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# Malolactic Fermentation by Engineered *Saccharomyces cerevisiae* as Compared with Engineered *Schizosaccharomyces pombe*

VIRGINIE ANSANAY, SYLVIE DEQUIN*, CAROLE CAMARASA, VALERIE SCHAEFFER, JEAN-PIERRE GRIVET†, BRUNO BLONDIN, JEAN-MICHEL SALMON AND PIERRE BARRE

*IPV-Laboratoire de Microbiologie et Technologie des Fermentation, INRA-ENSAM 2 Place Viala F-34060, Montpellier cedex 01, France*
*†Centre de Biophysique Moléculaire, CNRS, 1A Av. de la Recherche Scientifique, F-45071 Orléans Cedex 02, France*

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The ability of yeast strains to perform both alcoholic and malolactic fermentation in winemaking was studied with a view to achieving a better control of malolactic fermentation in enology. The malolactic gene of *Lactococcus lactis (mleS)* was expressed in *Saccharomyces cerevisiae* and *Schizosaccharomyces pombe*. The heterologous protein is expressed at a high level in cell extracts of a *S. cerevisiae* strain expressing the gene *mleS* under the control of the alcohol dehydrogenase (*ADH1*) promoter on a multicopy plasmid. Malolactic enzyme specific activity is three times higher than in *L. lactis* extracts. *Saccharomyces cerevisiae* expressing the malolactic enzyme produces significant amounts of L-lactate during fermentation on glucose-rich medium in the presence of malic acid. Isotopic filiation was used to demonstrate that 75% of the L-lactate produced originates from endogenous L-malate and 25% from exogenous L-malate. Moreover, although a small amount of exogenous L-malate was degraded by *S. cerevisiae* transformed or not by *mleS*, all the exogenous degraded L-malate was converted into L-lactate via a malolactic reaction in the recombinant strain, providing evidence for very efficient competition of malolactic enzyme with the endogenous malic acid pathways. These results indicate that the sole limiting step for *S. cerevisiae* in achieving malolactic fermentation is in malate transport. This was confirmed using a different model, *S. pombe*, which efficiently degrades L-malate. Total malolactic fermentation was obtained in this strain, with most of the L-malate converted into L-lactate and $CO_2$. Moreover, L-malate was used preferentially by the malolactic enzyme in this strain also.

KEY WORDS — *Saccharomyces cerevisiae*; *Schizosaccharomyces pombe*; *Lactococcus lactis*; malolactic enzyme; malolactic fermentation; heterologous expression; NMR

## INTRODUCTION

In winemaking, lactic acid bacteria carry out the malolactic fermentation (MLF), which usually occurs after alcoholic fermentation. Malolactic fermentation results in deacidification via the conversion of L-malate into L-lactate and $CO_2$ catalysed by the malolactic enzyme (MLE). The main advantages of MLF in winemaking are an improved organoleptic balance of wine and microbiological stability through the removal of the substrate L-malate. However, the poor development of lactic acid bacteria in wine often leads to delayed MLF or even prevents the reaction. One solution to achieving better control of the MLF would be to perform both the alcoholic and malolactic fermentations with a single wine yeast strain.

The gene *mleS*, encoding the MLE of *Lactococcus lactis*, has recently been cloned (Ansanay *et al.*, 1993; Denayrolles *et al.*, 1994). Despite the homologies between the MLE and the malic enzyme family, the former is a single bifunctional enzyme requiring $NAD^+$ and $Mn^{2+}$ and converts L-malate to L-lactate without free intermediates or net reduction of $NAD^+$.

The aim of the work reported was to study the potential of a yeast into which malolactic activity

*Corresponding author.

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was transferred to perform the MLF, allowing for the fact that complete L-malate degradation is an absolute requirement for winemaking. Preliminary results concerning the expression of *L. lactis* MLE in *Saccharomyces cerevisiae* under the control of alcohol dehydrogenase (*ADH1*) regulatory elements have been reported previously (Ansanay *et al.*, 1993), showing that the MLE was functional in *S. cerevisiae*. Significant amounts of L-lactate were produced with this strain expressing MLE in the presence of exogenous L-malate in the growth medium. These results were recently confirmed by other workers (Denayrolles *et al.*, 1995). In addition, when L-malate was absent from the growth medium, L-lactate production was also observed (Ansanay *et al.*, 1993) but to a lesser extent. It is therefore likely that the L-lactate produced by the recombinant *S. cerevisiae* strain was derived from the degradation of both endogenous and exogenous L-malate.

It was therefore of interest to assess the origin of L-lactate production from the available L-malate pools (exogenous and endogenous) and to evaluate the efficiency of competition of MLE with other malic acid degradation pathways. It is shown, through filiation of [$^{13}$C]glucose and [$^{14}$C]malic acid, that most of the L-lactate produced by the recombinant *S. cerevisiae* strain under enological conditions arises from glucose and only a small part from exogenous L-malate. Moreover, it is demonstrated that all the exogenous L-malate degraded by the recombinant strain is converted into L-lactate and $CO_2$, providing evidence for efficient competition of MLE with other enzymatic malate degradation pathways. In contrast, we report that expression of *mleS* in *Schizosaccharomyces pombe*, a yeast basically degrading L-malate by the malic enzyme with high efficiency, is sufficient to achieve total MLF. These results suggest that the limited ability of *S. cerevisiae* to achieve MLF is probably related to a limitation of malate transport.

## MATERIALS AND METHODS

### *Strains and culture conditions*

*Escherichia coli* DH5α was used for cloning experiments. The strain *L. lactis* IL1441 used as control for expression studies has been described previously (Renault and Heslot, 1987). The *S. cerevisiae* strain used in this study is V5 (*MAT***a** *ura3*) and is derived from a Champagne wine strain. The *S. pombe* strain *leu1-32h+* is from the Bern collection. *E. coli* cultivation and media have been described previously (Sambrook *et al.*, 1989). *S. cerevisiae* and *S. pombe* were grown in YPD medium (10 g/l yeast extract, 20 g/l Bacto peptone, 20 g/l glucose). Yeasts were cultivated under magnetic stirring in 250 ml fermentors containing 200 ml of minimal synthetic medium (6·7 g/l yeast nitrogen base without amino acids, 200 g/l glucose, 6·3 g/l citric acid, pH 3·5 and supplemented with amino acid if necessary) for fermentation experiments. Experiments using labelled compounds were performed at 28°C in 12 ml tubes filled with 10 ml of minimal medium containing 200 g/l glucose and, when necessary, 3 g/l L-malic acid (final concentrations). For the NMR study, 100 g/l [2-$^{13}$C]glucose (Isotec, U.S.A.) was used. The specific radioactivity of [U-$^{14}$]malic acid (Amersham, U.K.) used in $^{14}$C experiments was 0·04 mCi/mmol.

### *Transformation methods*

*E. coli* transformation was carried out by the $CaCl_2$/$RbCl_2$ method (Hanahan, 1985). Transformation of *S. cerevisiae* and *S. pombe* was performed using the lithium acetate procedure (Schiestl and Gietz, 1989).

### *DNA manipulation and cloning techniques*

Restriction and modification enzymes were used according to the manufacturers' instructions. *E. coli* plasmid DNA was prepared using standard protocols (Sambrook *et al.*, 1989). Oligonucleotides were synthesized by Eurogentec.

### *Plasmid constructions*

The construction of pM1 has been described already (Ansanay *et al.*, 1993). To obtain the plasmid pARTM4, introduction of *Bam*HI and *Sac*I sites respectively at the 5′ and 3′ ends of the coding region of the malolactic gene from *L. lactis* was achieved by PCR with oligonucleotides with the following sequences: TCGGATCCATGCGTGCACATGA and CTGAGCTCCCCTTAGTACTC. The 1643 bp fragment defined by the primers was PCR amplified using pM1 as template, digested by *Bam*HI and *Sac*I and ligated to pART1 (McLeod *et al.*, 1987) digested by *Bam*HI and *Sac*I and dephosphorylated. The plasmids pM1 and pARTM4 were used to transform *S. cerevisiae* and *S. pombe* respectively.

*Preparation of cell-free extracts*

*L. lactis* cells were grown in 500 ml of E medium (Barre, 1969) without tomato juice but supplemented with DL-malate. The cells were harvested after 12 h of growth at 30°C, washed with 9 g/l NaCl, suspended in 100 mM-phosphate buffer, pH 6·0 (90 mM-$KH_2PO_4$, 2 mM-$Na_2HPO_4$) and disrupted by sonication. The cell-free extract was separated from the bacterial debris by centrifugation (15 000 *g*, 15 min, 4°C).

Yeast cells ($10^8$–$10^9$) were prepared from stationary phase cells. Cells were washed with 9 g/l NaCl, suspended in 0·5 ml of the 100 mM-phosphate buffer, pH 6·0, disrupted with glass beads for 4 min and centrifuged at 15 000 *g* for 5 min at 4°C.

Crude extracts were kept at 4°C for immediate enzyme assay or stored at −20°C.

*Protein and Western blot analyses*

Proteins were analysed by SDS–PAGE. Gels were stained with 0·25% Coomassie blue R-250 (Sigma) in 40°C methanol and 7% acetic acid. Western blot analyses were carried out using a polyclonal serum raised against the MLE from *L. lactis* (Ansanay *et al.*, 1993) and the alkaline phosphatase detection method (Sambrook *et al.*, 1989). Protein concentration was determined with the BCA Protein Assay Reagent (Pierce).

*Determination of malolactic activity*

MLE activity was determined at 30°C by measuring L-lactate produced from L-malate. The reaction mixture (2·3 ml) contained 200 mM-L-malate, 5 mM-NAD, 0·35 mM-$MnCl_2$ and 100 mM-phosphate buffer pH 6·0. The reaction was started by addition of crude extract. Samples (50 µl) were collected throughout the reaction and diluted in 950 µl of 50 mM-sodium carbonate buffer pH 10 in order to stop the reaction.

The specific activity was expressed as µmoles of L-lactate produced per minute per milligram of protein.

*L-lactate and L-malate determination*

L-lactate and L-malate were enzymatically analysed using Boehringer kits.

*NMR spectroscopy*

Samples for NMR spectroscopy were prepared as follows. The pH of the supernatants was adjusted using KOH to the pH range 7–8. $D_2O$ (0·3 ml), containing dioxane (final concentration 5 mM-$^{13}C$, used as a frequency and intensity reference) and 0·3 ml of a solution of $Na_2Cr$-diethylenetri-aminepentaacetate in $D_2O$ (relaxation reagent, final concentration 4 mM) were added to 2 ml of supernatant.

Spectra were then recorded on a Bruker AM-300 instrument at 75·4 MHz, in the gated decoupling mode with a relaxation delay long enough to obtain quantitative results. About 1000 transients were accumulated for each sample using 16 K data points. Signals were zero-filled to 32 K and then processed with a Lorentz-Gauss filter before Fourier transformation.

*Radioactivity determination*

[$^{14}C$]malic acid and [$^{14}C$]lactic acid contained in culture supernatants were separated by paper chromatography (Salgues and Andre, 1978). The paper bands corresponding to the organic acids were collected and then assayed for radioactivity in the presence of 10 ml Fluoran Safe (BDH Limited, Poole, U.K.) scintillation liquid on a Beckman LS 6500 counter.

## RESULTS

*Expression of malolactic enzyme in* S. cerevisiae

The malolactic coding region was previously cloned into the multicopy yeast expression vector pVT100-U, between the promoter and terminator region of the *ADH1* gene, resulting in the plasmid pM1 (Ansanay *et al.*, 1993). *S. cerevisiae* V5 was transformed with pVT100-U and pM1.

In order to evaluate the production of the MLE in *S. cerevisiae*, proteins from whole cell extracts of *L. lactis* and *S. cerevisiae* were resolved by SDS–PAGE and analysed by Western blotting (Figure 1A and B) with polyclonal antisera raised against the MLE from *L. lactis* (Ansanay *et al.*, 1993). The 60 kDa protein previously shown to correspond to *L. lactis* MLE (Ansanay *et al.*, 1993) is detected for *L. lactis* by Western blotting (Figure 1B, lane 1) and its position indicated on a Coomassie-blue-stained SDS–PAGE (Figure 1A, lane 1). In a cell extract from yeast transformed with the multicopy plasmid a band of identical size was detected by Western blotting (Figure 1B, lane 3) and clearly visible on a Coomassie-blue-stained SDS–PAGE (Figure 1A, lane 3), while this band was not detected (Figure 1B, lane 2) or observed for the control strain (Figure 1A, lane 2).

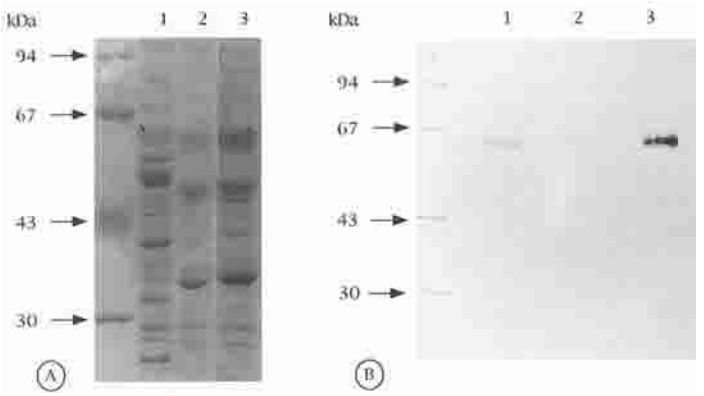


Figure 1. (A) Coomassie-blue-stained SDS–PAGE of *L. lactis* extract (lane 1), *S. cerevisiae* strain pVT100-U extract (lane 2) and pM1 recombinant strain extract (lane 3). Arrows show the position of MLE. (B) Immunoblot corresponding to (A). The amount of total protein loaded is 0·5 μg (B), or 50 μg (A). Molecular weight standards are indicated on the left.

Table 1. MLE activity of crude extracts of *L. lactis* and *S. cerevisiae* transformed with pVT100-U and pM1 Cells were collected at the stationary growth phase.

| Strains | MLE specific activity (μmol L-lactate released/ min per mg protein) |
|---|---|
| *L. lactis* IL1441 | 6 |
| *S. cerevisiae* V5/pVT100-U | nd |
| *S. cerevisiae* V5/pM1 | 18 |

nd, not detected.

MLE specific activity was determined in the different strains (Table 1). Under the growth conditions described for the experiment, the pM1 transformant showed MLE specific activity three times higher than that of *L. lactis*, while no detectable specific activity was observed in the control strain. The level of specific activity in the transformant is in good agreement with the results of gel analysis (Figure 1).

*Glucose fermentation and* L-lactate production

Fermentation experiments were conducted with *S. cerevisiae* V5 transformed with pVT100-U and pM1 in minimal medium with high glucose concentration (200 g/l), low extracellular pH (3·5) and 3 g/l L-malate (enological-like conditions) or without L-malate as control. The number of cells, L-lactate and L-malate concentrations and the MLE production were monitored during the experiment (Figure 2). About 3 g/l of L-lactate was produced during growth of the pM1 transformant in the presence of L-malate (Figure 2A). Most of the production took place in the first 200 h of fermentation. The MLE was detected in crude extracts during the early growth phase in close correlation with early lactate production and reached its maximal level in the stationary phase (Figure 2B). In contrast, the control strain showed no detectable L-lactate production (Figure 2C). When L-malate was absent from the growth medium (Figure 2D), the strain expressing *mleS* produced L-lactate with production kinetics similar to those observed in the presence of malate (Figure 2A). However, a 20% decrease in the final amount of L-lactate produced was observed. In addition, only a minor part of the initial external L-malate was degraded either by the control strain (0·4 g/l) or the recombinant strain (0·5 g/l; Figure 2A and C). A slight increase in malate degradation (0–10%, depending on the experiment) was observed for the recombinant strain compared to the control strain.

These results indicate that most L-lactate production observed with the strain transformed with pM1 probably arises from the endogenous L-malate. Moreover, this idea is supported by the observation that L-lactate production stopped after 200 h of growth (Figure 2A and D). At this stage, all the glucose had been consumed, even though a high level of MLE was still detected (data not shown). On the other hand, it is likely that part

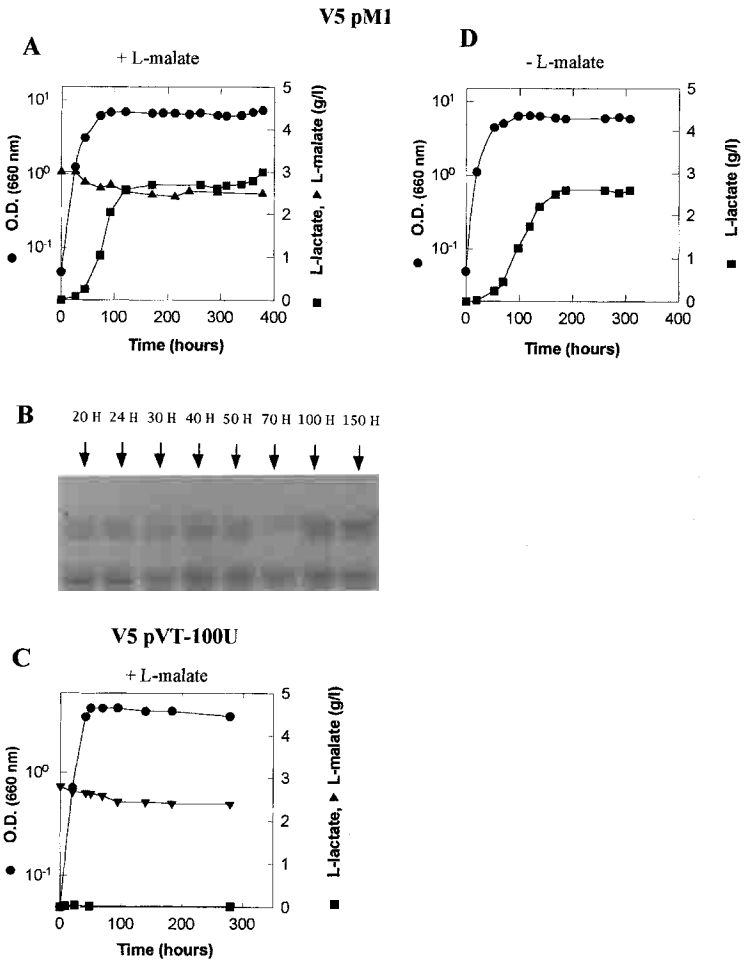


Figure 2. Growth, malate degradation and lactate production (A, C, D) of *S. cerevisiae* V5/pM1 (A, D) and of control strain V5/pVT100-U (C). Production of the MLE analysed by SDS–PAGE during growth of V5/pM1 (B). Fermentations were carried out on YNB medium containing 200 g/l of glucose and buffered to pH 3·5, as described in Materials and Methods. The amount of total protein loaded is 50 μg (B). Small arrows show the position of MLE.

of the exogenous L-malate entering the cells was converted into L-lactate via a malolactic reaction in the pM1 strain since a 20% increase in L-lactate production (0·5 g/l) is caused by the presence of exogenous L-malate.

*Filiation of $^{13}$C-labelling from glucose to L-lactate*

Filiation of [2-$^{13}$C]glucose to L-lactate was investigated to confirm that the major part of L-lactate produced originates from glucose. The recombinant strain was grown in the presence of 3 g/l L-malate in minimal medium containing 100 g/l [2-$^{13}$]glucose, 100 g/l non-labelled glucose. [$^{13}$C]-L-lactate and [$^{13}$C]-L-malate concentrations were determined in culture supernatants by MNR spectroscopy and compared to the whole concentrations of these organic acids (Figure 3, Table 2). A control experiment was performed under the same conditions without labelled glucose.

It was found that quantitative analysis of $^{13}$C-labelled organic acid concentrations was difficult to perform, taking into account the high discrepancy of concentrations of $^{13}$C-labelled compound: 1 M for glucose and ethanol against 0·2 M for malate. A small amount of [$^{13}$C]-L-malate was detected in this experiment (Figure 3B, Table 2).

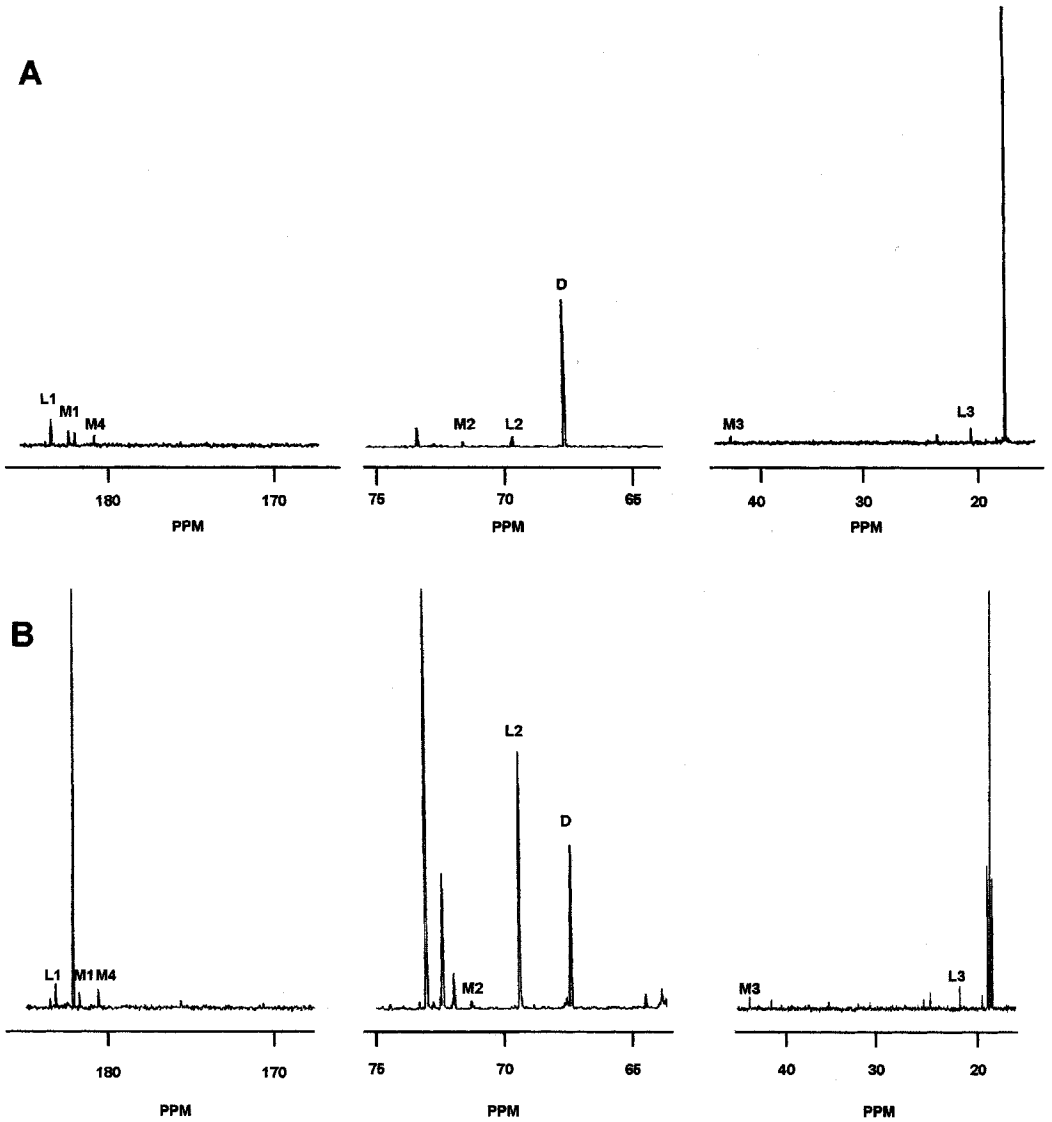


Figure 3. $^{13}$C-NMR spectra of supernatants obtained at the end of cultures performed (A) without $^{13}$C-labelled compound or (B) in the presence of 100 g/l [$^{13}$C]glucose. The three different regions of spectra are not recorded on the same scale. L1: Cl-lactate, L2: C2-lactate, L3: C3-lactate, D: dioxane, M1: C1-malate, M2: C2-malate, M3: C3-malate, M4: C4-malate.

This molecule has a uniform distribution of label and remains constant throughout the fermentation, corresponding to the natural 1% abundance of $^{13}$C isotope in L-malate. A significant part of the L-lactate was $^{13}$C-labelled (Figure 3B, Table 2): at the end of culture, about 30% of L-lactate produced included a $^{13}$C isotope, mostly located on C(2). The labelling yield obtained (30%) was higher than the maximal theoretical yield of $^{13}$C-labelled lactate (25%, corresponding to a total L-lactate production from glucose) expected under the experimental conditions. This may be explained by the low sensitivity of the NMR detection method under the experimental conditions (10% error due to background) and by the significant interference generated by the natural abundance of $^{13}$C isotope in the metabolites (Figure 3A, Table 2).

Table 2. Filiation of $^{13}C$-labelling from glucose to L-malate and L-lactate. pM1 recombinant strain was cultivated in minimal medium containing 10% of [2-$^{13}$]glucose and 10% non-labelled glucose with 0·3% L-malate. $^{13}C$ distribution in L-malate and L-lactate was determined by NMR spectroscopy (C1, C2, C3, C4). Corresponding total concentrations in organic acid (total) were measured by enzymatic dosage. Natural abundance of these compounds was determined during a control experiment realised with 200 g/l of unlabelled glucose.

| | Malate (mM) | | | | | Lactate (mM) | | | |
|---|---|---|---|---|---|---|---|---|---|
| Culture harvest time (h) | 1-$^{13}C$ labelling | 2-$^{13}C$ labelling | 3-$^{13}C$ labelling | 4-$^{13}C$ labelling | Total | 1-$^{13}C$ labelling | 2-$^{13}C$ labelling | 3-$^{13}C$ labelling | Total |
| Without labelled glucose | | | | | | | | | |
| 0 | 0·2 | 0·2 | 0·2 | 0·2 | 21 | 0 | 0 | 0 | 0 |
| 192 | 0·2 | 0·2 | 0·2 | 0·1 | 17 | 0·4 | 0·3 | 0·2 | 23 |
| With labelled glucose | | | | | | | | | |
| 0 | 0·3 | 0·3 | 0·2 | 0·3 | 20 | 0 | 0 | 0 | 0 |
| 192 | 0·4 | 0·3 | 0·3 | 0·3 | 15 | 0·3 | 9 | 0·4 | 29 |

Nevertheless, these results prove that L-lactate is mainly produced from glucose via the formation of endogenous L-malate. Distribution of $^{13}C$ in the carbon skeleton was consistent with the expectation that glucose was first converted into pyruvate, via glucolysis, and then transformed into L-malate by the intervention of pyruvate carboxylase. Such a conversion occurs in the presence or absence (data not shown) of exogenous L-malate. Because of the low sensitivity of the NMR technique in these assay conditions, it is difficult to judge whether the presence of exogenous L-malate interferes in L-lactate production from glucose and if a part, or all, of the consumed exogenous L-malate is converted into L-lactate.

### *Filiation of $^{14}C$-labelling from* L-*malate to* L-*lactate*

In order to determine the proportion of exogenous L-malate converted into L-lactate, cultures of *S. cerevisiae* V5 transformed with pVT100-U vector (control) and *S. cerevisiae* V5 transformed with pM1 vector strains were grown in minimal medium (glucose 200 g/l) containing 3 g/l [U$^{14}$C]-L-malate (specific radioactivity 0·04 mCi/mmol), and the $^{14}C$ filiation from L-malate to L-lactate was kinetically monitored during fermentation (Figure 4). The evolution of L-malate concentration during fermentation was identical for the two strains. Consumption of 4·5 mM exogenous L-malate was observed in both cases. During fermentation, the ratio between [$^{14}$C]-L-malate and total L-malate concentrations remained constant, at about 1.

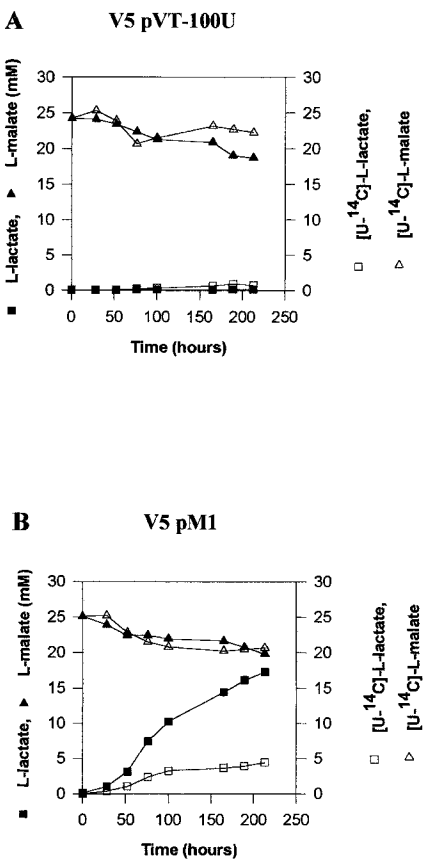


Figure 4. Filiation of $^{14}C$ from exogenous L-malate into L-lactate by *S. cerevisiae* strains pVT100-U (A) and pM1 (B). Cultures were realised on minimal medium containing 200 g/l of glucose and 3 g/l of [U-$^{14}$C]-L-malate (specific radioactivity: 0·04 mCi/mmol). Concentrations of total L-malate, [U-$^{14}$C]-L-malate, L-lactate and [U-$^{14}$C]-L-lactate were determined.

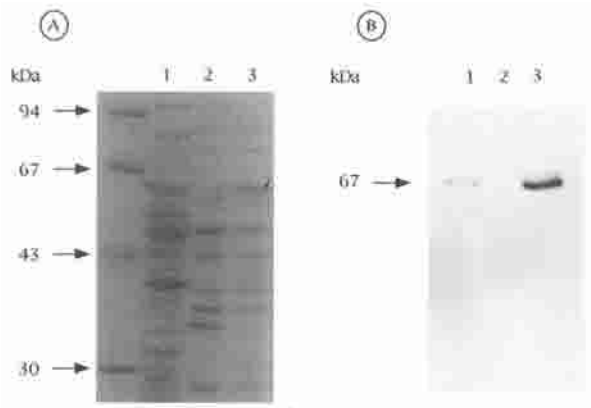


Figure 5. (A) Coomassie-blue-stained SDS–PAGE of *L. lactis* extract (lane 1), *S. pombe* strain pART1 extract (lane 2) and pARTM4 recombinant strain extract (lane 3). (B) Immunoblot corresponding to (A). The amount of total protein loaded is 0·5 μg (B), or 50 μg (A). Molecular weight standards are indicated on the left. Arrows show the position of MLE.

As expected, L-lactate production by *S. cerevisiae* transformed with the pVT100-U vector was very low. This minor formation corresponds to the basal yeast metabolism. In contrast, the strain expressing the bacterial malolactic activity exhibited significant L-lactate production (17 mM). At the end of the experiment, labelled L-lactate (4·4 mM) formed 25% of the total L-lactate produced. These observations confirm that, even in the presence of exogenous L-malate, most of the L-lactate produced by the recombinant strain derives from glucose. However, practically all the consumed exogenous malate is converted into L-lactate by the pM15 transformant, while such conversion was not observed in the control strain. The main pathway of transformation of L-malate by *S. cerevisiae* pM1 is therefore via the MLE.

### *Expression of MLE in* S. pombe

Recombinant vectors for expression in *S. pombe* were constructed by cloning the malolactic coding region into the yeast expression vector pART1 under the control of the promoter region of *S. pombe ADH1* gene, resulting in the plasmid pARTM4. *S. pombe* was transformed with pART1 and pARTM4.

Crude extracts of *L. lactis, S. pombe* pART1 and pARTM4 strains were resolved using SDS–PAGE and analysed by Western blotting (Figure 5A and B) with the polyclonal antiserum raised against the MLE. The 60 kDa band corresponding to MLE was immunodetected for the pARTM4 strain (Figure 5B, lane 3) and visible on the Coomassie-blue-stained gel (Figure 5A, lane 3), while this band was not detected in the control strain (Figure 5A and B, lane 2). Moreover, the intensity of the malolactic band detected in the recombinant *S. pombe* was comparable to that observed in *S. cerevisiae*, suggesting that the MLE was expressed at a similar level in both strains.

### *Malolactic fermentation by* S. pombe

The same fermentation experiments described for *S. cerevisiae* strains (Figure 2) were conducted for *S. pombe* pARTM4 and pART1 transformants (Figure 6). Only traces of L-lactate were detected during growth of *S. pombe* pARTM4 on glucose-rich medium without L-malate (Figure 6D), while a large amount of L-lactate (up to 1·6 g/l) was produced when L-malate was present in the growth medium (Figure 6A). The malolactic enzyme was detected in the recombinant strain at the same level throughout fermentation, as shown on Coomassie-blue-stained SDS–PAGE (Figure 6B). No detectable L-lactate production was observed in the control strain, *S. pombe* pART1 (Figure 6C). In all cases, L-malate was soon entirely consumed. It is, therefore, likely that most L-lactate production in the L-malate-grown transformant is produced from L-malate, with 70–80% of exogenous L-malate converted into L-lactate. Moreover, these results provide evidence for the preferential utilization of L-malate via MLE rather than by using the endogenous malic enzyme of *S. pombe*.

## DISCUSSION

*S. cerevisiae* and *S. pombe* transformants expressing the MLE from *L. lactis* at a high level were constructed, as displayed by Western blotting and *in vitro* determination of the specific activity. In order to compare the ability of both recombinant strains to perform malolactic fermentation, malate degradation and lactate production were monitored during fermentation with *S. cerevisiae* and *S. pombe* overexpressed strains.

When L-malate was absent from the growth medium, the *S. cerevisiae* pM1 strain displayed significant L-lactate production, while only traces were detected with the control strain, suggesting that the L-lactate produced arises from endogenous L-malate. This was further demonstrated by [$^{13}$C]glucose filiation. These results suggest that MLE competes strongly with potential

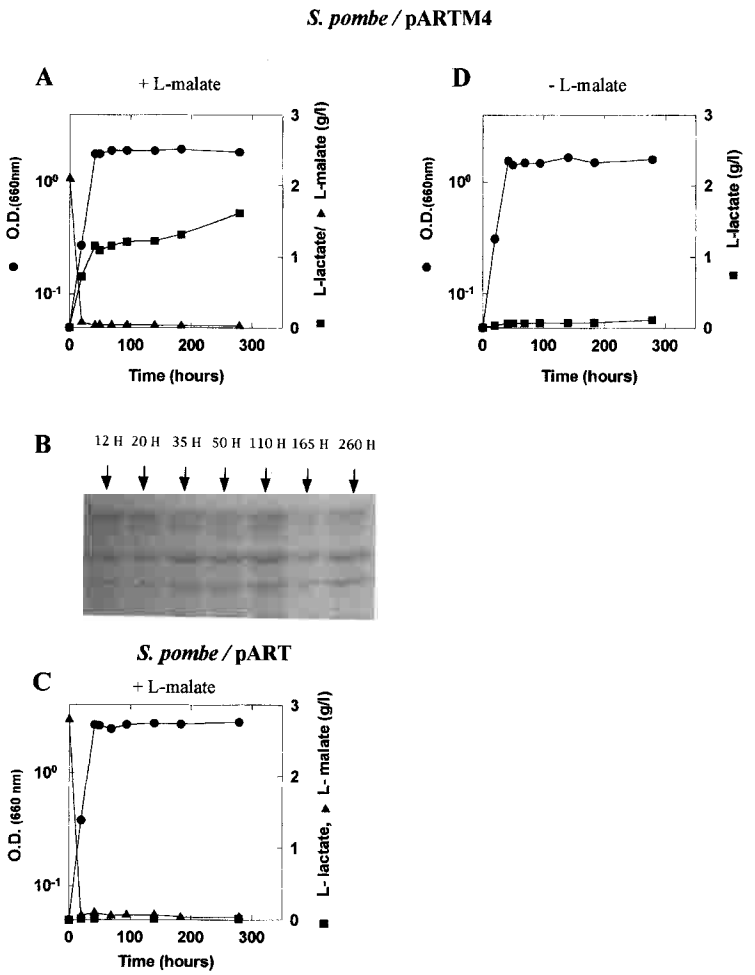


Figure 6. Growth, malate degradation and lactate production (A, C, D) of *S. pombe* pARTM4 (A, D) and of control strain pART1 (C). Production of the MLE analysed by SDS–PAGE during growth of *S. pombe* pARTM4 (B). Fermentations were carried out on YNB medium containing 200 g/l of glucose and buffered to pH 3·5, as described in Materials and Methods. The amount of total protein loaded is 50 μg (B). Small arrows show the position of MLE.

endogenous malate utilization systems in *S. cerevisiae*. Malate degradation in *S. cerevisiae* can be achieved via fumarase, malate dehydrogenase or malic enzyme; the main L-malate degradation pathway in anaerobiosis is the malic enzyme (Fuck and Radler, 1972), producing ethanol, although anabolism was shown to be achieved mainly by malate dehydrogenase (Salmon *et al.*, 1987a). The specific activities and L-malate $k_m$ values of malolactic enzyme (this work and Renault and Heslot, 1987), compared to those of malic enzyme (Fuck *et al.*, 1973) and malate dehydrogenase (Salmon *et al.*, 1987a) are in favour of the MLE.

The situation is quite different in the recombinant *S. pombe* strain since no L-lactate was observed in the absence of L-malate in the growth medium. These results may reflect either a lower intracellular L-malate concentration in *S. pombe* than in *S. cerevisiae* or greater efficiency of endogenous malate utilization systems in *S. pombe* in comparison with *S. cerevisiae*. The main degradation system of L-malate in *S. pombe* in anaerobiosis is malic enzyme; it exhibits very high affinity for L-malate ($K_m$ 3·2 mM). If significant endogenous L-malate levels are reached under these fermentation conditions, a preferential utilization of

L-malate via this enzymatic system cannot be excluded.

When exogenous L-malate is provided in the growth medium, a small amount (0·4–0·5 g/l) of exogenous L-malate is degraded by *S. cerevisiae* control or transformed strains. In this case, the pM1 transformant exhibits a 20% increase in lactate production (compared to growth without malate). By $^{14}C$ filiation of malic acid it was demonstrated that this increase in L-lactate production results from the conversion of exogenous L-malate. Seventy-five per cent of the final L-lactate amount produced in the described fermentation conditions arises from endogenous L-malate and 25% from exogenous L-malate. Moreover, all the degraded L-malate is converted into L-lactate, showing that the MLE is the most competitive enzyme in the utilization of L-malate. Different results have been reported recently (Denayrolles *et al.*, 1995). In this work, *mleS* gene from *L. lactis* was expressed in *S. cerevisiae* under the control of phosphoglycerate kinase promoter. In this recombinant strain, the MLE does not compete with other malic acid utilization enzymes since only a small part of the degraded exogenous L-malate was converted into L-lactate via MLE. This could be explained by the much lower specific malolactic activity obtained in this recombinant strain than that measured in V5/pM1. As a result, a much smaller L-lactate production is observed under the same experimental conditions (0·27 g/l against 3 g/l in this work). Moreover, it cannot be ruled out that part of the L-lactate produced is also derived from endogenous L-malate since the origin of L-lactate was not investigated by the authors.

In *S. pombe*, expressing MLE during fermentation in the presence of L-malate, L-lactate production was observed which may result from the degradation of 80% of exogenous L-malate. This is supported by two observations: (i) no L-lactate production arising from endogenous L-malate was observed in the control experiment without L-malate; (ii) the level of L-lactate produced increased in direct ratio with the L-malate concentration in the growth medium but was not dependent on glucose concentrations (data not shown).

The results show that the MLE of *L. lactis* is functional in both strains and is able to degrade most of the exogenous L-malate, provided that this substrate is available in the cell. It is thus highly probable that the differences in the ability to achieve a complete degradation of L-malate into L-lactate and $CO_2$ are related to the efficiency of L-malate transport systems of *S. cerevisiae* and *S. pombe*. Evidence for a carrier-mediated transport for malic acid has been reported in *S. pombe* (Osothsilp and Subden, 1986) and further characterized as a proton-dicarboxylate symport (Sousa *et al.*, 1992). On the other hand, the transport of malic acid in *S. cerevisiae* is not carrier-mediated (Salmon, 1987b), suggesting that influx of malic acid in *S. cerevisiae* is probably limited to a slow simple diffusion of the undissociated acid. Moreover, the existence of an efficient efflux system as suggested earlier (Salmon, 1987b) cannot be ruled out.

The installation of a new malate degradation pathway in *S. cerevisiae* and *S. pombe* does not alter the physiological characteristics of these yeasts. The growth kinetics, final biomass, and ability to ferment large amounts of sugar (200 g/l) are not modified in the recombinant strains, in spite of the large amount of L-lactate produced by the *S. cerevisiae* and *S. pombe* strains expressing *mleS* under the control of *ADH* regulatory elements. A small accumulation of intracellular L-lactate was observed in the V5/pM1 strain (5 mM, data not shown), while intracellular L-lactate was not detectable in the control strain. However, it was shown previously that a *S. cerevisiae* strain expressing a bacterial lacticodehydrogenase was able to produce a very large amount of lactate (Dequin and Barre, 1994) without dramatic physiological alterations. Although the mechanism by which lactate (mostly dissociated at the cytoplasmic pH) is extruded has not been elucidated, lactate efflux is probably not a limiting step.

It might be expected that the efficient conversion of endogenous L-malate into L-lactate in recombinant *S. cerevisiae* results in a lower intracellular concentration of malate. Indeed, the intracellular L-malate concentration drops from 5 mM (V5/VT100-U) to 1 mM (V5/pM1) (data not shown). Since no physiological modifications have been observed in the recombinant strains, it is likely that the malate level reached in the recombinant strain is sufficient to fulfil the requirements of the cell with C4 compounds.

From a technological point of view, *S. cerevisiae* is the most suitable microorganism for alcoholic fermentation in winemaking. Studies of L-malate uptake and efflux components potentially involved in the transport of malic acid during alcoholic fermentation are now in progress. However, the main disadvantage of using *S. pombe* in

winemaking is the drastic deacidification resulting from malo-alcoholic fermentation. For this reason, winemaking is sometimes performed by a mixed fermentation involving *S. pombe* and *S. cerevisiae*. The use of a pure strain of *S. pombe* expressing MLE is therefore now conceivable in winemaking. An alternative would be the use of reactors with immobilized genetically engineered *S. pombe* cells to achieve mild demalication of wine.

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