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Molecular Characterization of CcpA and Involvement of This Protein in Transcriptional Regulation of Lactate Dehydrogenase and Pyruvate Formate-Lyase in the Ruminal Bacterium *Streptococcus bovis*

Narito Asanuma,* Takahiro Yoshii, and Tsuneo Hino

Department of Life Science, College of Agriculture, Meiji University, Higashimita, Tama-ku, Kawasaki, Japan

Received 29 December 2003/Accepted 5 May 2004

A *ccpA* gene that encodes global catabolite control protein A (CcpA) in *Streptococcus bovis* was identified and characterized, and the involvement of CcpA in transcriptional control of a gene (*ldh*) encoding lactate dehydrogenase (LDH) and a gene (*pfl*) encoding pyruvate formate-lyase (PFL) was examined. The *ccpA* gene was shown to be transcribed as a monocistronic operon. A catabolite-responsive element (*cre*) was found in the promoter region of *ccpA*, suggesting that *ccpA* transcription in *S. bovis* is autogenously regulated. CcpA required HPr that was phosphorylated at the serine residue at position 46 (HPr-[Ser-P]) for binding to the *cre* site, but glucose 6-phosphate, fructose 1,6-bisphosphate, and NADP had no effect on binding. Diauxic growth was observed when *S. bovis* was grown in a medium containing glucose and lactose, but it disappeared when *ccpA* was disrupted, which indicates that CcpA is involved in catabolite repression in *S. bovis*. The level of *ccpA* mRNA was higher when cells were grown on glucose than when they were grown on lactose, which was in line with the level of *ldh* mRNA. When cells were grown on glucose, the *ldh* mRNA level was lower but the *pfl* mRNA level was higher in a *ccpA*-disrupted mutant than in the parent strain, which suggests that *ldh* transcription is enhanced and *pfl* transcription is suppressed by CcpA. The *ccpA*-disrupted mutant produced less lactate and more formate than the parent, probably because the mutant had reduced LDH activity and elevated PFL activity. In the upper region of both *ldh* and *pfl*, a *cre*-like sequence was found, suggesting that the complex consisting of CcpA and HPr-[Ser-P] binds to the possible *cre* sites. Thus, CcpA appears to be involved in the global regulation of sugar utilization in *S. bovis*.

Streptococcus bovis, an important amylolytic and lactate-producing bacterium, is one of the predominant bacteria in the rumen when ruminants are fed high-concentrate diets (18, 27). Rapid fermentation of starch often leads to an increase in ruminal lactate production, which causes a drop in the ruminal pH. *S. bovis* is relatively acid tolerant among ruminal bacteria (36) and produces higher percentages of lactate when the culture pH is low (37). Thus, *S. bovis* is likely to contribute to the progress of lactic rumen acidosis (37). Therefore, it is desirable to suppress the overproduction of lactate by *S. bovis* when high-concentrate diets are used.

On the other hand, lactate usually undergoes secondary fermentation in the rumen, and propionate is generally a major product (38). Propionate is important for ruminant nutrition as a glycogenic substance. Propionate is formed by accepting electrons, which generally leads to a reduction in ruminal methanogenesis. This is particularly important, because methanogenesis represents an energy loss for ruminants and, in addition, methane contributes to the greenhouse effect. Furthermore, lactate serves as an electron donor for sequential nitrate reduction to ammonia (20, 21), which also leads to a decrease in ruminal methanogenesis (19, 20). In addition, stimulation of nitrite reduction by lactate may alleviate toxic effects of nitrite (20, 21). Hence, it may be beneficial to modulate lactate production so that lactate is always present at appropriate levels in the rumen.

Lactate production by *S. bovis* is regulated by the ratio of lactate dehydrogenase (LDH) activity to pyruvate formate-lyase (PFL) activity (2, 7). The activities of LDH and PFL are regulated by allosteric effectors, such as fructose 1,6-bisphosphate (FBP), phosphoenolpyruvate, and triose phosphates, and also by the amounts of enzyme proteins (1, 2, 3). The synthesis of both enzymes is regulated at the transcriptional level (6, 7). *S. bovis* increases lactate production by increasing LDH synthesis and simultaneously decreases formate production by decreasing PFL synthesis in response to a drop in the pH and an excess energy supply (2, 6, 7). In addition, PFL activity is posttranslationally regulated by PFL activase and PFL deactivase (4). In *S. bovis*, PFL activase is usually present at a level that is high enough to activate PFL (4). However, since the amount of LDH is much larger than the amount of PFL, the ratio of formate to lactate produced is usually low (4). Therefore, control of LDH synthesis is important for the control of lactate production by *S. bovis*.

In *Streptococcus thermophilus* (41) and *Lactococcus lactis* (25), transcription of the gene encoding LDH (*ldh*) is regulated by catabolite control protein A (CcpA), which is a member of the LacI/GalR family of transcriptional repressors/activators (15). HPr (heat-stable protein), a component of the phosphoenolpyruvate-dependent phosphotransferase system, is phosphorylated at the Ser-46 residue by ATP-dependent HPr

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

kinase, which forms seryl-phosphorylated HPr (HPr-[Ser-P]) (9, 13, 17). CcpA forms a complex with HPr-[Ser-P], and then the complex binds to the catabolite-responsive elements (*cre*) (11, 13) that are located upstream of, or within, the 5′ region of many operons (17, 33). As a consequence, gene transcription is activated or repressed (9, 28).

In low-G+C-content gram-positive bacteria, such as bacilli, streptococci, and lactococci, the phosphotransferase system catalyzes the transport and phosphorylation of mono- and disaccharides and also plays a key role in the control of sugar metabolism by regulating the expression of catabolic genes, modulating the activity of key metabolic enzymes, and controlling the activity of sugar transport systems (14, 29, 35).

It has been reported previously that genes encoding HPr (*ptsH*) and HPr kinase (*hprK*) were identified in *S. bovis* (5). Expression of HPr was found to be regulated at the transcriptional level in response to the sugar supply (5). HPr kinase had both HPr-phosphorylating and -dephosphorylating activities, and these activities were affected by the concentration of inorganic phosphate (P_i) (5). Thus, HPr-[Ser-P] formation appears to be regulated by the bifunctional activity of HPr kinase in response to the intracellular P_i concentration in *S. bovis*. However, how HPr-[Ser-P] is involved in the transcriptional regulation through CcpA is unknown at present.

In order to elucidate the mechanism for transcriptional regulation by CcpA in *S. bovis*, we initially analyzed the gene encoding CcpA (*ccpA*) and then examined the transcriptional regulation of the gene. We also examined whether HPr-[Ser-P] affects the binding of CcpA to the *cre* site. In addition, we examined whether CcpA is involved in the transcriptional regulation of *ldh* and *pfl* (a gene encoding PFL) in *S. bovis*. To do these things, we constructed a *ccpA*-disrupted mutant. No report is available at present on the involvement of CcpA in *pfl* transcription even in other bacteria.

MATERIALS AND METHODS

Sources of bacterial strains and plasmids. The sources of *S. bovis* JB1 and 12U1 were described previously (4). Unless indicated otherwise, the JB1 strain was used. *Escherichia coli* DH5α was purchased from a commercial source (Toyobo, Tokyo, Japan). Plasmid pUC18 was purchased from the same commercial source in order to clone *S. bovis ccpA* and to construct a *ccpA*-disrupted mutant. Plasmid pGEX-6P-3 was purchased from Amersham Bioscience in order to express a glutathione *S*-transferase (GST) fusion protein, and plasmid pQE30 was purchased from QIAGEN in order to express a His-tagged fusion protein. The source of plasmid pSBE11, a shuttle vector between *E. coli* and *S. bovis* (32), was described previously (4).

Growth conditions. *S. bovis* was grown anaerobically in batch culture as described previously (1). The medium contained (per liter) 0.45 g of K_2HPO_4, 0.45 g of KH_2PO_4, 0.9 g of $(NH_4)_2SO_4$, 0.9 g of NaCl, 0.12 g of $CaCl_2 \cdot 2H_2O$, 0.19 g of $MgSO_4 \cdot 7H_2O$, 0.1 g of $Fe(NH_4)_2(SO_4)_2$, 1.0 g of Trypticase (BBL, Becton Dickinson), 1.0 g of yeast extract (Difco Laboratories), and 0.6 g of cysteine HCl. As an energy source, either glucose or lactose (3 g/liter) was provided. Culture incubation was performed in triplicate, and the pH was maintained between 6.8 and 7.0 (1). Cell growth was estimated by measuring the optical density at 600 nm. Unless indicated otherwise, *S. bovis* was grown until the late log phase. To prepare the fusion protein, *E. coli* was grown aerobically in Luria-Bertani medium.

Determination of sugars, fermentation products, and cellular nitrogen. Glucose and lactose (16), as well as organic acids (16), were analyzed by high-performance liquid chromatography as described previously. The cellular nitrogen (cell-N) content was determined by the Kjeldahl method as described previously (3).

Sequencing of the *ccpA* gene. Unless indicated otherwise, we used the standard methods for general cloning procedures (39). Genomic DNA was extracted from *S. bovis*, and nucleotide sequences were determined on both strands as described previously (6, 7). The sequence data were evaluated as previously described (7).

Based on the sequences of the *ccpA* gene in *Streptococcus mutans* (GenBank accession number AF014460), *L. lactis* (AF106673), *Lactococcus delbrueckii* (Z54205), and *Bacillus subtilis* (M85182), two oligonucleotide primers for PCR were designed and prepared commercially (Hokkaido System Science, Sapporo, Japan), and they were designated *ccpA*-p1 (5′-GTN TCN ATG GCA ACN GT-3′; from position 89 to position 105) and *ccpA*-p2 (5′-ACN GCW CCN ANR TCA TA-3′; from position 939 to position 923) (N is any base, W is A or T, and R is A or G). The PCR product obtained from the genomic DNA of *S. bovis* JB1 was a 851-bp fragment, which was homologous with a high degree of identity to part of the *ccpA* gene reported for the bacteria described above (BLAST search). Subsequently, the 851-bp fragment was labeled with a digoxigenin DNA-labeling and detection kit (Roche) and used as a hybridization probe for Southern blotting.

S. bovis genomic DNA was digested with EcoRI or Sau3AI, and the fragments were ligated to plasmid pUC18. Restriction and modification enzymes were used according to the recommendations of the manufacturer (TaKaRa Shuzo, Kyoto, Japan). The recombinant plasmids were then introduced into *E. coli* DH5α by electroporation. A fragment containing the upstream and downstream regions of the 851-bp fragment (part of a possible *ccpA* gene) was selected with the probe described above.

Primer extension analysis. Primer extension analysis was carried out with an IRD800-labeled primer, *ccpA*-PEX (5′-TCT TTA TCG TCG TCT TCA TCA CTT G-3′; from position 363 to position 339), and Moloney murine leukemia virus reverse transcriptase RNase H Minus (Toyobo). Products obtained from the primer extension analysis were separated by using a Li-cor DNA sequencer (Aloka, Tokyo, Japan) as previously described (7).

Northern blot analysis. Cultures were promptly frozen by immersing them in liquid nitrogen and were stored at −80°C to stop the degradation of mRNA after sampling (7). Northern blot analysis was performed as previously described (6). A probe specific for *ccpA* for Northern blotting was designed to cover the open reading frame (ORF) and was labeled with a digoxigenin DNA-labeling and detection kit (Roche). Probes specific for *S. bovis ldh* (accession number U60997) and *pfl* (AB014686) were prepared as described previously (6, 7). The amounts of *ccpA*, *ldh*, and *pfl* mRNA in 10 μg of total RNA were estimated from the peak areas and intensities of the bands on a 1.0% (wt/vol) agarose–0.6% (wt/vol) formaldehyde gel by using a Fluor-S Multi Imager (Bio-Rad) as described previously (6). Northern blotting was carried out three times.

Estimation of the rate of degradation of *ccpA* mRNA. Rifampin (100 μg/ml) was added to cultures at the mid-log growth phase, and cells were harvested every 2.5 min after the addition of rifampin. The rifampin concentration added was confirmed to be high enough to inhibit RNA synthesis (3). The rate of degradation of *ccpA* mRNA was estimated from the decay curve.

Preparation of recombinant proteins. The ORFs of *S. bovis ccpA*, *ptsH* (accession number AB027569), and *hprK* (AB027460) were amplified with primers having BamHI and SalI restriction sites at the 5′ and 3′ termini, respectively. Then a *ccpA*-containing fragment and a *ptsH*-containing fragment were ligated into plasmid pGEX-6P-3. A fragment containing *hprK* was ligated into plasmid pQE30. The recombinant plasmids were introduced into *E. coli* DH5α, as described previously (2). The GST fusion protein produced by *E. coli* was purified by affinity column chromatography with a glutathione-Sepharose 4B column (Amersham Bioscience) and then by chromatography with a Superdex 200 HR 10/30 column (1.0 by 30 cm; AKTA System; Amersham Bioscience). The GST part of the fusion protein was cut off with prescission protease (Amersham Bioscience). The His-tagged HPr kinase produced by *E. coli* was purified by affinity column chromatography with HiTrap Chelating HP (Amersham Bioscience) and by Superdex 200 HR 10/30 column chromatography. The purified proteins were confirmed to include the target proteins, as judged from the molecular weights estimated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (24).

Phosphorylation of HPr. The recombinant HPr was phosphorylated with recombinant HPr kinase as previously described (5). The reaction mixture contained 100 mM Tris-HCl (pH 7.0), 5 mM $MgCl_2$, 10 mM NaCl, 5 mM ATP, 10 μg of HPr, and an appropriate amount of His-tagged HPr kinase. The reaction was initiated by adding HPr kinase, and then incubation was carried out at 37°C for 10 min. The reaction was terminated by heating the mixture at 70°C for 5 min. After it was confirmed that HPr was completely converted to HPr-[Ser-P] by nondenaturing 15% PAGE, the reaction mixture was applied to a HiTrap Chelating HP column to separate His-tagged HPr kinase. The eluate containing HPr-[Ser-P] was further purified by Superdex 200 HR 10/30 column chromatography.

Electrophoretic mobility shift assay. A DNA fragment (48 bp; from position −83 to position −36) containing a *cre* sequence in *ccpA* was synthesized by Hokkaido System Science. Binding of CcpA to the DNA fragment containing a *cre* sequence was examined as follows. The reaction mixture contained 100 mM Tris-HCl (pH 7.5), 1 mM EDTA, 10 mM NaCl, a 48-bp *cre*-containing DNA fragment at a concentration of 5 μM, 5 μM CcpA (dimer), and 10 μM HPr-[Ser-P]. To distinguish specific binding from nonspecific binding, 50 μg of poly(dI-dC) per μl (an amount much larger than the amount of the *cre*-containing DNA fragment) was also added. Then the reaction mixture was incubated at 37°C for 15 min and subjected to nondenaturing 10% PAGE. The gel was stained with ethidium bromide and then with Coomassie brilliant blue.

Construction of *ccpA*-disrupted mutant of *S. bovis*. The upper and lower regions of *ccpA* were amplified by PCR with oligonucleotide primer pairs. One pair was *ccpA-pi1* (5′-AGA GTT GAA GCC GAA AAA-3′; from position −249 to position −232) and *ccpA-pi2* (5′-CAT TGA GAC GCC TGC TTC-3′; from position 97 to position 80), and the other pair was *ccpA-pi3* (5′-TTT GTT TCA GGA CCA CTT-3′; from position 590 to position 607) and *ccpA-pi4* (5′-TAG GTT TGG ACG AGT GTA-3′; from position 901 to position 884). The *ccpA-pi1* and *ccpA-pi4* primers were designed to introduce BamHI and SalI restriction sites, respectively, at the 5′ end of each primer. Both of the PCR products were blunt ended by using T4 DNA polymerase (TaKaRa Shuzo). An erythromycin resistance gene, *ermB* (8), was inserted between the *ccpA-pi1–ccpA-pi2* fragment and the *ccpA-pi3–ccpA-pi4* fragment with T4 DNA ligase (TaKaRa Shuzo). The ligated product was digested with BamHI and SalI and then introduced into plasmid pUC18. The recombinant plasmid was electroporated into a competent strain of *S. bovis*, strain 12U1, with a Genepulser (Bio-Rad) operated at 12.5 kV/cm and 200 Ω. Transformants were selected with 10 μg of erythromycin per ml.

Reintroduction of *ccpA* into the *ccpA*-disrupted mutant of *S. bovis*. The gene including the ORF of *ccpA* was amplified by PCR as described above. The PCR product was blunted with T4 DNA polymerase and phosphorylated with T4 polynucleotide kinase (TaKaRa Shuzo). The resulting fragment was introduced into SmaI-digested pSBE11. The recombinant plasmid was electroporated into the *ccpA*-disrupted mutant described above. Transformants harboring the recombinant plasmid were selected by Southern blotting with a *ccpA*-specific probe.

Assay for the activities of LDH and PFL. *S. bovis* cells collected by centrifugation (20,000 × *g*, 10 min, 4°C) were repeatedly disrupted by ultrasonication until approximately 95% of the cells were broken (1). After recentrifugation, the supernatant was immediately subjected to analysis. LDH activity was assayed as described previously (1, 2), and PFL activity was measured by using coupled reactions with malate dehydrogenase and citrate synthase (2, 23). Enzyme activity was expressed as specific activity (in micromoles of NADH changed per minute per microgram of cell-N). Since cell-N was proportional to cell mass or cell dry weight, the specific activity represented the enzyme content in cells.

Evaluation of data. Data were analyzed by the Tukey test or the Student *t* test by using the SigmaStat statistical analysis system (Jandel Scientific).

Nucleotide sequence accession number. The nucleotide sequence of *S. bovis ccpA* reported in this paper has been deposited in the GenBank database under accession number AB028599.

RESULTS AND DISCUSSION

Characterization of *S. bovis ccpA*. *S. bovis ccpA* consisted of 1,005 nucleotides, which included a putative ATG start codon (from position 38 to position 40) and a TAA termination codon (from position 1,040 to position 1,042) (Fig. 1A). The protein encoded by the *ccpA* ORF was deduced to consist of 333 amino acids with a molecular mass of 36,568 Da. The isoelectric point of *S. bovis* CcpA was 5.33. The amino acid sequence of CcpA in *S. bovis* showed high levels of identity and similarity to the sequences of CcpA of other bacteria. The levels of identity and similarity to streptococcal proteins were especially high (more than 70%) (BLAST search), and levels of identity and similarity to *B. subtilis* CcpA (accession number M85182) were 50 and 65%, respectively.

A part of the presumptive *pepQ* gene (from position −304 to position −1209) encoding a proline peptidase (PepQ; 301 amino acids) was found to be located 341 bp upstream of the *ccpA* ORF in *S. bovis*, which was present in the opposite strand. The levels of amino acid identity of part of *S. bovis* PepQ to the proteins reported for *S. mutans* (accession number AF014460) and *Lactobacillus plantarum* (AJ310777) were 68 and 43%, respectively. The arrangement of the *pepQ* and *ccpA* genes in *S. bovis* was the same as that in *S. mutans* (40). In contrast to streptococcal *pepQ*, the *pepQ* gene in *L. plantarum* has been reported to be located in an area downstream of *ccpA* on the same strand (31). In *L. plantarum*, however, *ccpA* and *pepQ* are transcribed as distinct transcriptional units.

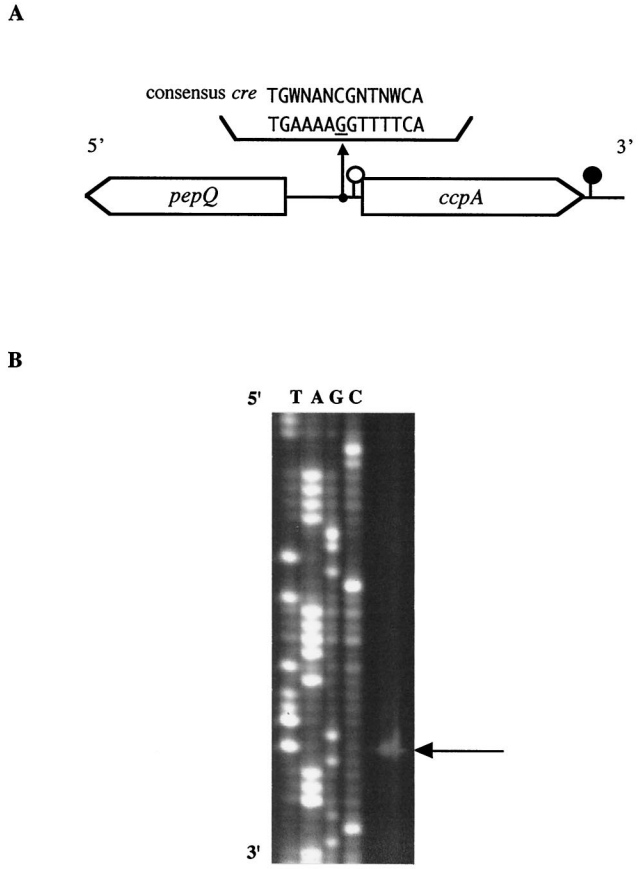

FIG. 1. (A) Schematic representation of the *ccpA* and *pepQ* genes in *S. bovis*. A putative transcriptional start site and a termination site for *ccpA* are indicated by open and solid circles, respectively. A possible *cre* sequence is shown along with the *cre* consensus sequence. (B) Primer extension analysis of *S. bovis ccpA*. Sequence ladders were electrophoresed with the same primer, and the transcriptional start site is indicated by an arrow.

Transcription of *ccpA* in *S. bovis*. A putative ribosome-binding site, the Shine-Dalgarno sequence (GAAAG, from position 22 to position 26), was detected 12 bp upstream from the ATG initiation codon. Primer extension analysis with a *ccpA*-PEX primer demonstrated that there was a single transcriptional start site 37 bp upstream of the *ccpA* start codon (Fig. 1B). Putative −35 (TTAAAA, from position −34 to position −29) and −10 (TAAAAT, from position −11 to position −6) promoter regions were also present. An inverted repeat sequence that is one of the characteristics of a transcriptional terminator was found to be located 40 bp downstream from the *ccpA* termination codon (TGA TTT TTT TTA GTC TTA

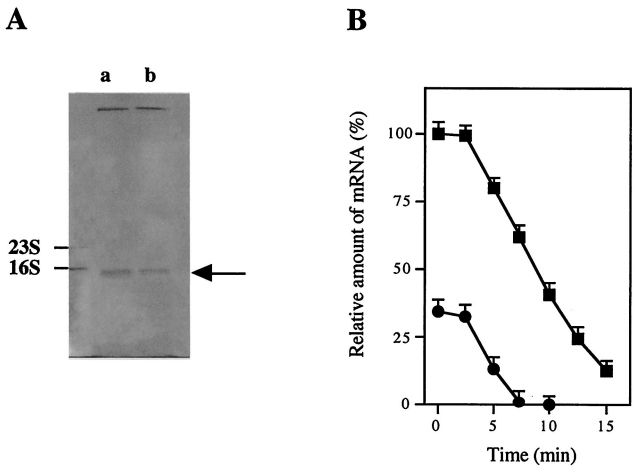

FIG. 2. (A) Level of *ccpA* mRNA in *S. bovis* grown on glucose (lane a) or lactose (lane b), as estimated by Northern blotting. The arrow indicates the position of 1.1-kb *ccpA* mRNA. The ratio of mRNA levels (level of *ccpA* mRNA in *S. bovis* grown on glucose/level of *ccpA* mRNA in *S. bovis* grown on lactose) was 3/1. (B) Decay of *ccpA* mRNA in *S. bovis* cells grown on glucose (■) or lactose (●).

GTG AAA AAA ATT A, from position 1,082 to position 1,112). Calculation of the free energy change for this region of the corresponding mRNA (−10.0 kcal/mol) suggested the presence of a stem-loop structure. Northern blot analysis gave a single band that hybridized with a *ccpA*-specific probe (Fig. 2A). The *ccpA* mRNA was estimated to be approximately 1.1 kb long, which is consistent with the length of the transcriptional unit of *ccpA* deduced from nucleotide sequence analysis (*ccpA* operon; 1,112 bp). These results indicate that the *ccpA* gene is transcribed as a monocistronic mRNA.

The Shine-Dalgarno sequence (GAAGG in the opposite strand, from position −293 to position −297) was located 7 bp upstream from the ATG initiation codon of the *pepQ* gene. Potential −35 (TTGATT in the opposite strand, from position −249 to position −254) and −10 (AAAAAT in the opposite strand, from position −272 to position −277) promoter regions for the *pepQ* gene were also present. As deduced from the gene arrangement, *pepQ* and *ccpA* may be transcribed separately in *S. bovis*. However, whether *S. bovis pepQ* is monocistronic or polycistronic is unknown at present.

A 14-bp palindromic sequence (TGAAAAGGTTTTCA, from position −68 to position −51) was found in the intergenic region between *pepQ* and *ccpA* in *S. bovis* (Fig. 1A). This sequence was 88 and 236 bp upstream of the ORFs of *ccpA* and *pepQ*, respectively. The sequence differed from the consensus *cre* sequence (TGWNANCGNTNWCA) defined by Weickert and Chambliss (42) by one nucleotide (indicated by underlining in the palindromic sequence). In *S. mutans*, a *cre* site has been reported to be present in the promoter region between *pepQ* and *ccpA* (40). Therefore, the palindromic sequence in *S. bovis* is probably a *cre* site. Autogenous regulation of *ccpA* has been described for *L. plantarum* (31), *Lactobacillus pentosus* (26), and *Staphylococcus xylosus* (12).

Binding of CcpA to the *cre* site. Binding of CcpA to the deduced *cre* site in the upper region of *ccpA* was examined by electrophoretic mobility shift analysis. The migration during nondenaturing PAGE was slower when a *cre*-containing fragment had been incubated with both CcpA and HPr-[Ser-P] previously than when the *cre*-containing fragment alone was loaded (Fig. 3A). However, when HPr-[Ser-P] was replaced by HPr or bovine serum albumin (used to distinguish specific binding from nonspecific binding) and then the preparation was incubated, the migration was similar to that when the

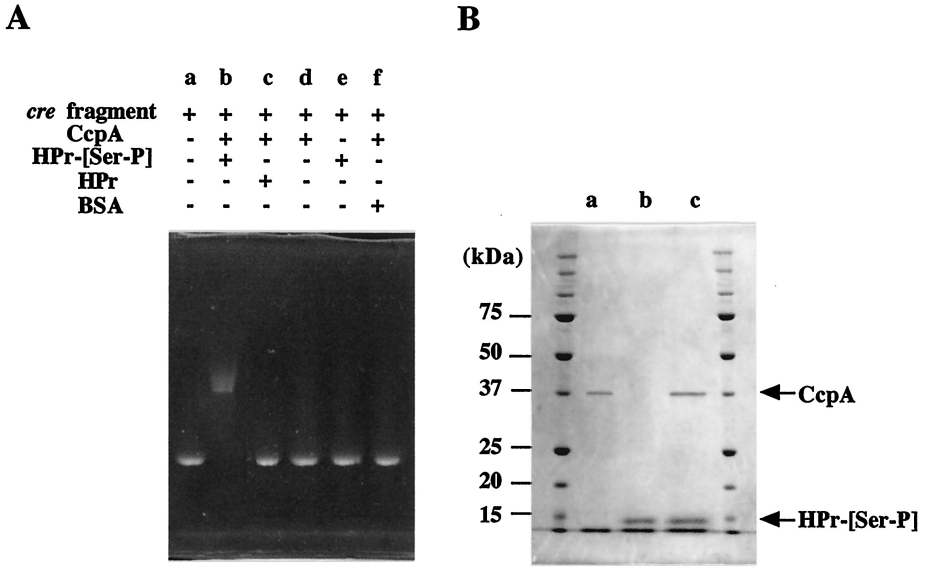

FIG. 3. (A) Gel shift assay showing the binding of the complex consisting of CcpA and HPr-[Ser-P] to the *cre* site. A *cre*-containing fragment was incubated in the presence (plus sign) or in the absence (minus sign) of CcpA, HPr, and/or HPr-[Ser-P]. Bovine serum albumin (BSA) was used as a nonspecific protein. (B) SDS-PAGE of the complex consisting of CcpA and HPr-[Ser-P] after the gel shift assay. Lanes a and b contained CcpA and HPr-[Ser-P], respectively. Lane c contained the complex consisting of CcpA, HPr-[Ser-P], and the *cre*-containing fragment.

TABLE 1. Effect of disruption of *ccpA* on the ratio of formate to lactate produced in *S. bovis*[a]

Strain	Energy substrate	Growth rate[b]	Relative amt of mRNA[c]		Sp act			Ratio of formate to lactate[e]
			ldh	*pfl*	LDH[d]	PFL[d]	PFL/LDH	
12U1	Glucose	0.98 A	1.0 A	1.0 C	61 A	1.8 C	0.03	0.07 D
	Lactose	0.78 B	0.5 B	3.6 B	29 B	5.5 B	0.19	0.48 B
12U1-*ccpA*$^-$	Glucose	0.94 A	0.5 B	9.4 A	27 B	14.2 A	0.53	0.39 C
	Lactose	0.82 B	0.5 B	9.2 A	31 B	14.5 A	0.47	0.59 A
12U1-*ccpA*$^+$	Glucose	0.97 A	1.1 A	1.2 C	59 A	1.9 C	0.03	0.09 D
	Lactose	0.80 B	0.5 B	3.4 B	31 B	5.1 B	0.20	0.49 B

[a] Different letters within a column indicate a significant difference ($P < 0.05$; $n = 3$).
[b] The growth rate is expressed as an increase in the amount of cell-N (in milligrams) per hour during the log stage.
[c] Relative amount of mRNA as estimated by Northern blotting.
[d] Increase or decrease in the amount of NADH (in micromoles per minute per 10 μg of cell-N).
[e] Ratio of the amount of formate to the amount of lactate produced in 1 h.

cre-containing fragment alone was applied. Incubation of the *cre*-containing fragment with either CcpA or HPr-[Ser-P] did not alter the migration. These results indicate that the complex consisting of CcpA and HPr-[Ser-P] binds to the *cre* site. In other words, HPr-[Ser-P] is needed for the binding of CcpA to the *cre* site. In addition, binding of the complex consisting of CcpA and HPr-[Ser-P] strongly supported the hypothesis that the deduced *cre* site is actually a *cre* site.

The ternary complex consisting of HPr-[Ser-P], CcpA, and the *cre*-containing fragment eluted from the gel after a nondenaturing PAGE gel was subjected to SDS-PAGE. The results showed that the ternary complex was dissociated into two proteins (Fig. 3B). The behavior on the gel indicated that the two proteins corresponded to CcpA and HPr-[Ser-P], confirming that the ternary complex contained HPr-[Ser-P], CcpA, and the *cre*-containing fragment.

Binding of the complex consisting of CcpA and HPr-[Ser-P] to the *cre*-containing fragment was not affected by 30 mM glucose 6-phosphate, FBP, or NADP (data not shown), which was different from the finding that these phosphates affected the binding of CcpA to the *cre* site at a concentration of 30 mM or less in *B. subtilis* (10, 22, 30). Because the concentration of glucose 6-phosphate, FBP, or NADP never exceeded 30 mM in *S. bovis* cells (3), it is unlikely that these compounds affect the binding in vivo.

Regulation of the transcription of *ccpA*. As shown in Fig. 2A, the level of intracellular *ccpA* mRNA was approximately threefold higher when *S. bovis* was grown on glucose than when it was grown on lactose. This result suggests that *ccpA* expression was greater when glucose was an energy source, which is in contrast to the previously reported observation that the amount of CcpA protein was at least twofold larger when *S. thermophilus* was grown on lactose than when it was grown on glucose (41). *S. bovis* ferments glucose more rapidly than it ferments lactose (4), whereas *S. thermophilus* utilizes lactose more rapidly than it utilizes glucose (34, 41). Therefore, it is conceivable that *ccpA* expression is higher when a more rapidly utilizable energy source is supplied.

The rate of degradation of *ccpA* mRNA when *S. bovis* was grown on glucose did not differ significantly from the rate when the organism was grown on lactose (Fig. 2B), suggesting that the level of *ccpA* mRNA reflects the rate of transcription. Therefore, CcpA synthesis in *S. bovis* appears to be regulated at the transcriptional level. However, why *ccpA* transcription is greater when a more rapidly utilizable energy source is supplied remains to be clarified.

Involvement of *ccpA* in catabolite repression. A clone of *S. bovis* 12U1 carrying a null mutation in the *ccpA* gene was constructed by a two-step homologous recombination process. Disruption of *ccpA* was confirmed by Southern blotting and PCR analysis (data not shown). The growth rate of the *ccpA*-disrupted mutant (12U1-*ccpA*$^-$) was similar to that of the parent strain (12U1), indicating that growth was not affected by the disruption of *ccpA* (Table 1).

Diauxic growth was observed when 12U1 was grown in a medium containing both glucose and lactose (Fig. 4A). Lactose was utilized after the glucose was exhausted, indicating that 12U1 utilizes glucose in preference to lactose; i.e., 12U1 has a system of catabolite repression for lactose utilization. However, when 12U1-*ccpA*$^-$ was grown in the same medium, glucose and lactose were utilized simultaneously, and diauxic growth was not observed (Fig. 4B). When a 12U1-*ccpA*$^-$ clone into which a *ccpA*-containing plasmid was introduced (12U1-*ccpA*$^+$) was grown, diauxic growth was observed again (Fig. 4C). These results indicate that CcpA is involved in catabolite repression in *S. bovis*.

Involvement of CcpA in the expression of LDH and PFL. The level of intracellular *ldh* mRNA was approximately twofold higher when 12U1 was grown on glucose than when it was grown on lactose (Table 1 and Fig. 5A). A similar trend was observed for the specific activity of LDH (amount of activity per unit of cell-N). These results are essentially the same as previous observations (4). However, when 12U1-*ccpA*$^-$ was used, there was no significant difference in the *ldh* mRNA level or the LDH specific activity between glucose-grown cells and lactose-grown cells. When 12U1-*ccpA*$^+$ was grown on glucose, the *ldh* mRNA level and the LDH specific activity were similar to the corresponding values for 12U1 (Table 1), suggesting that *ccpA* transcription was similar irrespective of whether *ccpA* was included in plasmid or chromosomal DNA.

The *ldh* mRNA level and LDH specific activity in glucose-grown 12U1-*ccpA*$^-$ were one-half of the corresponding values for 12U1 (Table 1). These results support the idea that CcpA positively regulates *ldh* transcription in *S. bovis*. However, there was no significant difference in the *ldh* mRNA level or LDH specific activity between 12U1 and 12U1-*ccpA*$^-$ when lactose was a substrate (Table 1), suggesting that *ldh* transcription is not enhanced by CcpA when cells are grown on lactose.

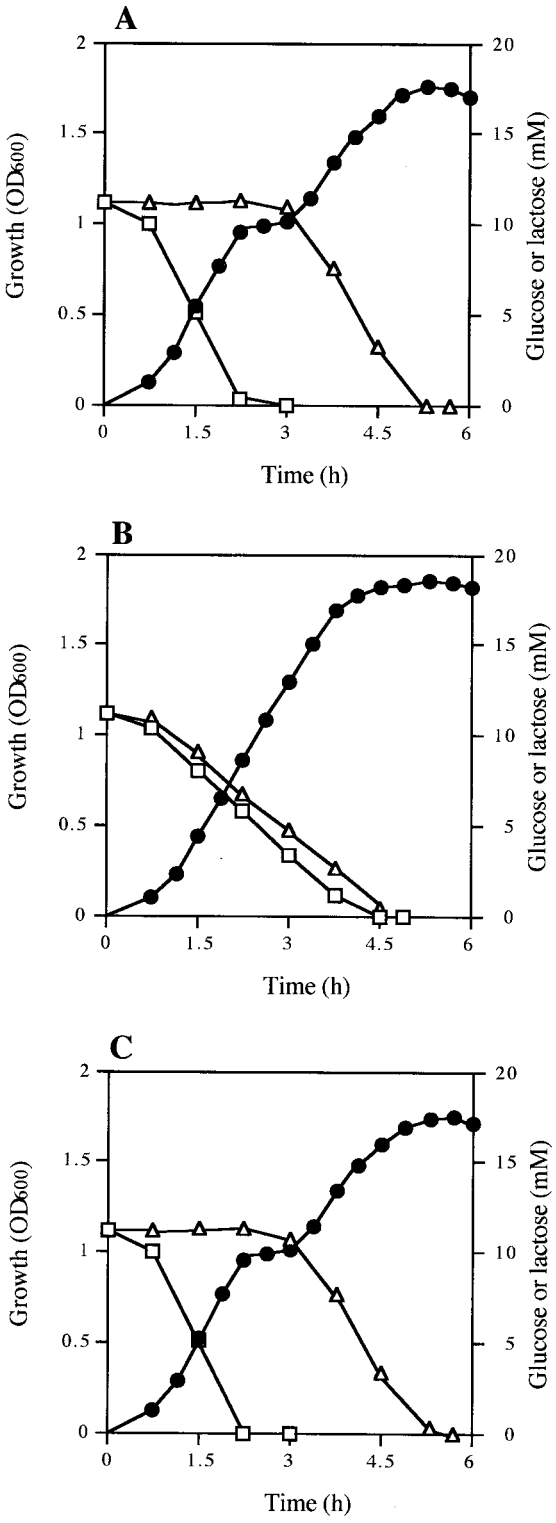

FIG. 4. Growth (●) and glucose (□) and lactose (△) concentrations in the medium when *S. bovis* 12U1 (A), 12U1-*ccpA*$^-$ (B), and 12U1-*ccpA*$^+$ (C) were grown in a medium containing both glucose and lactose. OD_{600}, optical density at 600 nm.

This is consistent with the finding described above that *ccpA* expression was much lower when lactose was an energy source.

In contrast to the *ldh* mRNA level, the *pfl* mRNA level was 3.6-fold higher when 12U1 was grown on lactose than when it was grown on glucose (Table 1 and Fig. 5B). The difference in PFL specific activity between lactose-grown 12U1 cells and glucose-grown 12U1 cells (3.1-fold) was similar to the difference in the *pfl* mRNA level. When glucose was an energy substrate, the *pfl* mRNA level and PFL specific activity of 12U1-*ccpA*$^-$ were 9.4- and 7.9-fold, respectively, higher than the values for 12U1. Even when lactose was a substrate, the *pfl* mRNA level and PFL specific activity in 12U1-*ccpA*$^-$ were 2.6-fold higher than the values for 12U1. These results strongly suggest that *pfl* transcription is suppressed by CcpA in *S. bovis*.

Thus, the impact of disruption of *ccpA* on the *pfl* mRNA level and PFL specific activity was opposite the impact on the *ldh* mRNA level and LDH specific activity, suggesting that CcpA negatively regulates *pfl* transcription in *S. bovis*. The *pfl* mRNA level and PFL specific activity in glucose-grown 12U1-*ccpA*$^-$ were comparable to the values for lactose-grown 12U1-*ccpA*$^-$. This result is in line with the finding described above that the *ldh* mRNA level and LDH specific activity in 12U1-*ccpA*$^-$ were not affected by the substrate supplied.

In the upper region of *ldh* and *pfl*, a *cre*-like sequence was found (Fig. 5C), suggesting that the complex consisting of CcpA and HPr-[Ser-P] binds to the possible *cre* sites. The levels of HPr[Ser-P] and CcpA are higher in glucose-grown cells than in lactose-grown cells. It is presumed that an increase in HPr[Ser-P] and CcpA causes an increase in the binding of the complex consisting of CcpA and HPr-[Ser-P] to the *cre* sites. Binding to the *cre* site in the upper region of *ldh* enhances *ldh* transcription directly or indirectly, whereas binding to the *cre* site in the upper region of *pfl* triggers suppression of *pfl* transcription. Experiments to confirm this presumption are in progress.

Based on these results, it is likely that CcpA is involved in the catabolite control of sugar utilization; in addition, the transcription of *ldh* and *pfl* is regulated through CcpA in opposite directions. How CcpA is involved in the regulation of *ldh* and *pfl* transcription remains to be clarified.

Impact of *ccpA* disruption on the fermentation pattern. The ratio of formate to lactate produced by 12U1 was much greater when cells were grown on lactose (0.48) than when cells were grown on glucose (0.07) (Table 1), which agreed with the previous results obtained with the JB1 and 12U1 strains (3, 4). When cells were grown on glucose, the formate-to-lactate ratio in 12U1-*ccpA*$^-$ (0.39) was much higher than that in 12U1 (0.07). The formate-to-lactate ratio appears to have been altered by a decrease in LDH specific activity and a simultaneous increase in PFL specific activity.

However, the formate-to-lactate ratio in 12U1-*ccpA*$^-$ grown on glucose (0.39) was lower than that in 12U1 grown on lactose (0.48), although the ratio of PFL specific activity to LDH specific activity was higher in the latter organism. This discrepancy may be explained as follows. Specific activity values reflect the amounts of enzymes, but they do not necessarily represent reaction rates. It is possible that the LDH reaction was actually faster than the PFL reaction (4), because the concentration of intracellular FBP, an activator of LDH, is much higher when cells are grown on glucose

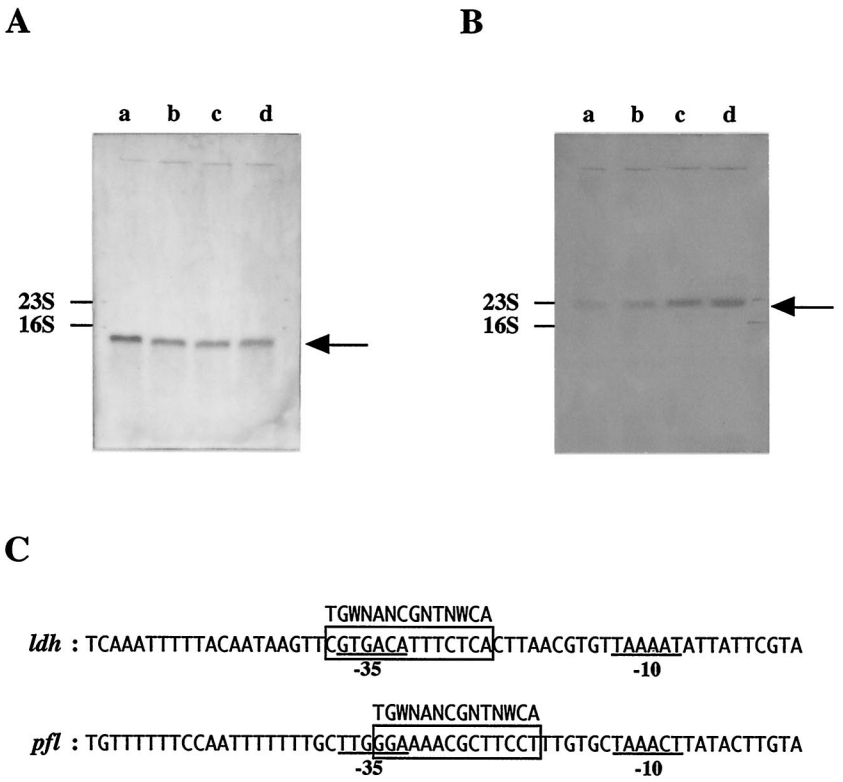

FIG. 5. (A) *ldh* mRNA level in *S. bovis* 12U1 grown on glucose (lane a) or lactose (lane b) and *ldh* mRNA level in *S. bovis* 12U1-*ccpA*$^-$ grown on glucose (lane c) or lactose (lane d), as estimated by Northern blotting. The arrow indicates the position of 1.0-kb *ldh* mRNA. (B) *pfl* mRNA level in 12U1 grown on glucose (lane a) or lactose (lane b) and *pfl* mRNA level in 12U1-*ccpA*$^-$ grown on glucose (lane c) or lactose (lane d). The arrow indicates the position of 2.3-kb *pfl* mRNA. (C) Alignment of the *ldh* (accession number U60997) and *pfl* (AB014686) promoter regions of *S. bovis*. The −35 and −10 sequences are underlined. Possible *cre* sites are enclosed in boxes, and the consensus *cre* sequence is indicated.

than when cells are grown on lactose and, in addition, the levels of dihydroxyacetone phosphate and D-glyceraldehyde-3-phosphate, which are inhibitors of PFL, are lower when cells are grown on lactose than when cells are grown on glucose (3). In 12U1-*ccpA*$^-$, the formate-to-lactate ratio was higher when cells were grown on lactose (0.59) than when cells were grown on glucose (0.39), although there was no difference in LDH or PFL activity. This difference is also likely to have been caused by the allosteric effects of glycolytic intermediates.

In conclusion, *S. bovis* has a *ccpA* gene, which is transcribed as a single operon. Transcription of *ccpA* may be autogenously regulated through a *cre* sequence that is present in the promoter region of *ccpA*. *S. bovis* CcpA requires HPr-[Ser-P] for binding to the *cre* site. Transcription of *ccpA* appears to be greater when a more rapidly utilizable energy source is supplied. *S. bovis* utilizes glucose in preference to lactose, indicating that it possesses a system of catabolite repression for lactose utilization. CcpA is probably involved in catabolite repression. Transcription of *ldh* and *pfl* is regulated reciprocally through CcpA, which alters the formate-to-lactate ratio. There is a *cre*-like sequence in the upper region of both *ldh* and *pfl*, suggesting that the complex consisting of CcpA and HPr-[Ser-P] binds to the *cre* sites. However, how CcpA acts in the global catabolite control system in *S. bovis* remains to be clarified. If it is possible to control CcpA synthesis, lactate production by *S. bovis* could be modulated.

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REFERENCES

1. **Asanuma, N., and T. Hino.** 1997. Tolerance to low pH and lactate production in rumen bacteria. Anim. Sci. Technol. **68:**367–376.
2. **Asanuma, N., and T. Hino.** 2000. Effect of pH and energy supply on the activity and amount of pyruvate formate-lyase in *Streptococcus bovis*. Appl. Environ. Microbiol. **66:**3773–3777.
3. **Asanuma, N., and T. Hino.** 2002. Fructose bisphosphate aldolase activity and glycolytic intermediate concentrations in relation to lactate production in *Streptococcus bovis*. Anaerobe **8:**1–8.
4. **Asanuma, N., and T. Hino.** 2002. Molecular characterization and expression of pyruvate formate-lyase-activating enzyme in a ruminal bacterium, *Streptococcus bovis*. Appl. Environ. Microbiol. **68:**3352–3357.
5. **Asanuma, N., and T. Hino.** 2003. Molecular characterization of HPr and related enzymes, and regulation of HPr phosphorylation in the ruminal bacterium *Streptococcus bovis*. Arch. Microbiol. **179:**205–213.
6. **Asanuma, N., M. Iwamoto, and T. Hino.** 1997. Regulation of lactate dehydrogenase synthesis in a ruminal bacterium, *Streptococcus bovis*. J. Gen. Appl. Microbiol. **43:**325–331.
7. **Asanuma, N., M. Iwamoto, and T. Hino.** 1999. Structure and transcriptional regulation of the gene encoding pyruvate formate-lyase of a ruminal bacterium, *Streptococcus bovis*. Microbiology **145:**151–157.
8. **Claverys, J. P., A. Dintilhac, E. V. Pestova, B. Martin, and D. A. Morrison.** 1995. Construction and evaluation of new drug-resistance cassettes for gene

disruption mutagenesis in *Streptococcus pneumoniae*, using an ami test platform. Gene **164:**123–128.
9. **Deutscher, J., and M. H. Saier, Jr.** 1983. ATP-dependent protein kinase-catalyzed phosphorylation of a seryl residue in HPr, a phosphate carrier protein of the phosphotransferase system in *Streptococcus pyogenes*. Proc. Natl. Acad. Sci. USA **80:**6790–6794.
10. **Deutscher, J., J. Reizer, C. Fischer, A. Galinier, M. H. Saier, Jr., and M. J. Steinmetz.** 1994. Loss of protein kinase-catalyzed phosphorylation of HPr, a phosphocarrier protein of the phosphotransferase system, by mutation of the *ptsH* gene confers catabolite repression resistance to several catabolic genes of *Bacillus subtilis*. J. Bacteriol. **176:**3336–3344.
11. **Deutscher, J., E. Kuster, U. Bergstedt, V. Charrier, and W. Hillen.** 1995. Protein kinase-dependent HPr/CcpA interaction links glycolytic activity to carbon catabolite repression in gram-positive bacteria. Mol. Microbiol. **15:** 1049–1053.
12. **Egeter, O., and R. Bruckner.** 1996. Catabolite repression mediated by the catabolite control protein CcpA in *Staphylococcus xylosus*. Mol. Microbiol. **21:**739–749.
13. **Fujita, Y., Y. Miwa, A. Galinier, and J. Deutscher.** 1995. Specific recognition of the *Bacillus subtilis* gut *cis*-acting catabolite-responsive element by a protein complex formed between CcpA and seryl-phosphorylated HPr. Mol. Microbiol. **17:**953–960.
14. **Hamilton, I. R.** 1987. Effect of changing environment on sugar transport and metabolism by oral bacteria, p. 94–133. *In* J. Reizer and A. Peterkofsky (ed.), Sugar transport and metabolism by Gram-positive bacteria. Ellis Horwood, Chichester, England.
15. **Henkin, T. M., F. J. Grundy, W. L. Nicholson, and G. H. Chambliss.** 1991. Catabolite repression of α-amylase gene expression in *Bacillus subtilis* involves a *trans*-acting gene product homologous to the *Escherichia coli lacI* and *galR* repressors. Mol. Microbiol. **5:**575–584.
16. **Hino, T., K. Shimada, and T. Maruyama.** 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.
17. **Hueck, C. J., W. Hillen, and M. H. Saier, Jr.** 1994. Analysis of a *cis*-active sequence mediating catabolite repression in Gram-positive bacteria. Res. Microbiol. 145:503–518.
18. **Hungate, R. E., R. W. Dougherty, M. P. Bryant, and R. M. Cello.** 1952. Microbiological and physiological changes associated with acute indigestion in sheep. Cornell Vet. **42:**423–449.
19. **Iwamoto, M., N. Asanuma, and T. Hino.** 1999. Effect of nitrate combined with fumarate on methanogenesis, fermentation, and cellulose digestion by mixed ruminal microbes *in vitro*. Anim. Sci. J. **70:**471–478.
20. **Iwamoto, M., N. Asanuma, and T. Hino.** 2001. Effects of pH and electron donors on nitrate and nitrite reduction in ruminal microbiota. Anim. Sci. J. **72:**117–125.
21. **Iwamoto, M., N. Asanuma, and T. Hino.** 2001. Effects of protozoa on nitrate and nitrite reduction in ruminal microbiota. Kanto J. Anim. Sci. **51:**9–15.
22. **Kim, J. H., M. I. Voskuil, and G. H. Chambliss.** 1998. NADP, corepressor for the *Bacillus* catabolite control CcpA. Proc. Natl. Acad. Sci. USA **95:**9590–9595.
23. **Knappe, J., and H. P. Blaschkowski.** 1975. Pyruvate formate-lyase from *Escherichia coli* and its activation system. Methods Enzymol. **41:**508–518.
24. **Laemmli, U. K.** 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature **227:**680–685.
25. **Luesink, E. J., R. E. M. A. van Herpen, B. P. Grossiord, O. P. Kuipers, and W. M. de Vos.** 1998. Transcriptional activation of the glycolytic *las* operon and catabolite repression of the *gal* operon in *Lactococcus lactis* are mediated by the catabolite control protein CcpA. Mol. Microbiol. **30:**789–798.
26. **Mahr, K., W. Hillen, and F. Titgemeyer.** 2000. Carbon catabolite repression in *Lactobacillus pentosus*: analysis of the *ccpA* region. Appl. Environ. Microbiol. **66:**277–283.
27. **Marounek, M., and S. Bartos.** 1987. Interactions between rumen amylolytic and lactate-utilizing bacteria in growth on starch. J. Appl. Bacteriol. **63:**233–238.
28. **Martin-Verstraete, I., J. Stulke, A. Klier, and G. Rapoport.** 1995. Two different mechanisms mediate catabolite repression of the *Bacillus subtilis* levanase operon. J. Bacteriol. **177:**6919–6927.
29. **Meadow, N. D., D. K. Fox, and S. Roseman.** 1990. The bacterial phosphoenolpyruvate:glucose phosphotransferase system. Annu. Rev. Biochem. **59:**497–542.
30. **Miwa, Y., K. Nagura, S. Eguchi, H. Fukuda, J. Deutscher, and Y. Fujita.** 1997. Catabolite repression of the *Bacillus subtilis gnt* operon exerted by two catabolite-responsive elements. Mol. Microbiol. **23:**1203–1213.
31. **Muscariello, L., R. Marasco, M. de Felice, and M. Sacco.** 2001. The functional *ccpA* gene is required for carbon catabolite repression in *Lactobacillus plantarum*. Appl. Environ. Microbiol. **67:**2903–2907.
32. **Nakamura, M., K. Ogata, T. Nagamine, K. Tajima, H. Matsui, and Y. Benno.** 2001. The replicon of the cryptic plasmid pSBO1 isolated from *Streptococcus bovis* JB1. Curr. Microbiol. **43:**11–16.
33. **Nicholson, W. L., and G. H. Chambliss.** 1985. Isolation and characterization of a *cis*-acting mutation conferring catabolite repression resistance to α-amylase synthesis in *Bacillus subtilis*. J. Bacteriol. **161:**875–881.
34. **Poolman, B.** 1993. Energy transduction in lactic acid bacteria. FEMS Microbiol. Rev. **12:**125–148.
35. **Postma, P. W., J. W. Lengeler, and G. R. Jacobson.** 1993. Phosphoenolpyruvate:carboxyhydrate phosphotransferase system of bacteria. Microbiol. Rev. **57:**543–594.
36. **Russell, J. B., and D. B. Dombrowski.** 1980. Effect of pH on the efficiency of growth by pure cultures of rumen bacteria in continuous culture. Appl. Environ. Microbiol. **39:**604–610.
37. **Russell, J. B., and T. Hino.** 1985. Regulation of lactate production in *Streptococcus bovis*: a spiraling effect that contributes to rumen acidosis. J. Dairy Sci. **68:**1712–1721.
38. **Russell, J. B., and R. J. Wallace.** 1997. Energy-yielding and energy-consuming reactions, p. 246–282. *In* P. N. Hobson and C. S. Stewart (ed.), The rumen microbial ecosystem, 2nd ed. Blackie Academic and Professional, London, England.
39. **Sambrook, J., E. F. Fritsch, and T. Maniatis.** 1989. Molecular cloning: a laboratory manual, 2nd ed. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.
40. **Simpson, C. L., and R. R. B. Russell.** 1998. Identification of a homolog of CcpA catabolite repressor protein in *Streptococcus mutans*. Infect. Immun. **66:**2085–2092.
41. **van den Bogaard, P. T. C., M. Kleerebezem, O. P. Kuipers, and W. M. de Vos.** 2000. Control of lactose transport, β-galactosidase activity, and glycolysis by CcpA in *Streptococcus thermophilus*: evidence for carbon catabolite repression by a non-phosphoenolpyruvate-dependent phosphotransferase system sugar. J. Bacteriol. **182:**5982–5989.
42. **Weickert, M. J., and G. H. Chambliss.** 1990. Site-directed mutagenesis of a catabolite repressor operator sequence in *Bacillus subtilis*. Proc. Natl. Acad. Sci. USA **87:**6238–6242.