The gene encoding IIAB^{Man} in *Streptococcus salivarius* is part of a tetracistronic operon encoding a phosphoenolpyruvate:mannose/glucose phosphotransferase system

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Glucose and mannose are transported in streptococci by the mannose-PTS (phosphoenolpyruvate:mannose phosphotransferase system), which consists of a cytoplasmic IIAB protein, called IIAB^{Man}, and an uncharacterized membrane permease. This paper reports the characterization of the *man* operon encoding the specific components of the mannose-PTS of *Streptococcus salivarius*. The *man* operon was composed of four genes, *manL*, *manM*, *manN* and *manO*. These genes were transcribed from a canonical promoter (*P_{man}*) into a 3.6 kb polycistronic mRNA that contained a 5'-UTR (untranslated region). The predicted *manL* gene product encoded a 35.5 kDa protein and contained the amino acid sequences of the IIA and IIB phosphorylation sites already determined from purified *S. salivarius* IIAB^{Man}. Expression of *manL* in *Escherichia coli* generated a 35 kDa protein that reacted with anti-IIAB^{Man} antibodies. The predicted ManM protein had an estimated size of 27.2 kDa. ManM had similarity with IIC domains of the mannose-EII family, but did not possess the signature proposed for mannose-IIC proteins from Gram-negative bacteria. From multiple alignment analyses of sequences available in current databases, the following modified IIC^{Man} signature is proposed: GX₃G[DNH]X₃G[LIVM]₂XG₂[STL][LT][EQ]. The deduced product of *manN* was a hydrophobic protein with a predicted molecular mass of 33.4 kDa. The ManN protein contained an amino acid sequence similar to the signature sequence of the IID domains of the mannose-EII family. *manO* encoded a 13.7 kDa protein. This gene was also transcribed as a monocistronic mRNA from a promoter located in the *manN*–*manO* intergenic region. A search of current databases revealed the presence of IIAB^{Man}, ManM, ManN and ManO orthologues in *Streptococcus mutans*, *Streptococcus pyogenes*, *Streptococcus pneumoniae* and *Enterococcus faecalis*. This work has elucidated the molecular structure of the mannose PTS in streptococci and enterococci, and demonstrated the presence of a putative regulatory protein (ManO) within the *man* operon.

Keywords: enzyme II, streptococci, *man* operon, phosphotransferase system

INTRODUCTION

Oral streptococci are among the most abundant micro-

Abbreviations: PEP, phosphoenolpyruvate; PTS, phosphotransferase system. The GenBank accession number for the sequence reported in this paper is AF130465.

organisms in the oral environment (Marsh & Martin, 1992). These Gram-positive eubacteria use sugars as their main source of energy, and transport several of them by the phosphoenolpyruvate:sugar phosphotransferase system (PTS) (Vadeboncoeur & Pelletier, 1997). The PTS concomitantly transports and phosphorylates sugars at the expense of phosphoenol-

pyruvate (PEP). It is composed of the general proteins HPr and Enzyme I (EI), which are found in the cytoplasm, as well as the sugar-specific proteins called Enzymes II (EII). EI catalyses the transfer of a phosphate group from PEP to HPr. HPr is a phosphate carrier that shuttles between EI and the EII complexes. These complexes are composed of three or occasionally four domains (IIA, IIB, IIC and IID) that can be located on one or multiple polypeptide chains. The IIA domain contains a histidine residue that is phosphorylated by HPr ~ P. IIA ~ P transfers its phosphate group to the IIB domain, usually on a cysteine, except for the IIB proteins of the mannose-PTS family, which are phosphorylated on a histidine residue. IIB ~ P then phosphorylates the incoming sugar. The IIC domain is part of an integral membrane protein responsible for the translocation of the sugar molecule, and does not undergo phosphorylation. The EII complex of the mannose-PTS family, unlike the other EII complexes, has an additional domain called IID. This domain forms the permease in association with IIC (Saier & Reizer, 1992; Postma *et al.*, 1993, 1996).

An analysis of several nucleotide sequences of EII genes allowed the PTS permeases to be classified into six families according to sequence similarity and domain organization (Postma *et al.*, 1993; Lengeler *et al.*, 1994). These families are: (a) glucose- and sucrose-PTS, (b) mannitol- and fructose-PTS, (c) lactose- and cellobiose-PTS, (d) mannose-PTS, (e) glucitol-PTS, and (f) galactitol-PTS. EIIs of the mannose-PTS family are considered to be evolutionarily distinct from the other EIIs (Reizer *et al.*, 1996). The members of the mannose-PTS family that have been identified are EII^{Man}, EII^{Aga} and EII^{Aga'} of *Escherichia coli* (Erni *et al.*, 1987; Reizer *et al.*, 1996), EII^{Lev} of *Bacillus subtilis* (Martin-Verstraete *et al.*, 1990), EII^{Sor} of *Klebsiella pneumoniae* (Wehmeier & Lengeler, 1994), EII^{Man/Glc} of *Vibrio furnissii* (Bouma & Roseman, 1996) and EII^{Man} of *Lactobacillus curvatus* (Veyrat *et al.*, 1996).

In *Streptococcus salivarius*, the mannose-PTS is constitutively produced (Vadeboncoeur, 1984), and is responsible for transporting mannose, glucose, fructose and 2-deoxyglucose (Vadeboncoeur, 1984). In addition to the permease moiety, this mannose-PTS was shown to contain two biochemically and antigenically related proteins, IIAB^{Man}_L and IIAB^{Man}_H, with molecular masses of 35.2 kDa and 38.9 kDa respectively (Bourassa *et al.*, 1990). Both forms of IIAB^{Man} are present only in *S. salivarius* and *Streptococcus vestibularis*; 15 other species of streptococci, *Lactococcus lactis* and *Lactobacillus casei* possess only one protein that reacts with anti-IIAB^{Man}_L or IIAB^{Man}_H polyclonal antibodies. It has been shown that mutations affecting the expression of *S. salivarius* mannose-PTS components, more specifically IIAB^{Man}_L, have pleiotropic consequences on the expression of a wide variety of membrane and cytoplasmic proteins (Gauthier *et al.*, 1990; Bourassa & Vadeboncoeur, 1992; Lapointe *et al.*, 1993; Brochu *et al.*, 1993), as well as on urease activity (Chen *et al.*, 1998).

The biochemical characterization of *S. salivarius*

IIAB^{Man}_L revealed that the IIA and IIB domains are phosphorylated on histidine residues, confirming that this enzyme belongs to the EII-mannose family (Pelletier *et al.*, 1998). Further characterization also showed that IIAB^{Man}_L is required for the phosphorylation of mannose and 2-deoxyglucose by the mannose-PTS, while the IIAB^{Man}_H is unable to fulfil this function (Pelletier *et al.*, 1998). The current work presents the isolation and characterization of the genes encoding the components of the mannose-PTS of *S. salivarius*.

METHODS

Bacterial strains, oligonucleotides, plasmids and growth conditions. *S. salivarius* ATCC 25975 was cultivated in TYE medium (per litre: 10 g Tryptone, 5 g yeast extract, 2.5 g sodium chloride and 2.5 g disodium phosphate) supplemented with 0.5% (w/v) glucose. *E. coli* strains were cultivated aerobically in Luria-Bertani medium at 37 °C. Antibiotics were sterilized by filtration through 0.22 µm membranes (Amicon) and added aseptically to media to the following final concentrations: ampicillin, 50 µg ml⁻¹; tetracycline, 10 µg ml⁻¹; kanamycin, 10 µg ml⁻¹. Plasmid pUC18 (Yanisch-Perron *et al.*, 1985) was used to construct genomic libraries, and pCRII (Invitrogen) was used for amplicon cloning. The oligonucleotides used for PCR amplifications were: E3N29 (5'-ATGATYGGIATYATYATYGCWIIICAYGG-3'), which corresponded to the N-terminal sequence of IIAB^{Man}_L (Pelletier *et al.*, 1998), E3I2205 (5'-TTIGCIGCWACTTGTYTCIGCR-TCIGCRTT-3'), where Y = C or T, W = A or T, I = deoxyinosine, which corresponded to the internal amino acid sequence of IIAB^{Man}_L, and the *lacZ* reverse universal primer RevlacZ (5'-GAGCGGATAACAATTTCACACAGG-3'). The primers used to amplify the *manL* probe were 624 (5'-CATTTAACCAAGCCAGT-3'), which corresponded to the 219th basepair of *manL*, and 1011R (5'-TCTGAAGCAAC-AATGAT-3'), which corresponded to the 605th basepair of *manL*. The *manO* probe was amplified using *manO* (5'-TATGGCACAATCACTAAACAA-3'), which corresponded to the 5' end of *manO*, and *manOR* (5'-GAATGAGAGTT-GGTAGCTTGA-3'), which corresponded to the 328th basepair of *manO*. Amplicons were labelled using the random-priming [³²P]DNA labelling technique.

DNA manipulations. The genomic DNA of *S. salivarius* was isolated as previously described (Lortie *et al.*, 1994). Unless otherwise mentioned, all DNA manipulations were performed using standard procedures (Ausubel *et al.*, 1990). DNA sequencing was completed on both strands by the DNA sequencing service of Université Laval using appropriate synthesized oligonucleotides. Computer-assisted DNA and protein analyses were performed using the Genetics Computer Group Sequence Analysis software package, Version 9.1 (Devereux *et al.*, 1984). At the time of writing, the relevant data from the genome sequencing project of *Streptococcus mutans*, *Streptococcus pyogenes*, *Streptococcus pneumoniae* and *Enterococcus faecalis* are available at <http://www.ncbi.nlm.nih.gov/BLAST/unfinishedgenome.html>.

RNA manipulations. Total RNA was extracted from *S. salivarius* as previously described (Gagnon *et al.*, 1995). Briefly, *S. salivarius* cells were harvested at mid-exponential phase by centrifugation and resuspended in 4 ml ice-cold 10 mM Tris (pH 8.0) containing 1 mM EDTA. Resuspended cells were mixed with a solution containing 3.5 g glass beads (150–212 µm diameter, Sigma), 3 ml phenol/chloroform/isoamyl alcohol (100:24:1, by vol.) and 0.25 ml 10% (w/v)

SDS, and vortexed for 3 min at 4 °C. Lysed cells were centrifuged and the supernatant was extracted five times with phenol/chloroform/isoamyl alcohol (100:24:1, by vol.). Ribonucleic acids were selectively precipitated by adding 0.1 vol. 10 M LiCl and 2 vols 95 % ethanol. The RNA pellet was washed with 70 % ethanol, air-dried, resuspended in 500 µl diethylpyrocarbonate-treated water, and stored in aliquots at -80 °C.

Denaturing formaldehyde agarose gels (1 %, w/v), buffers and samples were prepared according to Ausubel *et al.* (1990). RNA from *S. salivarius* ATCC 25975 (10 µg) was transferred to positively charged nylon membranes (Roche) using a PosiBlot pressure blotter according to the manufacturer's instructions (Stratagene). Total RNA was fixed by UV cross-linking. RNA molecular mass markers (Gibco-BRL) were used to determine the size of the transcripts. Primer extension analysis was performed as described by Ausubel *et al.* (1990) using primer 424R (5'-AATAATACCGATACCCATTCGT-GTT-3') labelled with T4 polynucleotide kinase and [γ -³²P]ATP. The radiolabelled oligonucleotide (10 ng) was hybridized with 20 µg *S. salivarius* total RNA, and the extension was performed using 200 U murine leukaemia virus (MLV) reverse transcriptase (Gibco-BRL) for 1 h at 42 °C. The extended product was denatured and analysed by electrophoresis on a 9 % polyacrylamide gel containing 7 M urea.

Western blot analysis. Crude extracts of *E. coli* XL-1 Blue bearing pML17D or pML18D were prepared by sonication as previously described (Gagnon *et al.*, 1995). Membrane-free cell extracts of *S. salivarius* were submitted to SDS-PAGE, electrotransferred to nitrocellulose membranes, and probed using rabbit anti-IIAB^{Man} polyclonal antibodies as previously described (Pelletier *et al.*, 1995).

RESULTS AND DISCUSSION

Cloning of the gene encoding IIAB_L^{Man}

IIAB_L^{Man} was extracted from two-dimensional gels and the sequence of the N-terminal amino acid residues was determined by Edman degradation. The resulting sequence was MIGIIASHGKFAEG (Pelletier *et al.*, 1998). The purified IIAB_L^{Man} was subsequently submitted to CnBr proteolysis, and the amino acid sequences of internal fragments were determined. The sequence of one internal peptide corresponded to ANATAEQVA-ANII. Two degenerate oligonucleotides, E3N29 and E3I2205 (see Methods), corresponding respectively to the N-terminal and the internal amino acid sequences, were designed. PCR using these oligonucleotides generated a 364 bp amplicon from the *S. salivarius* chromosome. Enriched genomic libraries of *S. salivarius* cloned into pUC18 failed to produce clones that hybridized with the 364 bp probe, suggesting that the chromosomal fragment bearing the IIAB_L^{Man}-encoding gene and/or the IIAB_L^{Man} protein was toxic for *E. coli* cells.

To circumvent this toxicity problem, PCR using the E3N29 primer and RevlacZ universal primer was performed on a ligation mixture of a 4–7 kb *Tsp509I* partial genomic library of *S. salivarius* cloned into pUC18. The amplicons were ligated to pCR-II (In-

Fig. 1. Western blot analysis of 5 µg of crude extracts of *E. coli* XL-1 Blue bearing pML17D (lane 1) or pML18D (lane 2), and 10 µg of membrane-free cell extracts of *S. salivarius* (lane 3) using IIAB_H^{Man}-specific polyclonal antibodies (Pelletier *et al.*, 1995). Note that the antibodies recognize IIAB_H^{Man} and IIAB_L^{Man} in *S. salivarius* cytoplasmic fractions (indicated by the arrows).

vitrogen) and transformed into *E. coli* XL-1 Blue. Screening using the 364 bp amplicon as a probe allowed the identification of one clone (pML17D) carrying a 1.2 kb amplicon. Analysis of the nucleotide sequence revealed the presence of 990 bp ORF encoding a 330 aa protein that shared a high degree of identity with IIAB proteins of the mannose-PTS family. The predicted molecular mass of this putative protein was 35.5 kDa, which was almost identical to the molecular mass of IIAB_L^{Man} (35.2 kDa) determined by SDS-PAGE (Bourassa *et al.*, 1990). Moreover, *E. coli* cells bearing pML17D produced a 35 kDa protein, which was recognized by anti-IIAB^{Man} antibodies. This protein was not found in extracts from *E. coli* cells bearing pML18D, which carried the same DNA fragment inserted in the opposite orientation (Fig. 1). This result proved that IIAB_L^{Man} is not toxic for *E. coli* under these growth conditions. The 35 kDa protein from pML17D was phosphorylated in the presence of PEP, EI and HPr proteins of *S. salivarius*, and allowed the phosphorylation of 2-deoxyglucose in the presence of membrane fractions from a *S. salivarius* mutant (G77) lacking IIAB_L^{Man} and IIAB_H^{Man} (Pelletier *et al.*, 1998). These results confirmed that the product of the gene (*manL*) is the IIAB_L^{Man} of *S. salivarius*.

manL is part of a tetracistronic operon

A Northern blot analysis of total RNA isolated from *S. salivarius* using a *manL* coding portion as a DNA probe allowed the detection of a single 3.6 kb mRNA transcript

Fig. 2. (a) Northern blot of total *S. salivarius* RNA using *manL* as a DNA probe. The arrow indicates the 3.6 kb *man* operon transcripts. (b) Northern blot of total *S. salivarius* RNA using *manO* as a DNA probe. The arrows indicate the 3.6 kb and 0.7 kb transcripts.

(Fig. 2a). This result suggested co-transcription of *manL* with other genes. Due to the toxicity problems previously encountered, the complete *man* operon was isolated using direct and inverse-PCR approaches. The amplicons obtained by inverse PCR were purified and sequenced on both strands, generating a total of 4070 bp DNA sequences that encompassed the entire *man* operon (Fig. 3). The *man* operon was composed of four

ORFs, which we named *manL*, *manM*, *manN* and *manO*. Two terminator-like structures flanked the *man* operon: one was located 155 bp upstream from the *P_{man}* promoter, and the second was 128 bp downstream from *manO*. The 5' end of an ORF, highly similar to the seryl-tRNA synthetase gene (*serS*) of *Staphylococcus aureus* (40% amino acid identity) and transcribed in the same orientation as the *man* operon, was located downstream from *manO*. An analysis of sequences currently available in the databases revealed that the same gene organization is found in *S. pyogenes*, i.e. *manL*, *manM*, *manN*, *manO*, *serS*, while in *S. mutans*, discontinuity of the sequences impeded complete interpretation of the 5' end of the operon. However, analysis of the 3' end of the *man* operon revealed that a 3.9 kb fragment bearing the *comA* gene is present between *manN* and *manO*, and that a 1.8 kb fragment bearing no identified genes is present between *manO* and *serS*. These data suggest that this region of the genome of *S. mutans* was originally identical to that of *S. salivarius* and *S. pyogenes*, but was subsequently subjected to chromosomal rearrangement. However, we cannot exclude the possibility that the *comA* gene was present in the chromosome of *S. salivarius* and *S. pyogenes* and was subsequently excised.

In the *S. pneumoniae* genome, the gene encoding the ManO homologue is not contiguous with PTS-related genes, but is downstream from the *thrA* gene encoding the aspartokinase. As previously described, the *manO* gene of *S. mutans* was separated from the *man* operon by the insertion of a 3.9 kb fragment bearing the *comA* gene. The chromosomal environment is unknown for the *Lc. lactis* gene. Northern blot analysis using the *manO* coding region as a DNA probe revealed the presence of two mRNAs, a long 3.6 kb transcript originating from *P_{man}* and encompassing the entire *man* operon, and a small 0.7 kb mRNA (Fig. 2b). This result confirms that *manO* is transcribed not only from *P_{man}* but also from another promoter in the *manN*–*manO* intergenic region. The putative *P_{manO}* had a consensus –10 box, while its putative –35 box had two mismatches with the consensus sequence, and was

Fig. 3. Schematic representation of the *S. salivarius* *man* operon. Promoters (P) and putative terminators (T) are indicated. The *manL*- and *manO*-specific DNA probes used in this study are indicated by black boxes above the corresponding ORFs.

Fig. 4. Mapping of the 5'-terminus of the *man* operon transcript. Primer extension experiments were performed using 20 µg *S. salivarius* RNA. The end-labelled primer (424R; lane P) was complementary to the beginning of the coding region of the *manL* gene. Lanes A, C, G, T correspond to the dideoxy sequencing reactions performed using the same oligonucleotide primer. The asterisk indicates the 5' terminus of the *man* transcript.

located 22 nt upstream from the Pribnow box. Even with this non-consensus promoter, the intensity of the *manO*-specific signal in Northern blots (Fig. 2b) suggested that this promoter was efficient. Interestingly, Waterfield *et al.* (1995) have reported that in *Lc. lactis* the gene encoding the ManO homologue is also expressed from an immediately adjacent promoter.

Characterization of *Pman*

Localization of the *man* operon promoter was confirmed by primer extension analysis (Fig. 4). The *Pman* promoter (TTGACA-N₁₇-TAGAAT) is only one nucleotide different from the *E. coli* σ^{70} and *Bacillus subtilis* σ^A -type consensus promoter sequences (TTGACA-N₁₇-TATAAT) (Lisser & Margalit, 1993; Moran *et al.*, 1982), which is consistent with the constitutive expression of IAB^{Man}_L in *S. salivarius* (Vadeboncoeur, 1984). Moreover, Szoke *et al.* (1987) have demonstrated that a promoter with this T/G substitution in the -10 region is the second strongest next to the perfect consensus promoter. The potential strength of the *Pman* promoter could explain the toxicity of this chromosomal portion for *E. coli* cells. Every attempt to clone the chromosome region containing *Pman* and the upstream terminator-like structure into *E. coli* has failed. The fact that pML17D, which contains the entire *manL* gene, is stable in *E. coli* led to the conclusion that the toxicity was caused by transcriptional determinants located

upstream from *manL*. Other authors have previously reported that DNA fragments from streptococci are toxic for *E. coli* due to the presence of strong promoters or promoter-like sequences (Stassi & Lacks, 1982; Chen & Morrison, 1987; Martin *et al.*, 1989; Dillard & Yother, 1991).

The coding region for *manL* is located 117 bp downstream from the transcriptional startpoint of the *man* operon. This portion of the transcript did not have an ORF preceded by an RBS. This 5'-untranslated region (5'-UTR) has several stretches of adenines and thymines, and is richer in A + T (70 mol%) than the rest of the chromosome (60 mol%) (Farrow & Collins, 1984). *In silico* studies revealed that 5'-UTR can form several alternative secondary structures with few unpaired nucleotides and energy levels as high as 29.5 kcal mol⁻¹ (123 kJ mol⁻¹) (results not shown). 5'-UTR sequences were detected in several mRNAs from different bacteria like *E. coli*, *Rhodobacter capsulatus* and *B. subtilis* (for reviews see Nierlich & Murakawa, 1996; Kushner, 1996; Coburn & Mackie, 1999), and mRNAs from cold-shock genes from *Lc. lactis* (Wouters *et al.*, 1998) and *E. coli* (Jiang *et al.*, 1996; Fang *et al.*, 1998). These structures have been shown to help stabilize the transcript (Coburn & Mackie, 1999) and to be important in the regulation of gene expression (Fang *et al.*, 1998). The function of this untranslated mRNA region is currently under investigation.

Characterization of the genes composing the *man* operon

***manL*.** The percentages of identity of the inferred amino acid sequence of IAB^{Man}_L with the corresponding domains of homologous proteins are presented in Table 1. It is interesting to note that the IIB domains share a slightly higher degree of conservation than the IIA domains, possibly reflecting structural constraints imposed for recognition of the IIC and IID mannose permeases. Analyses of sequences available in the databases revealed that in addition to *S. salivarius* and *E. coli*, genes encoding IAB^{Man} polypeptides are also present in the genomes of *S. mutans*, *S. pyogenes*, *S. pneumoniae* and *Ent. faecalis*. The IIA and the IIB domains of other mannose-PTSs are on separate polypeptides. A comparison of the amino acid sequences surrounding the IIA phosphorylation sites revealed a high level of identity (Fig. 5a). These levels of identity are particularly high among IIAs from streptococci and enterococci. The amino acid sequences of the IIA domains from *E. coli* and *S. salivarius* only shared 35% identity (Table 1, Fig. 5a). Nevertheless, their structures have numerous points in common. Among the residues determined to be in the vicinity of the IIA phosphorylated histidine (H₁₀) of the *E. coli* IAB^{Man} by crystallographic studies (M₂₃, L₂₄, D₆₇, G₇₁, S₇₂ and P₇₃) (Nunn *et al.*, 1996), several were conserved in the *S. salivarius* IAB^{Man}_L (M₂₃, I₂₄, D₆₇, G₇₁, T₇₂ and P₇₃). The hydroxyl group of S₇₂, which was shown to be essential for PTS activity (Nunn *et al.*, 1996), is replaced in the *S.*

Table 1. Level of identity of *S. salivarius* IIA^{Man} with the IIA^{Man} and IIB^{Man} domains from various bacteria

Organism	IIA domain		IIB domain		Source or accession no.
	Residues	Identity (%)	Residues	Identity (%)	
<i>S. salivarius</i>	1–150	100	151–330	100	This work
<i>S. pneumoniae</i>	1–145†	83.1	146–322	88.8	TIGR stp_4231
<i>S. pyogenes</i>	1–150	86.0	151–330	85.6	OUACGT Contig268
<i>S. mutans</i>	1–130‡	84.0	NA	–	OUACGT Contig163
<i>Ent. faecalis</i>	1–150	56.1	151–342	61.9	TIGR gef_6232
<i>Lb. curvatus</i>	1–146	28.0	1–162	40.5	U28163
<i>B. subtilis</i>	1–146	25.7	1–163	39.9	P26379 and P26380
<i>E. coli (man)</i>	1–150	35.0	151–322	44.8	P08186
<i>E. coli (aga)</i>	NA	–	1–158	29.3	P42909
<i>E. coli (aga')</i>	NA	–	1–157	34.4	P42904
<i>K. pneumoniae</i>	1–135	27.6	1–164	38.4	P37080 and P37081
<i>V. furnissii</i>	1–157	30.5	NA	–	U65015

NA, Not applicable.

† Incomplete sequence at 5' end of the corresponding gene.

‡ Incomplete sequence at 3' end of the corresponding gene.

salivarius homologue by another OH-bearing residue, T₇₂. In addition, in the *S. salivarius* IIA^{Man}, the hydrophobic L₂₄ residue is conservatively replaced by I₂₄. Consequently, the molecular environment of the IIA phosphorylation site of the *S. salivarius* IIA^{Man} should be highly similar to that of the *E. coli* of IIA^{Man}.

The amino acid sequences in the vicinity of the IIB phosphorylation sites also showed high levels of identity, especially for proteins from streptococci and enterococci (Table 1, Fig. 5a). It is important to note that the amino acid sequences of the two inferred phosphorylation sites of IIA^{Man} corresponded to those determined from the biochemical approach (Pelletier *et al.*, 1998). The five arginine residues reported to be involved in the mannose-PTS activity of the *E. coli* IIB^{Man} (R₁₆₈, R₁₇₂, R₁₉₀, R₂₀₄ and R₃₀₄) (Gutknecht *et al.*, 1998) are strictly conserved, with the exception of IIB^{Aga} and IIB^{Aga'}. Other workers have proposed a signature sequence for the IIB domain of the mannose-PTS family (Reizer *et al.*, 1996). This signature (R[LIVM]DXR[LIVMF]₂HGQ-[LIVM]X₃W) corresponds perfectly to the IIB phosphorylation domain of IIA^{Man}. Taken together, these results confirm that IIA^{Man} of *S. salivarius* is unequivocally a member of the mannose-PTS family.

manM. The *manM* gene was located 67 bp downstream from *manL* and coded for a hydrophobic 271 aa polypeptide (27.2 kDa). An analysis of database sequences revealed that ManM shared identity with the mannose-PTS IIC domains of *S. pyogenes* (79%), *S. pneumoniae* (77%), *Ent. faecalis* (71%), *S. mutans* (68%), *E. coli* (46%), *Lb. curvatus* (44%) and *K. pneumoniae* (41%), and with *B. subtilis* *levF* (43%), *E. coli* *agaC* (27%) and *E. coli* *agaW* (23%). A putative

RBS (AAAGGA) is present 9 bp upstream from the *manM* start codon. Sequence alignments of homologous IIC^{Man} proteins from different bacteria revealed that: (i) IIC^{Man} of streptococci and enterococci share high levels of identity (71–79%) and (ii) the residues that were previously identified by Reizer *et al.* (1996) as being strictly conserved among the IIC^{Man} enzymes of the *man*, *aga* and *aga'* systems of *E. coli* and the *lev* system of *B. subtilis* (G₂₀, P₃₃, G₄₁, G₄₅, G₅₀, G₅₄, G₅₅, P₇₃, D₁₂₆, P₁₇₆, G₁₈₂, G₁₈₇, V₁₉₃, G₁₉₄, M₂₀₂, P₂₀₉, G₂₁₄, F₂₁₅, L₂₂₆, G₂₃₃, A₂₃₇) were also conserved in the IIC^{Man} of *S. salivarius*, *S. mutans*, *S. pyogenes*, *S. pneumoniae*, *Ent. faecalis* and *Lb. curvatus* (data not shown). This strengthens the hypothesis that these residues are important for the structure and/or function of the enzyme. However, other residues reported by the same authors to be conserved in IIC^{Man} (L₇, Q₈, R₃₂, L₄₄, D₄₆, T₄₉, E₅₈, G₆₃, Q₁₀₈, T₁₈₀, G₁₈₈ and Y₂₄₀) were not maintained among streptococcal, enterococcal and lactobacillus homologues, suggesting that these residues are subject to more important evolutionary divergence than previously believed. None of the 12 variations occurred in the central cytoplasmic loop of IIC^{Man} (Huber & Erni, 1996; Reizer *et al.*, 1996). Among the 13 nonconserved residues, four (L₄₄, D₄₆, T₄₉, E₅₈) were part of the IIC^{Man} family signature proposed by Reizer *et al.* (1996) (Fig. 5b). Taking into account the sequences of Gram-positive bacteria genomes currently available, we propose a modified IIC^{Man} signature corresponding to GX₃G[DNH]X₃G[LIVM]₂XG₂[STL][LT][EQ]. Reizer *et al.* (1996) reported that E₅₈ is a candidate for a crucial role in enzyme activity, and could correspond to the glutamyl residue involved in sugar binding and the catalytic function of IIC proteins (Jacobson & Saraceni-

(a)		IIA		IIB	
Cons		HG	+++G	L+ R D R HGO + W	
Ssa man	1	MGIGIIASHGKFAEGIHQSGSMIFG	26	172 LA	RLDTRLHGGQVATNW T 189
Smu man	1	MAIGIVIASHGEFAAGIHQSGSMIFG	26		
Spy man	1	MGIGIIASHGKFAEGIHQSGSMIFG	26	172 LA	RVDTRLHGGQVATAW T 189
Spn man	?	ASHGEFAAGIHQSGSMIFG	?	? LA	RLDTRLHGGQVATAW T ?
Efa man	13	MVGIIILASHGEFAEGILQSGAMIFG	37	180 LA	RIDSRLHGGQVATAW T 197
Lcu man	1	MSKIIISGHGQYSIGLLNAFHMIFG	25	5 LA	RIDDRFIHGGQVLTWK V 22
Bsu lev	1	MISVIIISGHGDFPIALKESSGMIFG	25	6 LA	RIDDRFIHGGQILTRW I 23
Eco man	1	MTIAIVIGTHGWAAEQLLKTAEMLLG	26	166 LA	RIDDRLIHGGQVATRW T 183
Kpn sor	1	MVHAIFCAHGQLAGAMLDVCMVYG	25	5 LA	RIDDRLIHGGQVTTW S 22
Eco aga				8 LT	RIDNRLVHGGQVGTW T 25
Eco aga'				6 LS	RIDERLIHGGQVGVQW V 23
Vfu man	1	MLAVILSGHGAFASGMAQAVHQVIG	25		

(b)	Cons	+++	G G G +GG +	++ + + +	
Ssa man	37	CTLI	GAATGNLTAGIMLGGSLO	MIALAWANIG	68
Smu man	?	CTLI	GLASGHTVAGIMLGGLTQ	FLALGWANIG	?
Spy man	37	CTLI	GLVTGHLEAGVILGGTLQ	MLALGWANIG	68
Spn man	34	CTLI	GLVTGHLEAGIILGGSLO	MIALGWSNIG	65
Efa man	34	CTLI	GLVTGNLEAGIVLGGTLQ	MIALGWANIG	65
Lcu man	37	CTLV	GLILGDLQTGIILGGTLE	MMALGWMNVG	65
Bsu lev	34	CTLV	GLVLGDLKGTGIILGGTLE	LMALGWMNVG	65
Eco man	36	CTLV	GIVLGDMKTGIIIGGTLE	MIALGWMNIG	67
Kpn sor	36	CTVI	GLILGDLKGTGVMGGTLE	LIALGWMNVG	67
Eco aga	37	CTLT	GAILGDIQTGLITGGTLE	LAFAGLTPAG	68
Eco aga'	36	GPLV	GLVLGDLHTGILTGGTLE	LVWMGLAPLA	67

Proposed signature GX₃G[DNH]X₃G[LIVM]₂XG₂[STL][LT][EQ]

(c)	Cons	+S N ER + G	+P+ +++Y	I G	K + +GP AG GD+ +W	
Ssa man	24	GSWNYERMQLGWAYSLIPAICKLY	48	97 IQGV	KIGMMGPLAGIGDPVFW	F 118 180 DITKG
Smu man	24	GSWNYERMQLGWAYALIPAICKLY	48	97 IQGI	KIGMMGPLAGIGDPVFW	F 118 180 NITKG
Spy man	24	GSWNYERMQLGMWAYALIPAICKLY	48	97 IQGV	KIGMMGPLAGIGDPVFW	F 118 180 DITKG
Spn man	24	GSWNFERMQLGWAYTLIPAICKLY	48	97 IQGV	KIGMMGPLAGIGDPVFW	F 118 180 DITKG
Efa man	24	GSWNYERMQLGGWAFSMIPAICKLY	48	97 IQGV	KVGMMPPLAGVGDPIFW	F 118 180 DITKG
Bsu lev	24	GSNFERVQAMGYCYVMIPAICKLY	48	97 ISGM	KIGLMGPIAGVGDPIFW	G 118 180 <u>KLTEG</u>
Eco man	33	GSNFERMQALGFCSMVPARRLY	57	107 INGI	KVGLMGPLAGVGDPIFW	G 128 190 <u>KLTEG</u>
Kpn sor	23	ASFNFERIHLGFCYDMIPAICKLY	47	96 INGI	KVGLMGPLAGVGDPLVW	G 117 179 <u>KLTEG</u>
Eco aga	22	ASFNYERMQAGGFTWAMPLILKKIY	46	91 IKGL	KVALFGPIAGIGDAIFW	F 112 172 MIARS

Fig. 5. (a) Alignment of the IIA and IIB phosphorylation sites of the mannose-PTS family. The phosphorylated histidine residues are in bold and the signature sequence is boxed. (b) Alignment of the IIC domain portions located in the vicinity of the signature sequence. The glutamate residue previously reported to be essential is in bold. (c) Alignment of the IID domain portions located in the vicinity of the signature sequence. The strictly conserved glutamate residue (E₂₃) is in bold and the KLTEG motif is underlined. Strictly conserved residues are indicated in the consensus line (Cons) by capital letters. Residues conserved among the Gram-positive bacteria are indicated by '+'. The signature region is boxed. References for the sequences from the following organisms are presented in Table 1: Ssa, *Streptococcus salivarius*; Smu, *Streptococcus mutans*; Spy, *Streptococcus pyogenes*; Spn, *Streptococcus pneumoniae*; Efa, *Enterococcus faecalis*; Lcu, *Lactobacillus curvatus*; Bsu, *Bacillus subtilis*; Eco, *Escherichia coli*; Kpn, *Klebsiella pneumoniae*; Vfu, *Vibrio furnissii*.

Richards, 1993). This glutamate is substituted by a glutamine in the IIC^{Man} of the six Gram-positive species, suggesting that a negative charge at this position does not appear to be essential for IIC activity. Interestingly, the IIC^{Man} of all the Gram-positive bacteria possess strictly conserved glutamate (E₁₄₂) and aspartate (D₁₃₀) residues that might compensate for the absence of E₅₈ and maintain PTS activity. The Cys₃₇ residue proposed to be involved in the interactions of IIC^{Man} and IID^{Man} (Rhiel *et al.*, 1994) is conserved in IIC^{Man} proteins. However, despite its ubiquitous presence in IIC^{Man}

proteins, mutagenesis of this residue in the *E. coli* IIC^{Man} demonstrated that it is not essential for catalytic activity (Rhiel *et al.*, 1994).

manN. The *manN* gene is located 14 bp downstream from the *manM* stop codon. It encodes a putative 303 aa protein (33.4 kDa), which shared strong identity with the mannose-PTS IID domains of *S. pyogenes* (80%), *S. pneumoniae* (74%), *S. mutans* (74%), *Ent. faecalis* (68%), *E. coli* (55%), *K. pneumoniae* (49%), and with *B. subtilis levG* (48%) and *E. coli agaD* (37%). The gene

is preceded by a putative RBS (GGGGTG) 5 bp upstream from the ATG start codon. The KLTEG motif reported to be involved in permease–sugar interactions (Jacobson & Saraceni-Richards, 1993; Lengeler *et al.*, 1994; Wehmeier & Lengeler, 1994) was not conserved in streptococcal IID enzymes (Fig. 5c), as in IID^{Aga} of *E. coli* (Reizer *et al.*, 1996). The motif was replaced by a [DN]ITKG motif. A careful comparison of the motifs showed that the L residue was replaced by an I residue, a modification that could be regarded as conservative. Furthermore, with the exception of the *S. mutans* protein, the positively and negatively charged residues were inverted (K⁺LTE[−]G for D[−]ITK⁺G), leaving open the possibility that these residues are equally involved in the permease–sugar interaction. A highly conserved region (residues 24–48) on the cytoplasmic side of the permease (Huber & Erni, 1996) included a strictly conserved glutamate residue (E₂₉), which might be involved in the enzyme–substrate interaction. Interestingly, the IID^{Man} of several Gram-positive bacteria contains an additional segment of about 30 aa at the cytoplasmic-membrane junction. The previously reported IID^{Man} family signature (K[LIVM][GA][LIVM]₂GP[LIVM]AG[LIVM]GD[PA][LIVMF]₂W) (Reizer *et al.*, 1996) was strictly conserved among the IID^{Man} domains presented in Fig. 5(c). Thus, *manM* and *manN* encoded the permease portions of the mannose-PTS system of *S. salivarius*.

manO. The fourth and last gene of the *man* operon, *manO* is located 78 bp downstream from *manN*, and encodes a small basic (estimated pI 10.7) 124 aa protein (13.7 kDa). The basic pI suggested that it might interact with nucleic acids. However, *in silico* structure analyses of the ManO protein failed to assign the classical helix–turn–helix motif to a portion of the polypeptide. A BLAST search of databases revealed high levels of identity with putative proteins from *S. pneumoniae* (79%), *S. pyogenes* (65%), *S. mutans* (64%), *Ent. faecalis* (32%) and *Lc. lactis* (33%). No function has been assigned to these proteins, but the genes are also located downstream from the IID^{Man} genes in *S. pyogenes* and *Ent. faecalis*.

Conclusions

We have reported the first isolation and characterization of a complete *man* operon for a Gram-positive eubacterium. This operon was comprised of four genes encoding IIAB_L^{Man}, a IIC^{Man}, a IID^{Man} and a protein of unknown function. Identification of the precise functions of ManO and the putative regulatory roles of IIAB_L^{Man} is currently under investigation using insertional mutagenesis.

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