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

Agnès Groisillier and Aline Lonvaud-Funel

DNA sequences covering 36% of the mle gene that encodes the malolactic enzyme were determined for 13 strains of lactic acid bacteria, representing Pediooccus, 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. Pediooccus 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 & Holzapfel, 1997; Vandamme et al., 1996). DNA–DNA hybridization (Kawai et al., 1996), 16S rRNA sequencing (Collins et al., 1990, 1993; Lane et al., 1996) 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 parmesenteroides’ 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. (Martinez-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;
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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 Weisella, 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 Methanosarcinaeae) (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 (Ansany et al., 1993; Denayrolles et al., 1994) and ‘Leuconostoc oenos’ recently re-classified 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 Mn²⁺ 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 International Journal of Systematic Bacteriology 49
Table 1. Strains used for mle gene sequence phylogenetic studies

<table>
<thead>
<tr>
<th>Species</th>
<th>Source/strain no.</th>
<th>Other information</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lactococcus lactis</td>
<td>LRTL IL1441</td>
<td>Isolated from Porto</td>
</tr>
<tr>
<td>Oenococcus oeni</td>
<td>ATCC 23279&lt;sup&gt;T&lt;/sup&gt;</td>
<td></td>
</tr>
<tr>
<td>Leuconostoc mesenteroides</td>
<td>ATCC 8293&lt;sup&gt;T&lt;/sup&gt;</td>
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<td>Pediococcus acidilactici</td>
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<td></td>
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<tr>
<td>Pediococcus parvulus</td>
<td>ATCC 19371&lt;sup&gt;T&lt;/sup&gt;</td>
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<td>Lactobacillus salivarius</td>
<td>ATCC 11741&lt;sup&gt;T&lt;/sup&gt;</td>
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<td>Lactobacillus rhamnosus</td>
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<td>Lactobacillus plantarum</td>
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<td>Isolated from Muscat</td>
</tr>
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<td>Lactobacillus hilgardii</td>
<td>IOEB 9202</td>
<td>Isolated from Porto</td>
</tr>
<tr>
<td>Lactobacillus brevis</td>
<td>ATCC 14869&lt;sup&gt;T&lt;/sup&gt;</td>
<td></td>
</tr>
<tr>
<td>Lactobacillus brevis</td>
<td>IOEB 9647</td>
<td>Isolated from sweet white wine</td>
</tr>
</tbody>
</table>

Conserved amino acid regions of the mle<sub>S</sub> gene of Lactococcus lactis IL1441 (Ansanay et al., 1993; Denayrolles et al., 1994). These regions were NPYYVP (amino acids 86-92) and QGTGIV (amino acids 260-265) and the corresponding nucleotides sequences were ATCC-AGTTGTTATGATC (nucleotides 255-276) (sense) and AACAATACAGTTCTTG (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 MPVYTP (amino acids 109-115) and QGTAAV (amino acids 281-286) and the corresponding nucleotides sequences were TGCCGTGGATTTATACCC (nucleotides 324-345) (sense) and TACC-GCGCAGTGGCCTG (nucleotides 840-858) (antisense).

Approximately 100 ng of genomic DNA was amplified with 2.5 µl Taq polymerase (Stratagene) in a 50 µl reaction by using a MiniCycler (MJ Research). Conditions were 30 cycles of 95 °C (30 s), 38 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 EcoRV-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 EcoRI or HindIII [4 U (µg DNA)] 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 x SSC, 1% blocking reagent (Boehringer Mannheim), 0.02% SDS and 0.1% lauryl sarcosine. For hybridization, 10 ng ml<sup>-1</sup> of labelled and denatured probe were added. After incubation, membranes were washed twice at room temperature for 5 min with 2 x SSC and twice at 55 °C for 15 min in 2 x 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 mle<sub>S</sub> of Lactococcus lactis (Ansanay et al., 1993; Denayrolles et al., 1994) and mle<sub>A</sub> 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)
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 CO₂ 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 CO₂ 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...

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**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.

<table>
<thead>
<tr>
<th>Strain</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
<th>5</th>
<th>6</th>
<th>7</th>
<th>8</th>
<th>9</th>
<th>10</th>
<th>11</th>
<th>12</th>
</tr>
</thead>
<tbody>
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<td>1 <em>Escherichia coli</em></td>
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<td>32</td>
<td>33</td>
<td>34</td>
<td>34</td>
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</tr>
<tr>
<td>2 <em>Lactococcus lactis</em></td>
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<td>67</td>
<td>72</td>
<td>72</td>
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<td>69</td>
<td>68</td>
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<td>4 <em>Oenococcus oeni</em></td>
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<td>67</td>
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<td>38</td>
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<td>69</td>
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<td>11 <em>Lactobacillus fructivorans</em></td>
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<td>92</td>
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</tbody>
</table>

*Malic enzyme gene sequence.*
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 βββ 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 EcoRI and HindIII. 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 8290T. The sequence obtained for O. oeni ATCC 23279T 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...
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 et al., 1995; Van der Glezen et al., 1993; Denayrolles et al., 1994). Three of these four regions were available for any malic or malolactic enzymes, four functionally important domains have been identified (Bairoch, 1991; Viljoen et al., 1995). Four mutant enzymes substituted at Asp25s with glutamate, aspartate, paragine, 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 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., 1995; Denayrolles et al., 1994; Wierenga et al., 1994). Four mutant enzymes substituted at Asp25s with glutamate, aspartate, paragine, 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.

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**Table 3. Malic and malolactic sequences included in this study**

<table>
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<tr>
<th>Species</th>
<th>Subcellular location</th>
<th>Accession no.</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Amaranthus hypochondriacus</em></td>
<td>Mitochondrion</td>
<td>U01162</td>
<td>Long et al. (1994)</td>
</tr>
<tr>
<td><em>Anas platyrhynchos</em></td>
<td>Cytosol</td>
<td>X66648</td>
<td>Hsu et al. (1992)</td>
</tr>
<tr>
<td><em>Ascaris suum</em></td>
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<td>X81055</td>
<td>Kulkarni et al. (1993)</td>
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<td>L09233</td>
<td>Chou et al. (1994)</td>
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<tr>
<td><em>Escherichia coli</em></td>
<td>Mitochondrion</td>
<td>P26616</td>
<td>Mahayan et al. (1990)</td>
</tr>
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<td>Chloroplast</td>
<td>X57142</td>
<td>Borshc &amp; Weathoff (1990)</td>
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<td>?</td>
<td>U59300</td>
<td>Sanchez et al. (1996)</td>
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<td>L34035</td>
<td>Gonzalez-Manchon et al. (1995)</td>
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<td>Bagchi et al. (1986)</td>
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<td>U62041</td>
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<td>Fushimi et al. (1994)</td>
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<td>Wulter et al. (1990)</td>
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<td>Z28029</td>
<td>Boles et al. (1998)</td>
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<td>?</td>
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<td>Hrdy &amp; Müller (1995)</td>
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<td>Cytosol</td>
<td>L34836</td>
<td>Franke et al. (1995)</td>
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</table>

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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
This study shows that of the LAB we tested and which cross-hybridize with Lactobacillus sequences were identical to the 16S sequence of Lactobacillus hilgardii ATCC 8290. The result for Lactobacillus hilgardii was also exactly identical to the mleA of Lactobacillus hilgardii ATCC 8290 (Labarre et al., 1996). In a recent paper, mle-targeted primers were used for rapid identification and detection of Lactobacillus. Therefore, this enzyme appears to have an identical sequence at the species level. From this, Lactobacillus 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 mesenteroides. 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.
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 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 inter-relationships 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 Fe²⁺-ascorbate system inactivates the enzyme by cleavage at the peptide bond between Asp₁⁵⁸ and Ile₁⁵⁹ (Wei et al., 1997). Moreover, site-directed mutagenesis has confirmed that Asp₁⁵⁸ is one of the ligands of Mn²⁺ (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 enzymes. The consensus box I corresponds to the putative ADP-binding βββ 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; Spettioli et al., 1984) are NAD⁺-dependent. Indeed, they do not have cysteine but threonine or valine at 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 Saccharomyces cerevisiae 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 lactic without release of intermediate products, such as pyruvate or NADH.

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