Functional expression in *Saccharomyces cerevisiae* of the *Lactococcus lactis* *mleS* gene encoding the malolactic enzyme

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Abstract

Malolactic fermentation, a crucial step in winemaking, results mostly in degradation by lactic acid bacteria of L-mallic acid into L-lactic acid. This direct decarboxylation is catalysed by the malolactic enzyme. Recently we, and others, have cloned the *mleS* gene of *Lactococcus lactis* encoding malolactic enzyme. Heterologous expression of *mleS* in *Saccharomyces cerevisiae* was tested to perform simultaneously alcoholic and malolactic fermentations by yeast. *mleS* gene was cloned in a yeast multicopy vector under a strong promoter. Malolactic activity was present in crude extracts of recombinant yeasts. Malic acid degradation was tested during alcoholic fermentation in synthetic media and must. Yeasts expressing the *mleS* gene actually produced L-lactate from L-malate; nevertheless malate degradation was far from complete.

Keywords: Malolactic enzyme; Malic acid metabolism; *Saccharomyces cerevisiae*

1. Introduction

In most cases winemaking includes two steps: alcoholic fermentation performed by the yeast *Saccharomyces cerevisiae*, followed by a second step, malolactic fermentation (MLF), performed by lactic acid bacteria (LAB). The latter mainly consists in transformation of the major carboxylic acid of wine, L-malate, into L-lactate and carbon dioxide. This leads to a decrease in acidity, an increase in microbiological stability by depletion of this substrate for bacteria, and to modifications of wine flavours. In some cases during this fermentation, secondary metabolism of LAB is prejudicial to wine quality and can even lead to spoilage of wine. Moreover, MLF is often difficult to manage in winemaking. Different techniques to promote MLF or malate degradation have been suggested: inoculation of wine with selected strains of LAB, chemical or physical elimination of malate, and the use of the yeast *Schizosaccharomyces pombe* which is able to completely transform L-malate of must into ethanol and carbon dioxide. In the latter case, wine deacidification is too great, owing to the absence of lactic acid. Moreover, this yeast can also produce off-flavours [1]. In anaerobiosis, *S. cerevisiae* can also degrade malate via malic enzyme [2], but only partially as compared to *S. pombe*. An attractive idea would be to perform malolactic fermentation simultaneously with alcoholic fermentation with a genetically engineered yeast containing a gene encoding malolactic enzyme.
It is ten years since the first attempts to clone a malolactic enzyme gene from *Lactobacillus delbrueckii* [3] and *L. aenos* [4]. We, and others, have recently described the cloning and characterization of the *mleS* gene coding for the malolactic enzyme of *L. lactis* [5,6]. To test the feasibility of producing an enological yeast able to degrade malate through the malolactic enzyme, we describe here the expression of the *mleS* gene in two laboratory strains of *S. cerevisiae*.

2. Materials and methods

General procedures for cloning and DNA manipulations were performed as described by Sambrook et al. [7] with slight modifications.

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**Fig. 1.** Construction of pMDMAL0: insertion of *mleS* gene in the yeast multicopy plasmid pTG887 under the control of the PGK promoter (†) and terminator (†).
2.1. Yeast strains

Strains used for functional expression of mleS were the diploids FL(2n) (MATa/MATa, ura3-52/ura3-52) derived from the FL100 background (A.T.C.C. 28383), and LG16(2n) (MATa/MATa, ura3-/ura3-), a cross between LG16-1A and LG16-5A [8], derived from the S288C background (Yeast Genetic Stock Center, Berkeley, USA).

2.2. Amplification of mleS gene

Polymerase chain reaction (PCR) was performed in an automated DNA thermocycler (Biometra). The DNA template consisted of the pMDML plasmid containing the mleS gene carried on a 2.3 kb DNA fragment, as described previously [5]. The reaction mixture contained 100 pmol of each primer (OMDML1, 5'-TTTATCGATGTTGTACG-3'; OMDML2, 5'-TAAGGAAATCCCTTAG-3'), 10 ng of pMDML plasmid template DNA, 10 mM KCl, 20 mM Tris-HCl pH 8.8, 10 mM (NH₄)₂SO₄, 2 mM MgSO₄, 0.1% Triton X-100, 200 μM each dATP, dTTP, dGTP, dCTP and 2 U of Vent™ DNA polymerase (Biolabs) in a total volume of 100 μl. The DNA was amplified for 35 cycles at 94°C for 1 min, 45°C for 1 min, and 72°C for 1.5 min. The expected band of 1.6 kb DNA fragment was visualized after fractionation of PCR products by 0.8% agarose gel electrophoresis.

2.3. Cloning of mleS gene in pTG887 (see Fig. 1)

The 1.6 kb PCR fragment was ligated to the multicopy yeast plasmid pTG887 (Transgene S.A. and [9]). This procedure allowed insertion of the 1.6 kb fragment in the proper orientation for transcription of the mleS gene from the PGK promoter of pTG887. This material was used to transform E. coli XL1-Blue. The construction obtained, pMDMALO, was used to transform yeast by a method using DMSO [10].

2.4. Preparation of cell-free extracts and measurement of enzymatic activities

Cell-free extracts were prepared from S. cerevisiae cells transformed with pTG887 or pMDMALO grown aerobically at 25°C in the minimum medium YNB10 (10 g/l glucose, 5 g/l (NH₄)₂SO₄, 1.7 g/l yeast nitrogen base w/o amino acids and ammonium sulfate (Difco)) until log phase. Cells were disrupted using 0.4 μm diameter glass beads. Protein concentration was determined with the BCA Protein Assay Reagent (Pierce). MLE activity was determined at 37°C by measuring CO₂ released from l-malate with a specific CO₂ electrode (Eischeweiler and Co.) [11]. The specific activity was expressed as μmol of CO₂ released per min per mg of protein. Malic activity (malate to pyruvate) was measured as described elsewhere [11]. L-malate and l-lactate were determined by enzymatic methods (Boehringer Mannheim).

2.5. Microvinifications in synthetic media and in red grape must

Two different media were used to test degradation of malic acid during fermentation of sugar by yeasts: (i) a synthetic broth containing 100 g/l glucose, 100 g/l fructose, 4.5 g/l L-malic acid or not for the control, 0.3 g/l citric acid, 6 g/l tartric acid, 2 g/l (NH₄)₂SO₄, 1.7 g/l yeast nitrogen base w/o amino acids and ammonium sulfate (Difco); and (ii) a broth with red grape must, adjusted to 90 g/l glucose, 90 g/l fructose, 4.5 g/l L-malic acid. pH of all media was adjusted to 3.

Yeasts were grown in partial anaerobiosis at 25°C using 260 ml of media in 375-ml bottles which were obturated by a plug with a capillary. Inoculations were performed to ensure 10⁶ cells per ml and media were left to ferment with one shaking per day. Viable cell numbers were determined by plate counts. Sugars, malate and lactate were determined by enzymatic methods (Boehringer Mannheim).

3. Results and discussion

3.1. Cloning of mleS gene in yeast

The gene mleS was cloned in the multicopy plasmid pTG887 leading to pMDMALO, as described in Fig. 1 and according to Materials and Methods. Transcription of the mleS gene was then under the control of the strong promoter and termina-
Table 1
Specific malolactic activities of yeast cell crude extracts

<table>
<thead>
<tr>
<th>Strain</th>
<th>Plasmid</th>
<th>Malolactic specific activity a</th>
</tr>
</thead>
<tbody>
<tr>
<td>FL(2n)</td>
<td>pTG887</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>pMDMALO</td>
<td>0.70</td>
</tr>
<tr>
<td>LG16(2n)</td>
<td>pTG887</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>pMDMALO</td>
<td>0.05</td>
</tr>
</tbody>
</table>

a Specific activity: μmol of CO₂ per min per mg of protein.

The mleS gene, which codes for the malolactic enzyme, was introduced into two S. cerevisiae strains with different genetic backgrounds [12]. The diploid strains were preferred, because it was more representative of the chromosomal structure of enological strains.

To test the expression of the mleS gene in yeast, malolactic activity was first measured in crude extracts from yeast grown aerobically on a synthetic medium. Table 1 gives the results of L-malate decarboxylation. Only yeasts transformed with pMDMALO presented L-malate decarboxylation activity. No background activity was present in the extracts of yeast transformed with pTG887, thus reflecting, in these conditions, the inability to measure the in vitro malic activity (decarboxylation of L-malate to pyruvate) of S. cerevisiae. This was further confirmed by spectrophotometric measurement of malic activity.

To confirm that decarboxylation of L-malate in crude extracts of yeast transformed with pMDMALO was due to malolactic and not malic activity, the concentration of L-malate and L-lactate (substrate and product of malolactic reaction) was measured during incubations (Table 2). Significantly, only crude extracts of yeasts with the mleS gene transformed L-malate and produced L-lactate. L-lactate was not produced in stoichiometric amounts when L-malate was consumed, presumably owing to other endogenous malate-consuming activities.

In conclusion, the mleS gene was expressed in S. cerevisiae, thereby conferring a malolactic activity to yeast. The specific malolactic activity of the FL(2n) recombinant strain was in the same range as values obtained from malolactic bacteria, in particular with specific activity of Lactococcus lactis IL1441 (source of mleS gene) measured in the same conditions: 0.70 μmol of CO₂ per min per mg of protein for yeast versus 0.41 for this bacterium.

3.2. Activity of the mleS gene in vivo

Since L-malate enters S. cerevisiae cells by simple diffusion and particularly in enological conditions [13], external L-malate may be degraded during sugar fermentation.

To test this possibility, the strain FL(2n) which presented the highest in vitro malolactic activity (Table 1) was grown for 26 days in synthetic media at pH 3, mimicking composition of must (major sugars and acids of must, nitrogen, vitamins, bases). As shown in Fig. 2, the presence of mleS in FL(2n) did not influence the viability of cells or the fermentation kinetic. In all cases, the sugars were not totally degraded, which is common for laboratory strains with such high sugar concentrations. The ethanol yield was normal (data not shown). In all cases L-malate was partially degraded, but FL(2n) transformed with pMDMALO metabolized more malic acid than the control (15.6% of malate versus 5.6%).

Table 2
Degradation of L-malate and production of L-lactate during incubations of yeast cell crude extracts

<table>
<thead>
<tr>
<th>Strain</th>
<th>Time of incubation (h)</th>
<th>L-malate degraded (μmol)</th>
<th>L-lactate formed (μmol)</th>
<th>% of L-malate converted to L-lactate</th>
</tr>
</thead>
<tbody>
<tr>
<td>FL(2n) + pMDMALO</td>
<td>0</td>
<td>0</td>
<td>5</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>4</td>
<td>29</td>
<td>22</td>
<td>58.5</td>
</tr>
<tr>
<td>LG16(2n) + pMDMALO</td>
<td>0</td>
<td>0</td>
<td>2</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>4</td>
<td>13</td>
<td>10</td>
<td>61.5</td>
</tr>
<tr>
<td>FL(2n) or LG16(2n)</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>+ pTG887</td>
<td>4</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

200 μg of proteins were used in each incubation. Complete reaction mixture contained 51 μmol of L-malate, 1 μmol of NAD, 0.2 μmol of MnCl₂ and crude extracts in 2.3 ml of 0.2 M KH₂PO₄-Na₂HPO₄, pH 6.
Moreover this recombinant strain produced L-lactate, which was further metabolized in the control. The amount of L-lactate formed (0.23 g/l, 2.55 mM) was in stoichiometric amount with the excess of malate (0.40 g/l 2.98 mM) degraded by FL(2n) transformed with pMDMALO.

In conclusion, the presence of mleS gene did not interfere with the kinetics of fermentation in the synthetic medium. Moreover, the yeasts carrying the mleS gene had the new property of producing L-lactate from L-malate, albeit in small amounts.

To confirm this new property of yeast, fermenta-
tions in red grape must were performed. Must pH was adjusted to 3 as for synthetic medium and contained 4.5 g/l of l-malate. For this experiment, the two diploid strains were used and fermentations were monitored for 73 days. In this case, sugar fermentation was more complete than in the synthetic medium but was still incomplete, ending with residual sugar concentrations of 8.5 to 18.7 g/l. Both strains, whether containing the mleS gene or not, had a better malate utilization in must than in the synthetic medium (Fig. 3). The two pMDMALO-transformed strains degraded 1.1 g/l (8.2 mM) of L-malate, which represents 24.5% of the initial quantity, versus 0.8–0.9 g/l (5.9–6.7 mM; 17.8–20%) for control strains. Only these two strains produced l-lactate, and in the same quantities (0.27 g/l, 3 mM). Here, the stoichiometric balance between L-malic acid degradation and L-lactate produc-
tion was not respected. This was probably due to the
different balance of yeast endogenous malate-con-
suming activity versus malolactic activity.

In conclusion, these initial experiments show that
the bacterial mleS gene introduced into yeast S.
cerevisiae induces transformation of L-malate in L-
lactate. Nevertheless, the quantitative aspect must be
considered with caution. In winemaking, this trans-
formation must be complete. In our experiments, in
spite of the high in vitro MLE specific activity,
malate degradation via MLE was very low, although
present as shown by L-lactate production. In fact,
absolute malate depletion was quite similar for yeast
whether it contained the mleS gene or not. There-
fore, quantitative optimisation of the whole system is
necessary before a useful enological yeast able to
perform efficient malolactic transformation can be
obtained.

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