Stereoselectivity of the Membrane Potential-Generating Citrate and Malate Transporters of Lactic Acid Bacteria†

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ABSTRACT: The citrate transporter of *Leuconostoc mesenteroides* (CitP) and the malate transporter of *Lactococcus lactis* (MleP) are homologous proteins that catalyze citrate−lactate and malate−lactate exchange, respectively. Both transporters transport a range of substrates that contain the 2-hydroxycarboxylate motif, HO-CR_2-COO^- [Bandell, M., et al. (1997) *J. Biol. Chem. 272*, 18140−18146]. In this study, we have analyzed binding and translocation properties of CitP and MleP for a wide variety of substrates and substrate analogues. Modification of the OH or the COO^- groups of the 2-hydroxycarboxylate motif drastically reduced the affinity of the transporters for the substrates, indicating their relevance in substrate recognition. Both CitP and MleP were strictly stereoselective when the R group contained a second carboxylate group; the *S*-enantiomers were efficiently bound and translocated, while the transporters had no affinity for the *R*-enantiomers. The affinity of the *S*-enantiomers, and of citrate, was at least 1 order of magnitude higher than for lactate and other substrates with uncharged R groups, indicating a specific interaction between the second carboxylate group and the protein that is responsible for high-affinity binding. MleP was not stereoselective in binding when the R groups are hydrophobic and as large as a benzyl group. However, only the *S*-enantiomers were translocated by MleP. CitP had a strong preference for binding and translocating the *R*-enantiomers of substrates with large hydrophobic R groups. These differences between CitP and MleP explain why citrate is a substrate of CitP and not of MleP. The results are discussed in the context of a model for the interaction between sites on the protein and functional groups on the substrates in the binding pockets of the two proteins.

In the past decade, a growing number of secondary transporters have been discovered that generate rather than consume metabolic energy (*1*). These transporters have been termed precursor−product exchangers since they catalyze the coupled uptake of a substrate into the cell and exit of a metabolic end product into the medium. Well-studied examples are the oxalate transporter (OxlT) of *Oxalobacter formigenes* (*2*−*4*) and the citrate (CitP) and malate (MleP) transporters of the lactic acid bacteria *Leuconostoc mesenteroides* (*5*, *6*) and *Lactococcus lactis* (*6*, *7*), respectively. CitP exchanges divalent citrate and MleP divalent malate for monovalent lactate, an end product of both citrate and malate degradation (*7*, *8*). The net charge movement during this exchange results in a membrane potential of physiological polarity. Furthermore, decarboxylation reactions in the breakdown of citrate and malate consume scalar protons and thus generate a pH gradient of physiological polarity. The result of the combined activities of precursor−product exchange and decarboxylation is a proton motive force that is sufficiently high to drive ATP synthesis via F_0F_1-ATPase.

Citrate transporters (CitPs) from three different lactic acid bacteria have been cloned and sequenced, and the translated amino acid sequences were found to be 98% identical (*9*−*11*). Also, the transporters were functionally indistinguishable (*11*). More recently, the malate transporter MleP of *L. lactis* was cloned and sequenced and found to be homologous to CitP, with 48% of the residues being identical. Both transporters belong to the bacterial 2-hydroxycarboxylate transporter (2HCT) family that contains both membrane potential-generating and -dissipating members. A well-studied transporter of the latter group is the sodium ion motive force-driven citrate transporter of *Klebsiella pneumoniae*, CitS (*12*−*16*). Despite the different energetics, the homology between the proteins strongly suggests that the membrane potential-generating transporters are "classical" secondary transporters.

A study of the substrate specificity of CitP, MleP, and CitS revealed that all three transporters specifically transport substrates containing a 2-hydroxycarboxylate (HO-CR_2-COO^-) motif. The transporters were found to differ in their tolerance toward the two R substituents of the substrates (*6*). The Na^+ motive force-driven CitS was found to have a very narrow specificity, transporting mainly citrate. In contrast, the precursor−product exchangers MleP and CitP were found to have broad and overlapping specificities, the main difference being that CitP transports larger molecules than MleP. The largest substrate accepted by MleP is malate,

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whereas CitP, in addition to malate, also transports citrate. Both transporters translocate glycolate, the smallest 2-hydroxycarboxylate with the R groups representing H atoms. The ability to accept various R substituents that differ in size, and most importantly in charge, is essential for the function of CitP and MleP, since they catalyze exchange between a divalent precursor (i.e., citrate or malate) and a monovalent product (i.e., lactate).

In this study, we elaborate on the binding and catalytic properties of CitP and MleP for the substrates and a number of substrate analogues to determine the stereoselectivity of the transporters and, thus, gain insight into the nature of the substrate binding pockets of these proteins. The resulting models for substrate binding in CitP and MleP are similar and provide the basis for the physiological function of the transporters. Interactions with the OH and COO^- groups of the 2-hydroxycarboxylate motif are essential for recognition of the substrates by the transporters. In addition, a localized electrostatic interaction with the second carboxylate of the precursor molecules citrate and malate is responsible for high-affinity binding relative to the affinity for the product lactate. Steric restrictions during translocation in MleP are responsible for the inability of MleP to transport the larger citrate molecule.

EXPERIMENTAL PROCEDURES

Bacterial Strains, Plasmids, and Growth Conditions. *L. lactis* MG1363 is a malate and citrate fermentation negative strain that does not contain endogenous citrate and malate transport systems. *L. lactis* MG1363 was transformed with the *Escherichia coli/L. lactis* shuttle vectors pMB-citP or pMB-mleP (*6*) to express the citrate transporter CitP and the malate transporter MleP, respectively. In the vectors pMB-mleP and pMB-citP, the *mleP* gene from *L. lactis* IL1403 (*6*) and the *citP* gene from *Lc. mesenteroides* ssp. *mesenteroides* (*11*) were cloned downstream of the constitutive promotor of the *citP* gene cluster of *L. lactis* NCDO176 (*17*). The cells were grown in M17 broth (Difco) supplemented with 0.5% (w/v) glucose and 5 μg/mL erythromycin. The cells were grown at 30 °C in closed serum bottles without shaking.

Preparation of Membrane Vesicles. Cells of *L. lactis* MG1363 expressing either MleP or CitP were harvested at the end of the exponential growth phase at an optical density of 0.8 measured at 660 nm (OD_{660}),[1] washed with 50 mM potassium phosphate (pH 7), resuspended in the same buffer at an OD_{660} of 500, and 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. (*18*). (*S*)-Malate (L-malate) was present at a concentration of 5 mM throughout the procedure for loading the vesicles with (*S*)-malate. The vesicles were rapidly frozen in liquid nitrogen in 50 mM potassium phosphate (pH 6) containing 5 mM (*S*)-malate. The protein concentration was determined as described by Lowry et al. (*19*).

Exchange and Counterflow in Membrane Vesicles. Membrane vesicles of *L. lactis* MG1363 containing either CitP or MleP were washed in 50 mM potassium phosphate (pH 6) containing 5 mM (*S*)-malate and concentrated by centrifugation for 15 min in an Eppendorf centrifuge operated at full speed, followed by resuspension in the same buffer. For exchange measurements, the internal pool of (*S*)-malate was labeled with (*S*)-[^{14}C]malate by incubating the concentrated membranes with 186.7 μM L-[1,4(2,3)^{14}C]malate for 1 h at room temperature in the presence of 100 μM valinomycin and 50 μM nigericin. Aliquots of 2 μL were diluted into 200 μL of the potassium phosphate buffer containing various substrates at the indicated concentrations at 20 °C. For counterflow experiments, the (*S*)-malate-loaded concentrated vesicles were diluted 100-fold into 200 μL of buffer containing 9.8 μM L-[1,4(2,3)^{14}C]malate and different concentrations of various substrates when indicated. Valinomycin and nigericin were present at final concentrations of 1 and 0.5 μM, respectively. Final membrane protein concentrations in the assays were between 250 and 350 μg/mL. Reactions were stopped at the indicated times by addition of 4 mL of ice-cold 0.1 M LiCl and rapid filtration over 0.45 μm pore size cellulose nitrate filters (Schleicher & Schuell). The filters were rinsed once with 4 mL of ice-cold 0.1 M LiCl and transferred to scintillation vials to determine the internal radioactivity.

Evaluation of the Data. Initial rates of exchange were determined by fitting the data to an exponential decay as described previously (*6*) using nonlinear fitting procedures provided with the Sigma Plot software (Jandel Scientific, San Rafael, CA). The affinity constant for a substrate in the external buffer and the maximal rate of heterologous exchange between the substrate and internal (*S*)-malate were determined by measuring the initial rates of exchange at different concentrations of the substrate in the external buffer. The data were fitted to an equation describing competitive inhibition in which [S] is the concentration of the substrate in the dilution buffer, [I] the concentration of (*S*)-[^{14}C]malate in the external buffer caused by the dilution of the (*S*)-malate-loaded vesicles (i.e., routinely 50 μM), and K_i the affinity constant for (*S*)-malate determined from homologous (*S*)-malate exchange.

$$v = V_{max}\frac{[S]}{[S] + K_m\left(1 + \frac{[I]}{K_i}\right)} \quad (1)$$

Initial rates of counterflow were estimated from the amount of internalized label measured at the 3 and 5 s time points that were measured in triplicate. Data were used only when the rate of uptake increased proportionally with time to ensure initial rate conditions. The level of inhibition of (*S*)-malate counterflow by a substrate was measured at different concentrations of the substrate in the external buffer. The inhibition constant for the substrate was estimated by fitting the data to eq 1 in which [S] and K_m are the concentration and affinity constant for external (*S*)-malate, respectively, and [I] and K_i the concentration and affinity constants for the added substrate, respectively.

Chemicals. L-[1,4(2,3)^{14}C]Malate (51 mCi/mmol) was obtained from Amersham International (Buckinghamshire, U.K.). All other compounds were obtained from Fluca (Buchs, Switzerland) or Sigma (St. Louis, MO).

[1] Abbreviations: RSO, right-side-out vesicles; OD_{660}, optical density at 660 nm.

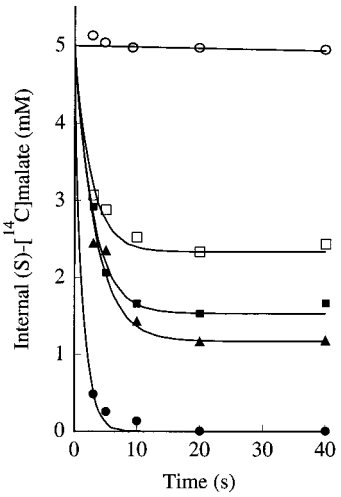
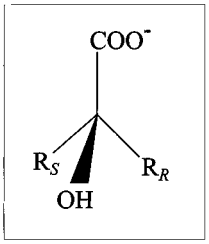


FIGURE 1: (*S*)-Malate−(*S*)-malate and (*R*)-malate−(*S*)-malate exchange catalyzed by MleP. RSO membrane vesicles of *L. lactis* MG1363 expressing MleP were preloaded with 5 mM (*S*)-[^{14}C]-malate. The membranes were diluted 100-fold into buffer containing no further additions (○), 5 mM (*S*)-malate (●), 0.1 mM (*S*)-malate (■), 0.05 mM (*S*)-malate (□), and 5 mM (*R*)-malate (▲).



	R_S	R_R
(*S*)-2-hydroxyglutarate	$-CH_2-CH_2-COO^-$	−H
(*S*)-malate	$-CH_2-COO^-$	−H
(*S*,*S*)-tartrate	$-CH(OH)-COO^-$	−H
(*S*)-mandelate	−C$_6$H$_5$	−H
(*S*)-2-hydroxyisovalerate	$-CH(CH_3)-CH_3$	−H
(*S*)-lactate	$-CH_3$	−H
(*S*)-citramalate	$-CH_2-COO^-$	$-CH_3$
citrate	$-CH_2-COO^-$	$-CH_2-COO^-$
2-hydroxyisobutyrate	$-CH_3$	$-CH_3$
glycolate	−H	−H

FIGURE 2: Stereochemistry of 2-hydroxycarboxylates. Throughout this paper, the position of the two R side chains of the 2-hydroxycarboxylate motif HO-CR_2-COO^- in the binding pocket will be denoted as R_S and R_R. By definition, the R_S and R_R positions correspond to the side occupied by the R group in the *S*- and *R*-enantiomers of monosubstituted 2-hydroxycarboxylates HO-CHR-COO^-, respectively. The *S*-enantiomers of the substrates used in this study are denoted in the table on the right. The corresponding *R*-enantiomers can be visualized by interchanging the R_R and R_S substituents.

RESULTS

Exchange of (S)- and (R)-Malate Catalyzed by MleP. Heterologous exchange provides a sensitive and unambiguous assay for transport of a compound by a secondary transporter. The assay measures the potency of a compound in inducing efflux of a radiolabeled substrate of the transporter from preloaded membrane vesicles. The assay depends on the condition that efflux down a concentration gradient is much slower than exchange with an external substrate.

Right-side-out membrane vesicles prepared from *L. lactis* cells in which the malate transporter MleP was expressed were loaded with 5 mM (*S*)-[^{14}C]malate. Release of the label was very slow when diluted 100-fold into buffer, indicating that efflux of malate down a concentration gradient is a slow process (Figure 1, ○). In contrast, equilibrium exchange which results from dilution into buffer containing 5 mM unlabeled (*S*)-malate was very rapid (●). At lower external (*S*)-malate concentrations of 50 (□) and 100 μM (■), the rate of exchange was slower. Also, the total amount of label released from the vesicles at equilibrium decreases with decreasing external substrate concentrations since this depends on the ratio of labeled and unlabeled exchangeable substrates at both sides of the membrane. When the 5 mM unlabeled (*S*)-malate in the external buffer was replaced by the same concentration of the stereoisomer (*R*)-malate, a rapid release to about 30% of the internal label was observed (Figure 1, ▲). This amount of released label was lower than expected and indicates an external substrate concentration of just above 100 μM [compare to the level obtained with 100 μM (*S*)-malate]. Therefore, the observed exchange is not caused by (*R*)-malate but by a transportable contamination present at about 2% in (*R*)-malate (100 μM/5 mM), most likely (*S*)-malate. The extent of heterologous exchange with (*R*)-malate can be estimated from the second phase of the curve (▲) and is undetectable in this experiment. In conclusion, MleP is highly selective for the *S*-enantiomer of malate. The presence of the contamination in (*R*)-malate limits the kinetic analysis of this substrate. A similar contamination of substrates of either MleP or CitP was found to be present in (*S*)-citramalate.

Stereoselectivity in Transport Catalyzed by CitP and MleP. Heterologous exchange rates of (*S*)-malate and the *R*- and *S*-enantiomers of a number of monosubstituted 2-hydroxycarboxylates, i.e., HO-CHR-COO^-, were determined for both MleP and CitP. The R substituents were either hydrophilic, i.e., malate, tartrate, and 2-hydroxyglutarate, or hydrophobic, i.e., lactate, 2-hydroxyisovalerate, and mandelate (see Figure 2).

For CitP, the substrates with hydrophilic and/or charged R groups yielded much higher exchange rates when in the *S*-enantiomeric than when in the *R*-enantiomeric form (Figure 3A). Relative to homologous (*S*)-malate exchange, heterologous exchange with (*S*)-2-hydroxyglutarate and (*S*)-tartrate was roughly 5 times slower under the conditions of the experiments. Remarkably, CitP exhibited a preference for the *R*-enantiomer of the substrates with the hydrophobic substituents. The stereoselectivity with the hydrophobic R groups was less stringent than with the hydrophilic R groups. (*S*)-Citramalate is like a "hybrid" of (*S*)-malate and (*R*)-lactate with a hydrophilic CH_2COO^- group at the R_S position and a hydrophobic CH_3 group at the R_R position (see Figure 2). As expected, (*S*)-citramalate was preferred over (*R*)-citramalate, but the rate of exchange with (*S*)-citramalate was low compared to that with (*S*)-malate.

MleP, like CitP, preferred the *S*-enantiomers of substrates bearing hydrophilic substituents and catalyzed homologous exchange of (*S*)-malate considerably faster than heterologous exchange with (*S*)-2-hydroxyglutarate and (*S*)-tartrate (Figure 3B). MleP clearly differed from CitP in that, in the case of substrates containing a hydrophobic substituent, it preferred to transport the *S*-enantiomers as well. The preference was most clear for lactate. The substrates mandelate and 2-hydroxyisovalerate, which have larger hydrophobic substituents, result in low exchange rates, but also for these substrates, the preference for the *S*-enantiomer was significant. In fact,

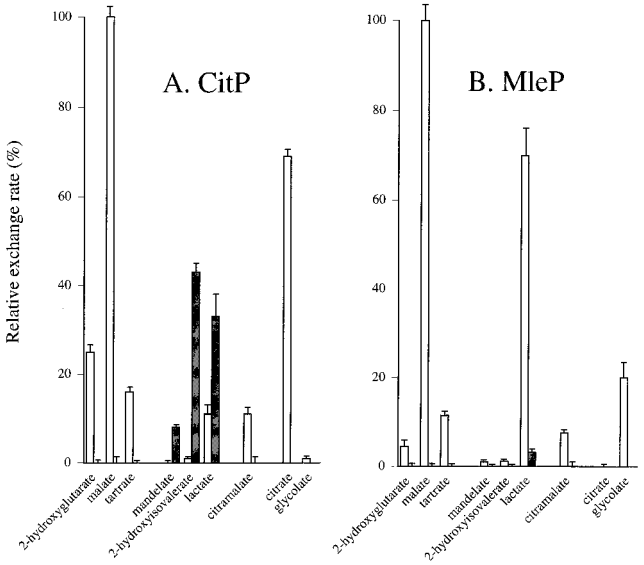


FIGURE 3: Heterologous exchange rates catalyzed by CitP (A) and MleP (B). RSO membrane vesicles of *L. lactis* MG1363 expressing CitP (A) and MleP (B) were preloaded with 5 mM (*S*)-[^{14}C]malate and diluted 100-fold into buffer containing the indicated 2-hydroxycarboxylates at 5 mM. When appropriate, the left bars (open) and the right bars (shaded) correspond to the *S*- and *R*-enantiomers of the substrates, respectively. Rates are given relative to the rate observed for homologous (*S*)-malate exchange that was set at 100%. The rates for homologous (*S*)-malate exchange varied per vesicle preparation between 1 and 1.2 mM/s for CitP and between 3 and 3.4 mM/s for MleP. Indicated rates and error bars represent average values of two to four independent measurements and the standard deviations, respectively.

no exchange with the *R*-enantiomers was observed even at concentrations as high as 20 mM and when assayed for longer periods of times (data not shown). MleP, like CitP, preferred (*S*)-citramalate over (*R*)-citramalate, indicating that the preference for a CH_2COO^- group at R_S prevails over the preference for a CH_3 at this position.

The exchange rates of the preferred enantiomers were compared to the rates of the two nonchiral substrates citrate and glycolate. CitP catalyzed exchange with (*S*)-malate at a higher rate than with citrate, which is the physiological substrate of CitP. As demonstrated previously, citrate is not transported by MleP and glycolate is a much better substrate for MleP than for CitP (*6*). For both transporters, the rates with the preferred enantiomers of lactate were higher than with glycolate, indicating the relevance of the methyl group at C2.

Counterflow of (S)- and (R)-Malate Catalyzed by MleP. Heterologous exchange does not discriminate between compounds for which the transporter has no affinity and compounds that bind to the transporter but are not translocated. This discrimination can be achieved by a counterflow assay in which uptake of external labeled substrate is driven by exchange with internal unlabeled substrate. The potency of an externally added compound to inhibit the uptake of the label is a measure of the affinity of the transporter for the compound.

Right-side-out membrane vesicles containing MleP were preloaded with 5 mM unlabeled (*S*)-malate and, subsequently, diluted 100-fold into buffer containing 50 μM labeled (*S*)-malate. The label rapidly entered the vesicles, and a steady state was reached in 1 min (Figure 4A, □). No subsequent release of label was observed during the time of the experiment, consistent with the low rate of efflux in the exchange experiments observed above. When the dilution buffer contained an additional 400 μM unlabeled (*S*)-malate, the initial rate of influx of the label was decreased due to competition between labeled and unlabeled (*S*)-malate. The final level of uptake was considerably lower because of the higher total external concentration of transportable substrates (Figure 4A, ●). Addition of 400 μM (*R*)-malate rather than (*S*)-malate had no significant effect on the uptake of the label (Figure 4A, ○). The lack of inhibition of the initial rate of uptake indicates that MleP has a low, if any, affinity for (*R*)-malate. Clearly, the lack of activity with (*R*)-malate in the heterologous exchange assay correlates with a lack of affinity of MleP for the *R*-enantiomer.

In some cases, an overshoot in internalized label was observed, for instance, with 400 μM (*S*)-tartrate (Figure 4A, ▲). Initially, the rate of exchange with labeled (*S*)-malate is much faster than with unlabeled (*S*)-tartrate, but eventually, internalized label will be exchanged for (*S*)-tartrate until the same final level of uptake is reached as observed with 400 μM (*S*)-malate. The experiment demonstrates that (*S*)-tartrate is transported by MleP, but with a lower affinity than observed for (*S*)-malate.

Stereoselectivity in Binding by CitP and MleP. The relative affinities of CitP and MleP for the *S*- and *R*-enantiomers of the substrates were determined from the inhibition of (*S*)-malate counterflow. An inhibitor concentration was selected for the different substrates that allowed estimation of the inhibition from the initial rates of label influx (Table 1).

For both CitP and MleP, the *S*-enantiomers of the compounds with a hydrophilic R group (i.e., malate, 2-hydroxyglutarate, and tartrate) caused a stronger inhibition than was found for the *R*-enantiomers (Table 1). The higher exchange rates observed above with the *S*- relative to those with the *R*-enantiomers correlated with a higher affinity of the transporters for the *S*-enantiomers. (*S*)-Citramalate, which combines both a hydrophilic substituent at R_S and a hydrophobic substituent at R_R, was found to be a potent inhibitor of both transporters, while the *R*-enantiomer did not result in any inhibition at the same concentration (Figure 4B). Citrate, which has the same hydrophilic R group at both R_S and R_R, was an equally potent inhibitor of CitP. Surprisingly, citrate also inhibited counterflow catalyzed by MleP that does not transport citrate, indicating that citrate binds to MleP but is not translocated.

For MleP the inhibition of counterflow by (*S*)-lactate and for CitP the inhibition by (*R*)-lactate could not be assessed because of a combination of high exchange rate and relatively low affinity. For instance, at 0.4 mM (*S*)-lactate, the final level of uptake of label was reached very rapidly, not allowing estimation of the initial rate (Figure 4C). Relatively high concentrations of the opposite enantiomers, (*R*)-lactate in the case of MleP and (*S*)-lactate in the case of CitP, were required to obtain significant inhibition, suggesting low affinities.

The *R*-enantiomers of the compounds with the larger hydrophobic substituents (i.e., 2-hydroxyisovalerate and mandelate) were the stronger inhibitors of counterflow catalyzed by CitP. For MleP, the inhibition by 2-hydroxyisovalerate and mandelate was the same for the two enantiomers. Since exchange activity catalyzed by MleP could

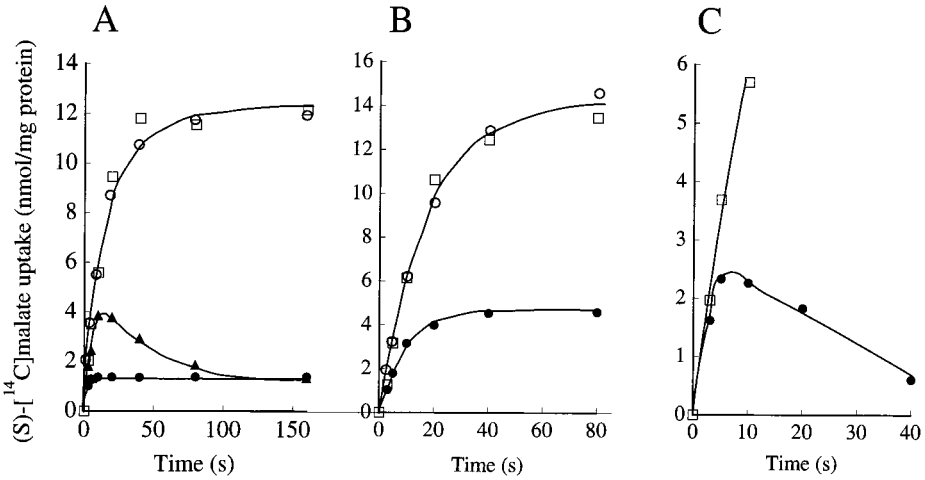


FIGURE 4: Inhibition of counterflow catalyzed by MleP. RSO membrane vesicles of *L. lactis* MG1363 expressing MleP were preloaded with 5 mM (*S*)-malate and diluted 100-fold into buffer containing 9.8 μM (*S*)-[^{14}C]malate without further additions (□) or with 400 μM (*S*)-malate (A, ●), 400 μM (*S*)-tartrate (A, ▲), 400 μM (*R*)-malate (A, ○), 200 μM (*S*)-citramalate (B, ●), 200 μM (*R*)-citramalate (B, ○), and 400 μM (*S*)-lactate (C, ●).

Table 1: Inhibition of (*S*)-Malate Counterflow Catalyzed by CitP and MleP[a]

	CitP			MleP		
	[I]	inhibition (%)		[I]	inhibition (%)	
inhibitor	(mM)	*S*-isomer	*R*-isomer	(mM)	*S*-isomer	*R*-isomer
malate	0.2	67	0	0.2	37	0
tartrate	4.5	27	0	0.8	39	0
2-hydroxyglutarate	5.0	27	0	10.0	33	0
lactate	6.0	13	–[b]	5.0	–[b]	25
2-hydroxyisovalerate	6.0	0	35	5.0	23	24
mandelate	5.0	0	30	2.5	43	47
citramalate	0.2	93	0	0.2	42	0
citrate	0.2	70		5.0	33	
2-hydroxyisobutyrate	1.0	33		1.25	30	
glycolate	15.0	20		5.0		16

[a] (*S*)-Malate counterflow was carried out as descibed in the legend of Figure 4 with the indicated concentration of the inhibitor [I] in the dilution buffer. The percent inhibition is given as the decrease of the initial rate in the absence of the inhibitor. [b] Initial rates could not be determined.

not be detected with (*R*)-mandelate and (*R*)-2-hydroxyisovalerate at concentrations of 5 mM, these substrates bind to MleP but are not translocated as was concluded for citrate. The stereoselectivity of MleP observed in the exchange reaction of these compounds appears to be related to differences in turnover rate rather than affinity.

The concentrations of the 2-hydroxycarboxylates with hydrophobic R groups that are required for significant inhibition are higher than those for the preferred *S*-enantiomers of the physiological substrates with hydrophilic R groups. This is also the case for the nonchiral glycolate. Nonchiral hydroxyisobutyrate, with methyl groups at R_R and R_S, seems to be the most potent inhibitor in the group of substrates with the hydrophobic R substituents.

Kinetic Parameters of CitP and MleP. The heterologous exchange assay and counterflow assay described above represent the same kinetic mode of the transporters, characterized by the same set of kinetic constants (see the Discussion). The K_m obtained for external (*S*)-malate in homologous exchange catalyzed by CitP was 0.1 mM which was in fair agreement with the K_i value of 0.08 mM obtained from the inhibition of counterflow (Table 2 and Experimental Procedures). A similar result was obtained with MleP, but the K_m and K_i values of 0.46 and 0.4 mM, respectively, showed that the affinity of MleP for (*S*)-malate was approximately 5 times lower than that of CitP. Estimation of the kinetic parameters for (*R*)-malate was hampered by the presence of a transportable contamination, most likely (*S*)-malate. No significant inhibition of counterflow was observed at concentrations up to about 1 mM. Moreover, in the heterologous exchange assay, using a concentration of 5 mM (*R*)-malate, the rate and extent of exchange correlated with the presence of about 100 μM (*S*)-malate in the external medium (Figure 1, ▲), suggesting no inhibition by (*R*)-malate at this concentration. The affinity of CitP and MleP for (*R*)-malate can be estimated to be at least 2 orders of magnitude lower than for (*S*)-malate.

The low exchange rates with (*S*)-citramalate did not allow a reliable estimation of the affinity of the transporters from a titration of the external (*S*)-citramalate concentration. Therefore, the affinity constant was estimated from the inhibition of counterflow at a range of concentrations. (*S*)-Citramalate is the substrate for which both CitP and MleP have the highest affinity with K_i values of 14 and 250 μM, respectively. The maximal exchange rates with (*S*)-citramalate were estimated from the exchange rate at an external concentration that was 20 times higher than the inhibition constants. The V_{max} values were roughly 10 times lower than those for (*S*)-malate. Analysis of the kinetics of (*R*)-citramalate was not possible because of the presence of contaminating (*S*)-citramalate. Similar experiments as described above for (*R*)-malate suggested that the affinity of both transporters for (*R*)-citramalate was very low.

The affinity of CitP and MleP for citrate was estimated from the inhibition of (*S*)-malate counterflow. The affinity of CitP (K_i = 56 μM) was in the same range as those observed for (*S*)-malate and (*S*)-citramalate. The maximal rate of heterologous exchange was determined at a citrate concentration of 5 mM and found to be somewhat slower than that with (*S*)-malate. Titration of the inhibition of counterflow catalyzed by MleP revealed an inhibition constant K_i of 9 mM for citrate. No exchange activity could be measured with citrate concentrations twice the K_i value, showing that citrate binds to MleP but is not translocated.

Table 2: Kinetic Parameters of CitP and MleP

	CitP			MleP		
substrate[a]	V_{max}[b] (%)	K_m[b] (mM)	K_i[c] (mM)	V_{max}[b] (%)	K_m[b] (mM)	K_i[c] (mM)
glycolate	–	–	≥ 50[d]	–	–	≥ 25[d]
(*S*)-lactate	110 ± 23	26 ± 8	–	140 ± 10	4.6 ± 0.9	–
(*S*)-malate	100 ± 3	0.10 ± 0.02	0.08 ± 0.02	100 ± 4	0.46 ± 0.15	0.40 ± 0.06
(*S*)-citramalate	12 ± 2[e]	–	0.014 ± 0.002	7.5 ± 0.5[e]	–	0.25 ± 0.03
citrate	69 ± 3[e]	–	0.056 ± 0.009	0	–	9 ± 2
(*R*)-citramalate	–	≥50[f]	–	–	≥50[f]	–
(*R*)-malate	–	≥50[f]	–	–	≥50[f]	–
(*R*)-lactate	380 ± 87	32 ± 9	–	15 ± 2.4	14 ± 5	–
glycolate	–	–	≥40[d]	–	–	≥25[d]

[a] With glycolate at the top and bottom, substrates were ordered according to increasing side chains up to citrate (see also Figure 2). [b] Maximal rate and affinity constant for the substrate in heterologous exchange with (*S*)-malate as described in the legend of Figure 1. Maximal rates were relative to the rate for homologous (*S*)-malate exchange that was set at 100. [c] Inhibition constant for the substrate inferred from the inhibition of (*S*)-malate counterflow as described in the legend of Figure 4. The K_i values were calculated using a K_m value for (*S*)-malate of 90 μM (CitP) or 0.43 mM (MleP). [d] Estimated from the data in Table 1. [e] Maximal rates were the exchange rates at a substrate concentration that was 20 times higher than the K_i value obtained from the inhibition of counterflow. [f] Lower limit of the affinity constants. No exchange activity could be measured.

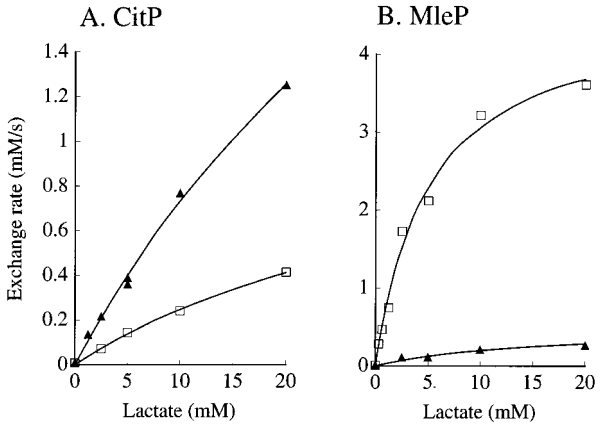


FIGURE 5: Kinetic differences between MleP and CitP for (*S*)- and (*R*)-lactate exchange. RSO membrane vesicles of *L. lactis* MG1363 expressing CitP (A) and MleP (B) were preloaded with 5 mM (*S*)-[^{14}C]malate. The membranes were diluted 100-fold into buffer containing concentrations of (*R*)-lactate (▲) and (*S*)-lactate (□) ranging from 1 to 20 mM. Heterologous exchange rates were plotted against the lactate concentrations. For the kinetic parameters derived from the plots, see Table 2.

The kinetic constants for both stereoisomers of lactate were estimated from heterologous exchange of (*S*)-malate with external lactate concentrations ranging from 0.5 to 20 mM (Figure 5). The affinity constants of CitP for (*S*)- and (*R*)-lactate were very similar, 26 and 32 mM, respectively, while the maximal rate with (*R*)-lactate was almost 4 times higher than with (*S*)-lactate (Table 2). The affinities of MleP for lactate were higher than those of CitP. (*S*)-Lactate resulted in a 3-fold higher affinity than (*R*)-lactate (4.6 vs 13.8 mM). The most prominent difference was the 10-fold higher maximal rate for (*S*)-lactate when compared to that of (*R*)-lactate.

Glycolate at a concentration of 5 mM exhibited significant rates of exchange with (*S*)-malate for both CitP and MleP. The level of inhibition of counterflow at this concentration was low, suggesting much higher affinity constants and high maximal rates. Precise measurement of K_m and V_{max} was not possible because higher concentrations started to inhibit exchange nonspecifically and due to the increased ionic strength of the buffer. The affinity constants for glycolate of both CitP and MleP can safely be estimated to be higher than 40 mM for CitP and 25 mM for MleP.

Role of the 2-Hydroxycarboxylate Motif in Binding. CitP and MleP translocate substrates with the 2-hydroxycarboxylate motif. Exceptions to the rule are oxaloacetate that is translocated efficiently by CitP and the 3-hydroxycarboxylates 3-hydroxybutyrate and 2,2-dimethyl-3-hydroxypropionate that are translocated by both transporters, albeit at a slow rate (*6*). Inhibition of counterflow showed that a similar degree of inhibition required a concentration of the 2-oxocarboxylate oxaloacetate 50 times higher than that of the corresponding 2-hydroxycarboxylate (*S*)-malate (not shown). A similar difference was obtained between 2-hydroxyisobutyrate and 2,2-dimethyl-3-hydroxypropionate. Replacing the hydroxyl in citrate, malate, and citramalate with hydrogen (yielding tricarballate, succinate, and methylsuccinate, respectively) resulted in the complete loss of affinity as inferred from the lack of inhibition of these compounds at concentrations of 20 mM in the counterflow assay. At the least, the hydroxyl group is essential for high-affinity binding.

Methylation of the carboxylate in (*S*)-lactate to methyl-(*S*)-lactate and reduction to glyceraldehyde and 1,2-propanediol resulted in the complete loss of affinity in the counterflow assay (not shown), indicating that the carboxylate group of the motif is essential for binding.

DISCUSSION

CitP and MleP are homologous transporters capable of transporting a range of 2-hydroxycarboxylates of different sizes and charges. Under physiological conditions, the proteins exchange a divalent precursor (citrate or malate) present in the medium for the monovalent metabolic product of the precursor (lactate) that is present in the cytoplasm. Consequently, they generate a membrane potential, supplying the cell with metabolic energy, and take part in the removal of the end product from the cell. CitP and MleP are believed to be "normal" secondary transporters that have been optimized to catalyze exchange (*1*). The transporters have been shown to be H^+/substrate symporters (*5*, *7*). Symport involves binding, translocation, and dissociation of the substrates in one direction and reorientation of the empty binding sites in the reverse direction. In the exchange mode, the second step is replaced by the same sequence as in the first step but in the opposite direction. In exchangers such as CitP and MleP, reorientation of the empty binding sites is slow relative to reorientation of the substrate-bound sites,

resulting in much higher rates for exchange than for unidirectional modes of transport, like efflux (for instance, see Figure 1). The heterologous exchange assay and counterflow assay used in this study both measure exchange activity of the transporters, but in different experimental setups. In the former, turnover is assessed by following the release of radiolabeled internal (*S*)-malate from the membranes, while in the latter, uptake of external radiolabeled (*S*)-malate is assessed. Turnover of the transporters was assessed at a constant internal (*S*)-malate concentration and a variable external substrate/inhibitor composition. Measurement of the exchange rates at different concentrations of the external substrates revealed both the affinity constant K_m for the external substrate and the maximal heterologous exchange rate V_{max}. Measurement of the extent of inhibition of counterflow at different external substrate concentrations revealed the inhibition constant K_i of the substrate. When corrected for the external (*S*)-malate concentrations (see Experimental Procedures), the affinity constants from the two measurements represent the same parameter as is nicely demonstrated for (*S*)-malate, in which case experimental conditions allowed the measurement of the affinity constant using both assays (Table 2).

The maximal initial rate of exchange is determined by the reorientation and dissociation of the enzyme−substrate complexes in the two directions. In the heterologous exchange and counterflow assays used here, translocation of (*S*)-malate from "in" to "out" is common to all measurements, while the different substrates in the dilution buffer result in different complexes that translocate from out to in. Since the maximal rate of (*R*)-lactate−(*S*)-malate exchange catalyzed by CitP was 4 times faster than that observed for homologous (*S*)-malate exchange, it can be concluded that the common translocation of (*S*)-malate from in to out is not rate-controlling (Table 2). The translocation and/or dissociation step of (*S*)-malate, citrate, and (*S*)-citramalate into the vesicle is rate-determining at saturating substrate concentrations. It was shown before that entrance of citrate into the cell catalyzed by CitP is the rate-controlling step in the citrate metabolic pathway in *Lc. mesenteroides* (*8*).

Citrate−lactate and malate−lactate exchange are the physiological modes of transport of CitP and MleP, respectively. The main metabolic activity of lactic acid bacteria is the conversion of carbohydrates in lactate that accumulates in the medium. Eventually, lactate in the medium will inhibit the citrate- and malate-degrading pathways since the transporter has affinity for both citrate or malate and lactate. This study shows that CitP and MleP have affinities for the di- and tricarboxylates malate and citrate that are at least 1 order of magnitude higher than the affinity for the monocarboxylate lactate at the external face of the membrane. The difference in affinity largely prevents the inhibition of the citrate and malate pathways by the accumulating lactate in the growth medium (see also ref *20*).

The affinity and translocation properties determined in this study result in a model for the substrate binding site of the CitP and MleP proteins. With few exceptions, both transporters were known to transport substrates containing the 2-hydroxycarboxylate motif (*6*). This study shows that the inability to transport substrates in which either the hydroxyl or the carboxylate of the motif was modified is due to the loss of binding affinity. It seems reasonable to assume that CitP and MleP recognize their substrates through an interaction with both functional groups. Under physiological conditions, the carboxylate group is negatively charged, and since removal of the charge resulted in the complete loss of affinity, the interaction is likely to be electrostatic. The hydroxyl group could, to some extent, be replaced by an oxo group (for instance, malate or oxaloacetate), be it with a significant loss of affinity. Possibly, the hydroxyl is hydrogen bonded to the protein with the oxygen atom acting as the H acceptor. Alternatively, the 2-oxo substrates are bound in their hydrated form in which the 2-hydroxy group is restored. Flexibility in the substrate molecule and/or the protein may account for the low activity observed with 3-hydroxycarboxylates such as 3-hydroxybutyrate (*6*).

The interactions of the transporters with the 2-hydroxycarboxylate motif fix the substrates in the binding pocket, allowing a detailed analysis of the effect of the two R groups. In the two enantomeric forms of monosubstituted 2-hydroxycarboxylates, i.e., HO-CHR-COO^-, the same R group has a different spatial orientation relative to the hydroxyl and carboxyl groups of the motif when bound to the protein (see Figure 2). For hydrophilic R groups, CitP and MleP interact efficiently with the *S*-enantiomers, while interaction with the *R*-enantiomers could not be detected. All the substrates in the micromolar affinity range [(*S*)-malate, (*S*)-citramalate, and citrate] have a CH_2COO^- group at the R_S position, suggesting that this group or, more particularly, the negatively charged carboxylate is essential for high-affinity binding via an electrostatic interaction with the protein. The high-affinity binding of di- and tricarboxylates as compared to monocarboxylates is physiologically relevant as discussed above. The lower affinities for the dicarboxylates (*S*)-tartrate and (*S*)-2-hydroxyglutarate are due to additional features of the R groups (Table 1 and Figure 2). Apparently, the additional hydroxyl in (*S*)-tartrate [compare to (*S*)-malate] results in a negative interaction with the proteins, while the extended length of the R group in (*S*)-2-hydroxyglutarate wrongly positions the carboxylate in the binding site. The inability of especially CitP to interact with substrates with a second carboxylate at the R_R position is not due to a spatial restriction in the binding site since citrate, with CH_2COO^- groups at both R_S and R_R, is a good substrate. Citrate however is known to be transported in its divalent negative form (*5*), leaving one of the three carboxylates uncharged. Since (*S*)-malate is also transported in its divalent form, the protonated carboxylate in citrate is likely to be at the R_R position. This suggests that specifically a negative charge is not tolerated at the R_R position in CitP, for instance, because of the presence of a hydrophobic surface in the binding pocket.

The affinity of both CitP and MleP for the substrate increases when a methyl group is present at the R_S and R_R positions as evidenced by the improved affinities of (*S*)- and (*R*)-lactate relative to those of glycolate (Table 2). The effects of the methyl groups at R_S and R_R are additive since 2-hydroxyisobutyrate with methyl groups at both positions exhibited higher affinity than (*R*)- or (*S*)-lactate. Addition of the methyl to (*S*)-malate at the R_R position, i.e., (*S*)-citramalate, also resulted in a significantly higher affinity. Apparently, the methyl groups give a better fit of the substrates in the binding site, resulting in an improved interaction with the protein.

The stereoisomers of the monosubstituted substrates with hydrophobic R groups behaved in a manner different from that of the substrates with the hydrophilic R groups. In addition, these substrates revealed differences between the binding pockets of MleP and CitP. In marked contrast to the substrates with the hydrophilic R groups, MleP bound the *R*- and *S*-enantiomers of lactate, 2-hydroxyisovalerate, and mandelate with similar affinities. The higher affinity of mandalate relative to those of the two other substrates indicates the presence of hydrophobic surfaces in the binding pocket and no steric restrictions for accommodating a benzyl group at both the R_S and R_R positions. A marked difference between the *R*- and *S*-enantiomers was evident in the translocation step. (*S*)-Lactate is translocated 10 times faster than (*R*)-lactate (Figure 5 and Table 2), while translocation of mandalate and 2-hydroxyisovalerate could only be detected in the *S*-enantiomer (Figure 3). Whereas the outward facing binding site of MleP does not discriminate between the stereoisomers, the transition complex is clearly more tolerant of R groups at the R_S position than at the R_R position. CitP binds (*S*)- and (*R*)-lactate with similar affinities, but no affinity was detected for the *S*-enantiomers of 2-hydroxyisovalerate and mandelate, suggesting that CitP is spatially restricted at the R_S position. In the transition complex, CitP is very tolerant of hydrophobic R groups at R_R as evidenced by the highest of all maximal rates obtained with (*R*)-lactate and rates similar to those obtained with malate and citrate for (*R*)-mandalate and (*R*)-2-hydroxyisovalerate. In conclusion, MleP has difficulties accepting hydrophobic R groups at the R_R position during translocation and CitP does not bind substrates with large hydrophobic R groups at the R_S site. The conclusion explains why citrate is a substrate of CitP and not of MleP. The CH_2COOH group of citrate at the R_R position is bound and translocated by CitP, while MleP can accept the group in the binding site, but does not allow translocation (Table 2).

In summary, the substrate binding pockets of CitP and MleP are found to be very much alike, especially where the properties are concerned that give the transporters their physiological function as precursor−product exchangers. The binding pocket contains sites that specifically interact with the carboxylate and the hydroxyl of the 2-hydroxycarboxylate motif that is common to precursors and product (Figure 6). A separate site in the pocket interacts with the second carboxylate of the *S*-enantiomers of di- and tricarboxylates, resulting in a high affinity for the precursors and a strong stereoselectivity for dicarboxylates. Both interactions with the carboxylate groups may be mediated by positively charged amino acid residues on the protein. On opposite sides in the pocket, at R_R, a hydrophobic surface rejects the carboxylates of the *R*-enantiomers of dicarboxylates and contributes to the affinity for substrates with large hydrophobic substituents. Optimal interaction with the substrate at the R_S and R_R positions in the pocket seems to require at least methyl groups. The difference in stereoselectivity between CitP and MleP for monocarboxylates was found to be due to differences in both affinity and translocation properties. CitP is quite tolerant of larger groups at the R_R position, and less tolerant of those at R_S, while MleP can accommodate larger groups at both R_S and R_R; however, the presence of a group at R_R prevents translocation.

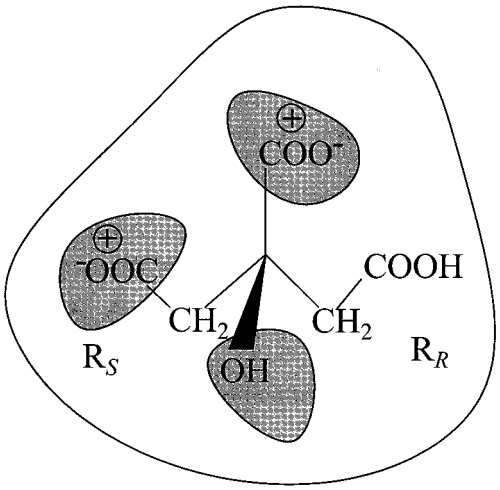


FIGURE 6: Model of the substrate binding site of CitP and MleP. Relevant interactions between substrate molecule and the protein are represented by gray surfaces. The substrate depicted in the pocket is citrate. Steric restrictions during translocation at R_R prevent turnover of citrate in MleP.

Substrate binding by MleP and CitP exhibits some interesting similarities to substrate binding by the Na^+/dicarboxylate cotransporter (NaDC-1) from renal brush borders (*21*, *22*). Simmilar to CitP and MleP, this transporter has a broad substrate specificity and its preferred substrates are divalent anions. Moreover, it was found that one carboxylate on the substrate is essential for binding and the other increases the affinity. Despite the absence of sequence homology between NaDC-1 and the MleP and CitP proteins, certain basic principles for binding and translocating carboxylic acids may apply.

Future studies on MleP and CitP will include a detailed investigation of mutant transporters for identifying the amino acid residues involved in the different interactions with the substrate as defined in this study.

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REFERENCES

1. Lolkema, J. S., Poolman, B., and Konings, W. N. (1996) in *Handbook of Biological Physics* (Konings, W. N., Kaback, H. R., and Lolkema, J. S., Eds.) pp 229−260, Elsevier, Amsterdam.
2. Anantharam, V., Allison, M. J., and Maloney, P. C. (1989) *J. Biol. Chem. 264*, 7244−7250.
3. Fu, D., and Maloney, P. C. (1997) *J. Biol. Chem. 272*, 2129−2135.
4. Fu, D., and Maloney, P. C. (1998) *J. Biol. Chem. 273*, 17962−17967.
5. Marty Teysset, C., Lolkema, J. S., Schmitt, P., Divies, C., and Konings, W. N. (1995) *J. Biol. Chem. 270*, 25370−25376.
6. Bandell, M., Ansanay, V., Rachidi, N., Dequin, S., and Lolkema, J. S. (1997) *J. Biol. Chem. 272*, 18140−18146.
7. Poolman, B., Molenaar, D., Smid, E. J., Ubbink, T., Abee, T., Renault, P. P., and Konings, W. N. (1991) *J. Bacteriol. 173*, 6030−6037.
8. Marty Teysset, C., Posthuma, C., Lolkema, J. S., Schmitt, P., Divies, C., and Konings, W. N. (1996) *J. Bacteriol. 178*, 2178−2185.
9. David, S., van der Rest, M. E., Driessen, A. J. M., Simons, G., and De Vos, W. M. (1990) *J. Bacteriol. 172*, 5789−5794.

10. Vaughan, E. E., David, S., Harrington, A., Daly, C., Fitzgerald, G. F., and De Vos, W. M. (1995) *Appl. Environ. Microbiol. 61*, 3172−3176.
11. Bandell, M., Lhotte, M. E., Marty Teysset, C., Veyrat, A., Prevost, H., Dartois, V., Divies, C., Konings, W. N., and Lolkema, J. S. (1998) *Appl. Environ. Microbiol. 64*, 1594−1600.
12. van der Rest, M. E., Siewe, R. M., Abee, T., Schwarz, E., Oesterhelt, D., and Konings, W. N. (1992) *J. Biol. Chem. 267*, 8971−8976.
13. Pos, K. M., and Dimroth, P. (1996) *Biochemistry 35*, 1018−1026.
14. van Geest, M., and Lolkema, J. S. (1996) *J. Biol. Chem. 271*, 25582−25589.
15. Pos, K. M., Bott, M., and Dimroth, P. (1994) *FEBS Lett. 347*, 37−41.
16. van Geest, M., Nilsson, I., von Heijne, G., and Lolkema, J. S. (1999) *J. Biol. Chem. 274*, 2816−2823.
17. Lopez de Felipe, F., Magni, C., de Mendoza, D., and Lopez, P. (1995) *Mol. Gen. Genet. 246*, 590−599.
18. Otto, R., Lageveen, R. G., Veldkamp, H., and Konings, W. N. (1982) *J. Bacteriol. 149*, 733−738.
19. Lowry, O. H., Rosebrough, N. J., Farr, A. L., and Randall, R. J. (1951) *J. Biol. Chem. 193*, 265−275.
20. Magni, C., de Mendoza, D., Konings, W. N., and Lolkema, J. S. (1999) *J. Bacteriol. 181*, 1451−1457.
21. Wright, S. H., Kippen, I., Klinenberg, J. R., and Wright, E. M. (1980) *J. Membr. Biol. 57*, 73−82.
22. Pajor, A. M. (1999) *Annu. Rev. Physiol. 61*, 663−682.

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