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Comparison of partial malolactic enzyme gene sequences for phylogenetic analysis of some lactic acid bacteria species and relationships with the malic enzyme

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DNA sequences covering 36% of the *mle* gene that encodes the malolactic enzyme were determined for 13 strains of lactic acid bacteria, representing *Pediococcus*, *Leuconostoc*, *Lactobacillus* and *Oenococcus* genera. The sequences were aligned with the corresponding region of *mleS* in *Lactococcus lactis*. The phylogenetic distance matrix tree of all *mle* sequences was compared with the 16S rRNA phylogenetic tree. The analysis showed that the *mle* fragment evolved more rapidly than the 16S gene and differently. *Pediococcus* and *Lactobacillus* species were intermixed in the 16S rRNA tree whereas they were separated in the *mle* tree. *Leuconostoc mesenteroides* and *Oenococcus oeni* were distinct from other species in the 16S rRNA tree, whereas they were intermixed with *Lactobacillus* species and *Lactococcus lactis* in the *mle* tree. The amino acid sequences deduced from partial *mle* genes were aligned with 22 malic enzyme sequences and the corresponding phylogenetic tree was constructed. Malic and malolactic enzymes were distinct at the phylogenetic level, except for malic enzymes of yeast and *Escherichia coli* which were nearer the malolactic enzymes than the other malic enzymes. The analysis of conserved sites showed several interesting amino acids specific to either malic enzyme or malolactic enzyme.

Keywords: malolactic enzyme, lactic acid bacteria, phylogeny

INTRODUCTION

Lactic acid bacteria (LAB) are extensively used in most food and beverage fermentation processes. Their main role is to acidify raw materials by producing large amounts of lactate. The classification of LAB into different genera was formerly based on their morphology, metabolism and physiological characteristics (Buchanan & Gibbons, 1986; Collins *et al*., 1987, 1990; Stiles & Holzapel, 1997; Vandamme *et al*., 1996). DNA–DNA hybridization (Kawai *et al*., 1996), 16S rRNA sequencing (Collins *et al*., 1990, 1993; Lane *et al*., 1985) and soluble protein patterns (Dicks *et al*., 1996) have led to the description of new genera. The *Leuconostoc* group has recently undergone taxonomic changes. '*Leuconostoc paramesenteroides*' and related species have been reclassified in a new genus *Weissella* (Collins *et al*., 1993) on the basis of their 16S rRNA sequences. Phylogenetic studies have also revealed that '*Leuconostoc oenos*' is distinct from other *Leuconostoc* spp. (Martínez-Murcia & Collins, 1990) and it has been suggested that this organism is an interesting case of a fast-evolving species (Yang & Woese, 1989). In 1995, the genus *Oenococcus* was proposed for these bacteria (Dicks *et al*., 1995). *Lactobacillus* is the most heterogeneous of the genera included in LAB. Its division into three groups (Buchanan & Gibbons, 1986) is not in agreement with results of phylogenetic analysis (Collins *et al*., 1991). rRNA sequencing is mostly used for phylogenetic studies of bacteria, but other approaches show it may sometimes not be sufficient for species identification (Fox *et al*., 1992). Both molecular and classical approaches are necessary for systematic bacterial studies.

In recent years, enzyme-encoding genes have been used for phylogenetic analysis (Birtles & Raoult, 1996;

Abbreviations: LAB, lactic acid bacteria; MLE, malolactic enzyme.

The GenBank accession number for the 16S rRNA sequence of *Lactobacillus salivarius* ATCC 11741^T is AF089108.

Christensen & Olsen, 1998; Morse *et al*., 1996; Roux *et al*., 1997; Springer *et al*., 1995). Sequencing of *rpoC* gene encoding the *β′* subunit of DNA-dependent RNA polymerase of the leuconostocs confirms that *Oenococcus oeni* is distinct from *Leuconostoc sensu stricto* and *Weissella*, but these bacteria have not evolved rapidly (Morse *et al*., 1996). Citrate synthase gene (of *Bartonella* and *Rickettsiae* spp.) (Birtles & Raoult, 1996; Roux *et al*., 1997) or methyl-coenzyme M reductase (from the family *Methanosarcinaceae*) (Springer *et al*., 1995) are also used as phylogenetic tools because differences between the 16S rRNA sequence are not sufficient to deduce evolutionary relationships. The choice of gene depends on the bacteria studied and also on the taxonomic level. Data on DNA and amino acid sequences are used for the construction of phylogenetic trees.

In most cases of winemaking, after alcoholic fermentation mainly performed by *Saccharomyces cerevisiae*, some LAB classified as *Lactobacillus*, *Leuconostoc*, *Pediococcus* and especially *Oenococcus* transform L-malate into L-lactate and carbon dioxide during malolactic fermentation. The nucleic acid sequence of the *mle* gene, which encodes the malolactic enzyme (MLE), has been determined only for *Lactococcus lactis* (Ansanay *et al*., 1993; Denayrolles *et al*., 1994) and '*Leuconostoc oenos*' recently reclassified as *O. oeni* (Labarre *et al*., 1996). MLE has been purified from several LAB (Caspritz & Radler, 1983; Lonvaud, 1976; Lonvaud-Funel & Strasser de Saad, 1982; Naouri *et al*., 1990; Spettoli *et al*., 1984). It is composed of two to four identical subunits of 60–70 kDa. The protein is strongly homologous to malic enzymes from different organisms, but its activity is similar to malic enzyme plus lactate dehydrogenase in the presence of NAD and Mn^{2+} without the release of intermediate products. A phylogenetic tree constructed from the sequences of the MLE from *Lactococcus lactis* and malic enzyme proteins shows that the MLE is most strongly related to the *Escherichia coli* malic enzyme, and that the bacterial proteins constitute a group distinct from the animal or plant group (Hrdy & Müller, 1995; Van der Glezen *et al*., 1997).

We report here the sequencing of a 580 bp fragment of the *mle* gene from different LAB species: *O. oeni*, *Leuconostoc mesenteroides*, two *Pediococcus* spp. and ten strains corresponding to six *Lactobacillus* spp. These sequences were analysed to build phylogenetic trees which were compared with 16S rRNA trees and with physiologic and phenotypic data. The corresponding amino acid sequences were compared with malic enzyme proteins. The analysis shows differences between the two proteins and points to the essential amino acids involved in the enzymic reaction.

METHODS

Bacterial strains and culture conditions. The sources of the LAB strains used in this study are shown in Table 1. Strains isolated in our laboratory were identified by using the API 50CH system (API bioMérieux) and DNA–DNA hybridization (Lonvaud-Funel *et al*., 1991). Cells were grown at 25 °C in a modified MRS medium: yeast extract, 4 g l^{-1}; beef extract, 8 g l^{-1}; bactopeptone, 10 g l^{-1}; glucose, 10 g l^{-1}; fructose, 10 g l^{-1}; DL-malic acid, 10 g l^{-1}; KH_2PO_4, 2 g l^{-1}; $MgSO_4.7H_2O$, 0·2 g l^{-1}; $MnSO_4.H_2O$, 0·1 g l^{-1}; Tween 80, 1 ml; NaOH, 10 M to adjust pH to 5.

Escherichia coli strain XL-1 Blue (Stratagene) (Bullock *et al*., 1987) was used for amplification of the malic enzyme, for Southern blot and cloning procedures. The culture medium was DYT (Miller, 1972).

Cell-free extract preparation. When OD_{600} measured in a 932 Uvikon Spectrophotometer (Kontron) reached 0·6, the LAB cells were harvested by centrifugation at 10000 ***g*** for 15 min at 4 °C. The pellet was suspended in 25 ml phosphate buffer (0·1 M, pH 6) or Tris/HCl buffer (0·1 M, pH 8) and cells were disrupted at 4 °C with an ultrasonic disintegrator (MSE Scientific Instruments) at 150 W for 15 min with alternating sonication and pause every 30 s.

For *E. coli*, an overnight culture was centrifuged and the pellet was suspended in 4 ml Tris/HCl buffer (0·1 M, pH 8). Cells were disrupted at 4 °C with an ultrasonic disintegrator (Heat Systems) at 12% output power for 4 min with alternating sonication and pause every 30 s.

The cell-free extract was separated from the bacterial debris by centrifuging at 14000 ***g*** for 20 min at 4 °C. The protein concentrations were determined using the BCA Protein Assay (Pierce Interchim) (Smith *et al*., 1985).

Measurement of enzymic activity. The enzymic activity of MLE was determined by measuring the CO_2 released from malic acid with a specific CO_2 electrode (Eischweiler and Co., Kiel, Germany), according to the method of Lonvaud & Ribereau-Gayon (1973). The reaction mixture contained malic acid (50 µmol), NAD^+ (1 µmol), $MnCl_2$ (0·2 µmol) in phosphate buffer (0·1 M, pH 6). The experiments were conducted at 25 °C and the reaction was started by adding the cell-free extract. Activity was expressed as µmol CO_2 released min^{-1} (mg protein)$^{-1}$.

For malic enzyme, the same reaction mixture was used, except for buffer that was either phosphate buffer (0·1 M, pH 6) or Tris/HCl buffer (0·1 M, pH 8). Activity was measured by the formation of NADH at 340 nm in a 932 Uvikon Spectrophotometer (Kontron). The activity was expressed as nmol NADH released min^{-1} (mg protein)$^{-1}$.

Nucleic acid preparation. For LAB, the procedure was derived from that described by Gasson & Davies (1980). The bacteria were treated with lysozyme (5 mg ml^{-1} final concn) and the resulting protoplasts were lysed with 20% (w/v) SDS. Nucleic acid and cell debris were separated by addition of NaCl (1 M final concn), incubated at 4 °C and centrifugation. DNA was extracted with phenol/chloroform (1:1), the upper phase was added with sodium acetate (3 M, pH 5·2) and ethanol. Then, the DNA pellet was suspended in sterile water with RNase (10 mg ml^{-1}).

For *E. coli*, cells were lysed with 3% SDS, DNA was extracted with phenol/chloroform (1:1) then treated in the same way as for the LAB. The DNA concentration was estimated after agarose gel electrophoresis by comparing the fluorescence of the bands after staining with ethidium bromide with amounts of standard DNA.

PCR amplifications. To amplify a fragment of the MLE gene of different LAB strains, the primers were chosen in two

Table 1. Strains used for *mle* gene sequence phylogenetic studies

ATCC, American Type Collection and Culture; IOEB, Institut d'Œnologie de Bordeaux; LRTL, Laboratoire de Recherches et Technologie Laitière de Rennes. [T]Type strain.

Species	Source/strain no.	Other information
Lactococcus lactis	LRTL IL1441	
Oenococcus oeni	ATCC 23279[T]	
Leuconostoc mesenteroides	ATCC 8293[T]	
Pediococcus acidilactici	ATCC 8042	
Pediococcus parvulus	ATCC 19371[T]	
Lactobacillus salivarius	ATCC 11741[T]	
Lactobacillus rhamnosus	ATCC 7469[T]	
Lactobacillus plantarum	ATCC 8014	
Lactobacillus fructivorans	ATCC 8288[T]	
Lactobacillus hilgardii	ATCC 8290[T]	
Lactobacillus hilgardii	IOEB 9101	Isolated from Muscat
Lactobacillus hilgardii	IOEB 9202	Isolated from Porto
Lactobacillus brevis	ATCC 14869[T]	
Lactobacillus brevis	IOEB 9647	Isolated from sweet white wine

conserved amino acid regions of the *mleS* gene of *Lactococcus lactis* IL 1441 (Ansanay *et al.*, 1993; Denayrolles *et al.*, 1994). These regions were NPVVYDP (amino acids 86–92) and QGTGIV (amino acids 260–265) and the corresponding nucleotides sequences were ATCCAGTTGTTTATGATC (nucleotides 255–276) (sense) and AACAATACCAGTTCCTTG (nucleotides 777–795) (antisense). For amplification of an analogous fragment of NAD-linked malic enzyme gene of *E. coli* (Mahayan *et al.*, 1990), the amino acids regions were MPVIYTP (amino acids 109–115) and QGTAAV (amino acids 281–286) and the corresponding nucleotides sequences were TGCCTGTTATTTATACCC (nucleotides 324–345) (sense) and TACCGCCGCAGTGCCCTG (nucleotides 840–858) (antisense).

Approximately 100 ng of genomic DNA was amplified with 2·5 U *Taq* polymerase (Stratagene) in a 50 µl reaction by using a MiniCycler (MJ Research). Conditions were 30 cycles of 95 °C (30 s), 38, 40 or 43 °C (30 s) according to the strain, and 72 °C (2 min). The amplification products were either extracted once with a phenol/chloroform mixture (1:1), precipitated with sodium acetate (3 M, pH 5·2) and ethanol, then treated with Klenow enzyme (Boehringer Mannheim) for cloning, or purified on QIAquick spin Columns (Qiagen) for direct sequencing.

Cloning. Purified PCR products were added to a ligation mixture containing *Eco*RV-cut pBluescriptII KS (Stratagene) and T4-Ligase (Biolabs) as recommended by the manufacturer. This solution was incubated at 16 °C overnight. Two microlitres were added to 40 µl competent cells (XL-1 Blue) and the preparation was electroporated in a Pulser Controller (Bio-Rad). After blue-white selection, plasmids containing DNA fragments were extracted by an alkaline-lysis method (Birnboim & Doly, 1979) and the inserted fragment length was verified by agarose gel electrophoresis.

Probe labelling and Southern blot hybridization. PCR fragments corresponding to the MLE gene of *Lactococcus lactis* and malic enzyme gene of *E. coli* were used as probes for Southern blot hybridization. Five hundred nanograms of DNA were labelled with digoxigenin-11-DUTP using the DIG DNA Labelling and Detection Kit (Boehringer Mannheim). For Southern blot, 1 µg genomic DNA isolated from each strain was digested with *Eco*RI or *Hin*dIII [4 U (µg DNA)$^{-1}$] and fragments were separated by 1 % agarose gel electrophoresis overnight at 35 V. Digested DNA was denatured then transferred to a nylon membrane using a rapid transfer method (Vacuum Blot; Bio-Rad). Prehybridization (1 h at 55 °C) and hybridization (overnight at 55 °C) were performed in a solution containing 5 × SSC, 1 % blocking reagent (Boehringer Mannheim), 0·02 % SDS and 0·1 % lauroyl sarcosine. For hybridization, 10 ng ml^{-1} of labelled and denatured probe were added. After incubation, membranes were washed twice at room temperature for 5 min with 2 × SSC and twice at 55 °C for 15 min in 2 × SSC, 0·1 % SDS. The detection was carried out by chemiluminescence using the DIG DNA Labelling and Detection Kit as recommended by the manufacturer.

Sequencing. Purified PCR products or positive inserts were sequenced by using the reagents of a Thermo Sequenase radiolabelled terminator cycle sequencing kit (Amersham Life Science). The primers described for PCR amplifications were used for sequencing. The sequencing reactions were performed by using a MiniCycler with DNA concentration as recommended by the kit. All samples were separated by electrophoresis in 6 % acrylamide gels. For each plasmid or PCR product, the two sense and antisense primers were used and the migration lasted 2 or 6 h. All these conditions made it possible to sequence a 580 bp fragment in double strand.

Analysis of sequence data and construction of phylogenetic trees. Nucleotide sequences of *mle* genes obtained in this study were compared with *mleS* of *Lactococcus lactis* (Ansanay *et al.*, 1993; Denayrolles *et al.*, 1994) and *mleA* of *O. oeni* (Labarre *et al.*, 1996). Sequences of 16S rRNA genes, nucleotide sequences of *mle* genes and partial amino acid sequences of malic and malolactic enzymes were aligned with the CLUSTAL W program (Thompson *et al.*, 1994). For phylogenetic tree construction, gaps (insertion–deletion)

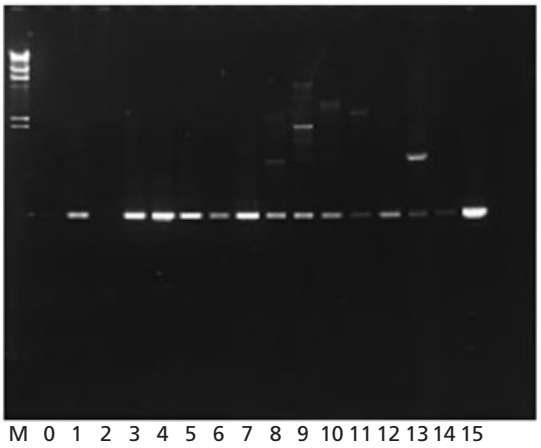

Fig. 1. Amplification profile of 580 bp *mle* fragment from 14 LAB and one strain of *E. coli*. Lanes: M, molecular mass marker λ/*Hin*dIII (23130, 9416, 6557, 4361, 2322, 2027, 564, 125 bp); 0, negative control; 1, *Lactococcus lactis* LRTL IL1441; 2, *E. coli*; 3, *Lactobacillus hilgardii* ATCC 8290^T; 4, *Lactobacillus hilgardii* IOEB 9101; 5, *Lactobacillus hilgardii* IOEB 9202; 6, *O. oeni* ATCC 23279^T; 7, *Leuconostoc mesenteroides* ATCC 8293^T; 8, *P. acidilactici* ATCC 8042; 9, *P. parvulus* ATCC 19371^T; 10, *Lactobacillus salivarius* ATCC 11741^T; 11, *Lactobacillus rhamnosus* ATCC 7469^T; 12, *Lactobacillus plantarum* ATCC 8014; 13, *Lactobacillus fructivorans* ATCC 8288^T; 14, *Lactobacillus brevis* ATCC 14869^T; 15, *Lactobacillus brevis* ATCC 9647.

were removed from alignments. Distance matrices for DNA and amino acid alignments were calculated by using DNADIST and PROTDIST programs in the PHYLIP software package (Felsenstein, 1989). Trees were inferred from the matrices by using the FITCH program in PHYLIP and then combined to yield a consensus tree (CONSENSE in PHYLIP). The data were also examined by using parsimony analysis (DNAPARS and PROTPARS in PHYLIP). The consensus tree was obtained by CONSENSE in PHYLIP. Bootstrap values were calculated from 100 replicates.

Nucleotide sequence accession numbers. The GenBank accession numbers of the malic enzyme nucleotide sequences, the *mleA* nucleotide sequence of *O. oeni* and the *mleS* nucleotide sequence of *Lactococcus lactis* are shown in Table 3. The *mle* sequences of the LAB studied have the following GenBank database accession numbers: *Lactobacillus salivarius*, AF098461; *Lactobacillus rhamnosus*, AF098777; *Lactobacillus brevis*, AF098778; *Lactobacillus fructivorans*, AF098779; *O. oeni*, AF098780; *Lactobacillus hilgardii*, AF098781; *Leuconostoc mesenteroides*, AF098782; *Lactobacillus plantarum*, AF098783; *Pediococcus parvulus*, AF098784; *Pediococcus acidilactici*, AF098785.

RESULTS

PCR amplification of 580 bp *mle* gene fragments and sequence determination

All LAB, except *Lactobacillus casei*, *Enterococcus faecalis* and more recently *Streptococcus bovis* are known to contain malolactic instead of malic activity (Batterman & Radler, 1990; Kawai *et al.*, 1996; London & Meyer, 1969). Before the present phylogenetic study, we confirmed that the strains studied had the MLE and not the malic enzyme, also no inactive malic gene. The malolactic activity was measured with a specific CO_2 electrode (see Methods). For all strains, the malolactic activity of cell-free extract was of the same order, between 0·2 and 0·5 μmol CO_2 min^{-1} mg^{-1}. As expected, these bacteria had no malic enzyme, unlike *E. coli* which was tested as a control.

The *mleS* gene from *Lactococcus lactis* was used to

Table 2. Percentage similarity between *mle* sequences and between 16S rRNA gene sequences

The values on the upper right are levels of *mle* sequence similarity, and the values on the lower left are levels of 16S rRNA sequence similarity.

Strain	1	2	3	4	5	6	7	8	9	10	11	12
1 *Escherichia coli**		33	32	33	34	34	28	31	33	31	31	38
2 *Lactococcus lactis*	75		67	72	72	69	38	36	69	66	70	73
3 *Leuconostoc mesenteroides*	77	81		69	68	67	42	38	72	72	64	67
4 *Oenococcus oeni*	73	80	84		67	67	38	38	64	65	64	69
5 *Pediococcus parvulus*	77	85	85	82		78	36	36	66	68	68	75
6 *Pediococcus acidilactici*	77	84	85	81	96		38	40	67	68	70	73
7 *Lactobacillus salivarius*	78	84	85	82	92	92		75	39	41	41	40
8 *Lactobacillus rhamnosus*	78	84	85	82	94	93	93		37	38	39	39
9 *Lactobacillus brevis*	73	82	82	79	90	90	87	88		77	63	66
10 *Lactobacillus plantarum*	77	84	85	82	93	93	91	92	90		66	69
11 *Lactobacillus fructivorans*	77	84	84	82	94	94	92	93	89	92		71
12 *Lactobacillus hilgardii*	76	84	84	82	94	93	92	93	90	92	93	

* Malic enzyme gene sequence.

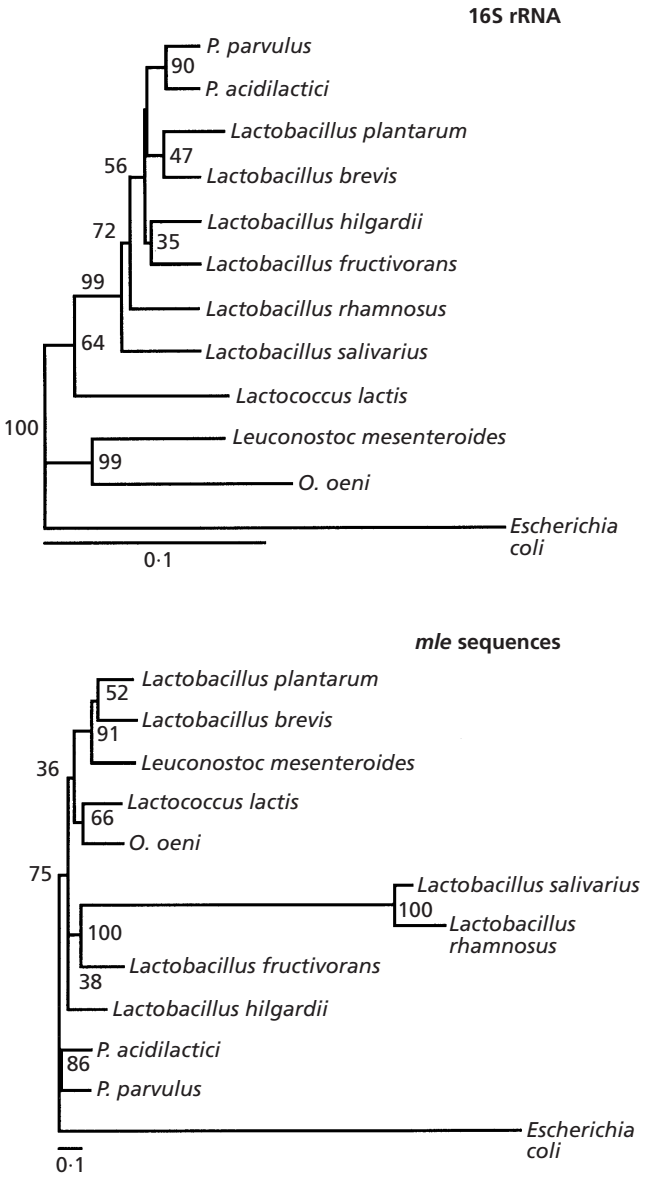

Fig. 2. Comparison of 16S rRNA and *mle* Fitch distance matrix trees. The numbers are parsimony bootstrap values. The scale bar indicates 10 estimated changes per 100 positions.

choose two primers for PCR amplification of an approximately 580 bp region of the *mle* gene from thirteen LAB strains. In *Lactococcus lactis*, this region consisted of 36% of *mleS* gene which contains the malate-binding site (Viljoen *et al*., 1994), the ADP-binding $\beta\alpha\beta$ fold region (Wierenga *et al*., 1985) and a region described as the malic enzyme signature in protein databases (Bairoch, 1991). Moreover, two corresponding primers were chosen from the *E. coli* malic enzyme gene. These two pairs of oligonucleotides were tested on the thirteen strains of bacteria, on *Lactococcus lactis* and on *E. coli*. Results of amplification with *mle* primers are shown in Fig. 1. For all the strains, an approximately 580 bp fragment was obtained, except for *E. coli*. In the same conditions, no fragment was obtained with malic enzyme primers, except for *E. coli* (data not shown). The 580 bp fragment of *mleS* in *Lactococcus lactis* and the 580 bp fragment of malic enzyme in *E. coli* were used as probes in Southern blot hybridization. Genomic DNA of bacteria were successively digested with *Eco*RI and *Hin*dIII. The malic enzyme probe did not hybridize with DNA of LAB, whereas only one signal was obtained for each lactic acid bacterium, with the *mle* probe (data not shown).

The purified PCR fragments were cloned in pBluescriptII KS and sequenced. Nucleotide sequences obtained for all strains were aligned with the *mleS* gene, which confirmed that part of the malolactic genes really had been amplified. For three strains isolated in our laboratory, *Lactobacillus hilgardii* IOEB 9101, *Lactobacillus hilgardii* IOEB 9202 and *Lactobacillus brevis* IOEB 9647, the same sequence was obtained. This sequence was identical to the *mle* of *Lactobacillus hilgardii* ATCC 8290^T. The sequence obtained for *O. oeni* ATCC 23279^T was compared to the MLE (*mleA*) of *Leuconostoc oenos* IOEB 8413 (named Lo84.13 by Labarre *et al*., 1996). Results show that these two sequences are exactly the same.

Comparisons and phylogenetic analysis

A pairwise comparison of *mle* sequences revealed similarity values between 36 and 78% for LAB (Table 2). There were two distinct groups: first, *Lactobacillus salivarius* and *Lactobacillus rhamnosus* which are only 36–42% similar to other *mle* sequences; and a second group composed of *Lactococcus lactis*, *Leuconostoc mesenteroides*, *O. oeni*, *P. parvulus*, *P. acidilactici*, *Lactobacillus brevis*, *Lactobacillus plantarum*, *Lactobacillus fructivorans* and *Lactobacillus hilgardii* (63–78%). The percentage of similarity between *mle* sequences and the malic enzyme sequence of *E. coli* was lower (28–34%), except for *Lactobacillus hilgardii* (38%). Table 2 shows a pairwise comparison of 16S rRNA sequences of the same strains. The similarity values were 79–96% for LAB and were between 73–78% when these Gram-positive bacteria were compared with *E. coli*.

Before phylogenetic tree construction and in order to minimize alignment ambiguities, all deletion and insertion differences between sequences were removed. The G+C percentages of these eleven species, ranging from 34 to 47 mol% (De Roissart & Luquet, 1994), do not influence tree analysis. Dendrograms were inferred by using matrix distance and parsimony methods. The analysis of the *mle* and 16S rRNA sequence data by these two methods yielded slightly different trees. Fig. 2 shows the trees obtained with the Fitch method and the parsimony bootstrap values for 100 replicates. *E. coli* was used as the outgroup. The 16S rRNA sequences tree showed three distinct groups of LAB: *Lactococcus lactis* alone, *Leuconostoc mesenteroides*

Table 3. Malic and malolactic sequences included in this study

Species	Subcellular location	Accession no.	Reference
Amaranthus hypochondriacus	Mitochondrion	U01162	Long *et al.* (1994)
Anas platyrhynchos	Cytosol	X66418	Hsu *et al.* (1992)
Ascaris suum	Mitochondrion	M81055	Kulkarni *et al.* (1993)
Colombia livia	Cytosol	L09233	Chou *et al.* (1994)
Escherichia coli		P26616	Mahayan *et al.* (1990)
Flavernia trinervia	Chloroplast	X57142	Borshc & Weathoff (1990)
Giardia intestinalis	?	U59300	Sanchez *et al.* (1996)
Homo sapiens	Cytosol	L34035	Gonzalez-Manchon *et al.* (1995)
Homo sapiens	Mitochondrion	M55905	Loeber *et al.* (1991)
Lactococcus lactis	*mleS*	X71897	Ansanay *et al.* (1993), Denayrolles *et al.* (1994)
Mesenbryanthenum crystallinum	Cytosol	X64434	Cushman (1992)
Mus musculus	Cytosol	M26756	Bagchi *et al.* (1986)
Neocallimastix frontalis	Hydrogenosome	U62041	Van der Glezen *et al.* (1997)
Oenococcus oeni	*mleA*	X82326	Labarre *et al.* (1996)
Oryza sativa	Chloroplast	D16499	Fushimi *et al.* (1994)
Phaesolus vulgaris	?	J03825	Walter *et al.* (1990)
Populus trichocarpa	Cytosol	X56233	Van Doorsselaered *et al.* (1991)
Saccharomyces cerevisiae	Mitochondrion	Z28029	Boles *et al.* (1998)
Schizosaccharomyces pombe	?	U00621	Viljoen *et al.* (1994)
Solanum tuberosum	Mitochondrion	Z23023	Winning *et al.* (1994)
Sus scrofa	Cytosol	X93016	Unpublished
Trichomonas vaginalis	Hydrogenosome	U16836	Hrdy & Müller (1995)
Vitis vinifera	Cytosol	L34836	Franke *et al.* (1995)
Zea mays	Chloroplast	J05130	Rothermel & Nelson (1989)

and *O. oeni*, then all the other species. In the *mle* sequence phylogenetic tree, the genus *Pediococcus* was separated from the other LAB. *Lactococcus lactis*, *Leuconostoc mesenteroides* and *O. oeni* were intermixed with the *Lactobacillus* spp.

A phylogenetic tree was constructed with amino acid sequences of *mle* fragments of LAB and malic fragments of species listed in Table 3 (Fig. 3). The analysis confirmed results obtained in previous studies (Hrdy & Müller, 1995; Van der Glezen *et al.*, 1997). Malic and malolactic enzymes were clearly separated. In addition, this tree shows that malic enzymes of yeast (*Saccharomyces cerevisiae* and *Schizosaccharomyces pombe*) and *E. coli* are nearer the MLEs than other malic enzymes.

Comparison of amino acid sequences between *mle* fragments and malic enzymes

The amino acid sequences of *mle* protein deduced from the ORF were compared with 22 related malic enzyme sequences available in databases (Table 3). This region had approximately the same size in all malic enzymes whereas the size of total proteins was not the same. The alignment shown in Fig. 4 demonstrates the similarity of MLEs with all these enzymes and shows identical and similar amino acids not only for malic and malolactic enzymes but also for each enzyme. Although a crystallographic three-dimensional structure is not yet available for any malic or malolactic enzymes, four functionally important domains have been identified (Bairoch, 1991; Viljoen *et al.*, 1994; Wierenga *et al.*, 1985). Three of these four regions were analysed in the present study and the corresponding consensus regions are presented in Fig. 5. The box III sequence is exactly the consensus between all malic and malolactic enzymes. Asp^{258} (second D in FNDDIQGT sequence) in pigeon liver malic enzyme is the metal coordinate (Wei *et al.*, 1994). Four mutant enzymes substituted at Asp^{258} with glutamate, asparagine, lysine or alanine were inactive. Taken together with these results, the fact that this aspartate is conserved in all malic enzymes and seven MLEs suggests that it is essential for metal binding and thus catalytic activity. Malate-binding site and box I were also highly conserved, but Fig. 5 shows some interesting differences. The cysteine in the malate-binding site, which is essential for the activity of several malic enzymes (Chang *et al.*, 1993; Gavva *et al.*, 1991), was replaced by isoleucine in yeast malic enzymes and all MLEs. Moreover, arginine in box I was replaced by glycine in yeast malic enzymes and malolactic enzymes.

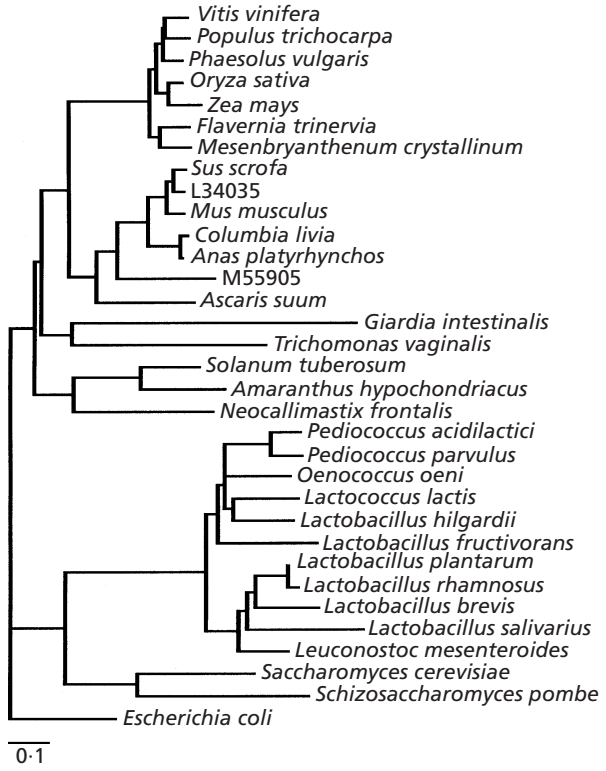

Fig. 3. Fitch distance matrix tree of amino acid sequences of *mle* and malic enzymes. The scale bar indicates 10 estimated changes per 10 positions.

DISCUSSION

This study shows that of the LAB we tested and which have no malic enzyme activity, none has any malic enzyme gene either mutated or disrupted. Amplification and sequencing of 13 *mle* fragments showed that this malolactic enzyme is highly conserved in LAB. The reliability of the sequence data generated from PCR analysis was examined. Products obtained from independent PCR for the *mleS* fragment and *E. coli* malic enzyme fragment were sequenced. Although *Taq* polymerase was used, sequencing results did not show any errors. Among the bacteria studied, three strains were from IOEB: *Lactobacillus hilgardii* 9101 and 9202 and *Lactobacillus brevis* 9647. Their malolactic sequences were identical to the *mle* fragment of *Lactobacillus hilgardii* ATCC 8290^T. The result for *Lactobacillus brevis* IOEB 9647 confirmed other current studies in our laboratory. Genomic DNA from strains which cross-hybridize with *Lactobacillus brevis* and *Lactobacillus hilgardii* were previously classified as *Lactobacillus brevis* due partly to their ability to ferment arabinose, unlike *Lactobacillus hilgardii*. However, fingerprinting and partial 16S rRNA sequencing has made it possible to classify arabinose-fermenting strains as *Lactobacillus hilgardii* spp. (Sohier *et al*., 1999). In addition, for four strains of the same species *Lactobacillus hilgardii*, we obtained four identical nucleotidic sequences, which suggests the conservation of *mle* within this species. The partial sequence of *O. oeni* ATCC 23279^T *mle* was also exactly identical to the *mleA* of *O. oeni* IOEB 8413 (Labarre *et al*., 1996). In a recent paper, *mle*-targeted primers were used for rapid identification and detection of *O. oeni* in wine (Zapparoli *et al*., 1998). Therefore, this enzyme appears to have an identical sequence at the species level. From this, *O. oeni* seems to be a homogeneous species (Zavaleta *et al*., 1997). It would be interesting to know if MLE is homogeneous or heterogeneous in species containing subspecies such as *Leuconostoc mesenteroides* or *Lactococcus lactis*.

16S rRNA phylogenetic analysis

Usually, an unknown strain is first identified and classified by conventional morphological, physiological and biochemical methods. In addition, molecular methods are now available. For example, close relationships (at species and subspecies level) can be determined with DNA–DNA homology studies (Johnson, 1984). Yet some species, such as *Lactobacillus hilgardii* and *Lactobacillus brevis*, can cross-hybridize and conventional techniques are not sufficient. Comparison of 16S rRNA sequences is currently the most powerful and accurate technique for determining phylogenetic relationships between micro-organisms (Woese, 1987). In addition, rRNA sequencing is a suitable tool for classification of LAB, as exemplified by the descriptions of new genera (Collins *et al*., 1990, 1993; Wallbanks *et al*., 1990). The LAB have been classified at the genus level in four groups (Salminen & Von Wright, 1998). In 1991, Collins *et al*. studied 16S rRNA sequences of 55 species of *Lactobacillus* genera and several bordering species (Collins *et al*., 1991). Results show that the genus *Lactobacillus* is separated into three groups; *Lactobacillus delbrueckii*, *Lactobacillus casei–Pediococcus* and *Leuconostoc paramesenteroides*. According to these authors, the strains of *Lactobacillus* and *Pediococcus* in our study are included in the second group. These species really form a supercluster within LAB (Fig. 2). Therefore, there are great differences between the 16S sequence phylogeny and the classification based on morphological, physiological and biochemical studies. For these reasons, the search for an alternative tool, such as a gene encoding a protein, is intensifying (Birtles & Raoult, 1996; Christensen & Olsen, 1998; Morse *et al*., 1996; Roux *et al*., 1997; Springer *et al*., 1995).

Comparison of 16S rRNA and *mle* phylogenetic analysis

Although the MLE is not described as existing outside LAB, not all LAB contain it. Comparisons of similarity values showed that this gene is less conserved than 16S rRNA gene in LAB. The dendrograms inferred from 16S rRNA and *mle* sequences had different profiles. *P. acidilactici* and *P. parvulus* were

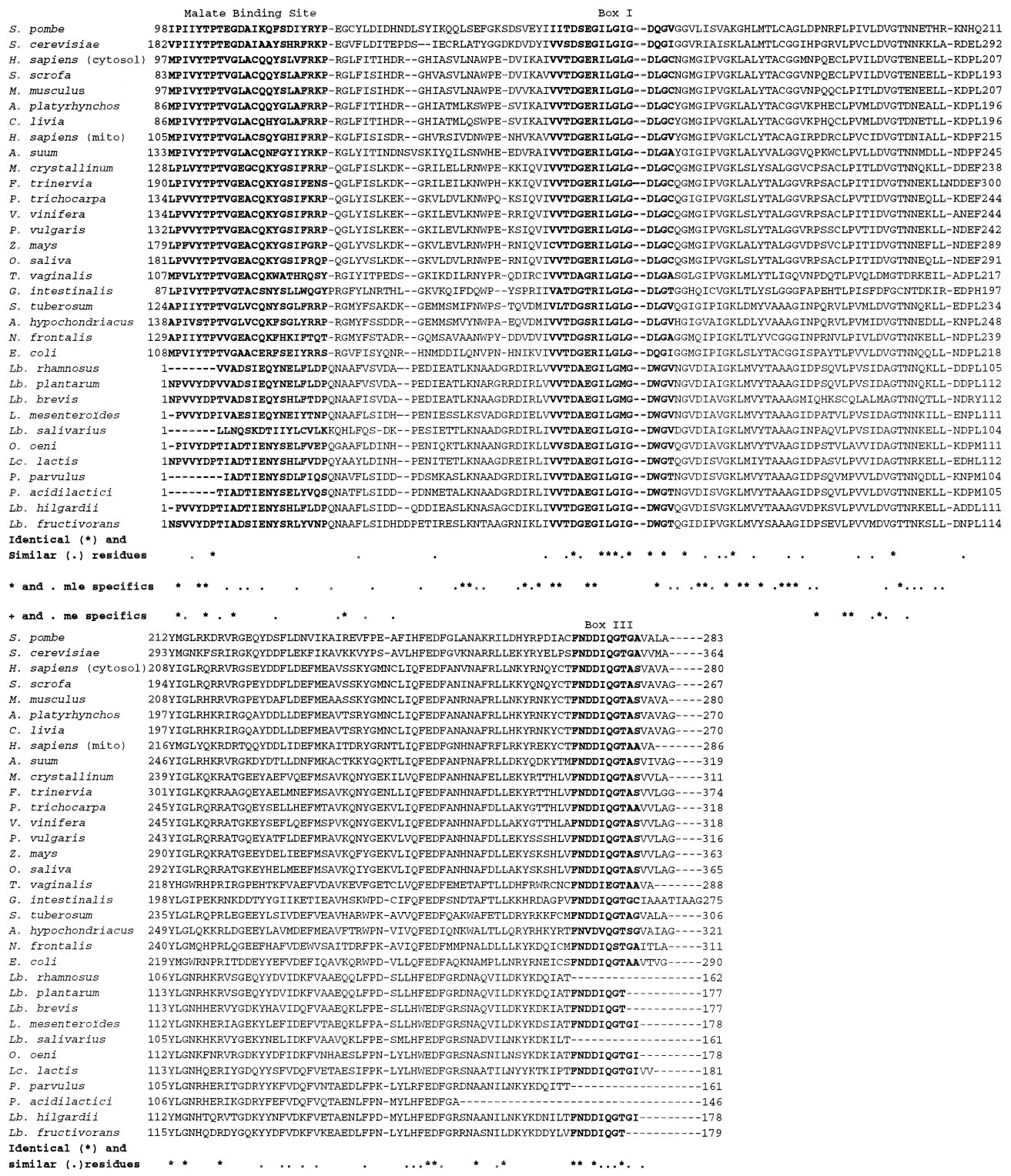

Fig. 4. Alignment of partial amino acid sequences of different malic and malolactic enzymes.

Consensus Malate Binding Site

M	P	I	V	Y	T	P	T	V	G	D	A	C	Q	K	Y	S	S	L	F	R	R	P
L		V	I		**D**		V	I	A	E	***S***	***I***	***E***	Q	F	G	L	I	Y	V	K	S
N								*E*		L	**T**		S	N		N	E	A		L	Q	Y
A																	H			Q	N	
I																	D			T	D	
V																	Y					
																	G					

Consensus Box I

V	V	T	D	G	E	R	I	L	G	L	G	D	L	G
		S		A	S	***G***				I			**W**	
				S						M			Q	

Consensus Box III

F	N	D	D	I	Q	G	T

Fig. 5. Analysis consensus sequences of malic and malolactic amino acid aligment. Malolactic specific residues are indicated in bold. Yeast-specific residues are indicated in italics.

distinct from other LAB, whereas *Leuconostoc mesenteroides*, *O. oeni* and *Lactococcus lactis* were included in the *Lactobacillus* group. This group itself is divided into two subclusters, the first containing *Lactobacillus hilgardii*, *Lactobacillus fructivorans*, *Lactobacillus salivarius* and *Lactobacillus rhamnosus*, the second containing *Leuconostoc mesenteroides*, *Lactobacillus plantarum*, *Lactobacillus brevis*, *Lactococcus lactis* and *O. oeni*. Moreover, the percentage of changes in sequences between *O. oeni* and the other LAB was lower for *mle* than for 16S rRNA. This result is in agreement with the phylogenetic tree of the amino acid sequences of the β' subunit of DNA-dependent RNA polymerase (Morse *et al.*, 1996), and the analysis of *mle* gene does not support the hypothesis inferred from 16S that *O. oeni* is a fast-evolving organism (Yang & Woese, 1989). On the contrary, *Lactobacillus salivarius* and *Lactobacillus rhamnosus* appear to be two fast-evolving organisms on the basis of *mle* sequence analysis. As suggested by Morse *et al.* (1996), since 16S rRNA is a neutral indicator of evolutionary change, the entire genome should evolve like it. However, for three species of LAB, there is no correlation between 16S and malolactic gene evolution. The 16S rRNA gene is always necessary for the survival of the bacteria, whereas a gene encoding a protein of metabolism is more dependent on selection pressure. This supports the hypothesis that MLE is less used by *Lactobacillus salivarius* and *Lactobacillus rhamnosus* than by the other species studied, and that its use depends on the evolution conditions of the bacteria.

In this study, the complete sequences of 16S rRNA were used but a stretch could be found in the 16S rRNA that would mirror the situation of the *mle* genes much more closely (data not shown).

Comparison of malic and malolactic enzymes

The malolactic protein sequence was very similar to the malic protein sequence. The phylogenetic tree obtained with amino acid sequences of LAB *mle* and amino acid sequences of different malic enzymes showed that these two enzymes might have a common ancestor. With regard to eukaryotes and bacteria, malolactic and malic enzymes are separated into two groups. However, the malic enzyme of *E. coli*, *Schizosaccharomyces pombe* and *Saccharomyces cerevisiae* is closer to the MLE of LAB than to other malic enzymes. Surprisingly, yeasts are closest to LAB. The phylogenetic tree of the amino acid sequences of malic and malolactic enzymes was constructed without bootstraps and with *E. coli* as outgroup. With 100 replicates, the result was not in agreement with previous studies (Hrdy & Müller, 1995; Van der Glezen *et al.*, 1997). The LAB trees obtained by comparing of amino acid and nucleotide sequences were different. The first shows that there are two groups where all bacteria species are intermixed. The amino acid sequence is not subject to degeneration of the genetic code, so only *Lactobacillus salivarius* appears to have a faster evolution than other LAB. Considering the different habitats of the species studied (De Roissart & Luquet, 1994), all species evolve in fermented food, fermented dairy products or wines except *Lactobacillus salivarius*, which is only found in the human oral cavity and the intestines. In addition, *Lactobacillus plantarum*, *Lactobacillus brevis* and *Lactobacillus rhamnosus* can also be identified in human samples and even associated with infectious endocarditis, septicaemia and meningitis (Harty *et al.*, 1994; Oakey *et al.*, 1995; Saxelin *et al.*, 1996). These four species are situated in the same group in the amino acid phylogenetic tree (a group also including *Leuconostoc mesenteroides*). The amino acid sequence comparison also sheds light on the relationships between malic and malolactic enzymes, and on the influence of habitat, whereas the nucleotide sequence comparison reveals phylogenetic interrelationships between LAB species.

In this study, malic and malolactic enzymes harboured the same conserved sites. The functions of these sites have been intensively studied in malic enzymes by different enzymic methods and by site-directed mutagenesis (Chang *et al.*, 1993; Gavva *et al.*, 1991; Wei *et al.*, 1995, 1997). The consensus box III was exactly identical between malic and all malolactic enzymes. In pigeon liver malic enzyme, a Fe^{2+}-ascorbate system inactivates the enzyme by cleavage at the peptide bond between Asp^{258} and Ile^{259} (Wei *et al.*, 1997). Moreover, site-directed mutagenesis has confirmed that Asp^{258} is one of the ligands of Mn^{2+} (Wei *et al.*, 1995). Our results suggest that this consensus aspartate, and thus consensus site, plays the same role of metal-binding site not only in all malic enzymes but also in all malolactic enymes. The consensus box I corresponds to the putative ADP-binding $\beta\alpha\beta$ dinucleotide fold (Wierenga *et al.*, 1985). The presence of a conserved cysteine in this site (Gonzalez-Manchon *et al.*, 1995) is

indicative of a $NADP^+$-dependent malic enzyme. All the MLEs studied in this report or previously purified (Caspritz & Radler, 1983; Lonvaud, 1975; Lonvaud-Funel & Strasser de Saad, 1982; Naouri *et al*., 1990; Spettoli *et al*., 1984) are NAD^+-dependent. Indeed, they do not have cysteine but threonine or valine at this consensus site. The importance of a cysteine in malate binding has been demonstrated by SH reagent inhibition studies in several malic enzymes (Chang *et al*., 1993; Gavva *et al*., 1991). This residue is replaced by isoleucine in MLEs (lysine for *Lactobacillus salivarius*), and there are very few cysteine residues in MLE sequences. The first hypothesis was that this cysteine is essential in understanding the different functions of these two similar enzymes. However, the malic enzymes of *Schizosaccharomyces pombe* and *Saccharomyces cerevisiae* also have isoleucine, like the MLEs. So, if an SH group intervenes since pyruvate is released by malic enzymes, another cysteine is involved in yeast malic enzymes. Amino acid sequence analysis of the two enzymes shows some other interesting residues that are specific either to malic enzymes or to MLEs. For example, in the malate-binding site a conserved aspartate in MLEs is replaced by a threonine in malic enzymes.

Therefore, it appears that malic and malolactic enzymes are close proteins. No crystallographic three-dimensional structure is yet available for these two enzymes. Therefore, site-directed mutagenesis studies are needed to find why the MLE, unlike malic enzyme, transforms malate to lactate without release of intermediate products, such as pyruvate or NADH.

REFERENCES

Ansanay, V., Dequin, S., Blondin, B. & Barre, P. (1993). Cloning, sequence and expression of the gene encoding the malolactic enzyme from *Lactococcus lactis*. *FEBS Lett* **332**, 74–80.

Bagchi, S., Wise, L. S., Brown, M. L., Sul, H. S., Bregman, D. B. & Rubin, C. S. (1986). Regulation and structure of murine malic enzyme mRNA. *Ann N Y Acad Sci* **478**, 77–92.

Bairoch, A. (1991). PROSITE: a dictionary of sites and patterns in proteins. *Nucleic Acids Res* **19** (suppl.), 2241–2245.

Batterman, G. & Radler, F. (1990). A comparative study of malolactic enzyme and malic enzyme of different lactic acid bacteria. *Can J Microbiol* **37**, 211–217.

Birnboim, H. C. & Doly, J. (1979). A rapid alkaline extraction procedure for screening recombinant plasmid DNA. *Nucleic Acids Res* **7**, 1513–1523.

Birtles, R. J. & Raoult, D. (1996). Comparison of partial citrate sythase gene (*glt*A) sequences for phylogenetic analysis of *Bartonella* species. *Int J Syst Bacteriol* **46**, 891–897.

Boles, E., de Jong-Gubbels, P. & Pronk, J. T. (1998). Identification and characterization of MAE1, the *Saccharomyces cerevisiae* structural gene encoding mitochondrial malic enzyme. *J Bacteriol* **180**, 2875–2882.

Borsch, D. & Westhoff, P. (1990). Primary structure of NADP-dependent malic enzyme in the dicotyledonous C4 plant *Flaveria trinervia*. *FEBS Lett* **273**, 111–115.

Buchanan, R. E. & Gibbons, N. E. (1986). *Bergey's Manual of Determinative Bacteriology*, 9th edn. Baltimore: Williams & Wilkins.

Bullock, W. O., Fernandez, J. M. & Short, J. M. (1987). XL1-Blue: a high efficiency plasmid transforming RecA *Escherichia coli* strain with β-galactosidase selection. *BioTechniques* **5**, 376–379.

Caspritz, G. & Radler, F. (1983). Malolactic enzyme of *Lactobacillus plantarum*. Purification, properties, and distribution among bacteria. *J Biol Chem* **258**, 4907–4910.

Chang, G. G., Satterlee, J. & Hsu, R. Y. (1993). Essential sulfhydryl group of malic enzyme from *Escherichia coli*. *J Protein Chem* **12**, 7–10.

Chou, W. Y., Huang, S. M., Liu, Y. H. & Chang, G. G. (1994). Cloning and expression of pigeon liver cytosolic $NADP^+$-dependent malic enzyme cDNA and some of its abortive mutants. *Arch Biochem Biophys* **310**, 158–166.

Christensen, H. & Olsen, J. E. (1998). Phylogenetic relationships of *Salmonella* based on DNA sequence comparison of *atpD* encoding the beta subunit of ATP synthase. *FEMS Microbiol Lett* **161**, 89–96.

Collins, M. D., Farrow, J. A. E., Phillips, B. A., Ferusu, S. & Jones, D. (1987). Classification of *Lactobacillus divergens*, *Lactobacillus piscicola* and some catalase-negative, asporogenous, rod-shaped bacteria from poultry in a new genus, *Carnobacterium*. *Int J Syst Bacteriol* **37**, 310–316.

Collins, M. D., Williams, A. M. & Wallbanks, S. (1990). The phylogeny of *Aerococcus* and *Pediococcus* as determined by 16S rRNA sequence analysis: description of *Tetragenococcus* gen. nov. *FEMS Microbiol Lett* **58**, 255–262.

Collins, M. D., Rodrigues, U., Ash, C., Aguirre, M., Farrow, J. A. E., Martínez-Murcia, A., Phillips, B. A., Williams, A. M. & Wallbanks, S. (1991). Phylogenetic analysis of the genus *Lactobacillus* and related lactic acid bacteria as determined by reverse transcriptase sequencing of 16S rRNA. *FEMS Microbiol Lett* **77**, 5–12.

Collins, M. D., Samelis, J., Metaxopoulos, J. & Wallbanks, S. (1993). Taxonomic studies on some leuconostoc-like organisms from fermented sausages: description of a new genus *Weissella* for the *Leuconostoc paramesenteroides* group of species. *J Appl Bacteriol* **75**, 595–603.

Cushman, J. C. (1992). Characterization and expression of a NADP-malic enzyme cDNA induced by salt stress from the facultative crassulacean acid metabolism plant, *Mesembryanthemum crystallinum*. *Eur J Biochem* **208**, 259–266.

Denayrolles, M., Aigle, M. & Lonvaud-Funel, A. (1994). Cloning and sequence analysis of the gene encoding *Lactococcus lactis* malolactic enzyme: relationships with malic enzymes. *FEMS Microbiol Lett* **116**, 79–86.

De Roissart, H. & Luquet, F. M. (editors) (1994). *Bactèries Lactiques. Aspects Fondamentaux et Technologiques*, vol. 1, pp. 133–137. Uriage: Lorica.

Dicks, L. M. T., Dellaglio, F. & Collins, M. D. (1995). Proposal to reclassify *Leuconostoc oenos* as *Oenococcus oeni* (corrig.) gen. nov., comb. nov. *Int J Syst Bacteriol* **45**, 395–397.

Dicks, L. M. T., Du Plessis, E. M., Dellaglio, F. & Lauer, E. (1996). Reclassification of *Lactobacillus casei* subsp. *casei* ATCC 393 and *Lactobacillus rhamnosus* ATCC 15820 as *Lactobacillus zeae* nom. rev., designation of ATCC 334 as the neotype of *L. casei* subsp. *casei*, and rejection of the name *Lactobacillus paracasei*. *Int J Syst Bacteriol* **46**, 337–340.

Felsenstein, J. (1989). PHYLIP: phylogeny inference package. *Cladistics* **5**, 164–166.

Fox, G. E., Wisotzkey, J. D. & Jurtshuk, P. (1992). How close is

close: 16S rRNA sequence identity may not be sufficient to guarantee species identity. *Int J Syst Bacteriol* **42**, 166–170.

Franke, K. E. & Adams, D. O. (1995). Cloning of a full-length cDNA for malic enzyme (EC 1.1.1.40) from grape berries. *Plant Physiol* **107**, 1009–1010.

Fushimi, T., Umeda, M., Shimazaki, T., Kato, A., Toriyama, K. & Uchimiya, H. (1994). Nucleotide sequence of a rice cDNA similar to a maize NADP-dependent malic enzyme. *Plant Mol Biol* **24**, 965–967.

Gasson, M. J. & Davies, F. L. (1980). Conjugal transfer of drug resistance plasmid pAMβ1 in lactic streptococci. *FEMS Microbiol Lett* **7**, 51–53.

Gavva, S. R., Harris, B. G., Weiss, P. M. & Cook, P. F. (1991). Modification of a thiol at the active site of the *Ascaris suum* NAD-malic enzyme results in changes in the rate-determining steps for oxidative decarboxylation of L-malate. *Biochemistry* **30**, 5764–5769.

Gonzalez-Manchon, C., Ferrer, M., Ayuso, M. S. & Parrilla, R. (1995). Cloning, sequencing and functional expression of a cDNA encoding a NADP-dependent malic enzyme from human liver. *Gene* **159**, 255–260.

Harty, D. W., Oakey, H. J., Patrikakis, M., Hume, E. B. & Knox, K. W. (1994). Pathogenic potential of lactobacilli. *Int J Food Microbiol* **24**, 179–189.

Hrdy, I. & Müller, M. (1995). Primary structure of the hydrogenosomal malic enzyme of *Trichomonas vaginalis* and its relationship to homologous enzymes. *J Eukaryot Microbiol* **42**, 593–603.

Hsu, R. Y., Glynias, M. J., Satterlee, J. & 7 other authors (1992). Duck liver 'malic' enzyme. Expression in *Escherichia coli* and characterization of the wild-type enzyme and site-directed mutants. *Biochem J* **284**, 869–876.

Johnson, J. L. (1984). Bacterial classification. III. Nucleic acids in bacterial classification. In *Bergey's Manual of Systematic Bacteriology*, vol. 1, pp. 8–11. Edited by N. R. Krieg & J. G. Holt. Baltimore: Williams & Wilkins.

Kawai, S., Suzuki, H., Yamamoto, K., Inui, M., Yukawa, H. & Kumagai, H. (1996). Purification and characterization of a malic enzyme from the ruminal bacterium *Streptococcus bovis* ATCC 15352 and cloning and sequencing of its gene. *Appl Environ Microbiol* **62**, 2692–2700.

Kulkarni, L. P., Cook, P. F. & Harris, B. G. (1993). Cloning and nucleotide sequence of a full-length cDNA encoding *Ascaris suum* malic enzyme. *Arch Biochem Biophys* **300**, 231–237.

Labarre, C., Guzzo, J., Cavin, J. F. & Divies, C. (1996). Cloning and characterization of the genes encoding the malolactic enzyme and the malate permease of *Leuconostoc oenos*. *Appl Environ Microbiol* **62**, 1274–1282.

Lane, D. L., Pace, B., Olsen, G. J., Stahl, D. A., Sogin, M. L. & Pace, N. R. (1985). Rapid determination of 16S ribosomal RNA sequences for phylogenetic analysis. *Proc Natl Acad Sci USA* **82**, 6955–6959.

Loeber, G., Infante, A. A., Maurer-Fogy, I., Krystek, E. & Dworkin, M. B. (1991). Human NAD^+-dependent mitochondrial malic enzyme: cDNA cloning, primary structure, and expression in *Escherichia coli*. *J Biol Chem* **266**, 3016–3021.

London, J. & Meyer, E. Y. (1969). Malate utilization by a group D Streptococcus, physiological properties and purification of a inductible malic enzyme. *J Bacteriol* **98**, 705–711.

Long, J. J., Wang, J. L. & Berry, J. O. (1994). Cloning and analysis of the C4 photosynthetic NAD-dependent malic enzyme of Amaranth mitochondria. *J Biol Chem* **269**, 2827–2833.

Lonvaud, M. (1975). *Recherches sur l'enzyme des bactéries lactiques du vin assurant la transformation du malate en lactate*. Thèse de 3ème cycle, Université de Bordeaux II.

Lonvaud, M. & Ribereau-Gayon, P. (1973). Utilisation d'une électrode CO_2 pour la détermination de l'activité de 'l'enzyme malique' des bactéries lactiques du vin. *C R Acad Sci D* **276**, 2329–2331.

Lonvaud-Funel, A. & Strasser de Saad, A. M. (1982). Purification and properties of a malolactic enzyme from a strain of *Leuconostoc mesenteroides* isolated from grapes. *Appl Environ Microbiol* **43**, 357–361.

Lonvaud-Funel, A., Fremaux, C., Biteau, N. & Joyeux, A. (1991). Speciation of lactic acid bacteria from wine by DNA hybridization with DNA probes. *Food Microbiol* **8**, 215–222.

Mahayan, S. K., Chu, C. C., Willis, D. K., Templin, A. & Clark, A. J. (1990). Physical analysis of spontaneous and mutagen-induced mutants of *Escherichia coli* K-12 expressing DNA exonuclease VIII activity. *Genetics* **125**, 261–273.

Martínez-Murcia, A. J. & Collins, M. D. (1990). A phylogenetic analysis of the genus *Leuconostoc* based on reverse transcriptase sequencing of 16 S rRNA. *FEMS Microbiol Lett* **58**, 73–83.

Miller, J. H. (1972). *Experiments in Molecular Genetics*. Cold Spring Harbor, NY: Cold Spring Harbor Laboratory.

Morse, R., Collins, M. D., O'Hanlon, K., Wallbanks, S. & Richardson, P. T. (1996). Analysis of the β' subunit of DNA-dependent RNA polymerase does not support the hypothesis inferred from 16S rRNA analysis that *Oenococcus oeni* (formerly *Leuconostoc oenos*) is a tachytelic (fast-evolving) bacterium. *Int J Syst Bacteriol* **46**, 1004–1009.

Naouri, P., Chagnaud, P., Arnaud, A. & Galzy, P. (1990). Purification and properties of a malolactic enzyme from *Leuconostoc œnos* ATCC 23278. *J Basic Microbiol* **30**, 577–585.

Oakey, H. J., Harty, D. W. & Knox, K. W. (1995). Enzyme production by lactobacilli and the potential link with infective endocartis. *J Appl Bacteriol* **78**, 142–148.

Rothermel, B. A. & Nelson, T. (1989). Primary structure of the maize NADP-dependent malic enzyme. *J Biol Chem* **264**, 19587–19592.

Roux, V., Rydkina, E., Eremeeva, M. & Raoult, D. (1997). Citrate synthase gene comparison, a new tool for phylogenetic analysis, and its application for the *Rickettsiae*. *Int J Syst Bacteriol* **47**, 252–261.

Salminen, S. & von Wright, A. (editors) (1998). *Lactic Acid Bacteria. Microbiology & Functional Aspects*, 2nd edn, pp. 1–22. New York: Marcel Dekker.

Sanchez, L. B., Hashimoto, T. & Müller, M. (1996). Sequence of a malic enzyme gene of *Giardia lamblia*. *Mol Biochem Parasitol* **82**, 145–151.

Saxelin, M., Chuang, N. H., Chassy, B., Rautelin, H., Makela, P. H., Salminen, S. & Gorbach, S. L. (1996). Lactobacilli and bacteremia in southern Finland, 1989–1992. *Clin Infect Dis* **22**, 564–566.

Smith, P. K., Khron, R. I., Hermanson, J. T. & 7 other authors (1985). Measurement of protein using bicinchoninic acid. *Anal Biochem* **150**, 76–85.

Sohier, D., Coulon, J. & Lonvaud-Funel, A. (1999). Molecular identification of *Lactobacillus hilgardii* and genetic relationships with *Lactobacillus brevis*. *Int J Syst Bacteriol* **49**, 1075–1081.

Spettoli, P., Nuti, M. P. & Zamora, A. (1984). Properties of malolactic activity purified from *Leuconostoc œnos* ML34 by affinity chromatography. *Appl Environ Microbiol* **48**, 900–903.

Springer, E., Sachs, M. S., Woese, C. R. & Boone, D. R. (1995).

Partial gene sequences for the A subunit of methyl-coenzyme M reductase (*mcr*I) as a phylogenetic tool for the family *Methanosarcinaceae*. *Int J Syst Bacteriol* **45**, 554–559.

Stiles, M. E. & Holzapfel, W. H. (1997). Lactic acid bacteria of foods and their current taxonomy. *Int J Food Microbiol* **36**, 1–29.

Thompson, J. D., Higgins, D. G. & Gibson, T. J. (1994). CLUSTAL W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice. *Nucleic Acids Res* **22**, 4673–4680.

Vandamme, P., Pot, B., Gillis, M., de Vos, P., Kersters, K. & Swings, J. (1996). Polyphasic taxonomy, a consensus approach to bacterial systematics. *Microbiol Rev* **60**, 407–438.

Van der Glezen, M., Rechinger, K. B., Svendsen, I., Durand, R., Hirt, R. P., Fevre, M., Embley, T. M. & Prins, R. A. (1997). A mitochondrial-like targeting signal on the hydrogenosomal malic enzyme from the anaerobic fungus *Neocallimastix frontalis*: support for the hypothesis that hydrogenosomes are modified mitochondria. *Mol Microbiol* **23**, 11–21.

Van Doorsselaered, J., Villaroel, N., Montagu, V. & Inze, D. (1991). Nucleotide sequence of a cDNA encoding enzyme from poplar. *Plant Physiol* **96**, 1385–1386.

Viljoen, M., Subden, R. E., Krizus, A. & van Vuuren, H. J. J. (1994). Molecular analysis of the malic enzyme gene (*mae*2) of *Schizosaccharomyces pombe*. *Yeast* **10**, 613–624.

Wallbanks, S., Martínez-Murcia, A. J., Fryer, J. L., Phillips, B. A. & Collins, M. D. (1990). 16S rRNA sequence determination for members of the genus *Carnobacterium* and related lactic acid bacteria and description of *Vagococcus salmoninarum* sp. nov. *Int J Syst Bacteriol* **40**, 224–230.

Walter, M. H., Grima-Pettenati, J., Grand, C., Boudet, A. M. & Lamb, C. J. (1990). Extensive sequence similarity of the bean CAD4 (cinnamoyl-alcohol dehydrogenase) to a maize malic enzyme. *Plant Mol Biol* **15**, 525–526.

Wei, C. H., Chou, W. Y., Huang, S. M., Lin, C. C. & Chang, G. G. (1994). Affinity cleavage at the putative metal-binding site of pigeon liver malic enzyme by the Fe^{2+}-ascorbate system. *Biochemistry* **33**, 7931–7936.

Wei, C. H., Chou, W. Y. & Chang, G. G. (1995). Identification of Asp^{258} as the metal coordinate of pigeon liver malic enzyme by site-specific mutagenesis. *Biochemistry* **34**, 7949–7954.

Wierenga, R. K., De Maeyer, M. C. H. & Hol, W. G. J. (1985). Interaction of pyrophosphate moieties with α-helixes in dinucleotide binding proteins. *Biochemistry* **24**, 1346–1357.

Winning, B. M., Bourguignon, J. & Leaver, C. J. (1994). Plant mitochondrial NAD^+-dependent malic enzyme. cDNA cloning, deduced primary structure of the 59- and 62-kDa subunits, import, gene complexity and expression analysis. *J Biol Chem* **269**, 4780–4786.

Woese, C. R. (1987). Bacterial evolution. *Microbiol Rev* **51**, 221–271.

Yang, D. & Woese, C. R. (1989). Phylogenetic structure of the 'Leuconostocs': an interesting case of a rapidly evolving organism. *Syst Appl Microbiol* **12**, 145–149.

Zapparoli, G., Torriani, S., Pesente, P. & Dellaglio, F. (1998). Design and evaluation of malolactic enzyme gene targeted primers for rapid identification and detection of *Oenococcus oeni* in wine. *Lett Appl Microbiol* **27**, 243–246.

Zavaleta, A. I., Martínez-Murcia, A. J. & Rodriguez-Valera, F. (1997). Intraspecific genetic diversity of *Oenococcus oeni* as derived from DNA fingerprinting and sequence analysis. *Appl Environ Microbiol* **63**, 1261–1267.