Molecular characterization, enzyme properties and transcriptional regulation of phosphoenolpyruvate carboxykinase and pyruvate kinase in a ruminal bacterium, *Selenomonas ruminantium*

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To elucidate the regulatory mechanism for propionate production in *Selenomonas ruminantium*, the molecular properties and gene expression of phosphoenolpyruvate carboxykinase (Pck) and pyruvate kinase (Pyk) were investigated. The Pck was deduced to consist of 538 aa with a molecular mass of 59.6 kDa, and appeared to exist as a monomer. The Pyk was revealed to consist of four identical subunits consisting of 469 aa with a molecular mass of 51.3 kDa. Both Mg^{2+} and Mn^{2+} were required for the maximal activity of Pck, and Pck utilized ADP, not GDP or IDP, as a substrate. Either Mg^{2+} or Mn^{2+} was required for Pyk activity, and the enzyme was activated by phosphoenolpyruvate (PEP) and fructose 1,6-bisphosphate (FBP). Pyk activity was severely inhibited by P_i , but restored by the addition of FBP. The K_m value of Pck for PEP (0.55 mM) was nearly equal to the K_m value of Pyk for PEP, suggesting that the partition of the flow from PEP in the fermentation pathways is determined by the activity ratio of Pck to Pyk. Both *pck* and *pyk* genes were monocistronic, although two transcriptional start sites were found in *pyk*. The level of *pyk* mRNA was not different whether glucose or lactate was the energy substrate. However, the *pck* mRNA level was 12-fold higher when grown on lactate than on glucose. The level of *pck* mRNA was inversely related to the sufficiency of energy, suggesting that Pck synthesis is regulated at the transcriptional level when energy supply is altered. It was conceivable that the transcription of *pck* in *S. ruminantium* is triggered by PEP and suppressed by ATP.

Keywords: *Selenomonas ruminantium*, phosphoenolpyruvate carboxykinase, pyruvate kinase, propionate

INTRODUCTION

It is important to reduce methanogenesis in the rumen, because methane production brings about an energy loss to the host animal, and in addition, methane is considered to contribute to global warming. In the rumen,

methane is mainly produced from H_2 (Hungate *et al.*, 1970; Asanuma *et al.*, 1998), which is formed by a reductive reaction. In theory, H_2 formation can be reduced by augmenting other reductive reactions through a competition for electrons.

One of the most important reductive reactions in the rumen is propionate formation, and a reciprocal relationship is generally observed between methanogenesis and propionate production. For example, the addition of ionophores brings about a decrease in methane and an increase in propionate (Bergen & Bates, 1984; Van Nevel & Demeyer, 1988), and feeding high-

Abbreviations: Pck, phosphoenolpyruvate carboxykinase; Pyk, pyruvate kinase; PEP, phosphoenolpyruvate; OAA, oxaloacetate; FBP, fructose 1,6-bisphosphate; Ldh, lactate dehydrogenase.

The GenBank accession numbers for the *S. ruminantium* *pck* and *pyk* sequences reported in this paper are AB016600 and AB037182, respectively.

concentrate diets usually decreases methane and increases propionate (Miller, 1995). Accordingly, the augmentation of propionate production can be an effective means to reduce methanogenesis. In addition, propionate is a glycogenic substance, which is important for the nutrition of ruminants.

Propionate is produced via either the succinate pathway or the acrylate pathway, depending on the species of ruminal bacteria. In the known species of ruminal bacteria, *Megasphaera elsdenii* and *Prevotella* species produce propionate from lactate via the acrylate pathway (Marounnek *et al.*, 1989; Stewart *et al.*, 1997). Other bacteria, such as *Selenomonas ruminantium*, *Succinimonas amylolytica*, *Propionibacterium acnes* and *Veillonella parvula*, are known to use the succinate pathway (Hungate, 1966; Wolin *et al.*, 1997). Among these propionate-producing bacteria, *Sel. ruminantium* is one of the most predominant bacteria in the rumen, and has been reported to account for 22–51% of the total viable bacterial counts in the rumen (Caldwell & Bryant, 1966). Therefore, augmentation of propionate production by *Sel. ruminantium* could possibly increase propionate production in the rumen.

In the fermentation of glucose by *S. ruminantium*, phosphoenolpyruvate (PEP) has been reported to be carboxylated to form oxaloacetate (OAA) by PEP carboxykinase (Pck), which can lead to propionate production via the succinate pathway (Melville *et al.*, 1988). PEP is also converted to pyruvate by pyruvate kinase (Pyk), leading to the production of lactate and acetate (Melville *et al.*, 1988). The proportion of propionate in total fermentation products is thus determined by the proportion of the flow from PEP to OAA versus the flow from PEP to pyruvate. Since propionate production is possibly affected by both Pck and Pyk activities, it is important to know how Pck and Pyk activities are regulated, especially in response to growth conditions.

The objective of this study is to clarify how the synthesis of Pck and Pyk in *S. ruminantium* is regulated, especially at the transcriptional level. For this purpose, we analysed the genes encoding Pck (*pck*) and Pyk (*pyk*) with primer extension analysis. Then we examined the effect of the alteration of the energy substrate on the levels of *pck* and *pyk* mRNAs. In addition, we examined the molecular and enzyme properties of Pck and Pyk.

METHODS

Bacterial strain and growth conditions. *S. ruminantium* TH1 was isolated in this laboratory (Asanuma *et al.*, 1998) and was grown in batch culture in 120 ml serum vials as described previously (Asanuma & Hino, 1997). The media contained (l^{-1}): K_2HPO_4 , 0.45 g; KH_2PO_4 , 0.45 g; $(NH_4)_2SO_4$, 0.9 g; NaCl, 0.9 g; $CaCl_2 \cdot 2H_2O$, 0.12 g; $MgSO_4 \cdot 7H_2O$, 0.19 g; Trypticase (BBL Microbiology Systems), 1.0 g; cysteine-HCl, 0.6 g; vitamin solution (Tiwari *et al.*, 1969), 10 ml; VFA solution (Tiwari *et al.*, 1969), 1.0 ml; and either 15 mM glucose, 75 mM lactate, or 30 mM fumarate. Culture incubation was performed in triplicate, maintaining the pH at

6.8–7.0, until late-log phase. Cell growth was estimated by measuring the OD_{600} . *E. coli* HB101 for preparing fusion protein was purchased from Toyobo and aerobically grown in LB.

Assay for the activities of Pck and Pyk. Cell extracts of *S. ruminantium* were prepared as described previously (Asanuma & Hino, 1997). Pck activity was assayed in both the forward and reverse directions by the method of Schocke & Weimer (1997). In PEP carboxylation, the reaction mixture contained 8 mM PEP, 4 mM $MgCl_2$, 0.02 mM $MnCl_2$, 25 mM $NaHCO_3$, 1 mM ADP, 0.3 mM NADH and 2 U malate dehydrogenase (from yeast; Oriental Yeast) in 50 mM sodium borate/succinate buffer (pH 7.0). The reaction was initiated by adding cell extracts [$10\text{--}20\text{ }\mu\text{g}$ protein (ml assay mixture) $^{-1}$] and incubation was carried out at room temperature for 5 min under a stream of O_2 -free CO_2 . The rate of NADH oxidation was measured as A_{340} . The effect of pH on enzyme activity was examined by increasing malate dehydrogenase to 6 U, which compensated for a drop in activity at extremes of pH. The rate of OAA decarboxylation was also measured. The reaction mixture contained 8 mM OAA, 4 mM $MgCl_2$, 0.02 mM $MnCl_2$, 25 mM $NaHCO_3$, 5 mM ATP, 5 U each pig heart Pyk and lactate dehydrogenase (Ldh) (Oriental Yeast), 1 mM ADP, 0.3 mM NADH, and cell extracts [$10\text{--}20\text{ }\mu\text{g}$ protein (ml assay mixture) $^{-1}$] in 50 mM HEPES (pH 7.0).

Pyk activity was assayed by the procedure described by Collins & Thomas (1974) in the PEP to pyruvate direction. The reaction mixture contained 8 mM $MgCl_2$, 80 mM KCl, 5 mM PEP, 5 mM ADP, 2 mM fructose 1,6-bisphosphate (FBP), 10 U Ldh, 0.2 mM NADH and cell extracts [$1\text{--}2\text{ }\mu\text{g}$ protein (ml assay mixture) $^{-1}$] in 50 mM Tris/HCl (pH 7.0). To examine the effect of pH on enzyme activity, Ldh was increased to 20 U. In the reverse reaction, which was used to determine K_m , the reaction mixture contained 150 mM $MgCl_2$, 25 mM pyruvate, 375 mM $NaHCO_3$, 300 mM ATP, 5 U malate dehydrogenase and PEP carboxylase (from *Escherichia coli*; Sigma), 0.3 mM NADH and cell extracts [$5\text{--}6\text{ }\mu\text{g}$ protein (ml assay mixture) $^{-1}$] in 75 mM sodium borate/succinate buffer (pH 7.0).

Cellular nitrogen (N) was determined by the Kjeldahl method, as described previously (Asanuma & Hino, 1997). Enzyme activity was expressed as μmol NADH oxidized min^{-1} (μg cellular N) $^{-1}$. The activity values represent enzyme activity per dry cell weight, because in each case approximately 95% of cells were disrupted by ultrasonication, and cellular N determined by the Kjeldahl method was parallel to dry cell weight. Since the rates of NADH oxidation in the absence of one of the substrates and auxiliary enzymes were extremely low, NADH oxidase activity was considered to be negligible.

Purification of Pck and Pyk. Pck and Pyk were purified by column chromatography with DEAE-Sephacrose CL-6B ($2.5 \times 50\text{ cm}$ column), Sephacryl S-200 HR ($2.5 \times 70\text{ cm}$), Resource Q (6 ml), Mono Q (1 ml) and Superdex 200 HR 10/30 ($1.0 \times 30\text{ cm}$). All the gels were purchased from Amersham Pharmacia Biotech and procedures for chromatography were described previously (Asanuma *et al.*, 1997). The purified Pck and Pyk were analysed by SDS-PAGE (Laemmli, 1970) to estimate the molecular masses of the enzymes and subunits.

Extraction of genomic DNA and sequencing procedure. Unless otherwise stated, the handling of DNA was carried out by the standard procedures described by Sambrook *et al.* (1989). Nucleotide sequence was determined by using a Big Dye Terminator Sequencing Kit (PE Applied Biosystems) and

an ABI PRISM 310 sequencer (PE Applied Biosystems). The sequence data were evaluated as described previously (Asanuma *et al.*, 1999).

PCR amplification. Based on the sequences of the genes encoding Pck (*pck*) from *E. coli*, *Anaerobiospirillum succiniciproducens* and *Haemophilus influenzae*, oligonucleotide primers for PCR were designed, and two primers, *pck*-1 (5'-⁺²⁴¹ACCGGCCGTTCTCCTAA⁺²⁵⁷-3') and *pck*-2 (5'-⁺¹²⁹⁶ACCGAAGCAAGCGGAGAAGGT⁺¹²⁷⁶-3'), were prepared commercially (Especc Oligo Service). The PCR product from genomic DNA of *S. ruminantium* was a 1056 bp fragment, which was highly homologous to *pck* of other bacteria (BLAST search). Subsequently, inverse PCR (Howard *et al.*, 1988; Triglieri *et al.*, 1988) was carried out on *Pst*I-digested and religated genomic DNA to sequence the regions upstream and downstream of the 1056 bp *pck* fragment. Sequence analysis showed that the inverse PCR product included the 5' and 3' ends of *pck*.

The nucleotide sequence of the *S. ruminantium* Pyk gene (*pyk*) was determined by the procedure described above. The oligonucleotide primers to amplify *pyk*, *pyk*-1 (5'-⁺²⁹²GAT-ACHAAAGGTCCDGAA⁺³⁰⁹-3') and *pyk*-2 (5'-⁺⁸²¹CCRCG-VGCWACCATRATGCC⁺⁸⁰²-3'), were designed from the *pyk* sequences of *E. coli*, *Salmonella typhimurium* and *Bacillus subtilis*. Inverse PCR was carried out on *Eco*RI-digested and religated genomic DNA. The nucleotide sequences of *Sel. ruminantium pck* and *pyk* were registered in the GenBank nucleotide sequence database with accession numbers AB016600 and AB037182, respectively.

Primer extension analysis. This was carried out with IRD41-labelled primers, 5'-⁺³⁹⁰CAGTTCCTTTTATAGCGATTTC-TTTC⁺³⁶⁵-3' for *pck* and 5'-⁺³⁴⁴ATAACTTTGCCATCT-TTGAACTCGC⁺³²⁰-3' for *pyk*, with a Li-Cor DNA sequencer (Aloka) as described previously (Asanuma *et al.*, 1999).

Northern blot analysis of *pck* and *pyk* mRNAs. Northern blot analysis was performed as described previously (Asanuma *et al.*, 1997). The probe specific to *S. ruminantium pck* mRNA was the 1056 bp fragment which had been amplified with *pck*-1 and *pck*-2. The PCR product prepared with *pyk*-1 and *pyk*-2 was used as a probe for *pyk* mRNA. The amounts of *pck* and *pyk* mRNAs in 10 µg total RNA were estimated from the peak area and intensity by using a Fluor-S Multi Imager (Bio-Rad). To make a standard curve of each mRNA, graded amounts of an identical RNA sample were similarly subjected to Northern blot analysis. The relative amounts of each mRNA in samples were determined from the standard curves.

Determination of the degradation rate of mRNA. Rifampicin (100 µg ml⁻¹) was added to cultures at the mid-log growth stage and cells were harvested every 5 min after the addition. The degradation rates of *pck* and *pyk* mRNAs were estimated from the slopes of plots obtained by Northern blot analysis.

Preparation of antiserum against Pck and Western-blot analysis. The *pck* gene was cloned in plasmid pGEX-4T-3, to express a GST fusion protein (Amersham Pharmacia Biotech), and this plasmid was transformed into *E. coli* HB101. The *E. coli* harbouring the recombinant plasmid overexpressed recombinant protein. The recombinant protein was purified with Glutathione-Sepharose 4B (Amersham Pharmacia Biotech) and a part of glutathione-S-transferase was cut off with thrombin (Amersham Pharmacia Biotech). Polyclonal antibody against Pck was prepared in a rabbit, and Western blot analysis was carried out with the antibody. All the procedures were as described previously (Asanuma & Hino,

2000). The polyclonal antibody against Pck was the primary antibody, alkaline phosphatase-conjugated anti-rabbit goat IgG (Bio-Rad) was the secondary antibody and 5-bromo-4-chloro-3-indolyl phosphate and nitro blue tetrazolium were the substrates to visualize Pck. The amounts of Pck were estimated with a Fluor-S Multi Imager (Bio-Rad) as described above.

Determination of PEP, pyruvate, adenine nucleotides and fermentation products. To determine the concentrations of intracellular PEP, pyruvate and adenine nucleotides, cultures were immediately frozen in liquid nitrogen, and 1% (v/v) perchloric acid was added to the frozen samples. After being thawed on ice, the samples were centrifuged (18000 g, 5 min, 4 °C), and the supernatant was neutralized with 50% (w/v) K₂CO₃. After removal of the precipitate by centrifugation, the supernatant was concentrated to one-tenth volume with a centrifuge evaporator. The concentrated samples were analysed for PEP and pyruvate by the enzymic methods described by Garrigues *et al.* (1997). ATP was assayed by measuring the light output from a luciferin/luciferase mix (Micro Tech Nichion) as recommended by the manufacturer. ADP and AMP were assayed by converting to ATP with Pyk and adenylate kinase plus Pyk, respectively, according to Kimmich *et al.* (1975). Organic acids produced by *S. ruminantium* were analysed by HPLC as described previously (Hino *et al.*, 1991).

Evaluation of data. Data were analysed by Tukey's test or Student's *t*-test using the SigmaStat Statistical Analysis System (Jandel Scientific).

RESULTS

Characterization of *pck* and *pyk*

The *pck* operon of *S. ruminantium* was found to consist of 1620 bp, beginning with ATG and terminating with a TAA codon. *pck* was deduced to encode a 538 aa protein with a molecular mass of 59573 Da. Primer extension analysis revealed that only one transcriptional start site exists 73 bp upstream from the *pck* start codon (Fig. 1a).

A putative ribosome-binding site, the Shine-Dalgarno sequence (⁺⁵⁴GAAAGA⁺⁵⁹), was found 19 bp upstream from the ATG initiation codon. The -35 and -10 promoter regions (⁻³⁵TTGACA⁻³⁰ and ⁻¹²TAATAT⁻⁷) were also present. An inverted repeat sequence characteristic of transcriptional terminators was detected between +1706 and +1739, being situated 14 bp downstream of the termination codon. A free-energy change of -10.0 kcal mol⁻¹ in this region of the corresponding mRNA suggested a stem-loop structure. These results suggest that *S. ruminantium pck* is monocistronic.

Analysis of *pyk* indicated that the operon consists of 1413 bp, beginning with ATG and terminating with TAA. The molecular mass of Pyk was deduced to be 51285 Da. Two unidentified ORFs, 789 bp and 1101 bp, were located 464 bp and 1241 bp, respectively, downstream of the *pyk* operon. Two products were found by primer extension analysis (Fig. 1b), which indicates that transcription starts from two sites, 93 bp and 120 bp upstream of the *pyk* ATG start codon. The extension

Fig. 1. Primer extension analysis of *pck* (a) and *pyk* (b). Sequence ladders were run with the same primer, and are shown on the left. Transcriptional start sites are indicated by arrows.

product with higher intensity was shown to end at G⁺¹ on the coding strand, while the other product ended at T⁻²⁷.

A possible ribosome-binding site, the Shine–Dalgarno sequence (⁺⁷¹AAAGA⁺⁷⁵), was identified at a position 19 bp upstream from the ATG initiation codon of the *pyk* gene. In addition, the potential ⁻³⁵(⁻²⁹CTGATG⁻²⁴ and ⁻⁶³TTTACT⁻⁵⁸) and ⁻¹⁰(⁻⁸TTAAAT⁻³ and ⁻⁴⁰TAGAAT⁻³⁵) regions, corresponding to each transcriptional start site, were found. An inverted repeat terminator sequence was situated 59 bp downstream from the termination codon of *pyk* (between +1565 and +1595). A stem–loop structure was inferred in this region of the *pyk* mRNA, strongly suggesting that *pyk* is monocistronic.

Molecular mass and the quaternary structure of Pck and Pyk

The molecular mass of purified Pck was estimated to be ~60 kDa by gel filtration (data not shown). SDS-PAGE of the protein gave a single band at ~60 kDa, which agreed with the value deduced from the amino acid sequence. These results indicate that *S. ruminantium* Pck is a monomer. Purified Pyk was approximately 200 kDa, as estimated by gel filtration. Only one peak with Pyk activity was always shown in all the steps of column chromatography, which suggests that *S. ruminantium* has only one type of Pyk. The SDS-PAGE of the Pyk fraction after gel filtration gave a single band at ~50 kDa, suggesting that Pyk is a tetramer. Based on its gene structure, *S. ruminantium* Pyk was concluded to consist of four identical subunits.

Properties of Pck and Pyk

No Pck activity was detected in the absence of Mg²⁺ or Mn²⁺ (data not shown), suggesting that *S. ruminantium* Pck is a typical Pck that requires a divalent transition metal ion (Utter & Kolenbrander, 1972; Cannata & de Flombaum, 1974). Similar to *A. succiniciproducens* Pck (Laivenieks *et al.*, 1997), both Mg²⁺ and Mn²⁺ were required for maximal activity. The requirement for Mg²⁺ and Mn²⁺ is consistent with the fact that *S. ruminantium* Pck has two metal-binding sites at Gly²⁴²–Thr²⁴⁹ and Leu²⁵⁹–Asp²⁶³ (Matte *et al.*, 1996; Tari *et al.*, 1996). Pck had a pH optimum of 7.0, with half-maximal activity being observed at pH 6.0 and 8.5 (data not shown).

From the Lineweaver–Burk plot of the Pck reaction, *K_m* values were estimated to be 0.55 mM for PEP and 0.46 mM for ADP (data not shown). When GDP and IDP were substituted for ADP, Pck activity was not detected. The specific requirement for ADP is typical of bacterial Pck enzymes (Teraoka *et al.*, 1970; Samuelov *et al.*, 1991). In the decarboxylation of OAA, the *K_m* values for OAA and ATP were 0.81 and 0.92 mM, respectively.

S. ruminantium Pyk required either Mg²⁺ or Mn²⁺, and was activated by 5 mM K⁺ but not by 100 mM Na⁺ or 100 mM NH₄⁺ (data not shown). The optimal pH of Pyk was 7.0 and a change in pH had a smaller effect on Pyk activity than on Pck activity. In the reaction from PEP to pyruvate, Pyk was homotropically activated by the substrate. The *K_{0.5}* (*K_m* for an allosteric enzyme) values for PEP and ADP were 0.11 and 0.24 mM, respectively, whereas the values for pyruvate and ATP in the reverse reaction were 0.48 and 0.35 mM, respectively.

Table 1. Effect of P_i and FBP on the activities of Pyk and Pck

Effector concn (mM)		Enzyme activity*	
P_i	FBP	Pyk	Pck
0	0	55 ± 4.1^d	61.4 ± 5.2
0	0.5	117 ± 7.5^c	
0	1.5	255 ± 22^a	61.5 ± 4.6
0.1	0	28 ± 2.2^e	
0.1	0.5	135 ± 11^b	
0.1	1.5	262 ± 24^a	60.4 ± 5.1
30	0	0 ± 0.1^f	
30	5	48 ± 3.7^d	
30	15	166 ± 12^b	
30	30	268 ± 22^a	
30	45	275 ± 25^a	59.6 ± 4.9

* $\mu\text{mol NADH oxidized min}^{-1}$ ($10 \mu\text{g cellular N}$) $^{-1}$. Means $\pm$ SE are shown. Different superscript letters indicate significant difference ($P < 0.01$, $n = 3$).

Pyk was markedly activated by FBP and the maximal activity was observed at 1.5 mM FBP (Table 1). Pyk activity was inhibited even by 0.1 mM P_i , but the activity was restored by 1.5 mM FBP. In the presence of 30 mM P_i , FBP acted in a dose-response manner and an FBP level higher than the level of P_i was needed for complete restoration of activity. Glucose 6-phosphate, which activates Pyk in *E. coli* (Waygood *et al.*, 1975) and *Streptococcus mutans* (Abbe & Yamada, 1982), had no effect on Pyk activity (data not shown). Neither FBP nor P_i affected Pck activity (Table 1).

Transcription of *pck* and *pyk*

The Pck activity per cellular N when cells were grown on lactate was 11-fold higher than the activity when grown on glucose (Table 2); a similar result was obtained by measuring the amount of Pck protein (12-

Fig. 2. Levels of Pck protein (a) and *pck* mRNA (b) in cells grown on glucose (lanes 1) or lactate (lanes 2). Arrows indicate Pck protein (a) and 1.6 kb *pck* mRNA (b). **Fig. 3.** Level of *pyk* mRNA in cells grown on glucose (lane 1) or lactate (lane 2). The arrow indicates 1.4 kb *pyk* mRNA.

fold higher) by Western blotting (Fig. 2a, Table 2). The level of *pck* mRNA in lactate-grown cells was also 12-fold higher than that in glucose-grown cells (Fig. 2b,

Table 2. Levels of intracellular Pck and Pyk, and *pck* and *pyk* mRNAs, and fermentation products in *S. ruminantium* grown on glucose or lactate

Energy substrate	Activity*		Amount of Pck protein†	Relative level mRNA†		Fermentation products‡			
	Pck	Pyk		mRNA†		Propionate	Succinate	Lactate	Acetate
				<i>pck</i>	<i>pyk</i>				
Glucose	5.6	236	1.0	1.0	1.0	27.7	11.9	151.1	19.2
Lactate	62.9§	250	12.3§	11.6§	1.2	121.9§	8.9§	0.0§	78.2§

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

† The bands shown in Figs 2 and 3 were quantified by using a Fluor-S Multi Imager. Values for glucose-grown cells are expressed as 1.0, and relative values are shown.

‡ mol (100 mol glucose or 200 mol lactate fermented) $^{-1}$.

§ $P < 0.01$ ($n = 3$).

Fig. 4. Changes in the levels of *pck* mRNA (a) and Pck protein (b) by the addition of glucose (▲) or fumarate (■) to cultures growing on lactate (●). The initial values (zero time) are expressed as 100%. Bars indicate SE ($n = 3$).

Fig. 5. Changes in the levels of ATP (a), PEP (b, black symbols), and pyruvate (b, white symbols) by the addition of glucose (▲, △) or fumarate (■, □) to cultures growing on lactate (●, ○). The initial values (zero time) are expressed as 100%. Bars indicate SE ($n = 3$).

Table 2). Since the decay rate of *pck* mRNA was not affected by the energy substrate (data not shown), Pck synthesis is considered to be regulated at the transcriptional level. On the other hand, neither Pyk activity per cellular N nor the level of *pyk* mRNA was affected by the energy substrate (Fig. 3, Table 2).

Glucose was mainly fermented to lactate, and propionate production was much greater from lactate than from glucose (Table 2). This result was substantially compatible with the data on enzyme activity, although the difference in propionate plus succinate was smaller than the difference in enzyme activity.

In Northern blot analysis, only one band was detected that hybridized with a *pck*-specific probe (Fig. 2b), which agreed with the result of primer extension analysis. These results indicate that *S. ruminantium pck* is transcribed in a monocistronic fashion. Similarly, the transcription of *S. ruminantium pyk* was shown to be monocistronic (Fig. 3).

Effect of energy substrates on the levels of *pck* and *pyk* mRNAs

S. ruminantium was grown with lactate until the mid-log stage of growth and then incubated for an additional 20 min after supplementing with 10 mM glucose or fumarate. Addition of glucose and fumarate in the presence of lactate reduced the level of *pck* mRNA to 11% and 56%, respectively, of the initial value (Fig. 4a). A similar trend was observed in the amount of Pck protein per cellular N, although the changes were smaller (Fig. 4b). These results confirm that Pck synthesis is regulated at the transcriptional level. However, the level of *pyk* mRNA was not affected by supplementing with glucose or fumarate (data not shown).

The concentration of intracellular ATP was raised by supplementing with glucose, and to a lesser extent, with fumarate (Fig. 5a). The levels of ADP and AMP were inversely related to the level of ATP, although the changes were smaller compared to ATP (data not

shown). Intracellular PEP and pyruvate were increased by the addition of glucose, but not by fumarate (Fig. 5b). These results suggest that fumarate was mainly metabolized to succinate, which coupled with ATP regeneration via electron transport phosphorylation, and little PEP and pyruvate were produced from fumarate. When *S. ruminantium* was grown with fumarate and H_2 , neither PEP nor pyruvate was detected (data not shown). Under this condition, intracellular ATP was below $0.1 \mu\text{mol (g cellular N)}^{-1}$ and neither *pck* mRNA nor Pck protein was detected.

DISCUSSION

Structures of Pck, Pyk, *pck* and *pyk*

S. ruminantium Pck was shown to possess putative functional residues, such as the sites to bind to ATP, Mg^{2+} , Mn^{2+} and PEP, which have been reported for ATP/ADP-dependent Pck in other bacteria (Matte *et al.*, 1996; Tari *et al.*, 1996). The amino acid sequence of *S. ruminantium* Pck was highly homologous to the sequences of *E. coli* (Medina *et al.*, 1990) and *A. succiniciproducens* Pcks (Laivenieks *et al.*, 1997), suggesting that the tertiary structure is also similar.

Pyk has been reported to be a homotropic enzyme in *S. ruminantium* and other bacteria (Kapoor & Venkatasubramanian, 1981; Garcia-Olalla & Garrido-Pertierra, 1987; Melville *et al.*, 1988). *E. coli* is known to have two types of Pyk (Waygood *et al.*, 1975, 1976; Mattevi *et al.*, 1996): one type is activated by FBP, and the other type is activated by AMP and sugar monophosphates. However, *S. ruminantium* was shown to possess only one type, which was activated by FBP.

Based on the primary structure of Pyk, bacterial Pyk enzymes can be divided into two groups (Sakai & Ohta, 1993): one group includes *Bacillus* spp. (Sakai & Ohta, 1993; Tanaka *et al.*, 1995), which has an extra C-terminal sequence, and the other group includes *E. coli* (GenBank accession numbers M24636 and M63703) and *Sal. typhimurium* (X99945), which do not contain such a sequence. *Sel. ruminantium* Pyk was found to belong to the latter group.

Two transcriptional start sites were identified in *S. ruminantium pyk*, which were close to each other (Fig. 1b). However, in Northern-blot analysis, only one 1.4 kb transcript hybridizing with a *pyk* probe was observed (Fig. 3). This discrepancy is probably due to the small difference in the length of the two transcripts (26 bp), which made it technically difficult to separate them by Northern blotting. Possibly, *S. ruminantium* Pyk is transcribed from the two sites, suggesting that this gene is efficiently transcribed. In *Lactobacillus delbrueckii* subsp. *bulgaricus* (Branny *et al.*, 1993), *Lactococcus lactis* (Llanos *et al.*, 1993), *Bacillus psychrophilus* and *Bacillus licheniformis* (Tanaka *et al.*, 1995), the gene encoding phosphofructokinase (*pfk*) exists adjacent to *pyk*, forming one operon. However, *pfk* was not found in the region adjacent to *pyk* in *S. ruminantium*, supporting the conclusion that *S. ruminantium pyk* is

monocistronic. In *Zymomonas mobilis*, *pyk* is a monocistronic gene (Steiner *et al.*, 1998).

Properties of Pck and Pyk

For the maximal activity of *S. ruminantium* Pyk, FBP was required at a level higher than 1.5 mM (Table 2). In addition, the level of FBP needed to be higher than the level of P_i , because P_i severely inhibited the activity. Since the physiological P_i concentration is possibly 20–40 mM (Bond & Russell, 1998), intracellular Pyk may be present in an inactive state in the absence of FBP. It is conceivable that the Pyk reaction is highly dependent on the concentration of intracellular FBP. Probably, Pyk never acts at the maximal activity in *S. ruminantium* because the intracellular level of FBP is unlikely to rise to the level of P_i (Garrigues *et al.*, 1997; Bond & Russell, 1998). The optimal pH of *S. ruminantium* Pck and Pyk was 7.0, and the effect of low pH on the activities of these enzymes was not great, which is consistent with the observation that fermentation pattern was not greatly affected by culture pH (data not shown).

Melville *et al.* (1988) reported that the Pck reaction exhibited a sigmoidal saturation curve, as measured with PEP as the substrate, and the $K_{0.5}$ value for PEP was 5.5 mM. In our experiments, however, Pck was not homotropically activated by PEP and the K_m value for PEP was 0.55 mM. This discrepancy is inexplicable at present, but the difference in K_m or $K_{0.5}$ may be explained by the difference in assay conditions: Melville *et al.* (1988) did not add CO_2 and Mn^{2+} to the reaction mixture, which possibly resulted in a much higher value. In other bacteria, e.g. *E. coli* (Krebs & Bridger, 1980), *A. succiniciproducens* (Laivenieks *et al.*, 1997) and *Ruminococcus flavefaciens* (Schocke & Weimer, 1997), homotropic activation of Pck was not observed, and the K_m values were rather close to our value. On the other hand, *S. ruminantium* Pyk was shown to be activated in a homotropic fashion, which agreed with the result of Melville *et al.* (1988). The $K_{0.5}$ value for PEP was 0.11 mM, which is comparable to the value (0.086 mM) reported by Melville *et al.* (1988).

The K_m value for PEP in the Pck reaction was not greatly different from the value for OAA in the reverse reaction, and the K_m value for ADP was half the value for ATP. Accordingly, the Pck reaction in *S. ruminantium* is possibly reversible, and Pck may be used for gluconeogenesis. However, the Pck is probably used for ATP formation, when *S. ruminantium* is actively growing.

In the Pyk reaction, the $K_{0.5}$ value for pyruvate was 4.4-fold higher than the value for PEP and the value for ATP was 1.5-fold higher than the value for ADP, which suggests that the reaction from PEP to pyruvate is favoured. The equilibrium of the reaction is probably far towards pyruvate, but the reverse reaction may be possible *in vivo*. Melville *et al.* (1988) suggested the existence of pyruvate carboxylase in *S. ruminantium*, but we could not demonstrate the activity of this enzyme by the same assay method. We presume at present that PEP can be formed from lactate by Ldh and Pyk.

Fig. 6. A presumptive mechanism for the regulation of fermentation pathways in *S. ruminantium*. ⊕ and ⊖ indicate the stimulation and inhibition of transcription, respectively. (+) and (−) indicate the activation and inhibition of enzyme activity, respectively.

Transcription of *pck* and *pyk*

When *S. ruminantium* was grown on glucose, Pyk activity was 40-fold higher than Pck activity, whereas the sum of the amounts of lactate and acetate was fourfold higher than the sum of the amounts of succinate and propionate (Table 2). This is probably because Pyk does not act at its maximal activity, as described above. Since K_m values were not greatly different between Pck and Pyk, the enhancement of the activity or amount of Pck possibly augments propionate production.

The level of *pck* mRNA in glucose-grown cells was much lower than that in lactate-grown cells (Table 2). The level was greatly lowered by supplementing with glucose in the presence of lactate, and to a lesser extent, by supplementing with fumarate (Fig. 4a). There was an inverse relationship between *pck* mRNA and the ATP level (Fig. 5a), and a positive correlation between *pck* mRNA and ADP, as well as between *pck* mRNA and AMP, was observed. Melville *et al.* (1988) observed in the continuous culture of *S. ruminantium* that Pck activity was detected at a dilution rate (D) of 0.1 h^{-1} , but not detected at a D of 0.6 h^{-1} . Hobson & Summers (1972) reported that the ATP pool in *S. ruminantium* increased with an increase in D . These results may suggest that the transcription of *S. ruminantium pck* is suppressed by ATP, or stimulated by ADP or AMP. When cells are deficient in ATP, the fermentation pathway is possibly regulated to increase the flow from PEP to propionate so that more ATP is regenerated.

In *S. ruminantium* grown with fumarate and H_2 , neither *pck* mRNA nor Pck protein was detected despite a low level of ATP and high levels of ADP and AMP. Since neither PEP nor pyruvate was detected under this condition, it could be presumed that PEP or pyruvate is required for the onset of *pck* transcription. As an activator, PEP may be a more likely candidate, because

it is a substrate of Pck and a phosphorylated compound. On the other hand, the level of intracellular PEP was enhanced by the addition of glucose (Fig. 5b), but the level of *pck* mRNA declined (Fig. 4a). This result may indicate that ATP suppresses *pck* transcription, and that the suppression is not relieved even by the high level of PEP.

Collectively, the fermentation pathway of glucose branches out at PEP to pyruvate by Pyk and to OAA by Pck, leading to propionate production (Fig. 6). The amount of propionate is proportional to the activity ratio of Pyk and Pck. The *pyk* gene is always transcribed, and Pyk is activated by FBP and PEP. FBP alleviates the inhibitory effect of P_i . *S. ruminantium* regulates Pck synthesis at the transcriptional level, possibly responding to the degree of energy supply. Presumably, ATP and PEP act as a suppressor and an activator, respectively, in the transcription of *pck*, although this presumption needs to be verified.

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