

The EIIAB^{Man} Phosphotransferase System Permease Regulates Carbohydrate Catabolite Repression in *Streptococcus gordonii*^{∇†}

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Commensal oral streptococci play critical roles in oral biofilm formation and promote dental health by competing with, and antagonizing the growth of, pathogenic organisms, such as *Streptococcus mutans*. Efficient utilization of the spectrum of carbohydrates in the oral cavity by commensal streptococci is essential for their persistence, and yet very little is known about the regulation of carbohydrate catabolism by these organisms. Carbohydrate catabolite repression (CCR) in the abundant oral commensal *Streptococcus gordonii* strain DL-1 was investigated using the exo- β -D-fructosidase gene (*fruA*) and a fructose/mannose sugar:phosphotransferase (PTS) enzyme II operon (*levDEFG*) as model systems. Functional studies confirmed the predicted roles of FruA and LevD in *S. gordonii*. ManL, the AB domain of a fructose/mannose-type enzyme II PTS permease, contributed to utilization of glucose, mannose, galactose, and fructose and exerted primary control over CCR of the *fruA* and *levD* operons. Unlike in *S. mutans*, ManL-dependent CCR was not sugar specific, and galactose was very effective at eliciting CCR in *S. gordonii*. Inactivation of the apparent *ccpA* homologue of *S. gordonii* actually enhanced CCR of *fruA* and *levD*, an effect likely due to its demonstrated role in repression of *manL* expression. Thus, there are some similarities and fundamental differences in CCR control mechanisms between the oral pathogen *S. mutans* and the oral commensal *S. gordonii* that may eventually be exploited to enhance the competitiveness of health-associated commensals in oral biofilms.

The human oral cavity provides a complex and substantial source of nutrients to support the growth of a diverse population of microorganisms, with more than 750 bacterial phylogenotypes already detected in the oral cavity (1, 17, 36). However, a comparatively small subset of genera account for the majority of the population, with the oral streptococci being among the earliest colonizers and the most abundant inhabitants of the human mouth (42). Oral commensal streptococci include members of the sanguinis, salivarius, mitis, and anginosus groups of streptococci, many of which have been associated with dental and periodontal health. In contrast, *Streptococcus mutans*, the major etiologic agent for human dental caries, is not generally found in significant proportions on healthy dental tissues. Instead, this organism thrives when the diet of the host is excessively rich in carbohydrates, due in large part to its enhanced acidogenic and aciduric capacities compared to many commensal streptococci (6, 31, 32). Notably, a number of commensal oral streptococci can inhibit *S. mutans* growth by producing hydrogen peroxide and other inhibitory substances (21, 25, 44, 49, 50). Since it is generally recognized that dental caries has an ecological basis (34, 44), developing a complete picture of the mechanisms that commensals use to gain a selective advantage over cariogenic microbes will help to fac-

ilitate the rational design of technologies that discourage colonization and pathogenesis by undesirable organisms.

Carbohydrates are the principal source of energy for growth of *Streptococcus* spp., which lack a complete respiratory chain. Although there are some examples of carbohydrates that are internalized by streptococci through ABC transporters (4, 5, 43) or symport systems (14), the dominant high-affinity, high-capacity pathway for uptake of carbohydrates is the phosphoenolpyruvate (PEP)-dependent sugar:phosphotransferase system (PTS) (5, 51). The PTS consists of the general component enzyme I (EI) and histidine phosphocarrier protein (HPr), and an array of substrate-specific enzyme II (EII) complexes (37). EI-catalyzed phosphorylation of His15 of HPr by PEP is followed by transfer of the phosphate group to the EIIA and then the EIIB domains of PTS permeases, followed by internalization and concurrent phosphorylation of mono- or disaccharides mediated by a membrane-associated EIIC domain and, in some cases for certain permeases, an EIID domain (37). As with most bacteria, when rapidly metabolizable carbohydrates are present, the genes for utilization of nonpreferred carbohydrates are repressed in streptococci. Thus, carbon catabolite repression (CCR) is common in these bacteria (11, 12), allowing the organisms to optimize energy generation (18).

The primary route for CCR in most low-G+C Gram-positive bacteria involves HPr of the PTS and catabolite control protein A (CcpA), which binds to catabolite response elements (CRE) found in promoter regions of CCR-sensitive genes and regulates their transcription (12). The DNA-binding activity of CcpA is enhanced by interactions with the Ser46-phosphorylated form of HPr (HPr-Ser-P) and in some cases can be stimulated by the presence of certain glycolytic intermediates,

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including fructose-1,6-bisphosphate (12). Although a CcpA homologue in *S. mutans* can affect the regulation of central carbon metabolism (3), CcpA does not play a dominant role in CCR in *S. mutans* (19, 47, 53). Instead, HPr-Ser-P and three EII complexes (EIIAB^{Man}, FruI, and EII^{Lev}) exert primary control over the transport and catabolism of nonpreferred carbohydrates (2, 54, 55) at the transcriptional and posttranscriptional levels.

The metabolic pathways, nutrient requirements, and genomic structure of commensal oral streptococci and *S. mutans* are similar, so they likely compete for common nutrients in the oral cavity. As one of the most abundant oral commensals and an early colonizer of the oral cavity, *Streptococcus gordonii* interacts with various oral bacteria during the formation of oral biofilms and is able to antagonize the growth of *S. mutans* (21, 24, 26, 27). Notably, some differences in regulation of CCR between *S. mutans* and *S. gordonii* are already apparent. In particular, whereas CcpA does not play dominant roles in CCR in *S. mutans* (2, 47, 53–55), a *ccpA* mutant of *S. gordonii* displayed a loss of CCR of the arginine deiminase pathway (15), aberrant expression of amylase-binding protein (41), and loss of penicillin tolerance (7). If, in fact, oral commensals and *S. mutans* manage CCR in fundamentally different ways, it may be possible to design therapeutics that disrupt the ability of *S. mutans* to persist without interfering with the growth of commensals. Using two CCR-sensitive model systems that we show to have similar functions in *S. mutans* and *S. gordonii*, we demonstrate that there are core similarities and notable differences in the global regulation of carbohydrate catabolism by these competing microorganisms.

MATERIALS AND METHODS

Bacterial strains and culture conditions. The wild-type *S. gordonii* strain DL1 and its derivatives used in the present study (see Table S1 in the supplemental material) were maintained in brain heart infusion broth (BHI; Difco Laboratories, Detroit, MI) at 37°C in 5% CO₂ and 95% air with antibiotics added at the following concentrations: kanamycin (1.0 mg ml⁻¹), erythromycin (10 µg ml⁻¹), and spectinomycin (500 µg ml⁻¹), when necessary. *S. gordonii* cultures for chloramphenicol acetyltransferase (CAT) assays and for measuring growth rates were prepared in TV-base medium (9) containing the specified amounts of glucose, fructose, mannose, galactose, or inulin. Cells for PTS assays were cultured in an anaerobic chamber maintained with 85% N₂, 10% H₂, and 5% CO₂. All chemical reagents and antibiotics were obtained from Sigma Chemical (St. Louis, MO). Growth curves of *S. gordonii* strains were generated by using a Bioscreen C monitor (OyGrowth Curves AB, Helsinki, Finland) with readings taken every 30 min. *Escherichia coli* strains were grown in Luria-Bertani medium supplemented with antibiotics, when needed, at the following concentrations: kanamycin (40 µg ml⁻¹), erythromycin (300 µg ml⁻¹), and spectinomycin (50 µg ml⁻¹).

DNA manipulation. Standard recombinant DNA techniques were performed to engineer plasmids. All restriction and modifying enzymes were purchased from Invitrogen (Carlsbad, CA) or New England Biolabs (Beverly, MA) and used as recommended by the supplier. DNA purification was carried out using QiaQuick DNA purification kits purchased from Qiagen (Valencia, CA). All primers (see Table S2 in the supplemental material) were synthesized by Integrated DNA Technologies (Coralville, IA). PCR-ligation mutagenesis was used to replace the coding sequences of *fruA*, *levD*, and *manL* with a nonpolar erythromycin resistance marker (57), as detailed elsewhere (29). To construct a *manL ccpA* double mutant, a 0.9-kbp internal fragment of *ccpA* was PCR amplified and cloned onto pUC19. A nonpolar spectinomycin resistance cassette was subsequently inserted into the *ccpA* gene at an EcoRV site, and the mutation was introduced by competent transformation into a *manL* mutant. All engineered strains were verified by PCR and DNA sequencing, including determining the sequence of the chromosomal content of the arms used for recombination to ensure that no undesired mutations were introduced outside of the gene of interest.

CAT reporter gene fusions to the promoters for *fruA* (*PfruA-cat*), *levD* (*PlevD-cat*), and *manL* (*PmanL-cat*) were constructed using plasmid pJL84 (54) by inserting a promoter-containing DNA fragment, including the cognate ribosome-binding site, in front of a promoterless *cat* gene from *Staphylococcus aureus* that lacked a ribosome-binding site (58). The promoter-*cat* fusion was released by restriction digestion with SphI and SacI and subcloned into the integration vector pMJB8, which allows for delivery in single-copy of DNA inserts into the *gtfG* gene on the chromosome of *S. gordonii* (10). All gene fusions were confirmed by DNA sequencing prior to transforming *S. gordonii* and the correct conformation of the integration cassette in the resultant isolates was confirmed by PCR.

Biochemical assays. Bacterial cultures were grown to mid-exponential phase in 30 ml of TV-base medium supplemented with various carbohydrates, and the cells were collected by centrifugation, washed once with the same volume of 10 mM Tris buffer (pH 7.8), resuspended in 750 µl of the same buffer, and then homogenized in a Bead Beater (Biospec Products, Bartlesville, OK) for 30 s, twice, with a 2-min interval on ice. After centrifugation at 18,000 × *g* for 10 min at 4°C, the supernatant fluid was recovered and used for measuring the CAT activity by the method of Shaw (45). The protein concentration of the lysates was determined by the Bradford (Bio-Rad) or bicinchoninic acid (BCA; Thermo Scientific) assay. CAT activity was expressed as nmol of chloramphenicol acetylated mg of protein⁻¹ min⁻¹.

PEP-dependent PTS assays were carried out according to protocols described elsewhere (30) with minor modifications. Briefly, 30 ml of a culture growing anaerobically in TV-base medium with 0.5% of the specified carbohydrate was harvested in late exponential phase (optical density at 600 nm [OD₆₀₀] = 0.5 to 0.6), washed twice with 30 ml of 100 mM Na-K-PO₄ buffer (pH 7.2) containing 5 mM MgCl₂, resuspended in 1 ml of the same buffer, and then permeabilized by vortexing with 50 µl of toluene-acetone (1:9, vol/vol) for 2 min, twice, with a 2-min interval on ice. Anaerobically grown cells were utilized in these assays because cells that were grown in the presence of oxygen had high levels of spontaneous oxidation of NADH that interfered with the assay, apparently associated with induction of NADH oxidases. Permeabilized cells were then subjected to PTS assays (30). A BCA assay was used to measure protein concentrations of the samples, and the PTS activity was expressed as nmol of NADH oxidized mg of protein⁻¹ min⁻¹ in a PEP-dependent manner.

RESULTS

Identification of *fruA*, *levD*, and *manL* homologues in *S. gordonii*. To find a homologous exo-β-D-fructosidase (*fruA*) gene in the genome of *S. gordonii* strain DL1, the protein sequence of *fruA* from *S. mutans* (SMU.78; GenBank locus tag) was used in a BLAST search against the *S. gordonii* Challis genome sequence at <http://www.oralgen.lanl.gov>. A homologous open reading frame (ORF) SGO_0385 (Oralgen gene ID), annotated also as an exo-β-D-fructosidase, was identified. The *S. gordonii fruA* gene was predicted to encode a polypeptide of 1,408 amino acid residues with 95, 58, and 56% identity to β-fructosidases from *Streptococcus sanguinis*, *S. mutans*, and *Streptococcus salivarius*, respectively. The second most closely related gene product identified during the BLAST search was a sucrose-6-phosphate hydrolase (SGO_1302). To verify the function of *fruA* as a fructosidase, a *fruA* mutant was constructed by allelic exchange with a nonpolar erythromycin resistance determinant (*em*) and tested for growth in TV medium containing 0.05% glucose and 0.5% of the β₂,1-linked fructose homopolymer inulin. Whereas the wild-type strain grew normally, presenting with a typical diauxic growth curve, the *fruA* mutant stopped growing after the glucose was exhausted (Fig. 1). Therefore, the phenomenon provided an indication that the *fruA* gene of *S. gordonii* encodes a functional fructan hydrolase. Additional tests confirmed that the *fruA* mutant had no defect in the utilization of glucose and could not grow at all if inulin was the only carbohydrate source in the medium (data not shown).

Similarly, the protein sequence of a fructose/mannose-spe-

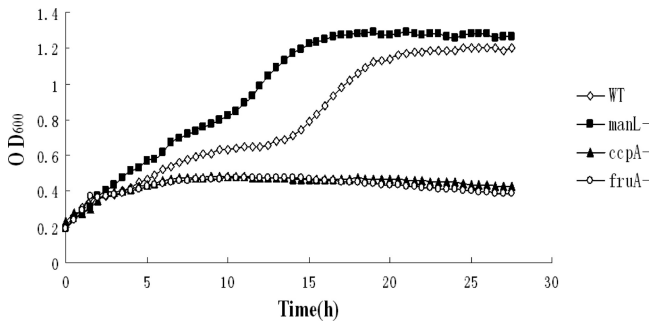


FIG. 1. Growth of *S. gordonii* wild-type strain DL1 (◇) and *manL* (■), *ccpA* (▲), and *fruA* (○) mutants in TV supplemented with 0.05% glucose and 0.5% inulin.

cific enzyme IIA component from *S. mutans* (*levD*, SMU.1961c) was used in a BLAST search against the *S. gordonii* genome sequence. An ORF, SGO_1893, annotated as a fructose (mannose)-specific IIA component of the PTS system was identified as the first gene in a four-gene operon encoding the IIA component (*levD*) and the IIB, IIC, and IID domains of a fructose/mannose-type permease, encoded by *levE*, *levF*, and *levG*, respectively. The *levD* gene was predicted to encode a polypeptide of 145 amino acids with 80, 68, and 53% identities to fructose PTS IIA components of *S. sanguinis*, *S. mutans*, and *Streptococcus uberis*, respectively. Also identified was a glucose/mannose-specific PTS IIB component (SGO_1679) that was designated *manL*. The *manL* gene of *S. gordonii* was predicted to encode a polypeptide of 329 amino acid residues with 94, 91, 87, and 83% identities to the gene for IIB^{Man} of *Streptococcus pneumoniae*, *S. sanguinis*, *S. salivarius*, and *S. mutans*, respectively, and was part of an apparent operon encoding the IIC and IID components of the EII^{Man} permease.

Characterization of *manL* and *levD* mutants of *S. gordonii*. To assess the function of *manL* and *levD* in *S. gordonii*, allelic replacement mutants were constructed in the background of strain DL1 using a nonpolar *em* cassette. To determine the role of *manL* and *levD* in carbohydrate transport, growth of the wild-type strain and the *manL* and *levD* mutants in TV medium supplemented with 0.2% of glucose, fructose, mannose, or galactose was monitored using a Bioscreen C. Compared to the wild-type strain, the growth rate of the *manL* mutant was slower in glucose and mannose, dramatically slower in galactose, but only modestly slower on fructose (Table 1), suggesting that ManL may participate in the uptake of glucose, mannose, and galactose by *S. gordonii*. In contrast, the *levD* mutant grew slower than DL1 in the presence of fructose or mannose,

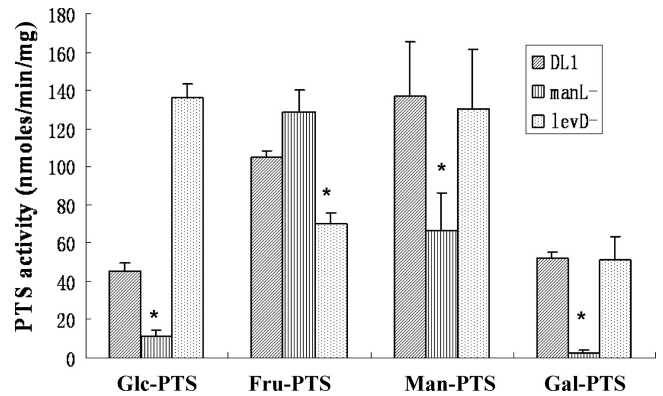


FIG. 2. PEP-dependent PTS activities of *S. gordonii* DL1 and *manL* and *levD* mutants grown anaerobically in 0.5% of glucose, fructose, mannose, or galactose were measured using 10 mM glucose, fructose, mannose, or galactose, respectively, as the substrate. An asterisk represents a *P* value of <0.05 (Student *t* test) compared to DL1. Glc, glucose; Fru, fructose; Man, mannose; Gal, galactose.

which is indicative of a role of LevDEFG in the transport of these hexoses. There were no significant differences in doubling times between the wild-type strain and the *levD* mutant when grown in glucose.

To verify the role in sugar transport by ManL and LevD, DL1 and the *manL* and *levD* mutants were cultured anaerobically in TV with 0.5% glucose, fructose, mannose, or galactose. Cells were collected in mid-exponential phase, and the PTS specific activities were measured by using glucose, fructose, mannose, or galactose as substrates. Compared to strain DL1, the *manL* mutant had significantly decreased PTS activities for glucose, mannose, and galactose but slightly increased activity for fructose. The *levD* mutant showed significantly lower PTS activity for fructose, significantly higher activity for glucose, and little change in mannose and galactose PTS activity (Fig. 2). These results further support that ManL participates in the transport of glucose, galactose, and mannose and may also be involved in the utilization of fructose, whereas LevD appears to be required primarily for the internalization of fructose.

CCR of the *fruA* and *levD* genes of *S. gordonii*. We examined how the expression of *fruA* and *levD* was regulated at the transcriptional level in response to different carbohydrates. A *PfruA-cat* and a *PlevD-cat* fusion were constructed by fusing a DNA fragment containing the *fruA* or *levD* promoters to a promoterless *cat* gene on an integration vector and establishing the promoter fusion in single copy at a remote site in the *S. gordonii* chromosome. Strain DL1 was transformed with the

TABLE 1. Doubling times and maximal OD₆₀₀ values for wild-type strain DL1 and *manL* and *levD* mutants growing in TV medium supplemented with 0.2% glucose, fructose, mannose, or galactose as the carbohydrate source

Strain	<i>T_d</i> (h) and OD ₆₀₀ (SD) ^a							
	Glucose		Fructose		Mannose		Galactose	
	<i>T_d</i>	OD ₆₀₀	<i>T_d</i>	OD ₆₀₀	<i>T_d</i>	OD ₆₀₀	<i>T_d</i>	OD ₆₀₀
DL1	2.03 (0.02)	0.51 (0.01)	2.30 (0.01)	0.49 (0.01)	2.29 (0.02)	0.55 (0.01)	5.92 (0.02)	0.79 (0.03)
<i>manL</i> mutant	2.70 (0.01)*	0.54 (0.01)	2.67 (0.02)*	0.46 (0.02)	3.98 (0.01)*	0.59 (0.01)	25.57 (0.02)*	0.43 (0.02)*
<i>levD</i> mutant	2.01 (0.01)	0.53 (0.01)	2.59 (0.01)*	0.47 (0.01)	2.67 (0.01)*	0.50 (0.01)	5.76 (0.01)*	0.78 (0.02)

^a *T_d*, doubling time; *, data significantly different from that in the DL1 background in corresponding carbohydrates, as verified by the Student *t* test (*P* < 0.01).

TABLE 2. CAT specific activities representing the expression levels of *PfruA-cat* and *PlevD-cat* fusions in the wild-type background growing on various carbohydrates

Fusion	CAT activity ^a (SD) in cells grown on the indicated carbohydrate						
	Glc	Fru	Gal	Inulin	Glc/inulin	Fru/inulin	Gal/inulin
<i>PfruA-cat</i>	0.0 (0.0)	0.0 (0.0)	0.0 (0.0)	24.8 (1.6)	7.5 (1.0)	0.0 (0.0)	9.5 (0.4)
<i>PlevD-cat</i>	14.9 (1.9)	8.9 (2.4)	21.0 (5.7)	630.1 (27)	235.6 (19)	12.9 (2.4)	338.7 (49)

^a CAT activity is expressed as nmol of chloramphenicol acetylated mg of protein⁻¹ min⁻¹, followed by the standard deviation in parentheses. The data are averages from at least three independent cultures, and all assays were performed in triplicate. The carbohydrate concentration in the growth medium was 0.5% (wt/vol). Glc, glucose; Fru, fructose; Gal, galactose.

gene fusion constructs, and the resultant strains were cultured in TV medium containing 0.5% each of glucose, fructose, galactose, or inulin or a combination of inulin and glucose, fructose, or galactose and then subjected to CAT assays. As shown in Table 2, the activities of the *fruA* and *levD* promoters were optimal when cells were growing on inulin but were lower when cells were growing on the preferred carbohydrates glucose or fructose. Expression from these promoter fusions was also repressed in cells growing on the combination of inulin and glucose, galactose, or fructose. Therefore, similar to the homologous systems in *S. mutans*, transcription of *fruA* and *levD* is inducible by the presence of a fructan polymer and repressed when preferred carbohydrates are present. Notably, CCR was most evident in cultures containing fructose, whereas CCR of *fruA* in *S. mutans* is elicited most effectively by glucose. Also of note, galactose caused apparent repression of the expression of *fruA*, but galactose is essentially a nonrepressing carbohydrate in *S. mutans*.

CCR is not abolished by inactivating *ccpA*. To determine the underlying basis for the sensitivity of *fruA* to CCR, *S. gordonii* DL1 and a *ccpA* mutant derived from DL1 (15) were grown in TV medium supplemented with 0.05% of glucose and 0.5% of inulin (Fig. 1). DL1 displayed classic diauxic growth, whereas the *ccpA* mutant achieved a level of growth comparable to that observed for the *fruA* mutant. The *ccpA* mutant of *S. gordonii* grows nearly as well as the parental strain on glucose alone (15), so it appears that the *ccpA* mutant lost its capacity to utilize inulin. To investigate the role of CcpA in CCR of the *fruA* and *levDEFG* genes in *S. gordonii*, *PfruA-cat* and *PlevD-cat* fusions were introduced into the *ccpA* mutant, and the CAT activity was measured in various growth carbohydrates (Tables 3 and 4). Consistent with the growth phenotype of the *ccpA* mutant on the combination of glucose and inulin, the expression of *fruA* was ~120-fold lower in the *ccpA* mutant

when cells were grown on inulin. Expression of *levD* was lower in the *ccpA* mutant across all five carbohydrates tested. Thus, consistent with the poor growth of the *ccpA* mutant of *S. gordonii* on inulin (Fig. 1), inactivation of CcpA might have led to strong inhibition of inulin-induced *fruA* and *levD* expression. Since it is known that CcpA can function as a transcriptional activator for certain genes and operons (12), it might be possible that loss of CcpA leads to lack of activation of *fruA* and *levD* expression.

A repressive effect of CcpA on expression of *fruA*, but not *levD*, can be observed. In *S. mutans*, the expression of the *fruA* and *levD* operons is controlled by a four-component regulatory system, LevQRST, which senses the presence of the inducing substrates fructose or mannose (54, 58). Due to relatively dominant regulation by CcpA-independent CCR mechanisms, CCR of *fruA* via CcpA in *S. mutans* was only apparent when expression was monitored in a fructose-pulsing experiment (3). That is, evidence of CCR by CcpA could only be observed when cells were grown to mid-exponential phase in a noninducing carbohydrate and then the cultures were pulsed with inducer for 1 to 3 h. In a similar experiment, *S. gordonii* carrying the *PfruA-cat* or *PlevD-cat* fusion was incubated in TV with 0.5% glucose to early exponential phase ($OD_{600} \cong 0.2$) and then treated for 3 h with various concentrations of fructose or inulin before being harvested for CAT assays. As shown in Table 5, there was an ~5-fold increase in *fruA* expression when the fructose concentration increased from 0.05 to 7.5 mM, with a clear dose-dependent response to the presence of fructose by the *PlevD-cat* strain (Table 5). Similarly, when inulin was used to pulse these strains, there was a concentration-dependent increase in the expression of *levD* in response to the addition of inulin.

Since inulin is a polymer of fructose, the induction effect on *fruA* and *levD* likely results from degradation of inulin by FruA

TABLE 3. *PfruA-cat* specific activities in the wild-type background and in *manL*, *ccpA*, and *manL ccpA* mutants growing on various carbohydrates^a

Strain	CAT activity (SD) in cells grown on the indicated carbohydrate				
	Glucose	Fructose	Galactose	Inulin	Mannose
DL1	0.0 (0.0)	0.0 (0.0)	0.0 (0.0)	24.8 (1.6) ^A	0.1 (0.1)
<i>manL</i> mutant ^B	1.9 (0.5)	1.1 (0.2)	2.1 (0.6)	42.5 (4.7)	50.0 (3.0)
<i>ccpA</i> mutant	0.0 (0.0)	0.0 (0.0)	0.0 (0.0)	0.2 (0.2) ^C	0.0 (0.0)
<i>manL ccpA</i> mutant ^D	1.2 (0.4)	0.8 (0.0)	0.9 (0.1) ^E	49.7 (12.1)	31.8 (2.6) ^E

^a CAT activity is expressed as nmol of chloramphenicol acetylated mg of protein⁻¹ min⁻¹, followed by the standard deviation in parentheses. The data are averages from at least three independent cultures, and all assays were performed in triplicate. The carbohydrate concentration in the growth medium was 0.5% (wt/vol). Superscript capital letters indicate significance as follows. Superscript A, data statistically different from that in other sugars in the DL1 background, as verified by the Student *t* test ($P < 0.01$). Superscripts B, C, and D, data statistically different from that in the DL1 background in the corresponding sugars, as verified by the Student *t* test ($P < 0.05$). Superscript E, data statistically different from that in the *manL* mutant, as verified by the Student *t* test ($P < 0.05$).

TABLE 4. *PlevD-cat* activities in the wild-type background and *manL*, *ccpA*, and *manL ccpA* mutants growing in various carbohydrates^a

Strain	CAT activity (SD) in cells grown on the indicated carbohydrate				
	Glucose	Fructose	Galactose	Inulin	Mannose
DL1	14.9 (1.9)	8.9 (2.7)	21.0 (5.7)	630.1 (27.1)	23.8 (2.7)
<i>manL</i> mutant ^A	44.2 (2.0)	19.4 (1.7)	108.6 (11.3)	1193 (243)	1340 (46)
<i>ccpA</i> mutant	0.0 (0.0)	3.6 (1.7)	0.1 (0.1)	0.7 (0.2)	0.02 (0.0)
<i>manL ccpA</i> mutant ^B	38.4 (7.7)	36.4 (5.9) ^C	26.1 (1.5) ^C	1055.0 (60)	966.3 (93) ^C

^a CAT activity is expressed as nmol of chloramphenicol acetylated mg of protein⁻¹ min⁻¹, followed by the standard deviation in parentheses. The data are averages from at least three independent cultures, and all assays were performed in triplicate. The carbohydrate concentration in the growth medium was 0.5% (wt/vol). Superscript capital letters indicate significance as follows. Superscript A and B, data are significantly different from that in the DL1 background in the corresponding carbohydrates, as verified by the Student *t* test (*P* < 0.05). Superscript C, data significantly different from that in the *manL* mutant, as verified by the Student *t* test (*P* < 0.05).

and release of free fructose, which serves as the inducing signal. A *fruA* knockout mutant was constructed, and the ability to induce *PlevD::cat* expression by inulin was tested in the mutant. As shown in Table 5, no induction above the level seen in the negative control (no inulin) was evident with inulin, although the strain remained responsive to induction by fructose (Table 5). Therefore, as in *S. mutans*, inulin itself does not appear to be an inducing signal for the expression of *levD*, and instead the fructose released from hydrolysis of inulin appears to function as the most effective inducing signal (Table 2). Notably, in both organisms, the steady-state levels of fructose liberated from inulin by FruA remain below those needed to elicit substantial CCR (9).

When the expression from the *PfruA-cat* and *PlevD-cat* fusions in response to pulsing by fructose was assessed there was a 2- to 5-fold derepression for *fruA* in the *ccpA* mutant (Table 5). However, expression of *levD* in the *ccpA* mutant under the same conditions was always lower than that measured in the wild-type background. Notably, no apparent CRE sequences could be detected near the promoter regions of either *fruA* or *levD*.

Disruption of enzyme II^{Man} (*manL*) eliminates CCR of *fruA* and *levD*. Given the importance of ManL in CCR of *S. mutans*, a *manL* deletion in *S. gordonii* was engineered, and its impact on CCR was investigated. When the mutant was tested for diauxic growth in TV medium containing 0.05% glucose and 0.5% inulin, diauxic growth was nearly eliminated (Fig. 1).

When *fruA* and *levD* gene expression was measured using the *PfruA-cat* and *PlevD-cat* fusions in the *manL* mutant background (Tables 3 and 4), both genes were derepressed relative to the expression levels in the wild-type background in all of the carbohydrates tested. The greatest level of derepression, at least according to data from *levD* expression, was seen in mannose cultures. Although a similar fold change in expression was seen with glucose and galactose, the amount of derepression seen in the ManL-deficient strain was lowest when fructose was the growth carbohydrate. When the cells were grown in TV-inulin, an ~3-fold derepression for both genes was observed in the *manL* mutant strain compared to the wild-type background.

CcpA negatively regulates *manL* expression. Since we have shown that disruption of CcpA results in decreased expression of *fruA* and *levD* under most conditions (Tables 3 and 4) and that loss of ManL generally leads to relieved CCR, it is possible that CcpA could indirectly influence CCR by regulating *manL*, as appears to be the case for *S. mutans* (54). A *ccpA* deletion was introduced into the *manL* mutant strain and expression of *PfruA-* and *PlevD-cat* fusions in the double mutant background was monitored. When grown in TV supplemented with 0.5% of glucose, fructose, galactose, inulin, or mannose, the *ccpA manL* double mutant produced CAT activities at significantly higher levels than in the wild-type strain or the *ccpA* mutant (Tables 3 and 4). Compared to the strain carrying the *manL* mutation alone, the level of derepression seen in the *ccpA*

TABLE 5. *PfruA-cat* and *PlevD-cat* activities measured in wild-type and *ccpA* and *fruA* mutant backgrounds under fructose or inulin pulsing^a

Strain and fusion	CAT activity (SD) in cells pulsed with the indicated concentration of fructose or inulin				
	0 mM	0.05 mM	3 mM	5 mM	7.5 mM
Pulsed with fructose					
DL1/ <i>PfruA-cat</i>	0.00 (0.00)	0.03 (0.02)	0.03 (0.01)	0.04 (0.02)	0.16 (0.10)
<i>ccpA</i> mutant/ <i>PfruA-cat</i>	0.00 (0.00)	0.08 (0.09)	0.16 (0.13)	0.15 (0.10)	0.49 (0.08)*
DL1/ <i>PlevD-cat</i>	6.13 (1.92)	7.46 (1.47)	19.52 (5.38)	23.07 (2.40)	34.98 (0.56)
<i>ccpA</i> mutant/ <i>PlevD-cat</i>	0.62 (0.12)	1.03 (0.17)	17.84 (3.31)	21.23 (1.19)	24.84 (0.78)
<i>fruA</i> mutant/ <i>PlevD-cat</i>	6.73 (0.19)	7.40 (0.48)	26.46 (3.37)	38.80 (5.20)	17.45 (2.69)
Pulsed with inulin					
DL1/ <i>PfruA-cat</i>	0.00 (0.00)	0.33 (0.02)	0.10 (0.01)	0.14 (0.02)	
DL1/ <i>PlevD-cat</i>	5.28 (2.80)	5.77 (1.41)	8.28 (0.44)	15.23 (2.64)	
<i>fruA</i> mutant/ <i>PlevD-cat</i>	6.73 (0.19)	7.02 (0.85)	6.65 (0.23)	6.68 (0.46)	

^a The cells were cultured in TV containing 0.5% glucose to an OD₆₀₀ of 0.2, and then different concentrations of fructose or inulin were added. Cells were incubated for 3 h and harvested for CAT assays as detailed in Materials and Methods. CAT activity is expressed as nmol of chloramphenicol acetylated mg of protein⁻¹ min⁻¹, followed by the standard deviation in parentheses. The data are averages from at least three independent cultures, and all assays were performed in triplicate. The carbohydrate concentration in the growth medium was 0.5% (wt/vol). *, data statistically different from that in the DL1 background, as verified by the Student *t* test (*P* < 0.05).

TABLE 6. *PmanL-cat* activities measured in wild-type and *ccpA*, *manL*, and *manL ccpA* mutant backgrounds growing on various carbohydrates^a

Strain	CAT activity (SD) in cells grown on the indicated carbohydrate		
	Glucose	Fructose	Galactose
DL1	16.1 (0.8)	12.6 (1.4)	51.6 (2.5)
<i>ccpA</i> mutant	48.9 (2.9)	58.3 (1.1)	67.9 (6.5)
<i>manL</i> mutant	22.0 (6.5)	21.5 (1.5)	131.5 (67.8)
<i>ccpA manL</i> mutant	416.4 (51.3)	375.1 (70.9)	449.4 (86.3)

^a CAT activity is expressed as nmol of chloramphenicol acetylated mg of protein⁻¹ min⁻¹, followed by the standard deviation in parentheses. The data are averages from at least three independent cultures, and all assays were performed in triplicate. The carbohydrate concentration in the growth medium was 0.5% (wt/vol).

manL double mutant was similar. However, the double mutant displayed lower activity when galactose or mannose was used to culture the cells (Tables 3 and 4). These results provide support for the idea that CcpA could influence expression of *fruA* and *levD* by affecting the amount of ManL in the cells.

To further test whether CcpA regulated *manL* expression, a *PmanL-cat* fusion was constructed and introduced into the wild-type and *ccpA* mutant strains. Upon growth in glucose, fructose, or galactose, expression of the *PmanL-cat* fusion was significantly higher in the *ccpA* mutant relative to that in the wild-type background (Table 6), a finding indicative of negative regulation of *manL* by CcpA. Also of note, *manL* expression was higher in galactose than in glucose or fructose in the wild-type background, possibly due to less efficient induction of CCR through CcpA by galactose, compared to glucose or fructose. Galactose should flow through the tagatose pathway in *S. gordonii*, yielding less glucose-6-phosphate (G-6-P) and fructose-1,6-bisphosphate (F-1,6-bP) than would growth on glucose or fructose. Since G-6-P and F-1,6-bP are activators of HPr kinase (12), CcpA-dependent repression of *manL* would then be less severe in cells growing on galactose. Consistent with this notion, there was little difference in the expression levels of *manL* in response to the various sugars tested in a strain lacking CcpA (Table 6).

Another possible cause for decreased activities of the *fruA* and *levD* promoters in the *ccpA* mutant is a potential shift in the phosphorylation status of the phosphocarrier protein HPr, since enhanced phosphorylation of HPr on Ser46 has been reported in a *ccpA* mutant of *Bacillus subtilis* (33). Also, increased seryl-phosphorylation of HPr in *S. mutans* negatively affected the expression of both *fruA* and *levD* independently of CcpA (55). Therefore, an anti-HPr antiserum was used in Western blots to detect the levels of seryl-phosphorylated and unphosphorylated HPr in the wild-type and various mutant backgrounds. Lysates were prepared from cells growing exponentially in BHI and separated by nondenaturing polyacrylamide gel after treatment at 100°C for 5 min to remove any phosphate from His15, as described elsewhere (16, 48, 55). Significantly higher levels of HPr-Ser-P were produced in the strain lacking CcpA (Fig. 3) than in the wild-type strain. The increases in Ser46-phosphorylated HPr were less pronounced in the *ccpA manL* double mutant background, despite a clear increase in the overall abundance of the HPr protein. This observation correlates well with the observed elevated CCR of

the *fruA* and *levD* genes in the *ccpA* mutant background and the alleviation of CCR when *manL* was deleted along with *ccpA* (Tables 3 and 4). Importantly, since little change in HPr-Ser-P levels was seen in the strain lacking only *manL*, loss of CCR in the *manL ccpA* double mutant cannot be attributed solely to the change in HPr phosphorylation status.

Autoregulation of *manL*. To investigate whether ManL of *S. gordonii* could influence *manL* expression, *PmanL-cat* fusion activity was measured in the *manL* mutant. The results (Table 6) showed that expression from the *manL* promoter was derepressed in the *manL* mutant relative to the wild-type strain when growing in galactose, although much smaller changes were seen when cells were growing in glucose or fructose. Again, this difference could have arisen from the variation in the efficiency of induction of CCR by these sugars. Indeed, when *manL* expression levels were determined in a *manL ccpA* double mutant, expression was highly derepressed in all of the sugars tested, indicating that the differential expression in the various sugars was related to more efficient CcpA-dependent CCR of *manL* in glucose and fructose, compared to galactose. A potential catabolite response element (CRE; TGAAAACG TTTTAT) was also identified in the promoter region of *manL*, which differs only slightly from the consensus CRE sequence (TGWANANCGNTNWCA) in Gram-positive bacteria (23). Notably, members of our group have recently made the observation that ManL in *S. mutans* can affect expression of the *manL* operon (L. Zeng and R. A. Burne, unpublished data).

DISCUSSION

Carbohydrate utilization and the global regulatory pathways modulating sugar catabolism are intimately related to persistence and bacterial virulence in a number of important pathogens, including *S. mutans*, *Streptococcus pyogenes*, and *S. pneumoniae* (2, 3, 20, 46). The microbial communities of the oral cavity harbor hundreds of bacterial phylotypes and intense interspecies competition exists between bacteria that depend on the limited nutrients available in these biofilms (8, 35, 38, 52). The interactions between oral commensals and oral patho-

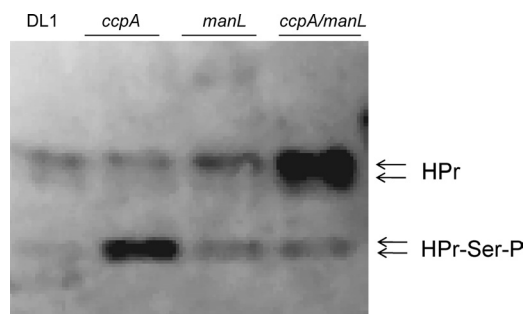


FIG. 3. Western blot analysis of HPr of *S. gordonii* DL1 and *ccpA*, *manL*, and *ccpA manL* mutants. Cells were cultured in BHI broth and cell lysates were prepared as described in the text. All samples were treated at 100°C for 5 min to eliminate His15-phosphorylated HPr prior to resolving the proteins on a 12.5% nondenaturing polyacrylamide gel. The doublets of both HPr (top) and HPr-Ser-P (bottom) represent the full-length protein and HPr without the N-terminal methionine (16, 48). The antisera, kindly provided by Christian Vadeboncoeur, was raised against purified HPr from *Streptococcus salivarius* and used as previously described (55).

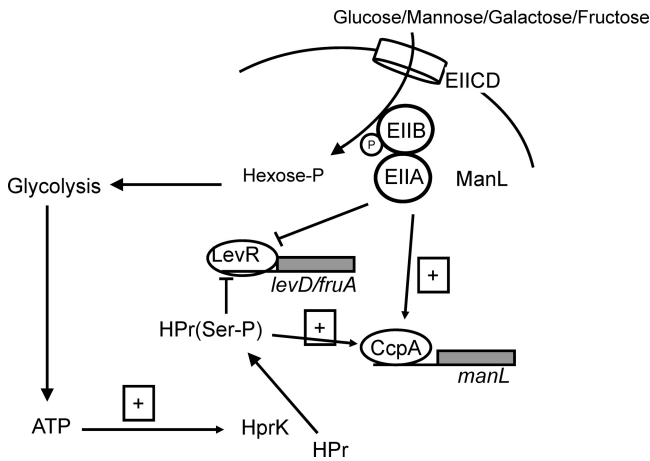


FIG. 4. Model depicting carbohydrate catabolite repression of the *levD*, *fruA*, and *manL* genes in *S. gordonii*. Binding of the transcriptional activator LevR to the *levD* (or *fruA*) promoter is negatively impacted by HPr(Ser-P) and underphosphorylated ManL. Conversely, negative regulation of *manL* by CcpA could be augmented by ManL and HPr(Ser-P). Similar to the findings in *S. mutans*, ManL in *S. gordonii* is capable of repressing the expression of *levD* and *fruA*, regardless of whether CcpA is present. Whereas CcpA-independent CCR in *S. mutans* is strongly elicited by glucose or mannose and but not particularly effective when fructose or galactose is the growth carbohydrate, ManL-dependent CCR in *S. gordonii* does not show such sugar specificity.

gens are critical in determining the “healthy” or “pathogenic” status of dental plaque (22, 24, 28, 39). Whereas in most low G+C Gram-positive bacteria, CcpA and its corepressor HPr-Ser-P are primary effectors of CCR, CcpA-independent CCR dominates the regulation of a variety of catabolic pathways in *S. mutans*, including *fruAB*, *levDEFG*, and the cellobiose and lactose operons (54, 55, 58). These observations with *S. mutans* raised the question of whether the evolution of CCR in this pathogenic organism was unique to *S. mutans* or perhaps reflected a general divergence in CCR control in organisms that have as their natural habitat the tissues of the mouth. From the present study, it appears that at least one abundant commensal governs the expression of two homologous CCR-sensitive operons in similar ways to *S. mutans* but that there are some notable differences in the roles of ManL and CcpA in regulation of carbohydrate utilization (Fig. 4).

Characterization of various mutants constructed in the present study demonstrated strong conservation in function and regulation of apparent homologues of *fruA*, *levDEFG*, and *manL* between *S. mutans* and *S. gordonii*. Similar to what has been found in *S. mutans*, FruA is required for utilization of fructose polymers by *S. gordonii*, and the *levDEFG* operon is primarily responsible for internalization of fructose. Also similar to *S. mutans*, the substrates of EII^{Man} appear to include glucose, mannose, galactose, and even fructose. Importantly, the LevQRST system required for the expression of both the *fruA* and the *levDEFG* operons in *S. mutans* is conserved in sequence and function in *S. gordonii*. Specifically, inactivation of the *levR* gene, encoding the response regulator of this four-component signal transduction system led to a complete loss of activation of both operons by inducing substrates (data not shown). Consequently, the high degree of conservation in

these model genes and their regulatory elements allowed for a reliable contrast of the CCR regulatory pathways between the two organisms.

A number of studies support a role for CcpA in catabolite repression and carbohydrate-dependent regulation of gene expression in *S. gordonii*, and it has been shown that CcpA can bind to CRE sequences in this organism to effect changes in transcription of target genes. These include the CCR-sensitive arginine deiminase (AD) genes, which are tightly repressed in the presence of glucose (15). Inactivation of CcpA resulted in a profound loss of glucose sensitivity for AD expression (15), as did deletion of the CRE sequence 5' to the *arcA* gene (57), supporting that the classic CcpA-dependent pathway for gene expression is functional in *S. gordonii*. Other examples of glucose-dependent repression of gene expression by CcpA in *S. gordonii* include the gene for amylase-binding protein A gene (*abpA* [41]). In contrast, although loss of CcpA has a global impact on gene expression in *S. mutans* (3), only a minor impact on CCR of genes in a *ccpA* mutant have been demonstrated and only under very specific *in vitro* conditions (3). Otherwise, there is no evidence for alleviation of CCR in a *ccpA* mutant during growth in the presence of combinations of inducing and repressing substrates, either at the gene expression level or by assessing diauxic growth of the mutant strains. This statement also applies to the agmatine deiminase (AgD) pathway of *S. mutans*. The AgD system is highly similar to the AD pathway of *S. gordonii* in biochemical terms and in regulation of expression, with both requiring substrate for induction and having sensitivity to CCR. However, unlike the AD gene in *S. gordonii*, inactivation of CcpA in *S. mutans* did not relieve CCR of AgD gene expression (19).

Although we were able to create conditions in which CcpA could be shown to have an impact on *fruA* and *levD* expression, the lack of a CRE sequence near the promoter regions of these genes in *S. gordonii* implies that the observed minor impact of CcpA on repression of these operons is indirect. In contrast, expression of *fruA* in *S. mutans* can be controlled by CcpA binding to two CREs near the *fruA* promoter (3). The work presented here provides the first evidence to demonstrate that CcpA-independent CCR plays major roles in regulating genes in response to carbohydrates in an oral commensal. Specifically, deletion of *manL* had the most profound effect on diauxic growth and resulted in high-level expression of *fruA* and *levD* in all carbohydrates tested. Notably, inactivation of *ccpA* in *S. gordonii* caused a lower level expression of the *fruA* and *levD* operons. Our data demonstrating that the effect of CcpA on repression could be eliminated in a ManL-deficient strain and the use of mRNA measurements and gene fusions to show that CcpA repressed *manL* expression demonstrated that the effects of CcpA on *levD* and *fruA* transcription were almost entirely attributable to the role of CcpA in the negative regulation of *manL*. The mechanism by which CCR is effected by ManL has not been definitively determined, but available evidence suggests that allosteric interactions with regulatory proteins or phosphorelay involving ManL and possibly other PTS components could be underlying mechanisms for ManL-dependent CCR (54, 55).

Another significant finding in the present study was that a dramatic increase in the amount of the Ser46-phosphorylated HPr protein was observed in the *ccpA* mutant. Introduction of

the *manL* mutation into the *ccpA* mutant strain reversed this effect, with the double mutant having lower levels of Ser-46-HPr, but also the double mutant had higher overall levels of HPr protein (Fig. 3) than the wild-type strain. Since CCR of the *fruA* and *levD* genes was enhanced in the *ccpA* mutant and abolished in the *ccpA manL* background, this finding raises the possibility that HPr-Ser-P is also an effector of CcpA-independent CCR of these model genes in *S. gordonii*, similar to what we recently reported for *S. mutans* (55). We propose that both ManL and HPr-Ser-P participate in CCR in *S. gordonii*, since deletion of *manL* alone did not elicit any changes in the levels of HPr-Ser-P (Fig. 3). The potential for ManL to influence CCR by modulating the expression and activities of the gene for HPr, *ptsH*, cannot be excluded at this time. However, our laboratory previously reported that altered expression of the *ptsH* and *ptsI* (EI) genes could be observed in a *manL* mutant of *S. mutans* (2).

Another important difference in the regulation of CCR between *S. mutans* and *S. gordonii* has also been disclosed by the present study. In *S. mutans*, CCR exerted through PTS permeases displays a clear sugar specificity, with greater effects on CCR being evident when the cognate or preferred carbohydrates for individual permeases are present. For example, EIIAB^{Man} confers CCR mainly in the presence of glucose and mannose (2), which is consistent with its role in glucose and mannose uptake, whereas FruI and EII^{Lev} only exert CCR efficiently in the presence of their preferred substrate, fructose (54). It has therefore been proposed that underphosphorylated EII proteins, which are present when EIIs are engaged in phosphotransfer to incoming carbohydrates, regulate the LevQRST signal transduction system through allosteric or other mechanisms. In contrast to *S. mutans*, significantly elevated expression of *fruA* and *levD* was seen in the *manL* mutant of *S. gordonii* growing in glucose, fructose, galactose, mannose, and even inulin (Tables 3 and 4). We propose that this observation arises from a broader substrate specificity of the EII^{Man} complex in *S. gordonii* than in *S. mutans*. In particular, PTS assays (Fig. 2) indicate that ManL could be involved in transport of glucose, mannose, and galactose, and there was a slight increase in fructose-PTS activity in the *manL* mutant, likely arising from elevated expression of the LevDEFG complex that transports fructose. However, since the *manL* mutant also grows slower on fructose, it is possible that ManL can also internalize fructose. Accordingly, expression of *fruA* and *levD* was enhanced in the *manL* mutant growing on inulin, which must be hydrolyzed into fructose before being used by the bacterium. Therefore, the comparative independence of CCR on the particular sugar source in *S. gordonii* may be associated with the capacity of ManL to efficiently internalize more substrates in *S. gordonii* than in *S. mutans*. It is notable that expression of the *fruA* and *levD* genes in the *manL* mutant growing on mannose was almost on the same level as inulin, which is probably induced by mannose transported via the fructose/mannose specific enzyme II LevDEFG complex in the *manL* mutant. It should be emphasized, though, that loss of ManL might also lead to a reduced ability to utilize multiple preferred sugars, which could result in a reduction in glycolytic intermediates that in turn lead to lower levels of HPr-Ser-P (40). Although this does not appear to be the case (Fig. 3), it cannot be excluded that the impact of ManL deficiency arises

in part from alterations in the amount or phosphorylation state of HPr.

Yet another dimension of complexity in CCR in oral streptococci disclosed in the present study was the ability of ManL to influence expression of its own operon. Thus, repression of the *manL* operon, encoding the EII^{Man} permease, is governed by both CcpA and ManL. Based on experiments described here and our studies with *S. mutans*, it seems that CcpA may repress *manL*, and other genes, when the concentration of carbohydrates is fairly high (3 to 5 mM). In this case, the classical CcpA model would apply in which cells monitor the flow of carbohydrate through the glycolytic pathway, regulating the amount of HPr-Ser-P through an ATP-dependent HPr kinase (13, 33). At lower carbohydrate concentrations, ManL could regulate its own production, and the levels of other permeases (2, 54, 56, 58), in response to the availability of its substrates outside of the cells, saving energy by not overproducing transport proteins. This two-tiered regulatory system (Fig. 4) is one that may have evolved in, and should be well suited to, oral bacteria, which confront extended periods of existing on low concentrations of the spectrum of carbohydrates released from glycoproteins, interspersed with high concentrations of preferred carbohydrates that are common in the modern diet. We propose that these subtle differences in the way in which the oral commensal *S. gordonii* and the oral pathogen *S. mutans* regulate catabolism of preferred carbohydrates reflects a niche adaptation that may give the commensal a competitive advantage in conditions conducive to dental health, whereas *S. mutans* optimizes sugar utilization more effectively under conditions that promote the emergence of a cariogenic flora and dental caries. Future studies will examine this hypothesis in more detail.

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