activity reached without malic acid was lower, except for M41 in which both maxima were similar. In this organism the presence of malic acid only advanced the synthesis of the malolactic enzyme. The maximum activity is reached at the end of the exponential growth phase.

The induction of malolactic enzyme in *L. oenos* implies a 1.5 to 9-fold increase in the basal levels of activity (Table 2). Two-fold increases in malolactic activities have previously been described for lactic acid bacteria (Henick-Kling, 1986). The fact that maximum values of malolactic activity in our study show a great heterogeneity depending on the strain, agrees with the findings of other authors (Lonvaud, *et al.*, 1977 and Lonvaud and Ribéreau-Gayon, 1975), and demonstrates the need of strain selection programs. *L. oenos* ML-34 shows a maximum activity of 2.28, slightly

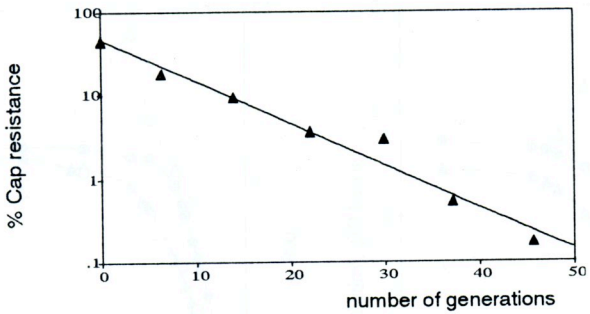

Figure 5.-Stability of pLO1 plasmid in *L. oenos* 86611 in MLO medium without chloramphenicol.

higher than values found by Spettoli *et al.* (1984). However, it must be noted that the level of activity depends on the growth stage. Almost all the wild *L. oenos* extracts show higher activities than the control strain ML-34, and strain 172 is a good candidate for a commercial use of its malolactic enzyme. The control strain *L. plantarum* CECT 220 shows an inducible malolactic enzyme, but its maximum activity (0.31) is lower than that of any *L. oenos* strain.

Isolation and characterization of MLF⁻ mutants of L. oenos

We have developed a selective medium for *L. oenos* mutants defective in malolactic fermentation (MMM). The colonies isolated after incubation in MMM were checked for malolactic activity in basal medium supplemented with L-malic acid, and those that showed no malolactic activity were selected as MLF⁻ mutants. 50% of the colonies isolated on MMM plates after UV mutagenesis were real MLF⁻. On the other hand, when total protein extracts of wild strains and several mutants were run on PAGE, it was observed that all the mutants lacked of a band present in the wild strains, the size of which corresponds to that described for the malolactic enzyme of *L. oenos* (Battermann and Radler, 1991). This suggests that the malolactic enzyme is not being produced by the mutants tested. At present we are checking whether the band absent in the protein pattern of the mutant strains is actually the malolactic enzyme.

Conjugation assays

Lactococcus lactis LM2301 harboring plasmid pVA797 (Evans and Macrina, 1983) was chosen as donor strain for conjugative plasmid transfer assays. To transfer pVA797 from *L. lactis* LM2301/pVA797 to *L. oenos* M42, the best conditions for incubating filters were achieved on plates of MLO at pH 5.5 and 28°C under anaerobic conditions. Selection was performed on MLO plates with chloramphenicol as selective antibiotic.

Plasmid pVA797 could be transferred to the *L. oenos* M42 strain, but at extremely low frequencies. A few *L. oenos* colonies appeared on the selection plates but they lacked chloramphenicol acetyl transferase (CAT) activity, and plasmids could not be detected in these colonies. Finally, we were able to isolate one colony, called 86611, which carries a plasmid (pLO1) and shows CAT activity. This means that the CAT gene from pVA797 is expressed in *L. oenos* 86611. However, we have observed that pLO1 is not stably maintained in *L. oenos* (Figure 5).

Restriction mapping of pLO1 revealed that this plasmid is a fragment of pVA797 that includes one replication origin and the Cap resistance gene.

Electroporation of L. oenos

Several methods for obtaining competent cells, different electroporation conditions and cloning vectors were assayed with M42 and CECT 4028 strains of *L. oenos*. Plasmids were pGK12, pGK13, pLO1, pVA797, and several constructions were obtained by inserting a cryptic plasmid isolated from *L. oenos* CECT 4028 into pHV60 or pHV60*repA* (these are derivatives of pBR322 with the Cap resistance gene from pC194). No transformants were isolated from these assays. We are now sequencing the cryptic plasmid of *L. oenos* CECT 4028.

Nuclease activity of *L. oenos* strains was evaluated in order to test whether degradation of incoming DNA could account for the lack of transformants. No nucleases could be detected under our assay conditions. Problems in the transformation of *L. oenos* may be due to the DNA entry into the cells, expression, and/or stabilization of genetic information. Since pLO1 can be replicated in *L. oenos* 86611, and CAP activity coded by this plasmid can be detected, the introduction of foreign DNA must be one of the keys to the problem.

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