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Two Gene Clusters Coordinate Galactose and Lactose Metabolism in *Streptococcus gordonii*

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Streptococcus gordonii **is an early colonizer of the human oral cavity and an abundant constituent of oral biofilms. Two tandemly arranged gene clusters, designated *lac* and *gal*, were identified in the *S. gordonii* DL1 genome, which encode genes of the tagatose pathway (*lacABCD*) and sugar phosphotransferase system (PTS) enzyme II permeases. Genes encoding a predicted phospho-β-galactosidase (LacG), a DeoR family transcriptional regulator (LacR), and a transcriptional antiterminator (LacT) were also present in the clusters. Growth and PTS assays supported that the permease designated EII^{Lac} transports lactose and galactose, whereas EII^{Gal} transports galactose. The expression of the gene for EII^{Gal} was markedly upregulated in cells growing on galactose. Using promoter-*cat* fusions, a role for LacR in the regulation of the expressions of both gene clusters was demonstrated, and the *gal* cluster was also shown to be sensitive to repression by CcpA. The deletion of *lacT* caused an inability to grow on lactose, apparently because of its role in the regulation of the expression of the genes for EII^{Lac}, but had little effect on galactose utilization. *S. gordonii* maintained a selective advantage over *Streptococcus mutans* in a mixed-species competition assay, associated with its possession of a high-affinity galactose PTS, although *S. mutans* could persist better at low pHs. Collectively, these results support the concept that the galactose and lactose systems of *S. gordonii* are subject to complex regulation and that a high-affinity galactose PTS may be advantageous when *S. gordonii* is competing against the caries pathogen *S. mutans* in oral biofilms.**

The human mouth accommodates a diverse and dynamic microbial ecosystem composed of hundreds of bacterial species, and the stability of these communities is associated with an array of antagonistic and cooperative interactions between constituents of the biofilms (26). Of particular importance to oral health and diseases are the viridans group streptococci, Gram-positive bacteria that are classified into the anginosus, mitis, salivarius, bovis, and mutans groups (25). The members of the mitis group, which includes *Streptococcus gordonii*, *Streptococcus mitis*, *Streptococcus oralis*, *Streptococcus parasanguinis*, and *Streptococcus sanguinis* (25), are among the first organisms to establish in the oral cavity and remain abundant members of the biofilms that colonize the hard and soft tissues of the mouth throughout the lifetime of the host. Many members of the mitis group, including the organism that is the focus of this study, *S. gordonii*, are frequently associated with dental health. In contrast, *Streptococcus mutans*, a member of the mutans group of streptococci and certain other lactic acid bacteria (e.g., lactobacilli), is generally regarded as the primary etiological agent of dental caries. In some cases, an inverse relationship of the proportions of *S. mutans* and *S. gordonii* (16, 27) in human plaque samples has been observed, consistent with the observation that *S. gordonii* can potently antagonize the growth of *S. mutans in vitro* (31).

The oral microbiome functions as a cooperative community to degrade complex macromolecules and to release a spectrum of compounds that can be readily transported and metabolized (40). Viridans group streptococci generate energy almost exclusively from the fermentation of carbohydrates that are obtained from the host diet, from other bacteria in the oral microbiome, from sloughed host cells, and from saliva (22). Saliva is a primary source of nutrients provided nearly continuously to the oral microbiome. Salivary glycoconjugates, particularly O- and N-linked oligosaccharides of glycoproteins, serve as the preferred carbon and energy sources for many of the more abundant members of the oral microbiome. Many bacteria produce secreted or surface-associated glycosidases that cleave glycoconjugates found in saliva (4, 11). Due to the intermittent eating patterns of most humans, the ability to scavenge and efficiently metabolize the collection of carbohydrates present in the oral cavity is critical for the persistence of the organisms and to the ecology of the biofilms (37). Notably, when carbohydrates are present in excess in the diet, a low-pH environment can be created, which favors the growth and persistence of acid-tolerant pathogens, like *S. mutans*, at the expense of less aciduric commensal organisms.

S. mutans encodes 14 putative sugar:phosphotransferase systems (PTSs) (2), including one for lactose (EII^{Lac}), while the genome of *S. gordonii* DL1 harbors a similar number of putative PTS gene clusters (http://oralgen.lanl.gov/). Lactose is transported and concomitantly phosphorylated by the PTS before it is processed by phospho-β-galactosidase (LacG) into galactose-6-phosphate (Gal-6-PO_4) and glucose (18, 46). The metabolism of Gal-6-PO_4 then occurs through the tagatose-6-phosphate (Tag-6-PO_4) pathway consisting of the LacABCD proteins (23). Galactose can also be catabolized by the Leloir pathway (3, 20), which usually involves a non-PTS transporter, such as GalP (18). Although the enzymes for the tagatose and Leloir pathways are expressed in *S. mutans*, this organism lacks a GalP homologue and a high-affinity PTS transporter for galactose (46). Instead, galactose is transported at a relatively low efficiency by PTS porters that have other carbohydrates as their cognate substrates and is subsequently pro-

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cessed through the tagatose pathway in *S. mutans* (46). Not surprisingly then, *S. mutans* needs relatively high concentrations of galactose to support growth, and the organism grows more slowly on galactose than on carbohydrates for which a higher-affinity uptake system is present.

Given the abundance of galactose and lactose entering the oral cavity in salivary secretions (12) and in the diet, respectively, we hypothesized that commensal streptococci may hold a selective advantage over the caries pathogen *S. mutans* by virtue of their ability to better utilize one or both of these carbohydrates. Accordingly, we identified and characterized two tandemly arranged gene clusters encoding tagatose pathway enzymes, two PTS enzyme II permeases, and two regulatory proteins. These gene products were shown to dominantly control galactose and lactose assimilation by *S. gordonii* DL1. Furthermore, competition assays provided evidence that the more efficient utilization of galactose by *S. gordonii* may confer a selective advantage over *S. mutans* UA159, particularly during periods of fasting by the host. The results reveal important differences in the regulation of, and capacity for, galactose and lactose metabolism between a strongly cariogenic isolate of *S. mutans* and this abundant oral commensal.

MATERIALS AND METHODS

Bacterial strains and growth conditions. *S. gordonii* and *S. mutans* cells were maintained on brain heart infusion (BHI) agar plates (Difco Laboratories, Detroit, MI), and broth cultures were routinely grown in BHI or in tryptone-vitamin (TV) medium (9); the latter medium supports the growth of *S. gordonii* or *S. mutans* only if it is supplemented with metabolizable carbohydrates. Unless stated otherwise, cultures were incubated at 37°C in a 5% CO_2 aerobic environment. Antibiotic-resistant strains were selected and maintained on BHI agar containing erythromycin (Em) (10 μg/ml) (BHI-Em agar), kanamycin (Km) (1 mg/ml), or spectinomycin (Sp) (1 mg/ml). BHI broth was supplemented with antibiotics at half those concentrations, when needed, or with 10% horse serum (Sigma-Aldrich, St. Louis, MO) to induce natural competence (35). *Escherichia coli* strain DH10B was used as a cloning host and was maintained on L agar with antibiotics added at the following concentrations: Em at 250 μg/ml, Km at 50 μg/ml, or Sp at 50 μg/ml.

To monitor growth on particular carbohydrate sources, individual colonies were inoculated in triplicate into BHI broth and grown overnight (17 h). Strains were then diluted 1:20 into fresh TV medium containing 0.5% (wt/vol) glucose (TV-glucose medium), galactose, or lactose and incubated at 37°C in a 5% CO_2 atmosphere. Growth was monitored hourly by measurements of the optical density at 600 nm (OD_{600}).

DNA manipulations. Coding sequences of the genes in the *lac* and *gal* clusters of *S. gordonii* were deleted, in whole or in part, and replaced with a nonpolar Em, Km, or Sp resistance cassette via allelic exchange (29). Briefly, two DNA fragments flanking the gene of interest were amplified via PCR using gene-specific primers with integrated restriction enzyme sites. These fragments, along with the appropriate antibiotic resistance determinant, were digested by using restriction enzymes and ligated together with T4 DNA ligase (New England BioLabs, Ipswich, MA). The ligation products were then used to transform genetically competent *S. gordonii* cells. Transformants were subjected to PCR verification and DNA sequencing before being used for phenotypic studies.

A *cat* (chloramphenicol acetyltransferase [CAT]) gene fusion to the *lacA1* promoter (P*lacA*1-*cat*) was constructed by cloning a 385-bp DNA fragment containing the promoter region of the *lacA1* gene into plasmid pYQ4, which was derived by modifying the integration vector pMJB8 to contain an Em resistance marker (47). The desired construct was then used to integrate a single copy of the gene fusion into the *gtfG* gene in the DL1 genome. To construct the P*lacA2-cat* fusion, a 308-bp sequence of the *lacA2* promoter region was first fused with the promoterless *cat* gene in pJL84 (48), and the fusion was then subcloned into pMJB8 for integration into the chromosome. The P*lacA2-cat* fusion carries a Km resistance marker.

A point mutation was generated in the *lacT* gene to convert the Met8 codon into a UAG stop codon to create the *lacT*(M8stop) strain by using a previously reported protocol (45). Briefly, a 2-kbp DNA fragment containing the *lacT*(M8stop) mutation was generated by using recombinant PCR and then used to transform *S. gordonii* DL1, along with the promoter fusion P*lacA2-cat* plasmid, which carries a Km resistance cassette (44). After plating, Km-resistant colonies were screened for the *lacT*(M8stop) mutation by using allele-specific mismatch amplification mutation analysis (MAMA) PCR (13). The desired transformants were then verified by PCR and sequencing.

Real-time RT-PCR. Total RNA was extracted from cultures of *S. gordonii* strains by using the RNeasy minikit (Qiagen, Germantown, MD), and specific mRNAs were quantified by using real-time reverse transcription (RT-PCR), as detailed previously (1). After the conversion of RNA into cDNA using random hexamers, the transcript levels of various genes were measured by using specific primers: 5′-GGT CAG GAT TTT GTT GAT GTG ACC C-3′ (forward) and 5′-GGA CCA GCC CCA TAA GCA TCG AT-3′ (reverse) for *lacA1*, 5′-GAC AGG CTA TGG AGA GGT CAA TC-3′ (forward) and 5′-TGG TGT ATC AAA GTG GTG AAG GG-3′ (reverse) for *lacG*, 5′-GGT GCA GAT GCT GCT GGA AAT-3′ (forward) and 5′-CAC CTC AGC TGC AAC TGC CAA T-3′ (reverse) for *lacA2*, 5′-GAT AAC AAC GGA GTA AGC CAA GG-3′ (forward) and 5′-TTG GAG CAT TTA GGA GGT CGT C-3′ (reverse) for the $EIIC^{Gal}$ gene, or 5′-CAC ACC GCC CGT CAC ACC-3′ (forward) and 5′-CAG CCG CAC CTT CCG ATA CG-3′ (reverse) for the 16S rRNA gene as a control.

CAT and sugar transport assays. CAT assays were performed by using exponentially growing bacterial cells in TV medium according to a previously reported protocol (38). Assays of galactose transport by the bacterial sugar:phosphotransferase system were carried out according to previously reported protocols (30), with minor modifications. Briefly, the assay couples pyruvate generation from phosphoenolpyruvate (PEP)-dependent sugar phosphorylation by the PTS to lactate generation via lactate dehydrogenase, which concurrently oxidizes NADH to NAD^+. However, the background level of spontaneous oxidation of NADH in wild-type (WT) *S. gordonii* cells in the absence of added PEP was unacceptably high. To circumvent this problem, we created strains of *S. gordonii* specifically for use in PTS assays which contained a deletion of the gene for NADH oxidase (encoded by *nox* [SGO_1167]), which reduced the background level of NAD generation (non-PEP dependent) in the PTS assay to acceptable levels (39). Cultures of *S. gordonii* that were grown overnight in BHI broth in a 5% CO_2 atmosphere were diluted 1:25 into fresh TV-galactose medium and incubated in an anaerobic chamber maintained with 85% N_2, 10% H_2, and 5% CO_2. The use of anaerobically grown cells further reduced the non-PEP-dependent NADH oxidation in the PTS assays. Cells were harvested at the mid-exponential phase, washed in phosphate buffer, and permeabilized by using toluene-acetone (1:9, vol/vol), and the PTS activity was measured and normalized to protein concentrations (30, 39).

Mixed-species liquid culture competition assay. *S. gordonii* DL1 and *S. mutans* UA159 were modified by replacing the *fruA* gene with Em and Km resistance markers, respectively. Strains were cultured in triplicate overnight in BHI broth. The next morning, bacterial cultures were diluted 1:50 into 10 ml of fresh BHI broth and cultured for 3 h. After the cell density was measured, *S. gordonii* and *S. mutans* samples were mixed together in a 1:1 ratio of 1×10^8 CFU into 10 ml fresh TV broth supplemented with either 0.5% glucose or galactose, with or without 50 mM potassium phosphate buffer (pH 7.5). This time point (t) was designated 0 h. The samples were incubated for 6 h ($t = 6$ h) before being diluted 1:50 into fresh TV medium. The rediluted (for viability) and original (for persistence) mixed cultures were then incubated overnight ($t = 22$ h). Subsequently, the persistence cultures were kept as-is, while the viability cultures were rediluted 1:50 into fresh medium. These samples were

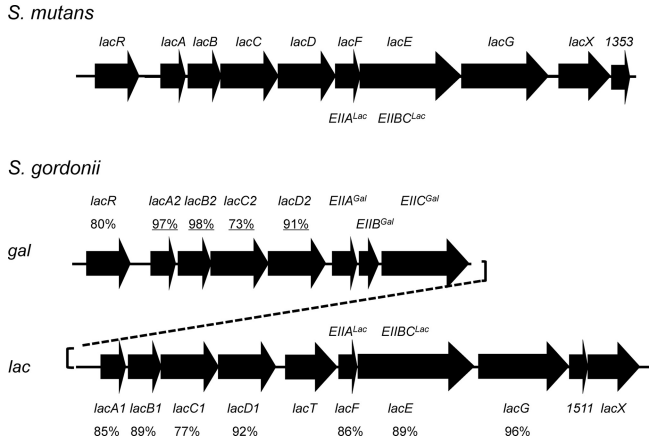

FIG 1 Diagrams depicting the tagatose-6-phosphate pathway clusters in *S. mutans* and *S. gordonii*. Two sets (*gal* and *lac*) of predicted tagatose pathway genes (*lacABCD*) and putative regulatory genes (*lacR* and *lacT*) are present in *S. gordonii* DL1, as opposed to one set in *S. mutans* UA159. Additional genes encoding a galactose-PTS enzyme II permease (EIIABC^{Gal}) are also present in the *gal* cluster of *S. gordonii*. Numbers indicate the percent similarities among homologous genes; underlined numbers denote similarity between two homologues within the *S. gordonii* genome, and others represent similarity to homologues in the *S. mutans* genome. Other homologous open reading frames with unknown functions, SGO_1511 and *lacX*, are also indicated.

incubated for an additional 8 h ($t = 30$ h). At each time point, the absorbance and pH were measured, and aliquots (100 μl) of the samples were removed, serially diluted, and plated onto BHI-Em and BHI-Km agar to enumerate *S. gordonii* and *S. mutans* bacteria, respectively. All plates were incubated for 2 days at 37°C in a 5% CO_2 atmosphere before the colonies were counted.

RESULTS AND DISCUSSION

Identification of the lactose and galactose gene clusters. Lactose and galactose are carbohydrates that are frequently encountered by the bacterial communities in the oral cavity. To identify lactose- and galactose-utilizing enzymes in the genome of *S. gordonii* strain DL1 Challis, a BLAST search was performed by using protein sequences of the tagatose-6-phosphate (Tag-6-PO_4) pathway (*lac*) of *S. mutans* UA159 (Fig. 1) (46). Two tandemly arranged gene clusters encoding the enzymes of the Tag-6-PO_4 pathway were identified, and the duplicated gene products shared high degrees of homology (Fig. 1). For example, the A subunits (LacA1 and LacA2) of the galactose-6-phosphate isomerase enzyme, which converts Gal-6-PO_4 into Tag-6-PO_4, were 97% identical. Similar to that of *S. mutans*, one of the apparent operons contained genes for a predicted phospho-β-galactosidase (LacG) and a lactose-specific EII permease (EII^{Lac}). The other operon harbored the genes for a putative galactose-specific EII permease (EIIABC^{Gal}) encoded by the genes SGO_1520 to SGO_1522 (http://oralgen.lanl.gov/). For convenience, the operon containing LacG and EII^{Lac} is referred to as the *lac* operon, and that encoding EII^{Gal} is referred to as the *gal* operon.

A gene for a transcriptional antiterminator (SGO_1515), designated here *lacT*, is present within the *lac* operon of *S. gordonii* but is absent in the *S. mutans* UA159 genome. LacT is a member of the BglG/SacY family of proteins commonly associated with the regulation of carbohydrate metabolism (15) and contains a coantiterminator (CoAT) RNA-binding domain (28) located in its amino terminus. CoAT domains facilitate binding to ribonucleic antiterminator (RAT) sequences in mRNA transcripts, preventing the formation of a terminator to allow RNA polymerase to continue the transcription of downstream genes (15). LacT also contains two PTS regulatory domains (PRDs), which serve as targets for phosphorylation by components of the PTS for the allosteric modulation of the DNA- or RNA-binding activity of the regulatory protein (42). Based on current models for the PTS-dependent regulation of antitermination (21), one would predict that when the PTS permease(s) for lactose or galactose is engaged in sugar transport, the PRD in LacT would be dephosphorylated, allowing for the antitermination of the *lac* or *gal* operon genes.

The transcriptional regulator 5′ to the *gal* gene cluster (Fig. 1) is designated LacR, based on similarities to known LacR proteins, and contains a helix-turn-helix domain of about 50 to 60 amino acids and a carboxy-terminal effector-binding domain. LacR-type proteins are present in multiple genera, including *Streptococcus*, *Staphylococcus*, *Lactococcus*, and *Bacillus*, and in many cases have been shown to negatively regulate sugar catabolism. Effector molecules for LacR-type regulators are often phosphorylated carbohydrates that are intermediates in the metabolic pathway controlled by the regulatory protein. In this case, Gal-6-PO_4 and Tag-6-PO_4 have each been suggested to bind to a LacR homologue to facilitate dissociation from its binding site (34, 41, 46). A protein alignment revealed 59% and 86% identities between LacR in *S. gordonii* and apparent homologues in *S. mutans* and *S. sanguinis*, respectively.

A survey of existing whole-genome sequences revealed that a number of other streptococci, lactococci, and lactobacilli retain various combinations of the *gal* and *lac* genes (5, 14, 32, 33, 36). For example, the *gal* and *lac* gene clusters of *Streptococcus pyogenes* strains closely resemble those of *S. gordonii*, encoding two sets of the tagatose enzymes, a lactose PTS, a predicted galactose PTS, and two homologous LacR proteins (32). *Lactococcus lactis*, *Lactobacillus casei*, *S. sanguinis*, *S. mitis*, *Streptococcus pneumoniae*, and *Staphylococcus aureus* also carry transporters that are similar to those in the *S. gordonii lac* and *gal* gene clusters.

Finally, it should be noted that the genes for the Leloir pathway (*galKTE*) for galactose utilization, as well as two predicted extracellular β-galactosidase enzymes, are present in the DL1 genome. However, as detailed below, the contribution of the Leloir pathway and these secreted enzymes to galactose or lactose catabolism appears to be nominal, so an analysis of the function or regulation of these genes was not pursued further in this study.

Growth phenotypes of the *S. gordonii lac* and *gal* gene mutants. (i) *lac* gene cluster. Various mutant strains were created via allelic exchange using nonpolar antibiotic resistance genes. Growth in TV broth supplemented with 0.5% of the desired carbohydrates was monitored (Table 1). When grown in TV medium supplemented with 0.5% galactose, *S. gordonii* DL1 cells grew to a final OD_{600} of 1.2 with a doubling time of 68 ± 2 min, while in TV-lactose medium, the organism grew with a doubling time of 67 ± 4 min and reached a final OD_{600} of 1.0. All of the mutations in the *lac* gene cluster caused severe defects in growth on lactose, but the effects of the same mutations on growth on galactose varied (Table 1). For example, the ΔEII^{Lac} strain, in which the presumed lactose-specific transporter was deleted, failed to grow on lactose but achieved a final OD on galactose similar to that of strain DL1, with a doubling time of 70 ± 4 min. In contrast, the Δ*lacA1B1* strain, lacking the subunits of the Tag-6-PO_4 isomerase encoded by the *lac* operon, showed no growth on lactose but grew

TABLE 1 Doubling times and final optical densities of wild-type strain DL1 and various mutants[a]

Strain	Glucose		Galactose		Lactose	
	Avg T_d (min) ± SD	OD_{600}	Avg T_d (min) ± SD	OD_{600}	Avg T_d (min) ± SD	OD_{600}
DL1 (wild type)	57.4 ± 6.7	1.08	67.5 ± 2.1	1.17	67.1 ± 4.3	0.97
ΔEII^{Lac}	57.5 ± 4.4	1.07	70.0 ± 4.2	1.13	ND	0.15
Δ*lacG*	72.4 ± 2.6	1.07	113.2 ± 7.9	1.16	ND	0.09
Δ*lacA1B1*	61.9 ± 1.2	1.03	201.0 ± 18.6	0.23	ND	0.20
lacT(M8stop)	64.8 ± 1.9	1.12	74.9 ± 0.4	1.16	ND	0.19
ΔEII^{Gal}	68.5 ± 8.4	0.86	107.6 ± 26.2	1.15	94.2 ± 6.5	0.88
Δ*lacR*	57.8 ± 1.5	1.16	64.1 ± 0.9	1.23	60.9 ± 2.3	0.93
Δ*lacA2B2*	57.0 ± 2.3	1.05	66.9 ± 2.5	1.27	69.4 ± 1.2	0.95
Δ*lacA1B1* Δ*lacA2B2*	55.7 ± 0.7	1.01	ND	0.10	ND	0.13
ΔEIIAB^{Man}	67.9 ± 1.7	0.89	109.7 ± 5.2	1.17	67.2 ± 3.0	0.77
ΔEII^{Gal}/ΔEIIAB^{Man}	61.4 ± 1.2	0.90	254 ± 47	0.59	61.2 ± 5.1	0.76

[a] Results (averages ± standard deviations) are each based on three manual growth curve analyses using TV medium containing the specified carbohydrate (0.5%) in the presence of 5% of CO_2. ND (not determined) is denoted when a strain displayed minimal growth and when the doubling time (T_d) was too large for accurate calculations.

slowly (201 ± 18 min) and achieved a final OD_{600} of 0.2 when galactose was the growth carbohydrate. Similarly, the Δ*lacG* strain, which produces no phospho-β-galactosidase enzyme and could not grow at all on lactose, grew with a doubling time of 113 ± 8 min on galactose. Notably, a similar phenotype was observed previously for a *lacG* mutant of *S. mutans* (46) and was associated with a requirement of LacG for the induction of the operon (see below). The requirement for the induction of the *lac* operon of *E. coli* by β-galactosidase, which produces the cognate inducer of the operon, allolactose, is well established (10).

Since the genome of *S. gordonii* also harbors two predicted extracellular β-galactosidases (BgaA [SGO_1486] and BgaC [SGO_0043]), the possibility exists that some of these enzymes could contribute to the growth of the bacteria on lactose by converting lactose into galactose and glucose in the supernatant fluid. In fact, a modest yet consistent growth defect on lactose was observed for a strain carrying deletions of both the *bgaA* and *bgaC* genes (data not shown). However, the phenotypes of the ΔEII^{Lac} and Δ*lacG* strains support that the predicted extracellular β-galactosidase enzymes are not sufficient to support growth on lactose, possibly reflecting that BgaA and BgaC may have relatively poor activity on lactose compared to, for example, galactose in glycoconjugates. Furthermore, when measured by using quantitative real-time RT-PCR, the transcript levels of both the *bgaA* and *bgaC* genes were relatively low in glucose-grown DL1 cells, and they remained so when cells were grown in lactose-based medium (data not shown). Also consistent with a lack of a significant contribution of BgaA or BgaC to lactose metabolism, when β-galactosidase assays were performed on intact cells of the wild type or the *lacG* mutant, using either 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-Gal) or *o*-nitrophenyl-β-D-galactopyranoside (ONPG) as a substrate, significant levels of activity were not detected in either genetic background (data not shown). These findings are in agreement with data from a previous study which showed that an apparent homologue of the *S. gordonii* BgaA enzyme in *S. pneumoniae* is not responsible for the hydrolysis of lactose in the extracellular environment (43).

(ii) *gal* gene cluster. Unlike the strain lacking EII^{Lac}, the growth of the EII^{Gal}-deficient strain on galactose was clearly impaired compared to the growth of the wild-type strain, with a minimum doubling time of 108 ± 26 min. Thus, EII^{Gal} appears to contribute significantly to the internalization of galactose. Consistent with these results, a PTS EII complex present in *S. pneumoniae* that has significant similarity to EII^{Gal} of *S. gordonii* was shown previously to be required for growth on galactose (24). However, we previously reported that a mutation of the *manL* gene, encoding the AB domains of a glucose/mannose-PTS enzyme II complex (EII^{Man}), contributed to galactose utilization by *S. gordonii* (39). When both EII^{Man} and EII^{Gal} were deleted from *S. gordonii*, the mutant displayed further reductions in levels of growth on galactose compared to the single mutants (Table 1). Therefore, in *S. gordonii*, EII^{Lac}, EII^{Man}, and EII^{Gal} all appear to have the capacity to contribute to galactose uptake.

The deletion of the *lacA2B2* genes (Fig. 1) did not significantly affect growth on galactose, but the mutant lacking *lacA1B1* and *lacA2B2* was unable to grow on galactose at all. These findings demonstrate that the tagatose pathway is the major pathway for *S. gordonii* to catabolize galactose for growth and that the Leloir pathway either is not involved in galactose catabolism or is regulated in such a way that it is not expressed in strains deficient in LacAB proteins. Consistent with these results, the deletion of the *galK* gene, encoding galactokinase in the Leloir pathway, had only a modest effect on growth on galactose (doubling time, 86 ± 4 min). Collectively, these data provide evidence that the GalKTE pathway is not a significant contributor to the catabolism of galactose by *S. gordonii* under the conditions tested and that the PTS is the main route of entry for galactose in this organism.

Consistent with the effects of the deletion of *lacA2B2* on growth on galactose, there was little impact of this mutation on growth on lactose. However, when both sets of isomerase (*lacAB*) genes were deleted, no growth was observed on lactose, even in the presence of an intact *lacG* gene. Since LacG is expected to cleave lactose-6-PO_4 to release Gal-6-PO_4 and glucose inside the cells, we propose that the failure of the mutant lacking both *lacAB* gene sets to grow is due to the growth-inhibitory effects of the accumulation of Gal-6-PO_4 in cells, as was observed previously for *S. mutans* and some other bacteria (46).

Interestingly, the mutant lacking the transcriptional antiterminator, *lacT*(M8stop), failed to grow on lactose and grew only slightly slower than the parental strain on galactose. The simplest interpretation of these results is that LacT functions in the antitermination of the genes for the EII^{Lac} permease. It appears less likely

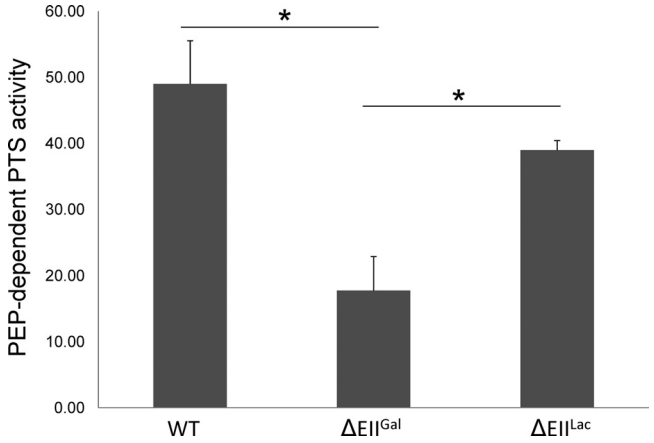

FIG 2 Galactose-PTS activities. *In vitro* PTS assays were performed on *S. gordonii* wild-type (WT) and ΔEII^{Gal} and ΔEII^{Lac} strains grown anaerobically in TV medium supplemented with 0.5% galactose. All three strains contain a *nox* (NADH oxidase) deletion. Values were obtained from triplicate assays of three individual cultures, and the asterisks indicate a *P* value of less than 0.005 according to the Student *t* test.

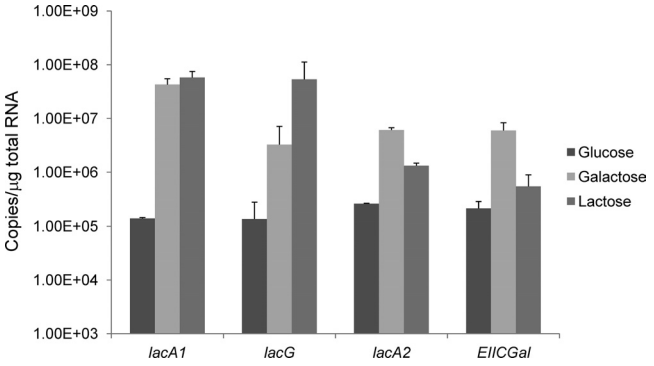

FIG 3 Expression levels of the *lacA1*, *lacG*, *lacA2*, and EIIC^{Gal} genes in cells grown on the indicated carbohydrates. Real-time quantitative RT-PCR was used to measure the transcript levels of each gene in DL1 cells grown in TV medium containing 0.5% glucose, galactose, or lactose. Results are the averages of data from three independent experiments, and the error bars represent standard deviations.

that LacT is required for the expression of the *lacA1B1C1D1* genes, since the inactivation of *lacA1B1*, but not *lacT*, caused a severe growth defect on galactose (Table 1). It is also of interest that EII^{Gal} has an influence on lactose utilization, perhaps reflecting a role in the regulation of LacT activity. Further analysis of the regulatory functions of LacT is under way in our laboratory. The loss of LacR, on the other hand, had relatively little influence on the growth of strains on lactose or galactose, consistent with its predicted role as a negative regulator of the tagatose pathway genes.

(iii) Effects of *lac* and *gal* mutations on growth on glucose. When grown in TV broth supplemented with 0.5% glucose, wild-type strain DL1 grew to a final OD_{600} of 1.1 with a doubling time of 57 ± 7 min. With the exception of the Δ*lacG* and ΔEII^{Gal} strains, which grew modestly slower, all other mutant strains exhibited similar growth rates and achieved final optical densities comparable to that of the wild-type strain when grown on glucose (Table 1). Also of note, the Δ*lacR* strain had a strong tendency to aggregate when grown in BHI broth, which contains 0.3% added glucose, and it formed much longer chains than the parental strain (about 15 to 20 cells per chain, compared to 2 to 5 cells per chain for the WT). Both LacR and LacG have been shown to play regulatory roles in the metabolism of galactose and lactose in *S. mutans* (46), and we propose that a perturbation of the cell wall biosynthetic pathways associated with aberrant galactose metabolism may account for the slower growth of the *lacG* mutant and the cell division defects of the *lacR* mutant. Consistent with this hypothesis was the observation that *lac* operon expression was altered in a *lacG* or *lacR* deletion strain grown on glucose (see below).

Transport of galactose via the PTS. To further substantiate that EII^{Gal} and, potentially, EII^{Lac} are the primary galactose transporters in *S. gordonii*, PTS assays were performed. Notably, *S. gordonii* appears to produce high levels of NADH oxidase activity when exposed to O_2 or when grown on galactose (39), which interferes with accurate determinations of PTS activity in the coupled assay employed here (30). To circumvent this problem, PTS assays were conducted in the wild-type, ΔEII^{Gal}, and ΔEII^{Lac} genetic backgrounds in strains carrying a deletion of the major NADH oxidase enzyme of *S. gordonii* (*nox*), as detailed in Materials and Methods. When grown anaerobically in 0.5% galactose, the ΔEII^{Gal} strain had a significantly lower level of galactose-PTS activity than did strains with an intact EII^{Gal} permease (Fig. 2). The strain lacking EII^{Lac} consistently had lower levels of PTS activity than those seen with the wild-type-background strain, although the differences were not statistically significant. However, the level of PTS activity in the strain lacking EII^{Gal} was significantly lower than that in the strain without EII^{Lac}. These results provide additional support that, in addition to EII^{Man} (39), EII^{Gal} is capable of transporting galactose in *S. gordonii*. We speculate that the EII^{Lac} complex is also involved in the transport of galactose albeit at levels too low to be demonstrated by our PTS assay in a statistically meaningful way.

Operon-specific gene expression in response to carbohydrate. Real-time RT-PCR was performed to evaluate the expressions of the *lacA1* and *lacG* genes of the *lac* operon and the *lacA2* and EIIC^{Gal} genes of the *gal* operon, which were chosen based on their positions in each gene cluster (Fig. 1). Total RNA from strain DL1 was extracted from cells that were grown exponentially in TV medium supplemented with 0.5% glucose, galactose, or lactose and converted into cDNA by using random hexamers. The amount of the *lacG* transcript was 24-fold higher in cells grown on galactose ($P = 0.04$) than in glucose-grown cells (Fig. 3). Even higher expression levels were noted when the cells were grown on lactose, with a 390-fold induction compared to that of cells grown on glucose ($P = 0.02$). The greater increase in the *lacG* expression level in lactose-grown cells than in galactose-grown cells, together with the lack of growth of the *lacT*(M8stop) strain on lactose, supports the idea that the EII^{Lac} complex modulates LacT activity, since *lacT* and the downstream *lacFEG* genes are likely cotranscribed.

Interestingly, *lacA1* transcript levels were elevated approximately 100-fold in cells grown on galactose or lactose, as opposed to glucose-grown cells. Additional tests of *lacA1* expression in the *lacT*(M8stop) background also indicated that LacT is not required for *lacA1* promoter activity (data not shown). On the other hand, the expression level of the gene for EIIC^{Gal} in the *gal* gene cluster was only 2.5-fold higher in cells grown on lactose, compared with a 28-fold ($P = 7.6 \times 10^{-5}$) increase in cells grown on galactose

TABLE 2 Expression levels of promoter-*cat* fusions in the wild type and *lacR*, *lacG*, and *ccpA* mutants[a]

	Avg CAT sp act (nmol mg of protein^{-1} min^{-1}) (SD)								
	P*lacA1-cat*			P*lacA2-cat*			P*lacA2cre-cat*		
Strain	Glc	Gal	Lac	Glc	Gal	Lac	Glc	Gal	Lac
DL1	83.6 (15)	2,253 (165)	1,886 (180)	0.72 (0.2)	41.0 (0.9)	5.2 (0.5)	43.5 (9.3)	196 (6)	231 (7)
lacR	13,908 (6,531)	14,431 (5,337)	22,546 (5,679)	7.4 (0.2)	46.1 (1.2)	4.6 (0.5)	428 (22)	305 (35)	135 (29)
lacG	45.1 (4)	1,203 (387)	ND	ND	ND	ND	ND	ND	ND
ccpA	43.6 (13.5)	3,189 (138)	1,724 (220)	24.6 (5.1)	129 (17)	123 (30)	ND	ND	ND

[a] Cells were harvested from exponentially growing cultures in TV medium supplemented with 0.5% glucose (Glc), galactose (Gal), or lactose (Lac). The data are the averages (standard deviations) of the activities based on three individual cultures. CAT specific activities are expressed as nmol of chloramphenicol acetylated mg of protein^{-1} min^{-1}. ND, not determined.

over those measured in glucose-grown cells. A similar pattern of expression was found for the *lacA2* gene, with increased transcript levels in cells grown on lactose and much higher levels in galactose-grown cells. These results are consistent with the observation that the *lac* genes appear to be dedicated mainly to lactose metabolism, whereas both the *lac* and *gal* operons were required for optimal galactose utilization.

In *S. mutans*, the inactivation of the apparent homologues of the *S. gordonii lacR* or *lacG* gene resulted in the aberrant expression of the *lac* gene cluster (46). To assess whether the loss of LacR or LacG impacted the expression of the *lac* or *gal* gene cluster in *S. gordonii*, promoter-*cat* fusions were constructed by using DNA fragments containing their respective promoters (P*lacA1* for the *lac* operon and P*lacA2* for the *gal* operon) and were integrated in a single copy into a distal site (*gtfG*) of the chromosome in both the wild-type and mutant strains (47). The loss of LacR led to a large increase in expression from the *lacA1* promoter in cells grown on glucose, galactose, or lactose, whereas the loss of *lacG* led to clear reductions in *lacA1* promoter activity in either glucose- or galactose-containing medium; *lacG* mutants cannot grow with lactose as the only carbohydrate. Interestingly, the deletion of *lacR* caused little change in the P*lacA2-cat* expression levels under the same conditions.

Further analysis of the promoter regions of the *lac* and *gal* gene clusters identified a conserved catabolite response element (*cre*), which is the binding site for catabolite control protein A (CcpA) (17), near the promoter of *lacA2*. Therefore, it was possible that the effect of the deletion of *lacR* on the *lacA2* promoter was masked by catabolite repression. To exclude the involvement of CcpA in our genetic analyses, point mutations were introduced to disrupt *cre* in the P*lacA2-cat* promoter fusion, resulting in a P*lacA2cre-cat* fusion. As predicted, the P*lacA2cre-cat* fusion produced higher levels of CAT activity than did the P*lacA2-cat* gene fusion in the wild-type genetic background (Table 2). When assayed in cells grown on glucose or galactose (Table 2), P*lacA2cre-cat* also showed much higher expression levels in the *lacR* mutant background than those seen in the wild-type genetic background. Collectively, these data indicate that LacR plays a role in the repression of both the *lac* and *gal* operons. Moreover, the *lacTFEG* genes are likely regulated by the lactose PTS exerting its influence through LacT-dependent antitermination, whereas the *gal* operon is dominantly regulated by carbon catabolite repression (CCR) via the CcpA protein. In the *in vitro* studies described here, CcpA-mediated CCR is active in cells, since they were grown with relatively high concentrations of carbohydrate. However, under carbohydrate-limiting conditions, such as those seen during periods of fasting by the host, one would predict that the *gal* operon, including the EII^{Gal} complex, could contribute significantly to the utilization of galactose in the human oral cavity, where carbohydrate is frequently a limiting nutrient and is present in concentrations that do not trigger CCR.

Comparison of growths of *S. gordonii* DL1 and *S. mutans* UA159 on galactose. The possession of a dedicated high-affinity galactose transport system and redundant galactose metabolic pathways in *S. gordonii*, which are lacking in *S. mutans* UA159, may reflect a niche adaptation by the commensal that imparts a growth advantage over the caries pathogen *S. mutans.* To begin to test this hypothesis, wild-type *S. gordonii* and *S. mutans* strains were grown in TV medium supplemented with either 0.5% glucose or galactose or 2% galactose (Table 3). When grown in TV medium supplemented with 0.5% glucose, both strains grew to similar final ODs, and *S. gordonii* had a doubling time of 57 ± 7 min, while *S. mutans* divided every 63 ± 1 min. When grown in TV medium supplemented with 0.5% galactose, *S. gordonii* grew to a final OD_{600} of 1.2 with a doubling time of 68 ± 2 min, whereas *S. mutans* grew to a final OD_{600} of 0.9 with a doubling time of 135 ± 11 min. Similarly, in TV medium with 2% galactose, *S. gordonii* again grew faster than *S. mutans* and achieved a higher final optical density. However, *S. mutans* cells grew at a significantly higher rate (doubling time of 84 ± 2 min) in 2% galactose than in 0.5% galactose.

Collectively, these data suggest that *S. gordonii* may be better

TABLE 3 Doubling times and final optical densities of cultures of *S. mutans* strain UA159 and *S. gordonii* strain DL1[a]

	0.5% glucose		0.5% galactose		2% galactose	
Strain	Avg T_d (min) ± SD	OD_{600}	Avg T_d (min) ± SD	OD_{600}	Avg T_d (min) ± SD	OD_{600}
S. mutans UA159	62.9 ± 1.2	1.09	135.3 ± 10.6	0.93	83.5 ± 2.4	0.91
S. gordonii DL1	57.4 ± 6.7	1.08	67.5 ± 2.1	1.17	70.3 ± 2.2	1.14

[a] Strains were grown in TV-based medium supplemented with various carbohydrates. Results for the doubling times are the averages and standard deviations derived from three independent cultures.

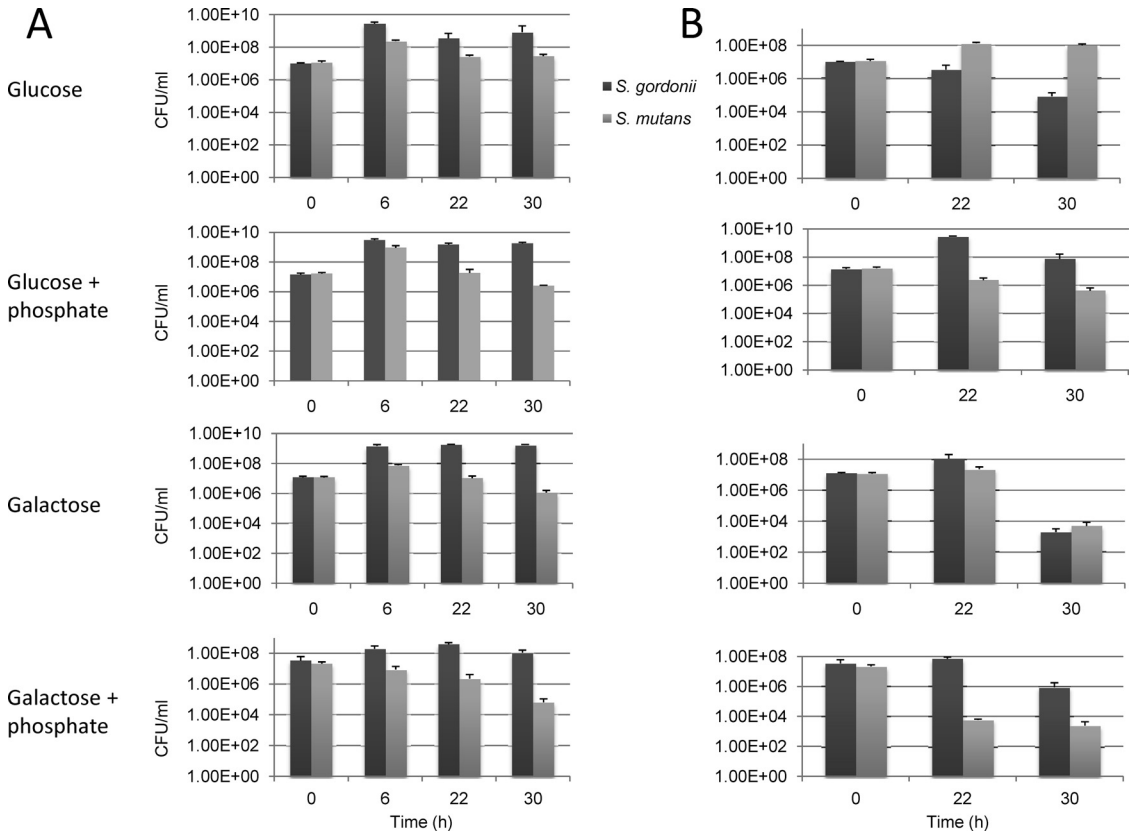

FIG 4 Mixed-species competition assay testing viability (A) and persistence (B) of *S. gordonii* and *S. mutans*. Exponentially growing cultures of DL1 and UA159, each containing an antibiotic marker (Em for DL1 and Km for UA159), were inoculated in a 1:1 ratio into TV medium supplemented with 0.5% glucose or galactose, with or without 50 mM potassium phosphate buffer (pH 7.5) (see Materials and Methods for details). At time points of 6, 22, and 30 h, the cultures for viability testing were diluted into fresh medium (excluding $t = 30$ h), while the pH was measured (Table 4), and CFU were determined by plating. Results are the averages of data from three independent experiments, and the error bars represent standard deviations.

equipped than *S. mutans* UA159 to utilize galactose, as evidenced by the higher growth rate, higher final yield, and apparently higher affinity for this hexose (46). The improved growth of *S. mutans* on 2% galactose compared with growth on 0.5% galactose likely reflects that it has a lower capacity and a lower affinity for the transport of this hexose (46). Given that galactose is probably present at low steady-state concentrations as it is liberated from host glycoproteins, the differences between these two organisms in their abilities to scavenge and efficiently catabolize galactose could have a significant impact on the ecological balance of dental biofilms, particularly during periods of fasting by the host.

Mixed-species liquid culture competition assay. While the production of bacteriocins and excess acid by *S. mutans* is detrimental to the survival of oral commensal bacteria, including *S. gordonii*, *S. gordonii* is capable of producing hydrogen peroxide, which is inhibitory to the growth of *S. mutans* (26). Here, a mixed-species liquid culture competition assay was used to assess the capacity of *S. gordonii* DL1 to compete with *S. mutans* UA159 in the presence of different carbohydrates. To be able to enumerate these two bacterial species accurately during plating, the *S. gordonii fruA* gene (39) was replaced with an Em resistance cassette, and the *fruA* gene of *S. mutans* (8, 48) was replaced with a Km resistance marker. FruA is an exo-β-fructosidase, and the loss of this enzyme affects growth only on sucrose or homopolymers of fructose (6–8, 39). Cells were grown to the mid-exponential phase to an OD_{600} of 0.5 and mixed together in a liquid culture in a 1:1 ratio ($t = 0$ h). All incubations were static in a 5% CO_2 atmosphere at 37°C. At the 0-h time point and at the 6-, 22-, or 30-h time point, the OD_{600} and pH were measured, serial dilutions were plated for CFU enumerations, and the samples were subcultured 1:50 into fresh medium with or without 50 mM phosphate buffer to assess viability. The original mixed culture was also incubated continuously and subjected to all of the same measurements at the same time points, but the medium was never changed, and buffer was not added to the samples. This allowed for the comparison of the abilities of these cells to persist in the same environment over time. All conditions were tested in triplicate, and the results are presented in Fig. 4 (CFU) and Table 4 (pH).

In the viability aspects of the competition assays, *S. gordonii* outcompeted *S. mutans* under all conditions tested. As shown in Fig. 4, both species reached their highest cell densities at 6 h, but the proportion of *S. gordonii* cells in the mixed culture was up to 20-fold higher than that of *S. mutans*. At subsequent time points, *S. gordonii* maintained largely similar viable counts, whereas the proportion of *S. mutans* cells consistently declined. When cultured without phosphate buffer, *S. mutans* maintained higher CFU counts throughout the test when grown in glucose than when grown in galactose, whereas *S. gordonii* showed little preference between these two sugars when viability was assessed. The addition of 50 mM phosphate buffer had little impact on the viability

TABLE 4 pH measurements at various time points of the mixed-species liquid cultures[a]

Assay and *t* (h)	Avg pH ± SD			
	Glucose	Glucose + phosphate	Galactose	Galactose + phosphate
Viability				
6	4.9 ± 0.2	6.3 ± 0.6	6.0 ± 0	6.8 ± 0.2
22	4.6 ± 0.1	5.6 ± 0.3	4.8 ± 0.1	5.5 ± 0.2
30	6.7 ± 0.5	6.6 ± 0.6	6.0 ± 0.1	6.9 ± 0.1
Persistence				
22	4.5 ± 0.1	5.7 ± 0.3	4.7 ± 0.1	5.4 ± 0.2
30	4.5 ± 0.1	5.7 ± 0.3	4.7 ± 0	5.4 ± 0.2

[a] *S. gordonii* and *S. mutans* cells were mixed at a 1:1 ratio in TV-glucose or TV-galactose medium supplemented with or without 50 mM potassium phosphate buffer (pH 7.5) and incubated continuously for 30 h (persistence assay) or diluted into fresh medium periodically (viability assay). Results are the averages and standard deviations from three individual experiments.

of *S. gordonii* in TV-glucose medium but resulted in a slight reduction in CFU in TV-galactose medium. Conversely, the buffering of the TV-glucose medium appeared to lead to a faster decline in the numbers of *S. mutans* cells than in nonbuffered medium, perhaps associated with an enhanced antagonism by *S. gordonii* against *S. mutans* in a less acidic environment.

Without phosphate buffer, both glucose- and galactose-containing media were acidified overnight to pH values of 4.6 and 4.8, respectively (Table 4). However, the next stage of culturing saw the pH of the cultures stabilize and rise above 6.0 by the end of the assay, a strong indication that a mixed culture dominated by *S. gordonii* could maintain a less acidic environment than *S. mutans* alone. The elevated pH in the *S. gordonii*-enriched cultures was almost assuredly due to the hydrolysis of arginine by the arginine deiminase pathway of *S. gordonii* (19), which is derepressed in galactose cultures compared with glucose cultures.

In the persistence portion of the competition assays, *S. mutans* was found at higher levels than *S. gordonii* under both galactose and glucose conditions without the addition of phosphate buffer. Specifically, in TV medium containing galactose alone, *S. gordonii* managed to maintain a slight dominance at the 22-h time point, but *S. mutans* was present in 2.5-fold-higher numbers at the 30-h time point (Fig. 4). In TV-glucose medium, *S. mutans* cells outnumbered *S. gordonii* cells by 38-fold at the 22-h time point and by 3 logs after 30 h of continuous incubation. When supplemented with 50 mM phosphate buffer, however, *S. gordonii* was able to maintain high levels of viability and to survive much better than *S. mutans*. This was especially clear in galactose cultures with phosphate buffer, where at end of the 30-h mixed coincubation, *S. mutans* was barely detectable. Thus, *S. gordonii* is able to outcompete *S. mutans* when galactose is present and when the environment is not extremely acidic.

Summary and conclusions. The genome of *S. gordonii* strain DL1 contains more than 16 genes dedicated to the metabolism of lactose and galactose, carbohydrates that are commonly found in the oral environment. The growths and PTS activities of various strains constructed for this study indicate that galactose and lactose are transported by PTS permeases and catabolized primarily through the tagatose pathway. Although *S. gordonii* has a *galKTE* pathway, this pathway either is not sufficiently active or is cross-regulated by the *lac* and *gal* systems in a way that does not allow for compensatory growth when certain mutations are present or under the particular conditions tested here. A quantitative assessment of gene expression showed that the *lac* operon, and *lacG* in particular, is strongly induced by lactose but less effectively induced by galactose. In contrast, the induction of the *gal* operon occurs mainly in the presence of galactose and when CCR is relieved. Growth comparisons and a mixed-species competition assay highlight a potentially important role for the *gal* operon in the ability of *S. gordonii* to compete for galactose with the caries pathogen *S. mutans*. Thus, the ability of *S. gordonii* to utilize galactose more efficiently than *S. mutans* may represent an ecological advantage for these, and possibly other, commensal bacteria. It is also notable that the production of hydrogen peroxide by *S. gordonii* is strongly inhibitory to the growth of *S. mutans* and that one of the major pathways for H_2O_2 production, pyruvate oxidase, is repressed by high glucose concentrations but is highly expressed in cells grown in galactose (49). Therefore, during fasting periods, when galactose is a primary carbohydrate for growth, *S. gordonii* may gain a selective advantage over *S. mutans* not only by growing more efficiently but also through more effective antagonism. Possibly, then, galactose or its derivatives may prove effective at promoting an oral flora that is less cariogenic.

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