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Membranepotential-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 fer-
dase-containing vesicle. Catalyzed by primary transport systems at the expense of some membrane potential and a pH gradient. Proton pumping is membrane which results in the two components of the pmf, a translocation of protons against the gradient across the cell membrane which leads to the formation of a membrane potential of phys-
ological 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 substrates (6–8). Recently, a more complex system termed citrolactic fermentation was found in Leuconostoc mesen-
teroides, another lactic acid bacterium. Similar to the malo-
lactic 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 glu-
cose metabolism (9, 10).

The membrane potential-generating secondary transporters involved in malolactic fermentation and citrolactic fermenta-
tion, 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-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). Strain LL108, kindly provided by K. 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* NCD0176 is a wild type citrate-fermenting strain obtained from the Dutch Institute of Dairy Research (Edse, 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. 

**Standard DNA Manipulation 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 and propagated in *E. coli* DH5α. Nucleotide sequences were determined on a Vistra 725 or Applied Biosystems 373A automated sequencer.

**Preparation of Membrane Vesicles and Hybrid Membranes**

Cells of *L. lactis* MG1363 or LL108 expressing either rMleP or CitP were harvested at the end of the exponential growth phase at an abs of 0.8, washed with 50 mM potassium phosphate, pH 7.0, resuspended in the same buffer at an abs 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) essentially as described by Driessen et al. (30). 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 a ratio of 10 mg of 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.

Rightside-out membrane vesicles of *E. coli* strain BL21(DE3) expressing CitS from pSKLAcitS (17) were prepared by the osmotic lysis procedure essentially as described by Kaback (33) with the following modification. Spherolectins 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).

**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* NCD0176 (27). The 1.572-base pair region was amplified by the plasmid by PCR. The forward primer, 5'-GGTGAATTCATAGTTCTCTACCTGTAATG-3' and the reverse primer, 5'-GGTTCTATCTCCATTGGTGGATCCAGTATGTCATGAC-3' overlapped with the citP start codon and introduced a unique *NcoI* site (ACATGT) 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 *BamHI* and *EcoRI* and ligated into plasmid pRK29 (28) digested with the same enzymes. The resulting plasmid pPM2 contains the *L. lactis* citP promoter region followed by a ribosomal binding site and an *AflIII* restriction site around the start codon. Downstream of the *AflIII* restriction site a number of unique restriction sites are present for cloning purposes.

The gene coding for the malate transporter *mleP* was amplified by PCR from chromosomal DNA isolated from *L. lactis* IL1403. The forward primer, 5'-TCTTAATCCAGGGAAAACTTAAAGACCGAATATACCGG-3' generated an NcoI site around the start codon, and the reverse primer, 5'-TGCTCTAGATTACCGGATATCAGAAATCCGATT-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 endonucleolytically plasmid-containing *L. mesenteroides* creating unique *NcoI* and XbaI restriction sites around the start codon and downstream of the stop codon, respectively. The forward primer was 5'-GATAAGAACCCTCTGGATACCCCGATTC-3' and the reverse primer 5'-CTTTAATTCGTTAATCGGATTTTGATCC-3'. The amplified fragments were digested with *NcoI* and XbaI and ligated into the *AflIII* and *NcoI* sites of pMB which have compatible overhangs. The resulting plasmid pMBmleP was digested with *NcoI* and *BamHI* 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 in *L. mesenteroides* while creating unique *NcoI* and XbaI restriction sites around the start codon and downstream of the stop codon, respectively.

**Transport Assays**

PmR-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. (1,14,23,34) 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* rightside-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 72.5 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 were 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
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 DNA 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 (CitPs) and to the Na+-dependent citrate carriers of *K. pneumoniae* (CitS), *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 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, ○). 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 (\( \Delta \phi \)) and a pH gradient (\( \Delta pH \)). The role of each component of the pmf in driving [14C]malate uptake was investigated by manipulating \( \Delta pH \) and \( \Delta \phi \) 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 pMB*mleP* 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.
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 is indicative of rapid homologous exchange (▲), with 1 mM NaCl (▲) showing that the exchange is electrogenic. The results are most likely caused by sodium ion contaminations in the buffer (15). Addition of 1 mM NaCl 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 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 pSKleitS (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 NaCl 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.

**TABLE I**

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

| Substrate   | R₁   | R₂    | Relative rate of exchange%^a | MleP | CitP | CitS |
|-------------|------|-------|-------------------------------|------|------|------|
| Isocitrate  | CH₆COO⁻| CH₂COO⁻| H                             | 0.4 ± 0.2 | 2.6 ± 0.4 | 1.5 ± 0.7 |
| Citrate     | CH₆COO⁻| CH₂COO⁻| H                             | 0.5 ± 0.3 | 100 ± 8 | 100 ± 6 |
| Citromalate | CH₆COO⁻| CH₂⁻   | H                             | 6.9 ± 1.0 | 32 ± 1 | 4.6 ± 0.5 |
| Tartarate   | CHOHOCH²⁻| H    | H                             | 18 ± 3 | 7.1 ± 0.2 | 0.6 ± 0.5 |
| Malate      | CH₆COO⁻| H     | H                             | 100 ± 8 | 142 ± 6 | 1.6 ± 0.8 |
| 2-Hydroxybutyrate | CH₆CH₃ | H     | 15 ± 3 | 54 ± 1 | 1.3 ± 0.4 |
| 2-Hydroxycisobutyrate | CH₆ | CH₂ | 16 ± 3 | 100 ± 1 | 1.9 ± 0.7 |
| Lactate     | CH₆   | H     | H                             | 26 ± 8 | 3.3 ± 0.5 | 1.7 ± 0.8 |
| Glycolate   | H     | H     | None                          | 0.5 ± 0.3 | 1.0 ± 0.5 | 1.0 ± 0.6 |

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 ms/s for MleP, CitP, and CitS, respectively. Rates are given as the average of two to four independent measurement and the S.D.
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 a hydrogen atom. 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 the 2-oxo analog of lactate, for both MleP and CitP. The position of the hydroxy group relative to the carboxyl group was investigated by comparing 2-hydroxybutyrate and 3-hydroxybutyrate. A low but significant transport activity with the latter substrate was observed with CitP but not with MleP. In conclusion, MleP and CitP have the highest activity with 2-hydroxycarboxylates but significant activity is observed with some 2-oxocarboxylates and in case of CitP even a 3-hydroxycarboxylate.

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 glyceraldehyde 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. 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 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 *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 $\text{R}_3\text{R}_4\text{COHCOO}^-$ (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

**Tables**

**Table II**

| Substrate        | OH substituent | Relative rate of exchange |
|------------------|----------------|---------------------------|
| Citrate          | OH             | MleP: 0.5 ± 0.3           |
| Tricarboxylate   | H              | MleP: 1.0 ± 0.2           |
| Malate           | OH             | MleP: 1.0 ± 0.2           |
| Succinate        | H              | MleP: 0.4 ± 0.1           |
| Oxaloacetate     | NH$_3^+$       | MleP: 0.7 ± 0.3           |
| Aspartate        | NH$_3^+$       | MleP: 1.3 ± 0.3           |
| Lactate          | OH             | MleP: 0.5 ± 0.3           |
| Propionate       | H              | MleP: 1.3 ± 0.3           |
| Glyoxylate       | O              | MleP: 1.2 ± 0.3           |
| Glycine          | OH$_3^-$       | MleP: 0.6 ± 0.2           |
| Glycolate        | O              | MleP: 3.3 ± 0.5           |
| Acetate          | OH$_3^-$       | MleP: 2.2 ± 0.2           |
| Glucose          | OH$_3^-$       | MleP: 1.1 ± 0.5           |
| 2-Hydroxybutyrate| OH on C2       | MleP: 0.5 ± 0.3           |
| 3-Hydroxybutyrate| OH on C3       | MleP: 1.1 ± 0.5           |
| None             |                | MleP: 0.5 ± 0.3           |

**Table III**

| Substrate        | COO$^-$ substituent | Relative rate of exchange |
|------------------|---------------------|---------------------------|
| Lactate          | COO$^-$             | MleP: 91 ± 14             |
| Methyl-L-lactate | COOCH$_3^-$         | MleP: 35 ± 1              |
| 1,2-Propanediol  | CH$_2$OH            | MleP: 0.1 ± 0.1           |
| Glyceraldehyde   | CHO                  | MleP: 0.9 ± 0.5            |
| None             |                     | MleP: 0.5 ± 0.3           |
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 keto group, the R groups of the transported substrates range in size from the smallest to the largest. Remarkably, the R groups 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 Na+ transport and smaller R groups are compensated for by the conformation of the translocation site.