

Effects of pH and Energy Supply on Activity and Amount of Pyruvate Formate-Lyase in *Streptococcus bovis*

NARITO ASANUMA AND TSUNEO HINO*

Department of Agriculture, Meiji University, Higashimita, Tama-ku, Kawasaki 214-8571, Japan

Received 6 January 2000/Accepted 9 May 2000

The enzyme system of pyruvate formate-lyase (PFL) in *Streptococcus bovis* was investigated by isolating PFL and PFL-activating enzyme (PFL-AE) from *S. bovis*, flavodoxin from *Escherichia coli*, and chloroplasts from spinach. In this study, the PFL and PFL-AE in *S. bovis* were found to be similar to those in *E. coli*, suggesting that the activating mechanisms are similar. The optimal pH of *S. bovis* PFL was 7.5, which is in contrast to the optimal pH of *S. bovis* lactate dehydrogenase, which is 5.5. The apparent K_m of *S. bovis* PFL was 2 mM. The intermediates of glycolysis, dihydroxyacetone phosphate (DHAP) and D-glyceraldehyde-3-phosphate (GAP), were shown to inhibit PFL activity. The concentrations of intracellular DHAP and GAP in *S. bovis* ranged from 1.9 mM to less than 0.1 mM and from 0.6 mM to less than 0.05 mM, respectively, depending on the energy supply. The wide variations in DHAP and GAP levels indicated that PFL activity is allosterically regulated by these triose phosphates in vivo. The amount of PFL protein, as determined by Western blot analysis with polyclonal antibody, changed in parallel with the level of *pfl*-mRNA, responding to the culture conditions. These observations confirm that PFL synthesis is regulated at the transcriptional level and support the hypothesis that *S. bovis* shifts the fermentation pathway from acetate, formate, and ethanol production to lactate production when the pH is low and when excess energy is supplied.

Streptococcus bovis is one of the prevailing microbes in the rumen, especially in animals receiving a high-starch diet. Its primary fermentation product is lactate (16, 19). In this organism, pyruvate is either converted to lactate by lactate dehydrogenase (LDH) or converted to acetyl coenzyme A and formate by pyruvate formate-lyase (PFL) (19) and the acetyl coenzyme A is then converted either to acetate or ethanol. The percentages of lactate in fermentation products differ under different growth conditions, and the percentage is determined by the relative activities of LDH and PFL (2, 4, 19).

We previously reported that *S. bovis* increases the percentage of lactate by increasing the amount of LDH in response to low pH and an excess energy supply (2–4) and that LDH synthesis is regulated at the transcriptional level (3, 4). The increase in lactate production at low pH is explained in part by the fact that the optimal pH of *S. bovis* LDH is 5.5, which is similar to the intracellular pH when the extracellular pH is 4.7 (2, 19). The enhanced lactate production in the presence of an excess energy supply can be explained in part by the activation of LDH by an increased concentration of intracellular fructose-1,6-bisphosphate (2, 19). Thus, lactate production by *S. bovis* is regulated by the ultimate activity of LDH, which includes the amount of enzyme protein and the function of the enzyme.

When the pH was low and the energy supply was in excess, the level of *pfl*-mRNA decreased, while the level of *ldh*-mRNA increased (4). The activity of PFL decreased with a decrease in the level of *pfl*-mRNA. Accordingly, PFL synthesis may also be regulated at the transcriptional level, but PFL activity does not accurately reflect the amount of PFL, because PFL is possibly synthesized as the inactive (nonradical) form and is posttranslationally converted to the active (radical) form by a PFL-activating enzyme (PFL-AE). In addition, the active form is

irreversibly inactivated by oxygen (27), which precludes accurate determination of PFL activity; i.e., it is difficult to relate the values of PFL activity determined to the amounts of PFL.

For this reason, to confirm that regulation at the transcriptional level actually changes the protein level, we performed Western immunoblot analysis with polyclonal antibody against *S. bovis* PFL. We also examined the enzyme system of the PFL reaction in *S. bovis* and the properties of *S. bovis* PFL. In addition, the effect of triose phosphates on PFL activity was examined. In this study, we hypothesized that the enzyme system of *S. bovis* is analogous to that of *Escherichia coli* (12).

MATERIALS AND METHODS

Bacterial strains and growth conditions. The source of *S. bovis* JB1 was as described previously (2), and *E. coli* K-12 (ATCC 10798) and HB101 were purchased from a commercial source. Batch culture of *S. bovis* in a glucose medium was carried out in triplicate as previously described (2).

Continuous culture of *S. bovis* was performed in duplicate as described previously (4). The apparatus and culture procedure were described in a previous paper (10), except that six 1-liter fermentors were used. When *S. bovis* was grown at pH 4.5, two cycles of culture were performed in order to collect approximately 10 g of cell pellet.

To prepare PFL and PFL-AE, *E. coli* K-12 was anaerobically grown in Luria-Bertani medium supplemented with glucose (5 g/liter) as described previously (11). *E. coli* HB101 for preparing fusion protein was aerobically grown in Luria-Bertani medium.

Determination of organic acids. Organic acids produced by *S. bovis* were analyzed by high-performance liquid chromatography as described previously (11).

Separation of PFL, PFL-AE, flavodoxin, and chloroplasts. Isolation of PFL and PFL-AE from *E. coli* K-12 and *S. bovis* was carried out under anaerobic conditions (under a stream of O₂-free N₂ or in an anaerobic glove box) by the method of Knappe and Blaschkowski (12), with minor modifications. The entire procedure was carried out at temperatures below 4°C. Cultures were transferred to centrifuge tubes in a glove box, and cells were harvested by centrifugation (20,000 × g, 10 min). Cells suspended in potassium phosphate (KPi) buffer (pH 7.0) containing 0.05% cysteine · HCl and 10 mM dithiothreitol (DTT) were disrupted by sonication as described previously (2). The homogenate was ultracentrifuged (200,000 × g, 30 min), and the supernatant was used as a source of enzymes.

The extract was diluted with 50 mM KPi buffer (pH 6.1) containing 10 mM DTT, and 2.5% (wt/vol) protamine sulfate was added. After the first centrifugation (20,000 × g, 15 min), the pH of the supernatant was adjusted to 7.0, and the preparation was centrifuged again. The precipitate was subsequently used for

* Corresponding author. Mailing address: Department of Agriculture, Meiji University, Higashimita, Tama-ku, Kawasaki 214-8571, Japan. Phone: 81-44-934-7825. Fax: 81-44-934-7825. E-mail: hino@isc.meiji.ac.jp.

isolation of flavodoxin. To the supernatant, ammonium sulfate was added to 40% saturation, and then the preparation was centrifuged ($20,000 \times g$, 10 min). The pellet, dissolved in 0.02 M KPi buffer (pH 6.5) with 10 mM DTT, was centrifuged ($20,000 \times g$, 10 min). The supernatant was loaded on a Sephacryl S-200 HR column (2.5 by 70 cm; Amersham Pharmacia Biotech) and eluted with 0.02 M KPi buffer (pH 6.5) with 10 mM DTT. The A_{370} of the eluate was monitored to obtain a convenient guideline for identification of the desired fractions (12). The PFL fraction appeared at 90 to 120 ml of the eluate (pool 1), and the PFL-AE eluted at 130 to 160 ml (pool 2).

Pool 1 was concentrated by ultrafiltration with a molecular weight cutoff of 13,000 and then centrifuged ($20,000 \times g$, 10 min). The supernatant was applied to a DEAE-cellulose DE-32 (Whatman) chromatography column (1.5 by 15 cm) and eluted with a linear 0.04 to 0.2 M KPi gradient (pH 6.5). The protein in each fraction was assayed with Coomassie brilliant blue (6). The PFL fraction, which eluted at KPi concentrations of 0.07 to 0.12 M, was concentrated by ultrafiltration and subjected to an assay for PFL activity. To estimate the molecular weight of PFL, the PFL fraction was further separated anaerobically by gel filtration (Superdex 200 HR 10/30; Amersham Pharmacia) with an elution buffer consisting of 0.1 M KPi with 10 mM DTT.

Pool 2 was processed like pool 1 and was eluted with a linear 0.01 to 0.12 M KPi gradient (pH 6.5) in a DEAE-cellulose column. The PFL-AE fraction appeared at KPi concentrations of 0.05 to 0.07 M.

Flavodoxin was isolated by the method of Vetter and Knappe (26), with a minor modification. Spinach chloroplast fragments were isolated, and the chlorophyll content was estimated by the method of Knappe and Blaschkowski (12).

Activation of PFL and coupled optical assay for PFL activity. Activation of PFL by PFL-AE was performed with flavodoxin and chloroplasts by the method of Knappe and Blaschkowski (12). The apparatus was essentially similar to that described by these authors. Two small reaction tubes (1 ml) were hand made from a glass tubing, and the mouth of each tube had a silicon rubber stopper for a gas chromatograph. This setup made it possible to stab an injection needle for complete replacement of air with O_2 -free N_2 by using a vacuum pump. The two tubes were connected to each other with plastic tubing.

The activity of PFL was measured by a coupled enzyme assay (12) with 1-ml cuvettes having silicone rubber stoppers; the cuvettes had been hand made from disposable cuvettes. The PFL reaction was initiated by injecting activated PFL into these cuvettes. The PFL activity was assayed at 30°C by monitoring the rate of NAD reduction at A_{340} . The reduction of NAD in blank cuvettes, in which one of the substrates and auxiliary enzymes (citrate synthase and malate dehydrogenase) were omitted, was negligible.

For assays to examine the effect of pH on PFL activity, the amounts of auxiliary enzymes were increased fivefold to compensate for their reduction in their activity at extremes of pH. The activity of PFL was confirmed to limit the rate of reaction.

To examine the effect of triose phosphates on PFL activity, dihydroxyacetone phosphate (DHAP) and D-glyceraldehyde-3-phosphate (GAP) were added to the assay mixture before injection of activated PFL. Since the rate of reaction in the presence of triose phosphates declined with time, as observed for *Streptococcus mutans* by Takahashi et al. (22), PFL activity was determined 5 min after initiation of the reaction; i.e., the mean rate of reaction during the initial 5 min was determined.

Assay for PFL activity by formate production. The activity of PFL was also assessed by spectrophotometric measurement of the formate produced as described by Matheron et al. (17). In this case, the amount of activated PFL was doubled, and incubation was performed for 15 min.

Preparation of recombinant PFL protein. The *pfl* gene of *S. bovis* (4) was amplified by PCR with chromosomal DNA and primers PFLBAMHI (5'-TTC GGATCCATGGCGACTGTAAAACA-3') and PFLSALI (5'-TATGTCGAC TTATTTAGCAGCAACAGT-3'). These primers were designed to introduce the *Bam*HI and *Sal*I restriction sites for ligation to expression vectors. The PCR product was cloned in *Bam*HI-*Sal*I-digested pGEX-4T-3 to express GST fusion protein (Amersham Pharmacia). *E. coli* HB101 was transformed with the recombinant plasmid. All the manipulations with recombinant DNA were carried out by standard procedures (20).

Upon induction with 0.6 mM isopropyl- β -D-thiogalactopyranoside (IPTG), *E. coli* HB101 that received the recombinant plasmid overexpressed recombinant protein. The amount of the recombinant protein was approximately 20% of the total cell protein, as estimated by scanning of a sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) gel. The recombinant protein was purified by affinity column chromatography with a Glutathione Sepharose 4 B (Amersham Pharmacia). In the final step of purification, the enzyme preparation was eluted through a Mono Q column (1 ml) (AKTA system; Amersham Pharmacia) with a linear 0 to 0.5 M NaCl gradient in phosphate-buffered saline containing 10.1 mM Na_2HPO_4 , 1.8 mM KH_2PO_4 , 2.7 mM KCl, and 140 mM NaCl (pH 7.0). The GST protein was cut off with Thrombin protease (Amersham Pharmacia).

Preparation of antiserum. The recombinant PFL solution was mixed with an equal volume of Freund's complete adjuvant (Behring-Werke, Marburg, Federal Republic of Germany), and a rabbit was immunized with four injections of the mixture (0.2 mg of protein per injection). Serum was collected 42 days after the first injection under light ether anesthesia. The rabbit was kept in a metal cage (50 by 50 by 43 cm) placed in an experimental animal room. All animal care

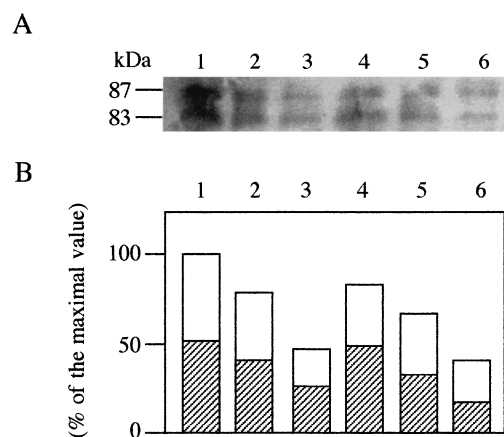


FIG. 1. Western immunoblot analysis and quantitation of PFL protein in extracts of *S. bovis* cells grown in continuous culture. (A) Western blot analysis. Lanes 1 through 3, glucose-limited culture (lane 1, pH 6.9, D of $0.1 h^{-1}$; lane 2, pH 6.9, D of $0.6 h^{-1}$; lane 3, pH 4.5, D of $0.1 h^{-1}$); lanes 4 through 6, ammonia-limited culture (lane 4, pH 6.9, D of $0.1 h^{-1}$; lane 5, pH 6.9, D of $0.6 h^{-1}$; lane 6, pH 4.5, D of $0.1 h^{-1}$). (B) Quantitation of Western blots. The sum of the values for the 87- and 83-kDa proteins is shown. The striped areas indicate the amounts of the 83-kDa protein.

procedures were carried out according to the *Guide for the Care and Use of Laboratory Animals* (18). A polyclonal antibody against *S. bovis* PFL was prepared from the rabbit antiserum by affinity column chromatography.

Immunological estimation of the amount of PFL. Cell extracts of *S. bovis* were prepared by ultrasonication as described previously (2). Ultrasonication was repeated until approximately 95% of the cells, as estimated by counting unbroken cells with a microscope, were disrupted. The protein content of the extract was determined by the Lowry method (15). For Western blot analysis, exactly 30- μ g portions of cell extracts were subjected to SDS-PAGE (14) and transferred to polyvinylidene difluoride membranes (Bio-Rad) by the procedure described by Towbin et al. (25). The polyclonal antibody against PFL was the primary antibody, alkaline phosphatase-conjugated anti-rabbit goat immunoglobulin G (Bio-Rad) was the secondary antibody, and 5-bromo-4-chloro-3-indolyl phosphate and nitroblue tetrazolium were the substrates used to visualize PFL. The amounts of PFL were estimated with a Fluor-S Multi Imager (Bio-Rad) as described previously (3).

Estimation of the concentrations of intracellular DHAP and GAP. Batch cultures of *S. bovis* were grown in glucose-limited medium at pH 7.0 or 4.5 as described previously (2), and cells were harvested at three stages of growth. Cell samples (2 ml) were quickly removed from the cultures and immediately frozen in liquid nitrogen. For DHAP and GAP analyses, 0.2 ml of 10% (vol/vol) perchloric acid was added to each frozen sample, and then the samples were thawed on ice. Each cell suspension was vigorously vortexed and centrifuged ($18,000 \times g$, 5 min, 4°C) to remove cell debris. The supernatant (2 ml) was neutralized with 50% (wt/vol) K_2CO_3 while it was agitated vigorously. After removal of the precipitate by centrifugation, 2 ml of the supernatant was concentrated to 0.4 ml with a centrifuge evaporator. The concentrated samples were analyzed for DHAP and GAP by the enzymatic method reported by Garrigues et al. (9). The concentrations of intracellular DHAP and GAP were calculated by assuming a ratio of 4.3 μ l of cytoplasmic fluid to 1 mg of cellular protein in *S. bovis* (5).

Determination of cellular protein. Cellular protein was determined by the method of Bond et al. (5). Cells were treated with 0.2 M NaOH (100°C, 10 min), and protein was determined by the Lowry method (15).

Molecular weight determination. The molecular weights of PFL and its cleavage product were determined by time of flight mass spectrometry (TOF-MS) with a Voyager RP mass spectrometer (PE Biosystems). The bands for the two proteins on SDS-PAGE gels (Fig. 1) were cut off and extracted with a matrix solution as described in a manufacturer's manual. The ionization mode was set to matrix-assisted laser desorption/ionization at an accelerating voltage of 20 kV with 2,5-dihydroxybenzoic acid as the matrix.

Evaluation of data. Data were analyzed by Tukey's test using the SigmaStat Statistical Analysis System (Jandel Scientific, San Rafael, Calif.); $P < 0.05$ was considered to be statistically significant.

RESULTS

Enzyme system of PFL in *S. bovis*. The activity of PFL was measured by using the assay system reported for *E. coli* PFL

TABLE 1. Activities of the PFL purified from *S. bovis* and *E. coli*

Enzyme source		Activity ^a
PFL	PFL-AE	
<i>E. coli</i>	Not added	17.2 ± 1.5 b
<i>E. coli</i>	<i>E. coli</i>	21.8 ± 2.1 a
<i>S. bovis</i>	Not added	4.5 ± 0.2 d
<i>S. bovis</i>	<i>E. coli</i>	9.5 ± 0.3 c
<i>S. bovis</i>	<i>S. bovis</i>	9.8 ± 0.3 c
<i>E. coli</i>	<i>S. bovis</i>	19.8 ± 1.7 a

^a The values are means ± standard errors ($n = 3$). Means followed by different letters differ significantly ($P < 0.05$). Activity is expressed as the increase in NADH (in micromoles per minute per milligram of protein).

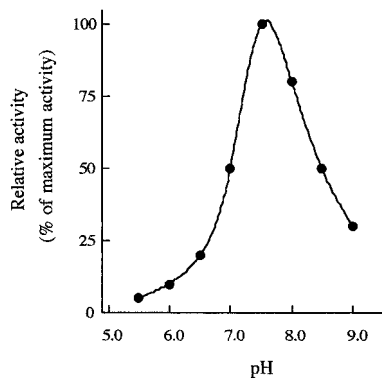
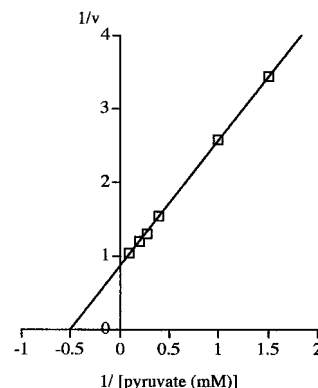
(12). The activity of *S. bovis* PFL was detected by replacing *E. coli* PFL with *S. bovis* PFL (Table 1). Even when PFL-AE was omitted, PFL activity was detected, but the activity was low. These results indicate that the inactive form of *S. bovis* PFL is activated by *E. coli* PFL-AE. The activity was also detected when both PFL and PFL-AE were of *S. bovis* origin. The PFL of *E. coli* was activated by *S. bovis* PFL-AE. These results indicate that the enzyme system of PFL in *S. bovis* is similar to that in *E. coli*.

Molecular mass of *S. bovis* PFL. Protein with PFL activity was eluted as the major peak by gel filtration of the PFL fraction from *S. bovis*. The molecular mass of the protein was estimated to be approximately 180 kDa from the behavior in the column. SDS-PAGE of the protein conducted under aerobic conditions gave two bands at 80 to 90 kDa, which were similar to the bands in Fig. 1. The molecular masses of the proteins in the two bands were estimated to be 87 and 83 kDa by TOF-MS.

Properties of *S. bovis* PFL. The optimal pH of *S. bovis* PFL was 7.5, and the activity decreased to less than 10% of the maximal activity at pH 6.0 (Fig. 2). When PFL activity was determined as formate production, similar pH dependency was observed (data not shown).

The affinity of PFL for substrate was estimated by measuring PFL activity with graded concentrations of pyruvate by the coupled optical assay. A Lineweaver-Burk plot indicated that the reaction of *S. bovis* PFL followed the Michaelis-Menten equation, and the apparent K_m was 2.0 mM (Fig. 3).

Effects of DHAP and GAP on PFL activity, and intracellular concentrations of DHAP and GAP. As shown in Fig. 4, DHAP and GAP inhibited PFL activity in a dose-response fashion. DHAP decreased the activity to 40% of maximum activity at a

FIG. 2. Effect of pH on the activity of *S. bovis* PFL.FIG. 3. Lineweaver-Burk plot of the reaction of *S. bovis* PFL.

concentration of 0.1 mM, and GAP inhibited the activity by more than 80% at a concentration of 0.1 mM.

The concentrations of triose phosphates in *S. bovis* cells grown in batch culture at pH 7.0 and 4.5 are shown in Table 2. At both pHs, the concentration of DHAP was highest at the middle exponential stage of growth and decreased as the growth rate decreased. No detectable amount of DHAP (concentration, less than 0.1 mM) was present immediately before growth cessation. Similarly, the concentration of GAP was highest at middle exponential stage and decreased with a decrease in growth rate to less than 0.05 mM. Thus, the intracellular levels of DHAP and GAP range so widely that these triose phosphates can actually affect PFL activity in *S. bovis*.

Amounts of PFL in cells and formate production rate. In the Western immunoblot analysis of the PFL of *S. bovis* grown in continuous culture, two PFL-specific bands were detected (Fig. 1A). As described below, these bands corresponded to the full-length PFL (87 kDa) and its cleavage product (83 kDa). Therefore, the sum of the amounts of the two proteins represents the amount of PFL in cells.

The amount of PFL protein decreased when the dilution rate (D) was increased from 0.1 to 0.6 per h at pH 6.9 in both glucose- and ammonia-limited cultures (Fig. 1). At the same D, the amounts of PFL were slightly larger in the glucose-limited culture than in the ammonia-limited culture. In both the glucose- and ammonia-limited cultures grown at a D of 0.1 per h, *S. bovis* cells grown at pH 4.5 contained approximately half the amounts of PFL present in the cells grown at pH 6.9.

The formate production rate was greatly increased by in-

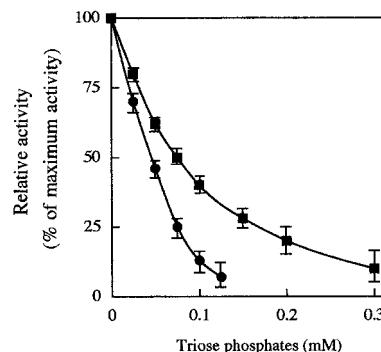
FIG. 4. Effects of triose phosphates on the activity of *S. bovis* PFL. Symbols: ●, GAP; ■, DHAP. Bars indicate the standard error ($n = 3$).

TABLE 2. Concentrations of DHAP and GAP in cells harvested at different growth stages in glucose-limited batch culture of *S. bovis*

Growth stage	pH	Intracellular concn (mM) ^a	
		DHAP	GAP
Middle exponential	7.0	1.7 ± 0.2	0.3 ± 0.02
Late exponential	7.0	0.5 ± 0.1	0.1 ± 0.01
Immediately before growth cessation	7.0	<0.1	<0.05
Middle exponential	4.5	1.9 ± 0.2	0.6 ± 0.05
Late exponential	4.5	0.7 ± 0.1	0.1 ± 0.02
Immediately before growth cessation	4.5	<0.1	<0.05

^a The values are means ± standard errors ($n = 3$).

creasing the D from 0.1 to 0.6 per h at pH 6.9 under both glucose- and ammonia-limited conditions (Table 3). The increase was not brought about by an increase in PFL, but the rate of formate production increased with an increase in the influx rate of glucose. On the other hand, the decrease in pH from 6.9 to 4.5 decreased the formate production rate, which was consistent with the decrease in the amount of PFL and the percentage of formate. The ratio of formate to lactate naturally reflected the ratio of the formate production rate to the lactate production rate. The change from glucose-limited conditions to ammonia-limited conditions decreased the formate production rate, which was again consistent with the decrease in PFL and the percentage of formate.

DISCUSSION

In this study, *S. bovis* PFL and PFL-AE were identified and characterized, and they were found to be homologous to *E. coli* PFL and PFL-AE. Probably, the activation mechanisms are also similar. However, the electron transport system in *S. bovis* is still unknown at present, because we used flavodoxin prepared from *E. coli*.

The molecular mass of *S. bovis* PFL was estimated to be approximately 180 kDa. The genetic analysis of *S. bovis* PFL indicated that the operon has only one open reading frame, which may encode a 87-kDa protein (4). *E. coli* PFL was reported to be a homodimer with an 85-kDa subunit (7), and its active form is irreversibly cleaved by exposure to O₂ at the position between Ser-733 and Gly-734, producing 82- and 3-kDa polypeptides (27). Analogous to *E. coli* PFL, the active form of *S. bovis* PFL is possibly cleaved by O₂ between Ser-736 and Gly-737 (4), giving rise to 83- and 4-kDa polypeptides. The presence of 87- and 83-kDa proteins was confirmed by TOF-MS. These results indicate that *S. bovis* PFL is a homodimer with a molecular mass of 174 kDa.

The amount of the 83-kDa protein was nearly equal to that of the 87-kDa protein (Fig. 1), and the activity of *S. bovis* PFL activated by PFL-AE was approximately twice the activity in the absence PFL-AE (Table 1). Assuming that the active form of *S. bovis* PFL is cut off by O₂, the amount of the active form was nearly equal to that of the inactive form, and approximately half the amount of the active form was irreversibly broken down during the separation of PFL. In *E. coli*, the PFL activity in the absence of PFL-AE was approximately 80% of the value in the presence of PFL-AE (Table 1), indicating that most of the PFL in *E. coli* cells was present as the active form. The specific activity of *S. bovis* PFL in the presence of PFL-AE was much lower than that of *E. coli* PFL (Table 2). This is partly because *S. bovis* contained a higher percentage of the active form, half of which was irreversibly inactivated. In addition, the properties of PFL may be different, or the PFL frac-

tion of *S. bovis* may have contained a larger amount of other protein.

The optimal pH of *S. bovis* PFL was 7.5, and the activity decreased greatly as the pH decreased (Fig. 2), explaining our previous observations that formate production decreased greatly when the pH was reduced (2, 4). The apparent K_m for PFL was 2.0 mM (Fig. 3), indicating that the affinity for pyruvate does not differ greatly between PFL and LDH (K_m 1.5 mM) (2). The K_m for *S. bovis* PFL is comparable to the K_m values reported for *E. coli* (2.0 mM) (13), *Streptococcus mutans* (2.6 mM) (22), and *Clostridium butyricum* (1.6 mM) (23). The small difference in affinity between PFL and LDH suggests that the relative amounts of PFL (active form) and LDH, as well as intracellular pH and the levels of effectors, affect the partition of flow from pyruvate in *S. bovis*.

In *Streptococcus lactis* (8), *Streptococcus cremoris* (24), and *S. mutans* (22), triose phosphates have been reported to inhibit PFL activity. As in these streptococci, the activity of *S. bovis* PFL was inhibited by GAP and DHAP (Fig. 4). The intracellular concentrations of these triose phosphates decreased greatly with the growth stage, but the DHAP concentration was not changed by culture pH (Table 2). The pool size of GAP increased at low pH, suggesting that the metabolic flow downstream from GAP in the glycolytic pathway is slowed down when the intracellular pH becomes low.

The amounts of DHAP and GAP in resting cells of *S. mutans* have been reported to be 12 and 5.6 nmol per mg of (dry weight) of cells, respectively (1). Assuming that 1 mg of dry cells contains 1.69 μl of cytoplasmic fluid (20), the intracellular concentrations of DHAP and GAP in *S. mutans* are calculated to be 7.1 and 3.3 mM, respectively. The intracellular concentrations of DHAP and GAP in *Lactococcus lactis* at the exponential stage of growth were 21 and 6 mM, respectively (9). These values are much higher than the values for *S. bovis*. Since *S. mutans* and *L. lactis* contained much higher concentrations of fructose-1,6-bisphosphate than *S. bovis* contained (5), the pool sizes of glycolytic intermediates in *S. bovis* are probably much smaller than those in *S. mutans* and *L. lactis*. The intracellular concentrations of DHAP and GAP decreased greatly with the growth stage, suggesting that the concentrations of these triose phosphates fluctuate greatly depending on the rate of glucose influx into cells. The fluctuation was great enough to exert an allosteric effect on PFL activity. Allosteric inhibition by triose phosphates is consistent with our previous observations that only a small amount of formate was produced when excess glucose was supplied (4, 18).

However, even when excess glucose was supplied, formate production continued (4), which indicates that PFL functions at any in vivo concentration of triose phosphates. PFL activity

TABLE 3. Production rates and percentages of formate and lactate in continuous culture^a

Culture condition	D (h ⁻¹)	pH	Production rate ^b		% of product	
			Formate	Lactate	Formate	Lactate
Glucose limited	0.1	6.9	86 ± 3	19 ± 1	82 ± 3	18 ± 1
Ammonia limited	0.1	6.9	63 ± 3	34 ± 2	65 ± 3	35 ± 1
Glucose limited	0.6	6.9	310 ± 9	286 ± 8	52 ± 2	48 ± 2
Ammonia limited	0.6	6.9	238 ± 7	315 ± 9	43 ± 2	57 ± 2
Glucose limited	0.1	4.5	35 ± 2	132 ± 4	21 ± 1	79 ± 3
Ammonia limited	0.1	4.5	30 ± 1	146 ± 4	17 ± 1	83 ± 3

^a The values are means ± standard errors ($n = 3$).

^b Expressed in micromoles per hour per milligram of cell N.

was not immediately inhibited by the addition of DHAP or GAP, but their inhibitory effect increased gradually with time. As described for *S. mutans* by Takahashi et al. (22), formation of the enzyme-inhibitor complex may be considerably slower than the reaction between the enzyme and the substrate. Therefore, it is possible that newly synthesized, or activated, PFL has activity even at high concentrations of triose phosphates. Moreover, since the inhibition by triose phosphates is not 100%, a reaction can occur if the amount of PFL is large. In addition, Takahashi et al. (22) found that the inhibitory effects of DHAP and GAP in *S. mutans* decreased with an increase in the amount of PFL.

The amount of PFL protein was decreased by increasing the D, and cells grown under glucose-limited conditions possessed larger amounts of PFL protein than cells grown under ammonia-limited conditions (Fig. 1). The cells grown at pH 4.5 contained approximately half the amount of PFL present in the cells grown at pH 6.9, apparently suggesting that PFL synthesis is suppressed at low pH. This observation is in marked contrast to LDH synthesis, which was enhanced at low pH (2, 4). The results obtained in this experiment were essentially consistent with our previous results showing that the level of *pfl*-mRNA changed with culture conditions (4). The synthesis of PFL appears to be regulated at the transcriptional level in response to growth conditions, as observed for LDH synthesis (3, 4). However, the changes in PFL activity reported in the previous paper (4) were much greater than the changes in PFL protein. This discrepancy was probably brought about by the difficulty of measuring PFL activity. Another possibility is that a small change in PFL protein is amplified during conversion to its active form.

The rate of formate production was roughly parallel to the amount of PFL at the same D (Table 3). However, the formate production rate is not exactly proportional to the amount of PFL protein, as shown by following five observations: (i) all the PFL protein is not in the active form; (ii) PFL activities are different at different intracellular pH values; (iii) the intracellular concentrations of DHAP and GAP are different; (iv) the amounts of LDH protein are different at different pH values (4); and (v) LDH activities are different at different intracellular pH values (2, 19). Thus, the rate of formate production is affected not only by PFL activity but also by LDH activity. When PFL activity was high, LDH activity was low (4). This reciprocal relationship is considered to amplify the change in the ratio of formate to lactate (Table 3). Under actively growing conditions, PFL is probably saturated, and glucose spills over into lactate.

In conclusion, the results of the present study proved the hypothesis that the fermentation pattern in *S. bovis* is determined not only by LDH activity but also by PFL activity, and the synthesis of the two enzymes is regulated at the transcriptional level in response to growth conditions. The regulation of the activation and inactivation of PFL remains to be clarified.

ACKNOWLEDGMENTS

This study was supported in part by a Grant-in-Aid for Scientific Research (no. 10460125 and 160132) from the Ministry of Education, Science, and Culture of Japan and by Research Fellowships (no. 8156) from the Japan Society for the Promotion of Science for Young Scientists.

REFERENCES

1. Abbe, K., S. Takahashi, and T. Yamada. 1982. Purification of pyruvate formate-lyase from *Streptococcus mutans* and its regulatory properties. *J. Bacteriol.* **152**:175–182.

2. Asanuma, N., and T. Hino. 1997. Tolerance to low pH and lactate production in rumen bacteria. *Anim. Sci. Technol.* **68**:367–376.
3. Asanuma, N., M. Iwamoto, and T. Hino. 1997. Regulation of lactate dehydrogenase synthesis in a ruminal bacterium, *Streptococcus bovis*. *J. Gen. Appl. Microbiol.* **43**:325–331.
4. Asanuma, N., M. Iwamoto, and T. Hino. 1999. Structure and transcriptional regulation of the gene encoding pyruvate formate-lyase of a ruminal bacterium, *Streptococcus bovis*. *Microbiology* **145**:151–157.
5. Bond, D. R., B. M. Tsai, and J. B. Russell. 1998. The diversion of lactose carbon through the tagatose pathway reduces the intracellular fructose 1,6-bisphosphate and growth rate of *Streptococcus bovis*. *Appl. Microbiol. Biotechnol.* **49**:600–605.
6. 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.
7. Conradt, H., M. Hohmann-Berger, H. P. Hohmann, H. P. Blaschkowski, and J. Knappe. 1984. Pyruvate formate-lyase-activating enzyme of *Escherichia coli*: isolation and structural properties. *Arch. Biochem. Biophys.* **228**:133–142.
8. Fordyce, A. M., V. L. Crow, and T. D. Thomas. 1984. Regulation of product formation during glucose or lactate limitation in nongrowing cells of *Streptococcus lactis*. *Appl. Environ. Microbiol.* **48**:332–337.
9. Garrigues, C., P. Loubiere, N. D. Lindley, and M. Coccagn-Bousquet. 1997. Control of the shift from homolactic acid to mixed-acid fermentation in *Lactococcus lactis*: predominant role of the NADH/NAD⁺ ratio. *J. Bacteriol.* **179**:5282–5287.
10. Hino, T., and T. Miwa. 1996. An improved apparatus for the continuous culture of rumen bacteria that require particularly strict anaerobiosis. *Bull. Fac. Agric. Meiji Univ.* **107**:59–64.
11. Hino, T., K. Miyazaki, and S. Kuroda. 1991. Role of extracellular acetate in the fermentation of glucose by a ruminal bacterium, *Megasphaera elsdenii*. *J. Gen. Appl. Microbiol.* **37**:121–129.
12. Knappe, J., and H. P. Blaschkowski. 1975. Pyruvate formate-lyase from *Escherichia coli* and its activation system. *Methods Enzymol.* **41**:508–518.
13. Knappe, J., H. P. Blaschkowski, P. Grobner, and T. Schmitt. 1974. Pyruvate formate-lyase of *Escherichia coli*: the acetyl-enzyme intermediate. *Eur. J. Biochem.* **50**:253–263.
14. Laemmli, U. K. 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature (London)* **227**:680–685.
15. Lowry, O. H., N. J. Rosebrough, A. L. Farr, and R. J. Randall. 1951. Protein measurement with the Folin phenol reagent. *J. Biol. Chem.* **193**:265–275.
16. Marounek, M., and S. Bartos. 1987. Interactions between rumen amylolytic and lactate-utilizing bacteria in growth on starch. *J. Appl. Bacteriol.* **63**:233–238.
17. Matheron, C., A. M. Delort, G. Gaudet, and E. Forano. 1997. Re-investigation of glucose metabolism in *Fibrobacter succinogenes*, using NMR spectroscopy and enzymatic assays. Evidence for pentose phosphates phosphoketolase and pyruvate formate lyase activities. *Biochim. Biophys. Acta* **1355**:50–60.
18. National Research Council USA. 1996. Guide for the care and use of laboratory animals. National Academy Press, Washington, D.C.
19. Russell, J. B., and T. Hino. 1985. Regulation of lactate production in *Streptococcus bovis*, a spiraling effect that contributes to rumen acidosis. *J. Dairy Sci.* **68**:1712–1721.
20. Sambrook, J., E. F. Fritsch, and T. Maniatis. 1989. Molecular cloning: a laboratory manual, 2nd ed. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.
21. Sjoberg, A., and B. Hahn-Hagerdal. 1989. β -Glucose-1-phosphate, a possible mediator for polysaccharide formation in maltose-assimilating *Lactococcus lactis*. *Appl. Environ. Microbiol.* **55**:1549–1554.
22. Takahashi, S., K. Abbe, and T. Yamada. 1982. Purification of pyruvate formate-lyase from *Streptococcus mutans* and its regulatory properties. *J. Bacteriol.* **149**:1034–1040.
23. Thauer, R. K., F. H. Kirchniaw, and K. A. Jungermann. 1972. Properties and function of the pyruvate-formate-lyase reaction in clostridia. *Eur. J. Biochem.* **27**:282–290.
24. Thomas, T. D., K. W. Turnere, and V. L. Crow. 1980. Galactose fermentation by *Streptococcus lactis* and *Streptococcus cremoris*: pathways, products, and regulation. *J. Bacteriol.* **144**:672–682.
25. Towbin, J., T. Staehlin, and J. Gordon. 1979. Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedure and some applications. *Proc. Natl. Acad. Sci. USA* **76**:4350–4354.
26. Vetter, H., Jr., and J. Knappe. 1971. Flavodoxin and ferredoxin of *Escherichia coli*. *Hoppe-Seyler's Z. Physiol. Chem.* **352**:433–446.
27. Wagner, A. F. V., M. Frey, F. A. Neugebauer, W. Schaffer, and J. Knappe. 1992. The free radical in pyruvate formate-lyase is located on glycine-734. *Proc. Natl. Acad. Sci. USA* **89**:996–1000.