CURRENT MICROBIOLOGY Vol. 49 (2004), pp. 4–9
DOI: 10.1007/s00284-003-4232-0

Current Microbiology
An International Journal

Molecular Characteristics of Phosphoenolpyruvate: Mannose Phosphotransferase System in *Streptococcus bovis*

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Received: 3 September 2003 / Accepted: 4 November 2003

Abstract. To elucidate the regulatory mechanism of catabolite control in *Streptococcus bovis,* we investigated the molecular properties and gene expression of the mannose-specific phosphoenolpyruvate (PEP)-dependent sugar: phosphotransferase system (PTS). The mannose PTS gene cluster (*man*) was found to comprise a gene encoding enzyme (E) II AB (*manL*) and genes encoding EIIC (*manM*), EIID (*manN*), and a putative regulator (*manO*). The gene cluster (*man* operon) was transcribed from one transcriptional start site, which was located 40 bp upstream of the *manL* start codon. However, two transcriptional start sites were found between *manN* and *manO* in primer extension analysis, and the *manO* may be transcribed independently from the *man* operon. The *man* operon and *manO* were constitutively transcribed without being affected by culture conditions, such as the sugar supplied (glucose, galactose, fructose, maltose, lactose, sucrose, or mannose), growth rate, or pH.

Streptococcus bovis is one of the prevailing lactate-producing bacteria in the rumen and often proliferates in ruminants receiving high-concentrate diets [20, 25]. Feeding high-concentrate diets generally leads to an increase in ruminal lactate production, which causes a drop in ruminal pH. Since *S. bovis* is relatively acid tolerant among ruminal bacteria [30], the proportion of *S. bovis* in ruminal microbiota may increase when the ruminal pH is low. In addition, *S. bovis* produces higher percentages of lactate when the culture pH is low [31], thus suggesting that *S. bovis* contributes to the progress of rumen acidosis. Therefore, it is desirable to suppress the overgrowth of *S. bovis* or the overproduction of lactate by *S. bovis*.

Streptococcus bovis utilizes starch and sugars to acquire energy, and the rate of sugar transport may affect its growth rate. In *S. bovis,* the high-affinity phosphoenolpyruvate (PEP)-dependent sugar:phosphotransferase system (PTS) is an important route for the transport of most sugars and is principally used at low sugar concentrations [23, 28, 35]. In some bacteria, glucose is transported and phosphorylated not only by the glucose PTS, but also by the mannose PTS, which is a typical system that has broad substrate specificity (mannose, glucose, fructose, and 2-deoxyglucose) [28, 34]. In *Streptococcus salivarius,* an enzyme $II^{Mannose}$ (E II^{Man}) complex in the mannose PTS is constitutively synthesized [34]. In some bacteria, the E II^{Man} has an additional domain designated as E II D, which forms a permease in association with E II C [28, 32].

The mannose PTS of *S. salivarius* contains two biochemically and antigenically related proteins, E II $AB_L{}^{Man}$ and E II $AB_H{}^{Man}$, with molecular masses of 35.2 kDa and 38.9 kDa, respectively [8]. The mannose PTS gene cluster (*man*) comprises a gene encoding E II $AB_L{}^{Man}$ (*manL*) and genes encoding E II C (*manM*), E II D (*manN*), and a putative regulator (*manO*).

The E II^{Man} complex has been assumed to be involved in catabolite repression. In fact, mutations rendering the E II^{Man} complex inactive resulted in a loss of the preferential use of glucose over other sugars, such as lactose and ribose in *Lactobacillus casei* [16, 36], and xylose in *Tetragenococcus balophila* [1]. It has also been shown in *S. salivarius* that mutations affecting the expression of mannose PTS components, especially the E II $AB_L{}^{Man}$ subunit, have a pleiotropic effect on the synthesis of several metabolic enzymes [15, 22], on urease activity [10], and on an inducible fructose PTS activity [7]. In *Lactobacillus pentosus,* the E II^{Man} complex provides a strong signal to the global catabolite control

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protein A (CcpA)-dependent catabolite repression pathway [9].

In low G + C Gram-positive bacteria, HPr protein, a component of PTS, is involved in the transcriptional regulation of a variety of genes [12, 18, 26], in addition to the involvement in sugar transport [13, 38]. This transcriptional control system is referred to as the CcpA-PTS system. In transcriptional regulation, HPr that is phosphorylated at residue Ser-46 binds to CcpA, and then the complex binds to catabolite response elements (*cre*) in chromosomal DNA [13, 14]. The *cre* sites are located upstream of, or within, the 5′ region of many operons [19, 27]. As a consequence, transcription of the genes is either activated or repressed [11, 12, 26]. We previously reported the presence of the CcpA-PTS system in *S. bovis* [3]. Expression of the *pts*HI operon encoding HPr and enzyme I (components of the PTS) was regulated at the transcriptional level in response to sugar supply [3].

This paper reports the presence of the mannose-PTS in *S. bovis* in addition to the glucose PTS. We initially analyzed genes encoding the components of the mannose-PTS, and then examined the regulation of the transcription of the genes. The final goal of our study is to clarify how the mannose-PTS is related to transcriptional control by the CcpA-dependent catabolite repression pathway in *S. bovis*.

Materials and Methods

Source and growth conditions of *S. bovis*. *S. bovis* JB1 (ATCC 700410) was obtained, and grown in batch culture as described previously [2]. Growth medium was so designed that glucose limits maximum cell yield, i.e., nutrients other than glucose were added in excess. Unless otherwise stated, *S. bovis* was grown until the late exponential growth phase. Culture incubations were performed in triplicate, maintaining the pH at 6.8–7.0 [5]. Cell growth was estimated by measuring the optical density at 600 nm (OD_{600}).

Extraction of genomic DNA and sequencing procedure. Unless otherwise noted, DNA was extracted and sequenced by the standard procedures described by Sambrook et al. [33]. Restriction and modification enzymes were used according to the recommendations by the manufacturers. Nucleotide sequence was determined by using a Big-Dye Terminator Sequencing Kit (Applied Biosystems, Foster City, CA) and ABI PRISM 310 automatic sequencer (Applied Biosystems). Sequence data were evaluated as described previously [5].

PCR amplification. On the basis of the sequences of enzyme II AB in *Streptococcus pneumoniae* (GenBank accession no. AE008407), *Streptococcus pyogenes* (AE006602), *S. salivalius* (AF130465), and *L. lactis* subsp. *lactis* (AE006401), a degenerate primer, E II ABF (5′-CAA GAR AAA GTT CAA GT-3′, from +122 to +138), was designed. Another primer, E II DR (5′-ACW GGG TCA CCN AYA CC -3′, from +2361 to +2345), was designed from a conserved region of enzyme II D of the bacteria described above. PCR was carried out with *S. bovis* genomic DNA as a template [6].

To seek other clustered genes, nested PCR was carried out on a genomic library. A genomic library of *S. bovis* was constructed according to Sambrook [33]. Chromosomal DNA digested with *Eco*RI, *Hin*dIII, or *Pst*I was ligated into pUC18 vector plasmid. Then, the regions upstream and downstream from the region that was amplified with primers E II ABF and E II DR were sequenced.

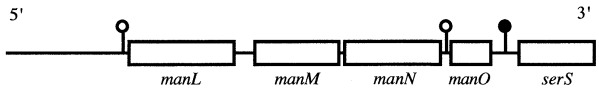

Fig. 1. Arrangement of *manL*, *manM*, *manN*, *manO*, and *serS* in *S. bovis*. A putative transcriptional start site and a termination site are shown as open circles and closed circles, respectively.

Primer extension analysis. Primer extension analysis was carried out with IRD800-labeled primers, *manL*-EX (5′-AGA CCT GTA ATG ATA GCC ATC TTG C-3′, from +324 to +300), *manN*-EX (5′- GAG TTA CAC CGA TGA TTG GAG C-3′, from +2255 to +2234), and *manO*-EX (5′-CCT GTT AGT TTT TTC ATA AGG ACT TG-3′, from +3359 to +3334), as previously described [5].

Northern blot analysis. Culture samples were immediately frozen by being placed in liquid nitrogen, and stored at −80°C [5]. Northern blot analysis was performed as previously described [4]. Probes specific to the *manL*, *manM*, *manN,* and *manO* genes were designed to cover the open reading frame (ORF) and prepared as described previously [4, 5]. The amount of mRNA in 15 μg of total RNA was estimated from the peak area and intensity by using a Fluor-S Multi Imager (Bio-Rad Laboratories Inc., Hercules, CA), as described previously [4].

Nucleotide sequence accession number. The GenBank accession number for the sequence of *S. bovis man* operon (Fig. 1) is AB114606.

Evaluation of data. Data were analyzed by Student's *t*-test with the SigmaStat Statistical Analysis System (Jandel Scientific, SanRafael, CA).

Results and Discussion

Cloning of the gene encoding enzyme II. The gene encoding enzyme II AB (*manL* homologs) in *S. bovis* was found to consist of 993 bp, beginning with ATG and terminating with TAA codon. ManL was deduced to be a 329-amino acid mature protein with a molecular mass of 35,296 Da. A putative ribosome-binding site, the Shine-Dalgarno (SD) sequence (GGAGG), was found 11 bp upstream from the ATG initiation codon. The deduced amino acid sequence of *S. bovis* ManL showed high similarity to that of *S. salivarius* (92%) and *S. pyogenes* (86%). Several amino acid residues that are essential for PTS activity were shown to be conserved in *S. bovis* ManL (E II AB), and especially the phosphorylation domain in both E II A and E II B had high similarity to E II A and E II B in enterococci and streptococci [24].

The gene encoding E II C (*manM* homologs) in *S. bovis* was located 168 bp downstream from *manL* (Fig. 1). The *manM* consisted of 804-bp nucleotides, beginning with ATG and terminating with TAG codon. The SD sequence (GAAAG) was present 12 bp upstream

from the ATG initiation codon. ManM was deduced to be a 266-amino acid mature protein with a molecular mass of 26,661 Da. The ManM contained many hydrophobic amino acids, which is consistent with the fact that ManM is a part of an integral membrane protein that is responsible for the translocation of sugar molecules [28]. On the basis of amino acid identity, *S. bovis* ManM resembles the ManM of *S. salivarius* (84%), *S. pyogenes* (80%), *S. pneumoniae* (80%), and *L. lactis* subsp. *lactis* (64%). The 55th glutamic acid residue, which was suggested to play a crucial role in enzyme activity [29], was replaced by glutamine in *S. bovis* ManM. Again, this is similar to the case of other streptococcal ManM [24].

The gene encoding *S. bovis* enzyme II D (*manN* homologs) was found to consist of 912 bp, beginning with ATG and terminating with TAA codon. The *manN* was located 16 bp downstream from *manM*. The SD sequence (GAAAG) was present 11 bp upstream from the ATG initiation codon. ManN was deduced to be a 302-amino acid protein (33,115 Da). The amino acid identity of *S. bovis* ManN to *S. salivarius* ManN and *S. pyogenes* ManN was 82% and 81%, respectively. The KLTEG motif in ManN has been proposed to be involved in permease-sugar interactions in some bacteria [21, 23, 37], but this motif was not found in *S. bovis* ManN, which is similar to the case of other streptococcal ManN [24]. This sequence was replaced by KITKG (180∼184) in *S. bovis,* and by DITKG (180∼184) in *S. salivarius* and *S. pyogenes* [24]. In the latter two streptococci, positively and negatively charged residues ($D^{-}ITK^{+}G$) have been presumed to be involved in permease–sugar interactions [24]. However, the KITKG sequence in *S. bovis* ManN may not be concerned in this function, because K (lysine) is positively charged.

In *S. bovis* ManN, several amino acid residues on the cytoplasmic side of the permease (residues 24–48) [17] were the same as those in the ManN of *S. salivarius* and *S. pyogenes* [24]. The 29th glutamate residue, which was supposed to be involved in the enzyme–substrate interaction [17], was also present in *S. bovis* ManN. In addition, the EII D^{Man} family signature (KIGMMGPLAGVGDPVFW; 101–117) [29] was strictly conserved in *S. bovis* ManN. These results suggest that *manM* and *manN* in *S. bovis* encode the permease portion of the mannose-PTS system.

The presence of *manO* homologs was shown in *S. bovis,* which was located 79 bp downstream of the *manN* (Fig. 1). In *S. salivarius* and *S. pyogenes*, *manO* has been reported to be located downstream from the *manN* [24]. The *manO* in *S. bovis* consisted of 372 bp, beginning with ATG and terminating with TAA codon. The SD sequence (AGAGG) was present 8 bp upstream from the ATG initiation codon. *S. bovis* ManO was deduced to be a 122-amino acid mature protein with a molecular mass of 13,580 Da. The deduced amino acid sequence of the ManO showed high identity (66%) to that of *S. salivarius* ManO (BLAST search). No function has been assigned to this protein.

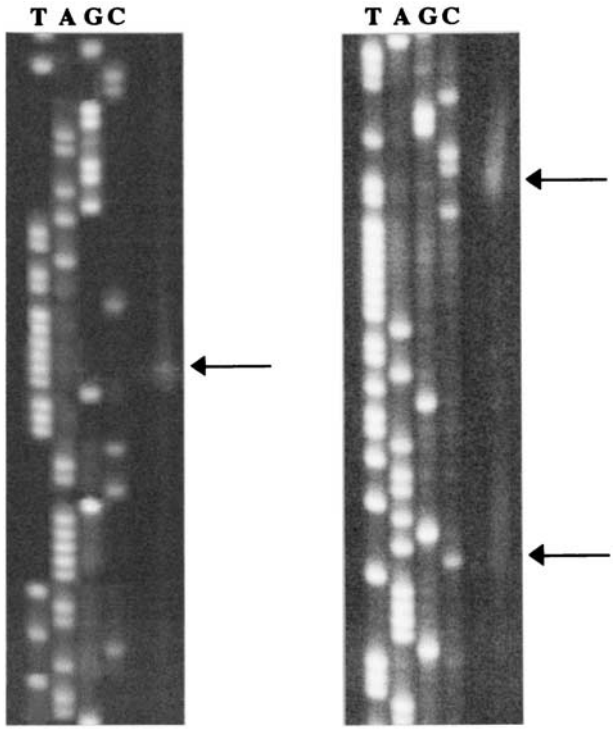

Fig. 2. Primer extension analysis of the *man* operon of *S. bovis* with *manL*-PEX primer (A) and *manO*-PEX primer (B). Sequence ladders were run with the same primer, and the transcriptional start sites are indicated by arrows.

It was suggested that a part of the gene encoding seryl-tRNA synthetase (*serS* homologs) was present 249 bp downstream of the *manO* in *S. bovis*. The *serS* gene was deduced to be transcribed in the same orientation as the *man* genes. The same gene organization was found in *S. pyogenes* and *S. salivarius* [24]. The SD sequence (GGAGG) was present 7 bp upstream from the ATG initiation codon. The amino acid identity of the part of *S. bovis* seryl-tRNA synthetase to the corresponding part of the enzyme in *S. pyogenes* and *E. coli* was 81% and 74%, respectively.

Transcription of the *man* operon. Primer extension analysis with *manL*-EX primer indicated that a single transcriptional start site is present 40 bp upstream of the *manL* start codon (Fig. 2A). Putative −35 (TTGCCG, from −34 to −29) and −10 (GATTAT, from −12 to −7) promoter regions were also present. When primer extension was carried out with *manN*-EX primer, no product was detected within a region between the *manL*

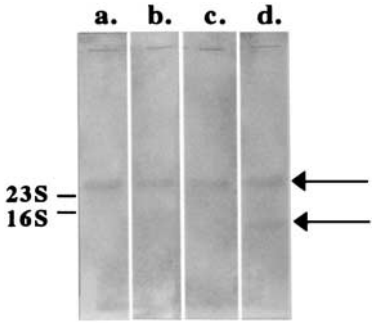

Fig. 3. Northern blot analysis of *man* operon-mRNA with probes specific to *S. bovis manL* (a), *manM* (b), *manN* (c), or *manO* (d). The upper arrow indicates the *man* operon-mRNA, and the lower arrow indicates the *manO*-mRNA.

Table 1. Relative amounts of *man* operon-mRNA and *manO*-mRNA

Sugar	Relative amount of mRNA[a]	
	man operon	*manO*
Glucose	100 ± 6	38 ± 3
Galactose	98 ± 6	39 ± 3
Fructose	105 ± 7	37 ± 2
Maltose	108 ± 7	39 ± 3
Lactose	94 ± 6	39 ± 3
Sucrose	96 ± 7	37 ± 2
Mannose	102 ± 5	39 ± 3

[a] Amount of mRNA per 15 μg of total RNA, as estimated by Northern blotting. Means ± SE are shown.

and *manM* (data not shown). This result suggests that the *manM* and *manN* genes were cotranscribed with *manL,* because an inverted repeat sequence, a potential rho-independent transcriptional terminator, was not found within a region between the *manL* start codon and *manO* termination codon.

On the other hand, two products were found by primer extension carried out with *manO*-EX primer (Fig. 2B), indicating that transcription starts from two sites, i.e., 26 bp and 52 bp upstream from the ATG start codon of the *manO*. One extension product with higher intensity was shown to end at T^{+2955} on the coding strand, while the other product ended at C^{+2981}. Neither putative −35 nor −10 promoter region was found within the regions 50 bp upstream of the two transcriptional start sites. Why promoter regions were absent in the usual sites (35 and 10 bp upstream of the transcriptional start site) is inexplicable at present, but it is conceivable that transcription of *manO* does not require a sigma factor. Alternatively, the *manO* transcript may be produced by an RNA processing event.

An inverted repeat sequence was found 144 bp downstream from the *manO* termination codon (GCT GGA TGG AAC CGC GCG ATT GCG CTC CAG C, from +3521 to +3551). Calculation of free-energy change for the corresponding region of mRNA (−15.4 kcal/mol) suggested the presence of a stem-loop structure. These results suggest that *manL*, *manM*, *manN*, and *manO* are cotranscribed, forming a single transcriptional unit (*man* operon). However, *manO* may be transcribed independently from the *man* operon.

Northern blot analysis with a *manL*-specific probe showed the presence of an approximately 3.5-kbp fragment (Fig. 3). This value is consistent with the assumption that *manL*, *manM*, *manN*, and *manO* are cotranscribed (*man* operon; 3,520 bp). A fragment with the same length was obtained, when a *manM*-specific probe or *manN*-specific probe was used instead of a *manL*-specific probe in Northern blot analysis (Fig. 3). These results confirm that the clustered genes are polycistronic (*man* operon).

On the other hand, in Northern blot analysis with a *manO*-specific probe, an approximately 0.6-kb fragment was detected in addition to a 3.5-kb fragment (Fig. 3). This result is consistent with the assumption that the *manO* gene is also transcribed from the promoter immediately upstream of the *manO*. If it is so, why the *manO* is transcribed from two promoter regions is unknown at present.

When *S. bovis* was grown on glucose, galactose, fructose, maltose, lactose, sucrose, or mannose, the amounts of 0.6 kb (*manO*-mRNA) and 3.5 kb (*man* operon-mRNA) transcripts were not significantly different (Table 1). This result suggests that the *manO* and *man* operon are constitutively transcribed without being affected by the kind of sugar. The amount of 0.6-kb transcript (*manO*-mRNA) was less than that of 3.5-kb transcript (Table 1 and Fig. 3). If ManO is a regulator protein, transcription of *manO* must be altered in response to some conditions. This problem remains to be investigated.

When *S. bovis* was grown on glucose, maintaining the pH at 7.0, the amounts of *man* operon-mRNA and *manO*-mRNA did not change with a decrease in growth rate (data not shown). The amounts of these mRNAs also did not change when the culture pH was reduced from 7.0 to 4.5 (data not shown). These results were different from the results that the levels of lactate dehydrogenase-mRNA and pyruvate formate lyase-mRNA changed with a change in growth rate or culture pH [4, 5].

In conclusion, the presence of *manL*, *manM*, *manN*, and *manO* was shown in *S. bovis*. The *man* gene cluster forms a single operon. However, the *manO* may also be transcribed independently from the *man* operon. The *man* operon and *manO* are constitutively transcribed

without being affected significantly by culture conditions. How the mannose-PTS is involved in the catabolite repression pathway in *S. bovis* remains to be clarified.

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