Membrane Potential-generating Transport of Citrate and Malate Catalyzed by CitP of Leuconostoc mesenteroides*

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Citrate uptake in Leuconostoc mesenteroides subsp. mesenteroides 19D is catalyzed by a secondary citrate carrier (CitP). The kinetics and mechanism of CitP were investigated in membrane vesicles of L. mesenteroides. The transporter is induced by the presence of citrate in the medium and transports both citrate and malate. In spite of sequence homology to the Na+-dependent citrate carrier of Klebsiella pneumoniae, CitP is not Na+-dependent, nor is CitP Mg2+-dependent. The pH gradient (ΔpH) is a driving force for citrate and malate uptake into the membrane vesicles, whereas the membrane potential (ΔΨ) counteracts transport. An inverted membrane potential (inside positive) generated by the generation of a membrane potential that contributes to the proton motive force across the cytoplasmic membrane, i.e. citrate metabolism in L. mesenteroides generates metabolic energy. Efficient exchange of citrate and D-lactate, a product of citrate/carbohydrate co-metabolism, is observed, suggesting that under physiological conditions, CitP function as an electrogenic precursor/product exchanger rather than a symporter. The mechanism and energetic consequences of citrate uptake are similar to malate uptake in lactic acid bacteria.

Citrate utilization in bacteria is mostly mediated by cation-dependent transport systems. Na+-dependent citrate transport has been described in Salmonella typhimurium and Klebsiella pneumoniae. The primary sequences of the genes coding for the transport proteins in these two organisms, CitC and CitS, respectively, are highly identical. CitP of K. pneumoniae transports citrate in symbiont with two sodium ions and one proton. The latter two observations place the citrate carrier of lactic acid bacteria in the class of secondary transporters that generate a membrane potential by translocating negative charge into the cell. Such transporters are involved in secondary metabolic energy-generating pathways that generate a proton motive force as a result of an electrogenic transport step and proton consumption in cytoplasmic metabolic steps (for a recent review, see Poolman (1993) and Konings et al. (1995)). Malate fermentation in lactic acid bacteria is a well known example. Internalized malate is decarboxylated by malolactic enzyme to yield lactate and carbon dioxide.

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‡ The abbreviations used are: kb, kilobase(s); cit, citrate; Hcit2+, divalent anionic citrate; H2cit1−, monovalent anionic citrate; pmf, proton motive force; COVs, proteoliposomes containing cytochrome c oxidase; TMPD, N,N,N′,N′-tetramethyl-p-phenylenediamine.

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Membrane vesicles of L. mesenteroides were prepared by the osmotic shock lysis procedure essentially as described previously by Otto et al. (1982). Lysis was improved by treating the cells with 18 mg/ml lysozyme (muramidase, Merck) in 100 mM potassium phosphate, 10 mM MgSO4, pH 7.0, for 1 h at 30°C. Cells were lyzed by rapid addition of 250 μg/ml DNase (deoxyribonuclease I from bovine pancreas, Sigma) and 250 μg/ml RNase (ribonuclease A from bovine pancreas, Sigma) in 100 mM potassium phosphate, 10 mM MgSO4, pH 7.0, for 30 min at 30°C. After incubation with 25 mM DTA for 10 min at 30°C and addition of 10 mM MgSO4, lysed cells were collected by centrifugation (48,200 × g for 30 min at 4°C) and resuspended in 50 mM potassium phosphate, pH 7.0, without MgSO4. Cells and debris were removed by a low spin (750 × g for 1 h), after which the vesicles were collected by spinning the supernatant at 48,200 × g for 30 min at 4°C and subsequently resuspended in 50 mM potassium phosphate, pH 7.0. Residual whole cells in the vesicle preparation were eliminated by extrusion through a 400-nm pore size polycarbonate filter (Avestin, Inc.). Membrane vesicles were rapidly frozen and stored in liquid nitrogen. The protein concentration was determined by the method of Lowry et al. (1951).

Preparation of Hybrid Membranes

L-α-Phosphatidylethanolamine was purified from 1 g of E. coli extract (Avanti Polar Lipids, Inc.) by successive washing with acetone and diethyl ether. L-α-Phosphatidylethanolamine concentration was determined as described by Driessen et al. (1991). Cytochrome c oxidase isolated from beef heart mitochondria was reconstituted into liposomes by detergent dialysis. The liposomes consisted of purified E. coli lipids and egg phosphatidylcholine in a 3:1 ratio (Driessen et al., 1985). Proteoliposomes containing cytochrome c oxidase (COVs) were fused with membrane vesicles of L. mesenteroides (1 mg of protein/10 mg of lipids) by freezing in liquid nitrogen followed by slow thawing at room temperature (Driessen et al., 1985). The resulting hybrid membranes were made unilamellar by sonication (eight cycles of 15 s on and 45 s off, amplitude of 4–6 mm) or by extrusion using successively 400- and 200-nm pore size polycarbonate filters (Mayer et al., 1986). Membrane vesicles were fused with liposomes lacking cytochrome c oxidase by the same procedure. Hybrid membranes were concentrated by centrifugation (250,000 × g for 45 min at 4°C).

Transport Assays

All transport studies were performed in 50 mM potassium phosphate, pH 6, at a membrane protein concentration of 0.25–0.3 mg/ml, unless otherwise stated, and at 30°C. Valinomycin and nigericin were used at final concentrations of 1 and 0.5 μM, respectively.

pmf-driven Uptake—The experiments were performed under a flow of water-saturated air. Membrane vesicles fused with COVs were incubated for 10 min in the presence or absence of ionophores. The hybrid membranes were energized by addition of 200 mM N,N,N′,N″-tetramethyl-p-phenylenediamine (TMPD), 20 μM cytochrome c (from horse heart, Sigma), and 10 mM potassium ascorbate. After incubation for 1 min, the radiolabeled substrates were added. Samples (100 μl) were taken at subsequent time points, transferred into 2 ml of ice-cold 0.1 mM LiCl to stop the reaction, and filtered through 0.45-μm pore size cellulose-nitrate filters (Schleicher & Schuell). Filters were rinsed with 2 ml of ice-cold 0.1 mM LiCl and transferred to scintillation vials, and the internalized radioactivity was determined. [1,5-14C]Citrate, L-[U-14C]leucine, and L-[U-14C]malate were used at final concentrations of 4.5, 1.6, and 7.8 μM, respectively. Control membranes were incubated with radiolabeled substrate in the absence of potassium ascorbate and without aeration.

Artificial Gradient-driven Uptake—Membrane vesicles fused with liposomes devoid of cytochrome c oxidase were energized at 30°C for 10 min in 50 mM potassium phosphate, pH 7, containing ionophores and the radiolabeled substrates. An artificial pH gradient was generated by adding a small aliquot of 0.2 N H3SO4, resulting in a pH drop of 1 unit. Uptake assays were performed as described above.

The generation of an inverted membrane potential (Δψ) (positive inside) was obtained by a thiocyanate diffusion potential. Hybrid membranes were prepared as described above and concentrated in 50 mM potassium phosphate, pH 6, containing 100 mM potassium thiocyanate. At the zero time point, concentrated hybrid membranes were diluted 100-fold into the same buffer without SCN− containing 4.5 μM [1,5-14C]citrate or 7.8 μM L-[U-14C]malate.

Exchange Measurements—Hybrid membranes obtained by fusion of membrane vesicles of L. mesenteroides with liposomes lacking cytochrome c oxidase were loaded with 5 mM citrate as described above. Concentrated hybrid membranes were incubated with 5 μM [1,5-14C]citrate for 30 min at 20°C. After incubation with valinomycin and nigericin, hybrid membranes were diluted 100-fold into buffer containing 5 mM unlabeled substrates.

Measurement of Δψ and ΔpH

The membrane potential was determined from the distribution of the lipophilic cation tetrathenylphosphonium+ using a tetrathenylphosphonium−selective electrode (Lokkena et al., 1982). The membrane potential was calculated from the Nerst equation after correction for concentration-dependent binding of the probe to the membrane. The estimated binding constant was 189. The specific internal volume of the hybrid membranes was assumed to be 8 μl/mg of protein (Driessen et al., 1985). The transmembrane pH gradient was measured with the fluorescent membrane-impermeable dye 8-hydroxy-1,3,6-pyrene trisulfonate (pyranine) as described previously by Dimiano et al. (1984). Conditions for the measurements were similar to those described for the transport experiment.

Chemicals

[1,5,14C]Citrate (110 mCi/mmol), L-[U-14C]leucine (319 mCi/mmol), [31P]potassium thiocyanate (55 mCi/mmol), and L-[U-14C]malate (48 mCi/mmol) were obtained from Amersham International (Buckinghamshire, United Kingdom). Pyranine was obtained from Eastman Kodak Co.

RESULTS

Substrate Specificity—Citrate (Fig. 1A) and malate (Fig. 1B) transport was studied in membrane vesicles derived from L. mesenteroides cit+ cells grown in the presence or absence of citrate and from L. mesenteroides cit− cells lacking the 22-kb plasmid containing the citP gene, grown in the presence of citrate. Membrane vesicles were fused with proteoliposomes containing cytochrome c oxidase as a protonotive force-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 (cytochrome c/TMPD/...
Fig. 1. Inducibility and specificity of CitP of L. mesenteroides 19D. Shown is citrate (A), malate (B), and leucine (C) uptake in membrane vesicles of L. mesenteroides fused with COVs. Membrane vesicles were prepared from L. mesenteroides cit⁻ cells grown in the presence (●) or absence (○) of citrate and from L. mesenteroides cit⁺ cells grown in the presence of citrate (●). Transport assays were performed in the presence of 1 μM valinomycin. Concentrations were 4.5 μM citrate (A), 7.8 μM malate (B), and 1.6 μM leucine (C), not energized.

Fig. 2. Heterologous exchange catalyzed by CitP. Shown is the exchange of [¹⁴C]citrate (A) and [¹⁴C]malate (B) with unlabeled citrate and malate. A control experiment with leucine is also shown (C). Membranes of L. mesenteroides fused with COVs were allowed to accumulate [¹⁴C]citrate (4.5 μM), [¹⁴C]malate (7.8 μM), and [¹⁴C]leucine (1.6 μM) in the presence (○) or absence (●) of the electron donor system cytochrome c/trimethylindophenol/ascorbate and 1 μM valinomycin. At the arrows, unlabeled citrate (●) or malate (○) was added. Citrate was added at a concentration of 1 mM, and malate at 5 mM (A and C) or 1 mM (B).

potassium ascorbate). The highest level of citrate and malate accumulation was observed in hybrid membranes derived from cit⁻ cells grown in the presence of citrate. For both substrates, the rates of uptake were significantly lower in membranes derived from cit⁻ cells grown in the absence of citrate. As expected, the L. mesenteroides cit⁻ strain did not take up citrate, but also malate uptake was not observed. The integrity of the different membrane preparations was checked by the accumulation of leucine (Fig. 1C), which is catalyzed by a secondary pmf-driven transport system (Winters et al., 1991).

These results show that both citrate and malate uptake systems are encoded by a gene located on the 22-kb plasmid and are induced by citrate, which is consistent with a single carrier for both substrates. This suggestion was substantiated by demonstrating heterologous citrate/malate exchange. Internally accumulated [¹⁴C]citrate (Fig. 2A) and [¹⁴C]malate (Fig. 2B) could be chased by addition of an excess of both unlabeled citrate and unlabeled malate. To exclude the possibility that efflux of the radiolabeled substrates in these experiments would be caused by dissipation of the pmf instead of heterologous exchange, the same concentrations of citrate and malate were added to membrane vesicles that had accumulated [¹⁴C]leucine. No release of leucine was observed (Fig. 2C). Furthermore, uptake of citrate was inhibited in the presence of 5 mM unlabeled malate, whereas leucine uptake was not affected by 5 mM citrate or malate (data not shown). Taken together, these results show that the citrate carrier of L. mesenteroides transports both citrate and malate.

Co-ion Specificity—In various bacteria, citrate is transported in symport with cations such as H⁺, Na⁺, or Mg²⁺. The citP gene is homologous to citS of K. pneumoniae, which is a Na⁺/citrate symporter (van der Rest et al., 1992a). The nature of ions symported with citrate by CitP was studied in membrane vesicles fused with proteoliposomes. Citrate uptake was measured in the presence of varying concentrations of sodium or magnesium ions. Precautions were taken to avoid Na⁺ ion contaminations by using special potassium phosphate buffers that contained low concentrations of sodium. Uptake of citrate was not affected by the absence or presence of Na⁺ up to 25 mM. Magnesium ions form a soluble complex with citrate. Uptake of citrate was increasingly inhibited by increasing magnesium concentrations, showing that citrate is not transported in a complex with magnesium (data not shown). It is concluded that neither Mg²⁺ nor Na⁺ is cotransported with citrate by CitP of L. mesenteroides.

Driving Force for Uptake of Citrate and Malate—The protonotive force generated by cytochrome c oxidation is composed of a membrane potential (Δψ) and a pH gradient (ΔpH). Uptake of citrate and malate (Fig. 3) and the magnitude of ΔpH and Δψ (Table I) were measured under the same experimental conditions. The role of each component of the pmf in driving citrate uptake was investigated in more detail by manipulating ΔpH and Δψ with the ionophores nigericin and valinomycin. In the absence of ionophores, when the pmf is highest (~125 mV), a low but significant citrate accumulation was observed. In the presence of nigericin, when the pmf is highest (~125 mV), no uptake of citrate occurred, indicating that the membrane potential is not a driving force for citrate transport. On the other hand, in the presence of valinomycin, when the pmf is only composed of a pH gradient of ~70 mV, a strong stimulation of citrate uptake was observed. In the lower portion of Table I, the driving force on citrate transport was calculated from the membrane potential and pH gradient assuming different proton/citrate transport stoichiometries (first column). The forces correlate with the equilibrium accumulation levels for citrate that are obtained with the different transport mechanisms. The final accumulation levels of citrate in Fig. 3A correlate with forces of about ~30 mV in the absence of ionophores, ~100 mV in the presence of valinomycin and a zero or positive force in the presence of nigericin.
These steady-state values fit qualitatively best with the Hct\(^2^+\)/H\(^+\) mechanism. The translocation of net negative charge by the carrier explains the poor uptake in the absence of ionophores when the driving force is low due to the counteracting membrane potential. Only after dissipation of the membrane potential was a high citrate uptake observed. Uptake of malate catalyzed by CitP was found to be qualitatively similar to citrate (Fig. 3B). Malate transport via CitP also is driven by \(\Delta\psi\) while \(\Delta\psi\) acts as a counteractive force. The effect of \(\Delta\psi\) on citrate and malate transport was further investigated with artificial gradients.

If indeed \(\Delta\psi\) (inside negative) acts as a counterforce for citrate uptake, \(\Delta\psi\) of opposite polarity (inside positive) should be able to drive citrate uptake. An inverted membrane potential was generated in membrane vesicles of \(L\). mesenteroides fused with liposomes lacking cytochrome \(c\) oxidase by the “\(pH \) jump” technique (Maloney and Hansen, 1982). The external \(pH\) was rapidly dropped from 7.0 to 6.0 by adding sulfuric acid, which created a \(pH\) gradient (inside alkaline). Since the membrane is less permeable to sulfate ions than to protons, the diffusion of protons through the membrane generates a diffusion potential (positive inside). At equilibrium, the pmf equals zero. The formation of inverted \(\Delta\psi\) is evidenced by uptake of the permeable anion SCN\(^-\) (Fig. 4B, ○). \(\Delta\psi = 62 \text{ mV} \) could be estimated from the level of SCN\(^-\) accumulation, which is in agreement with a \(pH\) jump of 1 unit. Under these conditions, no leucine uptake was observed, which is consistent with a zero pmf (Fig. 4A, ○). Only after quenching of the inverted membrane potential by valinomycin was \(\Delta\psi\)-driven leucine accumulation observed (Fig. 4A, ●). The highest level of citrate accumulation was observed when both a \(pH\) gradient (inside alkaline) and an inverted membrane potential were present (Fig. 4C, ○). Dissipation of the inverted membrane potential by valinomycin results in a strong reduction of citrate uptake (Fig. 4C, ●).

The complementary experiment, in which an artificial thiocyanate diffusion potential was generated, is shown in Fig. 5. Membrane vesicles of \(L\). mesenteroides fused with liposomes were loaded with potassium thiocyanate. Thiocyanate is negatively charged and diffuses passively out of the membranes upon dilution, generating an inverted membrane potential (inside positive). In response to \(\Delta\psi\), \(\Delta\psi\) of opposite polarity (inside negative) should develop by proton diffusion out of the hybrid membranes. Again, the highest level of citrate uptake was observed in the presence of inverted \(\Delta\psi\) and \(\Delta\psi\) of normal polarity (Fig. 5A, ○). The same result was obtained with malate as the substrate (Fig. 5B, ○). When the \(pH\) gradient was dissipated by addition of nigericin, still significant uptake of citrate and malate occurred, showing that both substrates are accumulated when an inverted membrane potential is the only gradient across the membrane (Fig. 5, ●). These results confirm that CitP translocates citrate and malate with net negative charge across the membrane. Under physiological conditions, the membrane potential is a counteractive force for citrate and malate accumulation.

![Image](https://example.com/image1.png)

**Fig. 3.** Effect of ionophores on citrate (A) and malate (B) uptake in the presence of a protonotive force. Citrate (4.5 \(\mu\)M) and malate (7.8 \(\mu\)M) uptake by membrane vesicles fused with COVs was assayed in the presence of no ionophores ( ●), valinomycin (○), and nigericin ( ▽ ). The electron donor system cytochrome c/ TMPD/potassium ascorbate was present in all samples. Control membranes were incubated with labeled substrates in the absence of potassium ascorbate was present in all samples. Control membranes were incubated with labeled substrates in the absence of potassium ascorbate and without aeration with water-saturated air ( ▽ ).

![Image](https://example.com/image2.png)

**Fig. 4.** Leucine (A), SCN\(^-\) (B), and citrate (C) uptake driven by a \(pH\) jump. Membrane vesicles fused with liposomes were equilibrated with 4.5 \(\mu\)M citrate, 1.6 \(\mu\)M leucine, 2.3 \(\mu\)M thiocyanate in 50 mM potassium phosphate, pH 7.0. Uptake was initiated by addition of sulfuoric acid (0.5 \(\mu\)l), which resulted in a drop of the external \(pH\) from 7 to 6, thereby generating \(\Delta\psi\). Experiments were performed in the absence (●) or presence (○) of 1 \(\mu\)M valinomycin. ▽, no acid was added.

![Image](https://example.com/image3.png)

**Fig. 4.** Leucine (A), SCN\(^-\) (B), and citrate (C) uptake driven by a \(pH\) jump. Membrane vesicles fused with liposomes were equilibrated with 4.5 \(\mu\)M citrate, 1.6 \(\mu\)M leucine, 2.3 \(\mu\)M thiocyanate in 50 mM potassium phosphate, pH 7.0. Uptake was initiated by addition of sulfuoric acid (0.5 \(\mu\)l), which resulted in a drop of the external \(pH\) from 7 to 6, thereby generating \(\Delta\psi\). Experiments were performed in the absence (●) or presence (○) of 1 \(\mu\)M valinomycin. ▽, no acid was added.

![Image](https://example.com/image4.png)

**Fig. 4.** Leucine (A), SCN\(^-\) (B), and citrate (C) uptake driven by a \(pH\) jump. Membrane vesicles fused with liposomes were equilibrated with 4.5 \(\mu\)M citrate, 1.6 \(\mu\)M leucine, 2.3 \(\mu\)M thiocyanate in 50 mM potassium phosphate, pH 7.0. Uptake was initiated by addition of sulfuoric acid (0.5 \(\mu\)l), which resulted in a drop of the external \(pH\) from 7 to 6, thereby generating \(\Delta\psi\). Experiments were performed in the absence (●) or presence (○) of 1 \(\mu\)M valinomycin. ▽, no acid was added.

![Image](https://example.com/image5.png)

**Fig. 4.** Leucine (A), SCN\(^-\) (B), and citrate (C) uptake driven by a \(pH\) jump. Membrane vesicles fused with liposomes were equilibrated with 4.5 \(\mu\)M citrate, 1.6 \(\mu\)M leucine, 2.3 \(\mu\)M thiocyanate in 50 mM potassium phosphate, pH 7.0. Uptake was initiated by addition of sulfuoric acid (0.5 \(\mu\)l), which resulted in a drop of the external \(pH\) from 7 to 6, thereby generating \(\Delta\psi\). Experiments were performed in the absence (●) or presence (○) of 1 \(\mu\)M valinomycin. ▽, no acid was added.
TABLE II

Effect of external pH on the kinetic parameters of citrate counterflow

Membrane vesicles fused with liposomes were loaded with 4 mM unlabeled citrate in the presence of ionophores and then diluted 100-fold into 50 mM potassium phosphate at different pH values and containing [14C]citrate ranging from 42 to 500 mM. The initial rate of citrate uptake was determined from the amount of label accumulated during the first 5 s. The three 

| pH   | V_max | K_m (app) | K_m | K_m | K_m |
|------|-------|-----------|-----|-----|-----|
|      | noml min⁻¹ mg⁻¹ | Total citrate | H₂cit⁻ | H₂cit⁻ | H₂cit⁻ |
| 5    | 52.4  | 42        | 0.2  | 15.7 | 25  | 1   |
| 5.5  | 38.8  | 13.8      | 0.008| 2.07 | 10.4| 1.3 |
| 6.0  | 36.7  | 18.2      | 0.001| 0.8  | 12.5| 4.9 |
| 6.5  | 36.8  | 47.2      | 0.00016| 0.41 | 20.7| 26  |

Kinetic Characterization of Citrate Transport—Counterflow experiments were used to kinetically characterize citrate uptake. In the pH range 5–6.5, the maximal rate is rather constant at ~40 nmol min⁻¹ mg⁻¹. Also, the affinity constant of CitP for citrate did not change a lot and appears to be quite high, e.g., at pH 5.0, K_m(app) = 42 μM (Table II). To determine the species of citrate transported by CitP, affinity constants for citrate were calculated at the different pH values for the different citrate species (Table II). The K_m(app) of the transport system was found to be almost constant in the pH range 5–6.5 for the monoprotonated form of citrate (H₄cit⁻). In contrast, the affinity constants calculated for cit⁻ and for H₂cit⁻ and H₂cit decrease drastically and systematically in this pH range. Assuming one of the ionic species is transported by the carrier, these results indicate that H₂cit⁻ is recognized and transported by the citrate carrier of L. mesenteroides.

Exchange with Metabolic Products—Transporters catalyzing translocation of net negative charge into the cell play a crucial role in secondary metabolic energy generation. They function either as uniporters or as exchangers that couple the uptake of a substrate to the excretion of a metabolic end product (precursor/product exchange). Typically, the latter class of transporters catalyzes exchange faster than unidirectional transport (Salama et al., 1994). Hybrid membranes were allowed to accumulate citrate driven by a pH gradient, after which nigericin (efflux) or nigericin plus unlabeled citrate (exchange) were added. Under these conditions, CitP performed homologous exchange faster than efflux (Fig. 6), suggesting that under physiological conditions, the carrier may function as an exchanger rather than a unidirectional transporter. The same technique cannot be used to show exchange with acetate or lactate since addition of these substrates at micromolar concentrations collapses the pH gradient by rapid diffusion of the protonated species across the membrane. To demonstrate heterologous exchange with metabolic end products, hybrid membranes were preloaded with [¹⁴C]citrate followed by dilution into medium containing the weak acids. Ionophores were present to prevent the generation of ΔpH by diffusion of protonated lactate or acetate. The experiments were performed at 20 °C to slow down the processes. An additional advantage of the lower temperature is that it affects efflux more strongly than exchange (Fig. 7, compare C and A). A slow release of citrate was observed in the absence of a counter-substrate (efflux) and in the presence of external acetate. Interestingly, exchange between citrate and lactate was observed. d-Lactate consistently resulted in faster exchange than l-lactate, suggesting higher affinity of the carrier for d-lactate than for l-lactate. These results show that, in addition to citrate and malate, CitP transports l- and d-lactate.
citP genes coding for citrate transporters have been cloned from Lactococcus lactis, Leuconostoc lactis, and L. mesenteroides and were found to be virtually the same. The citP genes are located on different endogenous plasmids in the three organisms. Regulation of expression may differ among the organisms. The Lactococcus lactis citP gene is located on a 5.6-kb plasmid. Magni et al. (1994) demonstrated that the transcript of citP and citrate uptake are independent of the presence of citrate in the growth medium, i.e., CitP is constitutively expressed. In L. mesenteroides, citP is located on a much bigger plasmid of 22 kb. The present results show that citrate acts as an inducer of CitP expression. Membrane vesicles from cells of L. mesenteroides grown in the absence of citrate were significantly more active in citrate uptake than those from cells grown in the absence of citrate (Fig. 1).

The primary sequence of CitP is 30% identical to the Na+-dependent citrate carrier of K. pneumoniae (CitS). Hydrophathy profiling reveals an even stronger structural similarity between the two proteins (van der Rest et al., 1992a; Lokkema et al., 1994b). Translocation of citrate by CitS is obligatory coupled to the translocation of Na+ ions (Lokkema et al., 1994a). However, CitP is not Na+ ion-dependent. As observed in other families of homologous secondary transporters, structural similarity does not necessarily correlate with cation specificity. Surprisingly, CitP not only catalyzes citrate transport, but also malate and lactate transport. Malate transport by CitP could be demonstrated by the correlation between the ability of membrane vesicles to accumulate malate and the expression of CitP (Fig. 1) and by heterologous exchange between citrate and malate (Fig. 2). Transport of lactate via CitP was demonstrated by its accelerating effect on citrate efflux, which is explained as citrate/lactate exchange (Fig. 7). Both d- and L-lactate showed the accelerating effect, indicating that the carrier is not stereoselective. Citrate (Structure 1, i), malate (ii), and lactate (iii) are structurally related, suggesting that CitP is a general carrier for hydroxycarboxylic acids presenting the motive R1R2CHOHCOOH.

\[
\begin{align*}
C-\text{COOH} & \\
\text{HO-C-}\text{COOH} & \\
\text{HO-C-}\text{COOH} & \\
\text{HO-C-}\text{COOH} & \\
\text{HO-C-}\text{COOH} & \\
\end{align*}
\]

Structure 1

The following results indicate that the citrate carrier of L. mesenteroides catalyzes translocation of net negative charge. (a) Citrate uptake driven by the proton motive force is largely stimulated when the membrane potential component is dissipated; (b) the highest accumulation level of citrate is observed in the presence of a pH gradient of physiological polarity and a membrane potential of opposite polarity; and (c) an inverted membrane potential by itself can drive the accumulation of citrate into the membranes. The analysis of the forces (see Fig. 3 and Table I) shows that a single unit of charge is translocated per catalytic cycle, i.e., Hcit2+/Hcit2−, or cit−2/2H+. Translocation of 2 units of charge would result in an outwardly directed force on citrate in the absence of ionophores, which is not observed. Kinetic analysis of citrate transport in membrane vesicles of L. mesenteroides shows that the dianionic form of citrate (Hcit2−) is recognized by CitP (Table II). Therefore, CitP catalyzes symport of Hcit2− with 1H+. The characteristics of malate transport were similar to those observed for citrate, i.e., CitP catalyzes malate2−/H+ symport. Divalent anionic citrate is also the species translocated by the Na+− and H+−-dependent citrate carriers of K. pneumoniae. However, these carriers translocate positive charge across the membrane, i.e., CitH catalyzes Hcit2−/3H+ symport, and CitS catalyzes Hcit2−/2Na+−/H+ symport. In L. oenos, citrate transport is catalyzed by a unipporter that recognizes Hcit−1 (Ramos et al., 1994). These observations suggest that the form of citrate that is recognized by the transporter is determined by the pH of the medium at which the organism normally grows. L. oenos grows in wine at low pH, where Hcit−1 is the predominant species, whereas L. mesenteroides and K. pneumoniae grow at more neutral pH values, where Hcit−2 is the most abundant protonation state.

Transport of citrate by CitP results in the generation of a membrane potential (inside negative) in L. mesenteroides. Inside the cell, citrate is split in oxaloacetate and acetate by citrate lyase. Subsequently, oxaloacetate is decarboxylated, yielding pyruvate and carbon dioxide, in a reaction that consumes a scalar proton from the cytoplasm. The overall result of citrate metabolism is the generation of a proton motive force in a similar way to that observed during malate fermentation in lactic acid bacteria. There appears to be a remarkable parallel between citrate and malate uptake in these bacteria. In L. oenos, both substrates are transported by a unipporter mechanism translocating net negative charge into the cell as Hcit−2 and monovalent malate (Ramos et al., 1994; Salema et al., 1994). In Lactococcus lactis, the malate transporter catalyzes in vivo negative charge translocation, most likely as malate2−/H+ symport. CitP of L. mesenteroides catalyzes symport of both Hcit2− and malate2− with one proton. However, in vivo, the malate carrier of Lactococcus lactis catalyzes exchange of divalent malate with monovalent lactate, which is the product of malate decarboxylation (precursor/product exchange). Exchange experiments showed that CitP has affinity for lactate as well (Fig. 7), and therefore, we hypothesize that in vivo CitP also functions as an electrogenic Hcit2−/lactate− exchanger. In Leuconostoc species, lactate is a product of citrate/carbohydrate co-metabolism. Pyruvate formed from oxaloacetate decarboxylation functions as an electron sink for the reducing equivalents generated by glycolysis and is stoichiometrically reduced to β-lactate (Starrenburg and Hugenholtz, 1991; Schmitt et al., 1992). Currently, we are investigating the in vivo mechanism of proton motive force generation by citrate metabolism in L. mesenteroides. Electrogenic citrate/lactate exchange catalyzed by CitP would make the analogy with malate metabolism complete. Already, it is clear that the citrate and malate carriers in lactic acid bacteria form a closely related family of secondary transporters. Recently, the mal operon of Lactococcus lactis containing the structural gene coding for the malate permease (malP) was sequenced. The primary sequences of MalP and CitP were found to be 50% identical.3

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