JOURNAL OF BACTERIOLOGY, June 1982, p. 1024–1032
0021-9193/82/061024-09$02.00/0

Vol. 150, No. 3

Arginine Metabolism in Lactic Streptococci

VAUGHAN L. CROW* AND TERENCE D. THOMAS

New Zealand Dairy Research Institute, Palmerston North, New Zealand

Received 26 October 1981/Accepted 13 January 1982

Streptococcus lactis metabolizes arginine via the arginine deiminase pathway producing ornithine, ammonia, carbon dioxide, and ATP. In the four strains of *S. lactis* examined, the specific activities of arginine deiminase and ornithine transcarbamylase were 5- to 10-fold higher in galactose-grown cells compared with glucose- or lactose-grown cells. The addition of arginine increased the specific activities of these two enzymes with all growth sugars. The specific activity of the third enzyme involved in arginine metabolism (carbamate kinase) was not altered by the composition of the growth medium. In continuous cultures arginine deiminase was not induced, and arginine was not metabolized, until glucose limitation occurred. In batch cultures the metabolism of glucose and arginine was sequential, whereas galactose and arginine were metabolized concurrently, and the energy derived from arginine metabolism was efficiently coupled to growth. No arginine deiminase activity was detected in the nine *Streptococcus cremoris* strains examined, thus accounting for their inability to metabolize arginine. All nine strains of *S. cremoris* had specific activities of carbamate kinase similar to those found in *S. lactis*, but only five *S. cremoris* strains had ornithine transcarbamylase activity.

The arginine deiminase pathway (formerly referred to as the arginine dihydrolase pathway) occurs in many bacteria, including some species of streptococci (1). This pathway involves the three enzymes arginine deiminase (EC 3.5.3.6), ornithine transcarbamylase (EC 2.1.3.3), and carbamate kinase (EC 2.7.2.2) which catalyze the following reactions, respectively

$$\text{L-arginine} + H_2O \rightarrow \text{L-citrulline} + NH_3$$
$$\text{L-citrulline} + P_i \rightleftharpoons \text{L-ornithine} + \text{carbamyl phosphate}$$
$$\text{carbamyl phosphate} + ADP \rightleftharpoons ATP + CO_2 + NH_3$$

Although there is considerable detail available concerning the properties of all three enzymes from *Streptococcus faecalis* (8, 9, 13–16, 22, 23) and to a lesser extent concerning ornithine transcarbamylase and carbamate kinase from *Streptococcus lactis* (10, 24, 25), the factors affecting enzyme synthesis in bacterial cells are ill defined. Although various reports indicate that some streptococci are induced for arginine metabolism when grown on galactose (2, 32), on low concentrations of glucose (20), or in the presence of high arginine concentrations (8, 23), there appear to be no specific activity data available for the enzymes concerned.

Lactic streptococci (*Streptococcus cremoris*, *S. lactis*, and *Streptococcus diacetylactis*) have a vital role as starters in commercial milk fermentations, where their essential function is the homolactic fermentation of lactose. *S. cremoris* strains are generally preferred to *S. lactis* as cheddar cheese starters (12). Lactic streptococci possess only limited catabolic and biosynthetic abilities, and their complex growth requirements include a fermentable sugar for carbon and energy supply. Orla-Jensen (21) in 1919 established *S. cremoris* as a type distinct from *S. lactis* on the basis of a number of characteristics including the tendency to form longer chains, lower maximum growth temperature, and less fermentative power. Sherman (27) described these differences between the two organisms as relative rather than definitive.

A definitive difference between the two "species" was established by a number of workers (5, 7, 20, 35) who showed that some streptococci such as *S. lactis* hydrolyzed peptone (and specifically arginine) with the production of ammonia, whereas *S. cremoris* strains lacked this ability. Agar media were subsequently developed (26, 28, 34) to differentiate *S. lactis* from *S. cremoris* on the basis of ability to produce ammonia from arginine; such media are important in selecting strains for use in milk fermentations. It is therefore surprising that arginine metabolism has not been more extensively investigated in lactic streptococci.

The present study was undertaken to examine the enzymes involved in arginine metabolism in lactic streptococci and to investigate why *S.*

cremoris strains are unable to metabolize arginine.

MATERIALS AND METHODS

Organisms and culture conditions. All strains were from the collection held at the New Zealand Dairy Research Institute. Arginine-negative mutants of *S. lactis* ML_8 and H_1 were kindly supplied by H. A. Heap and A. W. Jarvis, and the arginine-positive mutant of *S. cremoris* 166 was kindly supplied by L. E. Pearce.

Unless otherwise specified, static batch cultures were grown at 30°C in T_5 complex broth (30) which contained 28 mM galactose (or glucose) or 14 mM lactose with various amounts of arginine added (up to a final concentration of 48 mM). The basal concentration of free arginine in the complex broth was 0.6 mM, and the initial pH was 7.2. Standardized carbohydrate and arginine solutions were filter sterilized before they were added to autoclaved broth. Strains were transferred at least three times on the appropriate carbohydrate-arginine medium before cells were used in any experiments. Where indicated, *S. lactis* strains were also maintained and grown in a chemically defined medium (29). Continuous culture experiments involved anaerobic growth of *S. lactis* strains in defined medium containing 28 mM glucose and 5.5 mM arginine; the detailed culture conditions have been reported elsewhere (29).

Enzyme assays. Unless indicated otherwise, exponentially growing cells were harvested from batch cultures when the residual carbohydrate concentration was about half the initial value. Cells from 100 ml of medium were washed and suspended in 5 ml of 20 mM phosphate buffer (pH 6.5) containing 50 mM NaCl and 10 mM $MgCl_2$. Cell disruption was achieved by shaking with glass beads for 2 min at 0 to 5°C in a Mickle disintegrator, and debris was removed by centrifugation at 35,000 × *g* for 5 min. Arginine deiminase was assayed in a reaction mixture (1 ml) containing 50 mM phosphate buffer (pH 6.7), 50 mM L-arginine, and the appropriate amount of cell-free extract. At suitable time intervals, the reaction was stopped by the addition of 1 ml of 10% trichloroacetic acid, and precipitated protein was removed by centrifugation at 20,000 × *g* for 5 min. The citrulline formed was measured by a modification of the Archibald method (4). To 1 ml of the centrifuged trichloroacetic acid-treated sample, 1.4 ml of acid mixture (18 M H_2SO_4–14 M H_3PO_4; 1:3, vol/vol) and 0.5 ml of 3% diacetyl monoxime were added, mixed together, and boiled in the dark for 15 min. After cooling in the dark for 10 min, the absorbance of samples and citrulline standards (made in reaction mixture and treated in the same way) was measured at 490 nm. Ornithine transcarbamylase assay mixture (1 ml) contained 200 mM Tris-hydrochloride (pH 8.5), 10 mM carbamyl phosphate, 5 mM ornithine, and diluted cell-free extract. The reaction was stopped, and citrulline was measured as described above for arginine deiminase. Carbamate kinase was assayed by coupling ATP production from carbamyl phosphate and ADP to $NADP^+$ reduction via hexokinase and glucose 6-phosphate dehydrogenase. The reaction mixture (1 ml) contained 100 mM Tris-hydrochloride buffer (pH 8.5), 5 mM $MgCl_2$, 10 mM glucose, 0.5 mM $NADP^+$, 5 mM ADP, 5 U of glucose 6-phosphate dehydrogenase, 12.5 U of hexokinase, 10 mM carbamyl phosphate, and diluted cell-free extract. Controls lacking carbamyl phosphate were routinely included and deducted from values obtained for complete reaction mixtures. The amount of carbamyl phosphate used, as estimated with *Escherichia coli* carbamate kinase, was equal to the amount of NADPH produced when corrected for myokinase activity. All three enzymes were assayed at 25°C, and reaction rates were linear with time and extract concentration under the conditions used. Protein was determined by a modification (6) of the Lowry method, with bovine serum albumin as the standard.

Experiments with nongrowing cells. Cells from 100-ml exponential cultures were washed in 100 ml of 20 mM phosphate buffer (pH 6.5) containing 50 mM NaCl and 10 mM $MgCl_2$ and suspended in 10 ml of the same buffer at 3 to 6 mg (dry weight) of bacteria per ml. Portions of this suspension, appropriately diluted, were placed in a Radiometer pH-stat apparatus (TTA 31 titration assembly linked to an ABU 12 Autoburette, pH meter 26, Titrator 11, and Titrigraph SBR 2C). The stirred suspension (2.5 ml) was adjusted to pH 6.50 and maintained at 30°C. Arginine (50 μl, 0.5 M, pH 6.5) was added, and the rate of acid (0.1 M HCl) addition required for pH control at 6.50 was recorded for 5 min (see Table 3). In these experiments the activity is expressed as micromoles of arginine utilized per milligram (dry weight) of bacteria per minute. The arginine concentration was measured in some samples, and it was confirmed that 2 mol of H^+ was added per mol of arginine utilized. In other experiments (see Table 4), the cell suspension (10 ml) was maintained at pH 6.50 by the addition of either 1 M NaOH or 1 M HCl as appropriate. Samples (0.4 ml) were taken at intervals and added to an equal volume of 10% trichloroacetic acid. After spinning at 20,000 × *g* for 5 min, samples were stored at −20°C before assay of substrate and products. The rate of arginine utilized per milligram (dry weight) of bacteria per minute was determined from enzymatic analysis of NH_4^+ production. From a number of samples arginine was measured both enzymatically and by the amino acid analyzer. These analyses confirmed that 2 mol of NH_4^+ and 1 mol of ornithine were produced per mol of arginine utilized.

Experiments with growing cells. Cells previously adapted to grow on the appropriate sugar and arginine concentration were inoculated into 100 ml of medium and incubated at 30°C. During growth, 5-ml samples were removed and centrifuged at 20,000 × *g* for 5 min, and the supernatant was stored at −20°C before assay. For molar growth yield determination, three 20-ml samples were taken after the maximum cell density had been reached. The bacterial density in these samples was determined directly by using membrane filters (29).

Substrate and product analysis. Glucose was determined with Glucostat reagents (Worthington Diagnostics). For lactose measurement, the Glucostat reagents were modified by the addition of $MgCl_2$ (5 mM final concentration) and 0.8 U of β-galactosidase per ml of reconstituted Glucostat reagent. Galactose was assayed enzymatically by the method of Kurz and Wallenfels (11). Ammonia was measured via glutamate dehydrogenase in the following reaction mixture (1 ml) incubated at 25°C for 45 min: 0.25 mM NADH, 25 mM α-ketogluturate, 200 mM triethanolamine-hydrochlo-

ride buffer (pH 7.8), 9.6 U of glutamate dehydrogenase, and the sample. Arginine and ornithine were measured with a Locarte amino acid analyzer, although a more convenient arginine assay was developed by coupling the reaction of arginine kinase to pyruvate kinase and lactate dehydrogenase. The reaction mixture (1 ml) contained 2 mM phosphoenolpyruvate, 5 mM ATP, 10 mM $MgCl_2$, 50 mM KCl, 0.4 mM NADH, 200 mM triethanolamine-hydrochloride buffer (pH 7.8), 7 U of pyruvate kinase, 10 U of lactate dehydrogenase, 2.8 U of arginine kinase, and 0.1 to 0.2 mM arginine. After incubation at 25°C for 30 min, the absorbance was measured at 340 nm.

Materials. All biochemicals were obtained from Sigma Chemical Co., St. Louis, Mo., as the grades with highest analytical purity.

RESULTS

Properties of enzymes involved in arginine metabolism. The kinetic properties of arginine deiminase, ornithine transcarbamylase, and carbamate kinase were examined to ensure that saturating, noninhibiting substrate concentrations were chosen for routine assays. With cell-free extracts from *S. lactis* strains C_{10} and ML_3, a K_m value of 2.5 to 5.0 mM arginine was obtained from Lineweaver-Burk plots for arginine deiminase; 50 mM arginine did not inhibit activity. With carbamate kinase the K_m values were 1.2 to 1.5 mM for carbamyl phosphate and 0.4 to 0.5 mM for ADP; the near-saturating concentrations of substrates used in the standard assay caused no apparent inhibition. With ornithine transcarbamylase the K_m values were 2.1 to 2.5 mM for carbamyl phosphate and 3 to 5 mM for ornithine. However, saturating ornithine was not used in routine assays, as ~12 mM ornithine gave 50% inhibition of activity. Ornithine also inhibited the ornithine transcarbamylase from *S. faecalis* (19), but this substrate did not inhibit the enzyme from *S. lactis* strain 8039 (24), in contrast to the present results for strains C_{10} and ML_3.

The arginine deiminase activity from *S. lactis* was the least stable of the three enzymes studied. The activity of this enzyme in cell-free extracts maintained at 4°C had decreased by 40% after 8 h. In contrast, ornithine transcarbamylase activity fell by only 10%, and the carbamate kinase showed no loss of activity after storage for 8 h at 4°C. Arginine deiminase and ornithine transcarbamylase were assayed within 2 h of preparing the cell-free extracts. Carbamate kinase was assayed within 4 h of extract preparation.

Enzyme specific activities in *S. lactis* strains. (i) Batch culture experiments. With strain ML_3, the specific activities of arginine deiminase, ornithine transcarbamylase, and carbamate kinase were measured in extracts from cells grown in media of different composition (Table 1). The specific activity of carbamate kinase was independent of medium composition, whereas the specific activities of arginine deiminase and ornithine transcarbamylase varied considerably. Specific activities of both the latter enzymes were higher (i) when galactose, rather than glucose, was the growth sugar, and (ii) when arginine was added to the medium. Much lower specific activities of arginine deiminase and ornithine transcarbamylase were usually found in cells grown in defined medium compared with those in cells grown in the complex broth. This broth medium was therefore chosen for most studies. Use of broth also allowed examination of *S. cremoris* strains which do not grow in the defined medium.

Three other *S. lactis* strains (C_{10}, H_1, ML_8) grown in complex broth showed enzyme patterns (data not shown) similar to those of strain ML_3 (Table 1). In addition, the pattern of en-

TABLE 1. Effect of growth medium composition on activities of enzymes involved in arginine metabolism in *S. lactis* ML_3[a]

Growth medium			Enzyme sp act		
Type	Sugar	Arginine (mM)	Arginine deiminase[b]	Ornithine transcarbamylase[b]	Carbamate kinase[c]
Defined	Galactose	0.4	0.06	3.3	7.1
	Galactose	47.0	0.09	5.3	7.1
	Glucose	0.4	0.001	2.9	7.2
	Glucose	47.0	0.05	5.0	7.2
Complex broth	Galactose	0.6	0.05	7.9	5.6
	Galactose	47.0	0.28	59.3	6.6
	Glucose	0.6	0.01	0.9	6.0
	Glucose	47.0	0.14	25.5	5.0

[a] Culture conditions and enzyme assays were as described in the text. Mean values of enzyme specific activity from at least two separate experiments are given.

[b] Micromoles of citrulline formed per milligram of protein per minute.

[c] Micromoles of ATP formed per milligram of protein per minute.

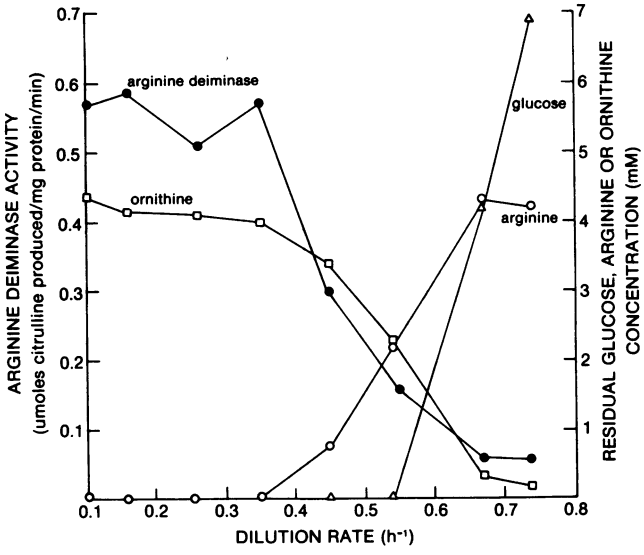

FIG. 1. Effect of residual glucose concentration (△) on the level of arginine deiminase (●) and on the metabolism of arginine (○) to ornithine (□) in steady-state continuous cultures of *S. lactis* ML_3 growing in defined medium containing 5.5 mM arginine.

zyme specific activities with all four *S. lactis* strains was the same on substituting lactose for glucose.

Arginine-negative mutants of strains H_1 and ML_8 grown in complex broth containing galactose, lactose or glucose, and 47 mM arginine contained no detectable arginine deiminase or ornithine transcarbamylase activity (data not shown). The activity of carbamate kinase in these mutants was similar to that found in strain ML_3.

Preliminary experiments (data not shown) in batch cultures indicated that the specific activities of arginine deiminase and ornithine transcarbamylase, but not carbamate kinase, were influenced by the concentration of either glucose or galactose in the medium at the point of cell harvest. Enzyme specific activities were higher in cells harvested when the residual sugar concentration was low. This trend was more marked for the glucose-grown cells. More precise experiments were therefore undertaken with continuous cultures where cells can be grown at defined sugar concentrations.

(ii) Continuous culture experiments. At high dilution rates (0.74 and 0.67 h^{-1}) where glucose was in excess (7 and 4 mM, respectively), the level of arginine deiminase was 0.06 μmol of citrulline formed per mg of protein per min (Fig. 1). At lower dilution rates, where glucose was limiting, the level of arginine deiminase increased markedly to reach a maximum of 0.6 μmol of citrulline formed per mg of protein per min.

Enzyme specific activities in *S. cremoris* strains. No arginine deiminase activity was detected in cell-free extracts from nine *S. cremoris* strains grown in complex broth, even when cells were grown on galactose in the presence of a high arginine concentration (Table 2). The possibility of there being inhibitory material in the *S. cremoris* extracts was checked. The addition of cell-free extract (0.5 to 2 mg of protein) from *S. cremoris* strains ML_1, E_8, HP, or 166 to assay systems containing extract from *S. lactis* C_{10}, H_1, or ML_3 did not alter the arginine deiminase activity. To check for possible enzyme instability, four other buffers having different pH values and containing 2 mM dithioerythritol were used to prepare cell-free extracts from *S. cremoris* strains ML_1, E_8, and 166. However, no arginine deiminase activity was detected in any of the freshly prepared extracts.

The nine *S. cremoris* strains were divided into two groups according to the specific activity of ornithine transcarbamylase found (Table 2). No activity of this enzyme was detected in the group II strains. The extracts from these strains did not alter the ornithine transcarbamylase activity of extracts from *S. lactis* strains C_{10}, H_1, and ML_3, and different procedures for the preparation of cell-free extracts failed to elicit any activity. The five *S. cremoris* strains in group I had low specific activities of ornithine transcarbamylase

TABLE 2. Activities of enzymes involved in arginine metabolism in *S. cremoris* strains grown in complex broth[a]

Strain	Sugar in medium[b]	Enzyme sp act		
		Arginine deiminase[c]	Ornithine trans-carbamylase[c]	Carbamate kinase[d]
Group I[e]	Galactose	ND[f]	0.1–0.3	1.0–3.0
	Glucose[g]	ND	0.02–0.08	0.8–1.5
Group II[e]	Galactose	ND	ND	1.0–3.0
	Glucose[g]	ND	ND	1.0–3.0

[a] Culture conditions and enzyme assays were as described in the text. Mean values of enzyme specific activity from at least two separate experiments are given.
[b] Similar data were obtained with 0.6 and 47 mM arginine in the growth media.
[c] Micromoles of citrulline formed per milligram of protein per minute.
[d] Micromoles of ATP formed per milligram of protein per minute.
[e] Group I strains are 104, 266, AM_2, E_8, and ML_1. Group II strains are 114, 166, C_{13}, and HP.
[f] ND, Not detectable (less than 0.001).
[g] The same results were obtained when glucose was replaced by lactose.

activity when grown on glucose or lactose. The specific activity increased when cells were grown on galactose but, in contrast to the *S. lactis* strains, activity was still low and was not increased by the presence of arginine.

The *S. cremoris* strains had specific activities of carbamate kinase (Table 2) similar to those found in *S. lactis* strains. Citrulline at concentrations up to 50 mM in the growth medium was not an inducer of the enzymes for arginine metabolism in either *S. cremoris* or *S. lactis* strains. The arginine-positive mutant of *S. cremoris* 166 showed high levels of arginine deiminase and ornithine transcarbamylase (specific activities of 0.3 and 14.7, respectively) when grown in complex broth containing galactose and 47 mM arginine. As in strain ML_3 (Table 1), the specific activities of these two enzymes decreased when the mutant was grown on glucose or with a low arginine concentration (0.6 mM), whereas the specific activity of carbamate kinase was not altered by the different growth conditions.

TABLE 3. Rates of arginine metabolism by nongrowing *S. lactis* ML_3 cells[a]

Growth medium composition		Arginine utilized (μmol/mg [dry wt] of bacteria/min)
Sugar	Arginine (mM)	
Galactose	0.6	0.011
Galactose	47.0	0.092
Glucose	0.6	0.003
Glucose	47.0	0.006
Lactose	0.6	0.003
Lactose	47.0	0.006

[a] Cells were grown in complex broth containing 28 mM galactose or glucose or 14 mM lactose with arginine at the concentrations indicated. Harvested cells were held in buffer, and the pH was maintained at 6.5 using the pH-stat apparatus described in the text.

Nongrowing cell experiments. The rate of arginine metabolism by nongrowing *S. lactis* ML_3 cells (Table 3) was consistent with the specific activities of arginine deiminase and ornithine transcarbamylase present in cell-free extracts (Table 1). Similar findings were made with the other three *S. lactis* strains (data not shown).

With *S. cremoris* strains E_8 and ML_1 grown under a variety of conditions, the nongrowing cells were unable to produce NH_4^+ from arginine. This is consistent with the absence of detectable arginine deiminase activity in cell-free extracts.

Various combinations of carbohydrates and arginine were incubated with *S. lactis* C_{10} cells which had been grown in a medium containing galactose plus arginine (Table 4). The presence of carbohydrate reduced the rate of arginine metabolism and vice versa.

Molar growth yield for arginine. To investigate the coupling of energy from arginine metabolism to growth, molar growth yields for arginine ($Y_{arginine}$) were measured with different fermentable sugars. The results for ML_3 on defined medium are shown in Fig. 2. With galactose as the fermentable carbohydrate the $Y_{arginine}$ derived from the data shown in Fig. 2 was 16 g (dry weight) of bacteria per mol of arginine utilized. In contrast there was only a slight increase in the yield of bacteria due to arginine metabolism when glucose was the fermentable carbohydrate and the apparent $Y_{arginine}$ was <2.0 g/mol. Lactose gave similar results to glucose with strain ML_3 (data not shown).

With complex medium containing either galactose or glucose, all four *S. lactis* strains gave results similar to those shown in Fig. 2. Howev-

TABLE 4. Initial rates of arginine and carbohydrate metabolism by nongrowing *S. lactis* C_{10} cells[a]

Addition to buffer (mM)	Rate of metabolism	
	Arginine[b]	Carbohydrate[c]
Arginine (10)	0.088	
Galactose (20) plus arginine (10)	0.050	0.047
Galactose (20)		0.081
Glucose (20) plus arginine (10)	0.050	0.055
Glucose (20)		0.089
Lactose (20) plus arginine (10)	0.050	0.030
Lactose (20)		0.043

[a] Cells were grown in complex broth containing 28 mM galactose and 47 mM arginine. Harvested cells were held in buffer, and the pH was maintained at 6.50 using the pH-stat apparatus described in the text.

[b] Micromoles of arginine utilized per milligram (dry weight) of bacteria per minute.

[c] Micromoles of carbohydrate utilized per milligram (dry weight) of bacteria per minute.

er, on lactose $Y_{arginine}$ values varied from 2.5 to 6.0 g/mol. All molar growth yield values were obtained in the absence of autolysis.

Concurrent or sequential metabolism of arginine. (i) Batch culture experiments. The previous data indicating that the apparent $Y_{arginine}$ varies with the growth sugar raises the question as to whether metabolism of sugar and arginine was concurrent or sequential in batch cultures. With *S. lactis* H_1 growing on glucose, the concentration of sugar dropped from an initial value of 28 mM to between 0 and 2 mM before arginine metabolism was initiated (Fig. 3A).

In contrast to the sequential metabolism of glucose and arginine, both galactose and arginine were metabolized concurrently (Fig. 3B).

(ii) Continuous culture experiments. In continuous cultures containing excess glucose, arginine was not appreciably metabolized to ornithine (Fig. 1). The decrease in arginine concentration in the glucose-excess cultures (i.e., from 5.5 mM in the medium to ~4 mM in the culture) presumably reflects the growth requirement for this essential amino acid. However, as the dilution rate was dropped and glucose limitation occurred, then arginine was metabolized until at a dilution rate of $\leq 0.35\ h^{-1}$ arginine was not detectable in the culture. Results similar to those shown in Fig. 1 were obtained with *S. lactis* strains H_1 and ML_8.

Determination of arginine, ornithine and NH_4^+ concentrations in samples from experiments described in Fig. 1, 2, and 3 and in Tables 3 and 4 showed the expected stoichiometry for arginine metabolism, i.e., 1 mol of ornithine plus 2 mol of NH_4^+ produced per mol of arginine utilized.

DISCUSSION

In *S. lactis* cells growing in batch cultures, the specific activities of the first two enzymes of the arginine deiminase pathway were dependent upon both the nature of the sugar present and on the arginine concentration. In contrast to the inducible nature of arginine deiminase and ornithine transcarbamylase (and the carbamate ki-

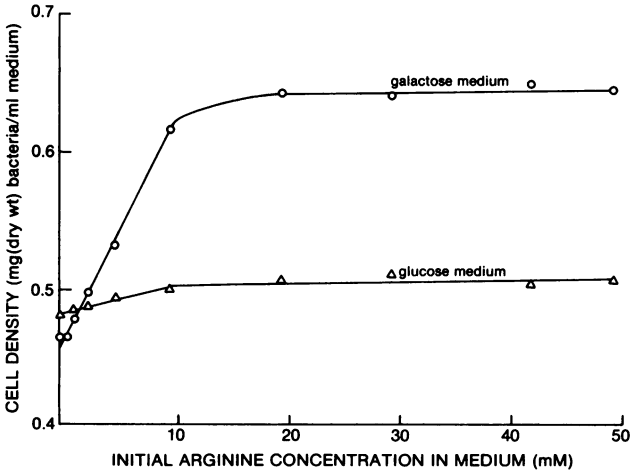

FIG. 2. Effect of the initial arginine concentration on the yield of *S. lactis* ML_3 cells growing in defined medium containing an initial sugar concentration of 11 mM galactose (O) or 11 mM glucose (Δ). When cell densities were determined, sugar was not detectable in the medium, whereas residual arginine concentrations were <0.5 mM.

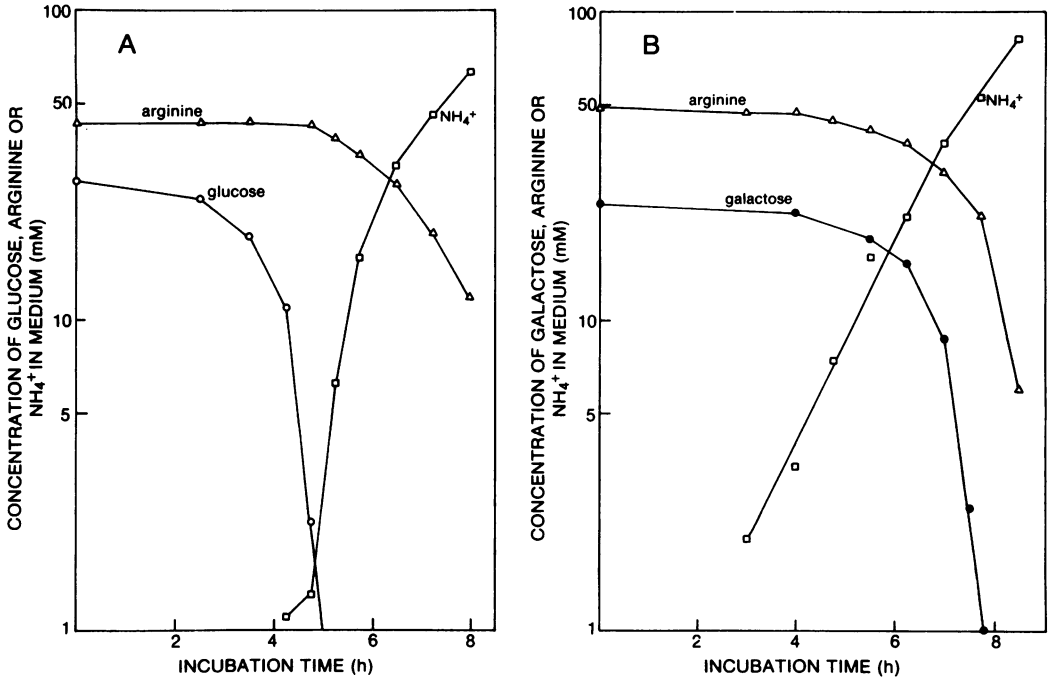

FIG. 3. Metabolism of glucose (A) or galactose (B) and arginine, together with NH_4^+ production, when *S. lactis* H_1 was grown in batch culture in complex broth medium. Symbols: ○, glucose; ●, galactose; △, arginine; □, NH_4^+.

nase from *S. faecalis* [8]), the carbamate kinase was constitutive in all four *S. lactis* strains.

Continuous culture experiments indicated that the glucose concentration in the growth medium has a marked effect on arginine deiminase with glucose-limited cells containing up to 10 times the specific activity of cells growing with excess glucose. It is interesting that growth of *S. lactis* under glucose-limiting (energy-limiting) conditions causes two phenotypic changes which both result in an increase in energetic efficiency, namely, (i) the induction of arginine metabolism, as shown in the present study, and (ii) a change from homo- to heterolactic fermentation with most strains (29). The results for continuous cultures are consistent with the finding that in batch cultures of *S. lactis* the metabolism of glucose and arginine was sequential (Fig. 3A). In these cultures, the energy derived from arginine metabolism was not efficiently coupled to growth, as shown by the low $Y_{arginine}$ values. This is presumably because the carbon source (glucose) was virtually exhausted before arginine metabolism commenced. Arginine cannot serve as the sole source of carbon; for its metabolism to proceed in the nongrowing cells, energy must be dissipated to regenerate ADP. Myokinase activity (1 to 4 μmol of ATP formed per mg of protein per min) was found in the strains of *S. lactis* studied and could play a role in the regeneration of ADP. However, when arginine metabolism was concurrent with sugar metabolism (i.e., in cells growing on galactose) energy was efficiently coupled to growth as shown by the $Y_{arginine}$ (Y_{ATP}) of 16 g/mol. This is similar to the $Y_{arginine}$ value (17.8) reported for *S. faecalis* (18).

The inability of *S. cremoris* strains to metabolize arginine appears to be due to the absence of arginine deiminase. Some of the *S. cremoris* strains examined also lacked ornithine transcarbamylase activity (group II, Table 2). It is considered unlikely that the absence of detectable activity was due to enzyme instability as different extraction procedures were used and enzymes were assayed immediately after preparation of cell-free extracts.

The specific activity of carbamate kinase was similar in *S. cremoris* and *S. lactis*. It is not clear whether this activity was due to the enzyme carbamate kinase or to a nonspecific kinase with a broad substrate specificity. For example, Thorne and Jones (33) separated two proteins from *S. faecalis* which both had activity with acetyl phosphate. One of these proteins, which had an unusually high K_m for acetate, also had carbamate kinase activity, whereas in *E. coli* a single protein appeared to have both kinase

activities (33). If carbamyl phosphate was a substrate for the acetate kinase in lactic streptococci and the specific activity varied with growth conditions, then the observed lack of regulation of carbamate kinase could be an artifact. However, the specific activity of acetate kinase did not vary with the sugar in the growth medium (31) or with various arginine concentrations (V. L. Crow, unpublished data), and it is reasonable to assume that the acetate kinase from lactic streptococci is similar to that from *S. faecalis* in not utilizing carbamyl phosphate as a substrate.

The activity of ornithine transcarbamylase in group I *S. cremoris* strains (Table 2) was increased when glucose was replaced by galactose. However, unlike the *S. lactis* strains, the addition of arginine to the growth medium did not increase the enzyme levels further. The enzyme pattern in group I strains was similar to that found in some *S. lactis* strains growing on glucose or lactose in the presence of low arginine concentrations. For example, no arginine deiminase activity and low ornithine transcarbamylase activity (specific activity of 0.2) were found in *S. lactis* C_{10}. The enzyme pattern of arginine-negative strains of *S. lactis* ML_8 and H_1 was the same as that of *S. cremoris* (group II) strains. *S. cremoris* strains may lack the structural genes coding for these two enzymes. Alternatively, the structural genes for arginine deiminase and ornithine transcarbamylase may be present in some strains of *S. cremoris*, but are not expressed because of some defect in a structural gene or a possible regulatory gene. This is supported by the finding of an arginine-positive mutant of *S. cremoris* which showed levels of arginine deiminase and ornithine transcarbamylase similar to those found in *S. lactis* strains. As in *S. lactis*, the specific activities of these two enzymes in the mutant were highest in cells grown with galactose and high arginine concentrations in the medium.

Arginine is an essential amino acid for many lactic streptococci (3). In *S. lactis*, the enzyme specific activities vary with growth conditions, including arginine concentration. In cells growing with low arginine concentrations the activity of the arginine catabolic pathway may be sufficiently low to ensure that depletion of the essential amino acid does not occur.

The metabolism of arginine by H_1 (Fig. 3) and C_{10} (data not shown) is concurrent with galactose fermentation, but is sequential with glucose fermentation. Only when the glucose concentration is between 0 and 2 mM does arginine metabolism occur to an appreciable extent. However, these same batch-grown cells, when harvested at 12 to 14 mM residual glucose, have high activities of the arginine enzymes and, when used in nongrowing cell experiments (Table 3), do metabolize arginine at an appreciable rate. This apparent contradiction could be explained if enzyme(s) of the arginine deiminase pathway were regulated by a fine control mechanism such as fluctuating metabolite concentrations. P_i is one metabolite that may be important in regulation of arginine metabolism. Ornithine transcarbamylase requires phosphate for the phosphorylytic cleavage of ornithine. The direction is thermodynamically unfavorable, and the K_m value for phosphate is high (47 mM) in *S. lactis* (10). Mason et al. (17) concluded that the intracellular concentration of P_i was a major factor in the control of glycolysis in nongrowing *S. lactis* cells, with phosphate being high in starved cells (47 mM) and low (4 mM) after glucose addition. In our experiments, cells growing on glucose or lactose fermented most of the carbohydrate before arginine was used. These cells, depleted of carbohydrate in the growth medium, are likely to contain high concentrations of inorganic phosphate (17). The possibility that with a high arginine concentration in the growth medium, cells growing on galactose contain high phosphate concentrations (which can drive the ornithine transcarbamylase) will be investigated.

As previously mentioned, arginine metabolism was believed to be a definitive difference that could be used to distinguish *S. lactis* and *S. cremoris*, which are otherwise closely related organisms. The present work, however, makes it clear that this character is also relative in view of the potential of *S. cremoris* to metabolize arginine, the regulatory nature of arginine metabolism in *S. lactis*, and the production of arginine-negative strains of *S. lactis*. The results lend further support to the view that *S. cremoris* is probably a variety of *S. lactis* better suited for use in many milk fermentations. Regardless of its taxonomic position, it is thus still important to differentiate the *S. cremoris* group from *S. lactis*, and this can be achieved by joint consideration of a number of relative characters. Further studies involving arginine mutants of lactic streptococci are likely to shed additional light on relationships within this group of organisms.

ACKNOWLEDGMENTS

We are grateful to Judith Cleland for excellent technical assistance and to Alan Matheson and Barry Richardson for operating the amino acid analyzer, and Lindsay Pearce for helpful discussions.

Continuous culture experiments were carried out at the PHLS Centre for Applied Microbiology and Research, Porton Down, England.

LITERATURE CITED

1. **Abdelal, A. T.** 1979. Arginine catabolism by microorganisms. Annu. Rev. Microbiol. 33:139–168.
2. **Abrams, A., J. B. Smith, and C. Baron.** 1972. Carbodii-

mide-resistant membrane adenosine triphosphatase in mutants of *Streptococcus faecalis*. J. Biol. Chem. **247**:1484–1488.
3. **Anderson, A. W., and P. R. Elliker.** 1953. The nutritional requirements of lactic streptococci isolated from starter cultures. I. Growth in a synthetic medium. J. Dairy Sci. **36**:161–167.
4. **Archibald, R. M.** 1944. Determination of citrulline and allantoin and demonstration of citrulline in blood plasma. J. Biol. Chem. **156**:121–142.
5. **Ayers, S. H., W. T. Johnson, Jr., and C. S. Mudge.** 1924. Streptococci of souring milk with special reference to *Streptococcus lactis*. Studies of the streptococci. J. Infect. Dis. **34**:29–48.
6. **Hartree, E. F.** 1972. Determination of protein: a modification of the Lowry method that gives a linear photometric response. Anal. Biochem. **48**:422–427.
7. **Hills, G. M.** 1940. Ammonia production by pathogenic bacteria. Biochem. J. **34**:1057–1069.
8. **Jones, M. E.** 1962. Carbamyl phosphate synthesis and utilization. Methods Enzymol. **5**:903–925.
9. **Kalman, S. M., and P. H. Duffield.** 1964. Purification and properties of carbamate kinase from *Streptococcus faecalis*. Biochim. Biophys. Acta **92**:498–512.
10. **Korzenovsky, M., and C. H. Werkman.** 1953. Conversion of citrulline to ornithine by cell-free extracts of *Streptococcus lactis*. Arch. Biochem. Biophys. **46**:174–185.
11. **Kurz, G., and K. Wallenfels.** 1974. D-Galactose UV assay with galactose dehydrogenase, p. 1279–1282. *In* H. U. Bergmeyer (ed.), Methods of enzymatic analysis, vol. 3. Verlag Chemie, Weinham.
12. **Lawrence, R. C., and T. D. Thomas.** 1979. The fermentation of milk by lactic acid bacteria, p. 187–219. *In* A. T. Bull, D. C. Ellwood, and C. Rutledge (ed.), Microbial technology: current state, future prospects. Symposium of the Society for General Microbiology. Cambridge University Press, Cambridge.
13. **Marshall, M., and P. P. Cohen.** 1966. A kinetic study of the mechanism of crystalline carbamate kinase. J. Biol. Chem. **241**:4197–4208.
14. **Marshall, M., and P. P. Cohen.** 1972. Ornithine transcarbamylase from *Streptococcus faecalis* and bovine liver. I. Isolation and subunit structure. J. Biol. Chem. **247**:1641–1653.
15. **Marshall, M., and P. P. Cohen.** 1972. Ornithine transcarbamylase from *Streptococcus faecalis* and bovine liver. II. Multiple binding sites for carbamyl-P and L-norvaline, correlation with steady state kinetics. J. Biol. Chem. **247**:1654–1668.
16. **Marshall, M., and P. P. Cohen.** 1972. Ornithine transcarbamylase from *Streptococcus faecalis* and bovine liver. III. Effects of chemical modifications of specific residues on ligand binding and enzymatic activity. J. Biol. Chem. **247**:1669–1682.
17. **Mason, P. W., D. P. Cabone, R. A. Cushman, and A. S. Waggoner.** 1981. The importance of inorganic phosphate in regulation of energy metabolism of *Streptococcus lactis*. J. Biol. Chem. **256**:1861–1866.
18. **Moustafa, H., and E. B. Collins.** 1968. Molar growth yields of certain lactic acid bacteria as influenced by autolysis. J. Bacteriol. **96**:117–125.
19. **Nakamura, M., and M. E. Jones.** 1970. Ornithine carbamyltransferase (*Streptococcus faecalis*). Methods Enzymol. **17**:286–294.
20. **Niven, C. F., Jr., K. L. Smiley, and J. M. Sherman.** 1942. The hydrolysis of arginine by streptococci. J. Bacteriol. **43**:651–660.
21. **Orla-Jensen, S.** 1919. The lactic acid bacteria, p. 1–196. Høst, Copenhagen.
22. **Pandey, V. N., and D. S. Pradhan.** 1981. Reverse and forward reactions of carbamyl phosphokinase from *Streptococcus faecalis* R. Participation of nucleotides and reaction mechanisms. Biochim. Biophys. Acta **660**:284–292.
23. **Petrach, B., L. Sullivan, and S. Ratner.** 1957. Behavior of purified arginine desiminase from *S. faecalis*. Arch. Biochem. Biophys. **69**:186–197.
24. **Ravel, J. M., M. L. Grona, J. S. Humphreys, and W. Shive.** 1959. Properties and biotin content of purified preparations of the ornithine-citrulline enzyme of *Streptococcus lactis*. J. Biol. Chem. **234**:1452–1455.
25. **Ravel, J. M., J. S. Humphreys, and W. Shive.** 1961. A study of carbamyl phosphate synthesis in *Streptococcus lactis*. Arch. Biochem. Biophys. **92**:525–531.
26. **Reddy, M. S., E. R. Vedamuthu, C. J. Washam, and G. W. Reinbold.** 1969. Differential agar medium for separating *Streptococcus lactis* and *Streptococcus cremoris*. Appl. Microbiol. **18**:755–759.
27. **Sherman, J. M.** 1937. The streptococci. Bacteriol. Rev. **1**:1–97.
28. **Thomas, T. D.** 1973. Agar medium for differentiation of *Streptococcus cremoris* from other bacteria. N. Z. J. Dairy Sci. Technol. **8**:70–71.
29. **Thomas, T. D., D. C. Ellwood, and V. M. C. Longyear.** 1979. Change from homo- to heterolactic fermentation by *Streptococcus lactis* resulting from glucose limitation in anaerobic chemostat cultures. J. Bacteriol. **138**:109–117.
30. **Thomas, T. D., B. D. W. Jarvis, and N. A. Skipper.** 1974. Localization of proteinase(s) near the cell surface of *Streptococcus lactis*. J. Bacteriol. **118**:329–333.
31. **Thomas, T. D., K. W. Turner, and V. L. Crow.** 1980. Galactose fermentation by *Streptococcus lactis* and *Streptococcus cremoris*: pathways, products and regulation. J. Bacteriol. **144**:672–682.
32. **Thompson, J.** 1976. Characteristics and energy requirements of an α-aminoisobutyric acid transport system in *Streptococcus lactis*. J. Bacteriol. **127**:719–730.
33. **Thorne, K. J. I., and M. E. Jones.** 1963. Carbamyl and acetyl phosphokinase activities of *Streptococcus faecalis* and *Escherichia coli*. J. Biol. Chem. **238**:2992–2998.
34. **Turner, N., W. E. Sandine, P. R. Elliker, and E. A. Day.** 1963. Use of tetrazolium dyes in an agar medium for differentiation of *Streptococcus lactis* and *Streptococcus cremoris*. J. Dairy Sci. **46**:380–385.
35. **Yawger, E. S., Jr., and J. M. Sherman.** 1937. *Streptococcus cremoris*. J. Dairy Sci. **20**:205–212.