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Citrate Fermentation by *Lactococcus* and *Leuconostoc* spp.

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Citrate and lactose fermentation are subject to the same metabolic regulation. In both processes, pyruvate is the key intermediate. *Lactococcus lactis* subsp. *lactis* biovar diacetylactis homofermentatively converted pyruvate to lactate at high dilution (growth) rates, low pH, and high lactose concentrations. Mixed-acid fermentation with formate, ethanol, and acetate as products was observed under conditions of lactose limitation in continuous culture at pH values above 6.0. An acetoin/butanediol fermentation with α-acetolactate as an intermediate was found upon mild aeration in continuous culture and under conditions of excess pyruvate production from citrate. *Leuconostoc* spp. showed a limited metabolic flexibility. A typical heterofermentative conversion of lactose was observed under all conditions in both continuous and batch cultures. The pyruvate produced from either lactose or citrate was converted to D-lactate. Citrate utilization was pH dependent in both *L. lactis* and *Leuconostoc* spp., with maximum rates observed between pH 5.5 and 6.0. The maximum specific growth rate was slightly stimulated by citrate, in *L. lactis* and greatly stimulated by citrate in *Leuconostoc* spp., and the conversion of citrate resulted in increased growth yields on lactose for both *L. lactis* and *Leuconostoc* spp. This indicates that energy is conserved during the metabolism of citrate.

Citrate-utilizing lactic acid bacteria play an important role in many dairy processes. They are responsible for the production of the flavoring compound diacetyl. Diacetyl is essential for the flavor of dairy products such as butter and buttermilk and, sometimes, young cheeses. Because of this property, these lactic acid bacteria are often referred to as aromabacteria. During citrate metabolism CO_2 is also produced, leading to eye formation in certain cheese types. The degradation of citrate and the production of diacetyl are both traits that are presently difficult to control. This is a result of a lack of knowledge about the exact mechanisms of both processes. Most research on citrate metabolism over the last decades has been limited to incomplete studies of the influence on citrate metabolism and diacetyl production of several environmental and biological factors such as pH (4), oxygen tension (16), the presence or absence of lactose (4, 11, 22), metal ions (8, 15), and the growth phase and type of the lactic acid bacterium (11, 12, 19, 30). These studies were mostly conducted as batch fermentations in milk, sometimes with mixed cultures, resulting in constant changes in culture conditions (such as pH, lactate concentration, cell density, and culture composition) during the measurements. Metabolic studies using continuous cultivation under controlled conditions have been reported for *Lactococcus lactis* subsp. *lactis* biovar diacetylactis (13) and for *Leuconostoc* spp., (22) providing valuable data on the energetics of citrate utilization and the relationship between citrate metabolism and acetoin and/or diacetyl production. These reports, however, did not provide complete fermentation balances of citrate, which are necessary for a complete description of the mechanism and regulation of citrate metabolism and diacetyl production in these bacteria. In this report, we have focused on the product formation resulting from citrate metabolism in growing cultures of *Lactococcus lactis* subsp. *lactis* var. diacetylactis and in *Leuconostoc* strains. Lactose metabolism is an essential part of the present investigation, since citrate and lactose metabolism are intertwined following the production of pyruvate (11–14). For the most part, the studies were performed with continuous cultures to allow for the individual adjustment of physical and biological factors such as pH, oxygen tension, and growth rate.

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MATERIALS AND METHODS

Organisms and growth conditions. *Lactococcus lactis* subsp. *lactis* var. diacetylactis strain Ru4 was isolated from the diacetyl-overproducing butter starter culture 4/25. Strain C17 was a culture from the NIZO collection. For the study on product formation during continuous fermentation, the lactococci were cultivated at 30°C in M17 medium (27). *Leuconostoc mesenteroides* 7-1 was obtained from T. M. Cogan, Moorepark Research Centre, Cork, Ireland (3). *Leuconostoc* sp. 60 was from the NIZO collection. Fermentation of the *Leuconostoc* strains was performed at 30°C in MRS medium (9) with 0.5% lactose and with or without 10 mM citrate. *Lactococcus* and *Leuconostoc* strains were routinely stored in litmus milk with 0.1% yeast extract at −40°C.

Continuous fermentation. For the study on product formation from lactose and citrate, continuous cultivation was employed, with 1-liter vessels (Applicon Dependable Instruments bv., Schiedam, The Netherlands) with a working volume of 0.5 liter. *L. lactis* C17 and Ru4 were grown on M17 medium (27) which was adjusted for our specific purposes by using 0.5% lactose as a growth-limiting substrate, reduced amounts of β-glycerophosphate (0.2%) since the cultures were pH controlled, and 10 mM citrate when needed. The dilution rate (D) of the cultures could be varied from 0.1 h^{-1} to 1.0 h^{-1} or higher by changing the pump rate of the medium inlet. Most experiments were performed under anaerobic conditions by using a continuous flow of nitrogen over the culture. Aeration was achieved by flushing air through the cultures at a rate of approximately 50 ml/min.

Determination of fermentation products. Lactose, citrate, lactate, acetate, formate, ethanol, pyruvate, and butanediol production was determined by high-performance liquid chromatography (HPLC) with an HPX-87P anion exchange column (Bio-Rad, Inc.) with 0.01 N H_2SO_4 as the elution fluid. CO_2 production was calculated from the fermentation pat-

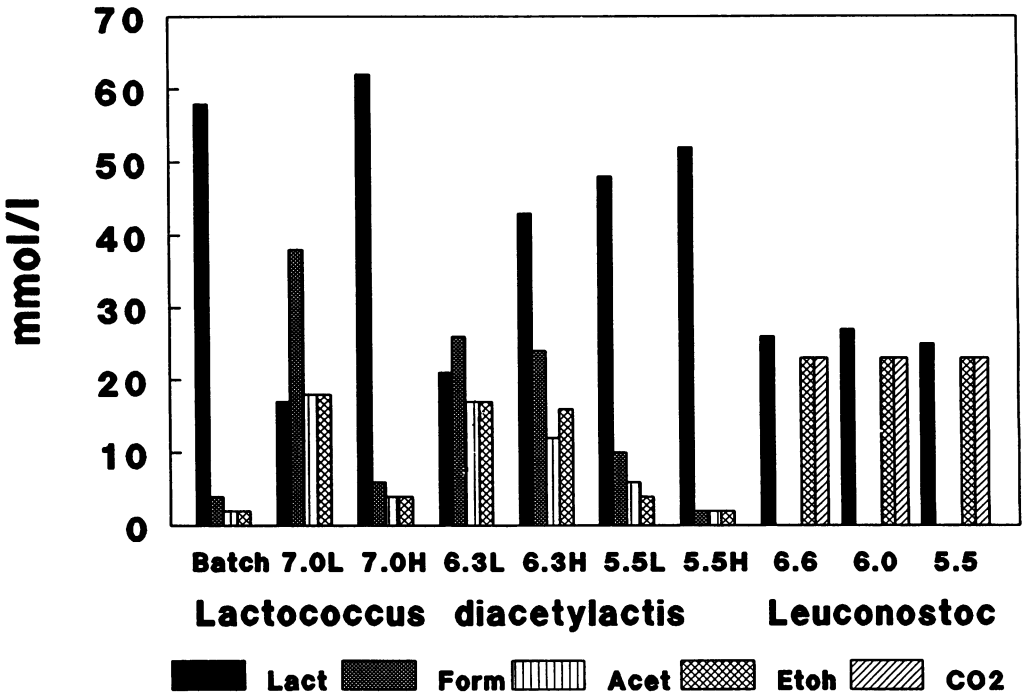

FIG. 1. Product formation from lactose (0.5%) by *L. lactis* and by *Leuconostoc* spp. in continuous culture at high (H [$D = 0.6\ h^{-1}$]) and low (L [$D = 0.2\ h^{-1}$]) dilution rates. The fermentations were performed at three different pH values (pH 7.0, 6.3, and 5.5 for *L. lactis* and pH 6.6, 6.0, and 5.5 for Leuconostoc spp.). For comparison, the fermentation pattern of *L. lactis* in batch culture is shown on the left.

terns. D-Lactate production was analyzed by an enzymatic assay measuring NAD^+ reduction catalyzed by D-lactate dehydrogenase (Boehringer; isolated from *Lactobacillus leichmannii*), as described by Cogan (3). α-Acetolactate production was determined either by HPLC as described previously (17) or colorimetrically by the method of Westerfeld (31) as adjusted by Veringa et al. (29). Acetoin production was measured by the same colorimetric method, and diacetyl production was determined by the method of Pien et al. (20) as adjusted by Veringa et al. (29). All measurements of metabolites were also conducted on M17 medium prior to fermentation. Some lactose (40 mg/liter) and glucose (100 mg/liter) were detected at levels that amounted to less than 3% of the added lactose during the experiments. Some organic acids (lactate and formate) were also present at low levels in nonfermented M17. Production data were corrected for these background values. Prior to the performance of the fermentation experiments, it was established that both *L. lactis* strains were unable to metabolize the buffer component of the M17 medium, β-glycerophosphate.

Growth (rate) measurement. Growth and growth rate were determined by the increase in optical density at 600 nm.

RESULTS

Product formation from lactose. *L. lactis* strains grown in batch culture homofermentatively converted more than 90% of the lactose to L-(+)-lactate, regardless of medium composition, medium pH, or gas atmosphere. In continuous culture under lactose limitation, the observed fermentation patterns were strongly dependent on the medium pH (Fig. 1). At pH values above 6.0, only 25% of lactose was converted to lactate at a dilution rate (D) of 0.2; even smaller fractions were converted at lower D values, while the rest of the lactose was converted by mixed-acid fermentation to acetate, formate, and ethanol. When the dilution rate was increased at these pH values, the lactococci switched to a homofermentative fermentation. At pH values below 6.0, lactococci were at least 80% homofermentative, even at low dilution rates. In all cases, the carbon recovered in the products amounted to 96 to 100% of the lactose consumed. This indicated that no medium components other than lactose were being metabolized and that no products other than those presented in Fig. 1 were formed during fermentation. Aeration of the cultures had a marked effect on fermentation in continuous culture. The mixed-acid fermentation was not observed, and mostly homofermentative fermentation was observed at all pH values. *L. lactis* strain Ru4 produced, besides lactate, considerable amounts of α-acetolactate (up to 2 mM; data not shown). At low pH and vigorous aeration, some acetate was also produced, presumably together with CO_2. For *Leuconostoc* spp., a heterofermentative conversion of lactose was observed under all conditions with D-(−)-lactate, ethanol, and CO_2 as fermentation products in a 1:1:1 ratio (Fig. 1).

Citrate utilization. The utilization of citrate by *Lactococcus* and *Leuconostoc* spp. was strongly dependent on the medium pH. In batch cultures, citrate was completely converted at pH values below 6.0. At higher pH values, decreasing amounts of citrate were converted by the lactic acid bacteria (Fig. 2). Also, citrate conversion during continuous fermentation was only complete at pH values below 6.0. At higher pH values, the fraction of citrate that was converted depended on the dilution rate (D) in the chemostat; high levels of conversion (0.1 to $0.2\ h^{-1}$) occurred at low D values and low levels of conversion (0.5 to $0.7\ h^{-1}$) occurred at high D values. When citrate was added to cultures, the highest

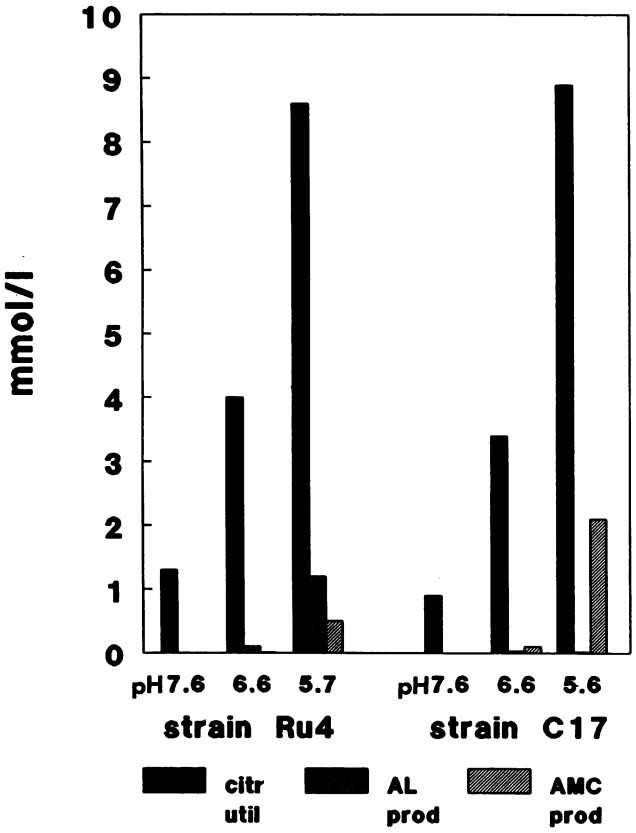

FIG. 2. Citrate utilization by *L. lactis* Ru4 and C17 in batch culture at different pH values of the growth medium (M17). α-Acetolactate (AL) and acetoin (AMC) production are also indicated.

conversion rates of citrate were observed at pH 5.5 to 6.0 (data not shown).

Product formation from citrate. The citrate-utilizing lactic acid bacteria were grown in media containing lactose and citrate. The amounts of products that were formed under these conditions were compared with the amounts of products that were formed in lactose medium without citrate (Fig. 1). By subtraction, the products formed by citrate metabolism could be established (Table 1). As a control, citrate was added separately to the cultures and the product formation was reanalyzed. This resulted in the same fermentation patterns. *Leuconostoc* spp. produced primarily D-(−)-lactate, acetate, and CO_2 from citrate under all conditions employed (Table 1). At pH values below 5.0, some acetoin and butanediol were formed. The combined products from citrate conversion were more reduced than the substrate (citrate). This was a result of a shift in heterofermentative lactose fermentation by *Leuconostoc* spp.; less ethanol and more acetate was produced from lactose (data not shown), with citrate acting as an electron acceptor. Similar observations have been reported for other *Leuconostoc* spp. in batch cultures (3).

L. lactis converts both lactose and citrate via pyruvate. Because of the more oxidized nature of citrate compared with lactose, more oxidized products were found; no lactate and no ethanol were produced from citrate. Instead, formate, acetate, CO_2, α-acetolactate, acetoin, and butanediol were produced in quantities that were dependent on the culture conditions (Table 1). Diacetyl was not produced by any of the lactic acid bacteria tested. The small amounts of diacetyl found during fermentation could all be explained by the chemical oxidation/decarboxylation of α-acetolactate (approximately 5%/h at pH 6.0 and 30°C). Some striking differences in product formation were observed between the two *Lactococcus* strains. Strain Ru4 produced considerable amounts of α-acetolactate from citrate, a process which was stimulated by aeration (unpublished results), while strain C17 accumulated acetoin and butanediol in culture but very little or no α-acetolactate (Table 1). Only after the addition of extra citrate was some α-acetolactate produced by this strain, but it was rapidly converted to acetoin and, subsequently, to butanediol (data not shown). This result clearly indicates that α-acetolactate is formed as an intermediate during the formation of acetoin and butanediol.

Effect of citrate on growth. Growth rates of *Lactococcus* and *Leuconostoc* spp. were determined in the absence and presence of 10 mM citrate. For *Leuconostoc* spp., growth (on lactose) was clearly stimulated by the presence of citrate; a 50 to 100% increase was observed in the maximum

TABLE 1. Product formation from 10 mM citrate by aromabacteria

Strain	pH	O_2	Formation (mM) of[a]:							
			Formate	Acetate	Ethanol	Lactate	Pyruvate	α-Acetolactate	Acetoin	Butanediol
L. lactis C17	5.5	−	1.8	11.8					1.8	2.3
	5.5	+		11.0			0.8		2.8	1.5
	6.3	−	7.6	18.1			1.0			0.8
L. lactis Ru4	5.8	−	4.3	14.1	0.9		1.0	2.0	0.3	
	5.7	+		11.4			1.5	3.1	0.8	
	6.3	−	8.3	17.5	1.6		1.2	0.2		
Leuconostoc 60	6.0	−		10.0		9.8	0.1			
	5.5	−		10.0		9.1	0.6		0.1	
	4.9	−		10.0		7.6	1.0			0.6

[a] CO_2 was also formed but was not quantified.

TABLE 2. Cell yield of *L. lactis* Ru4 and C17 and *Leuconostoc* 60 during lactose-limited continuous fermentation in medium with or without citrate

Strain	pH	Lactose concn (%)	Cell yield (OD_{600}) with:		
			No citrate	10 mM citrate	Citrate pulse (10 mM)[a]
L. lactis Ru4	5.8	0.5	1.99	2.06	2.16 (2.24)
	6.3	0.5	2.16	2.38	2.45 (2.54)
L. lactis C17	5.5	0.5	1.78	1.88	2.07 (2.19)
	6.3	0.5	2.46	2.65	2.67 (2.79)
	7.0	0.5	2.57	2.39	2.58 (2.44)
	6.0	0.25	1.35	ND[b]	1.62 (ND)
Leuconostoc 60	6.0	0.5	0.82	1.03	1.03 (1.26)
	5.5	0.5	0.75	0.87	0.97 (1.03)

[a] Numbers indicate cell yield after 10 mM citrate pulse added to cultures without or with (numbers in parentheses) 10 mM citrate in the initial fermentation.
[b] ND, not done.

specific growth rates at pH 6.0, which is in agreement with earlier observations by Cogan (3). For *L. lactis*, some growth stimulation (10 to 15%) by citrate was observed. However, at pH values below 6.0, citrate seemed to become increasingly toxic (data not shown). In continuous culture under lactose limitation, growth yields on lactose were higher in the presence of 10 mM citrate than they were in the absence of citrate for both *Lactococcus* and *Leuconostoc* spp. (Table 2), except at the higher pH values that do not allow citrate conversion. Furthermore, addition of extra citrate to the lactose-limited cultures resulted in an increase of cell material. This growth was a direct result of citrate metabolism and not of any other metabolic activity, since no lactose (or other carbohydrates) and no alternative growth substrates (arginine) were detected in the lactose-limited cultures.

DISCUSSION

In *Lactococcus lactis* subsp. *lactis* var. diacetylactis, lactose and citrate were both degraded via the intermediate pyruvate (Fig. 3A). The various products that are formed during lactose metabolism are a result of the relative activities of four pyruvate-dependent enzymes, which have been described for several lactic acid bacteria, including lactococci. These are (i) L-(+)-lactate dehydrogenase, with maximal activity at high lactose concentrations (i.e., high intracellular fructose-1,6-diphosphate levels), high intracellular NADH levels, and relatively low medium pH (14, 32), all of which are conditions present during batch fermentation; (ii) pyruvate-formate lyase, which is active at medium pH

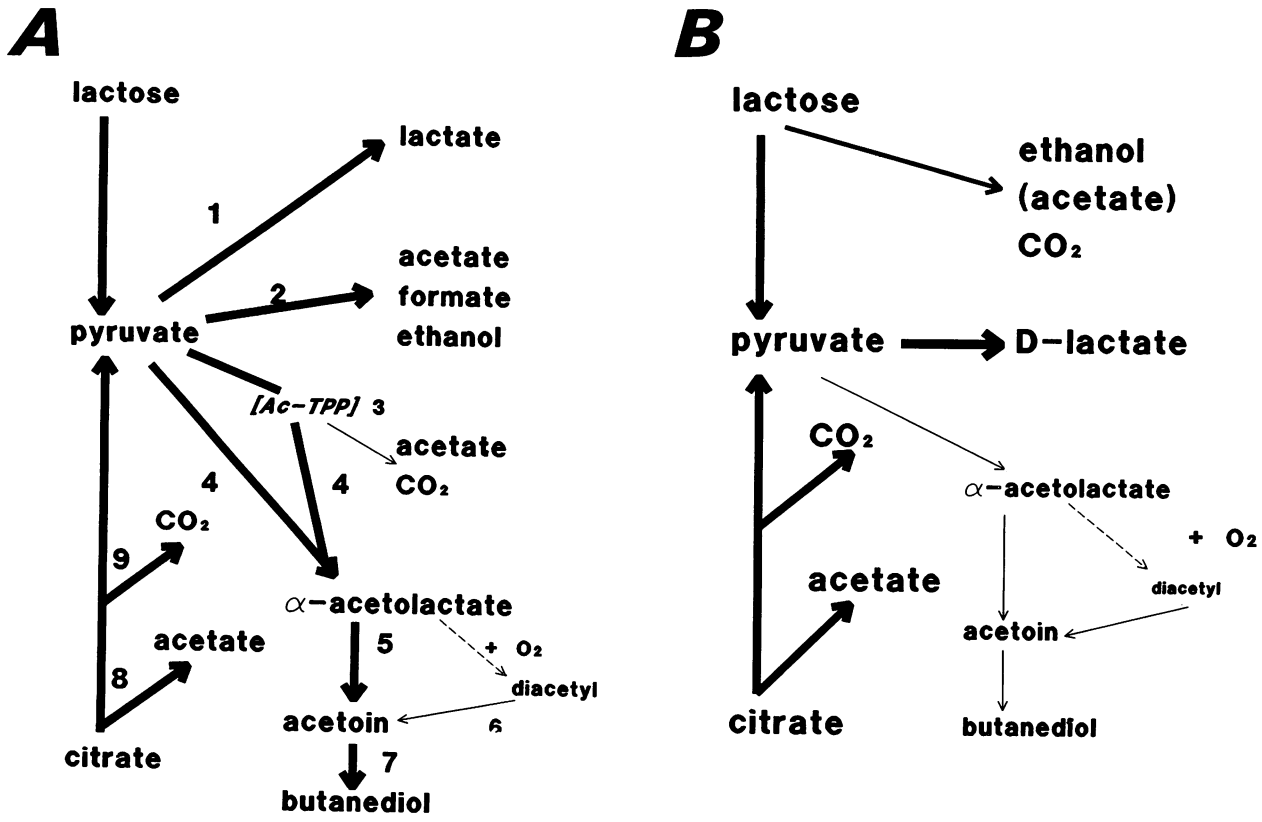

FIG. 3. Schematic presentation of lactose and citrate metabolism in *L. lactis* subsp. *lactis* var. diacetylactis (A) and *Leuconostoc* spp. (B). The enzymes involved are indicated as follows: 1, lactate dehydrogenase; 2, pyruvate/formate lyase; 3, pyruvate dehydrogenase; 4, α-acetolactate synthase; Ac-TPP, hydroxyethyl-thiamin-triphosphate; 5, α-acetolactate decarboxylase; 6, diacetyl reductase; 7, acetoin reductase; 8, citrate lyase.

higher than 6.0 and anaerobic conditions, as reported for *Streptococcus mutans* and *Enterococcus faecalis* (1, 18); (iii) pyruvate dehydrogenase, which is active under aerobic conditions and low pH (2); and (iv) α-acetolactate synthase, which is active at high pyruvate concentrations and low pH (13a). The change from homolactic fermentation to mixed-acid fermentation as a result of carbohydrate limitation in continuous culture has been described before for non-citrate-metabolizing lactococci (28). Also, clear effects of aeration on lactose metabolism by lactococci have been observed by other authors (5, 24).

When citrate is metabolized, the same enzymes are responsible for pyruvate conversion. However, lactate dehydrogenase plays a much less prominent role, since the activators of this enzyme, fructose diphosphate and NADH, are not formed during citrate metabolism. Consequently, citrate was converted to formate and acetate (and CO_2) and, especially at lower pH values and aerobic conditions, to acetoin and butanediol via α-acetolactate. The stimulation of α-acetolactate/diacetyl production by *L. lactis* Ru4 due to aeration could have important industrial implications. Diacetyl was not directly produced in any significant amounts by the whole cells or by cell extracts of *L. lactis* or *Leuconostoc* spp. These observations are in agreement with recent reports from Verrips et al. (30), who used nuclear magnetic resonance techniques for the study of citrate conversion. The observed diacetyl production in some of the fermentations reported here and, similarly, in some dairy products can be explained by the chemical oxidation/decarboxylation of α-acetolactate produced by the aromabacteria, as had been suggested earlier by de Man (7) and by Stadhouders (25).

The pH dependence of citrate utilization is determined by the activity of the citrate permease. The citrate permease of *L. lactis* has recently been cloned, sequenced, and characterized in our institute (6). A narrow optimum pH range of between pH 5.5 and 6.0 was observed for this protein, which coincides with the optimum pH for citrate conversion that is reported here.

The observed growth of *L. lactis* on citrate in the absence of lactose indicates that energy is conserved during citrate metabolism. Some of this energy is generated from the conversion of acetyl coenzyme A to acetate during the mixed-acid fermentation. The same process was responsible for the higher growth yields on lactose at pH 6.5 compared with those at pH 5.5 (Table 2). However, at pH 5.5, when citrate was converted to other products such as acetoin and α-acetolactate, significant growth on citrate was also observed. This suggests that energy is conserved during the breakdown of citrate to pyruvate (approximately 1 ATP equivalent per citrate molecule; data not shown). Possibly, a mechanism such as the one present in *Klebsiella aerogenes* is involved, in which decarboxylation of oxaloacetate results in the generation of a Na^+ gradient (10) which can serve as a driving force for several metabolic functions located in the cytoplasmic membrane (23). Some of these functions have been reported for lactic acid bacteria (21, 26), although they have not yet been reported for lactococci. Growth of lactic acid bacteria on citrate has not been reported previously. This is probably due to the fact that conditions that allow growth on citrate are very critical. Growth by *L. lactis* on citrate was only observed in chemostats at pH values between 5.5 and 6.0. At lower pH values citrate was growth inhibitory, and at higher pH values citrate was not utilized. Growth of *Leuconostoc* spp. on lactose was stimulated by citrate, but no growth on citrate alone was observed. The growth stimulation of *Leuconostoc* spp. in the presence of citrate can be explained by the action of citrate as an external electron acceptor, resulting in more acetate (and ATP) production and less ethanol production during the heterofermentative lactose conversion (Fig. 3B). This phenomenon has been suggested before by Condon (5) and was recently also described by Schmitt and coworkers (22).

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