Cloning and Characterization of the Genes Encoding the Malolactic Enzyme and the Malate Permease of *Leuconostoc oenos*

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Using degenerated primers from conserved regions of the protein sequences of malic enzymes, we amplified a 324-bp DNA fragment by PCR from *Leuconostoc oenos* and used this fragment as a probe for screening a *Leuconostoc oenos* genomic bank. Of the 2,990 clones in the genomic bank examined, 7 with overlapping fragments were isolated by performing colony hybridization experiments. Sequencing 3,453 bp from overlapping fragments revealed two open reading frames that were 1,623 and 942 nucleotides long and were followed by a putative terminator structure. The first deduced protein (molecular weight, 59,118) is very similar (level of similarity, 66%) to the malolactic enzyme of *Lactococcus lactis*; as in several malic enzymes, highly conserved protein regions are present. The synthesis of a protein with an apparent molecular mass of 60 kDa was highlighted by the results of labelling experiments performed with *Escherichia coli* minicells. The gene was expressed in *E. coli* and *Saccharomyces cerevisiae* and conferred “malolactic activity” to these species. The second open reading frame encodes a putative 34,190-Da protein which has the characteristics of a carrier protein and may have 10 membrane-spanning segments organized around a central hydrophilic core. Energy-dependent L-[¹⁴C]malate transport was observed with *E. coli* dicarboxylic acid transport-deficient mutants carrying the malate permease-expressing vector. Our results suggest that in *Leuconostoc oenos* the genes that encode the malolactic enzyme and a malate carrier protein are organized in a cluster.

Malolactic fermentation (MLF) of wine, which is the bacterial conversion of L-malic acid to L-lactic acid and carbon dioxide, results in a natural decrease in acidity (25), which leads to increased biological stability and changes in wine flavors. Usually, MLF develops naturally, but several factors, such as ethanol or sulfur dioxide concentration and pH, may affect this reaction. At the present time, this reaction is not entirely controlled, and processing problems, microbial wine alteration, and loss of time occur frequently in winemaking. To deal with such problems, MLF research, especially research concerning the malolactic enzyme (MLE) responsible for decarboxylation of L-malate, is performed.

MLEs have been purified from several lactic acid bacteria, including *Leuconostoc mesenteroides* (30, 45), *Lactobacillus plantarum* (9), and *Leuconostoc oenos* (34). The molecular masses of the subunits of the purified MLEs range from 60 to 70 kDa. The active form of each protein is a dimer or a tetramer consisting of identical subunits. The MLEs require NAD⁺ and Mn²⁺ as cofactors for activity (5).

The MLF system of *Lactococcus lactis* has been partially characterized. A positive regulatory gene called *mleR*, which is responsible for activation of the malolactic system was described first (38). Recently, cloning, sequencing, and heterologous expression of the MLE gene from *Lactococcus lactis* (1, 13, 14) have been described. A sequence alignment of the protein revealed that there are three highly conserved regions that are also present in proteins that belong to the malic enzyme family. The mechanism of L-malate transport, the other component of the MLF reaction, has also been characterized (37). As in *Lactobacillus plantarum* (36) and *Leuconos-*

toc oenos (42, 49), studies of L-malate transport revealed that carrier-mediated transport occurs. However, no L-malate permease gene has been described.

The limited genetic data available are data for *Lactococcus lactis*, which is not involved in MLF in wine. The MLE from *Lactobacillus delbrueckii* has been cloned, but a low level of expression was obtained (54). *Leuconostoc oenos* is the most common species in wine since it is the lactic acid bacterium that is best adapted to low pH values and high ethanol concentrations. Moreover, among the malolactic starters commonly used during winemaking, the most efficient industrial starters are produced from *Leuconostoc oenos* strains. Surprisingly, the genetic bases of the MLF in *Leuconostoc oenos* are still not known. Workers have attempted to clone the MLE structural gene from *Leuconostoc oenos* in *Escherichia coli* (28), but there were instability problems with the cloned DNA fragments and no further information was obtained.

In this paper, we describe the cloning and sequencing of the genes that encode MLE and a membrane protein in *Leuconostoc oenos*. Malolactic activity was obtained in *E. coli* and *Saccharomyces cerevisiae*. Transport experiments performed with *E. coli* demonstrated that the membrane protein is a malate carrier protein.

MATERIALS AND METHODS

Bacterial strains, plasmids, and media. The strains and plasmids used in this study are listed in Table 1. *E. coli* CBT313 was kindly provided by B. J. Bachmann, Department of Biology, Yale University, New Haven, Conn. *E. coli* strains were grown in Luria-Bertani broth or agar (6) at 37°C. Antibiotics were used at the following concentrations in the selective media: ampicillin, 50 µg · ml⁻¹; and erythromycin, 200 µg · ml⁻¹. *Leuconostoc oenos* Lo84.13 was grown at 30°C in FT80 medium (pH 5.3) (10) modified by adding meat extract instead of Casamino Acids. *S. cerevisiae* RH100-4 was grown in YPG medium (35) supplemented with 2% (wt/vol) galactose at 30°C with aeration. A selective medium (35) lacking uracil was used to select for transformant yeast strains. *S. cerevisiae* was transformed by a method in which lithium acetate and polyethylene glycol were used (20).

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TABLE 1. Strains and plasmids used

Strain or plasmid	Description	Source or reference
Strains		
<i>E. coli</i> TGI	<i>supE hsdΔ5 thi Δ(lac-proAB) F'[traD36 proAB⁺ lacI^q lacZΔM15]</i>	19
<i>E. coli</i> CBT313	<i>sdh-2 rpsL129 dctA4 thi-1</i>	29
<i>E. coli</i> DH5α	<i>supE44 ΔlacU169 (Δ80 lacZΔM15) hsdR17 recA1 endA1 gyrA96 thi1 relA1</i>	23
<i>E. coli</i> AR1062	<i>F⁻ thr leu ara azi fhuA lacY tsx minA gal rpsL xyl mtl thi hsdR</i>	15
<i>Leuconostoc oenos</i> Lo84.13	MLE ⁺	Oenological Institute of Bordeaux
<i>S. cerevisiae</i> RH100-4	<i>MATa ura3 his3 bar1-1 suc2Δ9</i>	35
Plasmids		
pJDC9	Em ^r Δ <i>lacZ</i>	11
pJ6D5	pJDC9 containing the 5-kb <i>Sau3A</i> DNA fragment from <i>Leuconostoc oenos</i>	This study
pJ2C3	pJDC9 containing the 3-kb <i>Sau3A</i> DNA fragment from <i>Leuconostoc oenos</i>	This study
pBluescript SK ⁺	Ap ^r Δ <i>lacZ</i>	Stratagene
pJF119EH/pJF119HE	<i>lacI^q rrmB Ap^r</i> (expression vector with <i>tac</i> promoter)	18
pCFED6/pCFHD6	pJF119EH containing the 2.7-kb <i>EcoRI-PstI</i> fragment of pJ6D5 inserted in both orientations containing <i>mleA</i>	This study
pCFEC3/pCFHC3	pJF119HE containing the 1.6-kb <i>Sall-XbaI</i> fragment of pJ2C3 inserted in both orientations containing <i>mleP</i>	This study
pUC19	Ap ^r Δ <i>lacZ</i>	56
pULKE6	pUC19 containing the 1.6-kb <i>KpnI-EcoRI</i> PCR fragment containing <i>mleA</i>	This study
pYeDP1/8-2	<i>ura3 Ap^r</i> (shuttle vector of <i>E. coli</i> and yeast) <i>Gal10 cycl</i> promoter <i>Pgk</i> terminator	12
pYMLE1	pYeDP1/8-2 containing the 1.6-kb <i>KpnI-EcoRI</i> PCR fragment containing <i>mleA</i>	This study

Construction of *Leuconostoc oenos* genomic bank. Total DNA from *Leuconostoc oenos* was prepared by the procedure of Byun et al. (8). The genomic DNA of *Leuconostoc oenos* was partially digested with restriction enzyme *Sau3A* and was size fractionated with a 10 to 40% (wt/vol) sucrose gradient by the method described by Sambrook et al. (43). Fractions containing DNA fragments that were 5 to 10 kb long were identified by electrophoresis in 0.6% agarose gels and were used to build the bank. DNA fragments were ligated to *Bam*HI-digested pJDC9 treated with bacterial alkaline phosphatase (Gibco BRL). Recombinant plasmids were transferred into *E. coli* TGI by electroporation (16).

Amplification of a specific probe. Oligonucleotide primers with opposite orientations were custom synthesized (Eurogentec, Seraing, Belgium) from two conserved regions of the malic enzymes (box I and box III) (1, 13). The 24-mer degenerated primers used were 5'-ATHYTNGGNATHGGNGAYTGGGGN-3' and 5'-NGTNCCTGATRTCTCTCTTAA-3' (R = A or G; Y = C or T; H = A, T, or C; N = A, G, C, or T). Amplification was performed with a Hybaid temperature cycler. Each reaction mixture contained 10 μl of 10× *Taq* DNA polymerase buffer, 2 mM MgCl₂, each deoxynucleoside triphosphate at a concentration of 50 μM, 50 ng of genomic DNA, 20 pmol of each primer, and 2.5 U of Goldstar *Taq* polymerase (Eurogentec) in a final volume of 100 μl. DNA amplification was performed for 35 cycles consisting of denaturation for 40 s at 92°C, annealing for 1 min at 37 or 42°C, and elongation for 1 min at 72°C, with a ramping rate of 5 s/1°C between the annealing and elongation steps. The PCR products were analyzed on a 1% agarose gel. The expected 324-bp DNA strand was recovered by using a DNA purification matrix kit (Bio-Rad), was made blunt ended with the *E. coli* DNA polymerase Klenow fragment (Boehringer Mannheim), and was ligated into *Sma*I-digested pBluescript SK⁺. After transformation of *E. coli* TGI cells with the plasmid, the sequence of the DNA was determined to check the identity of the PCR product (see below).

Insertion of restriction sites besides the *mleA* gene. The *mleA* gene was amplified by PCR by using plasmid pCFED6 as a template. The PCR primers used were 5'-GTTCTTGGGTACCAAATGACAGATC-3' and 5'-AAAG AATTCATTAGTATTTCCGATCCC-3', which included *KpnI* and *EcoRI* sites (underlined nucleotides), respectively. The PCR was performed with the high-fidelity *Pfu* polymerase (Stratagene) for 30 cycles by using the reaction mixture described above (denaturation for 1 min at 92°C, annealing for 4 min at 30°C, and elongation for 3 min at 72°C, with a ramping rate of 5 s/1°C between the annealing and elongation steps). The cloned gene was sequenced to verify its integrity. Using the unique restriction sites introduced by the PCR, we cloned the DNA fragment carrying the *mleA* gene in high-copy-number plasmids pUC19 and pYeDP1/8-2 (a shuttle vector for *E. coli* and *S. cerevisiae*). The resulting constructions were used to transform *E. coli* DH5α and *S. cerevisiae*.

DNA manipulations and sequence determination. Molecular procedures were carried out as described by Sambrook et al. (43). Double-stranded DNA from the recombinant plasmid was purified by using a Qiagen plasmid kit (Tip 100; Diagen) and was sequenced by the dideoxy chain termination method (44) by using a Sequenase version 2.0 kit (U.S. Biochemical Corp.). Both strands were sequenced by using synthetic oligonucleotide primers (Eurogentec) as recommended by the manufacturer.

Colony hybridization. Colony hybridization was carried out by transferring bacteria from a master plate to a nylon filter (pore size, 0.45 μm; Schleicher and

Schuell). The colonies on the filter were then lysed, and the liberated DNA was fixed to the filter by baking (21). Labelling of the DNA strand, hybridization, and detection were performed by using a DIG labelling and detection kit (Boehringer Mannheim) as recommended by the manufacturer.

Identification of plasmid-encoded proteins. Minicells were isolated from an overnight culture of *E. coli* AR1062 containing cloned DNA fragments in expression vectors pJF119EH and pJF119HE. Minicells were prepared, labelled with L-[³⁵S]methionine (Isotopchim, Ganagobie-Peyruis, France), and fractionated as described by Bally et al. (4). The expression products were analyzed by sodium dodecyl sulfate (SDS)-12% polyacrylamide gel electrophoresis (PAGE) by the method of Laemmli (27).

Preparation of cell extract and determination of malolactic activity. Cell extracts were prepared from *E. coli* and *S. cerevisiae* cultures. After growth, cells were concentrated and disrupted with a French press at 10⁸ Pa. After the *E. coli* AR1062 cell extract was prepared, the cellular proteins were precipitated with 30 to 60% (wt/vol) ammonium sulfate at 0°C.

The malolactic activity of a recombinant yeast culture was determined after cells were grown in YPG medium (pH 3) containing 1% (wt/vol) L-malate at 30°C under aerobic conditions. After centrifugation, the amount of L-lactate present in the supernatant was determined, and cells were used to prepare cell extracts.

The MLE activities of the cell extracts were determined by measuring the amount of L-lactate formed from the decarboxylation of L-malate at 37°C in phosphate buffer containing 0.1 mM MnCl₂, 0.5 mM NAD, and 37 mM L-malate (pH 5).

L-Lactate and L-malate contents were determined with Boehringer Mannheim enzymatic kits. Protein concentration was estimated by the method of Lowry et al. (31).

Transport assays. Cells were harvested from mid-log-phase cultures in Luria-Bertani medium after IPTG (isopropyl-β-D-thiogalactopyranoside) induction and were washed twice with 100 mM potassium phosphate (pH 5). The cells were resuspended in the same buffer containing 10 mM MgSO₄ to a final concentration of about 0.15 mg of protein per ml. Glucose (10 mM) was used as the energy source. The reaction was started by adding L-[U-¹⁴C]malate (Amersham, Les Ulis, France) at 25°C. The reaction was stopped by adding 2 ml of ice-cold 100 mM LiCl, and the mixture was immediately filtered through 0.45-μm-pore-size cellulose nitrate membrane filters (Whatman). The filters were washed twice with 2 ml of LiCl and placed into scintillation liquid, and the amount of radioactivity was determined.

Nucleotide sequence accession number and sequence analysis. The nucleotide sequence data reported in this paper have been deposited in the EMBL, GenBank, and DDBJ nucleotide databases under accession number X82326. A computer analysis and a comparison of the nucleotide and amino acid sequences were carried out with PC GENE and DNA Strider software.

RESULTS

Cloning of the *mle* locus from *Leuconostoc oenos*. We amplified a 324-bp DNA fragment from the genomic DNA of *Leu-*

FIG. 1. Restriction maps of the two overlapping insertions of recombinant plasmids pJ6D5 and pJ2C3. The restriction sites in parentheses belong to the multicloning site of pJDC9. The solid bar indicates the location of the probe used to screen the *Leuconostoc oenos* genomic bank. The large arrows indicate the positions and the directions of transcription of *mleA* and *mleP*. The small arrows indicate the primers used for PCR amplification to verify that the cloned genes belong to *Leuconostoc oenos* genomic DNA. pb, base pairs.

conostoc oenos. This fragment was subcloned in the sequencing vector pBluescript SK⁺, and its complete nucleotide sequence was determined. The deduced amino acid sequence of the cloned fragment revealed that it exhibited a high level of identity with the *Lactococcus lactis* MLE amino acid sequence (data not shown).

We constructed a genomic library of *Leuconostoc oenos* in vector pJDC9 as described above. Colony hybridization experiments were performed by using the 324-bp DIG-labelled DNA fragment of *Leuconostoc oenos* as the probe. Of the 2,990 clones in the genomic library, 7 hybridized with the probe. Restriction endonuclease mapping of the clones revealed the presence of overlapping insertions ranging from 3 to 5 kb long. Restriction maps of two overlapping insertions in recombinant plasmids pJ6D5 and pJ2C3 are shown in Fig. 1.

Nucleotide and protein sequences. The two insertions in recombinant plasmids pJ6D5 and pJ2C3 were sequenced on both strands. PCR was used to check that the cloned genes originated from *Leuconostoc oenos* (Fig. 2). The strands recovered by amplification of *Leuconostoc oenos* genomic DNA (Fig. 2, lanes 7 to 10) had the expected lengths (626, 719, 1,021, and 2,937 bp). No amplification was observed with *E. coli* genomic DNA (Fig. 2, lanes 2 to 5).

The nucleotide sequence contains two open reading frames (ORF) (Fig. 3). The coding region of the first ORF (designated *mleA*) is 1,623 nucleotides long starting at a putative ATG start codon (nucleotide 364). Preceding the start codon, there is a putative ribosome binding site (RBS) (5'-AGGAG-3'; nucleotides 353 to 359) that is complementary to the 3' end of *Leuconostoc mesenteroides* 16S rRNA 5'-CACCTCCTTCT-3' (complementary nucleotides are underlined) (55). The 3' end of *Leuconostoc mesenteroides* 16S rRNA was used as a reference since the 3' end of *Leuconostoc oenos* 16S rRNA has not been described. Thus, the putative RBS is quite homologous to the deduced RBS described for *Leuconostoc oenos* bacteriophage genes (47). The protein deduced from the *mleA* sequence is composed of 542 residues and has a calculated molecular mass of 59,118 Da and a predicted pI of 4.55. A comparison of the primary structure of the putative protein with data in the Swissprot and Pirprot protein sequence data banks revealed the following levels of identity with other en-

zymes: 66% with the MLE of *Lactococcus lactis* (1, 13), 41% with the malate dehydrogenase of *S. cerevisiae* (nucleotide sequence accession no. S37846), 38% with the bean cinnamyl alcohol dehydrogenase (52), and 39 and 37% with the malic enzymes of maize (40) and *E. coli* (32), respectively. Three highly conserved sequences (box I, box II, and box III) are present in the deduced protein sequence obtained from the *mleA* data, as in the five enzymes mentioned above (Fig. 4). Box I corresponds to an NAD binding domain (53); box II includes a motif that is homologous to a proposed nicotinamide coenzyme binding site (46); and box III is described as the "malic enzyme signature" in Prosit databases (2). A possible malate binding site, which has been described by Kulkarni et al. (24), is also present.

Putative start codon ATG (nucleotide 1989) of the second ORF (*mleP*) is found at the same level as the TAA stop codon (nucleotide 1987) that terminates *mleA*. There may be an RBS (5'-AAGTGGG-3'; nucleotide 1969) before the 945 nucleotides that constitute *mleP*. The sequence is partially complementary to the 3' end of *Leuconostoc mesenteroides* 16S rRNA 5'-CACCTCCTTCT-3' (complementary nucleotides are underlined). Downstream of the TAA stop codon (nucleotide 2931) of *mleP*, inspection of the noncoding region revealed an inverted repeat sequence with three unbounded bases (Fig. 3, convergent arrows) followed by a cluster of Ts that could form a stem and loop structure in mRNA with a calculated ΔG_f of -11.6 kcal/mol (-48.5 kJ/mol) (48). This structure probably functions as a rho-independent transcriptional terminator (39). The 314-amino-acid protein deduced from the nucleotide sequence of *mleP* has a predicted molecular mass of 34,190 kDa. An analysis of the amino acid composition revealed that there are 58% nonpolar residues and 42% polar residues. Among the charged residues, 63% are basic. This protein is thus a basic protein with a theoretical pI of 10.1. The deduced amino acid sequence was compared with data from the protein data banks mentioned above. This analysis revealed similarities with membrane proteins (data not shown). The identity scores were

FIG. 2. PCR amplification from *E. coli* (lanes 2 to 5) and *Leuconostoc oenos* (lanes 7 to 10) genomic DNAs by using the primers indicated below the restriction maps of pJ6D5 and pJ2C3 in Fig. 1 (primers A through D). A lambda-*HindIII* DNA ladder (lanes 1 and 11) and a DNA ladder (Gibco BRL) containing molecules from 100 to 1,500 bp long (lane 6) were used to determine the lengths of the amplified strands.

GAATTCAATTGTTGGTTGATTCATTCGGTCAATTCTCTGTTGGCGCGCAACCACTCTCTGAAGAATTTTTTGGCGTGTITTTCAAAAACTTTCCAGCT 100
 TCCGTCAAAGAAATCTGCCATCGCAGCGATTCGATTAATAGAGTTTTTTCATCTCGTTTCTAAACGCTGAATGGCAAAGAAATTTGCGGTTGAC 200
 TGACATTGAAAAAATAGCTGTTTTTGAAGAGATCAAGTTCCGATAAATTTGCGAAGTATTCGATATCGGTGAATTTTCATATAAGATAATTTTATCTC 300
 TTTTATAAGTTTGGACTATATTAATCCTAATGGAAATATGATAATGTGGTTCTTGGAGAGAAAATATGACAGATCCAGTAAGTATTTTAAATGATCCTTTT 400
 RBS M T D P V S I L N D P F
 ATTAACAAAGGAACGTCTTTTACGGAAGCGGAGAGAGAGAGGAGCTTGGTTTAAACGGTTTATTTACCGGCCAAGGTTTCAGGCTTTACAAGAGCAAGTTGATC 500
 I N K G T A F T E A E R E E L G L N G L L P A K V Q A L Q E Q V D
 AGACTTATGCTCAATTTCAAAGCAAGTTTCAAATCTCGAAAAACGATGTTTTTAAATGGAAATATTCATACGAATCAAGTGTGTTTTATAAGCTTTT 600
 Q T Y A Q F Q S K V S N L E K R L F L M E I F N T N H V L F Y K L F
 TTCACAACATGTTTGAATTTATGCCAATGTTTATGACCGCAAAATTTGCTGATACAAATGAAATATTTCCGAATATTTGTTGAACCGCAAGGTTGCC 700
 S Q H V V E F M P I V Y D P T I A D T I E N Y S E L F V E P Q G A
 ACTTTTGTGATATTAATCATCCGAAACATTCATCGACTCTGAAAAATCTGCTAATGGCGCGGATATCAAGCTGCTGCTGCTTTCTGATCGCGAAG 800
 A F L D I N H P E N I Q S T L K N A A N G R D I K L L V V S D A E G
 GTATTCTTGGGATGGGAGACTGGGCTGCTCAGGCTGTTGATATGCTGCTCGGCAAACTGATGGTTTATACAGTTGCGCGCGAATCGATCCATCAACAGT 900
 I L G I G D W G V Q G V D I A V G K L M V Y T V A A G I D P S T V
 TCTTGCAGTTGTTATGATGCTGGAACAAATAACGAAAGCTTTTGAAGATCCTATGATTTGGGAAATAAATTTAATCGTGTTCGTGGCGGATGAAGTAC 1000
 L A V V I D A G T N N E K L L K D P M Y L G N K F N R V R G D A K Y
 TATGATTTTATCGACAAATTTGTTAATCATGCCGAATCGCTTTTTCCTAATTTATATTTGCATTTGGGAAGATTTTGGCGTTCGAATGCTTCTATATCT 1100
 Y D F I D K F V N H A E S L F P N L Y L H W E D P G R S N A S N I
 TAAACAGCTATAAGATAAAATGCTACTTTTAAATGATGATTTCAAGGAATCGGATCGTCTTGGCGCGTTCCTTGGAGCGTTTGAAGATTTCCGG 1200
 L N S Y K D K I A T F N D D I Q G T G I V V L A G V L G A L K I S G
 TCAGAAATTAATGATCAAACTTACATGAGCTTCGGTGGCGGAATCGTGGAAATGGGAAATGTTAAACAGTTGATGAAGAAATGTTGAACAGGCTCTT 1300
 Q K L T D Q T Y M S F G A G T A G M G I V K Q L H E E M V E Q G L
 TCCGACGAAGAGGCTAAAGCAATTTCTCTTGTGACAAACAGGCTCTTGTGTCAGATGATCCGGATTTAATCCAGAGCAAAAGCTTTTCGCTG 1400
 S D E E A K K H F F L V D K Q G L L F D D D P D L T P E Q K P F A
 CTAAACGAAGTATTTCAAATAATGCTAATCAATGACCAATCTCCAGCAGCTGTTGAAGCTGTCACCCGACCATTTTGTGGGAACCTCGACACATCC 1500
 A K R S D F K N A N Q L T N L Q A A V E A V H P T I L V G T S T H P
 AAAATCTCTTACTGAAGAAATGTTAAAGATATGTCGTGTTACTGTAAGACCAATCAATTTTCCAATTTCCAATCCAACAAATTAGCGAAGCAAAA 1600
 N S F T E E I V K D M S G Y T E R P I I P P I S N P T K L A E A K
 GCCAAGATGTTTGAAGTGTCTAATGGAAAAGCTTATCGGTACTGCTGTTCCAGTTGACGATATGAATATGAGGGCAAGCTTACCAATCGGTC 1700
 A E D V L K W S N G K A L I G T G V P V D D I E Y E G N A Y Q I G
 AGGCCAACAATGCTTATCTATCCAGGCTTGGCTTGGTGGCAATGCGGCTCAATCAAAGCTGCTTACGCGTGAAGATGTTTCTGCTGCCCATAG 1800
 Q A N N A L I Y P G F G A I A Q S K L L T P E M I S A A H S
 TCTTGGAGGAATCGTTGATACAAACAAAGTTGGTGTGCTGTTTGGCACCAGTTTCAAATTTAGCCGACTTTTCGCGTACAGTCTGCTGCTGCTGCT 1900
 L G G I V D T T K V G A A V L P P V S K L A D F S R T V A V A V A
 AAAAAAGCTGTTGAACAGGCTCTAATCGCCAGCGATGATGATGTTGAAGGCGCTCGACGATTTGAAGTGGATCCGAAATACTATGATGATCTT 2000
 K K A V E Q G L N R Q P I D D V E K A V D D L K W D P K Y - -
 RBS M N A F
 TATTACAGTGTGAGAGTGTCTAGAAAATGTTCTAGTGAATGCTTTAGGTTATGTTTAAAGAGATCGGCTAAATAGCCGACTCTTTTAAAGGAAT 2100
 I T S V E S V V E I V L V I A L G Y V L R R S G K L A D S P K G N
 ATCTGATTTGATATGAATATGCGCTGCGCTGTCGATTTTGTGATGCTGCTGATTTATTTGACCCGCGATAAATAGTTAGTCTGCTGCGCGTT 2200
 I S Y L I M N I A L P L S I F V T L L T Y L T R D K L V S L S G G
 TGGTTTTCGCTGGGCTAGTTTGTGCTGAGCTACGTGCTGCTTATTTGATCACAATAATTTTAAATTCGCGGTGGTCTGCTGGAACATTTATCAA 2300
 L V L A A S F A V S Y V L A Y L I T K I F K I R V G R R G T F I N
 TATGTTGTTAAGCTAATACGATTTTATCGGTTTACCGCTAAATTCGCTTTTGTGCTGACGAAGTCTATGCTTATTTCTTAATTTATTAATGTTATG 2400
 M F V N A N T I F I G L P L N S A L F G T K S M P Y F L I Y Y V M
 AATACAATCTGACGTGGCAATGGCATCTTCTTTATTTATCCGATGATCAACGAGGGATAAGACGGAAGAAACAAAGTTTAAATGGAAAAAGTTAT 2500
 N T I S T W A I G I F I S S D D P T R D K T E K Q S F N W K K L
 TACCAATGCCATTAGTTGTTTCTGATTTGATTTGATTTGCTTGTGCAATCCGATTCGCGATGGGTTAGCACGACTTTTTCATCGTTGGCGG 2600
 L P M P L V G F L I S T V V L L L A I P I P G W V S T T F S M V G G
 AATGTTGACCGCGATGCTCTGATATATTCGGAATTAATTTGGCTGATGCTGGAATGAAATCGATTCATTTTCGATCGCGATTCGATTTTAGCTTAAATC 2700
 I V T P M S L I Y I G I I L A D A G L K S I H F D P H S I L A L I
 GGCGGTTTCATCATTTGTTCCGGGTTTATGATTATAATTTTAAATTTGTTGGCGGATCAATGCCAAGCTTGAATCTTCAACGCTGATTTTCAAGCAG 2800
 G R F I I V P A V M I I I L K L F G G S M P K L E S S T L I I Q A
 CTGCTCTGAGCTGGCTGTTTGGCGATCTTGGTTGGACAAATCATGTTGACGTTGAATATGCAACAAATGTCGTTACGACTTCAACGGTGTCTTTCGT 2900
 A A P G L A V L P I L V G Q N H G D V E Y A T N V V T T S T V L F V
 GATGTTGTTGCGGATCTTGTGATGATTTGCTAATGATAATTTACGATTTGAAGACTTACGCTCGTAAGCTTTTCTTGTACAAATTTTATCAGGT 3000
 I V V P I L M Q F V - -
 AGGAGGAAAAATGGCAGAGTGAAGTTTACATTTGATCAACAAAAGTTAAAGCACCATAGTGGCTTTGATTTGAAGAACAGAAAGGACCAAGGG 3100
 TGGCACAATGCCAAATTAATGATTAAGTTTGGCCGACGCAACCAAACTTCGATTTGAACAGGCGGCTTCAATACCTGGAAACATTTATTTGCTTCTTTGA 3200
 TGGCGACCGGCTTGTATGGAATTTATGATATTTCACTTTTGGTTGCGGAACGTTTTCATATGATTAGTTGGTATACCTACGATCTGAAACAGTTGCG 3300
 AAAAGCATTTGAAGAGACCTTAAACGGAATTTCCGATAGTATTTGAATTTCCGGATAATCTGGGCTCTGAAAAAACTTGTGTAATTTCAAGGACCAT 3400
 CTTTGTGGAGCGCTAAAACTGGGCAAGGTTATTTTGGATGAAGGTATCAGCAGCAT

FIG. 3. Nucleotide and deduced amino acid sequences of *mleA*, *mleP*, and flanking DNA from *Leuconostoc oenos*. The putative RBS are underlined. The putative start codons are enclosed in boxes, and the stop codons are indicated by dashes. The numbers on the right are the nucleotide positions. Convergent arrows indicate a putative terminator structure.

Malate Binding site		
OENOS	VLFYKLFSSQHVVEFMPIVYDPTIADTIENYSELFVEPQGAFLDINHPE	122
MLE.LACTIS	TLFYLFNKHIVEFNPVYDPTIADTIENYSHLFVDPQYAAFLDINHPE	121
MDH.YEAST	VLYFALIRRHKELVPIIYPTPTGDATAAYSHRFRKPE-GVFLDITEPDS	149
CYN.BEAN	RLFYKLLIDNVAELLFPVYPTPTVGEACQKYGSIFRRPQ-GLYISLKEKGK	146
ME.MAIZE	RLFYKLLIDNVVELLFPVYPTPTVGEACQKYGSIFRRPQ-GLYVSLKDKGK	146
ME.COLI	TLFYRLVNNHLDMMFVIYPTPTVGAACERFSEIYRRSR-GVFISYQNRHN	143
	I I I I I II	
BOX I		
OENOS	IQSTLKNAANGRDIKLLVVSDAEGILGIGDMGVQGVDAVAGKLMVYTAA	172
MLE.LACTIS	ITETLKSAAGDREIRLIVVTDAGILGIGDMGTQGVDSVAGKLMYITAA	171
MDH.YEAST	IECLATYGGDKDVYIVVSDSEGILGIGDQGGIGGVRIASKLALMTLCG	199
CYN.BEAN	ILEVLKNWPE-KSIQVIVVTDGERILGLGDLGCQGMGIPVGKLSLYTALG	195
ME.MAIZE	VLEVLRNWP-HRNIQVICVTDGERILGLGDLGCQGMGIPVGKLALYTALG	195
ME.COLI	MDDILQNVN-NNHNIKIVVTDGERILGLGDLGCQGMGIPVGKLSLYTACG	192
	I I I I I III II I I I II I	
OENOS	GIDPSTVLAVVIDAGTNNKELLKDPMYLGNKFNVRGDKYYDFIDKFVN	222
MLE.LACTIS	GIDPASVLPVVIDAGTNNKELLEHDHLYLGNHQRIYGDQYYSFVDQFVET	221
MDH.YEAST	GIHPGRVLPVCLDVGTNNKLLARDELYMGNKFSRIRGKQYDDFLEKFIKA	249
CYN.BEAN	GVRPSSCLPVTIDVGTNNKLLNDEFYIGLRQRATGQYATFLDEFMRA	245
ME.MAIZE	GVDPSVCLPITIDVGTNNKLLNDEFYIGLRQRATGEEYDELIEEFMSA	245
ME.COLI	GISPAYTLPVVDVGTNNQQLNDPLYMGWRNPRITDDEYEFVDEFIQA	242
	I I I I III I I I I I I	
BOX III		
OENOS	AESLFP-NLYLHWEDFGRSNASNILNSYKDKIATFNDDIQGTGIVVLAVG	320
MLE.LACTIS	AESIFP-KLYLHWEDFGRSNAATILNNYKTKIPTFNDDIQGTGIVVLGGI	319
MDH.YEAST	VKKVYP-SAVLHFEDFGVKNARRLLEKYRYELPSFNDDIQGTGAVVMASL	347
CYN.BEAN	VKQNYGEKVLVQFEDFANHNADFLLLEKYSSSHLVFNDDIQGTASVVLAVG	345
ME.MAIZE	VKQFYGEKVLVQFEDFANHNADFLLLEKYSSSHLVFNDDIQGTASVVLAVG	345
ME.COLI	VKQRPWD-VLLQFEDFAQKNAPLLNRYRNEICSFNDDIQGTAAVTVGTL	340
	III II I I I I I I I I	
BOX II		
OENOS	LGALKISGQKLTDDQTYMSFGAGTAGMGIVKQLHEEMVEQGLSD-EEAKKH	320
MLE.LACTIS	FGSLDITGEKLTDDQVLYCYGGSAGAGIAGRVHAEMVSEGLSE-EEAYKH	319 66%
MDH.YEAST	IAALKHTNRDLKDRVLIYAGSAGLGIADQIVNHMVTHGVDK-EEARKK	347 41%
CYN.BEAN	LASLKLVGGLADHTFLFLGAGEAGTGIAELIAVEVSKQTKAPVEETRK	345 38%
ME.MAIZE	LAALKMVGGLAEQTYLFLGAGEAGTGIAELIALEISKQTNAPIEECRKK	345 39%
ME.COLI	IAASRAAGGQLSEKKIVFLGAGSAGGIAEMIISQTQREGLSE-EAARQK	340 37%
	I I I I I I I I I I	

FIG. 4. Comparison of part of the deduced amino acid sequence of *mleA* of *Leuconostoc oenos* (OENOS) with parts of the sequences of the MLE of *Lactococcus lactis* (MLE.LACTIS; protein sequence accession no. S38728), the malate dehydrogenase of *S. cerevisiae* (MDH.YEAST; protein sequence accession no. S37846), the bean cynnamyl alcohol dehydrogenase (CYN.BEAN; protein sequence accession no. A31323), and the malic enzymes of maize (ME.MAIZE; protein sequence accession no. P16243) and *E. coli* (ME.COLI; protein sequence accession no. P26616). Identical amino acids are indicated by I. The conserved regions box I (NAD binding domain [53]), box II (nicotinamide coenzyme binding site [46]), and box III (malic enzyme signature [2]) and the possible malate binding site (24) are enclosed in boxes. The sequences were aligned by using the Clustal program. The numbers on the right are the amino acid positions in the protein sequences. The percentages on the right are the levels of identity between the entire protein sequence deduced from *mleA* and the sequences of the previously described proteins.

relatively low, but the similarity rates were high since all of these membrane proteins contain many hydrophobic amino acids. The hydrophobic profile of the deduced protein (Fig. 5) showed the alternation of hydrophobic and hydrophilic regions

FIG. 5. Hydropathy pattern of the putative protein deduced from the nucleotide sequence of *mleP* from *Leuconostoc oenos*. Hydropathy was calculated by the method of Kyte and Doolittle (26). The hydropathy values given on the y axis are normalized from -4.5 (hydrophilic) to +4.5 (hydrophobic). Ten possible membrane-spanning segments predicted by the method of Eisenberg et al. (17) are indicated by the solid bars. The hydrophilic core consisting of 24 amino acids is indicated by the solid peak.

typical of an integral membrane protein. A minimum of 10 membrane-spanning regions was predicted by the method of Eisenberg et al. (17); moreover, a 24-amino-acid hydrophilic core is centered among these segments.

Expression of the *mleA* and *mleP* genes in *E. coli* minicells. To identify the gene products, we used an *E. coli* minicell system which allows specific labelling of plasmid-encoded proteins.

The 2.7-kb *EcoRI-SalI* DNA fragment of pJ6D5 was subcloned in both orientations downstream of the strong inducible *tac* promoter by using expression vectors pJF119EH and pJF119HE (18). The 1.6-kb *SalI-XbaI* DNA fragment of pJ2C3 was subcloned in the same vectors. Minicells were prepared from *E. coli* AR1062 transformed with plasmid pCFED6 or pCFHD6 for *mleA* and with plasmid pCFEC3 or pCFHC3 for *mleP*. Proteins were labelled with L-[³⁵S]methionine during IPTG induction and were analyzed by SDS-PAGE (Fig. 6 and 7).

Minicells carrying pCFED6 and pCFHD6 (Fig. 6, lanes 2 through 5) expressed a protein that had a molecular mass of almost 60 kDa. This apparent molecular mass is consistent with the calculated molecular mass deduced from the nucleotide sequence. No protein other than the vector proteins was identified in the absence of the 2.7-kb DNA fragment (lane 1). The protein was present without IPTG induction (lane 2) and in

FIG. 6. SDS-PAGE of ^{35}S -labelled proteins synthesized in *E. coli* minicells containing pJF119EH (lane 1), pCFED6 (lanes 2 and 3), or pCFHD6 (lanes 4 and 5). Minicells were labelled in the absence (lanes 2 and 4) or in the presence (lanes 1, 3, and 5) of 1 mM IPTG. The position of the *mleA* gene product is indicated by an arrow. The positions of the proform and mature form of the β -lactamase are also indicated, as is the position of the *lac* repressor encoded by the *lacI^q* gene. The numbers on the left are the molecular masses of standards.

both orientations of the DNA fragment (pCFED6 and pCFHD6), suggesting that the *Leuconostoc oenos* promoter or a related sequence from the *E. coli* promoter was recognized by the *E. coli* RNA polymerase.

Minicells carrying the 1.6-kb DNA fragment (pCFHC3) expressed a protein that had a molecular mass of approximately 20 kDa (Fig. 7A). No protein was synthesized in the absence or presence of IPTG induction when minicells contained pCFEC3 (Fig. 7A, lanes 4 and 5) carrying the *SalI-XbaI* insertion in the opposite orientation. Surprisingly, the apparent molecular mass was lower than the value predicted from the nucleotide sequence. Such aberrant electrophoretic behavior has been described previously (7) and is typical of membrane-associated proteins. This behavior is probably due to the high affinity of the hydrophobic proteins for SDS. To localize the *mleP* gene product, labelled minicells were fractionated. Periplasmic, cytoplasmic, and membrane proteins were separated and loaded onto an SDS-12% PAGE gel (Fig. 7B). The 20-kDa protein was detected in the cytoplasmic and membrane fractions (Fig. 7B, lanes 3 and 4).

Malolactic activity in *E. coli* and *S. cerevisiae*. We attempted to determine the malolactic activity in *E. coli* TG1 and DH5 α containing the *mleA* gene subcloned under the strong *tac* (pCFED6) and *lac* (pULKE6) promoters, but low MLE activities were detected (data not shown).

Since we previously demonstrated that a 60-kDa protein was synthesized in *E. coli* AR1062 minicells, malolactic activity was determined by using this strain. No detectable activity was found in cells and purified minicells. Ammonium sulfate precipitation allowed us to fractionate and concentrate the proteins of cells and minicells. No activity was detected in several fractions of cells. However, significant activity was detected in the protein fraction precipitated from the minicells with 60% ammonium sulfate; up to 0.72 mg of L-lactate per mg of protein per h was produced by the strain carrying the recombinant plasmid. Only traces of L-lactate were detected with the control strain. No L-lactate was produced when the reaction mixture contained no L-malate.

To test the expression of the *mleA* gene in *S. cerevisiae*, we determined the malolactic activity in aerobically growing yeast cells in YPG medium at pH 3 in the presence of 10 g of

L-malate per liter (Table 2). A small amount of L-lactate was detected in the supernatant of the control strain, but large quantities of L-lactate ($1 \text{ g} \cdot \text{liter}^{-1}$) were produced by the transformant strain carrying plasmid pYMLE1. The specific activity in the crude extracts from the yeast culture was 0.12 mg of L-lactate per mg of protein per h. No L-lactate was produced by the control strain.

L-Malate uptake in *E. coli*. The uptake of L-malate was studied in the mutant *E. coli* CBT313, which is deficient in dicarboxylic acid transport. Plasmids pJF119HE and pCFHC3 (carrying the 1.6-kb DNA fragment containing *mleP*) were used to transform this strain, and transformants were plated onto MLD agar (10). On this medium, only CBT313 carrying pCFHC3 was able to increase the pH of the medium, and the colonies turned blue. The increase in the pH was the result of L-malate consumption. No color change was observed with CBT313 carrying pJF119HE. L-Malate uptake was checked by using L-[^{14}C]malate. A relatively low level of malate accumu-

FIG. 7. (A) SDS-PAGE of ^{35}S -labelled proteins synthesized in *E. coli* minicells containing pJF119HE (lane 1), pCFHC3 (lanes 2 and 3), or pCFEC3 (lanes 4 and 5). Minicells were labelled in the absence (lanes 2 and 4) or in the presence (lanes 1, 3, and 5) of 1 mM IPTG. The position of the *mleP* gene product is indicated by an arrow. The positions of the proform and mature form of the β -lactamase are also indicated, as is the position of the *lac* repressor encoded by the *lacI^q* gene. The numbers on the left are the molecular masses of the standards. (B) SDS-PAGE of ^{35}S -labelled proteins from various fractions of pCFHC3-containing minicells. Minicells were labelled in the presence of 1 mM IPTG. The extracts applied to the gel were extracts obtained from whole cells (lane 1), from the periplasm (lane 2), from the cytoplasm (lane 3), and from the total membrane (lane 4). The arrow indicates the position of the *mleP* gene product. The positions of the proform and mature form of the β -lactamase are also indicated, as is the position of the *lac* repressor encoded by the *lacI^q* gene. The numbers on the left are the molecular masses of the standards.

TABLE 2. Malolactic activity of transformant yeast strain *S. cerevisiae* RH100-4 containing the *mleA* gene subcloned in pYeDP1/8-2 between the *Gal10* *cyc1* promoter and the *Pgk* (phosphoglycerate kinase) terminator^a

Strain	Amt of L-lactate formed (g · liter ⁻¹) after:		Amt of residual L-malate (g · liter ⁻¹) after:	
	24 h	48 h	24 h	48 h
RH100-4(pYeDP1/8-2)	0.14	0.15	9.7	9.2
RH100-4(pYMLe1)	0.52	1	9.2	7.9

^a The amount of L-lactate formed and the amount of L-malate not consumed by the transformant strain were determined after 24 and 48 h of growth in YPG medium (pH 3) containing 10 g of L-malate per liter. No L-lactate was detected in the absence of L-malate in the medium. The *Gal10* *cyc1* promoter is described in reference 22.

lation was observed in the strain carrying pJF119HE (Fig. 8A). No effect of ionophores was observed, suggesting that passive efflux of L-malate occurs in the strain. A higher level of malate accumulation was observed in the strain containing pCFHC3 carrying the 1.6-kb DNA fragment (Fig. 8B). The presence of valinomycin, which dissipated the $\Delta\Psi$, enhanced the uptake of L-malate, while in the presence of nigericin, both inhibitors, or no inhibitor, the level of malate uptake was lower. This low level of L-malate uptake was certainly the result of the passive efflux observed in the strain carrying pJF119HE. These results suggest that the DNA fragment cloned in pCFHC3 encodes a protein that is responsible for L-malate uptake in the cells.

DISCUSSION

In this study the DNA fragment carrying the *mleA* gene of *Leuconostoc oenos* was subcloned, sequenced, and expressed in *E. coli* and *S. cerevisiae* cells. This is the first gene coding for an enzyme of biotechnological interest that has been cloned from *Leuconostoc oenos*, the malolactic bacterium. A comparison of the MLEs of *Leuconostoc oenos* and *Lactococcus lactis* revealed 66% identity in amino acids and strong similarities in the potential NAD binding domain, the nicotinamide coenzyme binding site, and the malic enzyme signature. Furthermore, the MLE of *Leuconostoc oenos* exhibits more than 30%

identity with previously described prokaryotic and eukaryotic malic enzymes, suggesting that there is a phylogenetic relationship between this MLE and malic enzymes. Recently, Viljoen et al. (51) described eight highly conserved regions present in malic enzymes. These regions are also present in the *Leuconostoc oenos* MLE.

The *mleA* gene was expressed in *E. coli*, and a protein with an estimated molecular mass of 60 kDa was found. The molecular mass predicted from the sequence and estimated on the basis of SDS-PAGE results and the pI are consistent with the characteristics previously described for the purified *Leuconostoc oenos* MLE (5, 34). No stable DNA fragment coding for malolactic activity in *Leuconostoc oenos* has been described previously. In our study, low levels of MLF activity were detected in *E. coli* transformants. Significant malolactic activity was observed in *E. coli* only after protein fractionation and concentration. No plasmid or strain effect was noticed.

Expression of the *mleA* gene in *S. cerevisiae* conferred malolactic activity to this organism. In a medium at pH 3, the internal pH values of *S. cerevisiae* and *Leuconostoc oenos* are about 5.6 (41), which is near the optimal pH of the *Leuconostoc oenos* MLE (5, 34). This could explain why there is good MLE activity in *S. cerevisiae*. However, about 20% of the L-malate in the medium was decarboxylated after 48 h of growth at pH 3 (Table 2) and the ratio of amount of L-lactate formed to amount of L-malate consumed was 1:1, which is consistent with the balance of the MLF. This incomplete MLF performed by *S. cerevisiae* could result in insufficient malate uptake.

A sequence analysis of a 2.57-kb fragment harboring the *mle* locus revealed that downstream of the *mleA* gene, there is a second ORF called *mleP*, which suggests that the two genes are organized in a cluster. PCR amplification of *Leuconostoc oenos* genomic DNA confirmed this hypothesis (Fig. 2, lane 10). No regions similar to consensus promoter transcription initiation sequences were found. Transcriptional analyses are currently being done to determine the existence of an operonic structure and the promoter transcription initiation sequences of *Leuconostoc oenos*, which remain unknown in this species.

The second ORF could code for a protein with a calculated molecular mass of 34 kDa. An apparent molecular mass of 20 kDa was estimated by SDS-PAGE. The difference between the predicted and apparent molecular masses could be the result

FIG. 8. Uptake of L-malate by whole cells of *E. coli* CBT313 carrying pJF119HE (A) or pCFHC3 (B). The inhibitors used were 4 μ M valinomycin and 4 μ M nigericin. Uptake of L-[¹⁴C]malate (final concentration, 29.2 μ M) was assayed at 25°C in the presence of valinomycin (●), nigericin (△), both inhibitors (+), or no inhibitors (◇) at pH 5. Cells were preincubated for 10 min in the presence of the inhibitors and were energized for 5 min with 10 mM glucose. The final protein concentration was about 0.15 mg · ml⁻¹.

of aberrant electrophoretic behavior due to the high hydrophobicity of the protein. The hydropathy profile resembles the profiles of other membrane-associated proteins. Moreover, the hydrophobic segments are arranged around a hydrophilic core as in several carrier proteins, including sugar transporters from both prokaryotes and eukaryotes (3) and citrate transporters (50). These results suggest that the *mleP* protein is an integral membrane protein; this is consistent with the location of the *mleP* expression product in the membrane.

The amino acid sequence RXGRR found between amino acids 94 and 100 in the protein has been described previously by Maiden et al. (33) and is highly conserved in six different transport proteins. In contrast to these proteins, this sequence is not duplicated but is in the same hydrophilic region. This sequence is found in transport proteins, as well as in other membrane proteins, suggesting that it plays an important role in the structure of the transport protein, as postulated by Maiden et al. (33), rather than in the mechanism of transport by this protein.

The *mleP* gene product was able to complement an *E. coli* strain affected in dicarboxylic acid transport, and L-[¹⁴C]malate uptake was clearly demonstrated. This protein is thought to be a malate carrier protein. No homology between the *Leuconostoc oenos* malate carrier protein and previously described carboxylic acid carrier proteins was found, suggesting that the former carrier protein could belong to a special class of carriers.

The presence of a gene coding for a malate carrier protein is consistent with the findings of Tourdot-Maréchal et al. (49) and Salema et al. (42), who found and characterized a malate carrier protein in *Leuconostoc oenos*. However, malate uptake experiments performed with *E. coli* vesicles are needed to determine if the *mleP* gene product is the malate carrier previously found in *Leuconostoc oenos* and characterized.

In conclusion, the molecular approach to studying MLF in *Leuconostoc oenos* should permit workers to elucidate the number of genes involved. A study of the regulation of MLF gene expression should aid in the development of an MLE-overproducing strain of *Leuconostoc oenos*.

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