

## Proton Motive Force Generation by Citrolactic Fermentation in *Leuconostoc mesenteroides*

CLAIRE MARTY-TEYSSET,<sup>1,2</sup> CLARA POSTHUMA,<sup>1</sup> JUKE S. LOLKEMA,<sup>1\*</sup>  
PHILIPPE SCHMITT,<sup>2</sup> CHARLES DIVIES,<sup>2</sup> AND WIL N. KONINGS<sup>1</sup>

Department of Microbiology, Groningen Biotechnology and Biomolecular Sciences Institute,  
University of Groningen, 9751NN Haren, The Netherlands,<sup>1</sup> and Departement de  
Microbiologie-Biotechnologie, ENS-BANA, Université  
de Bourgogne, 21000 Dijon, France<sup>2</sup>

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In *Leuconostoc mesenteroides* subsp. *mesenteroides* 19D, citrate is transported by a secondary citrate carrier (CitP). Previous studies of the kinetics and mechanism of CitP performed in membrane vesicles of *L. mesenteroides* showed that CitP catalyzes divalent citrate  $\text{Hcit}^{2-}/\text{H}^+$  symport, indicative of metabolic energy generation by citrate metabolism via a secondary mechanism (C. Marty-Teyssset, J. S. Lolkema, P. Schmitt, C. Divies, and W. N. Konings, *J. Biol. Chem.* 270:25370–25376, 1995). This study also revealed an efficient exchange of citrate and D-lactate, a product of citrate/carbohydrate cometabolism, suggesting that under physiological conditions, CitP may function as a precursor/product exchanger rather than a symporter. In this paper, the energetic consequences of citrate metabolism were investigated in resting cells of *L. mesenteroides*. The generation of metabolic energy in the form of a pH gradient ( $\Delta\text{pH}$ ) and a membrane potential ( $\Delta\Psi$ ) by citrate metabolism was found to be largely dependent on cometabolism with glucose. Furthermore, in the presence of glucose, the rates of citrate utilization and of pyruvate and lactate production were strongly increased, indicating an enhancement of citrate metabolism by glucose metabolism. The rate of citrate metabolism under these conditions was slowed down by the presence of a membrane potential across the cytoplasmic membrane. The production of D-lactate inside the cell during cometabolism was shown to be responsible for the enhancement of the electrogenic uptake of citrate. Cells loaded with D-lactate generated a  $\Delta\Psi$  upon dilution in buffer containing citrate, and cells incubated with citrate built up a pH gradient upon addition of D-lactate. The results are consistent with an electrogenic citrate/D-lactate exchange generating *in vivo* metabolic energy in the form of a proton electrochemical gradient across the membrane. The generation of metabolic energy from citrate metabolism in *L. mesenteroides* may contribute significantly to the growth advantage observed during cometabolism of citrate and glucose.

In bacteria, metabolic energy present in the form of ATP and ion gradients of  $\text{H}^+$  and  $\text{Na}^+$  are used to drive various endergonic reactions associated with cellular growth. The two forms of metabolic energy can be interconverted by the action of membrane-bound  $\text{F}_0\text{F}_1$ -ATPases that couple the translocation of an  $\text{H}^+$  (or  $\text{Na}^+$ ) to the hydrolysis/synthesis of ATP. In fermentative organisms, ATP is usually formed by substrate-level phosphorylation (glycolysis; arginine deaminase pathway), which is subsequently used to generate an electrochemical gradient of protons across the cytoplasmic membrane (proton motive force [pmf]) (16). Recently, a different mechanism of pmf generation was discovered that is of particular importance in the energetics of certain anaerobes and allows the  $\text{F}_0\text{F}_1$ -ATPase to function in the synthesis mode, i.e., the pmf drives the synthesis of ATP. The mechanism involves the action of secondary transporters and is therefore termed secondary metabolic energy generation (7). The pmf is formed indirectly during the metabolic breakdown of weak acids. The anionic forms of the acids are transported into the cell by an electrogenic secondary carrier that translocates net negative charge into the cell, generating a membrane potential. The internal degradation of the substrate involves a decarboxylation step that consumes a scalar proton, which results in the

formation of a pH gradient (11). The anions are taken up in exchange with a metabolic end product of the pathway (precursor/product exchange) or by a uniport mechanism, in which case the end product leaves the cell by passive diffusion.

Examples of pathways using the exchange type of uptake are oxalate fermentation in *Oxalobacter formigenes* (1), malolactic fermentation in *Lactococcus lactis* (17), and histidine decarboxylation in *Lactobacillus buchneri* (15), and an example of a pathway using the uniporter mechanism is malate and citrate fermentation in the acidophilic bacterium *Leuconostoc oenos* (18, 20).

Cometabolism of glucose and citrate by the lactic acid bacterium *Leuconostoc mesenteroides* results in a growth advantage relative to growth on glucose alone. The increased growth rate is usually attributed to a metabolic shift in the heterofermentative pathway for glucose breakdown, yielding additional ATP (2, 4, 9, 21). In the absence of citrate, acetyl-P formed from glucose is reduced to ethanol, which balances the redox equivalents produced in the other steps of the phosphoketolase pathway (see also Fig. 1). In the presence of citrate, the redox equivalents are shuttled to pyruvate produced from citrate, yielding D-lactate, and acetyl-P is converted into acetate via the acetate kinase pathway, which results in the production of ATP. In a previous study in this laboratory, the catalytic properties of the citrate carrier of *L. mesenteroides*, coded by the *citP* gene (8a), were analyzed by using isolated membrane vesicles (13). The *in vitro* studies showed that CitP catalyzed the translocation of divalent citrate,  $\text{Hcit}^{2-}$ , in symport with one proton. The translocation of negative charge into the cell

\* Corresponding author. Mailing address: Department of Microbiology, University of Groningen, Kerklaan 30, 9751NN Haren, The Netherlands. Phone: 31-50-3632155. Fax: 31-50-3632154. Electronic mail address: J.S.LOLKEMA@BIOL.RUG.NL.

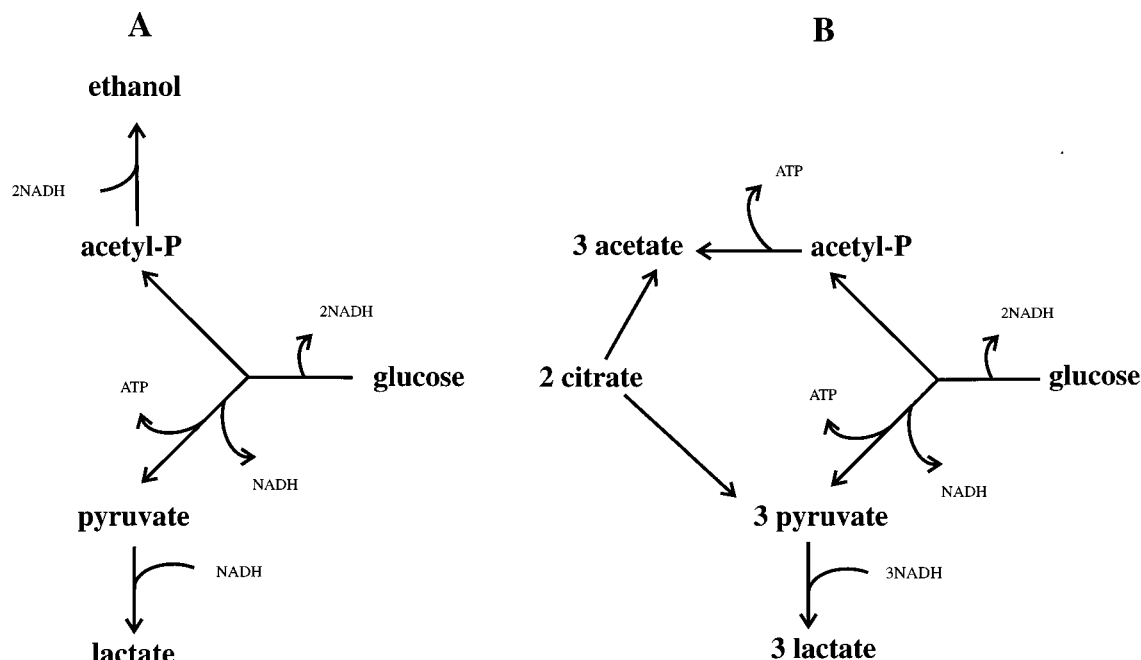


FIG. 1. Schematic representation of glucose metabolism (A) and glucose/citrate cometabolism (B) in *Leuconostoc* spp. The stoichiometries in panel B indicate the number of molecules necessary to balance the redox equivalents. Two of the three molecules of acetate and lactate originate from citrate; the other originates from glucose. Additionally, 1 molecule of  $\text{CO}_2$  is formed per molecule of citrate (not indicated).

suggests that *in vivo* citrate metabolism will be associated with secondary metabolic energy generation, which may contribute to the growth enhancement during cometabolism with glucose.

Energetically, the uptake of citrate via the  $\text{Hcit}^{2-}/\text{H}^+$  symport mechanism would be equivalent to the uptake of citrate via the  $\text{H}_2\text{cit}^-$  uniport mechanism observed in *L. oenos* (18). However, in addition to the symport reaction, it was shown that CitP catalyzed efficient exchange between citrate and lactate, opening up the possibility that the carrier may function *in vivo* as a precursor/product exchanger, since D-lactate is a product of citrate metabolism when cometabolized with glucose (citrolactic fermentation) (Fig. 1). It could not be concluded from the studies with the membrane vesicles whether or not the citrate/lactate exchange reaction is electrogenic.

In this study, we analyzed the energetic consequences of citrate metabolism in whole cells of *L. mesenteroides*. It will be shown that under physiological conditions, citrate consumption results in the generation of a pmf by a mechanism in which CitP functions as an electrogenic citrate/lactate exchanger. Secondary metabolic energy generation by citrate metabolism in *L. mesenteroides* is intimately associated with glucose metabolism.

#### MATERIALS AND METHODS

**Bacteria and growth conditions.** *L. mesenteroides* subsp. *mesenteroides* 19D was obtained from the Institut National de la Recherche Agronomique, Jouy en Josas, France. *L. mesenteroides* 19D Cit<sup>+</sup> was selected as blue colonies on citrate indicator plates (6). Cells were grown at 30°C in modified MRS broth without Tween 80 and acetate at pH 6.4 (3), containing ammonium citrate (2 g/liter) and glucose (20 g/liter). Cells were harvested in the exponential growth phase (optical density at 660 nm [ $\text{OD}_{660}$ ], 0.6 to 0.8), washed once, and resuspended in the appropriate buffer.

**Measurement of the membrane potential ( $\Delta\Psi$ ) and pH gradient ( $\Delta\text{pH}$ ).** The membrane potential in *L. mesenteroides* was calculated from the distribution of the lipophilic cation tetraphenylphosphonium ( $\text{TPP}^+$ ) that was measured with an ion-selective electrode as described elsewhere (10). The experiments were performed at 30°C in 20 mM potassium phosphate (pH 6.0) at a final protein concentration of 0.76 mg/ml (12). The membrane potential was calculated by using the Nernst equation after correction for concentration-dependent binding of the probe to cellular components (10). The estimated binding constant for

$\text{TPP}^+$  binding to cellular components was determined as described by Salema et al. (19) and found to be 8, with 58% of the binding site located in the cytoplasmic membrane and 42% located in the cytoplasm.

The transmembrane pH gradient was measured with the fluorescent indicator probe BCECF [2',7'-bis-(2-carboxyethyl)-5 (and-6) carboxyfluorescein] as described by Molenaar et al. (14). Cells concentrated in 50 mM potassium phosphate (pH 7.0) up to 7.6 mg/ml were loaded with the fluorescence probe BCECF by an acid shock. The experiments were performed in 100 mM potassium phosphate (pH 6.0) at 30°C.

In all measurements, citrate, pyruvate, and D-lactate were used at a final concentration of 2 mM, while the glucose concentration was 0.5 mM. Nigericin and valinomycin, which selectively dissipate the pH gradient and the membrane potential, respectively, were used at final concentrations of 1  $\mu\text{M}$ .

**Citrate utilization by resting cells of *L. mesenteroides*.** Cells were resuspended in 20 mM potassium phosphate (pH 6.0) and incubated for 10 min at 30°C. Citrate utilization was initiated in a total volume of 2 ml by addition of 2 mM citrate (final concentration) and lactic acid and glucose. At various time points, 200  $\mu\text{l}$  of cell suspension was immediately centrifuged for 30 s. Samples from the supernatant devoid of cells were analyzed for residual citrate and metabolic products. Citrate, pyruvate, and D-lactate concentrations were measured with commercially available enzyme kits (Boehringer, Mannheim, Germany). Pyruvate was determined with the kit for citrate determination, using a slightly adjusted protocol. The decrease in NADH concentration was measured at  $\text{OD}_{340}$  after addition of the sample of the supernatant to the buffer containing NADH, malate dehydrogenase, and L-lactate dehydrogenase. Pyruvate in the sample is converted by L-lactate dehydrogenase to L-lactate at the expense of NADH. Subsequent addition of citrate lyase converts citrate in the sample to oxaloacetate (and pyruvate) and results in an additional decrease in the NADH concentration which is equivalent to the citrate concentration. *L. mesenteroides* is known to produce exclusively D-lactate, which does not interfere with the assay.

**External pH measurement.** External pH was continuously measured with a pH electrode. Experiments were performed at 30°C in 20 mM potassium phosphate (pH 6.0). Concentrated cells (500  $\mu\text{l}$ ) were added to 4.5 ml of buffer containing the substrates. Citrate, D-lactate, and glucose were used at final concentrations of 2, 2, and 0.5 mM, respectively.

**Chemicals.** The fluorescent probe BCECF was obtained from Molecular Probes, Eugene, Oreg. All other chemicals were reagent grade and were obtained from commercial sources.

#### RESULTS

**Generation of a pmf by citrate metabolism in *L. mesenteroides*.** The two components of the pmf, the membrane potential,  $\Delta\Psi$  (Fig. 2), and the pH gradient,  $\Delta\text{pH}$  (Fig. 3), were

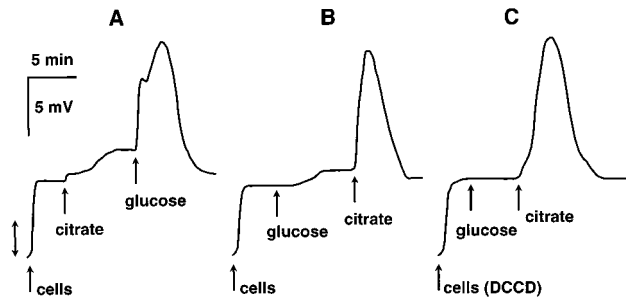


FIG. 2. Generation of a membrane potential by citrate metabolism in resting cells of *L. mesenteroides*. A 0.1-ml amount of untreated cells (A and B) or cells treated with 100  $\mu$ M DCCD for 30 min at 30°C (C) were added to 0.9 ml of 20 mM potassium phosphate (pH 6.0) containing 4  $\mu$ M TPP<sup>+</sup> to give a final protein concentration of 0.76 mg/ml. The uptake of TPP<sup>+</sup> by the cells was monitored with a TPP<sup>+</sup>-selective electrode. An increase in the response of the TPP<sup>+</sup> electrode indicates uptake of TPP<sup>+</sup> by the cells, which correlates with a negative membrane potential across the cytoplasmic membrane. The response caused by dilution upon the addition of cells is indicated by the arrow on the left. The immediate response upon addition of citrate in panel A is an artifact caused by citrate on the TPP<sup>+</sup> electrode. Arrows indicate the addition of 2 mM citrate or 0.5 mM glucose (final concentrations).

measured upon addition of citrate to resting cells of *L. mesenteroides*. The membrane potential was inferred from the distribution of the lipophilic cation TPP<sup>+</sup>, and the pH gradient was measured with the fluorescent pH indicator BCECF. Both methods allow instant monitoring of changes in the gradients. Resting cells of *L. mesenteroides* maintain a low pmf of -41 mV that is composed solely of a membrane potential (Table 1). Addition of citrate to the cells resulted in a small increase in the membrane potential to -53 mV without generation of a pH gradient (Fig. 2A and 3A). Successive addition of a low concentration of glucose (0.5 mM) led to a significant increase in both  $\Delta\Psi$  and  $\Delta$ pH. Both gradients were transient and relaxed to the resting potentials. Addition of nigericin (Fig. 3), a K<sup>+</sup>/H<sup>+</sup> antiporter, and valinomycin (not shown), a K<sup>+</sup> ionophore, resulted in immediate dissipation of the  $\Delta$ pH and the  $\Delta\Psi$ , respectively. The maximal magnitude of the  $\Delta\Psi$  and the  $\Delta$ pH was -87 and -32 mV, respectively, resulting in a pmf of -119 mV.

The increase in pmf upon addition of glucose might be explained by ATP formation from glucose metabolism, which

TABLE 1. Pmf generated by resting cells of *L. mesenteroides* in the presence of glucose and citrate<sup>a</sup>

Addition to medium		$\Delta\Psi$ (mV)	$\Delta$ pH (mV)	pmf (mV)
Glucose	Citrate			
-	-	-41	0	-41
+	-	-51	-15	-66
-	+	-53	0	-53
+	+	-87	-32	-119

<sup>a</sup> The magnitudes of the membrane potential and the pH gradient were calculated from the experiments presented in Fig. 2 and 3.

allows the generation of a pmf by F<sub>0</sub>F<sub>1</sub>-ATPase at the expense of ATP. However, the same glucose concentration in the absence of citrate results in  $\Delta\Psi$  and  $\Delta$ pH values of only -51 and -15 mV, respectively (Fig. 2B and 3B, respectively). Furthermore, addition of citrate following addition of glucose resulted in much higher increases in  $\Delta\Psi$  and  $\Delta$ pH than observed with citrate alone (compare panels B and A in Fig. 2 and 3). Therefore, the presence of both citrate and glucose is required for high pmf generation, suggesting that the generation of  $\Delta\Psi$  and  $\Delta$ pH from citrate metabolism is linked to glucose metabolism.

To specify the role of glycolysis in more detail, cells were pretreated with *N,N'*-dicyclohexylcarbodiimide (DCCD), which blocks F<sub>0</sub>F<sub>1</sub>-ATPases. As expected, addition of glucose to the pretreated cells resulted in neither a membrane potential nor a pH gradient (Fig. 2C and 3D, respectively). However, successive addition of citrate resulted in a membrane potential as high as observed in the untreated cells (Fig. 2C). In addition, a small but significant pH gradient was generated (Fig. 3C). The magnitude of the latter may be obscured by the acidifying glycolysis, which in DCCD-treated cells is not compensated for by the removal of protons from the cells by F<sub>0</sub>F<sub>1</sub>-ATPase. At any rate, a pmf consisting of both a membrane potential and pH gradient is generated without the involvement of F<sub>0</sub>F<sub>1</sub>-ATPase. In conclusion, efficient pmf generation by citrate metabolism does not follow from ATP synthesis but does require glycolysis activity.

**Rate of citrate metabolism and product formation.** Citrate metabolism by resting cells of *L. mesenteroides* was studied in the presence and absence of glucose. In *L. mesenteroides*, the first metabolic step in the pathway for citrate breakdown is the

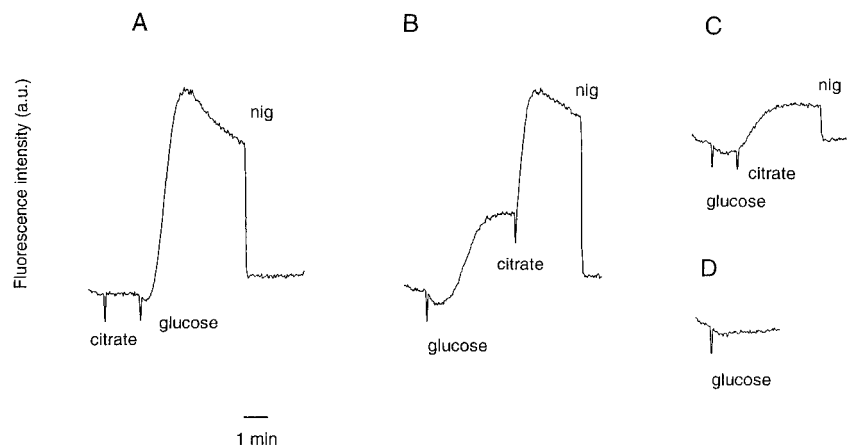


FIG. 3. Generation of a pH gradient by citrate metabolism in resting cells of *L. mesenteroides*. Cells were loaded with BCECF as described in the text, and the BCECF fluorescence was recorded. A 10- $\mu$ l amount of untreated cells (A and B) or cells treated with 100  $\mu$ M DCCD for 30 min at 4°C (C and D) was added to 3 ml of 100 mM potassium phosphate (pH 6.0). The dips in the traces indicate the points when citrate or glucose was added to final concentrations of 2 and 0.5 mM, respectively. Nigericin (nig) was used at a final concentration of 1  $\mu$ M.

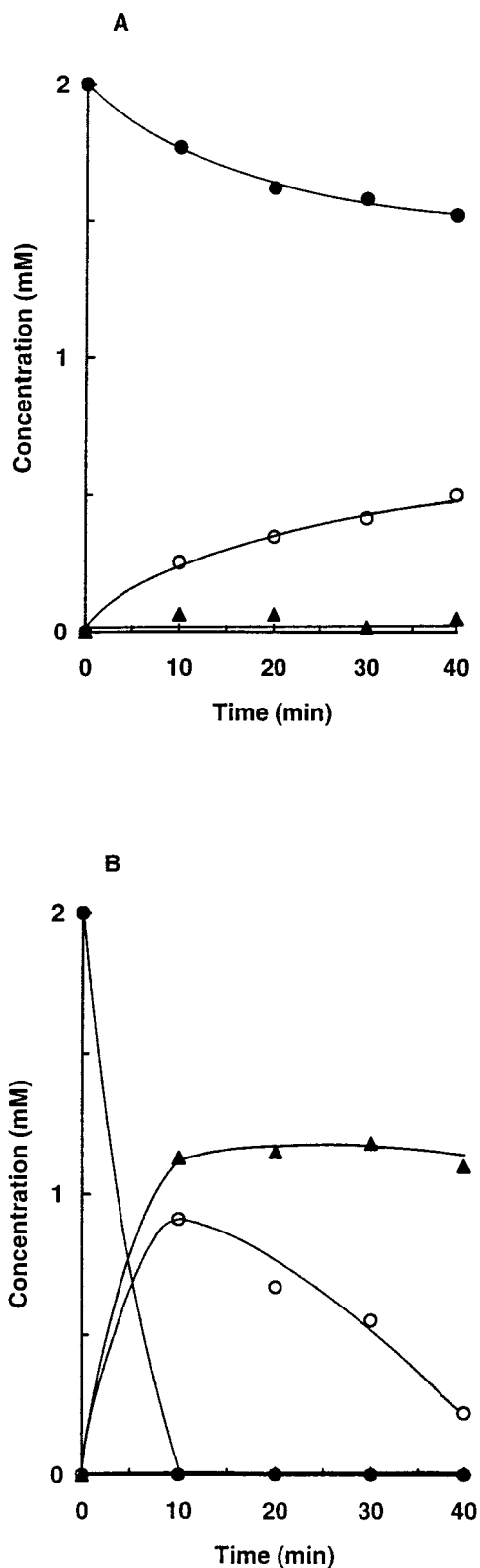


FIG. 4. Citrate consumption and pyruvate and lactate production by resting cells of *L. mesenteroides* in the absence (A) and presence (B) of glucose. Cells were incubated for 10 min in 100 mM potassium phosphate (pH 6.0) at 30°C at a cell protein concentration of 0.46 mg/ml. At time zero, 2 mM citrate was added alone (A) or simultaneously with 0.5 mM glucose (B). The external concentrations of citrate (●), lactate (▲), and pyruvate (○) were measured.

conversion into oxaloacetate and acetate by citrate lyase activity. Acetate leaves the cells, and oxaloacetate is decarboxylated, yielding pyruvate. Pyruvate is the precursor for D-lactate, butanediol, and aroma and flavor compounds like acetoin and diacetyl. Glucose metabolism leads to the production of D-lactate and ethanol by the phosphoketolase pathway (Fig. 1A). Cometabolism of glucose and citrate involves a metabolic shift from ethanol to acetate production in the conversion of acetyl-P (Fig. 1B). The reducing equivalents that in the absence of citrate are used for ethanol production are funneled to pyruvate produced from citrate, yielding D-lactate (citrolactic fermentation). The conversion of acetyl-P into acetate by acetate kinase yields an additional energy gain of 1 ATP per 2 molecules of citrate.

The rate of citrate utilization and the formation of the end products pyruvate and D-lactate were investigated in resting cells of *L. mesenteroides* (Fig. 4). In the presence of citrate alone, a slow rate of citrate consumption was observed (Fig. 4A). Citrate was stoichiometrically converted into pyruvate that was excreted into the medium (Fig. 4A). No lactate could be detected. Apparently under these conditions, further metabolism of pyruvate is blocked. In the presence of glucose at a concentration that is four times lower than the citrate concentration (0.5 and 2 mM, respectively), the rate of citrate utilization increased dramatically (Fig. 4B). Within 10 min, all citrate was consumed. The amount of D-lactate that is formed exceeds the concentration of glucose, which is consistent with the use of redox equivalents produced in the phosphoketolase pathway for the reduction of pyruvate formed from citrate. At first, pyruvate accumulated rapidly in the external medium, but subsequently, it was slowly metabolized by the cells, a conversion that was not observed in the absence of glucose. The rapid and complete metabolism of citrate correlates with the transient membrane potential that is generated under these conditions (Fig. 2). In fact, subsequent addition of new aliquots of citrate resulted in repetition of the transient membrane potential (data not shown).

**Alkalinization of the medium.** Citrate and glucose metabolisms are known to alkalinize and acidify the external medium, respectively. The stimulation of citrate metabolism by glucose metabolism can also be demonstrated by the effect on the medium pH. Figure 5 shows a slow and small alkalinization of the external medium after addition of citrate to resting cells of *L. mesenteroides*. This observation correlates with the slow consumption of citrate shown in Fig. 4A. In the presence of glucose alone, a rapid acidification of the medium occurred because of the production of lactic acid, followed by a slow alkalinization. At this moment, an explanation for the latter observation cannot be offered. In the presence of citrate and glucose, both metabolisms are involved. Again, a rapid acidification of the external medium was observed, but to a smaller extent than with glucose alone, indicative of the alkalinizing contribution of rapid citrate metabolism. The acidification was followed by slow alkalinization at a rate that correlates more or less with the pyruvate consumption rate in Fig. 4B.

**Effect of the membrane potential on citrate utilization.** The membrane potential generated in the transport step of citrate into the cells is a counteracting force on citrate uptake. The flux through the pathway as a whole will only be affected by the magnitude of the membrane potential when the transport step is significantly rate determining. Citrate consumption in the presence of glucose was monitored at a low cell density in a sodium phosphate buffer containing valinomycin, a  $K^+$  ionophore (Fig. 6). The presence of both citrate and glucose ensures the presence of a high membrane potential. Addition of 200 mM NaCl had no effect on the consumption rate. How-

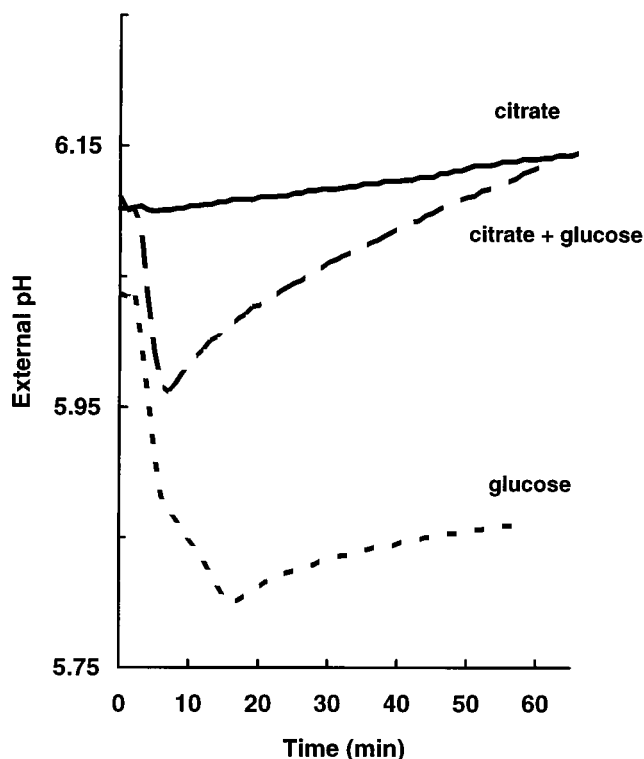


FIG. 5. Alkalinization of the external medium induced by citrate and glucose metabolism. Cells (0.5 ml) were added to 20 mM potassium phosphate (pH 6.0) containing 0.5 mM glucose, 2 mM citrate, or 0.5 mM glucose plus 2 mM citrate. The final protein concentration was 0.8 mg/ml. The external pH was measured continuously.

ever, addition of the same concentration of KCl caused an instantaneous dissipation of the membrane potential and resulted in a significant increase of the rate of citrate consumption. The result is consistent with the electrogenic nature of the citrate uptake step and shows that this step has significant control over the flux through the pathway under these conditions.

**Electrogenic citrate/D-lactate exchange.** The data so far show that in the presence of glucose, citrate metabolism efficiently generates metabolic energy in the form of  $\Delta\Psi$  and  $\Delta\text{pH}$  and that the rate of citrate metabolism is drastically increased. The rate enhancement must be a consequence of the formation of an intermediate or end product of glucose interacting with the citrate metabolic pathway. In vitro, the citrate carrier of *L. mesenteroides* is able to catalyze exchange between citrate and D-lactate, a product of glucose and citrate metabolism in *Leuconostoc* spp. (13). The citrate carrier CitP was shown to catalyze exchange faster than unidirectional transport of citrate, suggesting that the rate enhancement of citrate metabolism observed in whole cells in the presence of glucose may be a consequence of faster citrate uptake via citrate/lactate exchange. To demonstrate that the latter mode of transport is electrogenic, cells loaded with D-lactate were diluted in medium containing citrate. This resulted in the generation of a membrane potential of  $-91$  mV (Fig. 7B), which was significantly higher than the  $\Delta\Psi$  observed after dilution of unloaded cells or cells loaded with acetate ( $-61$  and  $-58$  mV in Fig. 7A and C, respectively).

Acetate is another main product of citrate/glucose metabolism (see Fig. 1) and has been proposed to be involved in pmf

generation by metabolism of citrate in *Lactococcus lactis* (5). However, the results presented here are at variance with this proposal and show that citrate/D-lactate exchange catalyzed by CitP is electrogenic and, most likely, responsible for the generation of the membrane potential during citrate/glucose co-metabolism.

**Enhancement of citrate metabolism in the presence of D-lactate.** The complete and rapid conversion of 2 mM citrate in the presence of only 0.5 mM glucose (Fig. 4B) shows that a low concentration of lactate, by itself, is already enough to speed up citrate metabolism. Indeed, simultaneous addition of 2 mM citrate and 2 mM D-lactate to cells results in a much higher rate of citrate metabolism than observed with citrate alone (compare Fig. 8 and 4A). Initially, citrate is stoichiometrically converted to pyruvate, but as the concentration in the external medium increases, the cells slowly start metabolizing it, as shown in Fig. 4B. The increase in the rate of citrate consumption was specific for D-lactate (and to a lesser extent for L-lactate) and not observed with acetate or pyruvate (data not shown). The rate of citrate utilization increased with the D-lactate concentration in the range from 1 to 4 mM (not shown). The effects observed on citrate consumption and pyruvate production with D-lactate were qualitatively the same as observed with glucose (Fig. 4B).

The enhancement of the rate of citrate metabolism in resting cells of *L. mesenteroides* in the presence of D-lactate is also evident from the alkalinization of the external medium. In the presence of citrate alone, only a slow alkalinization of the medium was observed. In the presence of both citrate and D-lactate, after a lag time, a much faster alkalinization was observed, whereas no pH change occurred in the presence of D-lactate alone (not shown).

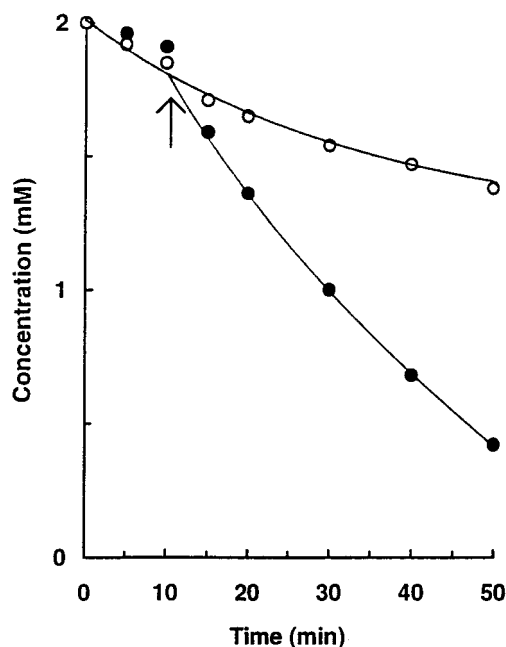


FIG. 6. Effect of the membrane potential on the rate of citrate utilization by resting cells of *L. mesenteroides*. Cells were resuspended in 20 mM sodium phosphate (pH 6.0) at a protein concentration of 0.076 mg/ml and incubated for 10 min in the presence of  $1 \mu\text{M}$  valinomycin. After energizing the cells for 5 min with 10 mM glucose, 2 mM citrate was added, and the citrate concentration in the medium was monitored. At the arrow, the membrane potential was dissipated by addition of 200 mM KCl (●). In the control experiment, 200 mM NaCl was added to the cells (○).

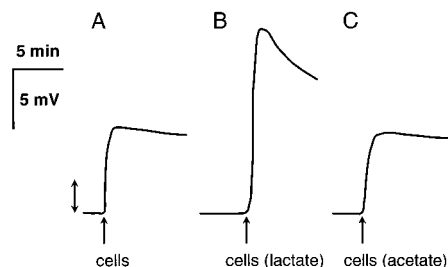
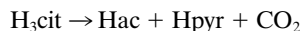


FIG. 7. Electrogenic citrate/D-lactate exchange in resting cells of *L. mesenteroides*. The recording of the TPP<sup>+</sup>-specific electrode is shown. Cells were incubated for 10 min in 20 mM potassium phosphate (pH 6.0) without further addition (control) or containing 20 mM lactate or acetate. At the arrow, 0.1 ml of the control cells (A) or cells loaded with lactate (B) or acetate (C) were diluted 10-fold into 0.9 ml of the same buffer supplemented with 2 mM citrate and 4  $\mu$ M TPP<sup>+</sup>. The recording of the TPP<sup>+</sup> electrode is shown. The arrow on the left indicates the response caused by the dilution upon the addition of the cells. The final protein concentration was 0.76 mg/ml.

The effect of citrate/D-lactate exchange on the internal pH was estimated from the fluorescence changes of intracellular BCECF. Addition of 2 mM citrate to resting cells of *L. mesenteroides* did not lead to internal alkalization (Fig. 3A), whereas a small but significant pH gradient was built up when D-lactate was also present (not shown). This effect was not due to the generation of a pH gradient by lactate diffusion into the cells, since addition of acetate had no effect. In conclusion, in the presence of D-lactate, citrate metabolism is fast enough to instantaneously alkalize the internal medium whereas, at first, the external pH remains the same. The result is a pH gradient. Subsequently, after a lag time, the external medium starts to alkalize.

## DISCUSSION

In this article, the energetic consequences of citrate metabolism in whole cells of *L. mesenteroides* were investigated. Citrate metabolism in resting cells of *L. mesenteroides* results in the generation of a pmf that is not due to ATP hydrolysis but is the result of citrate transport and metabolism. The magnitude of the pmf was largely dependent on the conditions. The different conditions are schematically presented in Fig. 9. Figure 9A shows the pathway for citrate breakdown by resting cells of *L. mesenteroides* in the absence of any other carbon and energy source. Under these conditions, citrate is stoichiometrically converted into pyruvate (Fig. 4) according to the following net reaction:



where H<sub>3</sub>cit, Hac, and Hpyr represent citric acid, acetic acid, and pyruvic acid, respectively. Addition of citrate to resting cells of *L. mesenteroides* results in the generation of a membrane potential (Fig. 2A). This observation is consistent with the results of an in vitro study performed in membrane vesicles of *L. mesenteroides*, which showed that citrate transport is an electrogenic process involving the translocation of net negative charge across the membrane (13). Citrate is transported in its dianionic form, Hcit<sup>2-</sup>, in symport with one proton (the Hcit<sup>2-</sup>/H<sup>+</sup> symport mechanism is equivalent to an Hcit<sup>2-</sup>/OH<sup>-</sup> antiport, but a reasonable discrimination between the two cannot be made at this point). To make the pathway as a whole electrogenic, the products should leave the cell in their protonated, uncharged states, which most likely proceeds by passive diffusion. The generation of a membrane potential and a pH gradient is coupled in a secondary metabolic energy-

generating pathway, as depicted in Fig. 9A (11). Nevertheless, generation of a pH gradient was not observed under these conditions (Fig. 3A), most likely because of the low rate of citrate metabolism, which is not capable of sustaining a significant gradient (Fig. 4A). However, proton consumption in the cytoplasmic metabolic steps is evidenced by the continuous rise in the pH of the medium when citrate is added to resting cells of *L. mesenteroides* (Fig. 5). Net proton consumption from the medium as a result of citrate metabolism is determined by the pKs of the substrates and products of the pathway and is dependent on the medium pH (11). At pH 6, citrate metabolism consumes only 0.28 proton per molecule of citrate converted (Fig. 9A).

The energetic consequences of citrate uptake by the symport mechanism in *L. mesenteroides* are equivalent to those observed with the H<sub>2</sub>cit<sup>-</sup> uniport mechanism described for *L. oenos* (18). The main difference between these two lactic acid bacteria is the form of citrate that is recognized by the transporter, which in turn seems to be determined by the pH of the growth medium of the organism. *L. oenos* grows in wine at low pH, where H<sub>2</sub>cit<sup>-</sup> is the dominant species, whereas *L. mesenteroides* grows at more neutral pH values, where Hcit<sup>2-</sup> is the most abundant protonation state. The in vitro studies with membrane vesicles of *L. mesenteroides* showed that CitP catalyzes homologous exchange much faster than unidirectional efflux and is able to catalyze efficient heterologous exchange between citrate and D-lactate. In vivo, the presence of D-lactate was shown to speed up the citrate metabolic pathway significantly without, at least initially, a change in the products of the pathway. Thus, in the presence of D-lactate, the rates of citrate utilization and pyruvate production were strongly increased

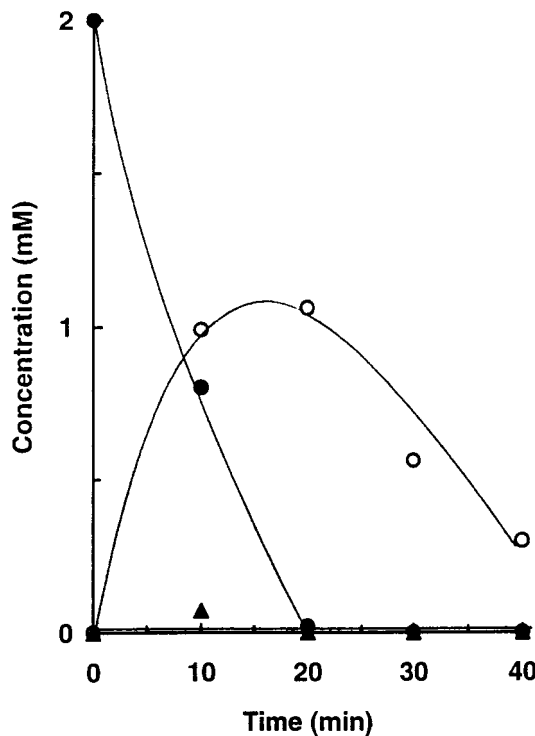


FIG. 8. Enhancement of citrate consumption and pyruvate production by resting cells of *L. mesenteroides* by D-lactate. At time zero, 2 mM citrate was added simultaneously with 2 mM D-lactate to cells resuspended in 100 mM potassium phosphate (pH 6.0) at a protein concentration of 0.46 mg/ml. The external concentrations of citrate (●), pyruvate (○), and lactate (▲) were measured.

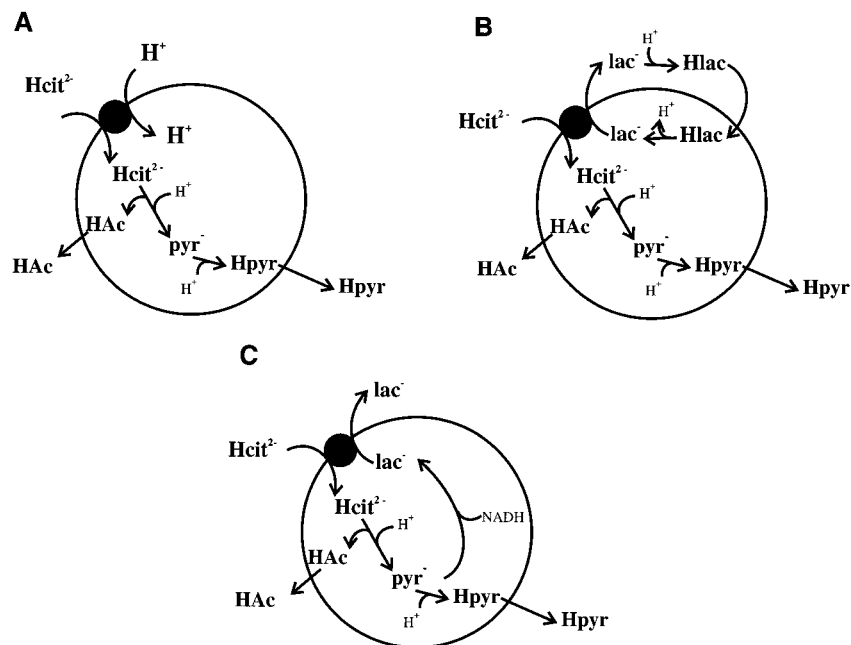


FIG. 9. Proposed mechanisms for the generation of metabolic energy by citrate metabolism in *L. mesenteroides* in the presence of citrate (A), in the presence of citrate and D-lactate (B), and in the presence of citrate and glucose (C). See the text for details.  $\text{Hcit}^{2-}$ , dianionic citrate;  $\text{Hpyr}$ , pyruvate;  $\text{HAc}$ , acetate;  $\text{Hlac}$ , lactate. For simplicity,  $\text{CO}_2$  formed in the conversion of citrate to pyruvate was omitted from the scheme. It is assumed that  $\text{CO}_2$  leaves the cell by passive diffusion.

(Fig. 8) and a much faster alkalization of the external medium was observed. The enhancement of the rate of metabolism is likely due to a shift in the transport mode of the citrate carrier from slow  $\text{H}^+$ /citrate symport to much faster citrate/D-lactate exchange. Figure 9B shows the citrate metabolic pathway in the presence of D-lactate. Lactate functions as a proton shuttle, bringing in a proton when it enters the cell in its protonated state by passive diffusion and leaving the cell in exchange with citrate in its deprotonated state. The transported proton is equivalent to the symported proton in the absence of lactate (Fig. 9A). Citrate/D-lactate exchange was shown to be an electrogenic mechanism *in vivo*. The electrogenicity was demonstrated by the generation of a membrane potential upon dilution of D-lactate-loaded cells into medium containing citrate (Fig. 7). The dianionic form of citrate is taken up by the cells in exchange with the monoanionic form of lactate. The net charge of the transport process is negative, meaning that citrate uptake generates a membrane potential (negative inside). As in the presence of D-lactate, the pathway of citrate breakdown is identical to the one shown in Fig. 9A, generation of a pH gradient is also expected. Indeed, the enhancement of the rate of citrate metabolism now results in the generation of a significant pH gradient.

The *in vivo* and *in vitro* studies of citrate transport and metabolism in the presence of D-lactate provide an explanation for pmf generation under physiological conditions, e.g., during cometabolism of citrate and glucose. The proposed mechanism is presented in Fig. 9C. Citrate enters the cell in exchange with D-lactate, ensuring efficient uptake. The difference with the mechanism in Fig. 9B is that D-lactate is produced inside the cell as an end product of citrate metabolism (citrolactic fermentation). In *Leuconostoc* species, during citrate/glucose cometabolism, a shift in glucose metabolism from ethanol to acetate production occurs (Fig. 1). Instead of being used to produce ethanol, the reducing power is used to convert pyruvate produced from citrate into D-lactate (2, 22). Citrate and

glucose metabolism maintains an outwardly directed D-lactate gradient, which, together with the inwardly directed citrate gradient, provides the best conditions for fast citrate/lactate exchange. Clearly, the highest rates of citrate metabolism (Fig. 4B) and the highest pmf consisting of both a membrane potential and pH gradient (Table 1) are observed during cometabolism of citrate and glucose. The flux through the pathway is inhibited by the membrane potential (Fig. 6), indicating that even under these conditions the transport step is controlling the rate to a large extent.

In conclusion, the pmf generated during cometabolism of citrate and glucose in *L. mesenteroides* is composed of a membrane potential generated by the uptake of divalent citrate in exchange with monovalent D-lactate and of a pH gradient generated by the consumption of scalar protons in the cytoplasmic breakdown of citrate. Citrate metabolism in *L. mesenteroides* and *L. oenos* represents the first-described example showing that secondary metabolic energy generation pathways are not restricted to simple decarboxylation pathways. Malolactic fermentation in *Lactococcus lactis* (17), histidine decarboxylation in *L. buchneri* (15), and oxalate decarboxylation in *Oxalobacter formigenes* (1) are simple systems consisting of an electrogenic transporter and a cytoplasmic decarboxylase. The corresponding decarboxylation products of the substrate leave the cell in exchange for the substrate. In contrast, citrate metabolism in the two *Leuconostoc* species consists of a number of metabolic steps that differ between the two organisms. Moreover, the citrate metabolic pathway in *L. mesenteroides* is intimately linked to glucose metabolism.

Finally, cometabolism of citrate and glucose by *L. mesenteroides* results in a growth advantage relative to growth on glucose alone. For the strain used in this study, the addition of citrate to the glucose broth led to an increase in the specific growth rate and molar growth yield from  $0.35 \text{ h}^{-1}$  and  $14.49 \text{ g/mol}$  to  $0.82 \text{ h}^{-1}$  and  $22.56 \text{ g/mol}$ , respectively (9). This growth stimulation is usually explained by ATP production via the

acetate kinase pathway (Fig. 1). This study also shows the generation of a pmf by a secondary metabolism, which can subsequently drive other metabolic energy-requiring processes and may contribute significantly to the growth stimulation.

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