APPLIED AND ENVIRONMENTAL MICROBIOLOGY, Aug. 1986, p. 359–365
0099-2240/86/080359-07$02.00/0
Copyright © 1986, American Society for Microbiology
Vol. 52, No. 2

Metabolism of Aspartate by *Propionibacterium freudenreichii* subsp. *shermanii*: Effect on Lactate Fermentation

VAUGHAN L. CROW
New Zealand Dairy Research Institute, Palmerston North, New Zealand

Received 5 March 1986/Accepted 20 May 1986

More than 90% of the aspartate in a defined medium was metabolized after lactate exhaustion such that 3 mol of aspartate and 1 mol of propionate were converted to 3 mol of succinate, 3 mol of ammonia, 1 mol of acetate, and 1 mol of CO_2. This pathway was also evident when propionate and aspartate were the substrates in complex medium in the absence of lactate. In complex medium with lactate present, about 70% of the aspartate was metabolized to succinate and ammonia during lactate fermentation, and as a consequence of aspartate metabolism, more lactate was fermented to acetate and CO_2 than was fermented to propionate. The conversion of aspartate to fumarate and ammonia by the enzyme aspartase and subsequent reduction of fumarate to succinate occurred in the five strains of *Propionibacterium freudenreichii* subsp. *shermanii* studied. The ability to metabolize aspartate in the presence of lactate appeared to be related to aspartase activity. The specific activity of aspartase increased during and after lactate utilization, and the levels of this enzyme were lower in cells grown in defined medium than levels in those cells grown in complex medium. Under the conditions used, no other amino acids were readily metabolized in the presence of lactate. The possibility that aspartate metabolism by propionibacteria in Swiss cheese has an influence on CO_2 production is discussed.

Propionibacteria are classically regarded as fermenting lactate and glucose to propionate, acetate, and CO_2 in a ratio of 2:1:1 (for reviews, see references 16, 19, and 29). However, fermentation of these substrates may give rise to different ratios (29). For example, the ratio of propionate to acetate varied from 2 to 14 for glucose fermentations by different strains of propionibacteria (31). A possible explanation has been suggested recently in which citrate plays a role in the anaerobic fermentation of glucose (29). With lactate as the substrate, the ratio of propionate to acetate varied from 1.7 to 2.2 for *Propionibacterium freudenreichii* subsp. *freudenreichii*, *Propionibacterium pentosaceum* (*Propionibacterium acidipropionici*) (7), and *Propionibacterium freudenreichii* subsp. *shermanii* (20) grown anaerobically in complex or synthetic medium. *P. freudenreichii* subsp. *shermanii* can adapt to aerobic metabolism with lactate oxidation mainly to acetate and CO_2 such that the propionate:acetate ratio decreases to 0.25:1.00 (20).

The formation of succinate may also explain why the ratio of propionate-acetate is not 2:1 under some circumstances (for a review, see reference 29). Between 7.9 and 26.1 mmol of succinate was produced per 100 mmol of glucose fermented with several *Propionibacterium* spp. (31). Utilization of CO_2 is involved in the formation of succinate from fermentation of glucose and glycerol by *Propionibacterium* spp. (32, 33). The main mechanism for CO_2 fixation in propionibacteria involves carboxytransphosphorylase (EC 4.1.1.38), with high concentrations of CO_2 favoring succinate as a fermentation product (24, 25, 30). Succinate is also produced from lactate by *P. freudenreichii* subsp. *freudenreichii* growing anaerobically in complex medium (6), although succinate is often found only in trace amounts (7). The possible contribution of carboxytransphosphorylase to the formation of succinate by propionibacteria growing on lactate has not been studied in detail.

In Swiss-type cheese, the molar ratio of propionate to acetate is usually less than 2:1. For example, the ratio ranged from 0.92:1.00 to 1.74:1.00 (average ratio, 1.38:1.00) in Swiss cheese analyzed by three groups of workers, the data being summarized by Langsrud and Reinbold (19). In another study (17), the propionate/acetate ratio ranged from 0.85:1.00 to 0.99:1.00. Succinate is present in Swiss cheese in concentrations ranging from 0.04 to 0.16 g/100 g of cheese (17, 22, 26). These low ratios of propionate to acetate in Swiss cheese and the mechanisms for succinate formation have not been adequately explained.

The present study was undertaken to examine the formation of succinate by five strains of *P. freudenreichii* subsp. *shermanii* growing on lactate. The data suggest that succinate can be produced from aspartate and that this influences lactate metabolism.

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 Diary Research Institute, Palmerston North, New Zealand. *P. freudenreichii* subsp. *shermanii* DRI and KFA were originally isolated from commercial Swiss cheese.

The complex and defined media used to grow *P. freudenreichii* subsp. *shermanii* have been described previously (4). The complex medium contained yeast extract and casein hydrolysate. The main nitrogen source in the defined medium was $(NH_4)_2SO_4$, with only two other amino acids present in low concentrations (2.5 mM cysteine and 0.5 mM tryptophan). Cultures were grown at 30°C either statically or anaerobically; in the latter case, N_2-CO_2 (95:5, vol/vol) was bubbled (~30 ml/min) through the medium. The N_2-CO_2 was passed over freshly regenerated, heated copper turnings to remove any traces of oxygen. In some experiments, the complex medium was modified (4) by pH adjustment to 5.25 (medium pH after autoclaving, 5.30), addition of 1% (wt/vol) NaCl, alteration of the ratio of lactate isomers, and incubation at 22°C.

Aspartase assays. Cell extracts were prepared as described previously (4). Aspartase activity was routinely assayed by measuring the production of fumarate. The standard assay (1

ml) contained 100 mM imidazole hydrochloride (pH 7.0), 50 mM L-aspartic acid (pH adjusted to 7.0 with NaOH), 1 mM $MgCl_2$, and diluted cell extract (added last to initiate reaction). The reaction was monitored at 240 nm (25°C) with a Gilford model 250 spectrophotometer. The molar extinction coefficient of fumarate at 240 nm (2,530 M^{-1} cm^{-1}) was used (10). Cell extracts were diluted to give an initial absorbance change of less than 1.0/min. The reaction rate was linear for up to 1 min before decreasing to a value between 40 and 60% of the initial rate due to interference from fumarase activity. The method described above was routinely used because of its convenience and sensitivity. Aspartase activity was also assayed by measuring the rate of ammonia production in the same reaction mixture (total volume, 5 ml). The reaction was stopped at 0, 5, 10, and 30 min by adding 1 ml of reaction mixture to 0.5 ml of 10% trichloroacetic acid. This mixture was subsequently assayed enzymatically (5) for ammonia. The initial rate of fumarate production was >95% of the rate of ammonia production. One unit of aspartase activity was defined as the amount of enzyme producing 1 μmol of fumarate per min under the standard assay conditions.

Substrate and product analyses. Enzymatic analyses were routinely used to assay for alanine (28), ammonia (5), aspartate (1), D-(−)-lactate (13), L-(+)-lactate (15), fumarate (14), malate (14), pyruvate (12), and succinate (*Methods of Enzymatic Food Analysis*, Boehringer, Mannheim, Federal Republic of Germany, 1980). The reaction mixture for succinate was modified (4). Propionate and acetate were routinely assayed by gas-liquid chromatography (4). Acetate, fumarate, lactate, malate, propionate, pyruvate, and succinate concentrations in culture supernatants were also measured by high-performance liquid chromatography (HPLC) (4).

The amino acid compositions of culture supernatants were determined by HPLC (with a Waters Associates, Inc., Liquid chromatograph composed of two 6000 A pumps, a WISP 710B autoinjector, a model 720 programmable system controller, and a model 420-AC fluorescence detector). A computing integrator (model 302; Lab Data Control, Riviera Beach, Fla.) was used for analysis of amino acids after postcolumn derivatization with *o*-phthalaldehyde. Culture supernatants were cleaned on a SEP-PAK C18 cartridge (*HPLC Amino Acid Analysis System Preliminary Manual*, Waters Associates, Inc., Milford, Mass., 1982) before injection (20 to 50 μl) into the column.

Other procedures. Protein was estimated by the method of Bradford (2).

Materials. All biochemicals were obtained from Sigma Chemical Co., St. Louis, Mo. The lactic acid (Sigma L-1375; Na^+ salt) added to the medium contained equal concentrations of the two isomers and is referred to as DL-lactate.

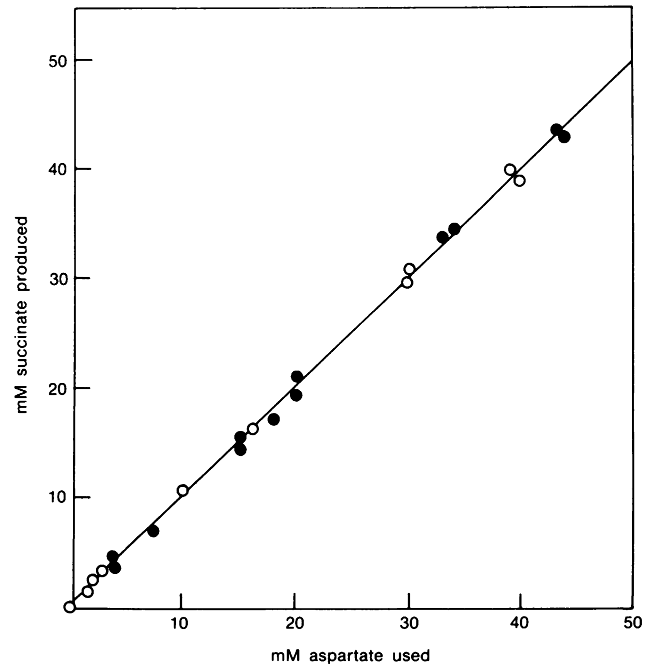

FIG. 1. Relationship between succinate produced and aspartate used by *P. freudenreichii* subsp. *shermanii* ATCC 9614 grown statically on 120 mM DL-lactate in defined (○) and complex (●) medium. Medium (pH 6.5) was supplemented with different amounts of aspartate (10 to 40 mM), and culture supernatants were analyzed at daily intervals for up to 5 days after inoculation (1% inoculum).

RESULTS

Aspartate utilization in static (semianaerobic) cultures. When *P. freudenreichii* subsp. *shermanii* was grown in complex medium, with or without the addition of 120 mM DL-lactate, 4 mM succinate was formed. In the first hour after lactate exhaustion, aspartate was the only amino acid that was utilized. After ATCC 9614 had been incubated for 60 h (1% inoculum) in complex medium with no added lactate, the only amino acids utilized were aspartate, alanine, serine, and glycine. When ATCC 9614 was incubated for up to 120 h in complex medium (containing 120 mM DL-lactate), succinate production was increased by the addition of 10 or 40 mM aspartate. No such increase in succinate production was observed in medium supplemented with similar amounts of alanine, glycine, or serine (data for the above not shown). The relationship between aspartate utilization and succinate production for ATCC 9614 grown on 120 mM DL-lactate in defined or complex medium is shown in Fig. 1. For every mole of aspartate used, 1 mol of succinate was formed. This relationship also held for four other strains (NCDO 566, DRI, KFA, and MNS; data not shown). Succinate was formed in excess (2 to 3 mM) of the concentration of aspartate utilized but only when higher initial DL-lactate concentrations (160 to 200 mM) were utilized by the different strains.

Aspartate utilization in anaerobic cultures grown in defined medium. Defined medium was used to obtain fermentation balances that were not influenced by undefined substrates present in the complex medium. With 41 mM aspartate initially present, ATCC 9614 had metabolized only 4 mM aspartate to 4 mM succinate by the time lactate fermentation was finished (Fig. 2). The remaining aspartate was subsequently metabolized to succinate (final concentration, 41.5 mM). There was an increase in turbidity, associated with this latter phase of aspartate metabolism, and a decrease in the propionate concentration (10 to 13 mM) equal to an increase in the acetate concentration. Thus, the final propionate/acetate ratio was 1.0:1.0 (Fig. 2), in contrast to a ratio of 2.0:1.0 observed during lactate fermentation in the absence of aspartate (data not shown). The succinate formed in excess of the aspartate used was reproducibly 0.5 mM. A similar concentration was formed when either 80 or 180 mM DL-lactate was fermented by ATCC 9614 in defined medium with no aspartate added. The ammonia concentration after aspartate exhaustion was 54 mM, rather than the expected

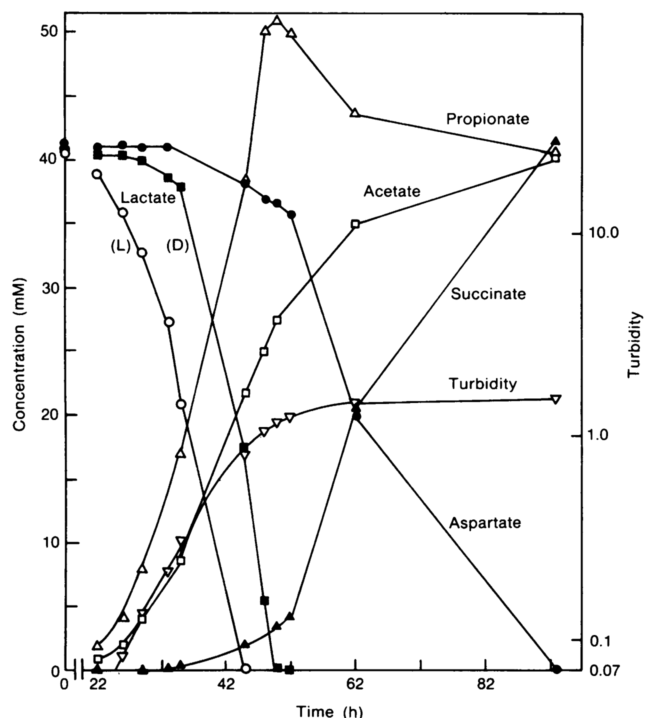

FIG. 2. Metabolism of L(+)-lactate, D-(−)-lactate, and aspartate by *P. freudenreichii* subsp. *shermanii* ATCC 9614 grown anaerobically at 30°C in defined medium containing 82 mM DL-lactate and 41 mM aspartate. A 2% inoculum was used. The concentration of the products acetate, propionate, and succinate are shown. Turbidity was monitored at 600 nm. The initial pH (6.6) increased to pH 7.1 after 95 h.

concentration of 58 mM, from metabolism of 41 mM aspartate (the initial concentration of NH_4^+ was 17 mM). There was a decrease in NH_4^+ (from 3 to 6 mM) after 80 or 180 mM lactate had been fermented in defined medium (no added aspartate) by ATCC 9614, suggesting that some NH_4^+ was utilized or lost in the gas stream.

The pattern of lactate and aspartate utilization was similar to that shown in Fig. 2 when ATCC 9614 and NCDO 566 were grown in defined medium with 80 or 180 mM DL-lactate and with any of four different aspartate concentrations (10, 20, 30, or 40 mM) added (data not shown). Under these conditions, the bulk of aspartate was metabolized in the absence of lactate in the following way: 3 mol of aspartate + 1 mol of propionate → 3 mol of succinate + 3 mol of ammonia + 1 mol of acetate + 1 mol of CO_2. NCDO 566 was slower in metabolizing aspartate than was ATCC 9614. For example, under the same experimental conditions described for ATCC 9614 (Fig. 2), less than 1 mM aspartate had been utilized by the time the lactate had been fermented by NCDO 566. At 40 h after the lactate had been fermented, ATCC 9614 had utilized all of the aspartate, in contrast to the utilization of only 5 mM aspartate by NCDO 566. It was only after a further 60 h that all of the aspartate had been utilized by NCDO 566, with the same final concentrations of fermentation products as produced by ATCC 9614 (Fig. 2).

Aspartate utilization in anaerobic cultures grown in complex medium. After an initial lag, aspartate was co-utilized with lactate when *P. freudenreichii* subsp. *shermanii* ATCC 9614 was grown in complex medium (Fig. 3). Throughout the growth, the rates of succinate and ammonia formation were similar to the rate of aspartate utilization, and at the end of fermentation, only up to 1 mM succinate was present in excess of the amount of aspartate utilized. The slight increase in ammonia, propionate, and acetate concentrations after lactate and aspartate utilization coincided with decreases in alanine and serine concentrations (data not shown). When the growth medium used in Fig. 3 was supplemented with alanine or serine (final concentrations, 36 and 34 mM, respectively), the concentrations of ammonia, propionate, and acetate showed further increases, but only after lactate and aspartate utilization was complete (data not shown). When all of the lactate and aspartate had been fermented, the culture turbidity was higher (1.3 times) for the experiment in Fig. 3 than for the same experiment without added aspartate.

The amount of aspartate utilized in complex medium during lactate fermentation was studied with two other strains (NCDO 566 and KFA; data not shown) under the same conditions described in Fig. 3. For NCDO 566, the initial aspartate concentration (33 mM) had decreased only by 2 and 6 mM by the time 50 and 90%, respectively, of the starting concentration of lactate had been fermented. Strain KFA gave results similar to those described for ATCC 9614 (Fig. 3), for which a faster rate of aspartate utilization was observed. The aspartate concentration had decreased by 8 and 20 mM by the time 50 and 90%, respectively, of the starting concentration of lactate had been fermented by strain KFA. At the end of the fermentation of 180 mM lactate and 33 mM aspartate, the ratio of propionate to acetate was 1.5:1.0 for NCDO 566, ATCC 9614, and strain

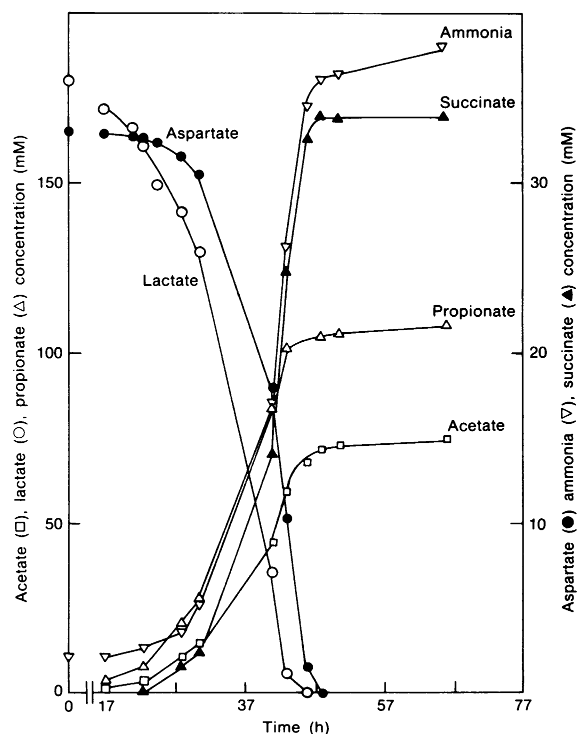

FIG. 3. Metabolism of lactate and aspartate by *P. freudenreichii* subsp. *shermanii* ATCC 9614 grown anaerobically at 30°C in complex medium containing 180 mM DL-lactate and 33 mM aspartate. A 2% inoculum was used. The concentrations of the products acetate, ammonia, propionate, and succinate are shown. The initial pH (6.5) increased to 7.0 after 67 h.

KFA (data shown only for ATCC 9614 [Fig. 3]). As has been noted in Fig. 3, the rates of succinate and ammonia formation by NCDO 566 and KFA were similar to the rate of aspartate utilization.

Strain KFA and ATCC 9614, grown in the complex medium without added aspartate, utilized the endogenous aspartate (4 mM) during the time that the lactate concentration was lowered from 180 to 100 mM. A ratio of propionate to acetate of 1.9:1.0 was obtained after lactate and aspartate had been fermented.

Aspartate and propionate utilization in complex medium without lactate. In complex medium with no lactate present, ATCC 9614 metabolized propionate and aspartate to acetate and succinate, respectively, and malate and fumarate were detected in culture supernatants (Fig. 4). The concentrations of malate and fumarate were approximately equal up to the peak concentration (at 50 h), and then the fumarate concentration decreased at a faster rate compared with malate (data not shown). Malate and fumarate were also detected in culture supernatants of NCDO 566, ATCC 9614, and strain KFA growing on lactate in defined or complex medium (data not shown), although the maximum concentrations formed (<1.8 mM) were not as high as those shown in Fig. 4 (8 mM). No pyruvate was detected in culture supernatants from Fig. 4. Maximum pyruvate concentrations of 0.5 and 2 mM were found in culture supernatants from Fig. 2 and 3, respectively (data not shown).

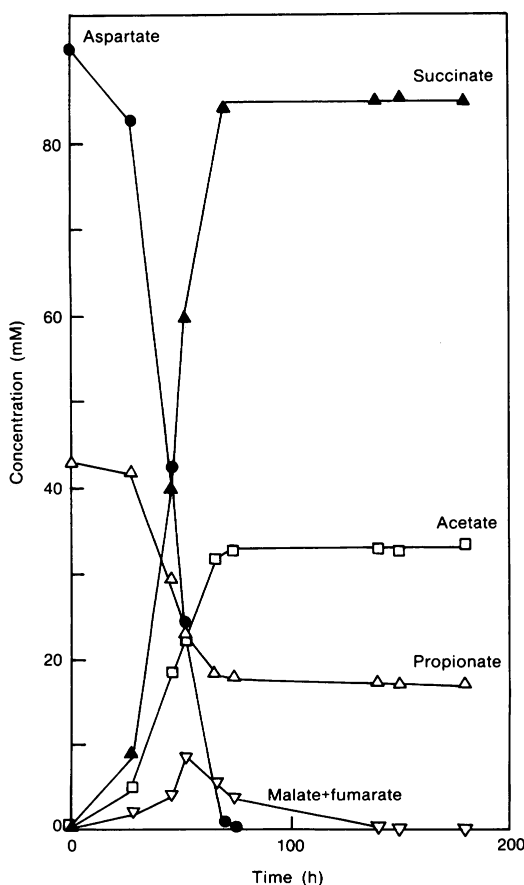

FIG. 4. Metabolism of 91 mM aspartate and 43 mM propionate grown statically at 30°C in complex medium. Complex medium contained half the normal concentration of casein hydrolysate and was supplied by GIBCO Laboratories, Grand Island, N.Y. Concentrations of acetate, malate-fumarate, and succinate are shown.

TABLE 1. Specific activity of aspartase in cell extracts of *P. freudenreichii* subsp. *shermanii* ATCC 9614[a]

Growth medium (initial aspartate concn [mM])	Concn (mM) at cell harvest of: Aspartate	Lactate	Aspartase sp act[b]
Defined			
30	30	155	0.01
	28	25	0.04
	27	1	0.07
	0	0[c]	0.08
0	0	132	0.01
	0	26	0.03
	0	0[d]	0.07
Complex			
34	34	165	0.05
	16	20	0.11
	0	0[e]	0.38
4	0	130	0.08
	0	0[f]	0.28

[a] Culture conditions: 30°C; N_2-CO_2 (95:5, vol/vol); initial pH, 6.5; and 180 mM DL-lactate. Cell extracts were prepared immediately, and aspartase was assayed within 3 h of cell harvest.
[b] μmol of fumarate produced per min per mg of protein.
[c] Concentration when time from lactate exhaustion to cell harvest was 40 h.
[d] Concentration when time from lactate exhaustion to cell harvest was 30 h.
[e] Concentration when time from lactate exhaustion to cell harvest was 50 h.
[f] Concentration when time from lactate exhaustion to cell harvest was 26 h.

The cell density was 1.23 mg (dry weight) of bacteria per ml (Fig. 4), compared with 0.16 mg (dry weight) of bacteria per ml for growth on endogenous substrates in complex medium with no added propionate or aspartate. The products of growth on endogenous substrates are propionate (5 mM), acetate (4.5 mM), and succinate (1.5 mM). After correcting for these endogenous products, there was a total of 31 mM propionate and 91 mM aspartate metabolized to 29 mM acetate, 85 mM succinate, and 92 mM ammonia (correcting for ammonia from alanine and serine). The final succinate concentration was 6 mM (range, 5 to 7 mM from six enzymatic and HPLC determinations) lower than the concentration of aspartate used. Another minor pathway for the metabolism of aspartate may have occurred under these conditions (Fig. 4) and may account for this lower succinate concentration.

Alanine and serine were fully utilized (present at initial concentrations of 3 and 2 mM, respectively) whether or not aspartate and propionate were added to the medium. These were the only amino acids, apart from aspartate, that were metabolized during the incubation time studied.

Aspartase activity in growing cells. In ATCC 9614, the specific activity of aspartase increased during and after lactate fermentation (Table 1). The specific activity values found in cell extracts were not altered significantly by the presence or absence of aspartate in defined medium or by a low (4 mM) or high (34 mM) concentration of aspartate present initially in complex medium. At comparable lactate conditions, the specific activity of aspartase was lower in cells grown in defined medium than that of cells grown in complex medium. Similar results were found for strain KFA (data not shown), except that specific activities were 1.5 to 2.0 times higher than those found for ATCC 9614. The other

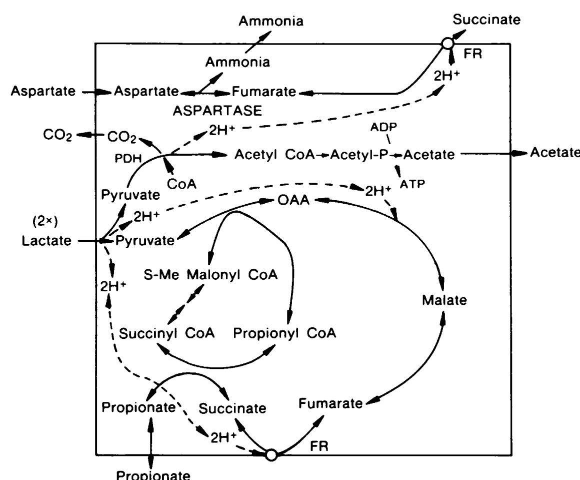

FIG. 5. Aspartate and lactate metabolism in propionibacteria showing how the O-R balance is maintained when 1 mol of aspartate is metabolized with 2 mol of lactate. PDH, Pyruvate dehydrogenase; FR, fumarate reductase.

strain studied (NCDO 566) utilized aspartate at a lower rate than did ATCC 9614 and strain KFA and had the lowest specific activity (data not shown). For example, the specific activity of aspartase reached a value of only 0.01 μmol of fumarate per min per mg of protein in cells of NCDO 566 grown in defined medium and harvested 2 h after lactate (180 mM) exhaustion and when the initial aspartate concentration (40 mM) had decreased to 38 mM. In complex medium a maximum value of 0.04 μmol of fumarate per min per mg of protein was obtained for NCDO 566 20 h after lactate exhaustion.

During metabolism of aspartate and propionate by *P. freudenreichii* subsp. *shermanii* ATCC 9614 in complex broth (Fig. 4), the specific activity of aspartase (2.8 μmol/min per mg of protein) was considerably higher than that found in cell extracts in which lactate was initially present (Table 1).

Effect of stressful conditions on aspartate metabolism. Complex medium, compared with defined medium, is more similar to the nutritional conditions found in a Swiss cheese environment. However, the routine growth conditions in complex medium (Fig. 3; also see Materials and Methods) are different from the Swiss cheese environment, particularly with respect to temperature, pH, and salt concentration (4). Therefore, to investigate the possibility of aspartate metabolism during lactate fermentation in Swiss cheese, two *P. freudenreichii* subsp. *shermanii* strains (ATCC 9614 and KFA) were incubated at 22°C in complex medium at a starting pH of 5.3 with 361 mM Na^+ present and 145 mM L-(+)- and 55 mM D-(−)-lactate added (data not shown). Both strains showed slower growth rates under these stressful conditions (4), and the endogenous aspartate (4 mM) was fully exhausted by ATCC 9614 and strain KFA when only 20 to 30 mM lactate was metabolized (data not shown). In comparison, under normal growth conditions, 80 to 85 mM lactate (initial lactate concentration, 180 mM) was metabolized by both strains by the time aspartate was completely utilized. ATCC 9614 was also grown under stressful conditions, with an initial aspartate concentration of 33 mM to compare with the results from Fig. 3 (normal growth conditions). When 50 and 90% of the starting lactate concentration had been fermented, the aspartate concentration had decreased by 15 and 32 mM, respectively, under stressful conditions, compared with 7 to 20 mM, respectively, under normal growth conditions (Fig. 3). For ATCC 9614 growing under stressful conditions, the addition of aspartate to a concentration of 33 mM (cf. with 4 mM endogenous concentration) decreased the final propionate/acetate ratio from 1.9:1.0 to 1.5:1.0 (i.e., after lactate and aspartate exhaustion). Throughout growth under these stressful conditions, the rates of succinate and ammonia formation were similar to the rate of aspartate utilization. However, 5 mM succinate was formed in excess of either of the two initial aspartate concentrations used. This excess succinate produced is likely to have been formed via CO_2 fixation by carboxytransphosphorylase. With cells harvested at comparable lactate concentrations in the medium, a two- to fourfold increase in the specific activity of aspartase was found in cell extracts of ATCC 9614 and strain KFA grown under stressful conditions, compared with strains grown under normal conditions.

DISCUSSION

The metabolism by *P. freudenreichii* subsp. shermanii strains of aspartate to succinate (via the intermediate formation of fumarate) and NH_4^+ influences the way lactate is fermented to propionate, acetate, and CO_2 (Fig. 5). Fumarate, provided externally or generated by intermediary metabolism, is a major terminal oxidant for bacteria (18). Thus, as a consequence of aspartate metabolism, more lactate is fermented to acetate and CO_2 than to propionate. The extra reducing equivalents, formed via pyruvate dehydrogenase (Fig. 5), are utilized stoichiometrically by fumarate reduction to succinate via fumarate reductase. The final fermentation products from lactate depend on the initial molar ratio of aspartate and lactate. The propionate/acetate ratio of 1:1 in the pathway (Fig. 5) is found for the experiment described in Fig. 2 in which the initial ratio of lactate to aspartate was 2:1. When less than 1 mol of aspartate is fermented with 2 mol of lactate, the propionate/acetate ratio will be between 1:1 and 2:1, as described in Fig. 3, in which the ratio is 1.5:1.0.

Carbon and oxidation-reduction (O-R) balances provide two important criteria for the evaluation of pathways of anaerobic metabolism (29). In the closed system of a true fermentation, the aggregate O-R state of the products corresponds to that of the substrates (34). The production of CO_2 was not measured in the present study but has been calculated to be equivalent to the acetate produced minus the succinate formed in excess of aspartate utilized. The contribution of CO_2 fixation (by carboxytransphosphorylase) to succinate formation was small under the anaerobic experimental conditions used. The flushing of N_2-CO_2 (95:5, vol/vol) through the medium presumably reduced the build-up of CO_2 and thus CO_2 fixation. With ATCC 9614 in defined medium (Fig. 2), the final products from 82 mM lactate and 41 mM aspartate were 40.3 mM acetate, 40.6 mM propionate, 41.5 mM succinate, and 39.8 mM CO_2 (calculated). With these data, an O-R number of 0.96 and a carbon recovery of 99% is obtained.

Similar values were obtained for ATCC 9614 and NCDO 566, which had fermented different combinations of aspartate and lactate in defined medium. In complex medium, the contribution of endogenous substrates to fermentation products was small compared with the concentration of products from added lactate and aspartate. In addition, utilization of endogenous substrates (mainly alanine and

serine) was negligible at the point at which lactate and aspartate had just been exhausted. With ATCC 9614 in complex medium (Fig. 3), the products from 33 mM aspartate and 180 mM lactate were 72 mM acetate, 105 mM propionate, 33.8 mM succinate, and 71.2 mM CO_2 (calculated). An O-R number of 1.05 and a carbon recovery of 98.8% were obtained for these products. Fermentation balances based on runs described in Table 1 (data not shown) gave similar values (O-R, 1.0 ± 0.08; carbon recovery, 100 ± 3%). Thus, the pathway for anaerobic metabolism of lactate and aspartate (Fig. 5) is supported by carbon and O-R balances.

Aspartase was identified in propionibacteria in 1932 (27) and was later partially purified and characterized from *Propionibacterium peterssonii* (8, 9). The metabolism by propionibacteria of amino acids, including aspartate, has been studied (3, 23). As early as 1923, aspartic acid was found to be a source of acetic acid and CO_2 when a propionibacterium strain was incubated in a 4% peptone solution with or without a 0.5% supplement of asparagine (23). Recently, four strains of propionibacteria were incubated with amino acid mixtures, including aspartic acid, and analyzed only for CO_2, ammonia, propionate, and acetate (3).

When propionibacteria were grown in defined or complex medium, although the final products from lactate and aspartate were the same, there was a major difference in the pathway leading to the formation of the final fermentation products. In complex medium (Fig. 3), 70% of the aspartate was metabolized during lactate fermentation; thus, some of the lactate was diverted from propionate to acetate and CO_2 formation to maintain the redox balance. In contrast, only 9% of the aspartate was metabolized in defined medium by the time lactate was exhausted (Fig. 2). As a consequence, the reducing equivalents are generated by propionate conversion to acetate and CO_2. Studies with radioactive compounds have shown that the reactions are reversible in propionic acid fermentation (for a review, see reference 29). The metabolism of aspartate in defined medium (Fig. 2) and in complex medium in which propionate, rather than lactate, was the other substrate (Fig. 4) also indicates the reversible nature of propionic acid fermentation in growing cells of propionibacteria.

For the two strains of *P. freudenreichii* subsp. *shermanii* studied, the specific activity of aspartase was between three- and eightfold lower in cells growing in defined medium than in cells growing in complex medium. This difference may explain why more aspartate was metabolized in the presence of lactate in complex medium. Glucose repression of aspartase was observed in *Aerobacter aerogenes* (11). Lactate repression of aspartase activity is probably an important control feature of aspartate metabolism because the level of enzyme activity in growing cells increased during and after lactate fermentation and was not altered by the aspartate concentration. The level of aspartase activity and the associated ability to metabolize aspartate in the presence of lactate varied in NCDO 566, ATCC 9614, and strain KFA.

Metabolism of a number of amino acids by propionibacteria may be important in the split defect in Swiss-type cheese (3). On the basis of this study, aspartate is the only amino acid likely to be readily metabolized by propionibacteria in a Swiss cheese environment when lactate is present and being rapidly metabolized in the warm room. Aspartate was more readily metabolized under stressful conditions similar to the Swiss cheese environment (e.g., low pH, 22°C, and in the presence of NaCl) than under the more optimum conditions of growth (pH 6.5, 30°C, and with no added NaCl) that were routinely used. If aspartate metabolism occurs during lactate fermentation in Swiss cheese in a manner similar to that found in complex broth, then the conversion of aspartate to succinate and ammonia is associated with altered lactate metabolism. More acetate and CO_2 are formed at the expense of propionate. Carboxytransphosphorylase and aspartase activity explain both the presence of succinate in Swiss cheese and the reason that the ratio of propionate to acetate is less than 2:1. However, CO_2 production is enhanced as a consequence of aspartase activity and is decreased by the CO_2 fixation step catalyzed by carboxytransphosphorylase. The variability of CO_2 production during lactate fermentation as a consequence of aspartase activity is dependent on the strain and also on factors that contribute to the availability of aspartic acid. Although there are data on the free amino acids in ripening Swiss cheese (for reviews, see references 18 and 21), the free amino acid content in the earlier stages of lactate utilization in the warm room has not been considered in detail.

ACKNOWLEDGMENTS

I thank Nicola Wilson for excellent technical assistance; Rex Humphrey and Barry Richardson for HPLC analysis of organic and amino acids, respectively; Russell Richardson for gas-liquid chromatographic analysis; and Graham Pritchard, John Smart, Terry Thomas, and Keith Turner for helpful discussion.

LITERATURE CITED

1. **Bergmeyer, H. U., E. Bernt, H. Möllering, and G. Pfleiderer.** 1974. L-Aspartate and L-asparagine, p. 1696–1700. *In* H. U. Bergmeyer (ed.), Methods of enzymatic analysis, 2nd ed., vol. 4. Verlag Chemie, Weinheim, Federal Republic of Germany.
2. **Bradford, M. M.** 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal. Biochem. **72**:248–254.
3. **Brendehaug, J., and T. Langsrud.** 1985. Amino acid metabolism in propionibacteria: resting cell experiments with four strains. J. Dairy Sci. **68**:281–289.
4. **Crow, V. L.** 1986. Utilization of lactate isomers by *Propionibacterium freudenreichii* subsp. *shermanii*: regulatory role for intracellular pyruvate. Appl. Environ. Microbiol. **52**:352–358.
5. **Crow, V. L., and T. D. Thomas.** 1982. Arginine metabolism in lactic streptococci. J. Bacteriol. **150**:1024–1032.
6. **de Vries, W., W. M. C. van Wyck-Kapteyn, and A. H. Stouthamer.** 1972. Influence of oxygen on growth, cytochrome synthesis and fermentation pattern in propionic acid bacteria. J. Gen. Microbiol. **71**:515–524.
7. **de Vries, W., W. M. C. van Wyck-Kapteyn, and A. H. Stouthamer.** 1973. Generation of ATP during cytochrome-linked anaerobic electron transport in propionic acid bacteria. J. Gen. Microbiol. **76**:31–41.
8. **Ellfolk, N.** 1953. Studies on aspartase. I. Quantitative separation of aspartase from bacterial cells and its partial purification. Acta Chem. Scand. **7**:824–830.
9. **Ellfolk, N.** 1953. Studies on aspartase. II. On the chemical nature of aspartase. Acta Chem. Scand. **7**:1155–1163.
10. **Emery, T. F.** 1963 Aspartase-catalyzed synthesis of *N*-hydroxyaspartic acid. Biochemistry **2**:1041–1045.
11. **Farley, M. A., and H. C. Lichstein.** 1963. Glucose inhibition of aspartase synthesis by *Aerobacter aerogenes*. Can. J. Microbiol. **9**:835–842.
12. **Fordyce, A. M., V. L. Crow, and T. D. Thomas.** 1984. Regulation of product formation during glucose or lactose limitation in nongrowing cells of *Streptococcus lactis*. Appl. Environ. Microbiol. **48**:332–337.
13. **Gawehn, K., and H. U. Bergmeyer.** 1974. D-(−)-Lactate, p. 1492–1493. *In* H. U. Bergmeyer (ed.), Methods of enzymatic

analysis, 2nd ed., vol. 3. Verlag Chemie, Weinheim, Federal Republic of Germany.
14. **Goldberg, N. D., and J. V. Passonneau.** 1974. L-Malate and fumarate. Fluorimetric determination, p. 1600–1603. *In* H. U. Bergmeyer (ed.), Methods of enzymatic analysis, 2nd ed., vol. 3. Verlag Chemie, Weinheim, Federal Republic of Germany.
15. **Gutmann, I., and A. H. Wahlefeld.** 1974. L-(+)-Lactate. Determination with lactate dehydrogenase and NAD, p. 1464–1468. *In* H. U. Bergmeyer (ed.), Methods of enzymatic analysis, 2nd ed., vol. 3. Verlag Chemie, Weinheim, Federal Republic of Germany.
16. **Hettinga, D. H., and G. W. Reinbold.** 1972. The propionic acid bacteria—a review. II. Metabolism. J. Milk Food Technol. **35**:358–372.
17. **Jager, H., and E. Tschager.** 1983. Die Bestimmung organischer Säuren in Käse mit der HPLC. Milchw. Berichte **75**:105–108.
18. **Kröger, A.** 1978. Fumarate as terminal acceptor of phosphorylative electron transport. Biochim. Biophys. Acta **505**:129–145.
19. **Langsrud, T., and G. W. Reinbold.** 1973. Flavor development and microbiology of Swiss cheese—a review. III. Ripening and flavor production. J. Milk Food Technol. **36**:593–609.
20. **Pritchard, G. G., J. W. T. Wimpenny, H. A. Morris, M. W. A. Lewis, and D. E. Hughes.** 1977. Effect of oxygen on *Propionibacterium shermanii* grown in continuous culture. J. Gen. Microbiol. **102**:223–233.
21. **Schormüller, J.** 1968. The chemistry and biochemistry of cheese ripening. Adv. Food Res. **16**:231–334.
22. **Schormüller, J., and H. Langner.** 1960. Uber die organischem Säuren verschiedener Käsearten. Z. Lebensm.-Unters. Forsch. **113**:289–298.
23. **Shaw, R. H., and J. M. Sherman.** 1923. The production of volatile fatty acids and carbon dioxide by propionic acid bacteria with special reference to their action in cheese. J. Dairy Sci. **6**:303–309.
24. **Siu, P. M. L., and H. G. Wood.** 1962. Phosphoenolpyruvic carboxytransphosphorylase, a CO_2 fixation enzyme from propionic acid bacteria. J. Biol. Chem. **237**:3044–3051.
25. **Siu, P. M. L., H. G. Wood, and R. L. Stjernholm.** 1961. Fixation of CO_2 by phosphoenolpyruvic carboxytransphosphorylase. J. Biol. Chem. **236**:PC21–22.
26. **Steffen, C., H. Glättli, and B. Nick.** 1979. Vergleichende Untersuchungen von Käsen mit und ohne Nachgärung. III. Bakteriologische und enzymatische Untersuchungen. Schweiz. Milchwirtsch. Forsch. **8**:19–26.
27. **Virtanen, A. I., and J. Tarnanen.** 1932. Die enzymatische Spaltung und Synthese der Asparaginsäure. Biochem. Z. **250**:193–211.
28. **Williamson, D. H.** 1974. L-Alanine. Determination with alanine dehydrogenase, p. 1679–1682. *In* H. U. Bergmeyer (ed.), Methods of enzymatic analysis, 2nd ed., vol. 4. Verlag Chemie, Weinheim, Federal Republic of Germany.
29. **Wood, H. G.** 1981. Metabolic cycles in the fermentation by propionic acid bacteria. Curr. Top. Cell. Regul. **18**:255–287.
30. **Wood, H. G., R. Stjernholm, and F. W. Leaver.** 1955. The metabolism of labeled glucose by the propionic acid bacteria. J. Bacteriol. **70**:510–520.
31. **Wood, H. G., and C. H. Werkman.** 1936. LXXXVIII. Mechanism of glucose dissimilation by the propionic acid bacteria. Biochem. J. **30**:618–623.
32. **Wood, H. G., and C. H. Werkman.** 1938. CLXVIII. The utilization of CO_2 by the propionic acid bacteria. Biochem. J. **32**:1262–1271.
33. **Wood, H. G., and C. H. Werkman.** 1940. 21. The relationship of bacterial utilization of CO_2 to succinic acid formation. Biochem. J. **34**:129–138.
34. **Wood, W. A.** 1961. Fermentation of carbohydrates and related compounds, p. 59–149. *In* I. C. Gunsalus and R. Y. Stanier (ed.), The bacteria, vol. 2. Academic Press, Inc., New York.