

## Utilization of Lactate Isomers by *Propionibacterium freudenreichii* subsp. *shermanii*: Regulatory Role for Intracellular Pyruvate

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Five strains of *Propionibacterium freudenreichii* subsp. *shermanii* utilized the L-(+) isomer of lactate at a faster rate than they did the D-(-) isomer when grown with a mixture of lactate isomers under a variety of conditions. ATCC 9614, grown anaerobically in defined medium containing 160 mM DL-lactate, utilized only 4 and 15% of the D-(-)-lactate by the time 50 and 90%, respectively, of the L-(+)-lactate was used. The intracellular pyruvate concentration was high (>100 mM) in the initial stages of lactate utilization, when either DL-lactate or the L-(+) isomer was the starting substrate. The concentration of this intermediate dropped during DL-lactate fermentation such that when only D-(-)-lactate remained, the concentration was <20 mM. When only the D-(-) isomer was initially present, a similar relatively low concentration of intracellular pyruvate was present, even at the start of lactate utilization. The NAD<sup>+</sup>-independent lactate dehydrogenase activities in extracts showed different kinetic properties with regard to pyruvate inhibition, depending upon the lactate isomer present. Pyruvate gave a competitive inhibitor pattern with L-(+)-lactate and a mixed-type inhibitor pattern with D-(-)-lactate. It is suggested that these properties of the lactate dehydrogenases and the intracellular pyruvate concentrations explain the preferential use of the L-(+) isomer.

During Swiss cheese manufacture, the starter culture (*Streptococcus thermophilus* and *Lactobacillus bulgaricus* or *Lactobacillus helveticus*) ferments lactose to L-(+) and D-(-)-lactate. Propionibacteria, which grow as the secondary flora during cheese ripening, ferment the lactate to propionate, acetate, and CO<sub>2</sub> (for reviews, see references 14 and 17). It has been suggested that the proportion of L-(+) and D-(-) isomers may be important for the growth of propionibacteria and the formation of CO<sub>2</sub> and volatile acids (18).

Some aspects of lactate metabolism by propionibacteria have been studied in detail, e.g., the membrane-bound electron transport system that is involved in transfer of reducing equivalents from lactate to fumarate and O<sub>2</sub> (6, 7, 20, 21). However, little is known about the mechanisms involved in the utilization of the two isomers. Only one strain of propionibacteria (*Propionibacterium pentosaceum*) has been studied (19) with respect to lactate-utilizing enzymes (9). The lactate dehydrogenase of *P. pentosaceum* is not linked to NAD<sup>+</sup> but can utilize fumarate as an electron acceptor (19).

In Swiss cheese, the concentration of the L-(+) isomer decreased more rapidly than the D-(-) isomer independently of the initial ratio of L-(+)- to D-(-)-lactate formed by different lactobacillus strains (for a review, see reference 17). CO<sub>2</sub> production from lactate by *Propionibacterium petersonii* showed a longer lag with the D-(-) isomer, but over time the rates with both isomers were similar (15). In the present study, five strains of *Propionibacterium freudenreichii* subsp. *shermanii* were investigated under a variety of culture conditions to determine the factors involved in the preferential use of the L-(+) isomer.

### MATERIALS AND METHODS

**Organisms and culture conditions.** The five strains of *P. freudenreichii* subsp. *shermanii* (NCDO 566, ATCC 9614, DRI, KFA, and MNS) used were from the collection held at the New Zealand Dairy Research Institute, Palmerston North, New Zealand. *P. freudenreichii* subsp. *shermanii*

DRI and KFA were originally isolated from commercial Swiss cheese.

Cultures were grown at 30°C in complex or defined medium under semi-anaerobic (static culture) conditions or anaerobically. For anaerobic growth, N<sub>2</sub>-CO<sub>2</sub> (95:5 [vol/vol]), which had been passed over freshly regenerated, heated copper turnings to remove any traces of oxygen, was bubbled (30 ml/min) through the medium.

The complex medium contained the following (per liter): 10 g of casein hydrolysate (Oxoid Ltd., London, England), 10 g of yeast extract (BBL Microbiology Systems, Cockeysville, Md.), 0.25 g of KH<sub>2</sub>PO<sub>4</sub>, 0.2 g of MgSO<sub>4</sub> · 7H<sub>2</sub>O, 0.05 g of MnCl<sub>2</sub> · 4H<sub>2</sub>O, and various amounts of DL-lactate (Sigma L-1375; Na<sup>+</sup> salt, containing equal proportions of L-(+) and D-(-) isomers). Medium was adjusted to the required pH (normally 6.8) and then autoclaved. The amino acid composition of a typical batch of autoclaved complex medium was as follows: 4.0 mM aspartate, 1.8 mM threonine, 3.1 mM serine, 6.7 mM glutamate, 2.4 mM proline, 1.8 mM glycine, 6.0 mM alanine, 1.4 mM valine, 0.3 mM methionine, 1.2 mM isoleucine, 2.5 mM leucine, 0.6 mM tyrosine, 1.0 mM phenylalanine, 1.9 mM histidine, 1.4 mM lysine, and 0.7 mM arginine.

The main nitrogen source in the defined medium was (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub> (final concentration, 10 mM). The chemically defined medium was sterilized as three separate solutions. Solution A contained the following (in a 986-ml volume): 0.5 g of KH<sub>2</sub>PO<sub>4</sub>, 0.5 g of K<sub>2</sub>HPO<sub>4</sub>, 0.15 g of MgSO<sub>4</sub> · 7H<sub>2</sub>O, 0.015 g of MnCl<sub>2</sub> · 4H<sub>2</sub>O, 0.01 g of CoCl<sub>2</sub> · 7H<sub>2</sub>O, 0.01 g of FeSO<sub>4</sub> · 6H<sub>2</sub>O, 0.01 g of NaCl, 0.4 g of cysteine hydrochloride, 0.1 g of L-tryptophan, 2 mg of pantothenic acid, 1 mg of *p*-aminobenzoic acid, 2 mg of riboflavin, 2 mg of nicotinic acid, and various amounts of DL-lactate. Medium was adjusted to pH 6.8. Solution B contained the following in a 30-ml volume of 0.03 M HCl: 30 mg of adenine, 30 mg of guanine, 30 mg of uracil, 30 mg of thiamine hydrochloride, and 20 mg of biotin. Solution C contained 30% (wt/vol) (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub>. Solutions A and B were autoclaved, and solution C was sterilized by passage through membrane filters

(0.22- $\mu\text{m}$  pore size; Millipore Corp., Bedford, Mass.). Complete defined medium was made by adding 10 ml of solution B and 4 ml of solution C to 986 ml of solution A.

**Cell extracts.** Cells were harvested at different stages of growth, as indicated in Results. Cells from 20 to 100 ml of medium were washed in 200 ml of Tris hydrochloride buffer (pH 7.5; 25 mM) containing 50 mM  $\text{MgCl}_2$  and 20% (vol/vol) glycerol before being disrupted in the same solution (5 ml) by shaking twice for 2 min each time at 0 to 5°C with 3 g of glass beads (75- to 150- $\mu\text{m}$  diameter) in a Mickle disintegrator. Debris was removed by centrifugation at 12,000  $\times g$  for 10 min.

**Assay of NAD<sup>+</sup>-independent LDHs.** The D(-)-lactate and L(+)-lactate dehydrogenase (LDH) activities were measured with 2,6-dichlorophenol indophenol (DCPIP) as the electron acceptor. The standard assay (1 ml) contained 50 mM sodium-potassium phosphate buffer (pH 7.2), 0.1 mM DCPIP, either 10 mM D(-)-lactate or 10 mM L(+)-lactate, and diluted cell extracts. The assay was initiated by the addition of lactate. Reduction of DCPIP was followed at 600 nm (25°C) in a Gilford model 250 spectrophotometer. The extinction coefficient for DCPIP at 600 nm of 20.6  $\text{mM}^{-1} \text{cm}^{-1}$  was used (2). One unit of LDH activity was defined as the amount of enzyme which oxidized 1  $\mu\text{mol}$  of lactate per min under the standard assay conditions. All assays reported were done on cell extracts which had been subjected to centrifugation (12,000  $\times g$  for 10 min) to remove unbroken cells and large cellular debris. When the supernatant was subjected to high-speed centrifugation (100,000  $\times g$  for 2 h), both LDH activities were found in the particulate fraction. However, during preparation, variable loss of the L(+)-LDH activity occurred relative to that of the D(-)-LDH. The L(+)-LDH activity in the particulate fractions was also more unstable (20).

**Extraction and assay of intracellular pyruvate.** Extracts were prepared by the methods developed for extraction of intermediates from lactic streptococci (23). Cells in 25- to 50-ml culture growing on lactate were collected rapidly by filtration through a membrane filter (diameter, 47 mm; pore diameter, 0.8  $\mu\text{m}$ ) and then placed in 5 ml of 0.6 N  $\text{HClO}_4$  at 0°C. After 15 min at 0°C, extracts were neutralized with KOH and centrifuged. Pyruvate was assayed enzymatically (8) within 12 h of extraction because prolonged storage (greater than 48 h) at 70°C resulted in a decrease in the pyruvate concentration.

The intracellular concentrations of pyruvate were calculated based on the fact that 1 g (dry weight) of cells was equivalent to 1.67 ml of intracellular fluid (cytoplasm). This value was estimated for *Streptococcus lactis* (24) and is similar to values reported for other bacteria (1, 4, 13). Up to half of the pyruvate concentration measured in  $\text{HClO}_4$  extracts was attributed to the carryover of culture fluid containing pyruvate. This carryover varied according to the concentration of cells filtered and of the pyruvate present in the culture fluid. The appropriate corrections were estimated at different time points at which duplicate samples of filtered cells were removed from suction at the same stage as samples for  $\text{HClO}_4$  extracts. They were immediately weighed wet and then weighed again after drying. The weight difference (with allowance for 1.67  $\mu\text{l}$  of water per mg [dry weight]) was the volume of culture supernatant that was carried over into the  $\text{HClO}_4$  extracts. The same corrections were obtained whether lactate was measured in the  $\text{HClO}_4$  extracts at the start of fermentation or propionate and acetate were measured in  $\text{HClO}_4$  extracts near the end of lactate fermentation. This assumes that there is no signifi-

cant contribution by intracellular lactate, propionate, or acetate. Therefore, culture filtrates were analyzed for pyruvate, and the appropriate subtractions were made from culture extracts to obtain intracellular pyruvate concentrations.

**Substrate and product analysis.** Culture samples (3 to 5 ml) taken during growth were centrifuged at 20,000  $\times g$  for 2 min (4°C), and the supernatants were stored frozen until analyzed. Enzymatic analysis was routinely used for the assay of D(-)-lactate (10), L(+)-lactate (12), pyruvate (8), and succinate (*Methods of Enzymatic Food Analysis*, Boehringer, Mannheim, Federal Republic of Germany, 1980). The following reaction mixture (1 ml) was used for succinate and incubated at 25°C for 1 h: 50 mM triethanolamine hydrochloride (pH 7.5), 10 mM  $\text{MgCl}_2$ , 1 mM ITP, 0.25 mM coenzyme A, 0.25 mM NADH, 1 mM phosphoenolpyruvate, 4 U of pyruvate kinase, 5 U of LDH, 0.2 U of succinyl-coenzyme A synthetase, and the sample. Succinate concentration was corrected for pyruvate.

Propionate and acetate were routinely assayed with a gas chromatograph (Varian 1400) and a glass column (1.8 m by 2 mm inside diameter) packed with Chromosorb 101, 100/120 mesh (Johns-Manville, Denver, Colo.). The injector and detector temperatures were 230°C, and the column was held at 180°C. The carrier gas was  $\text{O}_2$ -free  $\text{N}_2$  flowing at 30 ml/min. Detection was by flame ionization. Quantitation was carried out with an integrator (Hewlett-Packard HP3380A) preprogrammed after calibration with authentic standards. Culture supernatants (1 ml) were mixed with an equal volume of 20 mM butyric acid (internal standard) contained in 20% (vol/vol) formic acid before injection (5  $\mu\text{l}$ ) into the column.

Acetate, lactate, propionate, pyruvate, and succinate concentrations in culture supernatants were also measured by high-performance liquid chromatography. A liquid chromatograph (Waters Associates, Inc., Milford, Mass.), composed of an M6000A pump, an M441 detector (214-nm filter), a WISP 710B autoinjector, a model 721 programmable system controller, and a 730 data module, was used for analysis of these organic acids. A model Bio-Rad Laboratories HPX-87H cation exchange-ion exclusion column (300 by 7.8 mm) with a microguard precolumn was used in a temperature control unit (Waters Associates model III). The column was heated to 45°C, and 0.01 M  $\text{H}_3\text{PO}_4$  buffer was pumped through the column. Culture supernatants were mixed with equal volumes of 0.02 M  $\text{H}_3\text{PO}_4$  and then filtered before injection (50  $\mu\text{l}$ ) into the column. The concentrations of substrates and products, when measured by the two different methods, were similar ( $\pm 5\%$ ) and have been presented in the Results section as the average value.

**Other procedures.** Bacterial density was determined directly with membrane filters (22). Protein was estimated by the method of Bradford (3).

**Materials.** All biochemicals were obtained from Sigma Chemical Co., St. Louis, Mo., and were grades with highest analytical purity. For experiments in which only the D(-) isomer of lactate was added to the medium, the lithium salt of D(-)-lactic acid (Sigma L-1000) was converted to the  $\text{Na}^+$  form with cation-exchange resin (Dowex-50W hydrogen form).

## RESULTS

**Lactate isomer utilization in static cultures.** The five strains of *P. freudenreichii* subsp. *shermanii* studied grew to an  $A_{600}$  of between 0.4 to 0.5 in complex medium without an added

substrate such as lactate or carbohydrate. In this medium, propionate, acetate, and succinate were formed (4 to 6 mM each), and the amino acids aspartate, alanine, and serine were utilized (data not shown) (5).

An initial survey of the utilization of lactate isomers (Table 1) indicated that preferential use of the L-(+) isomer was common with all five *P. freudenreichii* subsp. *shermanii* strains studied in complex medium. This observation was independent of the initial pH, the initial lactate concentration, and the initial ratio of the isomers. The initial pH had changed by less than 0.1 U by the end of lactate utilization. The doubling times (data not shown) ranged from 3.8 to 4.6 h for the five strains and were not altered significantly by the different concentrations and ratios of the lactate isomers given in Table 1. Because all strains studied used the L-(+) isomer in preference to the D-(-) isomer, one strain (ATCC 9614) was chosen for more detailed study.

**Lactate isomer utilization in anaerobic cultures grown in defined medium.** The defined medium was used to obtain fermentation balances and to study the use of lactate isomers in the absence of other substrates that were present in the complex medium. No growth of the five *P. freudenreichii* subsp. *shermanii* strains was observed in the defined medium unless lactate or carbohydrate was added. In defined medium containing 80 mM L-(+)-lactate and 80 mM D-(-)-lactate, ATCC 9614 had utilized only 4 and 15% of the D-(-)-lactate by the time 50 and 90%, respectively, of the L-(+)-lactate had been used (Fig. 1). The propionate/acetate ratio was 2.0:1.0 at all sample points, and the final concentrations were 106 mM propionate and 53 mM acetate. This indicates that 3 mol of lactate are fermented to 2 mol of propionate, 1 mol of acetate, and 1 mol of CO<sub>2</sub>. The low concentration of succinate (0.3 mM) suggested that CO<sub>2</sub> fixation via phosphoenolpyruvate-carboxytransphosphorylase was minimal. In static cultures (semi-anaerobic), the pH did not change (pH 6.5 ± 0.1) during the fermentation of 160 mM DL-lactate, whereas in anaerobic cultures (Fig. 1), the pH increased from 6.5 to 7.0 at the end of lactate utilization. When stationary-phase static cultures were sparged with N<sub>2</sub>-CO<sub>2</sub> (95:5 [vol/vol]) for 5 min, the pH increased from 6.5 to 6.9. This suggests removal of the acidic product (CO<sub>2</sub>) by the gas stream.

Two other strains (NCDO 566 and KFA) studied in defined medium under the conditions described in Fig. 1 gave results similar to those found for ATCC 9614 (data not shown).

TABLE 1. Utilization of L-(+)-lactate and D-(-)-lactate by *P. freudenreichii* subsp. *shermanii* strains growing in static cultures in complex medium

Strains	Initial lactate concn [mM; L-(+)/D-(-)]	Initial pH	% D-(-)-lactate utilized when the following amt of initial L-(+)-lactate was utilized:	
			50%	90%
All five <sup>a</sup>	40/40	6.5	10-22	18-40
ATCC 9614, DRI	40/40	5.4	8-10	16-22
All five	10/10	6.5	6-14	10-20
ATCC 9614, DRI	10/10	5.4	8-10	16-20
All five	16/6	6.5	5-18	12-45
ATCC 9614, DRI	6/16	6.5	2-5	8-12

<sup>a</sup> *P. freudenreichii* subsp. *shermanii* strains NCDO 566, ATCC 9614, DRI, KFA, and MNS.

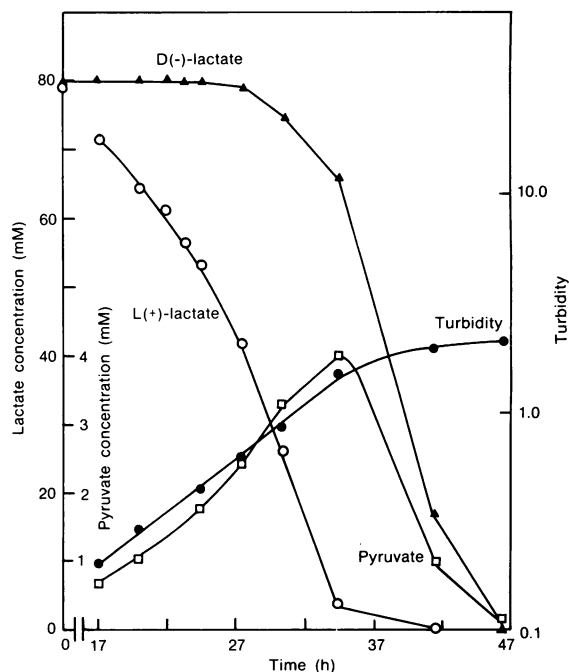


FIG. 1. L-(+)-lactate and D-(-)-lactate utilization by *P. freudenreichii* subsp. *shermanii* ATCC 9614 growing anaerobically at 30°C in defined medium containing 160 mM DL-lactate (initial pH, 6.5). A 1.5% inoculum was used. Turbidity was monitored at 600 nm. Pyruvate concentrations in culture supernatants are shown.

**Lactate isomer utilization in anaerobic cultures grown in complex medium.** In complex medium containing 176 mM DL-lactate, ATCC 9614 had utilized only 7 and 18% of the D-(-)-lactate by the time 50 and 90%, respectively, of the L-(+)-lactate had been used (Fig. 2). Two other strains (NCDO 566 and KFA) studied under the conditions described in Fig. 2 gave results similar to those found for ATCC 9614 (data not shown). Thus, the pattern of lactate isomer utilization by *P. freudenreichii* subsp. *shermanii* was similar in both defined and complex media.

Compared with defined medium, the complex medium is more similar to, but still not the same as, the rich nutritional medium provided by a Swiss-type cheese environment. In Swiss cheese, the pH is 5.25 to 5.60 during the warm-room incubation (22°C), and salt is present in the moisture phase at concentrations of 1.6 to 2.3%. The L-(+)-lactate is present at higher concentrations than the D-(-) isomer at the start of the warm-room incubation (25). Propionibacteria growing in liquid medium have a temperature optimum of 30°C and a pH optimum of 6.0 to 7.0. It has been found that 3% NaCl is necessary to reduce the growth rate of propionibacteria (for a review, see reference 16). Therefore, in some experiments *P. freudenreichii* subsp. *shermanii* was grown at 22°C in complex medium that was altered by (i) pH reduction to 5.25 before autoclaving (medium pH after autoclaving, 5.30), (ii) L-(+)-lactate addition and (iii) Na<sup>+</sup> addition (from NaCl and sodium lactate) so that the total Na<sup>+</sup> concentration was equivalent to 2.1% NaCl.

The rate of isomer utilization by *P. freudenreichii* subsp. *shermanii* ATCC 9614 was studied under these altered conditions (Fig. 3). Starting with an initial lactate isomer composition of 145 mM L-(+)-lactate and 55 mM D-(-)-lactate, the L-(+) isomer was used at a higher rate, such that an equimolar mixture of both isomers was present after

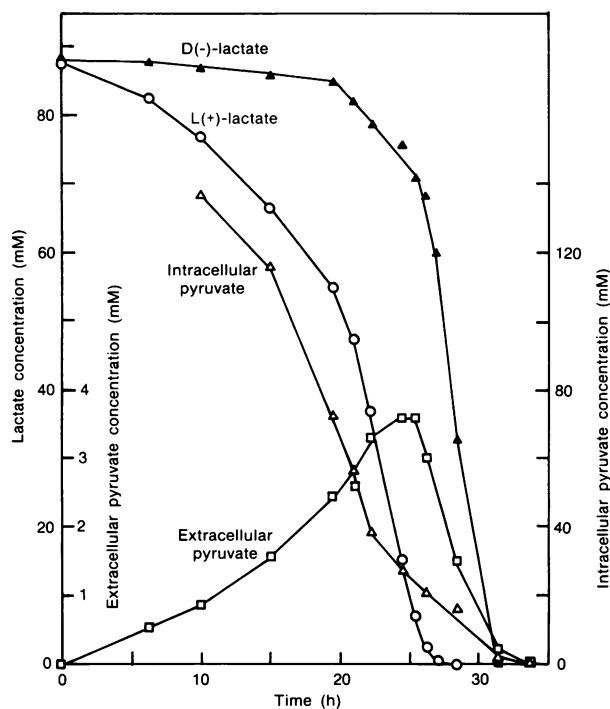


FIG. 2. Presence of intracellular and extracellular pyruvate during utilization of D(-)-lactate and L(+)-lactate by *P. freudenreichii* subsp. *shermanii* ATCC 9614 growing anaerobically at 30°C in complex medium containing 176 mM DL-lactate (initial pH, 6.5). A 3% inoculum was used. The scale for intracellular pyruvate is 20 times that for extracellular pyruvate.

116 h of growth. This indicates that L(+)-lactate was used at three times the rate of the D(-) isomer. During lactate utilization, the doubling time was 22 h in the first 70 h of growth and subsequently decreased to 16.5 h. Growth continued after lactate exhaustion, with turbidity at 600 nm increasing from 2.9 to 3.4. Associated with this increase was a decrease in some amino acids (alanine, serine, glycine, and glutamic acids) and an increase in  $\text{NH}_3$  from 6 to 23 mM. This suggests that growth after lactate depletion was due, at least in part, to amino acid utilization. The pH increased from 5.3 to 6.1 during lactate utilization. The rate of isomer utilization of *P. freudenreichii* subsp. *shermanii* KFA was similar to ATCC 9614 when studied under the same conditions as described in the legend to Fig. 3 (data not shown).

**Intracellular concentration of pyruvate.** In *P. freudenreichii* subsp. *shermanii* ATCC 9614 growing in complex medium, the intracellular concentration of pyruvate decreased during fermentation of 176 mM DL-lactate (Fig. 2). At the first sample point, at which only 10 mM L(+)-lactate had been used by the growing cells, the intracellular pyruvate concentration was high (137 mM). The concentration of this intermediate was only 19 mM by the time all of the L(+)-isomer was utilized, and there was still 60 mM D(-)-lactate in the medium. Similar data were found for ATCC 9614 growing in defined medium. Intracellular pyruvate concentrations were in the range of 40 to 70 mM for five strains (NCDO 566, ATCC 9614, DRI, KFA, and MNS) growing in complex medium under static conditions, with a starting concentration of 120 mM DL-lactate. In these experiments, cells were harvested after 40 to 60% of the L(+)-lactate had been utilized. In contrast, when lactate was replaced by glucose or lactose (starting concentration of either 3 or 10

mM), the intracellular pyruvate concentration during growth was only 1 to 3 mM in the five strains studied.

Extracellular pyruvate was detected in culture supernatants during lactate fermentation (Fig. 1, 2, and 3). The maximum concentration (3.7 mM) was reached at the point of L(+)-lactate exhaustion (Fig. 2). The extracellular pyruvate became undetectable soon after the D(-)-lactate was fermented. Similar observations were made with the other four strains. Extracellular pyruvate was not detectable when the added energy source was glucose or lactose (starting concentration of either 3 or 10 mM).

The intracellular pyruvate concentration was measured in *P. freudenreichii* subsp. *shermanii* ATCC 9614 growing in complex medium to which only one isomer of lactate was added (Fig. 4). The results with DL-lactate (Fig. 2) have been replotted in Fig. 4 for comparison. With L(+)-lactate and DL-lactate, the initial intracellular pyruvate concentration was >100 mM; this dropped off rapidly as lactate was utilized. In the early stage of D(-)-lactate utilization, the intracellular pyruvate concentration was low (~20 mM). In cells growing on L(+)-lactate, the intracellular pyruvate concentration only dropped below 20 mM when the L(+)-lactate concentration remaining in the culture supernatant was less than 10 mM.

The maximum concentration of pyruvate detected in culture supernatants was low (0.2 mM) when only the D(-)-isomer was added to complex medium in Fig. 4 (data not shown). However, the maximum concentration of pyruvate was higher (3 to 5 mM) in culture supernatants when L(+)-lactate (data not shown) was the initial isomer. The doubling time of ATCC 9614 growing on L(+)-lactate or DL-lactate was 4.3 to 4.5 h, compared with 8.0 h on D(-)-lactate.

**Levels of NAD<sup>+</sup>-independent D(-)- and L(+)-LDH activities in growing cells.** The specific activities of both D(-)- and

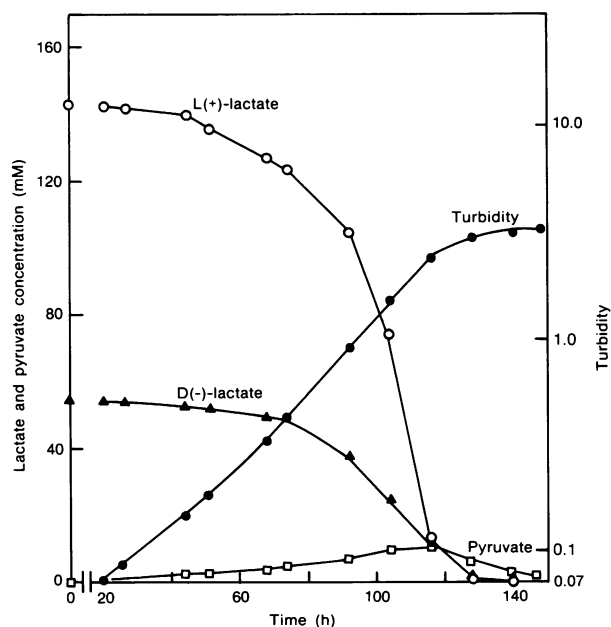


FIG. 3. L(+)-lactate and D(-)-lactate utilization by *P. freudenreichii* subsp. *shermanii* ATCC 9614 growing anaerobically at 22°C in complex medium containing 55 mM D(-)-lactate and 145 mM L(+)-lactate with 1.0% NaCl added (initial pH, 5.3). A 2% inoculum was used. Turbidity was monitored at 600 nm. Pyruvate concentrations in culture supernatants are shown.

L-(+)-LDH increased in ATCC 9614 during fermentation of DL-lactate in defined and complex media (Table 2). However, the ratio of D(-)- to L-(+)-LDH specific activities only varied between 1.2 and 1.7 during utilization of both isomers. Replacing DL-lactate with L-(+)-lactate in complex medium did not alter this ratio or the specific activities of the D(-)- and L-(+)-LDH. When cell extracts were reassayed after 20 h of storage at 4°C, the D(-)-LDH activity had not altered, but the L-(+)-LDH activity had decreased by 30 to 40%.

The ratio of D(-)- to L-(+)-LDH activities did not change significantly during fermentation of DL-lactate by the other strains studied (NCDO 566, DRI, KFA, and MNS) under anaerobic or static growth conditions (data not shown). For example, the ratio of D(-)- to L-(+)-LDH activities remained at 2.8:1.0 during lactate utilization by NCDO 566 growing anaerobically in complex medium with 170 mM DL-lactate added.

**Pyruvate inhibition of NAD<sup>+</sup>-independent L-(+)- and D(-)-LDH activities.** For all five *P. freudenreichii* subsp. *shermanii* strains studied with a saturating concentration of DCPIP (0.1 mM), the D(-)-LDH activity was more sensitive to pyruvate inhibition than that of L-(+)-LDH. Results are shown only for ATCC 9614 (Fig. 5). With 10 mM lactate as substrate, 20 mM pyruvate did not inhibit the L-(+)-LDH activity but caused 75% inhibition of D(-)-LDH activity, and 40 mM pyruvate caused 6 and 81% inhibition of L-(+)- and D(-)-LDH activity, respectively. With 1 mM lactate, L-(+)-LDH was sensitive to pyruvate but to a lesser extent than was D(-)-LDH.

The effects of pyruvate on the two LDH activities from ATCC 9614 (Fig. 6) were studied by varying the concentration of lactate isomer in the presence of fixed pyruvate

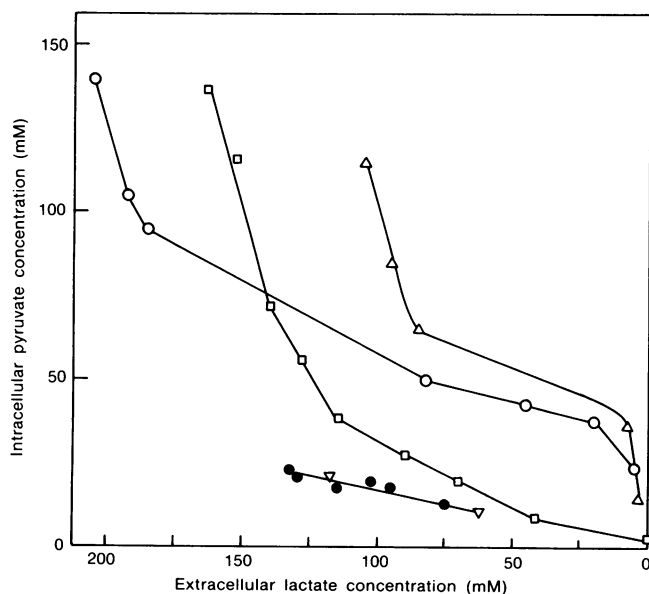


FIG. 4. Relationship between the extracellular lactate concentration and the intracellular pyruvate concentration with the following initial concentrations of lactate: 133 mM D(-)-lactate (●), 150 mM D(-)-lactate (△), 116 mM L-(+)-lactate (△), 221 mM L-(+)-lactate (○), and data from Fig. 3 with 176 mM DL-lactate (□). *P. freudenreichii* subsp. *shermanii* ATCC 9614 was grown anaerobically at 30°C in complex medium (initial pH, 6.5). The first points were determined from cells that had been growing between 10 and 18 h from a 1 to 3% inoculum.

TABLE 2. Specific activities of L-(+)- and D(-)-NAD<sup>+</sup>-independent LDHs in *P. freudenreichii* subsp. *shermanii* ATCC 9614 during anaerobic lactate fermentation<sup>a</sup>

Medium	Isomer composition of lactate at harvest [mM; L-(+)/D(-)]	Sp act of <sup>b</sup> :		Ratio of sp act [D(-)-LDH/L-(+)-LDH]
		L-(+)-LDH	D(-)-LDH	
Defined	55/81	0.64	0.82	1.3
	40/78	0.75	1.12	1.5
	8/63	0.91	1.14	1.3
	1/40	1.10	1.29	1.2
	0/16	1.40	1.90	1.3
	0/2	1.13	1.26	1.2
Complex	47/81	0.55	0.95	1.7
	15/76	0.94	1.37	1.5
	4/64	0.96	1.27	1.3
	0/34	0.92	1.42	1.5
Complex <sup>c</sup>	202/0	0.48	0.63	1.3
	180/0	0.74	1.04	1.4
	84/0	1.50	1.96	1.3
	46/0	1.72	2.25	1.3

<sup>a</sup> The medium initially contained 170 mM DL-lactate and had a pH of 6.5.

<sup>b</sup> Cell extracts prepared immediately and enzyme activities assayed within 2 h of cell harvest. Values given (in micromoles of lactate oxidized per minute per milligram of protein) are the means from at least three separate assays.

<sup>c</sup> Growth as described in footnote <sup>a</sup>, except that an initial concentration of 221 mM L-(+)-lactate was used.

concentrations and at a saturating concentration of DCPIP. Lineweaver-Burk plots, prepared by linear regression analysis, indicated that pyruvate gave a competitive inhibition pattern with L-(+)-lactate (Fig. 6A) and a mixed-type inhi-

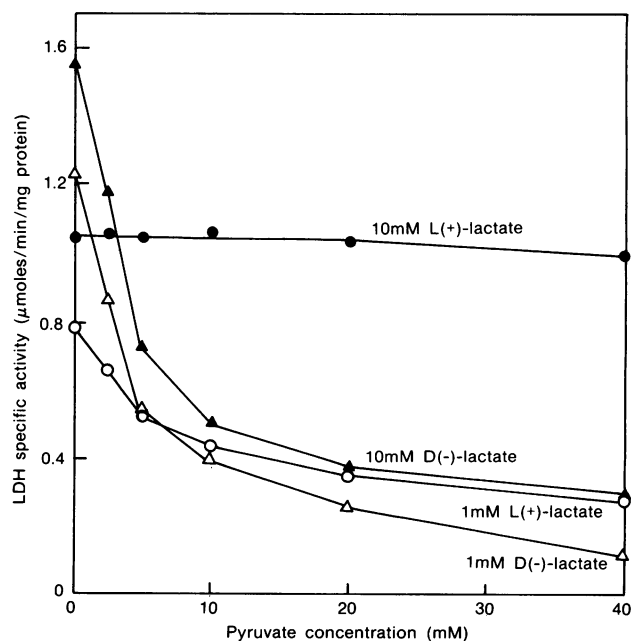


FIG. 5. Pyruvate inhibition of NAD<sup>+</sup>-independent LDH activity assayed in freshly prepared extract of *P. freudenreichii* subsp. *shermanii* ATCC 9614 by using components of standard reaction mixture (see Materials and Methods) with lactate concentrations of 10 mM L-(+)-, 1 mM L-(+)-, 10 mM D(-)-, and 1 mM D(-)- and pyruvate concentrations as shown.

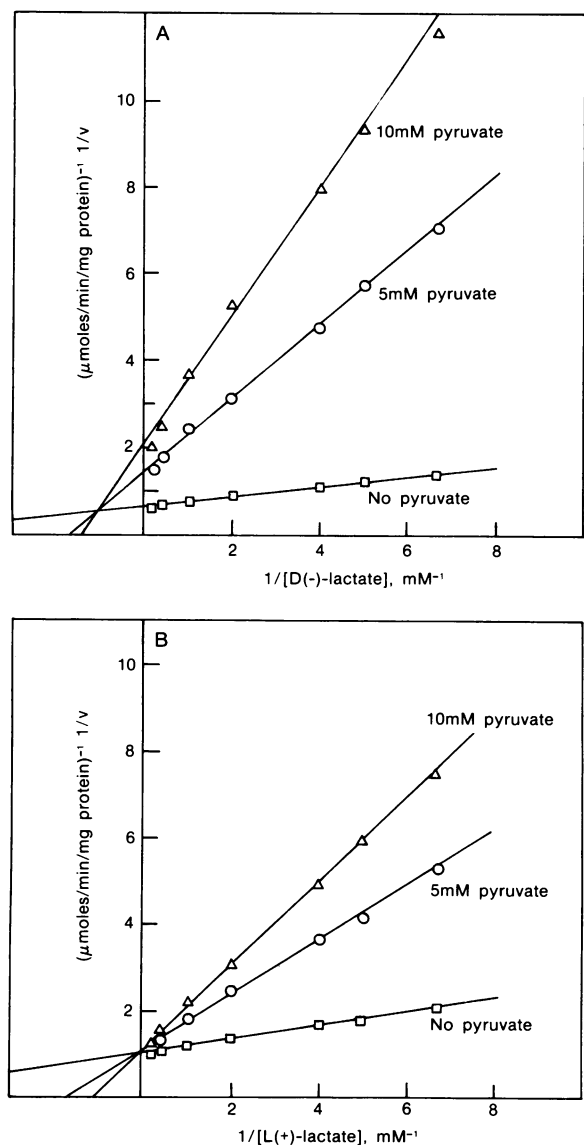


FIG. 6. Lineweaver-Burk plots of  $1/v$  versus (A)  $1/D(-)$ -lactate and (B)  $1/L(+)$ -lactate at pyruvate concentrations of 0, 5, and 10 mM. A freshly prepared extract of *P. freudenreichii* subsp. *shermanii* ATCC 9614 was used, and other components were the same as in the standard reaction mixture (see Materials and Methods).

hibition pattern with  $D(-)$ -lactate (Fig. 6B). The same inhibition patterns were found for strains 566 and DRI (data not shown). The enzyme affinities for the lactate isomers were similar for all three strains studied, with apparent  $K_m$  values of 0.16, 0.22, and 0.13 mM  $L(+)$ -lactate and 0.20, 0.17, and 0.23 mM  $D(-)$ -lactate for strains NCDO 566, ATCC 9614, and DRI, respectively.

## DISCUSSION

With all five strains of *P. freudenreichii* subsp. *shermanii*, the  $L(+)$  isomer of lactate was fermented in preference to the  $D(-)$  isomer when starting with a mixture of lactate isomers under a variety of growth conditions. The sequence of isomer utilization has been studied in detail with ATCC

9614. Under anaerobic growth conditions, 3 mol of lactate were fermented to 2 mol of propionate and 1 mol each of acetate and  $CO_2$  as found previously (for reviews, see references 14, 17, and 26).

The specific activities of both LDHs increased during growth on lactate in batch cultures. The ratio of  $D(-)$ -LDH to  $L(+)$ -LDH specific activity was between 1.2:1.0 and 1.7:1.0 and did not change appreciably during growth on either  $DL$ -lactate or  $L(+)$ -lactate (Table 2). Thus, the ratio of the two LDH activities does not explain why the  $L(+)$  isomer was utilized first during  $DL$ -lactate fermentation.

*P. freudenreichii* subsp. *shermanii* ATCC 9614 cells contained high concentrations ( $>100$  mM) of pyruvate when growing on lactate in defined medium, in agreement with an earlier report (J. B. Smart, Ph.D. thesis, Massey University, Palmerston North, New Zealand, 1980). During fermentation of  $DL$ -lactate by ATCC 9614 (Fig. 2), this high initial concentration decreased in parallel with the  $L(+)$ -lactate concentration in the medium. When only the  $D(-)$  isomer was present, the initial intracellular pyruvate concentration was much lower ( $<25$  mM) than when either  $L(+)$ - or  $DL$ -lactate was present. Therefore, this high level of pyruvate depends more on the concentration of the  $L(+)$  isomer than on the combined concentration of both isomers of lactate (Fig. 2 and 4). High intracellular pyruvate concentrations (40 to 70 mM) were found in all five strains of *P. freudenreichii* subsp. *shermanii* growing in static cultures on  $DL$ -lactate. Pyruvate was detected in culture supernatants at concentrations which reflected the intracellular pyruvate levels. Thus, with the  $D(-)$  isomer as the growth substrate, the maximum extracellular pyruvate concentration was low (0.2 mM) compared with the 3 to 5 mM pyruvate concentration found when  $L(+)$ -lactate was the only isomer present. During glucose or lactose fermentation, pyruvate was present at a low in vivo concentration (1 to 3 mM) and was not detected in culture supernatants of *P. freudenreichii* subsp. *shermanii*.

Pyruvate inhibited the  $NAD^+$ -independent  $D(-)$ -LDH activity more than the  $L(+)$ -LDH activity. This was evident in the extracts of all five *P. freudenreichii* subsp. *shermanii* strains studied. These differences are explained by the kinetic studies of LDH activity in extracts from three strains (NCDO 566, ATCC 9614, and DRI) which indicated that pyruvate showed a competitive inhibitor pattern with  $L(+)$ -lactate and a mixed-type inhibitor pattern with  $D(-)$ -lactate. Kinetic studies on the partially purified  $D(-)$ -LDH from *P. pentosaceum* indicated that pyruvate was a noncompetitive inhibitor of  $D(-)$ -lactate (19).

In the *P. freudenreichii* subsp. *shermanii* strains studied, the properties of the LDHs in combination with the high intracellular pyruvate concentration present during fermentation of  $L(+)$ -lactate can explain the preferential use of the  $L(+)$  isomer when  $DL$ -lactate was present. With use of the artificial electron acceptor (DCPIP), high  $D(-)$ -lactate concentrations did not completely overcome the pyruvate inhibition of LDH activity. In contrast, when  $L(+)$ -LDH was studied, pyruvate gave a competitive inhibitor pattern, indicating that increasing the  $L(+)$ -lactate concentration will eventually overcome inhibition by a particular concentration of pyruvate. Thus, during the initial stages of fermentation of  $DL$ -lactate, the high intracellular pyruvate concentration ( $>100$  mM) inhibits the  $D(-)$ -LDH to a greater extent than the  $L(+)$ -LDH, with the result that the  $L(+)$  isomer is preferentially utilized. As the  $L(+)$ -lactate concentration decreases, the intracellular pyruvate concentration also decreases, resulting in the oxidation of progressively more of

the D(-)-lactate to pyruvate by D(-)-LDH. The increase in the specific activity of the D(-)-LDH during lactate fermentation is likely to be particularly important for increasing the turnover of D(-)-lactate by D(-)-LDH in the presence of pyruvate. The doubling time for ATCC 9614 with D(-)-lactate was considerably longer than with L-(+)-lactate (8.0 and 4.4 h, respectively). This difference may be a consequence of rate-limiting oxidation of D(-)-lactate caused by the presence of inhibitory intracellular concentrations of pyruvate. In extrapolating the in vitro findings to the in vivo situation, it has been assumed that the pyruvate inhibition pattern for the LDHs found by using an artificial electron acceptor will be similar to that with acceptors in vivo. A membrane-bound electron transport system is involved in the transfer of reducing equivalents from lactate to fumarate in propionibacteria (7, 21).

The pattern of isomer utilization by *P. freudenreichii* subsp. *shermanii* ATCC 9614 and KFA under all growth conditions was similar to that found in Swiss-type cheese made with *P. freudenreichii* subsp. *shermanii* KFA (11, 25). The present results suggest that the preferential utilization of L-(+)-lactate in Swiss-type cheese results from pyruvate inhibition of D(-)-LDH in propionibacteria.

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