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

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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-containing vesicle. Functional analysis revealed the same properties as observed in membrane vesicles of a malolactic fermentation-positive strain. MleP belongs to a family of secondary transporters in which the citrate carriers from Leuconostoc mesenteroides (CitP) and Klebsiella pneumoniae (CitS) are found. Both CitP and CitS is also involved in membrane potential generation via electrogenic citrate/lactate exchange. MleP, CitP, and CitS were analyzed for their substrate specificity. The 2-hydroxycarboxylate motif R1R2COHCOOH, common to the physiological substrates, was found to be essential for transport although some 2-oxocarboxylates could be transported to a lesser extent. Clear differences in substrate specificity among the transporters were observed because of different tolerances toward the R substituents at the C2 atom. Both MleP and CitP transport a broad range of 2-hydroxycarboxylates with R substituents ranging in size from two hydrogen atoms (glycolate) to acetyl and methyl groups (citrate/malate) for MleP and two acetyl groups (citrate) or CitP. CitS was much less tolerant and transported only citrate and at a low rate citromalate. The substrate specificities are discussed in the context of the physiological function of the transporters.

The electrochemical gradient of protons across the cytoplasmic membrane is a major store of free energy in the bacterial cell. Usually, the proton motive force (pmf) is generated by translocation of protons against the gradient across the cell membrane which results in the two components of the pmf, a membrane potential and a pH gradient. Proton pumping is catalyzed by primary transport systems at the expense of some source of chemical energy or light. In certain anaerobes a different mechanism of pmf generation has evolved which involves the action of secondary transporters rather than primary ion pumps and, therefore, is termed secondary pmf generation (for reviews, see Refs. 1 and 2). An example of such a system is the malolactic fermentation pathway found in several lactic acid bacteria (3–5). In Lactococcus lactis the uptake of divergent malate is coupled to the exit of its decarboxylation product, monovalent lactate (precursor/product exchange), which leads to the formation of a membrane potential of physiological polarity. Furthermore, the intracellular decarboxylation of malate catalyzed by malolactic enzyme consumes a cytosolic proton which results in a pH gradient over 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 F0F1-ATPase (3). Similar pathways have been described for a number of other substances (6–8). Recently, another 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 glucone 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-dissipating (CitS) and -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-hydroxyxycarboxylates 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 LL1441 is a streptomycin-resistant derivative of *L. lactis* IL1403 (18). Strain LL108, kindly provided by R. Leenhouts, is a chloramphenicol-resistant derivative of MG1363 with multiple copies of the repA gene inserted in its chromosome which results in an increase in plasmid copy number (19). *L. lactis* NCDO176 is a wild type citrate-fermenting strain obtained from the Dutch Institute of Dairy Research (Ede, The Netherlands). The *L. lactis* strains were grown in closed serum bottles without shaking in M17 broth (Difco) supplemented with 0.5% (w/v) glucose and at 30 °C. Concentrations of 5 μg/ml erythromycin and 5 μg/ml chloramphenicol were used when indicated. *L. mesenteroides* ssp. *mesenteroides* 19D was grown at 30 °C in MRS medium without acetate and Tween and with 0.5% ammonium citrate (20). *Escherichia coli* strains DH5α, BL21(DE3), and MC1061 were grown aerobically at 37 °C in Luria broth supplemented with 100 μg/ml carbenicillin or 10 μg/ml chloramphenicol when indicated.

**Recombinant DNA Techniques**

Standard DNA manipulation techniques were carried out essentially as described by Sambrook et al. (21). *L. lactis* genomic DNA was isolated as described by Simon et al. (22). *L. lactis* and *L. mesenteroides* plasmid DNA was isolated as described by Leenhouts et al. (23). *L. lactis* was transformed by electroporation as described by Holo and Nes (24). For sequencing, plasmids were transformed to and propagated in *E. coli* DH5α. Nucleotide sequences were determined on a Vistra 725 or Applied Biosystems 373A automated sequencer.

**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-kb fragment obtained by *BamHI* digestion of chromosomal DNA isolated from *L. lactis* IL1441 overlapped with the insert in p153A. The fragment was cloned into the unique *BamHI* restriction site of plasmid pUC18 (26) yielding p191A. Unidirectional sets of nested deletions of p191A were constructed using exonuclease III (Pharmacia Biotech Inc.). Truncated fragments were sequenced using vector-specific primers after which internal primers were designed to sequence the ORF2 reconstructed from all of the fragments was confirmed by sequencing one strand of the pPME 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* NCDO176 (27). The 1,572-base pair region was amplified by the plasmid by PCR. The forward primer, 5′-GCTTAGATCTCCTGCATT-CAGTATGTC-3′, introduced an EcoRI restriction site in the fragment. The reverse primer, 5′-GCGGATCCTACATTTCTATCTCATCTT-3′, overlapped with the *citP* start codon and introduced a unique *Ai III* site (ACATGG) 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 pMB contains the *L. lactis* *citP* promoter region followed by a ribosomal binding site and an *Ai III* restriction site around the start codon. Downstream of the *Ai III* restriction site a number of unique restriction sites are present for cloning purposes.

The gene coding for the malate transporter *mleP* was amplified by *PCRM* using chromosomal DNA isolated from *L. lactis* IL1441 and the primers (22).

**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* 660 of 0.8, washed with 50 mM potassium phosphate, pH 7.0, resuspended in the same buffer at an *A* 660 of 500, and subsequently rapidly frozen in liquid nitrogen until use. Rightside-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) as described by Driessen et al. (30). Liposomes consisted of a mixture of purified *E. coli* lipids and egg yolk phosphatidylycholine 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 a lipid to protein ratio of 1:1. The liposomes 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.

Right-side-out membrane vesicles of *E. coli* strain BL21(DE3) expressing CitS from pSKLA101 (17) were prepared by the osmotic lysis procedure essentially as described by Kaback (33) with the following modification. Spheroplasts were lysed in 50 mM potassium phosphate, pH 7, containing 5 mM potassium citrate to load the vesicles with citrate. All subsequent steps were done in the presence of 5 mM potassium citrate. Before use the membrane vesicles were washed once in 15 volumes of potassium phosphate, pH 6, containing 5 mM citrate and appropriate concentrations NaCl and KCl and concentrated by centrifugation for 20 min in an Eppendorf tabletop centrifuge operated at full speed. Protein concentrations were determined as described by Lowry et al. (34).

**Transport Assays**

Pm-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 TMPD, 20 μM cytochrome *c* (horse heart, Sigma), and 10 mM potassium ascorbate. The assay volume was 100 μl and the membrane protein concentration 0.6–0.8 mg/ml. Valinomycin and nigericin were used at concentrations of 1 μM and 0.5 μM, respectively. -L1(1,4,2,3,5)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.](image-url)
fermentation pathway of L. lactis (25). One of the clones contained the 5’ 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 5’ 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 (CitP) and to the Na+-dependent citrate carriers of K. pneumoniae (CitC), Salmonella pullorum (CitC), and Salmonella dublin (CitC). Like the lactococcal CitPs, the Na+-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 hydropathy 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 pMBmleP (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 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). Control experiments with hybrid membranes prepared from membrane vesicles of strain MG1363 without pMBmleP 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 (\(\Delta \psi\)) and a pH gradient (\(\Delta p\)). The role of each component of the pmf in driving [14C]malate uptake was investigated by manipulating \(\Delta p\) and \(\Delta \psi\) with the ionophores nigericin, a K+/H+ antiporter, and valinomycin, a K+ 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 pMBmleP were fused with liposomes and preloaded with 5 mM L-[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. 2.** Hydropathy profile of MleP, CitP, and CitS. The individual profiles (thin) and average profile (bold) were calculated with a window of 21 residues and the normalized hydrophobicity scale of Kyte and Doolittle (42). Putative transmembrane segments are indicated by bars (17).

**Fig. 3.** Malate uptake in the presence of a pmf (panel A) and an exchange (panels B and C) catalyzed by MleP. Panel A, malate (13.1 \(\mu\)M) uptake by membrane vesicles of MG1363/pMBmleP fused with COVs was assayed in the presence of the electron donor system cytochrome c/TMPD/potassium ascorbate, \(\bigtriangleup \psi\), no ionophores; G, valinomycin; and N, nigericin. Panels B and C, membrane vesicles of L. lactis LL108 expressing MleP, fused with liposomes were preloaded with 5 mM L-[14C]malate with (panel C) and without (panel B) 100 mM KSCN and subsequently diluted 100-fold into buffer containing 5 mM lactate (C), malate (\(\bigtriangleup \psi\)), or no additions (\(\bigtriangleup \psi\)). Valinomycin (panel B) and nigericin (panels B and C) were present at 1 and 0.5 \(\mu\)M, respectively.
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.

TABLE I

Effect of the R substituents on the ability of MleP, CitP, and CitS to transport 2-hydroxycarboxylates

<table>
<thead>
<tr>
<th>Substratea</th>
<th>R₁</th>
<th>R₂</th>
<th>Relative rate of exchangeb</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>MleP</td>
</tr>
<tr>
<td>Isocitrate</td>
<td>CH₂COO⁻CH₂COO⁻</td>
<td>H</td>
<td>0.4 ± 0.2</td>
</tr>
<tr>
<td>Citrate</td>
<td>CH₃COO⁻</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>Malate</td>
<td>CH₂COO⁻</td>
<td>H</td>
<td>18 ± 3</td>
</tr>
<tr>
<td>2-Hydroxybutyrate</td>
<td>CH₃CH₂</td>
<td>H</td>
<td>100 ± 8</td>
</tr>
<tr>
<td>2-Hydroxyisobutyrate</td>
<td>CH₃</td>
<td>CH₃</td>
<td>15 ± 3</td>
</tr>
<tr>
<td>Lactate</td>
<td>CH₃</td>
<td>H</td>
<td>16 ± 3</td>
</tr>
<tr>
<td>Glycolate</td>
<td>H</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>Glycolate</td>
<td>H</td>
<td>H</td>
<td>0.5 ± 0.3</td>
</tr>
</tbody>
</table>

a Substrates were added at a concentration of 5 mM. Equal mixtures of the l- and d-isomers were used.

b 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 m/s for MleP, CitP, and CitS, respectively. Rates are given as the average of two to four independent measurements and the S.D.

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 (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 carboxylate 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 included in the assay. The results are summarized in Table I. The main conclusion is that CitS is very specific, transporting only citrate and to a low extent citromalate, whereas MleP and CitP transport a wide variety of these substrates. MleP has a preference for the smaller substrates, whereas CitP seems to prefer the larger molecules. In contrast to CitP, MleP does not transport citrate and only poorly citromalate, whereas CitP even transports isocitrate, which has the largest substituent, at a low but significant rate. At the other end of the spectrum, glycolate is a good substrate of MleP and a poor substrate of...
CitP. No clear discrimination is evident between substrates with a polar and a hydrophobic character.

The Hydroxyl Group— A set of compounds was selected in which the hydroxyl group of one of the transported substrates listed in Table I was replaced by another substituent. In line with the high specificity of CitS reported above, none of these compounds was transported by CitS (Table II). Substrates in which the hydroxyl group is replaced by a hydrogen atom are not transported. Similarly, the hydroxyl cannot be replaced by an amino group. Replacement of the hydroxyl group by a keto group resulted in significant transport in the case of oxaloacetate, especially by MleP. This transporter could also transport glyoxylic acid, the 2-oxo analog of glycolate, at a significant rate, whereas the rate catalyzed by CitP was very low but significant.

The Carboxylate Group—Three different analogs of lactate with different substituents replacing the carboxylate group were tested in the exchange assay (Table III). A common feature of the analogs 1,2-propanediol, methyllactate, and glycerol is that the charge of the carboxylate is removed. In addition to the aldehyde group replacing the carboxylate, glyceraldehyde has a hydroxyl group at the C3 position. This might have an additional effect on the suitability as a substrate, but, on the other hand, tartrate, an analog of malate, has the same feature and is transported both by MleP and CitP (Table I). None of the analogs was transported by any of the transporters, emphasizing the relevance of the carboxylate group in the motif.

**DISCUSSION**

Secondary pmf-generating pathways were discovered only in the last decade. Oxaloacetate 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 OxIT 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 was observed before in membrane vesicles of the malate-fermenting wild type strain IL403 (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 *oxIT* 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 hydrophathy 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 R1R2COHCOO" (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, unlike a hydrogen atom or an amino group, replacement of the hydroxyl group by a hydroxyl group or an aldehyde group resulted in transport activity with different R substituents. Remarkably, the R groups of CitS recognize the common 2-hydroxycarboxylate motif. At the same time, CitP activity 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). Wherein, MeP 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 glycocolate, 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 isocitrurate for CitP result in very poor translocation. Apparently, the translocation site is optimized for the physiological substrates, and smaller R groups 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 drastically, possibly because the R groups are essential for Na+ binding.

The members of the 2-hydroxycarboxylate transporter family are a potent experimental system to study the relation between the primary sequence and substrate specificity. Currently, we are dissecting the binding and translocation events kinetically, analyzing the stereo selectivity of the transporters, and identifying residues in the primary sequences which are located in the binding pockets of MleP, CitP, and CitS. Such studies will eventually give a detailed model of the binding sites and explain how the details in similar structures result in important functional differences.

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