Absence of Malolactic Activity Is a Characteristic of H+-ATPase-Deficient Mutants of the Lactic Acid Bacterium *Oenococcus oeni*

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The lack of malolactic activity in H+-ATPase-deficient mutants of *Oenococcus oeni* selected previously was analyzed at the molecular level. Western blot experiments revealed a spot at 60 kDa corresponding to the malolactic enzyme only in the parental strain. Moreover, the *mleA* transcript encoding the malolactic enzyme was not detected by reverse transcription (RT)-PCR analysis of mutants. These results suggest that the malolactic operon was not transcribed in ATPase-deficient mutants. The *mleR* gene encoding a LysR-type regulatory protein which should be involved in expression of the malolactic genes was described previously for *O. oeni*. Results obtained in this study show that the *mleR* transcript was not detected in the mutants by RT-PCR. No mutation in the nucleotide sequences of the *mleR* gene and the malolactic operon was found. The effect of a reduction in H+-ATPase activity on L-malate metabolism was then investigated by using other malolactic bacteria. Spontaneous H+-ATPase-deficient mutant strains of *Lactococcus lactis* and *Leuconostoc mesenteroides* were isolated by using neomycin resistance. Two mutants were selected. These mutants exhibited ATPase activities that were reduced to 54 and 70% of the activities obtained for the *L. lactis* and *L. mesenteroides* parental strains, respectively. These mutants were also acid sensitive. However, in contrast to the ATPase-deficient mutants of *O. oeni*, activation of L-malate metabolism was observed with the *L. lactis* and *L. mesenteroides* mutants under optimal or acidic growth conditions. These data support the suggestion that expression of the genes encoding malolactic enzymes in *O. oeni* is regulated by the *mleR* product, as it is in *L. lactis*. Nevertheless, our results strongly suggest that there is a difference between the regulation of expression of the malolactic locus in *O. oeni* and the regulation of expression of this locus in less acidophilic lactic acid bacteria.

Lactic acid bacteria are extensively used as starters in food and beverage fermentations, such as dairy fermentations, as well as in wine production. Although lactic acid bacteria are considered acid tolerant, the harsh environment resulting from accumulation of the lactic acid produced during carbohydrate catabolism limits their growth and their survival. On the other hand, an initial high level of acidity of the medium due to a significant concentration of organic acids can prevent bacterial growth. In the case of winemaking, the level of malic acid, which depends directly on grape maturity, affects the pH of the wine.

In order to withstand the low external pH values (pH_{ex}) of environments, bacteria have developed different strategies. When the pH_{ex} decreases, maintenance of a neutral cytoplasmic pH is essential for survival of the fermentative bacterium *Enterococcus faecalis* (23). Many acid-tolerant fermentative bacteria use another strategy: the internal pH (pH_{in}) decreases as the pH_{ex} decreases in order to maintain a constant transmembrane pH gradient rather than a constant pH_{in} (24).

Whatever the strategy, the most important mechanism by which fermentative bacteria can regulate pH_{in} is the proton-translocating ATPase. For *Enterococcus hirae*, this enzyme has been shown previously to be similar to the F_1-F_0 ATPase of aerobic bacteria, which synthesizes ATP coupled with the proton motive force (16, 23). However, the unique function of the H+-ATPase of enterococci is regulation of the cytoplasmic pH (9). This enzyme is also involved in regulation of pH_{in} in other lactic acid bacteria (18). An acid-sensitive variant of *Lactobacillus helveticus* was isolated as an H+-ATPase-deficient strain (27). More recently, Kullen and Klaenhammer (12) characterized the pH-inducible F_1F_0-ATPase operon of *Lactobacillus acidophilus*. An increase in *atp* mRNA was induced by low pH and was correlated with an increase in the activity of the H+-ATPase in membrane extracts. Genes encoding F_1F_0-ATPase in *Lactococcus lactis* have also been cloned and sequenced (11). Results obtained with a mutant strain in which expression of H+-ATPase on the chromosome is under control of the *nisA* promoter clearly demonstrated a requirement for nisin for growth of the mutant. The H+-ATPase was therefore essential for growth of this bacterium. Moreover, the acid sensitivity of a mutant of *L. lactis* with reduced membrane-bound H+-ATPase activity confirmed the major role of this enzyme in regulation of the cytoplasmic pH (1, 28).

Other mechanisms involving decarboxylase systems are thought to participate in pH_{in} regulation. Decarboxylation of amino acids may protect bacterial cells against intracellular and extracellular acidification, particularly when the main car-

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bon and energy sources have been consumed (4, 25). Decarboxylation of carboxylic acids also leads to internal consumption of protons. This is the case for *Oenococcus oeni*, the lactic acid bacterium responsible for malolactic fermentation in wine. Studies of the energetics of L-malic acid metabolism (5, 19, 21) have demonstrated that malolactic fermentation is an energy-producing pathway based on the electrogenic uptake of L-malate, intracellular decarboxylation of this compound by the malolactic enzyme (MLE), and efflux of the decarboxylation product L-lactic acid. Not only does this metabolic pathway result in the generation of a proton motive force sufficient to drive ATP synthesis via the membrane-bound F_1F_0-ATPase, but the proton consumption during the decarboxylation of L-malate also participates in the regulation of pH_{in} (22). We previously cloned and characterized the *mleA* and *mleP* genes encoding the MLE and the malate permease, respectively, in *O. oeni* (13). These two genes are organized in an *mle* locus. Upstream of the *mleA* gene, an open reading frame likely to encode a LysR-type regulatory protein was found (14). The role of this regulatory protein in malolactic gene expression in *O. oeni* has not been determined yet. On the other hand, it has been shown that activation of the malolactic system in *L. lactis* is mediated by *mleR* (20). The protein encoded by *mleR* is homologous to a class of positive regulatory proteins belonging to the LysR family of proteins.

In our laboratory, we isolated spontaneous ATPase-deficient mutants of *O. oeni* (26). The acid sensitivity of these mutants suggested a role for H^+-ATPase in the acid tolerance of *O. oeni*, like that demonstrated for other lactic acid bacteria. However, all of the mutants isolated lacked malolactic activity, suggesting that there is linkage between the ATPase and malolactic activities in *O. oeni*. In order to investigate more precisely the nature of the relationship between these two activities, we analyzed the absence of malolactic activity at the molecular level in ATPase-deficient mutant strains of *O. oeni*. Moreover, the lack of information about the influence of a deficiency in H^+-ATPase activity on L-malic acid metabolism in other malolactic bacteria led us to isolate ATPase-deficient mutant strains of *L. lactis* and *Leuconostoc mesenteroides* by using neomycin resistance as a positive marker. The metabolism of L-malic acid in these mutant strains under optimal or acidic growth conditions was also investigated.

MATERIALS AND METHODS

Bacterial strains and growth conditions. The parental strain *O. oeni* IOB84.13 (Oenological Institute of Bordeaux) and neomycin-resistant strains 9.01.07 and 6.27.06 obtained previously (26) were cultured at 30°C and pH 5.3 in FT80 medium modified (FT80m) as described by Labarre et al. (13). *L. mesenteroides* subsp. *mesenteroides* 19D isolated in our laboratory and *L. lactis* subsp. *lactis* biovar diacetylactis CNRZ 126 (INRA, Jouy en Josas, France) were used as other malolactic bacteria. *L. mesenteroides*, *L. lactis*, and neomycin-resistant strains obtained from both of these strains were cultured at 30°C and pH 6.5 in MRS medium (6) supplemented with 10 g of DL-malate per liter.

Isolation of neomycin-resistant mutants of *L. lactis* and *L. mesenteroides*. Spontaneous neomycin-resistant mutants of *L. lactis* and *L. mesenteroides* were isolated by the method of Yokota et al. (28), with some modifications. Bacteria were grown in 40 ml of MRS medium until the exponential phase was reached (optical density at 600 nm [OD_{600}], 0.3). Cells were harvested by centrifugation, concentrated in 2 ml of fresh medium, and spread onto MRS medium plates containing 300 μg of neomycin sulfate ml^{-1}. The plates were then incubated at 30°C for 48 h. Neomycin-resistant colonies were selected and inoculated onto plates containing 5 ml of MRS medium supplemented with 300 μg of neomycin sulfate ml^{-1}. A first screening, based on the bacterial growth characteristics, was

conducted with MRS medium at pH 6.5. The specific growth rates were calculated during the exponential phase (i.e., between 10 and 15 h for *L. lactis* strains and between 5 and 12 h for *L. mesenteroides* strains). Biomass concentrations were calculated for an initial OD_{600} of 0.06 after 27 and 38 h of growth for *L. lactis* and *L. mesenteroides*, respectively. Cell biomass was deduced based on a preliminary calibration, as follows: 1 OD_{600} unit = 0.4 mg (dry weight). Specific L-malate consumption was calculated by determining the amount of L-malate metabolized, expressed as millimoles per milligram of biomass at the end of culture.

Preparation of membranes and ATPase activity assay. Membranes were prepared and ATPase activity assays were performed as described by Tourdot-Maréchal et al. (26). The methods were adapted to *L. lactis* and *L. mesenteroides* as follows: the buffer used to break the cells was Tris-HCl buffer (50 mM Tris, 10 mM $MgSO_4$; pH 7.0); and ATPase activities were measured in Tris-HCl buffer (pH 7.0) with 6.25 mM ATP. ATPase activity was expressed as micromoles of P_i produced per minute per milligram of protein.

Western immunoblot analysis. Protein extraction was performed as described by Labarre et al. (15). Proteins were transferred from sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) gels to nitrocellulose membranes (PROTEAN; Schleicher and Schuell) by using a trans-blot semidry transfer cell (Bio-Rad, Richmond, Calif.), and immunoblot analysis was performed with a specific rabbit polyclonal antiserum directed against the 60-kDa MLE protein from *O. oeni* (15).

RNA isolation. *O. oeni* cells cultured at pH 5.3 were harvested by centrifugation for 10 min at 5,000 × *g* and resuspended in 1 ml of Tri reagent (Sigma). Cells were mechanically broken with 200 mg of glass beads (diameter, 70 to 110 μm) by using six 40-s periods of homogenization with 30-s intervals between the periods with a FastPrep cell disintegrator (FP120; Instrument Savant; Bio 101). Samples were then treated as recommended by the manufacturer.

RT-PCR. RNA samples were treated with RNase-free DNase (1 U μl^{-1}) as recommended by the manufacturer (Life Technologies, Gibco BRL). Reverse transcription (RT) was done in a 25-μl mixture containing 4 μl of 5× reverse transcriptase buffer, 2 μl of 0.1 M dithiothreitol, and 4 μl of a preparation containing each deoxynucleoside triphosphate at a concentration of 2.5 mM. The reaction mixture was incubated at 42°C for 2 min. Then 200 U of *Escherichia coli* SuperScript II Rnase H$^-$ reverse transcriptase (Life Technologies, Gibco BRL) was added. Incubation was continued for 50 min at 42°C and then for 15 min at 70°C for enzyme denaturation. Amplification was performed by using a 50-μl (final volume) reaction mixture containing 50 ng of cDNA, 30 pmol of each primer, each deoxynucleoside triphosphate at a concentration of 2 μM, and 2.5 U of *Taq* DNA polymerase. Specific cDNA was amplified for 40 cycles consisting of denaturation for 45 s at 92°C, annealing for 45 s at 55°C, and elongation for 1 min at 72°C. The PCR products were analyzed on a 1.6% agarose gel. The primers used for this experiment were 23- and 24-mer oligonucleotides for the *mleA* gene fragment (*mleA* probes 60440 [5′-ACC AAA ATG GTC GGG TGG ACA GC-3′] and 43406 [5′-GGA AGA TTT TGG CCG TTC GAA TGC-3′]) and 18- and 22-mer oligonucleotides for the *mleR*-like gene fragment (mleR4 [5′-CGA TAA AAC CAA TCC CGG-3′] and Reg1 [5′-GGT TTG GAA ACA ATT GAA ATC G-3′]). The gene encoding the thioredoxin gene *trxA*, used as an internal control, was amplified by using 20- and 21-mer oligonucleotides (Trx1 [5′-TTG CCG AAT TTA ACC CTC GA-3′] and Trx5 [5′-AGG AGG AAT TAT ATG GCA AT-3′]).

Other procedures. Protein concentration was determined by the Bradford method (3) by using a Bio-Rad protein assay kit (Bio-Rad Laboratories, Richmond, Calif.) with bovine serum albumin as the standard. L-Malic acid concentrations were determined enzymatically by using Boehringer Mannheim kits.

RESULTS

Immunoblot analysis of MLE in *O. oeni* ATPase-deficient strains. The absence of malolactic activity in both of the H^+-ATPase-deficient strains of *O. oeni* used (26) led us to first determine the amounts of MLE in the mutant strains. Proteins (10 μg) obtained from disrupted cells grown on FT80m medium were loaded on SDS-PAGE gels. An immunoblot experiment was then performed with specific polyclonal antibodies directed against the MLE of *O. oeni*. The MLE corresponds to a 60-kDa protein (15), and it was detected only in the parental strain (Fig. 1, lane 1). No cross-reaction signals were obtained with protein extracts from the H^+-ATPase-deficient mutants
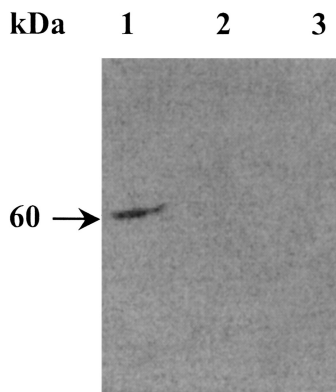
FIG. 1. Immunodetection of MLE by Western blotting. Harvested cells in the mid-exponential growth phase were washed in 10 mM of Tri-HCl buffer (pH 8) and disrupted as described by Labarre et al. (15). Protein extracts (10 μg) were analyzed by SDS-PAGE (10.5% acrylamide) and immunoblotting. Lane 1, total proteins of the wild-type strain; lanes 2 and 3, total proteins of strains 9.01.07 and 6.27.06, respectively. The arrow indicates the position of the MLE.

(Fig. 1, lanes 1 and 3). This result led us to hypothesize that the MLE was not synthesized in the two mutant strains. However, we cannot exclude the possibility that the enzyme was unstable or there was defective enzyme synthesis, which prevented a cross-reaction with the antiserum. To answer these questions, a transcriptional study of *mleA* gene expression was carried out.

Transcriptional analysis of malolactic gene expression. In order to detect *mleA* mRNA, RT-PCR experiments were performed. The RT-PCR experiments were performed with total RNA from exponentially growing cells of strains IOB84.13 and 9.01.07. Probes for *mleA* were used to amplify a 417-bp DNA fragment of the MLE gene (Fig. 2). The thioredoxin gene, *trxA*, was used as a PCR positive control. This gene was detected at a significant level and was estimated to be at roughly the same level during the exponential phase (10). A DNA strand of the expected length (417 bp) was amplified by PCR from the reverse transcriptase product only for the parental strain (Fig. 2,
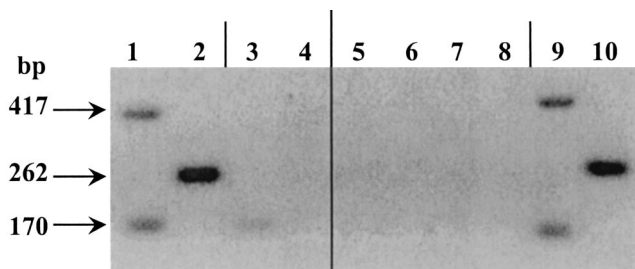

FIG. 2. RT-PCR analysis of malolactic gene expression. One microliter of the product was amplified by PCR. Lanes 1 and 3, amplification of *mleA* and *trxA* genes of strains IOB 84.13 and 9.01.07, respectively; lanes 2 and 4, amplification of the *mleR* gene of strains IOB 84.13 and 9.01.07, respectively. Amplification was also carried out with total DNA of the parental strain as a positive control (lanes 9 and 10). To check that there was no DNA contamination in the RNA sample, total RNA not treated with reverse transcriptase was amplified (lanes 5 and 6, strain IOB 84.13; lanes 7 and 8, strain 9.01.07). The thioredoxin gene *trxA* was used as a PCR positive control.
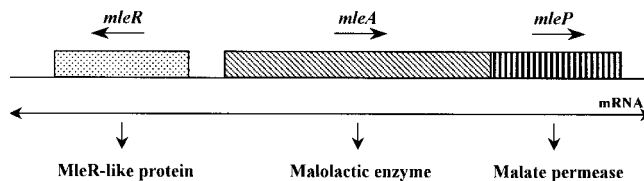

FIG. 3. Genetic organization of the *mle* genes of *O. oeni*. The *mleA* and *mleP* genes encoding the MLE and the malate permease of *O. oeni*, respectively, are transcribed in an operon. Upstream of the *mle* operon, another gene encoding an MleR-like protein is transcribed divergently. This protein is related to the LysR-type regulatory protein family (14).

lane 1). No amplification was observed for mutant strain 9.01.07 (Fig. 2, lane 3). The positive results observed for amplification of a 170-bp DNA fragment of the *trxA* gene (control) from the parental strain and mutant 9.01.07 validated our experiments. A lack of amplification with the *mleA* probes was also observed with strain 6.27.06 (data not shown). These data suggest that either there was no transcription or the *mleA* messenger was more unstable in the two mutants.

An RT-PCR analysis was conducted in order to detect *mleR* mRNA in the parental strain and the H$^+$-ATPase-deficient mutants. The RT-PCR experiment was performed with total RNA from exponentially growing cells. Primers mleR4 and Reg1 were used to amplify the 262-bp DNA fragment of the *mleR*-like gene. A DNA strand of the expected length was amplified only from the parental strain (Fig. 2, lane 2). No amplification was obtained for mutant strain 9.01.07 (Fig. 2, lane 4). The same result was obtained with mutant strain 6.27.06 (data not shown).

According to Labarre et al. (14), the *mleR*-like gene of *O. oeni* is transcribed divergently from the *mle* operon (Fig. 3), and the promoter regions of the *mleR* gene and the *mle* operon certainly overlap. To further investigate the lack of putative *mleR* gene expression in H$^+$-ATPase-deficient mutants, the *mleR*-like gene and the hypothetical region of overlapping promoters were sequenced. No change in the nucleotide sequence of the promoter region and the *mleR*-like gene was observed. Moreover, the complete *mle* operon was sequenced for both mutant strains. No mutation was observed (data not shown). These data led us to conclude that the malolactic deficiency phenotype is not due to a mutation in the malolactic locus in H$^+$-ATPase-deficient mutants.

Isolation of H$^+$-ATPase-deficient mutants of *L. lactis* and *L. mesenteroides*. The effect of a reduction in the H$^+$-ATPase activity on L-malate metabolism in other lactic acid bacteria was also investigated. We used *L. lactis* and *L. mesenteroides* for this analysis. In *L. lactis*, the *mleS* gene encoding the MLE was cloned and sequenced (2, 7), and activation of the malolactic system was shown to be mediated by *mleR* (20). Moreover, mutants of *L. lactis* with reduced membrane-bound ATPase activity were previously obtained by selection for neomycin resistance (1). In contrast, little information is available about genes implicated in malolactic activity in *L. mesenteroides*. However, this bacterium is phylogenetically closely related to *O. oeni* (8), and it is one of the heterofermentative cocci of wines (17). To isolate spontaneous neomycin-resistant mutants of *L. lactis* and *L. mesenteroides*, two sets of cultures

TABLE 1. Growth parameters and membrane-bound ATPase activities for wild-type strains of *L. lactis* and *L. mesenteroides* and neomycin-resistant mutants cultured in MRS medium (pH 6.5)

Strain	Specific growth rate $(h^{-1})^a$	Final biomass concn (g [dry wt] liter^{-1})^b	Membrane-bound ATPase sp act (μmol of P$_1$ min^{-1} mg of protein^{-1})
L. lactis CNRZ 126 (wild type)	0.46	1.04	1.27
Mutant 5.16	0.16	0.36	0.59
L. mesenteroides 19D (wild type)	0.45	1.26	0.33
Mutant 5.15	0.35	0.56	0.10

^{a} The specific growth rates are the averages for two independent cultures.
^{b} Calculated for an initial OD$_{600}$ of 0.06, after 27 h of growth for *L. lactis* and after 38 h of growth for *L. mesenteroides*.

containing 2×10^{10} CFU for each strain were treated with a lethal concentration of neomycin sulfate (300 μg ml^{-1}). A total of 1,682 spontaneous neomycin-resistant mutants were obtained from *L. lactis* with a frequency of 1.9×10^{-7}. A total of 345 mutants were obtained from *L. mesenteroides* with a frequency of 4.5×10^{-6}. Fifty *L. lactis* colonies and 30 *L. mesenteroides* colonies were randomly picked and first analyzed to determine their growth profiles under optimal growth conditions. We selected neomycin-resistant mutants of *L. lactis* and *L. mesenteroides* whose growth profiles were most affected at pH 6.5. Mutant 5.16 of *L. lactis* and mutant 5.15 of *L. mesenteroides* had the lowest final biomass (35 and 44% of the wild-type final biomasses, respectively) (Table 1). The membrane-bound ATPase activities of the mutants were also determined. As shown in Table 1, the membrane-bound ATPase activities of mutant strain 5.16 (*L. lactis*) and mutant strain 5.15 (*L. mesenteroides*) were reduced to 54 and 70%, respectively, of the activities of the parental strains.

Growth of ATPase-deficient mutants of *L. lactis* and *L. mesenteroides* under acidic conditions. The parental strains, the *L. lactis* mutant, and the *L. mesenteroides* mutant were cultured in MRS medium with an initial pH of 5.0 for *L. lactis* and an initial pH of 4.5 for *L. mesenteroides*. Under these conditions, the specific growth rates of the *L. lactis* and *L. mesenteroides* parental strains were 0.16 and 0.12 h^{-1}, respectively, compared with specific growth rates of 0.46 and 0.45 h^{-1}, respectively, at pH 6.5 (Fig. 4). The 5.16 mutant strain of *L. lactis* did not grow at pH 5.0. Mutant strain 5.15 of *L. mesenteroides* had an 85% lower specific growth rate than the parental strain when the organisms were cultured at pH 4.5. A significant decrease (43%) in the final biomass obtained after 38 h of growth was also observed (Fig. 4B).

L-Malate metabolism in H$^+$-ATPase-deficient mutants of *L. lactis* and *L. mesenteroides*. The residual malate was assayed enzymatically after growth on MRS medium under optimal growth conditions or under acidic conditions (Table 2). At pH 6.5, the ATPase-deficient mutants of *L. lactis* and *L. mesenteroides* and the wild-type strains used approximately 90% of the 37 mM L-malic acid initially present in MRS medium. Moreover, the H$^+$-ATPase-deficient mutants exhibited greater consumption of L-malate (expressed as millimoles of L-malate consumed per milligram of final biomass) than the parental

strains exhibited. The *L. lactis* and *L. mesenteroides* mutants consumed three and two times more L-malate than the wild-type strains consumed, respectively.

Similar measurements were obtained after growth under acidic conditions (Table 2). At pH 5.0, the wild-type strain of *L. lactis* metabolized 90% of the L-malate, whereas for the ATPase-deficient mutant, in spite of an absence of significant growth, 50% of the L-malate was still present in MRS medium after 27 h of culture. At pH 4.5, the wild-type strain of *L. mesenteroides* metabolized 90% of the L-malate, compared with 70% for the ATPase-deficient mutant, after 38 h of growth. Compared with the results obtained at pH 6.5, the consumption of L-malate increased four- and fivefold for the *L. lactis* and *L. mesenteroides* wild-type strains, respectively. The same results were obtained with the ATPase-deficient mutant strains. Moreover, for the mutant strains of *L. lactis* and *L. mesenteroides*, consumption of L-malate increased 3.5- and 1.5-fold, respectively, compared with consumption by the wild-type strains when the organisms were cultured under acidic conditions.

These results show that the ATPase-deficient mutants of *L. lactis* and *L. mesenteroides* metabolized L-malic acid, in contrast to the ATPase-deficient mutant of *O. oeni*. Moreover, the consumption of L-malate was greater than that calculated for wild-type strains. In the same way, under acidic growth conditions the L-malate consumption by the parental strains and also by the ATPase-deficient mutants clearly increased.

DISCUSSION

In this study the lack of malolactic activity previously observed with H$^+$-ATPase-deficient mutants of *O. oeni* (26) was analyzed at a molecular level to clarify the link between the malolactic system and the membrane-bound H$^+$-ATPase. The 60-kDa MLE was not detected in H$^+$-ATPase-deficient mutants. We can exclude the possibility that the enzyme was not detected by immunoblotting due to weak synthesis by the mutants. Indeed, significant quantities of total proteins (10 μg) were loaded on the gel, and the polyclonal antibodies against MLE prepared by Labarre et al. (15) are highly specific. Moreover, the malolactic activity of *O. oeni* was previously detected during the log phase of growth with or without L-malate added to the growth medium (15). These findings eliminate the possibility that there was not induction of MLE in ATPase-deficient mutant strains under our experimental conditions. The lack of detection of MLE in mutants was due to either a lack of gene expression or instability of the enzyme. Another possibility is synthesis of a defective MLE that did not cross-react with the antiserum. To examine these hypotheses, a transcriptional study of the malolactic operon was carried out. In a previous report it was shown that a structure in the operon harbors the *mleA* and *mleP* genes, which encode the MLE and the malate permease of *O. oeni*, respectively (13). The *mleA* transcript was not detected by RT-PCR analysis of two H$^+$-ATPase-deficient mutants when they were compared with the *O. oeni* parental strain. This result indicates that the *mle* operon does not seem to be transcribed in ATPase-deficient mutants. However, we cannot exclude the possibility that the malolactic mRNA is unstable, preventing detection by RT-PCR.
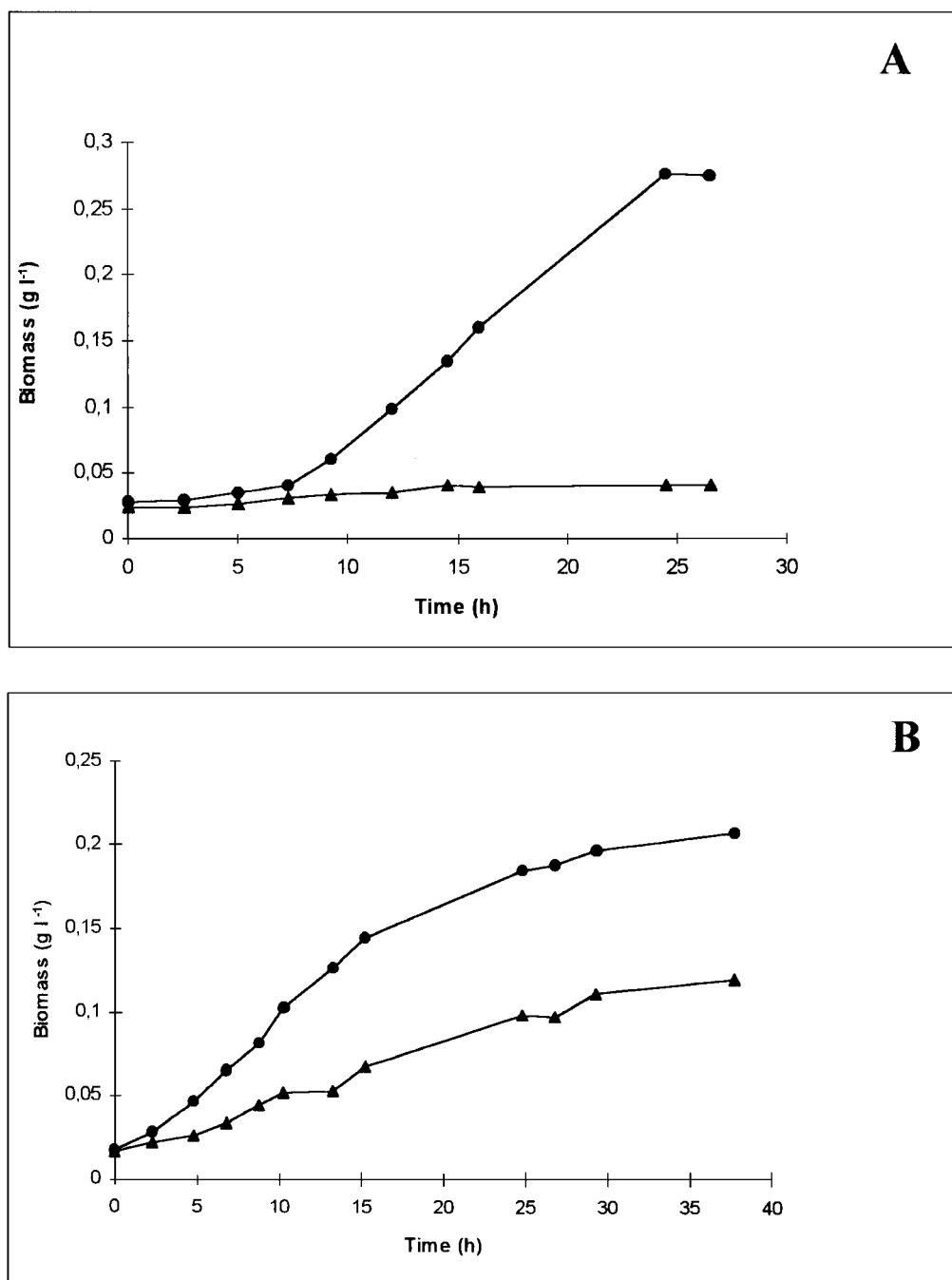
FIG. 4. Growth profiles of H$^+$-ATPase-deficient mutants of *L. lactis* (strain 5.16) and *L. mesenteroides* (strain 5.15) under acidic conditions. MRS medium at pH 5.0 was used for *L. lactis* (A), and MRS medium at pH 4.5 was used for *L. mesenteroides* (B). Symbols: ●, parental strain; ▲, mutant strain.

It has been proven that in *L. lactis* induction of the genes necessary to perform malolactic fermentation occurs only in bacteria with a functional copy of *mleR*. This gene encodes a LysR-type regulatory protein that acts as a positive regulator of the expression of the *mleA* gene (20). The *mleR* gene of *L. lactis* is not clustered with the *mleA* gene. In contrast, in *O. oeni*, an *mleR*-like gene was previously found upstream of the *mle* operon. This gene is transcribed divergently from the

operon (14). The translation initiation sites of *mleR* and *mleA* are very close to each other, and the promoter region of the *mleR* gene and the *mle* operon certainly overlap (14). The role of *mleR* in malolactic gene expression in *O. oeni* remains unknown. Results obtained in this study show that the *mleR*-like transcript was not detected by RT-PCR in H$^+$-ATPase-deficient mutants. Moreover, sequencing data proved that there is no mutation in the *mleR*-like gene and promoter region or in

TABLE 2. Effect of the growth medium pH on the L-malate concentration and L-malate consumption in wild-type strains of *L. lactis* and *L. mesenteroides* and H$^+$-ATPase-deficient mutant strains cultured in MRS medium

Strain	Residual L-malate concn (mM)[a]		L-malate consumption (mol of L-malate g of final biomass^{-1})	
	pH 6.5	Acidic conditions[b]	pH 6.5	Acidic conditions[b]
L. lactis CNRZ 126 (wild type)	2.9	2.6	33	126
Mutant 5.16	3.4	19.2	95	451
L. mesenteroides 19D (wild type)	3.2	5.6	27	153
Mutant 5.15	3.7	10.7	60	221

[a] Concentration after 27 h of growth for *L. lactis* and after 38 h of growth for *L. mesenteroides*.
[b] The initial pH values were 5.0 for *L. lactis* and 4.5 for *L. mesenteroides*. The results are means for two independent experiments.

the *mle* operon in the H$^+$-ATPase-deficient mutants. Taken together, these results are in accordance with positive regulation of *mle* operon expression by the *mleR*-like gene in *O. oeni*, like the situation in *L. lactis*. Further genetics experiments are needed to confirm the role of the *mleR* gene in *mle* operon regulation. Disruption of the *mleR* gene in *O. oeni* would be of great interest and would certainly allow us to understand regulation of malolactic gene expression. Unfortunately, DNA transfer techniques are only at the experimental stage at this time for this type of genetic approach with *O. oeni*. Mainly for this reason we chose to investigate L-malate metabolism in H$^+$-ATPase-deficient mutants in another lactic acid bacterium, *L. lactis*. The MLE of this bacterium and the MLE of *O. oeni* are encoded by similar genes. Spontaneous neomycin-resistant mutants of *L. lactis* with reduced membrane-bound ATPase activity were obtained previously (1, 28). We likewise chose *L. mesenteroides*. This bacterium, in contrast to *L. lactis*, is one of the heterofermentative cocci of wines (17), and it is phylogenetically closely related to *O. oeni*.

First, investigations were carried out to select neomycin-resistant mutants of *L. lactis* and *L. mesenteroides* on the basis of their growth profiles under optimal conditions. Selected mutants were found to be acid sensitive and had ATPase activities that were significantly reduced. Similar results were obtained previously with spontaneous neomycin-resistant mutants of *O. oeni* that were acid sensitive and exhibited twofold-reduced H$^+$-ATPase activity (26). The acid sensitivity of the ATPase-deficient mutants of *L. lactis* and *L. mesenteroides* confirms the suggestion that in lactic acid bacteria, the major role of the H$^+$-ATPase is maintenance of pH$_{in}$ (1, 27).

Previous data indicated that there is a relationship between a reduction in ATPase activity and a lack of malolactic activity in *O. oeni* (26). This study showed that there were unexpected increases in L-malate consumption in H$^+$-ATPase-deficient mutants of *L. lactis* and *L. mesenteroides* under both optimal and acidic growth conditions compared to the metabolism of the parental strains. In contrast to ATPase-deficient mutants of *O. oeni*, activation of L-malate metabolism for pH$_{in}$ homeostasis could be a compensatory consequence of the H$^+$-ATPase deficiency in these mutants.

In conclusion, the relationship between the H$^+$-ATPase deficiency and the lack of expression of the malolactic system seems to be a characteristic of mutants of *O. oeni*. It has been shown previously that expression of the MLE in *O. oeni* is not effectively modified by the presence of L-malate in the medium (14). In contrast, the malolactic activity in *L. lactis*, like that in *L. mesenteroides*, is inducible by L-malate (15). It appears that in *O. oeni* regulation of the expression of the malolactic operon may involve another regulatory factor that is presumably linked to the metabolic energy. Determination of the amount of ATP in relation to the level of transcription of malolactic genes should certainly help us elucidate the link between the ATPase and malolactic activities in *O. oeni*.

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