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Characterization of *Streptococcus mutans* Strains Deficient in EIIAB^{Man} of the Sugar Phosphotransferase System

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The phosphoenolpyruvate:sugar phosphotransferase system (PTS) is the major sugar uptake system in oral streptococci. The role of EIIAB^{Man} (encoded by *manL*) in gene regulation and sugar transport was investigated in *Streptococcus mutans* UA159. The *manL* knockout strain, JAM1, grew more slowly than the wild-type strain in glucose but grew faster in mannose and did not display diauxic growth, indicating that EIIAB^{Man} is involved in sugar uptake and in carbohydrate catabolite repression. PTS assays of JAM1, and of strains lacking the inducible (*fruI*) and constitutive (*fruCD*) EII fructose, revealed that *S. mutans* EIIAB^{Man} transported mannose and glucose and provided evidence that there was also a mannose-inducible or glucose-repressible mannose PTS. Additionally, there appears to be a fructose PTS that is different than FruI and FruCD. To determine whether EIIAB^{Man} controlled expression of the known virulence genes, glucosyltransferases (*gtfBC*) and fructosyltransferase (*ftf*) promoter fusions of these genes were established in the wild-type and EIIAB^{Man}-deficient strains. In the *manL* mutant, the level of chloramphenicol acetyltransferase activity expressed from the *gtfBC* promoter was up to threefold lower than that seen with the wild-type strain at pH 6 and 7, indicating that EIIAB^{Man} is required for optimal expression of *gtfBC*. No significant differences were observed between the mutant and the wild-type background in *ftf* regulation, with the exception that under glucose-limiting conditions at pH 7, the mutant exhibited a 2.1-fold increase in *ftf* expression. Two-dimensional gel analysis of batch-grown cells of the EIIAB^{Man}-deficient strain indicated that the expression of at least 38 proteins was altered compared to that seen with the wild-type strain, revealing that EIIAB^{Man} has a pleiotropic effect on gene expression.

Oral streptococci depend on dietary carbohydrates and carbohydrates presented in oral secretions for growth and persistence in the mouth. The ability of oral streptococci to metabolize a wide variety of carbohydrates to produce organic acids is directly related to their ability to elicit dental caries. The phosphoenolpyruvate (PEP):sugar phosphotransferase system (PTS) is the primary sugar transport system in oral streptococci, especially under carbohydrate-limiting conditions, and plays important roles in global control of gene expression (20, 29, 30, 37, 38). The PTS consists of two proteins that are common to all PTS substrates, enzyme I (EI) and the heat-stable phosphocarrier protein HPr, as well as a variety of sugar-specific permeases, known as EII complexes, which catalyze the transport and concomitant phosphorylation of the substrate. The EII complexes usually consist of three domains, A, B, and C, but sometimes a fourth domain, D, is required (20, 29). The A and B domains participate in phosphorylation of the cognate substrates, whereas the C and D domains comprise the membrane permeases. The EII domains can either be covalently linked as a single protein or can be present in various combinations of individual polypeptides.

The mannose PTS appears to play central roles in sugar transport and global regulation of gene expression in oral streptococci. Most of the research on the mannose PTS of streptococci has focused on the EII^{Man} complex of *Streptococcus salivarius*, which consists of two forms of EIIAB^{Man} (EIIAB$_L^{Man}$ and EIIAB$_H^{Man}$) proteins and of EIIC^{Man} and EIID^{Man} proteins (27, 37). EIIAB$_L^{Man}$ is responsible for the phosphorylation of mannose, fructose, glucose, and the nonmetabolizable glucose analog 2-deoxyglucose (2-DG), whereas the function of EIIAB$_H^{Man}$, which can accept a phosphate group from HPr, has not yet been determined (27). Spontaneous mutants of *S. salivarius* selected for resistance to 2-DG that were deficient in EIIAB$_L^{Man}$ had aberrant growth characteristics and alterations in the expression of multiple cytoplasmic and membrane components (37). Some of these mutants were still able to grow with mannose as the sole carbohydrate source, although at a slower rate than the wild-type strain, suggesting the potential for a second, lower-affinity mannose transport system (13, 37). Subsequently, the lower affinity mannose transporter was shown to be a fructose-inducible PTS that was derepressed in the absence of a functional EIIAB$_L^{Man}$ (26), indicating that EIIAB$_L^{Man}$ also has the capacity to regulate other sugar permeases (37). In addition to transporting glucose, fructose, and mannose, the EII^{Man} of *Lactobacillus pentosus* has been shown to be involved in the transport of xylose (9). Finally, the involvement of EII^{Man} in carbon catabolite repression (CCR) was previously suggested for *S. salivarius*, *Streptococcus bovis*, and *L. pentosus* (8, 12, 17). Clearly, then, EII^{Man} proteins are central to genetic and physiologic circuits in gram-positive bacteria with low levels of G+C.

Lortie et al. (22) demonstrated that *Streptococcus mutans* possesses a mannose-specific PTS that is comprised of an EIIAB^{Man} domain (with 84% identity to the EIIAB$_L^{Man}$ form of *S. salivarius*) as well as EIIC^{Man} and EIID^{Man} proteins. Previously, spontaneous mutants of *S. mutans* that were deficient in EII^{Man} grew as well as the parent strain on mannose, so the presence of an inducible mannose PTS was therefore suggested for this organism (23). A variety of studies have

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TABLE 1. *S. mutans* strains used in this study

Strain	Relevant genotype	Description	Source or reference
UA159	*manL*$^+$ *fruI*$^+$ *fruCD*$^+$	Wild type	
JAM1	*manL*$^-$	*manL*::*kan*	This study
TW30	*fruI*$^-$	*fruI*::*erm*	40
TW31	*fruCD*$^-$	*fruCD*::*tet*	40
TW32	*fruI*$^-$ *fruCD*$^-$	*fruI*::*erm fruCD*::*tet*	40
JAM11	*manL*$^-$ *fruI*$^-$	*manL*::*kan fruI*::*erm*	This study
JAM12	*manL*$^-$ *fruCD*	*manL*::*kan fruCD*::*tet*	This study
JAM13	*manL*$^-$ *fruI*$^-$ *fruCD*$^-$	*manL*::*kan fruI*::*erm fruCD*::*tet*	This study
JAM7	*manL*$^+$ *fruI*$^+$ *fruCD*$^+$ *Pgtfcat*[a]	UA159 harboring *Pgtfcat*	This study
JAM8	*manL*$^-$ *Pgtfcat*	JAM1 harboring *Pgtfcat*	This study
JAM9	*manL*$^+$ *fruI*$^+$ *fruCD*$^+$ *Pftfcat*[b]	UA159 harboring *Pftfcat*	This study
JAM10	*manL*$^-$ *Pftfcat*	JAM1 harboring *Pgtfcat*	This study

[a] *gtf* promoter fused to chloramphenicol acetyltransferase gene (*cat*).
[b] *ftf* promoter fused to *cat.*

highlighted the importance of carbohydrate source and availability in the regulation of expression of critical virulence attributes of *S. mutans*. The synthesis and catabolism of exopolysaccharides, acid production, levels of resistance to environmental stress, and PTS activity are among the various factors that have been shown to be influenced by the amount and type of carbohydrate on which the organisms are grown (2, 3, 4, 5, 21, 37, 38). In light of the important roles of EII^{Man} proteins in related organisms, we initiated an investigation of the function of the putative EII^{Man} protein of *S. mutans* UA159 in carbohydrate transport, in regulation of known virulence determinants, and in global control of gene expression.

MATERIALS AND METHODS

Bacterial strains and growth conditions. The *S. mutans* strains listed in Table 1 were grown in brain heart infusion (BHI) at 37°C in a 5% CO_2 atmosphere. When required, antibiotics were added at concentrations of 10 μg ml^{-1} for erythromycin (Em), 10 μg ml^{-1} for tetracycline (Tc), and 1 mg ml^{-1} for kanamycin (Km). Recombinant *S. mutans* strains were grown in BHI or, when cells were to be assessed for diauxic growth or enzymatic assays, in tryptone-vitamin (TV) base medium (7) supplemented with the desired carbohydrates. *Escherichia coli* strains were cultured in Luria-Bertani broth supplemented, when needed, with 100 μg of ampicillin ml^{-1}, 40 μg of Km ml^{-1}, 5 μg of Em ml^{-1}, 20 μg of chloramphenicol (Cm) ml^{-1}, or 10 μg of Tc ml^{-1}. To isolate spontaneous 2-DG-resistant mutants, cells were grown overnight in BHI broth and streaked onto BHI plates containing 5 mM 2-DG. Growth was observed after incubation for 24 h at 37°C in a 5% CO_2 atmosphere. MICs of 2-DG were tested in BHI broth supplemented with 1, 2, 5, 7, 10, 15, 20, 30, or 40 mM 2-DG.

Recombinant *S. mutans* strains carrying *cat* gene fusions were grown in a BioFloIII chemostat apparatus (New Brunswick Scientific, Edison, N.J.) with a working volume of 600 ml in TY base medium (3% tryptone, 0.5% yeast extract) supplemented with 10 μg of Tc ml^{-1}. The pH of the culture was controlled at 5, 6, or 7 pH by automated addition of 2.0 N KOH. Glucose was present in the growth medium at a concentration of 20 mM for carbohydrate-limiting conditions or 200 mM for carbohydrate excess conditions. Cells were grown for a minimum of 10 generations at a dilution rate of $D = 0.3$ h^{-1} to achieve a steady state at each parameter studied.

DNA manipulations. Total chromosomal DNA was isolated from *S. mutans* as previously described (6). Using the QIAprep Spin Miniprep kit (Qiagen, Chatsworth, Calif.), plasmid DNA was isolated from *E. coli*. Restriction and DNA-modifying enzymes were obtained from Life Technologies, Inc. (Gaithersburg, Md.), New England Biolabs (Beverly, Mass.), or MBI Fermentas (Amherst, N.Y.). Using Vent$_R$ DNA polymerase (New England Biolabs), PCRs were carried out with 100 ng of chromosomal DNA. DNA was introduced into *S. mutans* by natural transformation (28) and into *E. coli* by electroporation (31). Southern blot analyses were carried out at high stringency (31).

Construction of a *manL* mutant and reporter gene fusions. The *manL* gene (0.99 kbp), which encodes EIIAB^{Man}, and flanking sequences were amplified from *S. mutans* UA159 chromosomal DNA by recombinant PCR (15) to introduce *Sac*I and *Sph*I restriction sites at the 5′ and 3′ ends, respectively, and a *Bam*HI site at the center of the *manL* structural gene. Briefly, the primary PCRs used the primers designated EIIManS8SacI (5′-CGGAATCGAGCTCGCCAG TCATG-3′) and EIIManAS478BamHI (5′-TAAGTTTGCCGGATCCAATGA CAGTGCC-3′) to generate a product of approximately 500 bp containing engineered *Sac*I and *Bam*HI sites. The second set of primers, EIIManS478BamHI (5′-GGCACTGTCATTGGATCCGGCAAACTTA-3′) and EIIManAS989SphI (5′-GGACATGTGCATGCTCAATGAGTTC-3′), generated another 500-bp product containing *Bam*HI and *Sph*I sites. Equimolar quantities of each amplicon (50 ng) were mixed and used as templates in another PCR using the primers EIIManS8SacI and EIIManAS989SphI to generate a full-length *manL* gene. The final product was directly cloned into *Sac*I- and *Sph*I-digested pGEM5Zf(+) (Promega, Wis.) to generate pJA1. To inactivate the *manL* gene, a *Bam*HI fragment containing a promoterless nonpolar Km resistance marker (18) was introduced into pJA1 in the unique internal *Bam*HI site located 500 bp downstream of the *manL* start codon. The resulting construct, pJA2, was used to inactivate the *manL* gene of *S. mutans* UA159 by allelic exchange. The mutant was designated JAM1.

DNA fragments containing the promoter regions and ribosomal binding sites of the genes encoding the GTF-I and -SI enzymes (*gtfBC* [34, 36]) and fructosyltransferase (*ftf* [33]) of *S. mutans* UA159 were amplified by PCRs and cloned into pCW24 (10) such that transcription and translation of a promoterless *E. coli* Cm acetyltransferase (*cat*) gene were driven by the cognate *S. mutans* regulatory elements. DNA sequencing was performed to confirm that the amplification and cloning had yielded the desired constructs. Fragments containing the gene fusions were released by digestion with *Sac*I and *Hin*dIII, blunt ended, and subsequently cloned into *Hin*cII-digested pSF143 (35), which carries a Tc resistance determinant and replicates in *E. coli* but not in *S. mutans*. The resulting plasmids, pJA11 (carrying the *Pgtf-cat* promoter fusion) and pJA12 (carrying the *Pftf-cat* promoter fusion), were introduced into *S. mutans* UA159 and JAM1 (*manL*$^-$). Chromosomal DNA was extracted from the recombinant *S. mutans* strains carrying the gene fusions. PCR products containing the full-length promoter fusions were obtained, and nucleotide sequencing was used to confirm that the strains contained intact gene fusions in the correct orientation.

Proteomic analysis. *S. mutans* strains were grown in 100 ml of BHI broth to an optical density at 600 nm of 0.5. Cells were collected by centrifugation at 4,000 × *g* for 10 min, washed twice with Tris-buffered saline (10 mM Tris-HCl [pH 7.4], 0.9% NaCl), and resuspended in a total volume of 1 ml of 60 mM Tris-HCl (pH 6.8)–5% sodium dodecyl sulfate–10% glycerol. Cell lysates were obtained by mechanical disruption with a Bead Beater (BioSpec, Bartlesville, Okla.) in the presence of chilled glass beads (0.1-mm diameter) for 2 cycles of 30 s, with cooling on ice for 2 min during the interval. The protein concentration of each sample was determined using bicinchoninic acid assay reagent (Sigma, St. Louis, Mo.) with bovine serum albumin as the standard. Two-dimensional (2-D) electrophoresis was performed according to the method of O'Farrell (25) at Kendrick Labs, Inc. (Madison, Wis.), and the gels were stained with either silver or Coomassie blue. Densitometric analysis was used to compare the intensities of the spots. The identity of a selected protein obtained from the 2-D gels was determined by N-terminal sequencing at the Protein Core at the University of Florida (Gainesville, Fla.) followed by BLAST searches at National Center for Biotechnology Information.

Biochemical assays. To measure gene fusion activity in chemostat-grown cells, aliquots were rapidly aspirated from the culture vessel and cells were harvested by centrifugation, washed with 10 mM Tris-HCl (pH 7.8), and resuspended in 750 μl of the wash buffer. Cells were homogenized as described above, and the cleared lysates were used for determining Cm acetyltransferase (CAT) activity by the spectrophotometric method of Shaw et al. (32). To measure PTS activity, *S. mutans* strains were grown to an optical density at 600 nm of 0.6 in TV medium supplemented with 0.5% (wt/vol) mannose or glucose and PTS-specific activity with glucose, fructose, or mannose as substrate was determined as detailed by LeBlanc et al. (19). Briefly, 50 ml of late exponential phase cells was harvested, washed twice with 0.1 M sodium-potassium phosphate buffer (pH 7.2), and suspended in 5 ml of the same buffer. This cell suspension was permeabilized with 250 μl of toluene-acetone (1:9), and 10 to 50 μl of permeabilized cells was used in the assay. The assay measures the oxidation of NADH in a PEP-dependent manner, and the assay mixture contained 0.1 mM NADH, 10 U of lactic acid dehydrogenase, 5 mM PEP, 10 mM NaF, and a 10 mM concentration of the sugar of interest in a final volume of 0.1 M sodium-potassium phosphate buffer, pH 7.2. The rate of NADH oxidation was monitored for 3 min at 340 nm. No PEP was added in the reference tubes. PTS activity was expressed as nano-

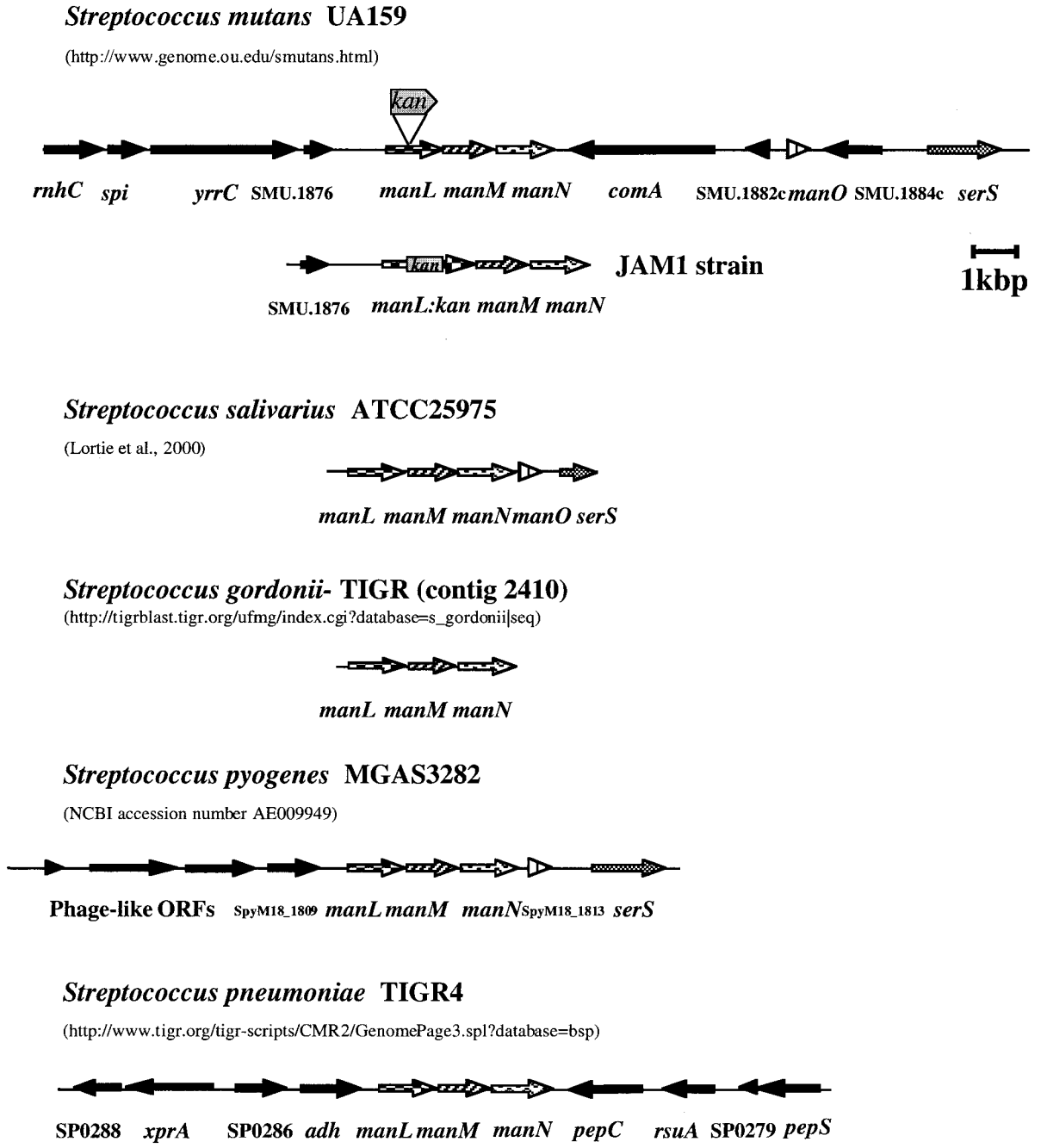

FIG. 1. The arrangement of *man* gene clusters and flanking regions in *S. mutans* UA159 and other streptococci. The location of the insertion of a nonpolar Km cassette within the *manL* gene to generate the strain JAM1 is indicated. Patterned arrows represent similar genes. The websites or references from which the *man* gene clusters and flanking regions were obtained are indicated.

moles of NADH oxidized in a PEP-dependent manner per minute per milligram of protein.

RESULTS AND DISCUSSION

Arrangement of the *man* gene cluster and flanking regions. Using the *S. salivarius manL* sequence, computer algorithms identified a putative *man* gene cluster in the *S. mutans* UA159 genome (1), the sequence of which was completed at the Advanced Center for Genome Technology at the University of Oklahoma (http://www.genome.ou.edu/smutans.html). The organization of the *man* gene cluster and flanking regions in *S. mutans* and other streptococci is shown in Fig. 1.

The arrangement of the *manL* gene cluster of *S. mutans* is significantly different than that of *S. salivarius*, which is composed of four open reading frames (ORFs) (22), *manLMNO*, but lacks *comA* and SMU.1882c-like genes between *manN* and *manO*. In both species, however, the *serS* gene is located downstream of *manO*. Lortie et al. (22) suggested that this region of the genome of *S. mutans* was probably subjected to chromosomal rearrangement, as suggested by the presence of addi-

TABLE 2. Doubling time of *S. mutans* strains in TV broth supplemented with 0.2% (wt/vol) of glucose, mannose and fructose

Strain	Doubling time (min) ± SD in broth with:		
	Glucose	Mannose	Fructose
UA159	82 ± 1	255 ± 12	73 ± 6
JAM1	92 ± 4	180 ± 17	74 ± 4
TW30	88 ± 2	243 ± 30	85 ± 4
TW31	89 ± 3	234 ± 15	72 ± 6
TW32	79 ± 8	244 ± 15	80 ± 6
JAM11	102 ± 0	190 ± 23	77 ± 5
JAM12	108 ± 5	201 ± 26	77 ± 6
JAM13	110 ± 14	187 ± 8	73 ± 2

tional genes and relatively long noncoding regions that are not present in the *S. salivarius* and *Streptococcus pyogenes man* operons and flanking regions. Of potential significance, the presence of phage-like ORFs directly upstream of the *manL* gene cluster was observed in the *S. pyogenes* MGAS8232 genome. In *Streptococcus pneumoniae* TIGR4, this gene cluster is also composed of *manLMN* but *manO* was not observed in the flanking regions. Also, the flanking regions of the *man* cluster of *S. pneumoniae* differed significantly from those of *S. mutans*, *S. salivarius*, and *S. pyogenes*. Lortie et al. (22) suggested that the *manO* gene has a regulatory function in *S. salivarius* because this ORF can be also cotranscribed with the *man* operon. The presence and location of this gene differ among the streptococci; however, the importance of *manO* in the functionality of EII^{Man} is unclear.

Characteristics of $EIIAB^{Man}$-deficient *S. mutans*. An otherwise isogenic *manL* mutant of *S. mutans* UA159 was constructed by allelic exchange with a nonpolar Km resistance determinant inserted 0.5 kbp downstream of the *manL* start codon, as detailed in Materials and Methods. Insertion of the marker into *manL* was confirmed by Southern blot analysis using probes specific for *manL* and for the Km resistance element. To determine the role of *S. mutans* $EIIAB^{Man}$ in glucose and mannose uptake, growth rates in TV medium containing glucose or mannose as the sole carbohydrate source were determined and sugar-specific PTS activities were measured for the parental strain and JAM1, the *manL* mutant. *S. mutans* JAM1 grew more slowly than UA159 in TV medium with 0.2% glucose, with a doubling time of 92 min for JAM1 compared to 82 min for UA159 (Table 2). Growth in 0.2% mannose was faster for JAM1 (doubling time ≅180 min) than for the wild-type strain (doubling time ≅255 min). This finding is in contrast with that reported for *S. mutans* GS-5, in which a spontaneously arising EII^{Man}-deficient strain grew more slowly than the parent in glucose but growth in mannose was unaffected (23, 24). Of note, no significant differences in growth rates were observed between the wild-type strain and JAM1 when 0.2% fructose was used as the sole carbohydrate source (Table 2). Thus, the behavior of the JAM1 strain also differs from that of an $EIIB^{Man}$ mutant of *L. pentosus*, which was shown to grow faster on fructose but which displayed slower growth on glucose and mannose than the wild-type strain (8). Therefore, it seems that $EIIAB^{Man}$ of *S. mutans* UA159 may constitute an ortholog of the $EIIAB_L{}^{Man}$ of *S. salivarius* and EII^{Man} of *L. pentosus*.

To assess PTS activity, UA159 and JAM1 were grown in TV medium with various carbohydrate sources. Glucose-specific PTS activity of JAM1 grown in glucose was markedly lower ($P \leq 0.001$) than that of the wild-type strain grown under the same conditions (Fig. 2A), indicating that EII^{Man} of *S. mutans* is probably involved in glucose transport and confirming the observation of Néron and Vadeboncoeur with strain GS-5 (23). Fructose PTS activity of JAM1 was not affected by the lack of a functional $EIIAB^{Man}$ (Fig. 2A), a finding consistent with the similar growth rates of UA159 and JAM1 in 0.2% fructose. These results indicate that unlike the case seen with *S. salivarius*, the *S. mutans* $EIIAB^{Man}$ does not appear to take up fructose. However, we cannot exclude the possibility that EII^{Man} is able to repress either fructose PTS gene expression or permease activity.

Lower levels of glucose-specific PTS activity were observed in the wild-type strain grown in mannose than were seen for

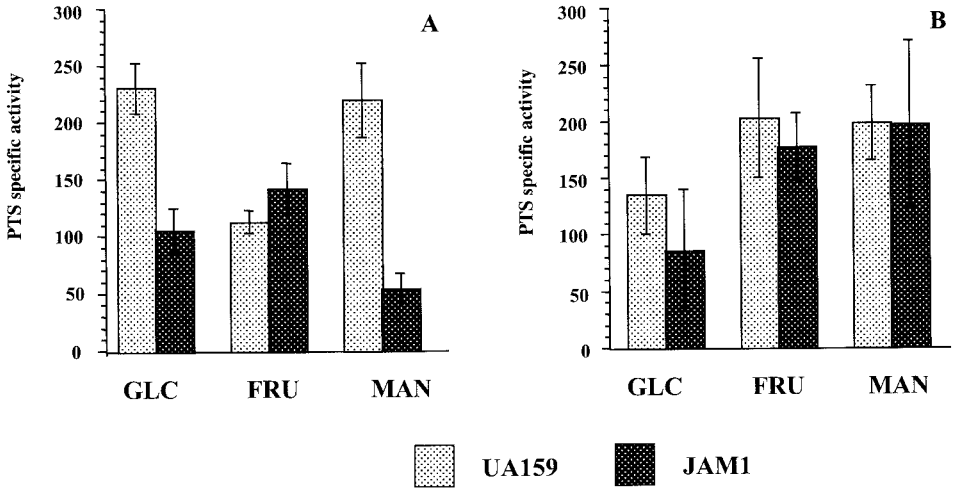

FIG. 2. Sugar-specific PTS activity of *S. mutans* UA159 and JAM1 grown in glucose (A) and mannose (B). The glucose-, fructose-, and mannose-specific PTS activities are indicated as GLC, FRU, and MAN, respectively. The values are the means ± standard deviations (SD) from at least three individual experiments.

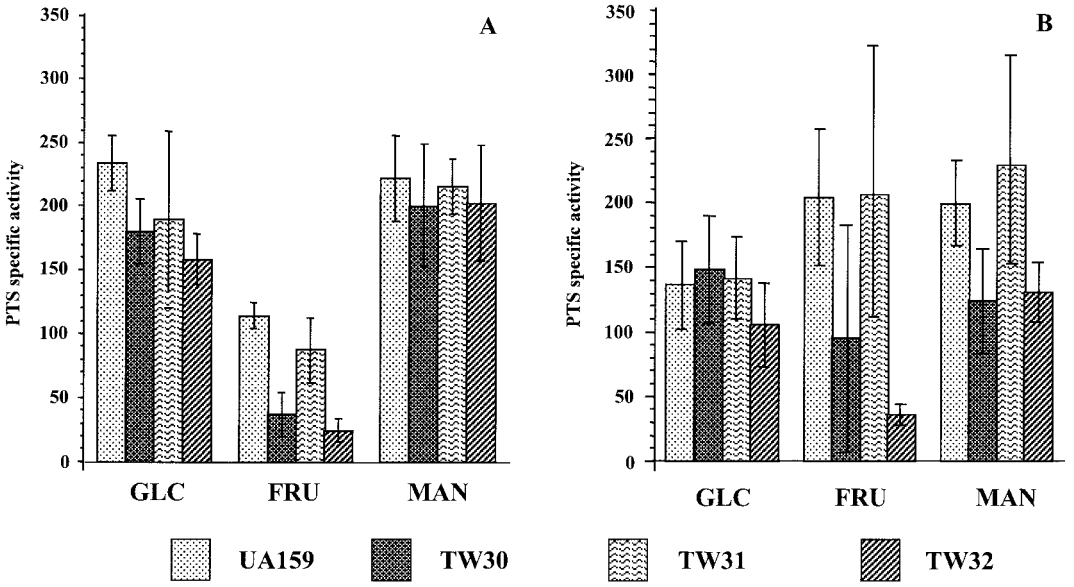

FIG. 3. Sugar-specific PTS activity of *S. mutans* UA159 and EII^{Fru-} strains (TW30, TW31, and TW32) grown in glucose (A) and mannose (B). The glucose-, fructose-, and mannose-specific PTS activities are indicated as GLC, FRU, and MAN, respectively. The values are the means ± SD from at least three individual experiments.

the strain grown in glucose (Fig. 2B), suggesting the presence of both a constitutive EII^{Glu} and a glucose-inducible, or mannose-repressible, EII^{Glu} in *S. mutans*, which is consistent with the findings of Néron and Vadeboncoeur (23). When JAM1 was grown in mannose, the mannose PTS activity was restored to the levels seen in the wild-type strain grown under the same conditions (Fig. 2B), providing compelling evidence for the presence of a mannose-inducible mannose PTS. Alternatively, EIIAB^{Man} may down-regulate this second mannose PTS, which may explain why the absolute level of mannose PTS activity does not differ markedly between UA159 grown in glucose or mannose and JAM1 grown in mannose. The fructose PTS activity of the wild-type strain was enhanced when cells were grown in mannose as substrate ($P \leq 0.02$) (Fig. 2B), suggesting that the fructose porters may also be glucose repressible or mannose inducible. If the latter is the case, a role for EII^{Fru} in mannose uptake would be logical.

Role of fructose-specific II enzymes. In *S. salivarius*, an inducible EII^{Fru} was shown to be able to transport mannose in spontaneously arising, EII^{Man}-deficient strains (13). To explore whether EII^{Fru} enzymes of *S. mutans* could be responsible for the increased growth rate of JAM1 in mannose and for the restored mannose PTS activity of JAM1 grown in mannose (Fig. 2B), *S. mutans* strains TW30 (deficient in the inducible fructose PTS [*fruI*$^-$]), TW31 (deficient in the constitutive fructose PTS [*fruCD*$^-$]), and TW32 (lacking both EII^{Fru} enzymes) (40) were examined in this study. Growth in 0.2% glucose was only slightly slower for TW30 and TW31 than for the wild-type strain, whereas strain TW32 showed no change in its growth rate on glucose. The doubling times of the fructose PTS mutants TW30, TW31, and TW32 growing on mannose were indistinguishable from that of UA159, the parent strain (Table 2).

Strains that were deficient in the fructose-inducible EII^{Fru} (TW30 and TW32) expressed modestly lower levels of glucose PTS activity than the wild-type strain when grown in glucose (Fig. 3A). For strain TW30, this was shown not to be statistically significant, although the differences between the wild-type strain and TW32 were ($P \leq 0.05$). The strain that was deficient in the constitutive EII^{Fru} only (TW31) did not show any alteration in glucose-specific PTS activity compared to the wild-type strain growing under the same conditions (Fig. 3A). At this stage, therefore, it cannot be ruled out that that the II^{Fru} enzymes, and in particular FruI, might be capable of transporting glucose. No differences were observed for glucose PTS activity when all strains were grown in mannose as the sole carbohydrate source, albeit glucose PTS activity was consistently lower than in glucose-grown cells (Fig. 3). As expected, all strains that carried mutations in the inducible fructose PTS genes (*fruI*) expressed lower levels of fructose-specific PTS activity, with the lowest level observed for strains that carried mutations in both of the fructose-specific EII genes (TW32) ($P \leq 0.001$). Interestingly, fructose-specific PTS activity was up-regulated in all strains growing in mannose (Fig. 3B) compared to that of glucose-grown cells (Fig. 3A), with the exception of TW32 (*fruI*$^-$/*fruCD*$^-$). This result indicates that growth in mannose can induce, or more likely allow for derepression of, the fructose PTS. In *L. pentosus*, cells grown on substrates for EII^{Man} had repressed fructose PTS activity, suggesting that EII^{Man} does have the capacity to negatively regulate EII^{Fru} (8), at least in some lactobacilli.

The *manL* gene was insertionally inactivated in strains TW30, TW31, and TW32 to generate JAM11 (*manL*$^-$/*fruI*$^-$), JAM12 (*manL*$^-$/*fruCD*$^-$), and JAM13 (*manL*$^-$/*fruI*$^-$/*fruCD*$^-$). The strains JAM11, JAM12, and JAM13 displayed the same growth pattern as the strains carrying only the EII^{Fru} mutations when fructose was provided as the sole carbohydrate (Table 2). Growth of JAM11-13 in glucose and mannose was essentially identical to that of the parental *manL*$^-$ strain, JAM1 (Table 2). The levels of fructose PTS activity in JAM11, -12, and -13 (Fig. 4) generally paralleled those observed in the parental

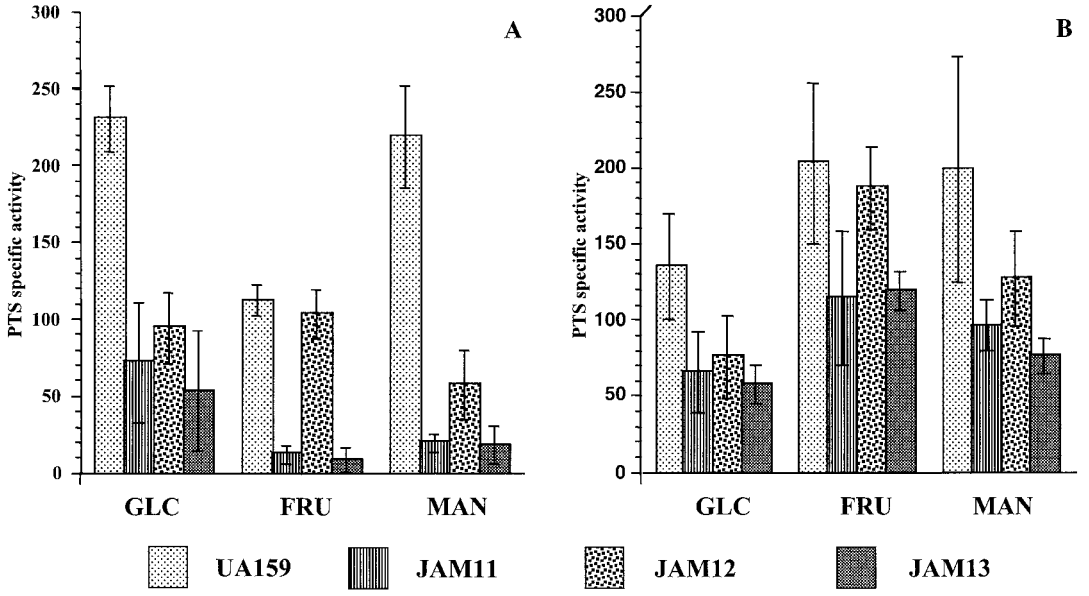

FIG. 4. Sugar-specific PTS activity of *S. mutans* UA159 and EIIAB^{Man-}/EII^{Fru-} strains (JAM11, JAM12, and JAM13) grown in glucose (A) and mannose (B). The glucose-, fructose-, and mannose-specific PTS activities are indicated as GLC, FRU, and MAN, respectively. The values are the means ± SD from at least three individual experiments.

strains TW30, -31, and -32 (Fig. 3). However, strain TW32 did not show enhanced fructose PTS activity when grown in mannose (Fig. 3B), whereas JAM13 (*manL*$^-$/*fruI*$^-$/*fruCD*$^-$) did ($P \leq 0.0007$) (Fig. 4B). These results suggested that *S. mutans* possesses yet another EII (different from FruI, FruCD, and EII^{Man}) that is capable of transporting fructose and that the expression of this gene is probably negatively regulated by EIIAB^{Man}.

Another interesting finding arising from this series of experiments was that JAM13, which is deficient in EIIAB^{Man}, FruI, and FruCD, showed enhanced mannose PTS activity when grown in mannose. This result indicated that there might be an inducible PTS permease that is capable of transporting mannose (one that is distinct from II^{Man} and the known II^{Fru} enzymes). Also of interest, when the strain that was deficient in EIIAB^{Man} and FruI (JAM11) was grown in glucose (Fig. 4A), mannose PTS activity was lower than in the EIIAB^{Man}-deficient strain (JAM1) growing under the same conditions (Fig. 2A). The strains TW30 and TW32 (*fruI*$^-$ and *fruI*$^-$/*fruCD*$^-$), when grown in glucose (Fig. 3A), had the same mannose PTS activity levels as the wild-type strain, although these same strains exhibited lower mannose PTS activity when grown in mannose (Fig. 3B). In light of these findings, and because there was no enhancement of mannose transport activity in strains that were grown in mannose and carried the *fruI* mutation, it is possible that FruI is capable of transporting mannose.

Resistance to 2-DG. EIIAB^{Man} of *S. salivarius* participates in the transport of 2-DG, the nonmetabolizable glucose analog. In *S. salivarius*, spontaneous mutants can generally be selected using 5 mM 2-DG on rich solid medium with a variety of PTS substrates. In contrast, both UA159 and JAM1 grew well on BHI agar supplemented with 5 mM 2-DG. The inherent high level of resistance of *S. mutans* UA159 to 2-DG suggests either that the affinity of EII^{Man} of *S. mutans* for 2-DG is markedly lower than that of ManL of *S. salivarius* or that EIIAB^{Man} of *S. mutans* does not transport 2-DG. We then tested the ability of *S. mutans* UA159 and JAM1 to grow in BHI broth containing 2-DG in concentrations ranging from 1 to 40 mM. Under these conditions, there was some inhibition of growth of *S. mutans* UA159 at 1 mM 2-DG and growth was completely inhibited at 15 mM 2-DG (data not shown). In contrast, there was no evidence of inhibition of growth of JAM1 at concentrations of 2-DG as high as 5 mM, although complete inhibition was also observed in BHI with 15 mM 2-DG. These findings would seem to indicate that EII^{Man} of *S. mutans* is capable of transporting 2-DG, perhaps with lower affinity than EII^{Man} of *S. salivarius*. Also, since complete inhibition of growth of JAM1 could only be achieved in the presence of relatively high concentrations of 2DG, it is likely that there is another low-affinity 2-DG transporter in *S. mutans*. Whether a similar secondary pathway for 2-DG uptake at elevated concentrations of the analog exists in *S. salivarius* has not been tested to our knowledge.

Involvement of EIIAB^{Man} in catabolite repression. Strains of *S. mutans* that lack ManL (JAM1 and JAM11-13) did not exhibit diauxic growth in the presence of glucose and a nonpreferred carbohydrate source (Fig. 5). Thus, as has been observed with *S. salivarius* and *L. pentosus*, EIIAB^{Man} of *S. mutans* appears to be an effector of CCR. Whether EIIAB^{Man} is able to effect CCR through phosphorylation of regulatory proteins, as has been observed in a variety of saccharolytic operons of *B. subtilis*, or through direct interaction with sugar permeases, similar to EII^{Glu} of *E. coli*, remains to be determined. Interestingly, a putative catabolite response element (CRE), which is a conserved DNA sequence to which the CcpA-HPr complex binds to effect catabolite repression in gram-positive bacteria (4, 11, 14, 16), was found 103 bp upstream of the *S. mutans manL* start codon. This putative CRE differs from the consensus sequence in only the last two bases (TGTAAACG TTTTAC); thus, residues that have been shown to be neces-

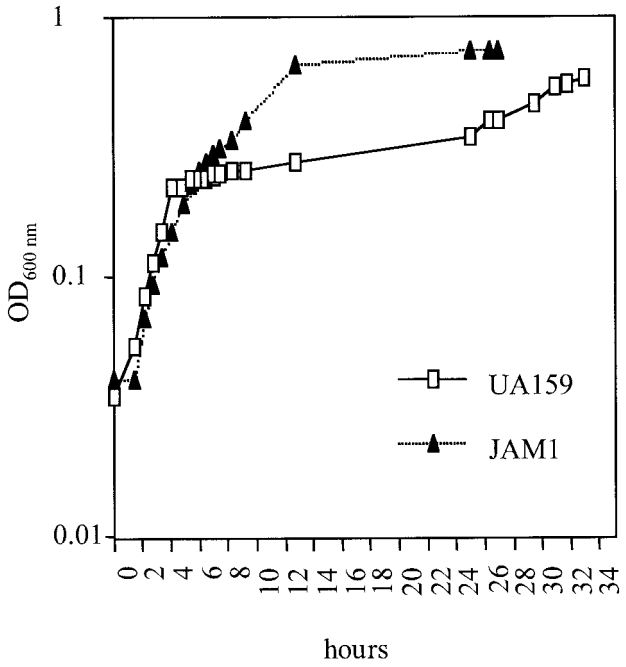

FIG. 5. Growth of UA159 and JAM1 in TV medium supplemented with 0.05% glucose and 0.5% inulin. The empty squares represent the wild-type strain UA159, whereas the filled triangles represent JAM1, the EIIAB^{Man-} strain. The results shown are representative of three independent experiments.

sary for efficient CRE function are conserved (14, 39). If this sequence represents a functional CRE, the EII^{Man} operon may be under the control of CCR via the CcpA pathway. Such an arrangement could allow for CcpA to act as a master regulator of CCR and for proteins such as EII^{Man} to act to fine tune gene expression in response to more subtle fluctuations in carbohydrate availability or at lower overall carbohydrate concentrations. Alternatively, the cells could utilize EII^{Man} to respond specifically to cognate sugars of this transporter, whereas the CcpA pathway, which senses carbon flow through fructose-1,6-bisphosphate, may govern gene expression in a less specific fashion in response to total available carbohydrate, thus allowing for monitoring of levels of sugars other than mannose and glucose.

Protein expression patterns differ in the *manL* mutant. The effect on expression of the products of a variety of genes in cells lacking a functional EIIAB^{Man} was evident in 2-D gels that were silver stained, revealing that at least 11 proteins were up-regulated and 27 were down-regulated in the *manL*$^-$ strain (Fig. 6). Thus, *manL* inactivation has a pleiotropic effect on gene expression. This effect could be exerted through the pathways for catabolite repression, through indirect effects caused by alterations in carbon catabolism, or through direct involvement of ManL in the regulation of expression (or allosteric regulation of activity) of various genes or their products. In any case, it is clear that ManL is of major importance in global regulation of gene expression in *S. mutans*. This finding is consistent with those reported for *S. salivarius* that showed alterations in the 2-D gel patterns of cytoplasmic and membrane preparations from wild-type cells and a spontaneously arising EII_L^{Man} mutant (37). A spot (which was present in the wild-type strain and absent in the *manL* mutant in Coomassie blue-stained gels) that we suspected on the basis of molecular weight and pI measurements to be EIIAB^{Man} was extracted and analyzed by N-terminal sequencing. The sequence data (XIGIV IAXHGEF) demonstrated that this protein was EIIAB^{Man}.

Expression of the exopolysaccharide machinery of *S. mutans* is influenced by EIIAB^{Man}. The ability to synthesize and degrade extracellular glucans and fructans has been shown to be a primary virulence attribute of *S. mutans* (5, 6, 7, 21). The use of sucrose to produce α1,3- and α1,6-linked glucans via glucosyltransferases is an integral part of formation of tenacious oral biofilms and is an essential component of the elicitation of smooth surface caries. Similarly, conversion of sucrose to fructan homopolymers by fructosyltransferase allows the organisms to create substantial stores of extracellular polysaccharides, which augment the acid challenge to the tooth surface. Previous studies by Li and Burne have revealed a strong relationship between carbohydrate source and availability and the expression of genes involved in exopolysaccharide synthesis (21). To determine whether EIIAB^{Man} was involved in regulation of *gtfBC* or *ftf* gene expression, *S. mutans* strains carrying *cat* gene fusions to the promoters of these genes (created as detailed in the Materials and Methods section) were grown in continuous chemostat culture to a steady state under glucose-limiting or glucose excess conditions (Fig. 7). In agreement with previous studies (21, 41), in the wild-type background (JAM7), *gtfBC* expression was markedly enhanced under conditions of excess carbohydrate, with optimal expression at pH 6. At pH 5, a considerably lower level of expression from the *gtfBC* promoter was observed in the wild-type background under both glucose excess and glucose-limiting conditions (Fig. 7A). JAM9, which is the *manL*$^-$ strain carrying the *gtfBC* promoter-*cat* fusion, appears to display a 2- or 2.8-fold reduction in CAT activity expressed from the *gtfBC* promoter in cells growing under glucose-limiting conditions (20 mM) at pH 7 or 6, respectively, compared with the expression levels in the wild-type background (Fig. 7A). In cells grown under glucose excess conditions, CAT activity was 2.9- and 3.1-fold lower in the mutant at pH 7 and 6 than in the wild-type strain grown under the same conditions. The decrease in expression from *PgtfBC* observed in the EIIAB^{Man} mutant strain grown under carbohydrate-limiting or carbohydrate excess conditions at pH 7 and 6 suggests that EIIAB^{Man} is required for optimal expression of these known virulence genes of *S. mutans*.

There are a number of observations that could account for these findings. First, ManL could effect changes in expression of *gtfBC* through phosphorylation of (or through direct interaction with) a *gtf* regulatory protein or through the modulation of expression of factors needed for optimal *gtf* transcription. Alternatively, alterations in the transport of carbohydrates induced by ManL deficiency could be relayed to the *gtf* regulator(s) via phosphorelay circuits or through other proteins that participate in global regulation of genes in response to carbohydrate availability. Since the factors required for differential expression of *gtfBC* have not yet been identified, information from the 2-D gels on the *manL* mutant cannot yet be used to disclose a likely pathway for altered regulation of *gtf* in the JAM8 mutant.

Expression of *ftf* as measured by monitoring CAT activity in both strains (JAM9 and JAM10) under glucose-limiting con-

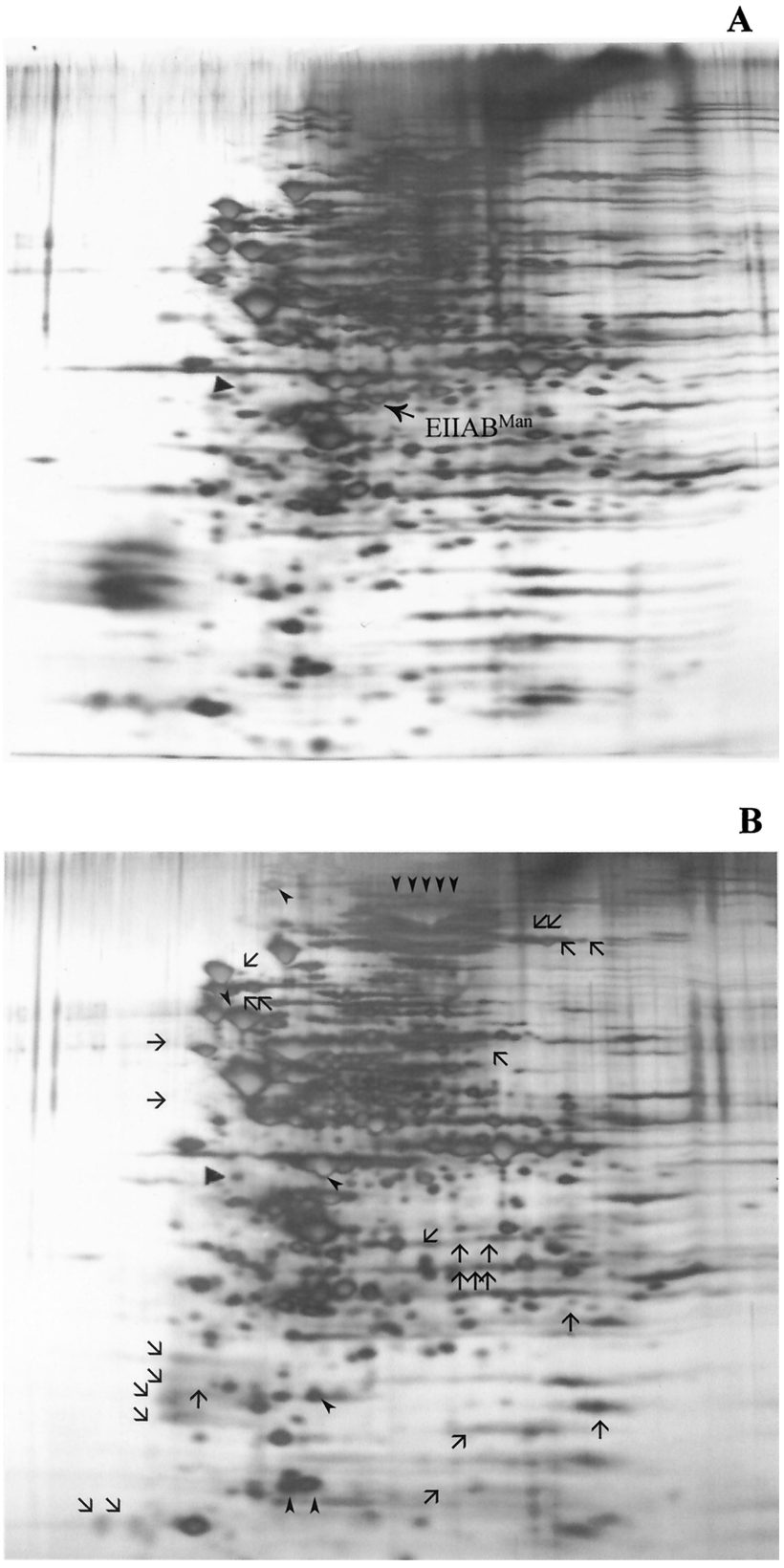

FIG. 6. Silver stained 2-D gels of total cell lysates of UA159 (A) and JAM1 (B) grown in BHI. (A) EIIAB^{Man} (arrow) is indicated in a 2-D gel of proteins from the wild-type strain. (B) Thin arrowheads represent up-regulated proteins, whereas down-regulated proteins are represented by thin arrows. Tropomyosin, the internal control, is indicated by a wide arrowhead in each of the panels.

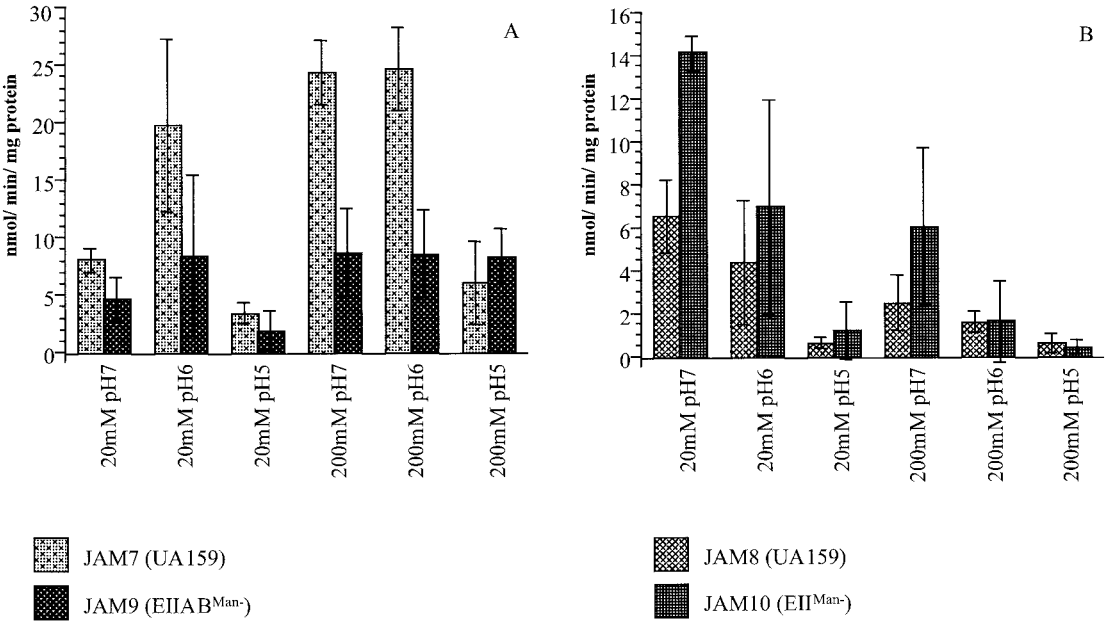

FIG. 7. CAT-specific activity driven by *gtfBC* and *ftf* promoters in the wild-type and *manL*$^-$ backgrounds under carbohydrate excess (200 mM glucose) and carbohydrate-limiting conditions (20 mM glucose) at pH 7, 6, and 5. Values shown are means ± SD from three independent chemostat runs. The results are expressed as nanomoles of Cm acetylated per minute per milligram of protein. (A) CAT activity driven by *gtfBC* promoter; (B) CAT activity driven by *ftf* promoter.

ditions was highest at pH 7, and expression diminished as the pH was lowered, with almost no detectable CAT activity at pH 5 (Fig. 7B). Expression of *ftf* was not significantly influenced by the lack of a functional EIIAB^{Man} under the conditions tested, with one exception. At pH 7 under glucose-limiting conditions, JAM10 had a 2.2-fold increase in CAT activity expressed from the *ftf* promoter (Fig. 7B). The possible reasons for these observations could be the same as those detailed for altered *gtfBC* expression. However, since it seems that loss of ManL does not consistently alter *ftf* expression, it is likely that indirect consequences of *manL* inactivation result in changes in *ftf* expression at neutral pH. It is noteworthy that PTS activity in oral streptococci is optimal around pH 7 and at low carbohydrate concentrations and that activity declines at lower pH values (38), so it is not completely surprising that the effects of *manL* inactivation are manifested when PTS activity is optimal.

Summary. It has been demonstrated that EIIAB^{Man} of *S. mutans* is involved in the transport of glucose and mannose as well as in the uptake of 2-DG. Also, it appears that an additional, lower-affinity 2-DG transporter is operable in *S. mutans*. The use of the ManL-deficient strain in growth and PTS assays revealed a possible role in mannose transport for the inducible fructose PTS permease (FruI) as well as an as-yet-unidentified, mannose-inducible PTS permease. Further, our results suggest the presence of a glucose-inducible glucose PTS and a mannose-inducible fructose PTS. Major changes in protein expression were revealed by proteomic analysis, and the participation of EIIAB^{Man} in CCR in *S. mutans* was unequivocally demonstrated. Finally, the participation of ManL in the expression of two essential virulence genes, *gtfBC* and *ftf*, revealed the importance of ManL in regulation of genes that are affected by carbohydrate source and availability.

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