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## The Citrate Metabolic Pathway in *Leuconostoc mesenteroides*: Expression, Amino Acid Synthesis, and $\alpha$ -Ketocarboxylate Transport

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**Citrate metabolism in *Leuconostoc mesenteroides* subspecies *mesenteroides* is associated with the generation of a proton motive force by a secondary mechanism (C. Marty-Teyssset, C. Posthuma, J. S. Lolkema, P. Schmitt, C. Divies, and W. N. Konings, *J. Bacteriol.* 178:2178–2185, 1996). The pathway consists of four steps: (i) uptake of citrate, (ii) splitting of citrate into acetate and oxaloacetate, (iii) pyruvate formation by decarboxylation of oxaloacetate, and (iv) reduction of pyruvate to lactate. Studies of citrate uptake and metabolism in resting cells of *L. mesenteroides* grown in the presence or absence of citrate show that the citrate transporter CitP and citrate lyase are constitutively expressed. On the other hand, oxaloacetate decarboxylase is under stringent control of the citrate in the medium and is not expressed in its absence, thereby blocking the pathway at the level of oxaloacetate. Under those conditions, the pathway is completely directed towards the formation of aspartate, which is formed from oxaloacetate by transaminase activity. The data indicate a role for citrate metabolism in amino acid biosynthesis. Internalized radiolabeled aspartate produced from citrate metabolism could be chased from the cells by addition of the amino acid precursors oxaloacetate, pyruvate,  $\alpha$ -ketoglutarate, and  $\alpha$ -ketoisocaproate to the cells, indicating a broad specificity of the transamination reaction. The  $\alpha$ -ketocarboxylates are readily transported across the cytoplasmic membrane.  $\alpha$ -Ketoglutarate uptake in resting cells of *L. mesenteroides* was dependent upon the presence of an energy source and was inhibited by inhibition of the proton motive force generating  $F_0F_1$  ATPase and by selective dissipation of the membrane potential and the transmembrane pH gradient. It is concluded that in *L. mesenteroides*  $\alpha$ -ketoglutarate is transported via a secondary transporter that may be a general  $\alpha$ -ketocarboxylate carrier.**

Cometabolism of glucose with citrate results in growth stimulation of *Leuconostoc* species that has been explained by a shift in glucose metabolism from ethanol to acetate production via acetate kinase, yielding additional ATP (2, 30). However, in recent studies, citrate metabolism in *Leuconostoc mesenteroides* was shown to be a secondary proton motive force (PMF)-generating pathway (14, 18, 21, 22). The mechanism of PMF generation is similar to that observed during malolactic fermentation in *Lactococcus lactis* (25), and by analogy the pathway was termed citrolactic fermentation. Studies of the mechanism of the citrate transporter CitP performed in membrane vesicles of *L. mesenteroides* showed that CitP catalyzes two modes of transport, symport of dianionic citrate with one proton ( $\text{CitH}^{2-}/\text{H}^+$ ) and exchange of citrate and D-lactate. D-Lactate is a product of citrate-carbohydrate cometabolism, which suggested that CitP might function as a precursor/product exchanger (21). Subsequent studies of the energetics of citrate metabolism in resting cells of *L. mesenteroides* showed that citrate/lactate exchange was indeed the physiologically relevant mode of transport. Electrogenic  $\text{CitH}^{2-}/\text{Lac}^-$  exchange efficiently generates a membrane potential, inside negative. Moreover, a pH gradient (inside alkaline) is formed through the consumption of scalar protons in the intracellular conversion of citrate into pyruvate (17, 22). Together the membrane

potential and pH gradient constitute the PMF that contributes significantly to the growth advantage observed during cometabolism of citrate and glucose. A similar mechanism of secondary PMF generation during citrate metabolism has been reported in *Leuconostoc oenos* (27).

Cometabolism of citrate and glucose increases the production of aromatic compounds by increasing the intracellular pool of pyruvate. Lactate dehydrogenase cannot cope with the rate of pyruvate production, and consequently pyruvate is degraded by other enzymes, such as  $\alpha$ -acetolactate synthase and pyruvate decarboxylase, yielding acetoin and diacetyl. The regulation and the characterization of these enzymes have been extensively studied in *Leuconostoc* and *Lactococcus* species (for a review, see reference 11). In contrast, few studies were performed in *Leuconostoc* species on the enzymes involved in the earlier steps of citrate breakdown to pyruvate, where the PMF is generated. Internalized citrate is cleaved by citrate lyase, yielding acetate and oxaloacetate, and the latter is decarboxylated by oxaloacetate decarboxylase, yielding carbon dioxide and pyruvate. Citrate lyase is regulated by acetylation via the action of citrate lyase ligase, as described for *Klebsiella pneumoniae* and *Rhodospseudomonas gelatinosa* (6, 28). Oxaloacetate decarboxylase of *Lactococcus lactis* subsp. *lactis* biovar diacetylactis has been purified (12) and shown to be a soluble cytoplasmic enzyme, in contrast to the oxaloacetate decarboxylase of gram-negative bacteria like *K. pneumoniae*, which functions as a membrane-bound primary sodium ion pump (4).

This study focuses on the citrate metabolic pathway in *L. mesenteroides* up to pyruvate. Studies on the control of expres-

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sion of the citrate transporter, citrate lyase, and oxaloacetate decarboxylase by citrate in the growth medium reveal a branching of the pathway at the level of oxaloacetate towards the production of aspartate via transamination. Subsequently, it is demonstrated that amino acid synthesis by transamination is a more general phenomenon in *Leuconostoc* species and that the  $\alpha$ -ketocarboxylate precursors are readily taken up from the medium by a PMF-driven transporter(s) in the cytoplasmic membrane.

#### MATERIALS AND METHODS

**Bacterial strain and growth conditions.** *L. mesenteroides* subsp. *mesenteroides* 19D (*L. mesenteroides*) was obtained from the collection of the Institut National de la Recherche Agronomique, Jouy en Josas, France. Citrate-positive *L. mesenteroides* 19D clones were selected via their formation of blue colonies on citrate indicator plates (13). Cells were grown in modified MRS broth at pH 6.4 without acetate and Tween 80 (3) and containing glucose (20 g/liter) and citrate (5 g/liter) when indicated. Cells were harvested in the late exponential growth phase (optical density at 660 nm, 0.8 to 1.2) (Universal Photometer, Vitatron), washed, and resuspended in the appropriate buffer.

**Uptake and metabolism in whole cells.** All experiments were performed at 30°C in 50 mM potassium phosphate, pH 5.0, at a final protein concentration of 0.76 mg of cell protein per ml. Cell protein concentration was determined as described by Lowry et al. (19). At time zero, labeled citrate was added to the cell suspension at a final concentration of 4.5  $\mu$ M. For uptake experiments, samples of 100  $\mu$ l 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- $\mu$ m-pore-size cellulose nitrate filters (Schleicher & Schuell). Filters were rinsed once with 2 ml of ice-cold 0.1 mM LiCl and transferred to scintillation vials for measurement of the internalized radioactivity. Citrate conversion was measured by the release of  $^{14}$ CO<sub>2</sub> from the suspension as described by Lokkema et al. (16). In parallel with the uptake experiments, 10- $\mu$ l aliquots of cell suspension were removed and transferred directly into scintillation liquid. The samples were vortexed intermittently to facilitate the release of  $^{14}$ CO<sub>2</sub>.

**Thin-layer chromatography (TLC).** Cells were allowed to take up labeled substrates for 30 to 60 min, after which the cells were separated from the medium by silicon oil centrifugation for 10 min in a tabletop centrifuge (Eppendorf 5415C) operated at maximum speed. Cells were collected in 10% perchloric acid supplemented with 10 mM EDTA. The perchloric acid fraction was neutralized with a 3 N KOH-KHCO<sub>3</sub> solution and centrifuged for 10 min in a tabletop centrifuge at maximum speed. Intracellular and extracellular fractions were spotted on silica gel 60 plates and eluted with two different eluents. Eluent A was a slight modification of the eluent described by Hansen (8) and was composed of a mixture of *n*-pentyl formate, chloroform, and formic acid at a ratio of 12:3:5. Eluent B consisted of a mixture of *n*-butanol, acetic acid, and water at a ratio of 4:1:1. After about 2 and 6 h of migration, the plates were dried with warm air and the spots were visualized by autoradiography. In addition to the cellular fractions, the  $R_f$  values of the following radiolabeled substrates were determined in both eluents: [1,5- $^{14}$ C]citrate (115 mCi/mmol), L-[U- $^{14}$ C]leucine (311 mCi/mmol), [1- $^{14}$ C]pyruvic acid (26 mCi/mmol), L-[2- $^{14}$ C]glutamic acid (260 mCi/mmol), L-[U- $^{14}$ C]aspartic acid (228 mCi/mmol), and [1- $^{14}$ C] $\alpha$ -ketoglutarate (51 mCi/mmol).

**Chemicals.** All radiolabeled substrates were obtained from Amersham (Buckinghamshire, United Kingdom) except for [1- $^{14}$ C] $\alpha$ -ketoglutarate, which was obtained from NEN (Du Pont de Nemours). All other chemicals were reagent grade and were obtained from commercial sources.

#### RESULTS

**Citrate uptake and metabolism in *L. mesenteroides* cells grown in the presence or absence of citrate.** Addition of citrate to resting cells of *L. mesenteroides* grown in the presence of citrate results in stoichiometric conversion to acetate, pyruvate, and CO<sub>2</sub> (22). The pathway for citrate degradation is presented in Fig. 1. Divalent anionic citrate is taken up in symport with one proton by the citrate transporter CitP, and the three end products leave the cell by passive diffusion (21). The two radioactive  $^{14}$ C atoms originally present in [1,5- $^{14}$ C]citrate end up in  $^{14}$ CO<sub>2</sub> and [ $^{14}$ C]acetate. The former is released from the suspension.

Addition of [1,5- $^{14}$ C]citrate to resting cells of *L. mesenteroides* grown in the presence of citrate resulted in very low levels of radioactivity inside the cells and a rapid release of radioactive CO<sub>2</sub> from the suspension (Fig. 2A). Within 5 min, about 50% of the label had disappeared from the suspension,

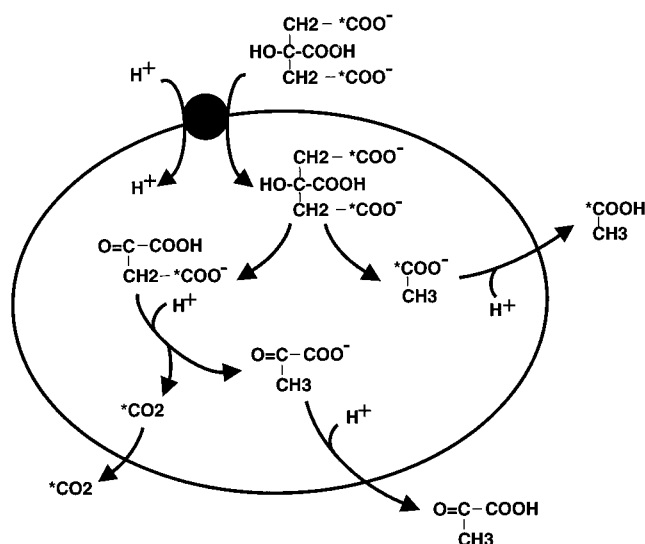


FIG. 1. Citrate metabolism in resting cells of *L. mesenteroides*. The  $^{14}$ C atoms in [1,5- $^{14}$ C]citrate are indicated by \*C. See the text for details.

indicating complete conversion of citrate (Fig. 1). The low level of accumulation of radioactivity indicates that the activities of the citrate-metabolizing enzymes, citrate lyase and oxaloacetate decarboxylase, are much higher than the citrate uptake activity, and therefore the transport step catalyzed by CitP is the rate-determining step in the pathway. In contrast, cells of *L. mesenteroides* grown in the absence of citrate accumulate significant radioactivity inside the cells upon addition of [1,5- $^{14}$ C]citrate while little  $^{14}$ CO<sub>2</sub> is produced (Fig. 2B). Apparently, the relative activities of uptake and metabolism have changed dramatically under these conditions and the expression of the enzymes involved is controlled differently. Uptake of citrate by cells grown in the presence or absence of citrate shows that CitP is constitutively expressed, as was shown before by citrate transport studies in membrane vesicles derived from *L. mesenteroides* (21). Consequently, the insignificantly low levels of CO<sub>2</sub> produced by the cells grown in the absence of citrate indicate that the pathway is blocked at the level of citrate lyase or oxaloacetate decarboxylase and that one of these two enzymes is not expressed under these conditions.

According to the metabolic pathway shown in Fig. 1, citrate accumulates either as citrate or oxaloacetate in cells grown in the absence of citrate, when no CO<sub>2</sub> is produced (Fig. 2B). Uptake of citrate catalyzed by CitP is driven by the pH gradient and counteracted by the membrane potential (CitH<sup>2-</sup>/H<sup>+</sup> symport). Accumulation of citrate in the cells will occur only when the pH gradient exceeds the membrane potential. Dissipation of the pH gradient by the ionophore nigericin should result in the release of accumulated citrate, as is observed in membrane vesicles, in which metabolism is completely absent (21). However, the label accumulated by the cells grown in the absence of citrate is not released upon addition of nigericin (Fig. 3), indicating that the labeled product is not citrate. In addition, dissipation of both the pH gradient and the membrane potential by the simultaneous addition of the ionophores nigericin and valinomycin did not result in the release of the labeled product, showing that it is also not a substrate of a PMF-driven secondary transporter. The results were the same when performed at pH 5 or 6, thereby excluding the possibility of a malfunctioning of the transporters at the lower internal pH induced by the addition of nigericin (data not shown).

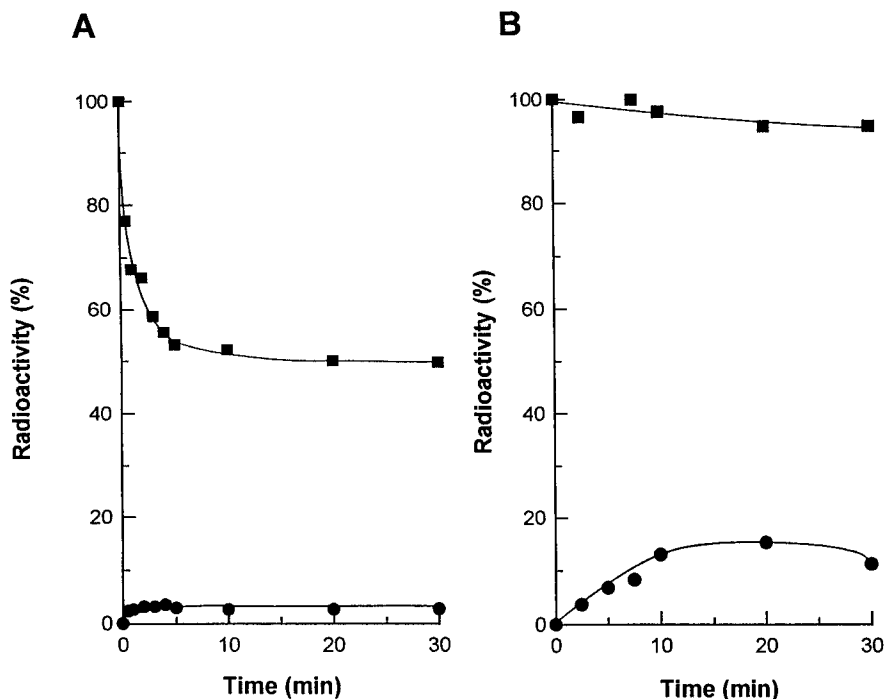


FIG. 2. Uptake and metabolism of citrate in resting cells of *L. mesenteroides* grown in the presence (A) or in the absence (B) of citrate. Cells were incubated for 10 min in the presence of 10 mM glucose at 30°C, after which 5  $\mu$ M [1,5- $^{14}$ C]citrate was added. Samples were taken at subsequent time points and analyzed for residual radioactivity (■, metabolism) and internalized radioactivity (●, uptake). The data are presented as the percentage of the radioactivity present at the start of the experiment.

Surprisingly, the internally accumulated label could be chased out of the cells by an excess of unlabeled citrate. Addition of 1 mM cold citrate at the steady level of label accumulation led to a decrease in internalized radioactivity (Fig. 3). Moreover, the labeled product could also be chased with the  $\alpha$ -keto acids  $\alpha$ -ketoglutarate, oxaloacetate, and pyruvate but not with succinate, malate, D-lactate, glutamine, and aspartate (Table 1).

**Identification of the accumulated labeled compound.** The labeled product that accumulated in response to [1,5- $^{14}$ C]citrate uptake by *L. mesenteroides* cells grown in the absence of citrate was analyzed by TLC. Two eluents were necessary to unequivocally identify the internalized label as aspartate. In both eluents A and B (see Materials and Methods) the internalized label migrated as a single spot, indicating a single product. In eluent B, citrate and the internal label clearly migrated differently (Fig. 4A), confirming the above conclusions. The  $R_f$  values for the internalized label and [ $^{14}$ C]aspartate were identical with eluents A and B (0.11 and 0.14, respectively). Equally important, it was demonstrated that a number of related substrates and potential products migrated with  $R_f$  values different from that of the internalized label in at least one of the two eluents (data not shown). These compounds included lactate, pyruvate, phosphoenolpyruvate, malate, succinate, glutamine, glutamate, and acetate.

Aspartate is formed from oxaloacetate in a single step by transamination. Therefore, since oxaloacetate is formed from citrate by the action of citrate lyase, we conclude that when citrate is absent from the growth medium, the pathway for citrate metabolism in *L. mesenteroides* is blocked at the level of oxaloacetate decarboxylation. The chasing of the label in the internalized aspartate pool by excess unlabeled citrate (Fig. 3) is explained as follows. Unlabeled citrate is converted to oxaloacetate. Excess intracellular unlabeled oxaloacetate, or

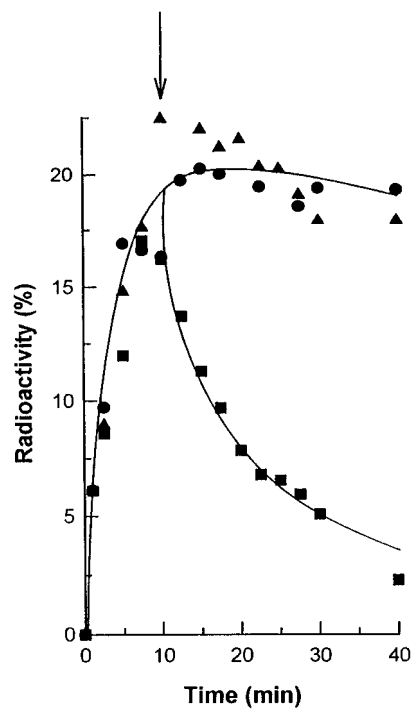


FIG. 3. Effect of ionophores and excess unlabeled citrate on accumulated radioactivity after [1,5- $^{14}$ C]citrate uptake in resting cells of *L. mesenteroides*. Cells grown in the absence of citrate were incubated for 10 min in 50 mM potassium phosphate, pH 5.0, with 5  $\mu$ M [1,5- $^{14}$ C]citrate, after which a mixture of 2.5  $\mu$ M valinomycin and 2.5  $\mu$ M nigericin (▲) or 1 mM unlabeled citrate (■) was added or no further additions were made (●). Internalized radioactivity is given as the percentage of the radioactivity present at the start of the experiment.

TABLE 1. Chasing of accumulated radioactivity with different substrates<sup>a</sup>

| Addition                      | Internal radioactivity (%) |
|-------------------------------|----------------------------|
| None .....                    | 22.3                       |
| Citrate.....                  | 3.6                        |
| $\alpha$ -Ketoglutarate ..... | 3.7                        |
| Oxaloacetate .....            | 4.1                        |
| Pyruvate.....                 | 4.4                        |
| Succinate .....               | 19.6                       |
| Malate.....                   | 18.5                       |
| Lactate .....                 | 18.8                       |
| Glutamine .....               | 25.4                       |
| Aspartate .....               | 24.4                       |

<sup>a</sup> The experiments were performed as described in the legend to Fig. 3. The cells were allowed to accumulate the label for 15 min. Subsequently, the substrates were added at 1 mM concentrations and the internal radioactivity was measured after another 30 min of incubation.

other keto acids, like pyruvate and  $\alpha$ -ketoglutarate (Table 1), can chase the label back to the oxaloacetate pool via transaminase activity and, subsequently, out of the cell. TLC of the extracellular fraction after addition of cold citrate resulted in  $R_f$  values of 0.15 and 0.14 with eluents A and B, respectively, which are different from the values obtained for aspartate, citrate, or any of the substrates tested. Unfortunately, oxaloacetate is not commercially available as a radiolabeled substrate, preventing the unequivocal identification of the chased product as such.

**Uptake and conversion of keto acids by *L. mesenteroides*.** The chasing of radiolabeled aspartate by externally added unlabeled pyruvate, oxaloacetate, or  $\alpha$ -ketoglutarate implies that these  $\alpha$ -keto acids are transported across the cytoplasmic membrane of *L. mesenteroides* and converted into the corresponding amino acids by transaminase activity.  $\alpha$ -Ketoglutarate is the precursor of glutamate, and addition of [<sup>14</sup>C] $\alpha$ -ketoglutarate to resting cells of *L. mesenteroides* resulted in the accumulation of label inside the cells (see below). TLC of the cell contents showed that the internal label is different from  $\alpha$ -ketoglutarate and that it runs at an  $R_f$  value similar to that of glutamate (Fig. 4B).

Pyruvate, which is the precursor of alanine in amino acid synthesis, is also taken up and converted readily by cells of *L. mesenteroides*. TLC of the cell contents after incubation with [<sup>14</sup>C]pyruvate revealed that a single product is formed inside the cells that, however, is not alanine (data not shown). Pyru-

vate is a key metabolite in lactic acid bacteria and is converted into a variety of end products, such as lactic acid, acetic acid, acetaldehyde, ethanol, diacetyl, and acetoin. No further attempts to identify the compound were made.

$\alpha$ -Ketoisocaproic acid is the precursor of leucine. Its uptake and conversion to leucine in resting cells of *L. mesenteroides* were shown by the following experiment. L-[U-<sup>14</sup>C]leucine is rapidly taken up by *L. mesenteroides* cells to equilibration via a secondary transport mechanism (33) (Fig. 5). Simultaneous addition of excess unlabeled  $\alpha$ -ketoisocaproic acid together with L-[U-<sup>14</sup>C]leucine resulted in a transient uptake of label (Fig. 5). The explanation for this phenomenon is that the rapid uptake of leucine is followed by a slower uptake of  $\alpha$ -ketoisocaproic acid followed by the internal conversion of the latter into unlabeled leucine, which chases the accumulated L-[U-<sup>14</sup>C]leucine out of the cells.

In conclusion, the  $\alpha$ -keto acids oxaloacetate,  $\alpha$ -ketoglutarate, and  $\alpha$ -ketoisocaproic acid are transported into the cells and converted into the corresponding amino acids (aspartate, glutamate, and leucine, respectively). Pyruvate is also a substrate in the transaminase reaction (Table 1), but alanine is not a major product upon entry of pyruvate into the cell.

**Mechanism of uptake of the  $\alpha$ -keto acids.** The rate of uptake of [1-<sup>14</sup>C] $\alpha$ -ketoglutarate by resting cells of *L. mesenteroides* is inhibited 2.5-fold by 2 mM  $\alpha$ -ketoisocaproic acid, suggesting that both substrates are taken up by a common transporter, but interaction at the transaminase level cannot be excluded (data not shown). The uptake of  $\alpha$ -ketoglutarate in cells of *L. mesenteroides* was independent of the presence of the endogenous 22-kb plasmid carrying the *citP* gene, showing that the citrate carrier CitP is not involved in  $\alpha$ -ketoglutarate transport.

Citrate metabolism in *L. mesenteroides* is a metabolic-energy-generating system, and the uptake of citrate in resting cells is independent of an additional energy source. In contrast, the uptake of  $\alpha$ -ketoglutarate is dependent on the presence of an energy source like glucose. At pH 5.0, a fourfold stimulation of the rate of [1-<sup>14</sup>C] $\alpha$ -ketoglutarate uptake was observed when cells were energized with 10 mM glucose (data not shown). Moreover, pretreatment of the cells with 100  $\mu$ M *N,N'*-dicyclohexylcarbodiimide, which inhibits  $F_0F_1$  ATPase and, thereby, the generation of a PMF (22), completely prevented [1-<sup>14</sup>C] $\alpha$ -ketoglutarate uptake into the cells (Fig. 6). Selective dissipation of the pH gradient across the membrane by nigericin also completely inhibited [1-<sup>14</sup>C] $\alpha$ -ketoglutarate uptake, whereas dissipation of the membrane potential by valinomycin resulted in partial inhibition (Fig. 6).

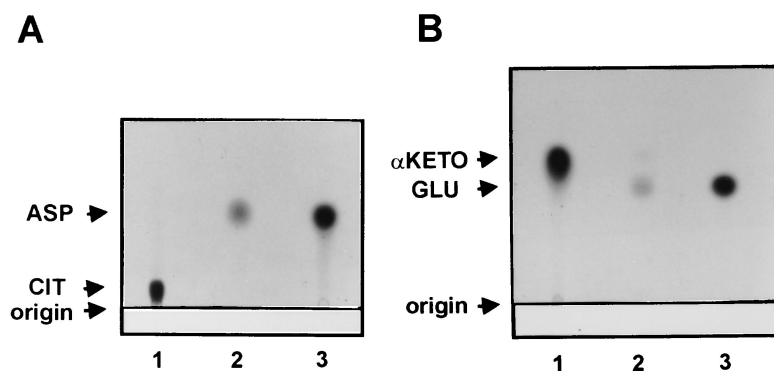


FIG. 4. TLC of the internalized label after uptake of [1,5-<sup>14</sup>C]citrate or [1-<sup>14</sup>C] $\alpha$ -ketoglutarate in resting cells of *L. mesenteroides*. Cells of *L. mesenteroides* grown in the absence of citrate were allowed to take up 5  $\mu$ M [1,5-<sup>14</sup>C]citrate (A) or 8  $\mu$ M [1-<sup>14</sup>C] $\alpha$ -ketoglutarate (B) for 1 h. The intracellular components (lanes 2) were spotted on silica gel 60 TLC plates and eluted with eluent B. Lanes 1, [1,5-<sup>14</sup>C]citrate (CIT) (A) and [1-<sup>14</sup>C] $\alpha$ -ketoglutarate ( $\alpha$ KETO) (B); lanes 3, L-[U-<sup>14</sup>C]aspartic acid (ASP) (A) and L-[2-<sup>14</sup>C]glutamate (GLU) (B).

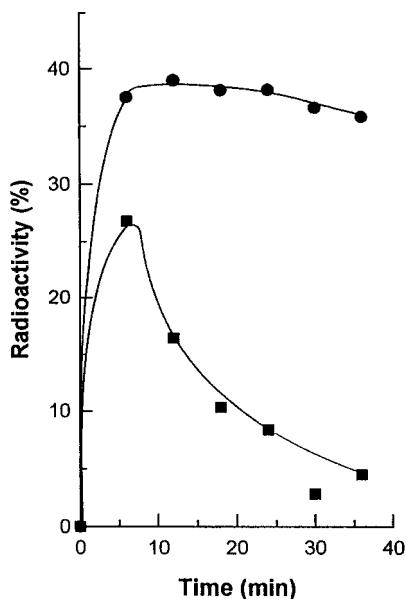


FIG. 5. Uptake of [ $^{14}\text{C}$ ]leucine in cells of *L. mesenteroides* in the presence and absence of  $\alpha$ -ketoglutarate. Cells of *L. mesenteroides* grown in the absence of citrate were resuspended in 50 mM potassium phosphate, pH 5.0, and energized by addition of 10 mM glucose for 5 min at 30°C. L-[U- $^{14}\text{C}$ ]leucine, at a concentration of 3.2  $\mu\text{M}$ , was added alone (●) or together with 2 mM  $\alpha$ -ketoglutarate (■).

Effectively, all internalized  $\alpha$ -ketoglutarate is converted to glutamate when added at micromolar concentrations, and no release of label is observed when the PMF is dissipated. At much higher concentrations of  $\alpha$ -ketoglutarate, the internal pool of amino acids necessary for the conversion of  $\alpha$ -ketoglu-

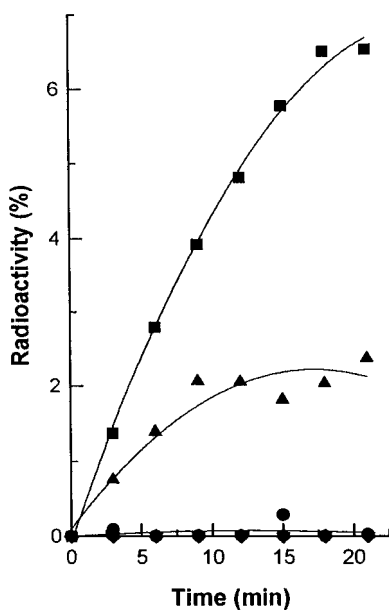


FIG. 6.  $\alpha$ -Ketoglutarate uptake in cells of *L. mesenteroides*. Cells grown in the absence of citrate were either preincubated at 30°C for 10 min with no further additions (■) or in the presence of 1  $\mu\text{M}$  valinomycin (▲) or 4  $\mu\text{M}$  nigericin (●) or were pretreated for 20 min at 4°C with 100  $\mu\text{M}$  *N,N'*-dicyclohexylcarbodiimide (▼). Cells were energized for 5 min by addition of 10 mM glucose, after which 8  $\mu\text{M}$  [ $^{14}\text{C}$ ]  $\alpha$ -ketoglutarate was added.

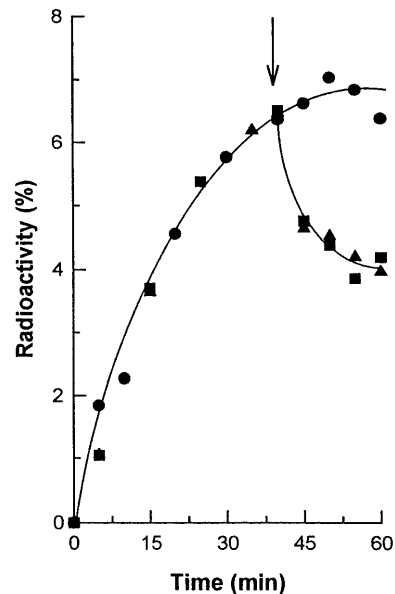


FIG. 7. Efflux of  $\alpha$ -ketoglutarate from resting cells of *L. mesenteroides*. Cells grown in the absence of citrate were energized for 5 min by addition of 10 mM glucose at 30°C. Uptake was monitored after the addition of 2 mM [ $^{14}\text{C}$ ]  $\alpha$ -ketoglutarate (●). At the time point marked by the arrow, a mixture of 1  $\mu\text{M}$  nigericin and 4  $\mu\text{M}$  valinomycin was added alone (▲) or together with cold 25 mM  $\alpha$ -ketoglutarate (■).

tarate to glutamate is depleted, resulting in a pool of free  $\alpha$ -ketoglutarate inside the cell. Then, dissipation of the PMF results in partial release of label from the cells (Fig. 7), showing the reversibility of the transport process. The presence of 25 mM unlabelled  $\alpha$ -ketoglutarate in the external medium in addition to the uncouplers did not affect the rate of efflux. The results suggest that transport of  $\alpha$ -ketoglutarate is catalyzed by a secondary transporter.

## DISCUSSION

**Regulation of the expression of the enzymes involved in the first steps of citrate metabolism in *L. mesenteroides*.** Citrate is taken up by resting cells of *L. mesenteroides* via a secondary citrate transporter, CitP, after which it is converted by citrate lyase into acetate and oxaloacetate. Acetate leaves the cells, and oxaloacetate is decarboxylated by oxaloacetate decarboxylase, yielding pyruvate and  $\text{CO}_2$  (Fig. 1). Expression of the enzymes is controlled by the presence of citrate in the growth medium. The initial rate of citrate uptake in membrane vesicles prepared from *L. mesenteroides* cells grown in the absence of citrate was significant but was lower than that of cells grown in the presence of citrate (21), showing that CitP is constitutively expressed but that the level of expression increases in the presence of citrate. Nevertheless, when *L. mesenteroides* is grown on citrate, uptake is the rate-determining step in the citrate breakdown pathway; i.e., the lyase and decarboxylase activities are higher than the uptake activity (Fig. 2A). In contrast, when cells are grown in the absence of citrate, the citrate metabolic pathway is blocked at the decarboxylation step, indicating that the expression of oxaloacetate decarboxylase is under stringent control of the citrate present in the medium (Fig. 2B and 8A). The only product observed inside the cells under these conditions was aspartate, indicating high citrate lyase and transaminase activities relative to transport

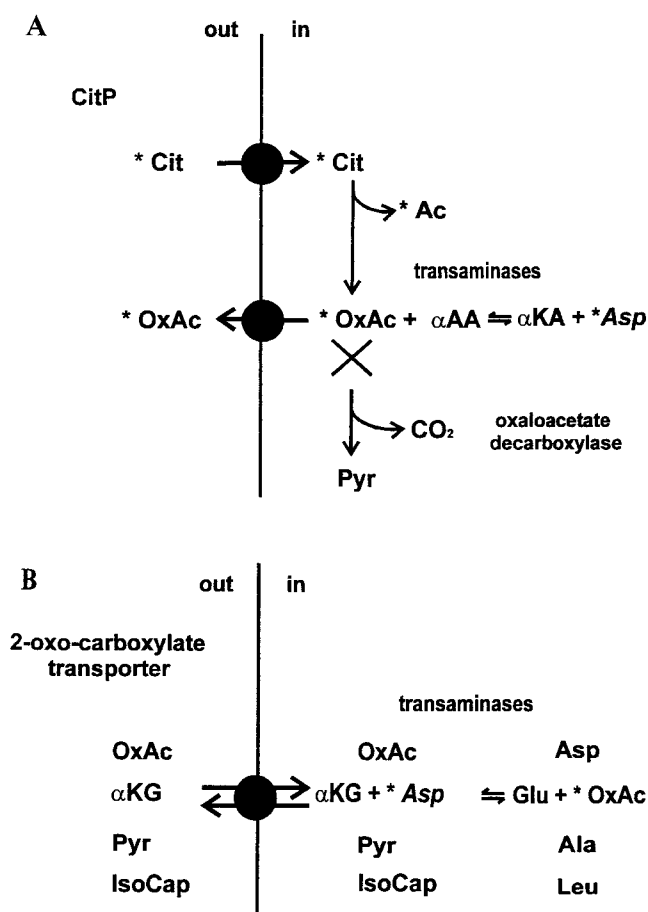


FIG. 8. The citrate metabolic pathway and amino acid synthesis in *L. mesenteroides*. (A) The pathway for citrate degradation in resting cells of *L. mesenteroides* grown in the absence of citrate. The pathway is blocked at the level of oxaloacetate decarboxylase. (B) 2-Oxo-carboxylic acid transport and transamination. The single 2-oxocarboxylate or  $\alpha$ -ketocarboxylate transporter is hypothesized. Cit, citrate; CitP, citrate transporter; Ac, acetate; OxAc, oxaloacetate;  $\alpha$ AA,  $\alpha$ -amino acid;  $\alpha$ KA,  $\alpha$ -keto acid;  $\alpha$ KG,  $\alpha$ -ketoglutarate; Asp, aspartate; Pyr, pyruvate; Ala, alanine; Glu, glutamate; IsoCap,  $\alpha$ -ketoisocaproic acid; Leu, leucine.

activity. Other studies have demonstrated that citrate lyase is an inducible enzyme in *Leuconostoc* species (9, 15, 23, 29).

In the related lactic acid bacterium *Lactococcus lactis*, the citrate transporter CitP is encoded on an endogenous plasmid in a different genetic context. In *Lactococcus lactis*, citrate transport is not affected by the presence of citrate in the growth medium, suggesting that expression of the transporter is not controlled by citrate (20). Similarly, no regulation of citrate lyase expression was found in *Lactococcus lactis* strains (1, 9). Therefore, the expression of the enzymes involved in the first steps of citrate metabolism seems to be less controlled by citrate in *Lactococcus* than in *Leuconostoc* species.

**Branching of the citrate metabolic pathway in *L. mesenteroides*.** Blocking of the citrate breakdown pathway at the level of oxaloacetate decarboxylase by growing *L. mesenteroides* in the absence of citrate revealed an alternative route to the aspartate, indicating a role for citrate metabolism in amino acid synthesis. Citrate enters the cells and is converted by citrate lyase into acetate and oxaloacetate. Oxaloacetate is not decarboxylated to pyruvate but is converted by a transaminase into aspartate (Fig. 8A). The alternative pathway was detected

by blocking the main pathway, but it is likely that the pathway towards aspartate is also present when citrate is present in the growth medium. In the wine bacterium *L. oenos*, it was shown by nuclear magnetic resonance that a small fraction of citrate was converted to aspartate by cells that were grown in the presence of citrate (26). Aspartate has been shown to be an essential amino acid for certain strains of *Leuconostoc* (5). The present study suggests that this may correlate with the inability to metabolize citrate, which in fact is the case for many lactic acid bacteria. In *Lactococcus lactis*, an alternative route has been described for the synthesis of aspartate: the condensation of pyruvate and carbon dioxide by pyruvate carboxylase followed by amination (10).

The transaminase in the pathway towards aspartate appears to have a rather broad substrate specificity since accumulated aspartate could be chased from the cells by a variety of keto acids, i.e., pyruvate, oxaloacetate,  $\alpha$ -ketoglutarate, and  $\alpha$ -ketoisocaproate (Fig. 8B). The formation of the amino acids glutamate and leucine upon addition of the last two keto acids to the medium could be demonstrated (Fig. 4B and 5) and suggests a more general role of transaminase activity in amino acid biosynthesis in *L. mesenteroides*.

Accumulated aspartate and glutamate was not released from the cells upon dissipation of the PMF. This indicates that no secondary transporters for these two amino acids are present in the cytoplasmic membrane of *L. mesenteroides*, which is consistent with the observation that the aspartate and glutamate transporters that have been described in lactic acid bacteria are all ATP-dependent systems (24).

**$\alpha$ -Ketocarboxylate transport.** An important spin-off of the detection of the alternative citrate degradation pathway towards aspartate is the detection of  $\alpha$ -ketocarboxylate transport across the cytoplasmic membrane of *L. mesenteroides*. Oxaloacetate, pyruvate,  $\alpha$ -ketoglutarate, and  $\alpha$ -ketoisocaproate are readily translocated across the membrane. Transport of  $\alpha$ -ketoglutarate was studied in more detail, and the following results suggest that transport proceeds via a secondary mechanism: (i) inhibition of the generation of an electrochemical proton gradient by inhibition of  $F_0F_1$  ATPase activity prevents any uptake; (ii) selective dissipation of the pH gradient and the membrane potential prevents and reduces uptake, respectively; and (iii) uptake is reversible. Possibly, a general transporter for  $\alpha$ -ketocarboxylates is present in the membrane of *L. mesenteroides* that transports substrates that present the motif R-CO-COOH (Fig. 8B). Such a transporter would resemble the citrate transporter CitP, which is a general transporter for substrates that present the motif  $R_1R_2COHCOOH$ , i.e., citrate, lactate, and malate (21).  $\alpha$ -Ketocarboxylate transporters have not been described in *Leuconostoc* species before but have been reported in other bacteria. Examples are  $\alpha$ -ketoglutarate transport in *Escherichia coli* (31),  $\alpha$ -ketoisocaproate transport in *Bacillus subtilis* (7), and pyruvate transport in *Lactobacillus plantarum* (32).

**Benefits of citrate metabolism.** In *Leuconostoc* species, co-metabolism of citrate and glucose (citrolactic fermentation) leads to a growth stimulation which used to be explained by a metabolic shift yielding additional ATP via the acetate kinase pathway (2, 30). Recently, we showed that metabolic energy in the form of a PMF is generated by citrate metabolism through a secondary mechanism that may contribute significantly to growth stimulation (22). In this study, citrate metabolism in *L. mesenteroides* is shown to be directly involved in amino acid biosynthesis. Citrate is a precursor of aspartate, an essential amino acid in many *Leuconostoc* species. Aspartate is involved in the synthesis of another amino acid, asparagine, but it also participates in the biosynthesis of pyrimidines and purines.

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