Volume 332, number 1,2, 74–80 FEBS 13080 October 1993

Cloning, sequence and expression of the gene encoding the malolactic enzyme from *Lactococcus lactis*

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Received 27 July 1993; revised version received 30 July 1993

Many lactic acid bacteria can carry out malolactic fermentation. This secondary fermentation is mediated by the NAD- and Mn^{2+}-dependent malolactic enzyme, which catalyses the decarboxylation of L-malate to L-lactate. The gene we call *mleS*, coding for malolactic enzyme, was isolated from *Lactococcus lactis*. The *mleS* gene consists of one open reading frame capable of coding for a protein with a calculated molecular mass of 59 kDa. The amino acid sequence of the predicted MleS gene product is homologous to the sequences of different malic enzymes. Bacterial and yeast cells expressing the malolactic gene convert L-malate to L-lactate.

Gene cloning; Malolactic enzyme; Malic enzyme; *Lactococcus lactis*; *Saccharomyces cerevisiae*

1. INTRODUCTION

Gram-positive lactic acid bacteria are used extensively in industrial processes, on the basis of their ability to generate lactate during growth. Knowledge of the genetics of lactic acid bacteria is still limited, as until recent years work has mostly focused on plasmid-encoded characteristics.

In this work, we report on malolactic fermentation (MLF, see [1,2] for review), a secondary fermentation that lactic acid bacteria genera such as *Lactobacillus*, *Lactococcus*, *Leuconostoc* and *Pediococcus* can carry out when L-malate is present in the medium. The decarboxylation of L-malate to L-lactate and CO_2 is of considerable technological interest: MLF is the second step in most wine-making processes, usually occurring after yeast alcoholic fermentation. MLF results in significant de-acidification of wine, and increases the microbial stability of wine by removal of the fermentable substrate L-malate. The initiation of MLF is not well controlled as wine medium is not favourable to the development of malolactic bacteria. An attractive solution would be to introduce in yeast the ability to perform both malolactic fermentation and alcoholic fermentation.

The different steps of MLF are catalyzed by enzymes encoded by chromosomal genes; components that seem to be involved in the reaction were identified from genetic [3–5] and physiological studies [6–8]: malolactic enzyme (MLE), malate transport and/or lactate efflux, and a regulation component. A positive regulator for induction of the genes necessary for MLF was cloned from *Lactococcus lactis* [9].

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The malolactic reaction is catalysed by a single enzyme, MLE, which is thought to be bifunctional: L-malate may first be decarboxylated to pyruvate with the reduction of NAD (malic enzyme activity), and then pyruvate reduced to L-lactate with reoxidation of NADH to NAD (lactate dehydrogenase activity), without release of intermediate products [10–12]. MLE has been purified from *Lactobacillus casei* [13], *Leuconostoc mesenteroides* [10], *Lactobacillus plantarum* [11], *Leuconostoc oenos* [12], *Lactobacillus* sp. [14], and *L. lactis* (Lonvaud, personal communication). It is composed of two to four identical subunits of 60 to 70 kDa.

Attempts have been made to clone the malolactic gene from *Lactobacillus delbrueckii* in *Escherichia coli* [15]. A DNA fragment of 5 kb was isolated, and shown through maxicell experiments to code for a 65 kDa protein. Although this fragment was shown to allow a weak conversion of L-malate to L-lactate in *E. coli*, the expression was not detectable in yeast, and no sequence has been reported since. Another group failed to clone any stable DNA fragment encoding malolactic activity from *Leuconostoc oenos* in *E. coli* [16].

We report here the cloning and sequence analysis of the gene encoding the MLE from *L. lactis*, and its expression in *E. coli* and in *Saccharomyces cerevisiae*. Comparison of the protein sequence of the *mleS* gene product with database protein sequences reveals that MLE is strongly homologous to malic enzymes from different organisms.

2. MATERIALS AND METHODS

2.1. *Strains, media and vectors*

L. lactis IL1441 (wild type), which was used as the source for the

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malolactic gene, and the mutant SO1 which is defective in MLE, have been described [4]. The cloning host was *E. coli* DH5α. *S. cerevisiae* V5 (Mat a, ura3) was derived from a Champagne wine strain. *L. lactis* was grown at 30°C in complex medium E without tomato juice [17] but supplemented with DL-malate (15 g/l), or in M17 medium [18]. *E. coli* cultivation and media were as described previously [19]. The culture media used for *S. cerevisiae* were YPD medium (1% bacto yeast extract, 2% bactopeptone, 2% glucose) or defined minimal synthetic medium (yeast nitrogen base without amino acid DIFCO 0.67%, glucose 2%, buffered to pH 3 or 6 with citric acid (0.63%)). For some experiments malate (1%) was added.

The plasmid used in *E. coli* was pUC18 [20]. The shuttle bacterial-yeast vector pVT100-U, containing an expression cassette consisting of *S. cerevisiae* ADHI promoter and terminator, has been described [21].

The phage λgt11 and the host strain Y1090 were provided by Amersham.

2.2. *Preparation of polyclonal antibodies and protein analysis*

MLE was purified from *L. lactis* (Ansanay and Lonvaud, unpublished) and antibodies were obtained by injecting rabbits with purified protein as previously described [22].

Proteins were analysed by SDS-PAGE. Gels were stained either with silver salts or 0.25% Coomassie blue R-250 (Sigma) in 40% methanol and 7% acetic acid. Western blot analysis was carried out using the polyclonal serum; the alkaline phosphatase detection method was used.

2.3. *Recombinant DNA techniques*

DNA manipulations were carried out using standard methods [19]. *L. lactis* DNA was prepared as described previously [23]. *E. coli* was transformed according to Hanahan [24]. Transformation of *S. cerevisiae* was performed using the lithium acetate procedure [25]. DNA probes were radio-labelled using the megaprime DNA labelling system (Amersham). Oligonucleotides were synthesized by Eurogentec.

2.4. *Construction and screening of the DNA library*

L. lactis DNA was partially digested by *Alu*I and *Hae*III and fractionated on a 10–40% sucrose gradient. DNA fractions ranging from 1.5 to 4 kb were cloned into the unique *Eco*RI λgt11 vector site using the λgt11 cloning kit from Amersham, according to the manufacturer's instructions, to produce a library of recombinant phage. 75,000 plaque-forming units were screened with the polyclonal antiserum [19]. The detection of false positives was limited by preliminary treatment of antiserum, to reduce the concentration of antibodies reacting with the bacterial vector and host [19]. Antibodies bound on the phage lift filters were detected as described for Western experiments. Positive clones were carried through two rounds of plaque purification, and inserts were subcloned in plasmid pUC18.

2.5. *DNA sequence determination*

Unidirectional sets of nested deletions were produced for each clone on both DNA strands using exonuclease III (Pharmacia), according to the supplier's instructions. DNA sequences were determined on double-strand templates from pUC18 subclones using the chain termination method. The Taq Dye Primer Cycle sequencing kit from Applied Biosystems was used.

Analysis of DNA and deduced protein sequence were assisted by the DNA Strider program [26]. Protein homologies were sought in peptide sequence databases at the National Center for Biotechnology Information using the BLAST network service. The scores of identity and similarity were calculated using the MaxHome program [27]. Protein alignements as shown in Fig. 4 were performed using the CLUSTAL program [28]. Secondary structures were predicted through Profile network prediction PHD [29].

2.6. *Construction of the MLE expression vector*

Introduction of *Xho*I and *Xba*I sites respectively at the 5′ and 3′ ends of the coding region was achieved by site-directed mutagenesis with oligonucleotides having the following sequences: GGCTCGAGATGCGTGCACATGA and TGTCTAGATATTCCCCTTAGTA. The 1624 bp fragment defined by the two primers was amplified, digested by *Xho*I and *Xba*I and ligated to pVT100-U, previously digested by *Xho*I and *Xba*I and dephosphorylated, to give the recombinant plasmid pM1.

2.7. *Lactate and malate determination*

Lactate and malate levels were determined enzymatically using Boehringer kits.

3. RESULTS

3.1. *Preparation of polyclonal serum against MLE*

Antibodies were prepared against the *L. lactis* MLE by injecting rabbits with 200 μg of the purified protein. To study the specificity of the polyclonal serum, the crude extract of *L. lactis* and the purified fraction were resolved using SDS-PAGE (Fig. 1A), and analysed by Western blotting (Fig. 1B). A major band around 60 kDa was detected for both the crude extract (lane 1) and the purified fraction (lane 2).

Confirmation of the identity of the purified enzyme was obtained by performing the same analysis (SDS-PAGE (Fig. 1C) and immunoblot (Fig. 1D)) with the wild type strain (lane 1) and a mutant (SO1) defective in MLE [4] (lane 2). The MLE band is indicated on the SDS-PAGE for the wild type strain. In contrast this band was not observed for the mutant SO1. On the corresponding immunoblot, the 60 kDa band observed for the wild type strain was no longer detected with the mutant.

3.2. *Cloning of the malolactic gene*

A DNA genomic library of *L. lactis* constructed in λgt11 vector was screened using the specific polyclonal serum. Positive clones, obtained by screening 75,000 plaques, were subcloned in pUC18 vector, and subjected to restriction and Southern analyses. Five inserts, carrying overlapping fragments, were together shown to represent 4.5 kb from the same genomic region of *L. lactis*. The restriction map of the 2.7 kb insert from the clone p153A is shown in Fig. 2.

Oligonucleotide mixtures corresponding to the NH_2 terminal sequence of MLE [30] were hybridised with a Southern blot of the different inserts. The oligonucleotide ATG(A,C)G(G,A,T,C)GC(G,A,T,C)CA(T,C)GA-(G,A)AT representing all the possible sequences coding for amino acid residues 1 to 6, gave a positive signal with the five overlapping clones (not shown). This suggests that these clones all contain the 5′ coding region of the malolactic gene.

3.3. *Sequence analysis*

The entire nucleotide sequence of the 2.7 kb insert from p153A was determined. Computer analysis detected two open reading frame (ORF's) (Fig. 2), one of 1620 nucleotides (ORF1), and the other of 582 nucleo-

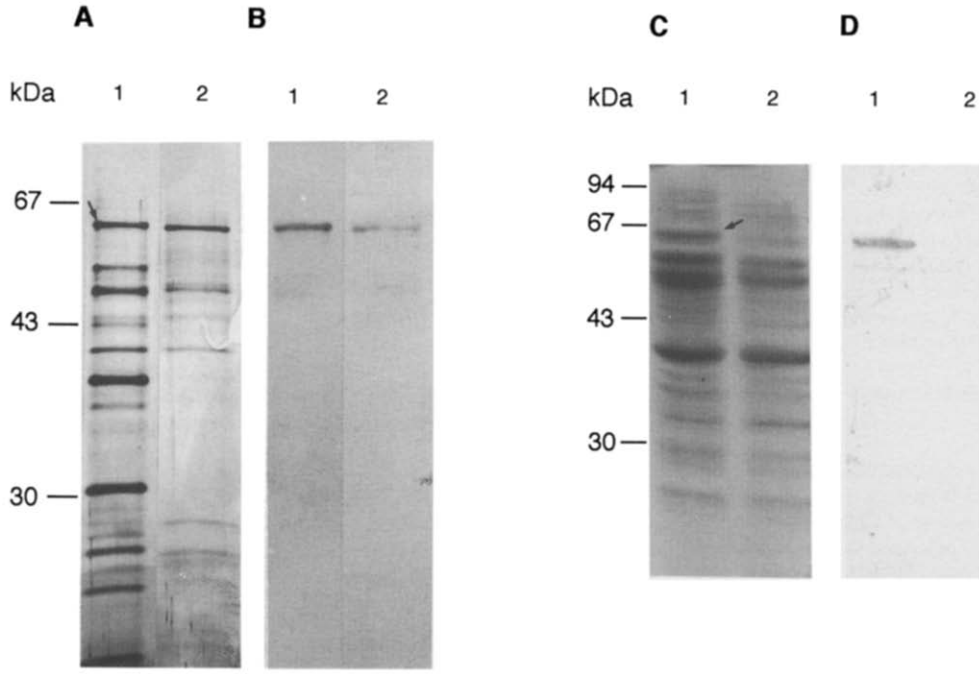

Fig. 1. (A) Silver-stained SDS-PAGE of *L. lactis* extract (lane 1) and purified malolactic fraction (lane 2). (B) Immunoblot corresponding to A. (C) Coomassie blue-stained SDS-PAGE of *L. lactis* extract (lane 1) and SO1 mutant extract (lane 2). (D) Immunoblot corresponding to C. The amount of total protein loaded is 0.5 mg (A,B,D), or 50 mg (C). Sizes of molecular weight markers (kDa) are indicated. Arrows show the position of MLE for the wild-type strain.

tides corresponding to the beginning of ORF2. Two of the cloned inserts do not contain ORF2, but part of ORF1. The MLE detected by immunoscreening is therefore encoded by ORF1. Moreover, the NH_2 terminal protein sequence deduced from the nucleotide sequence of ORF1 corresponds to the NH2 terminal sequence of *L. lactis* MLE [30].

The nucleotide sequence of the gene encoding the MLE of *L. lactis* and the deduced protein sequence are presented in Fig. 3. The coding region is 1620 nucleotide long. A possible Shine–Dalgarno sequence (GGAGG) is observed seven nucleotides upstream from the putative ATG start codon, providing a strong consensus with sequences previously described for *E. coli* and *L. lactis* [31]. Putative −10 (TATAGT) and −35 (TTGACT) promoter regions [31], separated by 18 nucleotides, were found 44 nucleotides upstream from the ATG start codon. No terminator structure was found at the 3′ end of ORF1.

The first 582 nucleotides of ORF2 were sequenced (not shown). A putative start codon is found 15 nucleotides after the stop codon TAA for MLE (Fig. 3). A possible Shine–Dalgarno-like sequence (AAGG) [31] upstream from the start codon is indicated. No typical promotor was found. These results strongly suggest that at least the two genes are organised in an operon structure.

Codon usage in MLE was compared with that of proteins from *E. coli* or *L. lactis*. Codons with a high AT content are always favoured, as observed in other *L. lactis* proteins [32]. The malolactic nucleotide sequence can be translated into a polypeptide of 640 amino acid residues, with a predicted molecular weight of 59 kDa. This is in good agreement with the molecular weight estimated using SDS-PAGE (60 kDa, Fig. 1).

3.4. *Homology comparison of malolactic enzyme*

Homology comparison of the MLE sequence with database protein sequences was performed by computer at the NCBI using the Blast network service. The proteins showing relatedness with MLE all belong to the malic enzyme family. Highest homology scores were found with malic enzymes from *E. coli* [33] (42% identity, 47% similarity), *Flaveria trinervia* [34] (37%, 47%), maize [35] (38%, 48%), rat [36] (35%, 47%), human [37] (35%, 45%), mouse [38] (35%, 47%), *Ascaris suum* (Swiss-Prot database, accession number P27443) (35%, 46%), and bean cinnamyl alcohol dehydrogenase (39%, 49%). This last one is an NADP-dependent enzyme in-

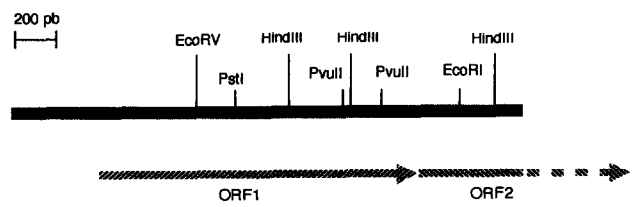

Fig. 2. Genetic and restriction map of the 2.7 kb cloned insert of p153A.

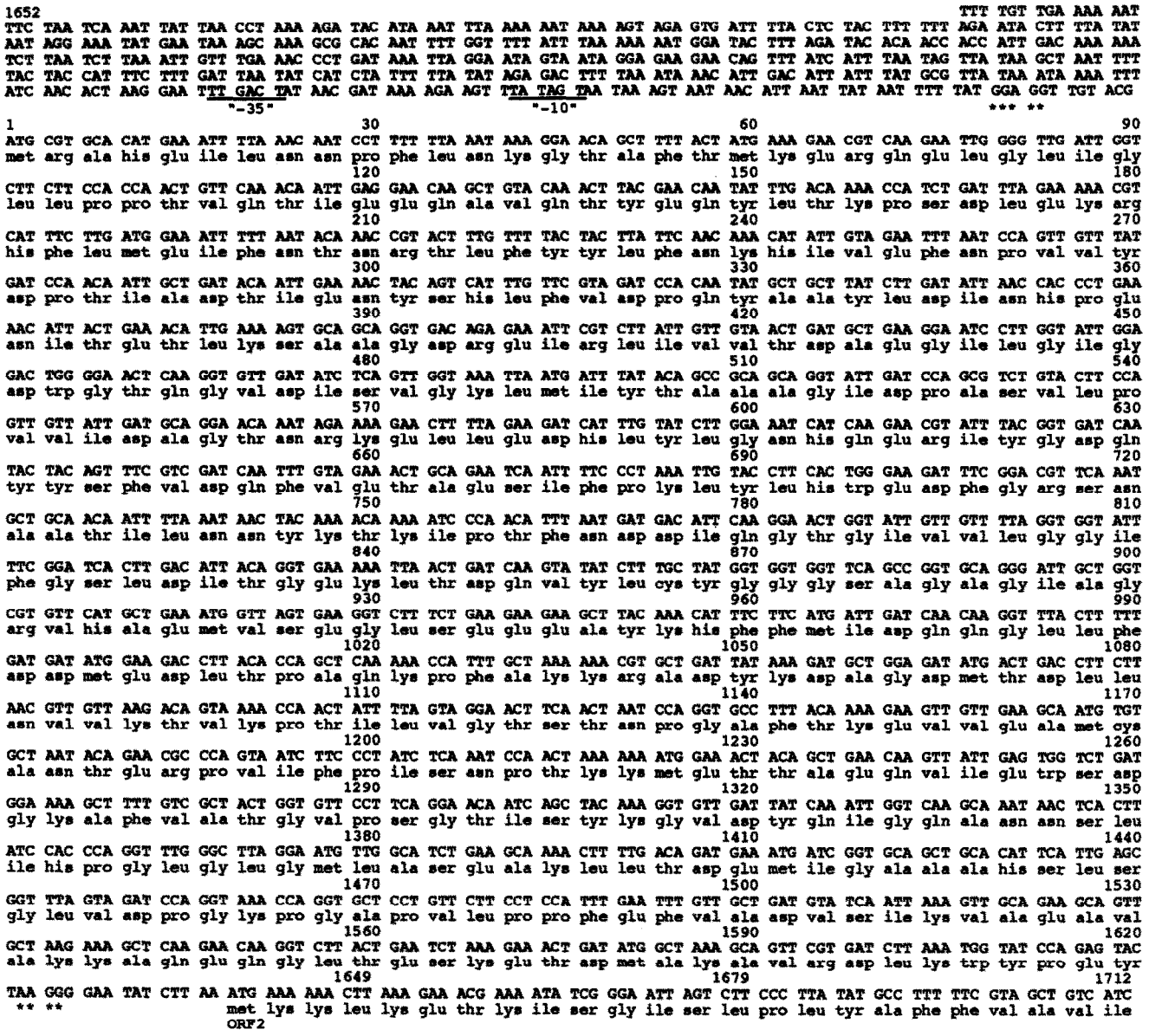

Fig. 3. Nucleic acid sequence and predicted protein sequence of the *mleS* gene. The putative '−10' and '−35' promoter (underlined) and Shine–Dalgarno (*) regions are indicated. The beginning of ORF2 and the putative corresponding Shine–Dalgarno region (*) are indicated.

volved in lignin synthesis having a high sequence homology with the malic enzymes [39]. Less homology was found with malic enzyme from *Bacillus stearothermophillus* [40], previously shown to be poorly related to all other known malic enzymes.

Multiple alignment of the *L. lactis* MLE sequence against the sequences of the five most homologous proteins reveals important homologies over the entire sequence length (Fig. 4), with highly conserved stretches interrupted by poorly conserved segments. Strong sequence conservation is found in the NH_2 terminal region between amino acids 143 and 152 (box I in Fig. 4). This sequence matches the consensus sequence for the NAD binding domain, as proposed by Wierenga et al. [41]. This 'ADP binding $\beta\alpha\beta$ fold' region, which is also present in different NAD or NADP-dependent dehydrogenases is thought to bind the ADP moieties of the dinucleotides. A region between amino acids 290 and 322 (box II in Fig. 4) includes a motif that is also homologous to a proposed nicotinamide coenzyme binding site [42]. Another sequence (FNDDIQGT) (box III in Fig. 4), described as the malic enzyme signature in Prosite databases [43], is also found in MLE. The functional significance of this region has not been determined.

The secondary structure of MLE, predicted using information from alignments of nine malic enzyme sequences (PHD program [29]) was shown to consist

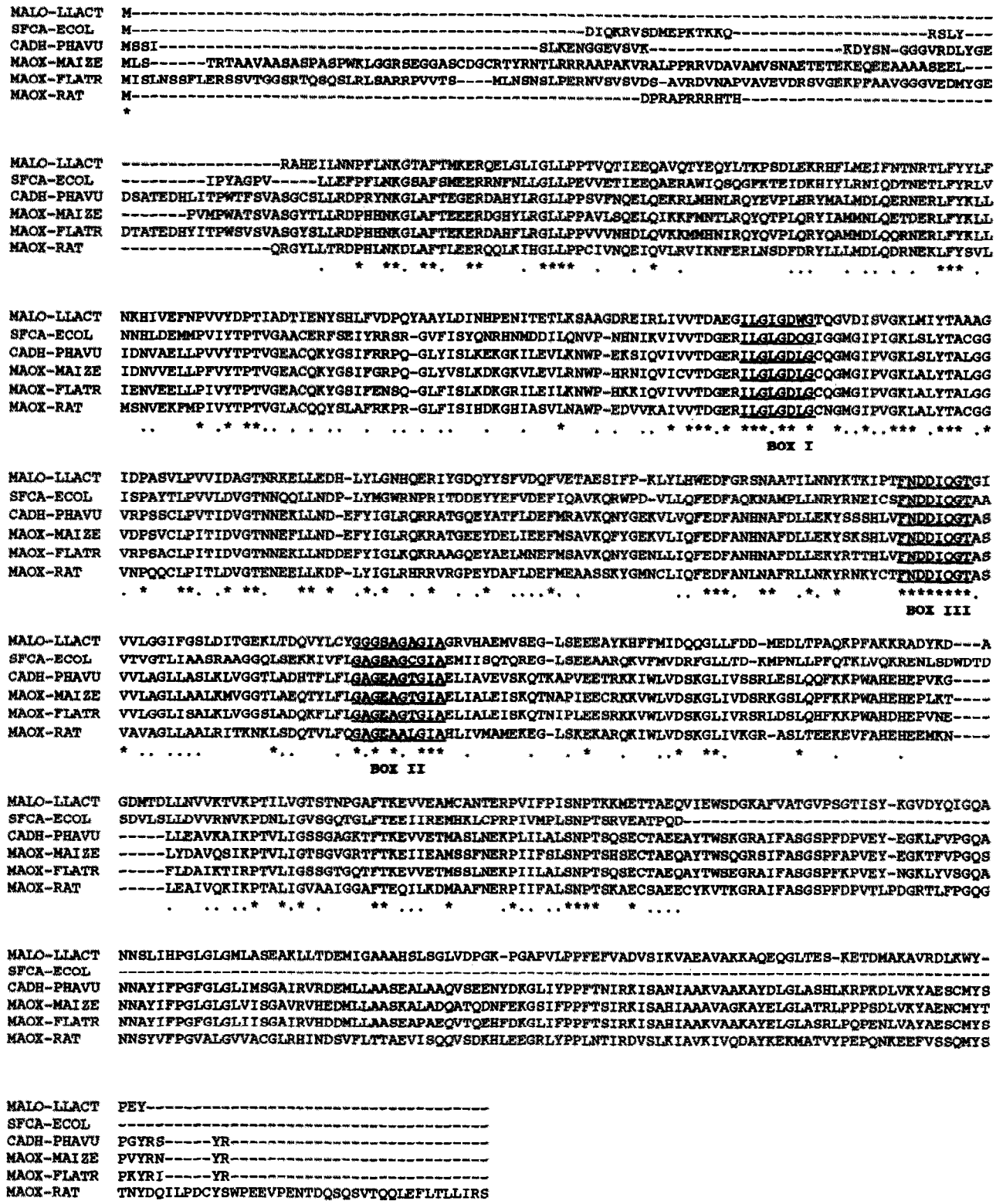

Fig. 4. Malolactic aminoacid sequence (malo-Llac) and five sequences presenting the best homology score (BLAST program) were aligned using the CLUSTAL program. Sequences aligned are malic enzymes from rat (Maox-Rat), *Flaveria trinervia* (Maox-Flat), maize (Maox-Maize), *E. coli* (Sfca-E.col), and bean cinnamyl alcohol dehydrogenase (Cadh-Phavu). Identity (*) and similarity (.) are indicated. Conserved regions (box I, II, III) are underlined.

mainly of α helix (46.7%), with a small proportion of β sheet (17%). Similar secondary structures have been found for the malic enzymes aligned previously (not shown).

Table I

Expression of MLE in *E. coli* and *S. cerevisiae* transformants

Strain	L-lactate (g/l)[a]	
	0% malate	1% malate
E.coli/pUC 18	0	0.01
E.coli/p153A	0.01	0.31
S.cerevisiae/pVT1OO-U	0	0.01
S.cerevisiae/pM1	0.15	0.52

[a] L-lactate level was determined in the supernatant of 48 h culture medium. Results are the mean of at least 3 separate cultures.

The phylogenetic tree constructed from the sequences of MLE and nine malic enzyme proteins (not shown) showed that MLE is most strongly related to *E. coli* malic enzyme, and that the bacterial proteins constitute a group distinct from the animal or plant groups.

3.5. *Expression of MLE in* E.coli *and* S.cerevisiae

The production of L-lactate from L-malate in malolactic transformants of *E. coli* and *S. cerevisiae* was monitored (Table I). The *E. coli* clones transformed with the plasmids pUC18 (control) and p153A were grown in M9 media containing 1% of L-malate. Under these growth conditions, only traces of L-lactate (0.01 g/l) are detected in the supernatant with the control strain, while 0.31 g/l of L-lactate were produced with p153A after 48 hours growth.

In order to assess expression in *S. cerevisiae*, the malolactic coding region was cloned into the multicopy yeast expression vector pVT100-U, between the promoter and terminator region of the *ADHI* gene, resulting in the plasmid pM1. The *S. cerevisiae* V5 strain was transformed with pVT100-U (control) and pM1. The transformant pM1, when grown on YNB plus 1% L-malate, buffered to pH 3, produced significant amounts of L-lactate (0.52 g/l), whereas only traces were detected with the control strain. With the pM1 strain only, a small but significant lactate amount (0.15 g/l) was produced, when no malate was added. Hence it is likely that lactate produced under these conditions is metabolised from endogenous malate, via MLE.

4. DISCUSSION

A molecular clone containing *mleS*, the gene encoding MLE, was isolated from *L. lactis*. Analysis of the amino acid sequence derived from the *L. lactis mleS* gene showed that the malolactic protein had important structural homologies with malic enzymes.

The identity of MLE (first called malic enzyme) has remained a puzzling question for many years (see [1] for review). However, direct conversion of L-malate to L-lactate and CO_2 with no release of intermediate compounds, and the preparation of purified MLE further proved that the so-called malic enzymes were in effect MLEs. Of the lactic acid bacteria, *L. faecalis* [44] and then *Lactobacillus casei* [13] were described as possessing a true malic enzyme. *L. casei* is the only organism known to contain both malic enzyme and MLE (see [1,2,45] for reviews). No true malic enzyme has been described in *L. lactis*. Futhermore, the MLE has already been purified from this bacteria [30], and a collection of mutants affected at differents stages of MLF (MLE, regulation, malate permeation) have been obtained [4].

Several lines of evidence led us to think that ORF1 encodes for MLE. Firstly, the antibodies we obtained did not react with the crude extract of a mutant that showed no detectable malolactic activity in vitro and was affected in the structural gene for MLE [4]. Secondly, the deduced protein sequence has the same NH_2 terminal sequence as the MLE purified from *L. lactis* [30]. Finally, both the *E. coli* and *S. cerevisiae* strain transformed by p153A were able to convert L-malate into L-lactate directly. In the past it was hypothesised that the bifunctionality of MLE might arise from an ancestral rearrangement of a malic enzyme and of an LDH sequence. However, in this work, no significant homologies were found between MLE and LDH sequences. This implies that the MLE and malic enzyme have acquired different functions by the way of minor sequence modifications. Further studies such as crystallographic analysis of the two proteins, and mutagenesis experiments, should lead to understanding of the evolutionary mechanism and of the structural features responsible for the funtional specificity of both enzyme types.

Additional information was obtained from Southern experiments. Although homology of MLE and malic enzyme was shown to be high also at the nucleotide level, no cross-hybridisation was observed during Southern experiments (not shown) with genomic DNA from *L. lactis*, using a malolactic probe. This result suggests that no true malic enzyme homologous to malolactic protein exists in addition to MLE in this strain.

Another ORF (ORF2) was found 15 nucleotides after the stop codon of MLE. The protein sequence deduced from the 582 nucleotides of ORF2 has important homologies with a citrate permease from *L. lactis* (Ansanay et al., unpublished). Malate transport was described as a component of MLF reaction; the product of ORF2 may therefore correspond to a malate permease. The organisation of MLE and malate permease in an operon structure suggests that the MLE and malate transport system in *L. lactis* are regulated in the same way. Although *L. lactis* MLE has been shown to be malate-inducible [9], data on the regulation of a malate permease in this strain are not, to our knowledge, available. Analysis of the operon and of its regulation are in progress.

Expression of malolactic enzyme was obtained in *S.*

cerevisiae. However more work is needed to achieve the high degradation of malate into lactate that would be required by a wine yeast for MLF.

Acknowledgements: We thank P. Renaud for providing us with the mutant SO1, oligonucleotides and helpful comments.

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