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

Narito Asanuma, Miwa Iwamoto and Tsuneo Hino

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 (Marounek & 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/DDBJ 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 lactate by Ldh or to acetyl-CoA and formate by pyruvate fermentation. In *S. bovis*, formate production in the rumen implies that formate 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 (Tauer et al., 1972; Wood & Jungermann, 1972), *Streptococcus faecalis* (Lindemark 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 O, functions under anaerobic conditions. In *E. coli*, Pfl consisting of two identical 85 kDa subunits is synthesized as the inactive, nonradical form (Ei), and is post-translationally converted to the active, radical form (Ea). 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 Ei to Ea by donating an electron. Ea possesses an organic free radical at the active site, on the a-carbon of Gly (84), 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 Ea to Ei, 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; yeast extract (Difco), 1; cytochrome-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 OD₆₅₀.

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, 30; Trypticase (BBL), 0.5; yeast extract (Difco), 0.5; cytochrome-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 340 nm. 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, 14 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
Pyruvate formate-lyase gene and mRNA of S. bovis

Genomic DNA was isolated from S. bovis as described previously (Asanuma et al., 1997). The DNA was digested and ligated with the enzymes shown in Fig. 1 from Takara Shuzo under conditions recommended by the supplier.

The nucleotide sequence was determined on both strands using an Applied Biosystems PRISM Terminator cycle sequencing kit and an Applied Biosystems 373S sequencer. The sequence data were evaluated on the basis of sequence homology to pfl genes in GenBank using BLASTN and BLASTP (NCBI), and analysed using GENETYX-MAC version 8.0 (Software Development).

PCR amplification. The sequences of the pfl genes from E. coli, Clostridium pasteurianum and S. mutans were used to design degenerate oligonucleotide primers for a PCR reaction. A 17mer oligonucleotide (5' ACHGGATTCCGCAGATGC 3', +549 to +565) and a 21mer oligonucleotide (5' ACHGGGTTTWACWKTGATCAT 3', +1670 to +1690) were synthesized with a DNA synthesizer (model 380A, Applied Biosystems) and a 1142 bp fragment from genomic DNA of S. bovis was amplified. Subsequently, inverse PCR was carried out on EcoRI-digested and religated genomic DNA to clone the upstream region of the pfl gene. Inverse PCR was carried out again with Xbal-digested genomic DNA to clone the downstream region of the pfl gene. PCR amplification was conducted with Takara LA Taq polymerase. The PCR products were purified with a QIAquick PCR purification kit (Qiagen) and directly sequenced.

**Primer extension analysis.** Primer extension analysis was carried out with approximately 10 µg total RNA and a IRD41-labelled primer, pfl-EX (5' CTTTTTCTTTCCAGTCTAGTACCTTTTA 3', from +104 to +129), with MMLV Reverse Transcriptase RNase H Minus (Toyobo). Products of the primer extension reaction were separated on sequencing gels alongside sequence reactions performed on the corresponding PCR product including the upstream region of the pfl gene using pfl-EX. Sequencing reactions were carried out with a SequiTherm Long-Read Cycle Sequencing kit (Aloka) and a Li-cor DNA sequencer (Aloka).

**Northern-blot analysis of ldh and pfl mRNAs.** The cell samples for Northern-blot analysis were centrifuged (20000 g, 10 min, 2°C) and the pellet was immediately frozen by immersing in cold ethanol (-80°C) and stored at -80°C. Total RNA was extracted from S. bovis cells as described previously (Asanuma et al., 1997). Exactly 10 µg RNA was separated in a 1.0% agarose/0.6% formaldehyde gel. Blotting, hybridization, and washing were carried out as described previously (Asanuma et al., 1997). Similarly, the S. bovis pfl-specific probe was designed as shown in Fig. 1. The amounts of ldh and pfl mRNA in 10 µg total RNA were estimated from peak area and intensity by using a Fluor-S Multi Imager (Bio-Rad). To make a standard curve for each mRNA, graded amounts of an identical RNA sample were subjected to Northern-blot analysis. The standard curve of each mRNA was not a straight line but a hyperbolic curve. The relative amounts of each mRNA in the samples were determined from the standard curves.

**Evaluation of data.** Data were evaluated by a Tukey's test at P < 0.05 (Snedecor & Cochran, 1967).

**RESULTS AND DISCUSSION**

**Nucleotide sequence of the S. bovis pfl gene**

A 1142 bp segment of the pfl gene (+549 to +1690) was amplified from genomic DNA by PCR with degenerate primers. This region showed high levels of homology to the pfl genes of other bacteria (BLAST search). To obtain the segment upstream of this region, inverse PCR was performed on EcoRI-digested and religated genomic DNA by using primers on the 5' and 3' ends of the 1142 bp fragment. The inverse PCR product was approximately 2-6 kb long. Sequence analysis showed that this fragment contained the 5' end of the pfl gene. Then, inverse PCR was carried out on Xbal-digested DNA by using two primers designed on the 5' terminal sequence of the pfl gene and the 3' terminal sequence of the 2.6 kb fragment. This inverse PCR product was approximately 3.0 kb and contained the 3' end of the pfl gene. The S. bovis pfl operon was found to consist of 2325 bp, beginning with ATG and terminating with a TAA codon, and encodes a 774 amino acid Pfl protein with a deduced molecular mass of 87513 Da.

The deduced amino acid sequence of S. bovis Pfl showed more than 40% similarity to those of E. coli, C.
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Primer extension analysis revealed that only one tran-

sional 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
transcript.

Fig. 4. The levels of 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 (OD660) 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).

<table>
<thead>
<tr>
<th>Culture</th>
<th>D (h&lt;sup&gt;-1&lt;/sup&gt;)</th>
<th>pH</th>
<th>Glucose</th>
<th>Ammonia level (mM)</th>
<th>Lactate produced (mmol l&lt;sup&gt;-1&lt;/sup&gt;)&lt;sup&gt;†&lt;/sup&gt;</th>
<th>Formate produced (mmol l&lt;sup&gt;-1&lt;/sup&gt;)&lt;sup&gt;†&lt;/sup&gt;</th>
<th>Ldh activity&lt;sup&gt;‡&lt;/sup&gt;</th>
<th>Relative amount of ldh mRNA&lt;sup&gt;‡&lt;/sup&gt;</th>
<th>Pfl activity&lt;sup&gt;‡&lt;/sup&gt;</th>
<th>Relative amount of pfl mRNA&lt;sup&gt;‡&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glucose limited</td>
<td>0.1</td>
<td>6.9</td>
<td>&lt;0.1</td>
<td>16.5</td>
<td>23</td>
<td>28&lt;sup&gt;a&lt;/sup&gt; (91.2)</td>
<td>28&lt;sup&gt;a&lt;/sup&gt;</td>
<td>1.00&lt;sup&gt;‡&lt;/sup&gt;</td>
<td>9.4&lt;sup&gt;‡&lt;/sup&gt;</td>
<td>1.00&lt;sup&gt;‡&lt;/sup&gt;</td>
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<tr>
<td>Ammonia limited</td>
<td>0.1</td>
<td>6.9</td>
<td>7.0</td>
<td>20.8</td>
<td>0.1</td>
<td>114&lt;sup&gt;a&lt;/sup&gt; (25.2)</td>
<td>27&lt;sup&gt;a&lt;/sup&gt;</td>
<td>1.60&lt;sup&gt;‡&lt;/sup&gt;</td>
<td>5.3&lt;sup&gt;‡&lt;/sup&gt;</td>
<td>0.88&lt;sup&gt;‡&lt;/sup&gt;</td>
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<tr>
<td>Glucose limited</td>
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<td>&lt;0.1</td>
<td>16.5</td>
<td>24</td>
<td>12.2&lt;sup&gt;a&lt;/sup&gt; (28.8)</td>
<td>19.0&lt;sup&gt;a&lt;/sup&gt;</td>
<td>1.04&lt;sup&gt;‡&lt;/sup&gt;</td>
<td>2.7&lt;sup&gt;‡&lt;/sup&gt;</td>
<td>0.66&lt;sup&gt;‡&lt;/sup&gt;</td>
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<tr>
<td>Ammonia limited</td>
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<td>6.9</td>
<td>7.3</td>
<td>20.3</td>
<td>0.1</td>
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<td>&lt;1&lt;sup&gt;‡&lt;/sup&gt;</td>
<td>0.36&lt;sup&gt;‡&lt;/sup&gt;</td>
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* αmol NADH oxidized min<sup>-1</sup> (10 µg cellular nitrogen)<sup>-1</sup>.  
† 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.
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