Structure and transcriptional regulation of the gene encoding pyruvate formate-lyase of a ruminal bacterium, *Streptococcus bovis*

Narito Asanuma, Miwa Iwamoto and Tsuneo Hino

Author for correspondence: Tsuneo Hino. Tel: +81 44 934 7825. Fax: +81 44 934 7825.
e-mail: hino@isc.meiji.ac.jp

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

The gene (*pfl*) encoding pyruvate formate-lyase (Pfl) from *Streptococcus bovis* was sequenced. The deduced amino acid sequence of Pfl was similar to *Streptococcus mutans* Pfl, and included the conserved regions necessary for free-radical formation and a catalytic site. The Pfl of *S. bovis* appeared to be a free-radical-containing enzyme because of its dioxygen sensitivity and its amino acid sequence similarity with the *Escherichia coli* enzyme. The *pfl* mRNA of *S. bovis* was approximately 2·3 kb and was transcribed in a monocistronic fashion. When cells were grown in batch culture at pH 6·9, the level of *pfl* transcript increased as the growth phase changed from exponential growth to stationary phase. This result was in contrast to the previous observation that the level of lactate dehydrogenase (*Ldh*) mRNA decreased during the later stages of growth. Continuous culture experiments conducted at pH 6·9 under glucose-limited and ammonia-limited conditions revealed that *pfl* mRNA was decreased by an excess supply of glucose, as well as by a high growth rate. On the contrary, *ldh* mRNA increased when excess glucose was supplied and the growth rate was high. The amount of *pfl* mRNA in cells was lower at pH 4·5 than pH 6·9, whereas the level of *ldh* mRNA was higher at pH 4·5. This result was consistent with the amounts of Pfl and Ldh in cells and the proportion of formate and lactate produced. These results support the hypothesis that *S. bovis* regulates Pfl and Ldh synthesis at the transcriptional level in response to growth conditions.

Keywords: *Streptococcus bovis*, pyruvate formate-lyase, rumen bacteria, *pfl* gene, transcription

INTRODUCTION

Streptococcus bovis often predominates in the rumen when high-starch diets are fed (Marounnek & Bartos, 1987; Hungate, 1966) and produces lactate as the major fermentation product when excess energy source is present and the growth rate is high (Asanuma *et al.*, 1997). The increased lactate production brings about a drop in ruminal pH. *S. bovis* is acid resistant compared with other main ruminal bacteria, and is able to grow at pH 4·5 (Asanuma & Hino, 1997). The percentage of lactate in the fermentation products of *S. bovis* also

increases when the environmental pH is low (Russell & Hino, 1985; Asanuma & Hino, 1997). Thus, *S. bovis* continues to produce lactate, and potentially contributes to the progress of rumen acidosis (Russell & Hino, 1985).

The increase in lactate production at low pH can be explained by the fact that *S. bovis* allows its intracellular pH to decrease to around pH 5·5 when the extracellular pH decreases to 4·7 (Russell & Hino, 1985; Russell, 1991); the pH optimum of its lactate dehydrogenase (*Ldh*) is pH 5·5 (Russell & Hino, 1985; Asanuma & Hino, 1997). In addition, *S. bovis* increases the synthesis of *Ldh* in response to low pH (Asanuma *et al.*, 1997; Asanuma & Hino, 1997). The enhanced lactate production by the excess supply of energy source is in part caused by the increase in the intracellular concentration of fructose-1,6-diphosphate which enhances *Ldh* activity (Russell & Hino, 1985).

Abbreviations: D, dilution rate; *Ldh*, lactate dehydrogenase; *Pfl*, pyruvate formate-lyase

The GenBank/EMBL/DBJ accession number for the *S. bovis pfl* sequence reported in this paper is AB014686.

The percentage of lactate in fermentation products depends on the partition of the flow from pyruvate, which is a branching point in the pathways of sugar fermentation. In *S. bovis*, pyruvate is either converted to lactate by Ldh or to acetyl-CoA and formate by pyruvate formate-lyase (Pfl). Therefore, it is also important to understand how Pfl activity is affected by pH and energy supply. Allosteric regulation of Pfl by triose phosphate is important in *Streptococcus mutans* (Thakahashi *et al.*, 1982), *Streptococcus lactis* (Fordyce *et al.*, 1984), *Streptococcus cremoris* (Thomas *et al.*, 1980), and *S. bovis* (unpublished observation). However, it may also be possible that the synthesis of Pfl is regulated in response to changes in culture conditions.

Formate production in the rumen implies that formate is used as a substrate for methane production. Since methane may contribute to the greenhouse effect, it is important to control formate production. In the main ruminal bacteria which produce formate, it appears to be formed by the Pfl reaction (Asanuma *et al.*, 1998). However, no reports on Pfl activity of ruminal bacteria are currently available.

So far, Pfl has been investigated for several bacteria such as *Escherichia coli*, Clostridia (Thauer *et al.*, 1972; Wood & Jungermann, 1972), *Streptococcus faecalis* (Lindmark *et al.*, 1969), *S. mutans* (Thakahashi *et al.*, 1982) and *Micrococcus lactilyticus* (McCormick *et al.*, 1962a, b). Of these bacteria, the Pfl of *E. coli* has been studied in detail (Knappe & Blaschkowski, 1975; Kessler & Knappe, 1996). *E. coli* Pfl, which loses activity in contact with O₂, functions under anaerobic conditions. In *E. coli*, Pfl consisting of two identical 85 kDa subunits is synthesized as the inactive, nonradical form (E_i), and is post-translationally converted to the active, radical form (E_a). The activating enzyme, which itself must be activated for the activation of Pfl, is activated by reduction through reduced flavodoxin. The active form of the activating enzyme converts E_i to E_a by donating an electron. E_a possesses an organic free radical at the active site, on the α -carbon of Gly⁷³⁴, and the mechanism of its action has been proposed to be a homolytic process with the participation of the organic free radical (Urking *et al.*, 1989). The reversible formation of the active-site radical is tightly regulated by the Pfl-activating enzyme and the deactivating enzyme catalysing the conversion of E_a to E_i, both of which are sensitive to the metabolic status of the micro-organism (Wong *et al.*, 1993).

The objective of this study was to clarify how the synthesis of Pfl in *S. bovis* is regulated, especially at the transcriptional level. For this purpose, we analysed the nucleotide sequence of the gene encoding Pfl (*pfl*) with the primer extension analysis. In addition, we examined the effect of culture conditions on the level of *pfl* mRNA, together with the effect on the *ldh* mRNA level.

METHODS

Bacterial strain and growth conditions. The source of *S. bovis* JB1 was as described previously (Asanuma & Hino, 1997). Batch culture of *S. bovis* was carried out in 120 ml serum vials

as described previously (Asanuma & Hino, 1997). The medium for batch culture contained (g l⁻¹): K₂HPO₄, 0.45; KH₂PO₄, 0.45; (NH₄)₂SO₄, 0.9; NaCl, 0.9; CaCl₂·2H₂O, 0.12; MgSO₄·7H₂O, 0.19; glucose, 3; Trypticase (BBL), 1.0; yeast extract (Difco), 1.0; cysteine-HCl, 0.6. The medium was adjusted to pH 6.9 with 8% (w/v) Na₂CO₃. Incubation was performed in triplicate, maintaining the pH approximately constant by adding anaerobic Na₂CO₃ (Hino *et al.*, 1992). Cell growth was estimated by measuring the OD₆₀₀.

Apparatus and general procedures for continuous culture were as described previously (Hino & Hamano, 1993; Hino *et al.*, 1993; Hino & Miwa, 1996). The apparatus consisted of four sets of a 5 l reservoir bottle, a peristaltic pump, an overflow-type fermenter with a capacity of 500 ml and a 5 l drainage bottle. The glucose-limited medium contained (g l⁻¹): K₂HPO₄, 0.45; KH₂PO₄, 0.45; (NH₄)₂SO₄, 1.8; NaCl, 0.9; CaCl₂·2H₂O, 0.12; MgSO₄·7H₂O, 0.19; glucose, 3.0; Trypticase (BBL), 0.5; yeast extract (Difco), 0.5; cysteine-HCl, 0.6. In the ammonia-limited medium, glucose was increased to 5 g l⁻¹ and (NH₄)₂SO₄ was decreased to 0.1 g l⁻¹. Continuous culture was performed in duplicate fermenters at 39 °C under a stream of CO₂+N₂ (1:9) for 4 d, repeating the same experiment twice. The dilution rate (D) was set at 0.1 or 0.6 h⁻¹ and the pH was maintained at pH 6.9 or 4.5.

Samples for the determination of fermentation products and bacterial cells for the measurement of the enzyme content and Northern-blot analysis were taken from the fermenters on the last day of the culture. Unless otherwise stated, the cultures (500 ml) were immediately cooled in an ice bath and cells were collected by centrifugation (20000 g, 10 min, 2 °C).

Determination of fermentation products, glucose, ammonia and cellular nitrogen. Organic acids were analysed by HPLC (Hino *et al.*, 1994) and glucose was determined as described previously (Asanuma & Hino, 1997). Ammonia was collected by the Conway's micro-diffusion method (Conway & Byrne, 1933) and assayed by the indophenol method (Chaney & Marbach, 1962). Cellular nitrogen was determined by digesting the cells by the Kjeldahl method, followed by the quantification of ammonia by the indophenol method.

Assay for Ldh and Pfl activity. Cell-free extracts of *S. bovis* for the assay of Ldh and Pfl activity were prepared as described previously (Asanuma & Hino, 1997), except that all the treatments were performed in an anaerobic glove box because no Pfl activity was detected when extracted in air. From the extracts, Pfl and Pfl-activating enzyme were isolated by the combination of protamine sulfate and ammonium sulfate precipitation, and then by two successive column chromatographies essentially according to the method of Knappe & Blaschkowski (1975). Flavodoxin was isolated by the method of Vetter & Knappe (1971) with a minor modification. The isolation of spinach chloroplast fragments and the estimation of chlorophyll content were carried out according to the method of Whatlay & Arnon (1963). The activation of Pfl was performed essentially by the method of Knappe & Blaschkowski (1975) using a device for anaerobic treatments. The details of the device will be reported elsewhere. The activity of Ldh (Russell & Hino, 1985; Asanuma & Hino, 1997) and Pfl (Knappe & Blaschkowski, 1975) was estimated by measuring the rate of NADH oxidation at A₃₄₀. The reaction mixture for the Pfl assay contained 20 mM sodium pyruvate, 0.08 mM CoA, 1 mM NAD, 6 mM sodium malate, 2 mM DTT, 1.4 U citrate synthase ml⁻¹, 14 U malate dehydrogenase ml⁻¹ and cell extract in 100 mM potassium phosphate buffer (pH 7.6). Again, the details of the determination of Pfl activity will be reported elsewhere. One unit of activity of each enzyme was

153

(a)	
<i>S. bovis</i>	PFL 409:SCISCCVSPL 418
<i>S. mutans</i>	PFL 409:SCISCCVSPL 418
<i>L. lactis</i>	PFL 412:SCISCCVSPL 421
<i>E. coli</i>	PFL 414:YAIACCVSPM 423
<i>C. paste.</i>	PFL 401:YAIACCVSAM 410
<i>H. influ.</i>	PFL 417:YAIACCVSPM 426
	* ****
(b)	
<i>S. bovis</i>	PFL 734:RISGYCV 740
<i>S. mutans</i>	PFL 734:RISGYCV 740
<i>L. lactis</i>	PFL 746:RISGYCV 752
<i>E. coli</i>	PFL 731:RVSGYAV 737
<i>C. paste.</i>	PFL 712:RVSGYAV 718
<i>H. influ.</i>	PFL 744:RVSGYAV 750
	* *** *

Fig. 2. Alignment of Pfl proteins at the catalytic site (a) and the radical-forming site (b). The sequence of *Streptococcus bovis* Pfl is aligned with all Pfl entries available in the databases by using the GENETYX-MAC 8.0. Asterisks in the consensus line represent identical amino acid residues.

pasteurianum and *Haemophilus influenzae* Pfls. The similarity was especially high to the Pfls of *S. mutans* (90%) (Yamamoto *et al.*, 1996) and *Lactococcus lactis* (80%) (Arnau *et al.*, 1997). The *S. bovis* Pfl included the conserved regions necessary for free-radical formation and a catalytic site (Fig. 2) (Knappe *et al.*, 1984; Wagner *et al.*, 1992; Parast *et al.*, 1995). The Gly⁷³⁴ residue, which is involved in free-radical formation in the active form of the enzyme in *E. coli* (Wagner *et al.*, 1992), was conserved at residue 737 in *S. bovis*, flanked by Ser⁷³⁶ and Tyr⁷³⁸. Cys⁴¹⁸ and Cys⁴¹⁹, which play an auxiliary role in the C–C bond cleavage of pyruvate and catalyses hydrogen exchange of the glycyl radical of the enzyme in *E. coli* (Parast *et al.*, 1995), were conserved in *S. bovis* Pfl at residues 413 and 414. These regions are conserved in all Pfl proteins characterized to date (Fig. 2).

S. bovis Pfl appeared to be a free-radical-containing enzyme because of its dioxygen sensitivity and its similarity to the *E. coli* enzyme. It is probable that *S. bovis* Pfl is activated by a mechanism similar to that of *E. coli*, because *S. bovis* Pfl was activated by the activating system for *E. coli* Pfl with S-adenosyl methionine, flavodoxin and chloroplasts (Knappe *et al.*, 1984), in which Pfl prepared from *E. coli* was replaced by Pfl from *S. bovis* (unpublished results).

A potential ribosome-binding site (GGAGA, +37 to +41) was identified 10 bp upstream of the ATG initiation codon of the *pfl* gene. In addition, potential –35 and –10 promoter regions (TTGGGA and TAAACT, –36 to –31 and –12 to –7, respectively) were found. The structure of these promoter regions suggests that the expression of *pfl* in *S. bovis* requires a sigma factor analogous to *E. coli* σ^{70} .

Primer extension analysis revealed that only one tran-

Fig. 3. Primer extension analysis of the *S. bovis pfl* gene. Primer extension was carried out with primer pfl-EX on *S. bovis* total RNA. A sequencing ladder was run with the same primer and is shown on the left. The transcriptional start site is indicated with an arrow beside the sequence.

scriptional start site existed 50 bp upstream of the *pfl* start codon (Figs 1 and 3). This is in contrast to *E. coli*, in which seven promoter regions have been implicated in the regulation and expression of *pfl* (Sawers, 1993). An inverted repeat sequence characteristic of transcriptional terminators was detected between bases +2385 and +2416, situated 10 bp downstream of the termination codon of *S. bovis pfl*. The formation of a stem-loop structure in this region of the mRNA could be presumed by a free-energy change of $-8.1 \text{ kcal mol}^{-1}$. These results suggest that the *pfl* gene from *S. bovis* is monocistronic.

Transcription of the *S. bovis pfl* and *ldh* genes

Batch culture experiment

S. bovis was grown in batch culture, maintaining the pH at approximately 6.9. Cell samples were taken at 2 h (mid-exponential phase), 5 h (late-exponential phase) and 6 h (just before the cessation of growth) after inoculation. Formate production per unit fermented glucose increased as the growth phase changed, from 2–6 h (Table 1). Parallel with formate production, Pfl activity per unit cellular nitrogen, which was considered to represent the cellular content of Pfl, increased with time. This result was in contrast to the previous observation that lactate production per unit glucose fermented and Ldh activity per unit cellular nitrogen decreased as the growth phase changed during the later stages of growth (Asanuma *et al.*, 1997).

Northern-blot analysis indicated that a single transcript hybridizing with the *pfl* probe was present in cells at any growth phase, which was estimated to be approximately

Table 1. The change in formate production, Pfl activity and the relative amounts of *pfl* mRNA at three growth stages in the batch culture of *S. bovis*

Values are presented as the mean $\pm$ SE ($n = 3$). Values within columns with no common superscript letters differ significantly ($P < 0.05$, $n = 3$).

Time (h)	Formate produced*	Pfl activity†	Relative amount of <i>pfl</i> mRNA‡
2	36 $\pm$ 7 ^c	0.9 $\pm$ 0.1 ^c	1.0 $\pm$ 0.1 ^c
5	78 $\pm$ 6 ^b	2.0 $\pm$ 0.3 ^b	1.8 $\pm$ 0.2 ^b
6	124 $\pm$ 10 ^a	4.4 $\pm$ 0.3 ^a	3.4 $\pm$ 0.3 ^a

* mol (100 mol glucose fermented)⁻¹. The amounts produced during 0–2, 2–5 and 5–6 h are shown.

† μ mol NADH oxidized min⁻¹ (10 μ g cellular nitrogen)⁻¹.

‡ The bands shown in Fig. 4, separated from 10 μ g total RNA, were analysed by using a Fluor-S Multi Imager and are expressed as relative values.

Fig. 4. The levels of *pfl* mRNA in *S. bovis* cells at three growth stages in batch culture. Cells were grown for 2 h (lane 1), 5 h (lane 2) or 6 h (lane 3). The arrow indicates the 2.3 kb *pfl* transcript.

2.3 kb (Fig. 4). This result agreed with the nucleotide sequence analysis, suggesting that *S. bovis pfl* has a monocistronic mode of transcription. In addition, primer extension analysis with the *pfl*-EX primer showed that the transcriptional start site did not change with growth stage (data not shown).

As shown in Table 1, the level of *pfl* mRNA increased during the late stages of growth. This result was in agreement with results showing that formate produced per unit glucose fermented and the content of Pfl increased with time (Table 1), and was in contrast to previous results showing that the level of *ldh* mRNA decreased during the late stages of growth (Asanuma *et al.*, 1997).

Continuous culture experiment

When *S. bovis* was grown in batch culture, excess glucose was initially present and the glucose concentration decreased as cells grew. The growth rate was considered to depend upon the availability of glucose. To examine whether the transcription of the *pfl* and *ldh* genes is regulated by growth rate or the abundance of glucose in cells, glucose-limited and ammonia-limited continuous culture was conducted.

As shown in Table 2, when *S. bovis* was grown at pH 6.9 under glucose limitation, little glucose (<0.1 mM) was present irrespective of dilution rate. The levels of ammonia were high (23–25 mM), indicating that virtually all the glucose introduced into the fermenters was consumed immediately and the growth rate was dependent on the rate of glucose supply. However, under ammonia limitation, approximately 7 mM glucose remained in the fermenters, indicating that excess glucose was supplied to the cells. A small amount of ammonia (approx. 0.1 mM) remained in the fermenters, but this concentration of ammonia was probably too low for optimal growth. When the ammonia concentration in the reservoir was doubled, the cell density (OD₆₀₀) increased approximately twofold, indicating that the rate of ammonia supply limited the growth rate under ammonia-limiting conditions.

The percentage of lactate produced was greater under ammonia limitation than glucose limitation, at either a D of 0.1 or 0.6 (Table 2), suggesting that excess glucose increased lactate production. This is contrary to the formate production, which was lower under the ammonia limitation than glucose limitation. The Ldh activity per unit cellular nitrogen, which was considered to reflect the amount of Ldh per cell mass, paralleled the amount and percentage of lactate produced. Similarly, Pfl activity paralleled formate production.

When intracellular Ldh increased, Pfl decreased, indicating that the regulation of Ldh synthesis was in the opposite direction to Pfl synthesis. This result may be explained as follows. When intracellular glucose was abundant, glucose must have been fermented to lactate with a small amount of ATP regenerated; under glucose-limited conditions cells needed to obtain a larger amount of ATP by fermenting glucose to acetate, ethanol and formate. Therefore, it is possible that a high ATP concentration or a high value of adenylate energy charge enhances Ldh synthesis and reduces Pfl synthesis. Another explanation may be that the intracellular pool of the intermediate products of glucose fermentation, fructose-1,6-diphosphate and triose phosphates, which affect the activity of Ldh (Asanuma *et al.*, 1997) and Pfl (unpublished observation) respectively, may also affect Ldh and Pfl synthesis.

In both glucose- and ammonia-limited cultures, the percentage of lactate and Ldh activity were increased when D was increased (Table 2). Inversely, the percentage of formate and Pfl activity were decreased as D was increased. This suggested that growth rate *per se*

Table 2. Effect of the abundance of glucose, D and pH on lactate and formate production, Ldh and Pfl activity, and the relative amounts of *ldh* and *pfl* mRNAs in *S. bovis* grown in continuous culture

Data from the last day of the culture, in duplicate, from two sets of cultures ($n = 4$). Figures in parentheses indicate percentages in lactate plus formate. Values within columns with no common superscript letters differ significantly ($P < 0.05$).

Culture	D (h^{-1})	pH	Glucose		Ammonia level (mM)	Lactate produced (mmol l^{-1})	Formate produced (mmol l^{-1})	Ldh activity*	Relative amount of <i>ldh</i> mRNA†	Pfl activity*	Relative amount of <i>pfl</i> mRNA†
			Level (mM)	Consumed (mmol l^{-1})							
Glucose limited	0.1	6.9	<0.1	16.5	23	2.7 ^d (8.8)	28.0 ^a (91.2)	28 ^d	1.00 ^d	9.4 ^a	1.00 ^a
Ammonia limited	0.1	6.9	7.0	20.8	0.1	11.4 ^e (25.2)	27.6 ^a (74.8)	90 ^e	1.60 ^e	5.3 ^b	0.88 ^b
Glucose limited	0.6	6.9	<0.1	16.5	24	12.2 ^c (38.4)	19.6 ^b (61.6)	104 ^c	1.64 ^c	2.7 ^c	0.66 ^c
Ammonia limited	0.6	6.9	7.5	20.3	0.1	20.4 ^b (45.2)	20.0 ^b (54.8)	139 ^b	2.50 ^b	2.5 ^c	0.60 ^c
Glucose limited	0.1	4.5	<0.1	16.5	25	23.0 ^b (73.0)	8.5 ^c (27.0)	250 ^a	3.40 ^a	<1.0 ^d	0.42 ^d
Ammonia limited	0.1	4.5	8.8	18.9	0.1	28.5 ^a (75.4)	9.0 ^c (24.6)	263 ^a	3.68 ^a	<1.0 ^d	0.36 ^d

* $\mu\text{mol NADH oxidized min}^{-1}$ (10 μg cellular nitrogen) $^{-1}$.

† Data were obtained from the bands as indicated in Table 1.

also affected the fermentation pathway downstream of pyruvate. Why Ldh and Pfl synthesis are affected by growth rate is inexplicable at present. The extent of the glucose-excess effect on Ldh synthesis was smaller at a D of 0.6 than 0.1, suggesting that lactate production had already been enhanced at the higher D.

Northern-blot analysis showed that a single 1.0 kb transcript that hybridized with the *ldh* probe was present (data not shown). The relative amounts of *ldh* mRNA are summarized in Table 2. The amount of *ldh* mRNA increased when D, i.e. growth rate, increased at pH 6.9 in both the glucose- and ammonia-limited cultures. At the same D, the level of *ldh* mRNA was higher in the ammonia-limited than the glucose-limited culture. Cells having a larger amount of Ldh also had a larger amount of *ldh* mRNA (Table 2). However, the magnitude of the change in *ldh* mRNA content was smaller than that in Ldh content. It is not surprising that the amount of mRNA was not exactly proportional to the amount of protein translated from it. Possibly, the turnover rate of mRNA is much higher than that of protein, and as a result the protein accumulates in cells. Regulation of Ldh synthesis at the translational level may also be possible, but the present results appeared to indicate that Ldh synthesis was at least in part regulated at the transcriptional level, responding to both growth rate and the abundance of glucose.

Northern-blot analysis with the *pfl* probe showed a single 2.3 kb transcript (data not shown). As shown in Table 2, the amount of *pfl* mRNA decreased when D was increased at pH 6.9 in both glucose- and ammonia-limited cultures. At the same D, the level of *pfl* mRNA tended to be slightly higher in the glucose-limited than the ammonia-limited culture. The Pfl content in the cells was consistent with the result of the Northern-blot analysis.

When glucose-limited continuous culture was performed at pH 4.5, the percentage of lactate and the

content of Ldh greatly increased, compared to the values for pH 6.9 (Table 2). Similar to the observations described above, the ammonia-limited culture did not have further effect on the percentage of lactate and Ldh content.

Northern-blot analysis indicated that the cells grown at pH 4.5 possessed a larger amount of *ldh* mRNA than cells grown at pH 6.9 in both glucose- and ammonia-limited cultures (Table 2). There was little difference in the amount of *ldh* mRNA between the glucose- and ammonia-limited cultures. These results confirmed the observations obtained in batch culture (Asanuma *et al.*, 1997). On the other hand, cells grown at pH 4.5 possessed a smaller amount of *pfl* mRNA than cells grown at pH 6.9 in both glucose- and ammonia-limited cultures (Table 2). *S. bovis* cells grown at pH 4.5 contained a much smaller amount of Pfl than cells grown at pH 6.9 (Table 2), apparently suggesting that Pfl synthesis is suppressed at low pH. These results suggest that *S. bovis* regulates Ldh and Pfl synthesis at the transcriptional level in response to a change in pH, probably internal pH (Russell & Hino, 1985; Russell, 1991).

Collectively, including all the data in Table 2, cells having a larger amount of Ldh and Pfl generally had a larger amount of *ldh* mRNA and *pfl* mRNA, respectively. These results show that the synthesis of Ldh and Pfl is regulated at the transcriptional level in response to growth conditions. In addition to allosteric regulation (see above), transcriptional regulation appears to be important.

As previously described for Ldh (Asanuma *et al.*, 1997), the level of mRNA mainly reflects the rate of transcription, but it is also possible that the concentration of mRNA is affected by the rate of its degradation. The problem as to the life of mRNA remains to be solved. The mechanism of regulation at the transcriptional level, as well as the translational level, also remains to be clarified.

ACKNOWLEDGEMENTS

This work was supported in part by Research Fellowships of the Japan Society for the Promotion of Science for Young Scientists (No. 8156) and Grants-in-Aid for Scientific Research from the Japanese Ministry of Education, Science, Sports and Culture.

REFERENCES

- Arnaud, J., Jorgensen, F., Madsen, S. M., Vrang, A. & Israelsen, H. (1997). Cloning, expression, and characterization of the *Lactococcus lactis* *pfl* gene, encoding pyruvate formate-lyase. *J Bacteriol* 179, 5884–5891.
- Asanuma, N. & Hino, T. (1997). Tolerance to low pH and lactate production in rumen bacteria. *Anim Sci Technol* 68, 367–376.
- Asanuma, N., Iwamoto, M. & Hino, T. (1997). Regulation of lactate dehydrogenase synthesis in a ruminal bacterium, *Streptococcus bovis*. *J Gen Appl Microbiol* 43, 325–331.
- Asanuma, N., Iwamoto, M. & Hino, T. (1998). Formate metabolism by ruminal microorganisms in relation to methanogenesis. *Anim Sci Technol* 69, 576–584.
- Chaney, A. L. & Marbach, E. P. (1962). Modified reagents for the determination of urea and ammonia. *Clin Chem* 8, 130–132.
- Conway, E. J. & Byrne, A. (1933). An absorption apparatus for the micro-determination of certain volatile substances. I. The micro-determination of ammonia. *Biochem J* 27, 419–429.
- Fordyce, A. M., Crow, V. L. & Thomas, T. D. (1984). Regulation of product formation during glucose or lactate limitation in non-growing cells of *Streptococcus lactis*. *Appl Environ Microbiol* 48, 332–337.
- Hino, T. & Hamano, S. (1993). Effects of readily fermentable carbohydrate on fiber digestion by rumen microbes in continuous culture. *Anim Sci Technol* 64, 1070–1078.
- Hino, T. & Miwa, T. (1996). An improved apparatus for the continuous culture of rumen bacteria that require particularly strict anaerobiosis. *Bull Fac Agric Meiji Univ* 107, 59–64.
- Hino, T., Mukunoki, H., Imanishi, K. & Miyazaki, K. (1992). Necessity of ready electron disposal and interspecies hydrogen transfer for the utilization of ethanol by rumen bacteria. *Asian-Australas J Anim Sci* 5, 511–517.
- Hino, T., Sugiyama, M. & Okumura, K. (1993). Maintenance of protozoa and methanogens, and fiber digestion in rumen-simulating continuous culture. *J Gen Appl Microbiol* 39, 35–45.
- Hino, T., Shimada, K. & Maruyama, T. (1994). Substrate preference in a strain of *Megasphaera elsdenii*, a ruminal bacterium, and its implications in propionate production and growth competition. *Appl Environ Microbiol* 60, 1827–1831.
- Hungate, R. E. (1966). *The Rumen and its Microbes*, pp. 60–64. New York: Academic Press.
- Kessler, D. & Knappe, J. (1996). Anaerobic dissimilation of pyruvate. In *Escherichia coli and Salmonella: Cellular and Molecular Biology*, 2nd edn, vol. 1, pp. 199–205. Edited by F. C. Neidhardt and others. Washington, DC: American Society for Microbiology.
- Knappe, J. & Blaschkowski, H. P. (1975). Pyruvate formate-lyase from *Escherichia coli* and its activation system. *Methods Enzymol* 41, 508–518.
- Knappe, J., Neugebauer, F., Blaschkowski, H. P. & Ganzler, M. (1984). Post-translational activation introduces a free radical into pyruvate formate-lyase. *Proc Natl Acad Sci USA* 81, 1332–1335.
- Lindmark, D. G., Paoletta, P. & Wood, N. P. (1969). The pyruvate formate-lyase system of *Streptococcus faecalis*: purification of the formate-pyruvate exchange enzyme. *J Biol Chem* 244, 3606–3612.
- McCormick, N. G., Ordal, E. J. & Whiteley, H. R. (1962a). Degradation of pyruvate by *Micrococcus lactilyticus*. I. General properties of the formate-exchange reaction. *J Bacteriol* 83, 887–898.
- McCormick, N. G., Ordal, E. J. & Whiteley, H. R. (1962b). Degradation of pyruvate by *Micrococcus lactilyticus*. II. Studies of cofactors in the formate-exchange reaction. *J Bacteriol* 83, 899–906.
- Marounek, M. & Bartos, S. (1987). Interactions between rumen amylolytic and lactate-utilizing bacteria in growth on starch. *J Appl Bacteriol* 63, 233–238.
- Parast, C. V., Wong, K. K., Lewisch, S. A. & Kozarich, J. W. (1995). Hydrogen exchange of the glycyl radical of pyruvate formate-lyase is catalyzed by cysteine 419. *Biochemistry* 34, 2393–2399.
- Russell, J. B. (1991). Intracellular pH of acid-tolerant ruminal bacteria. *Appl Environ Microbiol* 57, 3383–3384.
- Russell, J. B. & Hino, T. (1985). Regulation of lactate production in *Streptococcus bovis*; a spiraling effect that contributes to rumen acidosis. *J Dairy Sci* 68, 1712–1721.
- Sawers, G. (1993). Specific transcriptional requirements for positive regulation of the anaerobically inducible *pfl* operon by ArcA and FNR. *Mol Microbiol* 10, 737–747.
- Snedecor, G. W. & Cochran, W. G. (1967). *Statistical Methods*, 5th edn. Ames: Iowa State University Press.
- Thakahashi, S., Abbe, K. & Yamada, T. (1982). Purification of pyruvate formate-lyase from *Streptococcus mutans* and its regulatory properties. *J Bacteriol* 149, 1034–1040.
- Thauer, R. K., Kirschniawy, F. H. & Jungermann, K. A. (1972). Properties and function of the pyruvate formate-lyase reaction in clostridia. *Eur J Biochem* 27, 282–290.
- Thomas, T. D., Turnere, K. W. & Crow, V. L. (1980). Galactose fermentation by *Streptococcus lactis* and *Streptococcus cremoris*: pathways, products, and regulation. *J Bacteriol* 144, 672–682.
- Urking, V., Neugebauer, F. A. & Knappe, J. (1989). The free radical of pyruvate formate-lyase: characterization by EPR spectroscopy and involvement in catalysis as studied with the substrate analogue hypophosphite. *Eur J Biochem* 184, 723–728.
- Vetter, H., Jr & Knappe, J. (1971). Flavodoxin and ferredoxin of *Escherichia coli*. *Hoppe-Seyler's Z Physiol Chem* 352, 433–446.
- Wagner, A., Frey, M., Neugebauer, F., Schafer, W. & Knappe, J. (1992). The free radical in pyruvate formate-lyase is located on glycine-734. *Proc Natl Acad Sci USA* 89, 996–1000.
- Whately, F. R. & Arnon, D. I. (1963). Photosynthetic phosphorylation in plants. *Methods Enzymol* 6, 308–313.
- Wong, K. K., Murray, B. W., Lewisch, S. A., Baxter, M. K., Ridky, T. W., Ulissi-DeMario, L. & Kozarich, J. W. (1993). Molecular properties of pyruvate formate-lyase activating enzyme. *Biochemistry* 32, 14102–14110.
- Wood, N. P. & Jungermann, K. (1972). Inactivation of the pyruvate formate-lyase of *Clostridium butyricum*. *FEBS Lett* 27, 49–52.
- Yamamoto, Y., Sato, Y., Takahashi-Abbe, S., Abbe, K., Yamada, T. & Kizaki, H. (1996). Cloning and sequence analysis of the *pfl* gene encoding pyruvate formate-lyase from *Streptococcus mutans*. *Infect Immun* 64, 385–391.

Received 3 July 1998; revised 8 September 1998; accepted 7 October 1998.