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Membrane potential-generating Malate (MleP) and Citrate (CitP) Transporters of Lactic Acid Bacteria Are Homologous Proteins

SUBSTRATE SPECIFICITY OF THE 2-HYDROXYCARBOXYLATE TRANSPORTER FAMILY*

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Membrane potential generation via malate/lactate exchange catalyzed by the malate carrier (MleP) of Lactococcus lactis, together with the generation of a pH gradient via decarboxylation of malate to lactate in the cytoplasm, is a typical example of a secondary proton motive force-generating system. The mleP gene was cloned, sequenced, and expressed in a malolactic fermentation-positive strain. Proton pumping is membrane which results in the two components of the pmf, a translocation of protons against the gradient across the cell membrane. The combined activities of electrogenic exchange and proton-consuming decarboxylation result in a pmf that is sufficiently high to drive ATP synthesis via the F,F,ATPase (3). Similar pathways have been described for a number of other substrates (6–8). Recently, a more complex system termed citrolactic fermentation was found in Leuconostoc mesenteroides, another lactic acid bacterium. Similar to the malolactic fermentation pathway a secondary transporter catalyzes the uptake of divergent citrate in exchange for monovalent lactate, but the pathway in the cytosol converting citrate into lactate requires three different enzymes and is coupled to glucose metabolism (9, 10).

The membrane potential-generating secondary transporters involved in malolactic fermentation and citrolactic fermentation, MleP and CitP, respectively, differ from “usual” secondary transporters in two aspects: (i) they translocate net negative charge across the membrane, and (ii) they catalyze efficient heterologous exchange of two structurally related substrates (the precursor and the product). Functionally, MleP and CitP are quite similar because lactate is a substrate of both, and, moreover, it was shown that malate is a substrate of CitP as well (9). The structural gene coding for CitP was cloned from different organisms (11, 12) and shown to be homologous to the Na, dependent citrate carrier CitS of Klebsiella pneumoniae (13). CitS is a usual secondary transporter driven by the pmf and sodium ion motive force (14–16). Recently, CitS was shown to represent a new structural class of secondary transporters with a nine-helix bundle motif (17).

Here, we report the cloning and sequencing of the mleP gene coding for the malate transporter of L. lactis which is involved in malolactic fermentation. In line with the functional similarities of MleP and CitP the gene was found to be homologous to the citP and citS genes. The three proteins are part of a family of secondary transporters in which both metabolic energy-dissipation (CitS) -generating (CitP and MleP) members are found. Essential for MleP and CitP is the ability to transport two differently charged but structurally related molecules,
which suggests a wide substrate specificity. It is shown that a broad range of 2-hydroxycarboxylates can be transported by members of the family. In line with its physiological function, the substrate specificity of CitS is much more restricted than observed for the two precursor/product exchangers.

**EXPERIMENTAL PROCEDURES**

### Bacterial Strains and Growth Conditions

*L. lactis* strains IL1403 and IL1441 are wild type malate-fermenting strains; strains MG1363 and LL108 do not ferment malate. Strain IL1441 is a streptomycin-resistant derivative of IL1403 (18). *L. lactis* strains were grown in closed serum bottles without shaking in M17 broth (Difco) supplemented with 0.5% (w/v) glucose and 10 mM potassium phosphate, pH 7.0, resuspended in the same buffer at an *A*\textsubscript{600} of 500, and subsequently rapidly frozen in liquid nitrogen until use. Right-side-out membrane vesicles were prepared by the osmotic shock lysis procedure essentially as described by Otto et al. (29). The membranes were fused to liposomes or to proteoliposomes containing beef heart cytochrome *c* oxidase (COVs) essentially as described by Driessen et al. (31). Liposomes consisted of a mixture of purified *E. coli* lipids and egg yolk phosphatidylcholine at a ratio of 3:1. *E. coli* lipids were purified by successive washing of an *E. coli* extract (Avanti Polar Lipids) with acetone and diethyl ether, after which the concentration was determined as described by Driessen et al. (31). Cytochrome *c* oxidase isolated from beef heart mitochondria was reconstituted into liposomes by detergent dialysis. Liposomes or COVs were fused with the membrane vesicles of *L. lactis* at various lipid and 1 mg of protein by a single freeze/thaw step (30). The buffer contained 50 mM potassium phosphate, pH 6. In case of fusion with liposomes 5 mM l-malate or citrate was included in the buffer to load the vesicles. The resulting hybrid membranes were made unilamellar by subsequent extrusion through 400- and 200-nm pore size polycarbonate filters (32). Hybrid membranes were concentrated by ultracentrifugation at 250,000 × *g* for 20 min at 10 °C.

### Preparation of Membrane Vesicles and Hybrid Membranes

Cells of *L. lactis* MG1363 or LL108 expressing either MLeP or CitP were harvested at the end of the exponential growth phase at an *A*\textsubscript{600} of 0.8, washed with 50 mM potassium phosphate, pH 7.0, resuspended in the same buffer at an *A*\textsubscript{600} of 500, and subsequently rapidly frozen in liquid nitrogen until use. Right-side-out membrane vesicles were prepared by the osmotic shock lysis procedure essentially as described by Otto et al. (29). The membranes were fused to liposomes or to proteoliposomes containing beef heart cytochrome *c* oxidase (COVs) essentially as described by Driessen et al. (31).

**Cloning and Sequencing of the mleP Gene**

Plasmid p153A has been described before (25) and was shown to contain *mleS* encoding malolactic enzyme and the 5' part of a second open reading frame ORF2, presumably *mleP* encoding the malate transporter. A 2.5-kb fragment obtained by BamHI digestion of chromosomal DNA isolated from *L. lactis* IL1414 overlapped with the insert in p153A. The fragment was cloned into the unique BamHI restriction site of pMB which have compatible overhangs. The resulting plasmids were constructed using exonuclease III (Pharmacia and LKB). Truncated fragments were sequenced using vector-specific primers. The sequence of the mleP fragment was used to probe a 1,068 base pairs of ORF2. To clone the missing 3' end of each strand. The insert contained the first 0.8-kb fragment of ORF2 on pPNJ was used to probe a HindIII restriction site of *L. lactis* genomic DNA. The sequence of a 2.5-kb fragment that hybridized with the probe was determined using inverse polymerase chain reaction (PCR). Genomic DNA of *L. lactis* IL1441 digested with HindIII was ligated with T4 DNA ligase under conditions that favored the formation of monomeric circles. The circular fragments were used as a template for a PCR using oligonucleotides based on the sequence of the EcoRI/HindIII fragment of p191A. The primer sequences were 5'-TGCCAGG-TATTGGCTCTG-3' and 5'-CCATAAATCTGACATC-3'. A 2-kb fragment presenting a head to tail structure was amplified and cloned into pGEM-T (Invitrogen) to give vector pPNJ. The missing 0.4-kb fragment of ORF2 on pPNJ was sequenced on both strands. ORF2 was amplified from genomic DNA using a forward primer based on the sequence of p153A (5'-ATCTCAGGATATAAGAAATTAAAAG-3') and a reverse primer based on the sequence of pPNJ (5'-TGAGGGCATATTGAGT-3') and ligated into pGEM-T, yielding pPNJ. The sequence of ORF2 reconstituted from all of the fragments was confirmed by sequencing one strand of the pPNJ insert.

### Construction of Expression Vectors

An expression vector was constructed containing a promoter region that is located in front of the *citP* gene on the 7.9-kb plasmid of *L. lactis* NCO176 (27). The 1,572-base pair region was amplified by the plasmid by PCR. The forward primer, 5'-GCTTGAATTCCTTGCTAT-3', introduced an EcoRI restriction site in the fragment. The reverse primer, 5'-GATAGGCGATAT-3', overlapped with the *citP* start codon and introduced a unique *A*\textsubscript{III} site (CATGTT) around the start codon and a BamHI site at the end. Bases introducing mutations are underlined, and the start codon is indicated in bold. The PCR product was digested with EcoRI and BamHI and ligated into plasmid pGK13 (28) digested with the same enzymes. The resulting plasmid pGME contains the *L. lactis* *citP* promoter region followed by a ribosom binding site and an *A*\textsubscript{III} restriction site around the start codon. Downstream of the *A*\textsubscript{III} re- site a number of unique restriction sites are present for cloning purposes.

The gene coding for the malate transporter *mleP* was amplified by PCR using p191A chromosomal DNA isolated from *L. lactis* IL1441. The forward primer, 5'-TCCTTACATGAAGAAATTAAAAGAAACGTTATTAGG-3', generated an *NcoI* site around the start codon, and the reverse primer, 5'-GCTTGGATTCCCGATATCGGATTACCTTGCAC-3', generated an *XbaI* site downstream of the stop codon. Start and stop codons are indicated in bold. Similarly, the gene coding for the citrate transporter *citP* was amplified from an endogenous plasmid by PCR using p191A chromosomal DNA isolated from *L. lactis* IL1441. The forward primer was 5'-GATTAGAACCATTGATATCGCGCAGATTCCAC-3' and the reverse primer 5'-CTTAAAAATTGATATCGGATTACCTTGCAC-3'. The amplified fragments were digested with *NcoI* and *XbaI* and ligated into the *A*\textsubscript{III} and *XbaI* sites of *pMB* which have compatible overhangs. Truncated fragments were sequenced using vector-specific primers pMBpMleP and pMBpCitP code for *mleP* and *citP* under control of the *citP* promoter. In case of MLeP, the cloning procedure resulted in a Gly insertion after position 1, and the COOH-terminal Tyr residue was replaced by Val-Tyr-Ala. In *CitP* Met2 was replaced by Val. The sequence of the inserts was confirmed by automated sequencing.

### Transport Assays

**PmnD-driven Uptake in Hybrid Membranes**—The experiments were performed in 50 mM potassium phosphate, pH 6.0, under a flow of water saturated air and continuous stirring at 30 °C. Membrane vesicles fused with COVs were incubated for 1 min in the presence of 200 μM TMDP, 20 μM cytochrome *c* (horse heart, Sigma), and 10 mM potassium ascorbate. The assay volume was 100 μl and the membrane protein concentration 6.0–8.0 mg/ml. Valinomycin and nigericin were used at concentrations of 1 μM and 0.5 μM, respectively. (1,4,2,3,5)C]malate or...
[1,5-14C]citrate was added at a concentration of 13.1 or 4.4 μM, respectively. Uptake was stopped at different time intervals by adding 2 ml of ice-cold 0.1 M LiCl to a sample and rapid filtration over 0.45-μm pore size cellulose nitrate filters (Schleicher & Schuell). Filters were rinsed once with 2 ml of ice-cold 0.1 M LiCl and transferred to scintillation vials, and the internalized radioactivity was determined.

Exchange in Membrane Vesicles—Membrane vesicles of L. lactis LL108 fused to liposomes and E. coli right-side-out membrane vesicles preloaded with 5 mM L-malate or citrate were concentrated by centrifugation. Strain LL108 was used for this assay because of the higher expression of MleP and CitP in this strain. Concentrated hybrid membranes were incubated in 50 mM potassium phosphate, pH 6, with 50 μM nigericin with 72.5 μM [1,5-14C]citrate or 186.7 μM L[1,4(2,3)14C]malate for 30 min at room temperature. When indicated, 100 μM valinomycin or 100 μM KSCN was present in the assay mixture. Similarly, the concentrated E. coli membranes were incubated in 50 mM potassium phosphate, pH 6, in the presence of 100 μM valinomycin and 50 μM nigericin with 217.5 μM [1,5-14C]citrate and the appropriate concentrations of NaCl and KCl for 2 h. Aliquots of 2 μl were diluted 100-fold into buffer of 20 C containing various substrates at a concentration of 5 mM. The buffer contained 100 mM KCl in the case the membranes where preloaded with KSCN. Final membrane protein concentrations in the assays were 0.1–0.15 mg/ml and 0.23 mg/ml for L. lactis hybrid membranes and E. coli membranes, respectively. Samples were stopped and processed as described above. The data were fitted to an exponential decay. Within one set of experiments, the zero time point was determined from the curve representing efflux which is slow enough to allow a linear back extrapolation. The infinite time point was estimated from curves representing rapid exchange, usually homologous exchange.

Chemicals

[1,5-14C]Citrate (115 mCi/mmol) and L-[1,4(2,3)14C]malate (51 mCi/mmol) were obtained from Amersham International (Buckinghamshire, U.K.). Oligonucleotides were obtained from Eurosequence (Groningen, The Netherlands) and from Eurogentec (Seraing, Belgium). All other compounds were obtained from commercial sources.

RESULTS

Cloning and Sequencing of mleP—In a previous paper the cloning and sequencing were reported for the gene mleS encoding malolactic enzyme, the decarboxylase in the malolactic

FIG. 1. Alignment of the primary sequences of MleP, CitP, and CitS. Identical residues are printed in gray boxes. Residues conserved in all three transporters are indicated by an asterisk. Putative transmembrane segments (17) are indicated by bars.
fermentation pathway of *L. lactis* (25). One of the clones contained the 5E end of a second ORF starting 15 base pairs downstream of the stop codon of *mleS*. It was suggested that this ORF would be *mleP* coding for the malate transporter, the second protein of the pathway. The complete second reading frame, 1,278 base pairs in length, was cloned as described under “Experimental Procedures,” and the nucleotide sequence was determined. A putative Shine-Dalgarno sequence AAGG is found 16 nucleotides upstream of the start codon (ATG). The stop codon (TAA) is followed closely by an inverted repeat that has features typical of a putative rho-independent transcription termination signal (35). This organization suggests that the two genes are organized in an operon. To verify this, total RNA was isolated from *L. lactis* IL1441 grown in medium on glucose with and without additional malate. Malolactic enzyme coded by *mleS* is an inducible enzyme (18). The results demonstrated that in malate-grown cells a single transcript of approximately 3 kb hybridized with a probe specific for *mleP* and with plasmid p191A containing *mleS* and a 5E fragment of *mleP*. No band was detected when malate was omitted from the growth medium (not shown). The *mleP* sequence is available under accession number X75982.

Analysis of the deduced MleP amino acid sequence reveals a hydrophobic protein of 425 amino acid residues with a predicted mass of 46.7 kDa. Screening of the available data bases showed that the protein revealed homology to the citrate transporters of lactic acid bacteria (CitPs) and to the NaE-dependent citrate carriers of *K. pneumoniae* (CitS), *Salmonella pullorum* (CitC), and *Salmonella dublin* (CitC). Like the lactococcal CitPs, the NaE-dependent transporters form a group of proteins with almost identical primary sequences (>95%). The alignment of MleP with representative sequences from these two groups is shown in Fig. 1. MleP is most similar to CitP with 48% identical residues and shares 30% sequence identity with CitS. Overall, the alignment shows 86 (19%) conserved residues with an additional 85 similar residues. A glycine-rich region around residue 175 in MleP and approximately the COOH-terminal 60 residues are the most conserved regions in the family. Fig. 2 shows the hydrophy profiles of the individual members (thin lines) and the average profile of the family (bold). The profiles are remarkably similar, indicative of the same global structure.

**Functional Expression of MleP**—To determine whether the cloned gene identified as *mleP* is the malate transport protein involved in malolactic fermentation, the gene was expressed in *L. lactis* MG1363 and LL108, strains not able to ferment malate. Cytoplasmic membranes with a rightside-out orientation prepared from *L. lactis* MG1363 harboring pMB*mleP* (see “Experimental Procedures”) were fused to proteoliposomes reconstituted with purified beef heart cytochrome *c* oxidase (COVs) as a pmf-generating system. In these hybrid membranes a pmf (inside negative and alkaline relative to the outside) is generated in the presence of the electron donor system potassium ascorbate, TMPD, and cytochrome *c*. In the presence of a pmf the hybrid membranes took up a low but significant amount of [*14C*]malate (Fig. 3A, C). Control experiments with hybrid membranes prepared from membrane vesicles of strain MG1363 without pMB*mleP* showed no uptake under identical conditions (not shown). Therefore, the product of the *mleP* gene is a malate transporter. The pmf generated by cytochrome *c* oxidation is composed of a membrane potential (Δψ) and a pH gradient (ΔpH). The role of each component of the pmf in driving [*14C*]malate uptake was investigated by manipulating ΔpH and Δψ with the ionophores nigericin, a KE/H*+* antiporter, and valinomycin, a KE pore. In the presence of nigericin, when the pmf consists solely of a membrane potential, no uptake was observed, indicating that the membrane potential is not a driving force for malate transport (>). On the other hand, in the presence of valinomycin, when the pmf is composed solely of a pH gradient, a strong stimulation of malate uptake was observed, indicating that the membrane potential counteracts malate transport (>) and that net negative charge is translocated across the membrane during turnover.

A second important feature of the malate carrier involved in malolactic fermentation is the physiological mode of transport, i.e. heterologous malate/lactate exchange (3). Rightside-out membrane vesicles of *L. lactis* LL108 harboring pMB*mleP* were fused with liposomes and preloaded with 5 mM [*14C*]malate. 100-fold dilution of the membranes in buffer did not result in significant release of label within the first 40 s, indicating that efflux of malate down a concentration gradient is a slow process.
Fig. 4. Effect of the sodium ion concentration on homologous exchange and efflux catalyzed by CitS. RSO membrane vesicles of E. coli BL21(DE3) expressing CitS were preloaded with 5 mM [14C]citrate and with no further additions (panel A), with 1 mM NaCl (panel B), and with 75 mM NaCl (panel C). The Cl− concentration was kept constant by adding compensating amounts of KCl. The membranes were diluted 100-fold into buffer containing the same NaCl and KCl concentrations, in the presence (●) or the absence (○) of 5 mM citrate. Valinomycin and nigericin were present at 1 and 0.5 μM, respectively.

In the assay it is essential that efflux is much slower than exchange. This condition is a property of precursor/product exchangers (2) and has also been demonstrated for CitP (9). In case of CitS, conditions of rapid exchange/slow efflux were sought by varying the concentration of the symported Na+ ion (Fig. 4). With no additional Na+ added, efflux and exchange in rightside-out membrane vesicles of E. coli BL21(DE3) harboring plasmid pSK1citS (17) were observed at comparable rates. Since no effort was made to work “sodium free” the observed activities are most likely caused by sodium ion contaminations in the buffer (15). Addition of 1 mM Na+ increased both the rate of efflux and exchange. However, at 75 mM added Na+ the rate of exchange increased further, but the rate of efflux decreased. This behavior is typical for a solute/co-ion symporter (36). The latter condition was used for the substrate specificity assay.

From previous studies it was known that MleP transports both malate and lactate, CitP transports citrate, lactate, and malate (9), whereas for CitS no substrate other than citrate has been reported. These substrates all share the 2-hydroxycarboxylate motive, R1R2COHCOO−. The effect of the R1 and R2 groups, the hydroxyl group, and the carboxylic group on the ability of the three transporters to translocate the substrates was investigated subsequently.

The R Substituents—Nine different 2-hydroxycarboxylates with R substituents which differ both in size and polarity were used in these experiments. Preloading of the membranes with the membrane permeable ion SCN− results in the generation of a diffusion potential, negative outside, upon dilution when valinomycin is omitted from the assay mixture. The diffusion potential significantly inhibited malate/lactate exchange whereas for CitS no substrate other than citrate has been reported. These substrates all share the 2-hydroxycarboxylate motive, R1R2COHCOO−. The effect of the R1 and R2 groups, the hydroxyl group, and the carboxylic group on the ability of the three transporters to translocate the substrates was investigated subsequently.

Table I

<table>
<thead>
<tr>
<th>Substrate†</th>
<th>R1</th>
<th>R2</th>
<th>Relative rate of exchange‡</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>MleP</td>
</tr>
<tr>
<td>Isocitrate</td>
<td>CHCOO−</td>
<td>CH2COO−</td>
<td>0.4 ± 0.2</td>
</tr>
<tr>
<td>Citrate</td>
<td>CH2COO−</td>
<td>CH2COO−</td>
<td>0.5 ± 0.3</td>
</tr>
<tr>
<td>Citromalate</td>
<td>CH2COO−</td>
<td>CH2</td>
<td>6.9 ± 1.0</td>
</tr>
<tr>
<td>Tartarate</td>
<td>HCHOHCOO−</td>
<td>H</td>
<td>18 ± 1</td>
</tr>
<tr>
<td>Malate</td>
<td>CH2COO−</td>
<td>H</td>
<td>100 ± 8</td>
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<tr>
<td>2-Hydroxybutyrate</td>
<td>CH2CH3</td>
<td>H</td>
<td>15 ± 3</td>
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<tr>
<td>2-Hydroxyisobutyrate</td>
<td>CH3</td>
<td>H</td>
<td>16 ± 3</td>
</tr>
<tr>
<td>Lactate</td>
<td>CH3</td>
<td>H</td>
<td>91 ± 14</td>
</tr>
<tr>
<td>Glycolate</td>
<td>H</td>
<td>H</td>
<td>26 ± 8</td>
</tr>
<tr>
<td>None</td>
<td></td>
<td></td>
<td>0.5 ± 0.3</td>
</tr>
</tbody>
</table>

† Substrates were added at a concentration of 5 mM. Equal mixtures of the L- and D-isomers were used. ‡ Rates are relative to the rate observed for homologous exchange, which was set at 100%. These rates varied per vesicle preparation between 2.7 and 4.8, 0.7 and 0.8, and 2.3 and 2.6 μM/s for MleP, CitP, and CitS, respectively. Rates are given as the average of two to four independent measurement and the S.D.

(Fig. 3B). In contrast, dilution of the preloaded membranes in buffer containing an equimolar concentration of unlabeled malate resulted in rapid release of internal labeled malate, indicative of rapid homologous exchange (●). Most importantly, the same rapid release of label was observed upon dilution into buffer containing an equimolar concentration of lactate (○). To prevent the formation of ΔpH or Δψ which would counteract efflux, the ionophores valinomycin and nigericin were included in these experiments. Preloading of the membranes with the membrane permeable ion SCN− results in the generation of a diffusion potential, negative outside, upon dilution when valinomycin is omitted from the assay mixture. The diffusion potential significantly inhibited malate/lactate exchange whereas malate/malate exchange was not affected (Fig. 3C) showing that heterologous exchange is electrogenic. The results are consistent with those found for malate transport in membrane vesicles of the wild type malate-fermenting L. lactis IL1403 (3) showing that the mleP gene product is the malate transporter involved in malolactic fermentation.

Substrate Specificity of MleP, CitP, and CitS—The heterologous exchange assay demonstrated in Fig. 3B provides a sensitive and unambiguous assay for the substrate specificity of a transporter in general and was used to determine the substrate specificity of MleP and the two other representatives of the family, CitP of L. mesenteroides and CitS of K. pneumoniae. To use the assay it is essential that efflux is much slower than exchange. This condition is a property of precursor/product exchangers (2) and has also been demonstrated for CitP (9). In case of CitS, conditions of rapid exchange/slow efflux were sought by varying the concentration of the symported Na+ ion.
2-Hydroxycarboxylate Transporters

**Table II**

<table>
<thead>
<tr>
<th>Substrate</th>
<th>OH substituent</th>
<th>Relative rate of exchange</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>MleP</td>
<td>CitP</td>
</tr>
<tr>
<td>Citrate</td>
<td>0.5 ± 0.3</td>
<td>100 ± 8</td>
</tr>
<tr>
<td>Tricarbamate</td>
<td>1.0 ± 0.2</td>
<td>2.2 ± 0.7</td>
</tr>
<tr>
<td>Malate</td>
<td>100 ± 8</td>
<td>124 ± 6</td>
</tr>
<tr>
<td>Succinate</td>
<td>0.4 ± 0.1</td>
<td>0.5 ± 0.3</td>
</tr>
<tr>
<td>Oxaloacetate</td>
<td>27 ± 4</td>
<td>14 ± 1</td>
</tr>
<tr>
<td>Aspartate</td>
<td>0.7 ± 0.3</td>
<td>1.3 ± 0.3</td>
</tr>
<tr>
<td>Lactate</td>
<td>91 ± 14</td>
<td>36 ± 1</td>
</tr>
<tr>
<td>Propionate</td>
<td>0.5 ± 0.1</td>
<td>0.7 ± 0.3</td>
</tr>
<tr>
<td>Pyruvate</td>
<td>1.2 ± 0.3</td>
<td>4.2 ± 0.7</td>
</tr>
<tr>
<td>Alanine</td>
<td>NH$_3$</td>
<td>0.6 ± 0.2</td>
</tr>
<tr>
<td>Glyoxylate</td>
<td>26 ± 8</td>
<td>3.3 ± 0.5</td>
</tr>
<tr>
<td>Acetate</td>
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<td>0.3 ± 0.2</td>
</tr>
<tr>
<td>Glyoxylate</td>
<td>13 ± 2</td>
<td>2.2 ± 0.2</td>
</tr>
<tr>
<td>Glycine</td>
<td>NH$_3$</td>
<td>0.7 ± 0.3</td>
</tr>
<tr>
<td>2-Hydroxybutyrate</td>
<td>15 ± 3</td>
<td>54 ± 1</td>
</tr>
<tr>
<td>3-Hydroxybutyrate</td>
<td>0.5 ± 0.5</td>
<td>3.9 ± 0.2</td>
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**Table III**

<table>
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<tr>
<th>Substrate</th>
<th>COO$^-$ substituent</th>
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</thead>
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<td>MleP</td>
<td>CitP</td>
</tr>
<tr>
<td>Lactate</td>
<td>91 ± 14</td>
<td>36 ± 1</td>
</tr>
<tr>
<td>Methyl-L-lactate</td>
<td>COOCH$_3$</td>
<td>1.0 ± 0.1</td>
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<tr>
<td>1,2-Propanediol</td>
<td>CH$_2$OH</td>
<td>0.4 ± 0.3</td>
</tr>
<tr>
<td>Glycerinaldehyde</td>
<td>CHO</td>
<td>0.9 ± 0.5</td>
</tr>
<tr>
<td>None</td>
<td>0.5 ± 0.3</td>
<td>1.0 ± 0.5</td>
</tr>
</tbody>
</table>

**DISCUSSION**

Secondary pmf-generating pathways were discovered only in the last decade. Oxalate decarboxylation in *Oxalobacter formigenes* and malolactic fermentation in *L. lactis* were the first systems described in detail (3, 6). Both pathways consist of only two enzymes, a secondary transporter and a cytoplasmic decarboxylase. The secondary transporters that take up the substrate in exchange for the decarboxylation product (precursor/product exchange) and, thereby, generate the membrane potential play a central role in the pathways. Recently, the structural gene coding for the oxalate/formate exchanger OxlT of *O. formigenes* was cloned and sequenced (37). Here, we report the cloning and sequencing of MleP, the malate/lactate exchanger of *L. lactis*. The cloned gene was expressed in the malolactic fermentation-negative *L. lactis* strains MG1363 and LL108. Functional characterization in membrane vesicles derived from these cells showed that the gene product conferred the same transport characteristics as observed before in membrane vesicles of the malate-fermenting wild type strain *IL1403* (3). The cloned transporter catalyzed efficient heterologous malate/lactate exchange, and unidirectional uptake into the membranes was counteracted by the membrane potential.

The *mleP* gene of *L. lactis* is not homologous to the *oxlT* gene of *O. formigenes*, indicating that genes coding for membrane potential-generating secondary transporters do not form a separate gene family. *MleP* was found to be homologous to the membrane potential-generating citrate transporter *CitP* of lactic acid bacteria and the Na$^+$-dependent citrate transporters *CitS* (13) and *CitC* (38) of *K. pneumoniae* and *Salmonella* species. The homology to *CitS* and *CitC*, which are metabolic energy-dissipating transporters, suggests that *MleP* and *CitP*, and membrane potential-generating secondary transporters in general, are conventional secondary transporters. The membrane topology of *CitS* was recently reported to be quite different from the transmembrane 12-helix motif usually observed for secondary transporters (17). *CitS* traverses the membrane 9 times (the bars in Fig. 1) with a cytoplasmic amino terminus and a periplasmic carboxyl terminus. The sequence homology and the highly conserved hydropathy profile of the members in the family strongly suggest that *MleP* and *CitP* fold in a similar fashion in the membrane. In this structural model the two most conserved regions in the alignment shown in Fig. 1 are located in the periplasmic loop between helices V and VI and the cytoplasmic loop preceding the COOH-terminal helix IX. The alignment shows 6 conserved positively charged amino acid residues of which only Arg-407 (MleP numbering) is predicted to be located in the membrane, in putative helix IX. Since *MleP*, *CitP*, and *CitS* transport negatively charged substrates this Arg residue could play a role in substrate binding and/or transport.

Previous studies had shown that *MleP*, *CitP*, and *CitS* transport one or more of the structurally related substrates citrate, malate, and lactate (3, 9, 14), and it was noted that these substrates all contain the motif R$_1$R$_2$COHCOO$^-$ (9). In the present study the importance of the hydroxyl and carboxylate groups of the substrates was investigated. None of a limited number of lactate analogs in which the carboxylate group was...
methylated or replaced by an hydroxyl or aldehyde group could be translocated by any of the transporters. This suggests that the carboxylate and possibly the negative charge of this group are essential. A larger number of analogs showed that replacement of the hydroxyl group by a hydrogen atom or an amino group completely abolished transport activity (Table II). However, by replacing a keto group resulted to some extent in activity with MleP and CitP, especially with oxaloacetate. Moving the hydroxyl to the C3 position resulted in a low transport activity by CitP. Possibly, hydrogen bonding between a residue on the transporter and the hydroxyl or keto group on the substrates is essential for translocation. The transport activity of CitP with oxaloacetate is remarkable since oxaloacetate is the first metabolic intermediate in the citrate degradation pathway in lactic acid bacteria (39). Since the 2-hydroxycarboxylates are the physiological and preferred substrates of these carriers we have termed the family the 2-hydroxycarboxylate transporter family.

A typical feature of membrane potential-generating secondary transporters like MleP and CitP is the ability to translocate two structurally related substrates, i.e. malate/lactate and citrate/lactate, respectively. The transporters specifically recognize the common 2-hydroxycarboxylate motif. At the same time, MleP and CitP need to be quite tolerant toward the R1 and R2 groups since the cytoplasmic conversion of the substrate into the product not only results in a smaller molecule but also removes the charge on one of the R groups. Charge removal is crucial and results in the generation of the membrane potential. A similar tolerance toward the R groups is not a physiological requirement for the Na+–dependent citrate transporter CitS that functions as a Na+/H+ symporter (14–16). Consistent with this observation, the results in Table I show that CitS has a very narrow substrate specificity (mainly citrate), whereas MleP and CitP transport a wide range of 2-hydroxycarboxylates with different R substituents. Remarkably, the R groups of the transported substrates range in size from the smallest possible, i.e., hydrogen atoms in glycolate, to the R groups of the physiological substrates, i.e., malate for MleP and citrate for CitP. Larger R groups as in citromalate for MleP and isocitrate for CitS are compensated for by the conformation of the protein or a varying amount of cotransported water (40, 41). In the case of CitS smaller R groups reduce transport activity, but for many helpful suggestions.

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REFERENCES