Involvement of *Streptococcus gordonii* Beta-Glucoside Metabolism Systems in Adhesion, Biofilm Formation, and In Vivo Gene Expression

Ali O. Kiliç,1 Lin Tao,1* Yongshu Zhang,2 Yu Lei,2 Ali Khammanivong,2 and Mark C. Herzberg2,3

Department of Oral Biology, College of Dentistry, University of Illinois at Chicago, Chicago, Illinois 60612,1 and Department of Oral Sciences, School of Dentistry,2 and Mucosal and Vaccine Research Center,3 University of Minnesota, Minneapolis, Minnesota 55455

Received: 4 November 2003/Accepted: 15 March 2004

*Streptococcus gordonii* genes involved in beta-glucoside metabolism are induced in vivo on infected heart valves during experimental endocarditis and in vitro in biofilm formation on saliva-coated hydroxyapatite (sHA). To determine the roles of beta-glucoside metabolism systems in biofilm formation, the loci of these induced genes were analyzed. To confirm the function of genes in each locus, strains were constructed with gene inactivation, deletion, and/or reporter gene fusions. Four novel systems responsible for beta-glucoside metabolism were identified, including three phosphoenolpyruvate-dependent phosphotransferase systems (PTS) and a binding protein-dependent sugar uptake system for metabolizing multiple sugars, including beta-glucosides. Utilization of arbutin and esculin, aryl-beta-glucosides, was defective in some mutants. Esculin and oligosaccharides induced genes in one of the three beta-glucoside metabolism PTS and in four other genetic loci. Mutation of genes in any of the four systems affected in vitro adhesion to sHA, biofilm formation on plastic surfaces, and/or growth rate in liquid medium. Therefore, genes associated with beta-glucoside metabolism may regulate *S. gordonii* in vitro adhesion, biofilm formation, growth, and in vivo colonization.

*Streptococcus gordonii* is a pioneer colonizer on the surface of human teeth, initiating the formation of dental plaque (25). Although not directly associated with dental or gingival diseases in the oral cavity, *S. gordonii* colonizes damaged heart valves once it enters the bloodstream in the experimental rabbit (15) and is considered to cause endocarditis in humans (1, 8, 12). Thus, the virulence of *S. gordonii* is reflected in its ability to adhere, colonize, and survive in the heart, where it is a pathogen, and on teeth in the oral cavity, where it is not. Its virulence in a specific environment may reflect expression of genes uniquely in that environment.

Using an in vivo expression technology (IVET) library constructed in *S. gordonii* V288, at least 13 genes were shown to be expressed in vivo on infected heart valves during experimental endocarditis, but they were unexpressed in vitro under laboratory conditions (15). Since expression occurred only on heart valves, these genes were suggested to contribute to virulence and perhaps pathogenicity. Similarly, *S. gordonii* genes expressed during in vitro colonization of saliva-coated hydroxyapatite (sHA) were identified using the IVET library (17). Among the genes expressed during colonization of sHA and damaged heart valves in the rabbit were beta-glucoside metabolism-encoding genes. We therefore initiated in vitro studies to understand how the beta-glucoside metabolism genes might contribute to colonization of heart valves in infective endocarditis and on sHA.

Bacteria ferment beta-glucoside sugar substrates, including cellobiose, arbutin, salicin, and esculin. While common in plants and useful in establishing phenotypic fermentation patterns of bacteria, the aryl-beta-disaccharides are not found in mammalian tissues and fluids. Mammalian extracellular matrix, however, is rich in glycosaminoglycans (GAGs), which contain beta-linked disaccharide repeating units (22, 33). Structural analogues of cellobiose or *N*,*N*-diacetylchitobiose ([GlcNAc]$_2$), beta-linked disaccharides are released when GAGs degrade. In the rabbit model of endocarditis, *S. gordonii* may have metabolized GAGs from the injured heart valves. Metabolism of beta-glucosides by other species involves known genes. In *Escherichia coli*, the *cel* (cellobiose) operon is induced by (GlcNAc), and has been renamed the *chb* (*N*,*N*-diacetylchitobiose) operon (14). *Listeria monocytogenes* expresses beta-glucoside permease in vivo (10). Hence, metabolism of beta-glucosides, perhaps in the form of GAG-derived oligosaccharides, may be an important adaptive response to survival in vivo.

In the present study, we compared the gene sequences of the IVET clones with the *S. gordonii* genomic data (www.tigr.org) to identify the proximal genes in each locus. Five beta-glucoside-associated genes expressed in vivo during experimental endocarditis were shown to localize with other beta-glucoside metabolism genes comprising two different novel phosphoenolpyruvate-dependent phosphotransferase systems (PTS) and a binding protein-dependent sugar transport and metabolism. We also used this IVET system to identify *S. gordonii* genes critical to and perhaps induced during biofilm formation under in vitro conditions. Among the newly identified genes, many are associated with the metabolism of beta-glucosides through yet another PTS. Therefore, four novel systems for beta-glucoside metabolism have been identified in *S. gordonii* which may contribute to virulence during colonization in mammalian environments.

* Corresponding author. Mailing address: Department of Oral Biology, College of Dentistry, University of Illinois at Chicago, 801 South Paulina St., Chicago, IL 60612. Phone: (312) 355-4077. Fax: (312) 996-6044. E-mail: ltao@uic.edu.
Bacterial strains, media, and chemicals. Bacterial strains used in this study are listed in Table 1. S. gordonii V288 and its reporter gene-fusion strain library based on the streptococcal IVET vector pKpX6 (15) were grown in Todd-Hewitt broth (THB; Difco) or FMC medium (40) at 37°C under CO₂-rich conditions. Tetracycline (TET) was added to a concentration of 15 μg/ml to maintain the selective pressure for biofilm-induced genes. Bacteria were mixed with 1% crystal violet (CV) solution was added into each well. The plates were incubated at 37°C and incubated at 37°C for 24 h, and then 25 μl of a 1% crystal violet (CV) solution was added into each well. The plates were incubated at room temperature for 15 min and rinsed thoroughly with water. Biofilm formation was quantified by the addition of 200 μl of 95% ethanol to each CV-stained well, of which 125 μl was transferred to a new microtiter plate. The absorbency at 568 nm was determined with a plate reader. Bacteria with weak CV staining were picked from the duplicate plates as putative biofilm formation-defective mutants.

Selecting mutants defective in beta-glucoside fermentation. Single colonies of the Streptococcus V288 reporter gene library were plated onto modified Jordan agar supplemented with esculin and ferrous salt (4). The clones that did not show black pigmentation were tested again for their abilities to ferment cellulose, arbutin, and salicin.

PCR procedures. Arbitrary PCR was used to determine the DNA sequence flanking the inserted IVET vector (2, 28). In the first-round PCR, a primer unique to the 5' end of pACYC184 (oriT, 5'-AGTACAGGAAACGTTA AAAAAAACC-3') and an arbitrary primer (ARB1, 5'-GGACAAGCCTGCTG CTAATGACNNNINNNNGATAT3') were used in 50-μl PCR mixtures (1X Vent polymerase buffer, MgSO₄ [1 mM], deoxynucleosine triphosphates [0.25 mM], and Vent [exo⁻] DNA polymerase [1 U] with 5 μl of an overnight THB-grown culture as the DNA template). The first-round PCR conditions were 95°C for 5 min; 6 cycles of 90°C for 30 s, 80°C for 30 s, and 72°C for 1 min; and a final step at 72°C for 10 min. The second-round PCR was performed with the same conditions as the first round, except 5 μl of the first-round PCR product was used as DNA template with primers ARB2 (5'-GGACAAGCCTGCTGACTAGTAC-3') and oriT (5'-CAAGAGATTACGCAGACC3'). The ARB2 sequence was identical to the 5' end of the ARB1 primer. The oriT primer was derived from a sequence of pACYC184 located closer than the oriT sequence to the junction between the inserted pKpX6 and the S. gordonii chromosome (15)

Nucleotide sequence determination and genetic loci identification. The PCR products were purified using the QiAquick PCR purification kit (QIAGEN). The purified PCR products were sequenced using the oriT primer at the Advanced Genetic Analysis Center, University of Minnesota. The sequences were compared with the GenBank database using the BLAST program. The DNA sequence data that matched genes encoding the utilization of beta-glucoside sugars from our previous IVET study (15) and those from the present study were used for BLAST analysis against the partially completed S. gordonii genome (The Institute for Genomic Research [TIGR]; www.tigr.org). In case complete sequence data were not available for a certain locus, the inverse PCR technique (26) was used to complete the sequence data.
Biofilm and sHA adhesion assays. Mutants and previously isolated IVET clones were analyzed for their abilities to form biofilms on microtiter plates as described above. For the sHA adhesion assay, bacteria were cultured overnight (16 to 18 h) in 3 ml of FMC with [3H]thymidine (10$^{9}$Ci/ml). Cells were centrifuged and resuspended to a final optical density at 620 nm of 0.32 (10$^9$ cells/ml). Suspensions (1 ml) were added to tubes with sHA and rotated for 1 h at room temperature. The sHA with bound bacteria was allowed to settle, washed three times, and transferred to scintillation vials. Radioactivity associated with sHA was counted with a scintillation counter. Triplicates of each sample were analyzed. Statistical analysis was performed by using the two-tailed, unequal variance t test with comparisons between the values for the wild type and each mutant.

Sugar induction assay. To analyze whether the expression of the genes encoding beta-glucoside metabolism and/or other genes induced during endocarditis can be induced by any beta-glucoside sugars, strains with the pAK36 plasmid reporter gene fusion were tested on Jordan agar plates supplemented with 0.5% starch and 0.5% cellobiose, arbutin, salicin, esculin, or oligochitosaccharide. Mid-exponential-phase cultures in THB were diluted 10-fold with THB and spotted (5 µl) onto the starch agar plate. After incubation for 16 h, the plates were flooded with the iodine solution to detect the expression of amylase. The wild-type strain V288 was used as a control.

RESULTS

Identification of genes and loci associated with the utilization of beta-glucosides. By homology analysis, DNA sequences of S. gordonii clones induced during experimental endocarditis (15), genes induced during biofilm formation on sHA, and genes required for in vitro biofilm formation on plastic, indicated by diamonds, triangles, and circles, respectively. The star indicates the gene induced by shifting S. gordonii from mildly acidic (oral) to neutral (blood) pH (42). The small bending arrows represent promoter-like sequences. U, overlapping reading frames.

FIG. 1. Genetic loci encoding beta-glucoside metabolism in S. gordonii. Shown are genes induced specifically in the rabbit heart during experimental endocarditis (15), genes induced during biofilm formation on sHA, and genes required for in vitro biofilm formation on plastic, indicated by diamonds, triangles, and circles, respectively. The star indicates the gene induced by shifting S. gordonii from mildly acidic (oral) to neutral (blood) pH (42). The small bending arrows represent promoter-like sequences. U, overlapping reading frames.
*S. gordonii* genome and showed high homology to the mercury resistance gene (*merA*) of many bacteria.

**Nucleotide sequence analysis.** (i) The *bgl* locus. The *bgl* locus includes five open reading frames (ORFs). The first two ORFs, separated by 14 nucleotides, encode two putative transcriptional regulators, BglD (249 amino acids [aa]) and BglE (322 aa). The remaining three ORFs are highly homologous to genes encoding three components, BglA (107 aa), BglB (109 aa), and BglC (433 aa), of a PTS permease (enzyme II) for transporting beta-glucosides in *Streptococcus pyogenes* (Q8NZ62) and *Streptococcus pneumoniae* (Q97SS4). *bglA* is 223 nucleotides downstream of *bglE* and has its own putative promoter. The two downstream ORFs, *bglB* and *bglC*, are separated by only 11 and 2 nucleotides, respectively, from their immediate upstream ORFs. However, a gene encoding a phospho-beta-glucosidase is not found in this locus.

(ii) The *esc* locus. The *esc* locus includes six ORFs. The first ORF encodes the PTS permease (enzyme II), EscP (629 aa), for transporting beta-glucosides. The second ORF encodes a hypothetical protein, EscB (420 aa), which is homologous to the CapA protein associated with capsule synthesis of *Streptococcus agalactiae* (Q8E4G0). The third and fourth ORFs encode two conserved hypothetical proteins, EscC (75 aa) and EscD (55 aa). The fifth ORF, transcribed in the opposite direction, encodes a protein EscR (186 aa) homologous to the *Streptococcus mutans* regulator BglC (Q9K7J8). The last ORF, *escA*, encodes a protein (478 aa) homologous to *S. mutans* phospho-beta-glucosidase BglA (Q9K7J6). These ORFs, except ORF4, have their own putative promoters. A putative RNA antiterminator (RAT) sequence, GGATTGTACTGAGTCACGGCGCAAAAC CTA, is located 125 bases upstream of the initiation codon for the *escP* gene. It matches the consensus sequence of RATs (5).

(iii) The *bfb* locus. The *bfb* locus includes seven ORFs. The first ORF, *bfbF*, encodes a protein (488 aa) homologous to *S. pneumoniae* phospho-beta-glucosidase BglA (Q8D8R9). The second ORF encodes a putative protein, BfbG (79 aa). The third ORF, *bfbB*, encodes the B subunit of a beta-glucoside permease (106 aa; PTS enzyme IB). The fourth ORF, *bfbR*, encodes a BglG-like antiterminator (656 aa). The fifth ORF, *bfbA*, encodes the A subunit of a beta-glucoside permease (105 aa). The sixth ORF, *bfbD*, encodes a hypothetical protein (161 aa), possibly the D subunit of the PTS enzyme II. The seventh ORF, *bfbC*, encodes the C subunit of the PTS permease enzyme II (451 aa) for transporting beta-glucosides.

(iv) The *gom* locus. The *gom* locus has 15 ORFs, including *fucA* (α-1,3/4 fucosidase; 576 aa), *manA* (α-1,2-mannosidase; 697 aa, in opposite orientation), *gomA* (hypothetical protein; 435 aa), *manB* (α-1,6 mannosidase; 877 aa), *gomR* (regulator; 292 aa), *bhsA* (N-acetyl-beta-hexosaminidase; 627 aa), *gomF* and *gomG* (binding protein-dependent sugar transporter membrane components; 311 aa each), *gomE* (sugar-binding protein; 515 aa), *gomB* (hypothetical protein; 204 aa), *gomH* (two-component sensor histidine kinase; 529 aa), *gomD* (two-component response regulator; 427 aa), *bgL* (beta-glucosidase; 461 aa), *gomL* (VirJ-like sensor histidine kinase; 176 aa), and *glmS* (glucosamine-6-P synthase; 603 aa). An ATP-binding protein for the ABC sugar transport cassette is not found in this locus.

(v) The *bfr* locus. Although the *bgl* locus does not have a beta-glucosidase, a gene encoding 6-phospho-beta-glucosidase (Bgf; 479 aa) was identified in a separate locus, the *bfr* locus. Immediately downstream of *bglF* are two genes, *bfrA* and *bfrB*, encoding a biofilm-related two-component system (44).

**Phylogenetic analysis.** *S. gordonii* may use four different systems to metabolize beta-glucosides during biofilm formation and/or in vivo growth. To determine their genetic similarities and relationships with related proteins of other bacterial species, phylogenetic trees were constructed (data not shown). *S. gordonii* BglI is more homologous to beta-glucosidasises of *S. pneumoniae* (BglA2; Q8DRA9) and *S. pyogenes* (BglA2; Q8K735) than its three other beta-glucosidasises, BglF, BfbF, and EscA. Likewise, *S. gordonii* EscP is more similar to BglP of *S. mutans* than to its two other beta-glucoside permeases, BglC and BfbC, which contain three or four subunits. *S. gordonii* EscB is highly homologous to capsule synthases of a range of species and genera, including CapA of *S. agalactiae* (Q8E4G0).

**Phenotypes of various strains.** In addition to the reporter gene-fusion strains (some also created mutations by splitting target genes), insertion or deletion mutants were constructed to assign phenotypes to the genes located in these loci. When sugar fermentation patterns were compared, the mutants were similar to the wild-type except for hydrolysis of two beta-glucosides, arbutin and salicin. Most mutants defective in one gene involved in beta-glucoside metabolism appeared to ferment these two sugars more slowly. The mutants and wild-type strains, in contrast, fermented two other beta-glucoside sugars, esculin and cellobiose, similarly. Only two mutants, MG1015 (*bfr amyl cat*) and CG532 (*merA amyl cat*), were defective in arbutin, salicin, and esculin fermentation, suggesting suppression of multiple beta-glucoside regulons in these mutants. Regardless of insertion sites, all 13 strains with pAK36 insertions isolated by in vivo selections and about one-third of random clones of the pAK36 library displayed glucose inhibition of esculin hydrolysis, suggesting a possible artifact introduced by the plasmid. Therefore, strains (all CG strains except CG532) constructed with pSF151 or pSA891 were used to test for esculin inhibition. The wild-type and the tested mutant strains, except CG423 (*bglD*) and CG495 (*gom*), fermented esculin in the presence of glucose.

The wild-type strain did not ferment α-1,2- or α-1,3-d-mannobiose, α-1,3-α-1,6-d-mannopentose, heparin, chondroitin sulfate, or lichenan, but it fermented Lewis-X oligosaccharide, N,N',N' triacetylchitotriose (chitin oligosaccharides), α-1,6-d-mannobiose, and GlcNAc-β-1,2-mannose. All mutants fermented Lewis-X oligosaccharide and chitin oligosaccharides. Mutants defective in *manB*, *gomH*, or *gomR* showed reduced fermentation of GlcNAc-β-1,2-d-mannose and no fermentation of α-1,6-d-mannobiose.

To determine if selected genes were induced by any β-glucoside sugars, α-amylase activities of the reporter gene-fused clones were detected in agar containing starch (Fig. 2). Based on amylase reporter activities, cellobiose, arbutin, and salicin did not induce expression of any genes tested. Esculin and oligochitosaccharides induced genes in the *esc* system but not any of the other three systems for β-glucoside metabolism. Also, by testing other genes induced during endocarditis (15) or required for biofilm formation (44), four additional genetic loci were induced by the same two sugars. These were *iviE* (located upstream of *dnaX*), *iviH* (an amino acid transporter), *iviK* (*msrA*, peptide methionine sulfoxide reductase), and
bfrAB. Interestingly, mutants defective in these esculin-inducible genes showed iodophilic polysaccharide (IPS), as detected on plates containing glucose and starch. IPS was not seen in the wild type or strains with plasmid insertions in other beta-glucoside regulons. The escA (CG420 and CG425) and escP (CG421 and CG426) mutants constructed with plasmids pSF151 or pVA891 without the amy-cat reporter gene also showed IPS on agar containing glucose and starch (data not shown). This ruled out the possibility that IPS accumulation in these strains was an artifact introduced by the amy reporter gene.

To ascertain if genes in these loci were associated with functions in vitro, adhesion to sHA, biofilm formation on plastic, and growth rates of these strains were analyzed in vitro (Table 2). Mutation of many genes in these four presumptive regulons resulted in decreased biofilm formation and/or growth rate. Remarkably, mutation in escA, escR, or gomH and deletion of the gom regulon resulted in cells with defective abilities to adhere to sHA in buffer. In some cases, these mutations were accompanied by defects in growth rate or in biofilm formation (bglC and escA). Other genes associated with defective adhesion did not manifest defects in biofilm formation or growth rate (gomH and Δgom). While defective in adhesion, the escR mutant formed biofilm and showed a growth rate similar to that of wild type. Mutation in escB2 showed no adhesion defect, but biofilm formation was substantially reduced.

DISCUSSION

Several S. gordonii surface components probably mediate adhesion to the tooth surface, including sialic acid-binding adhesin (37), amylase-binding protein (30), CshA/B (23), SspA/B (6), and ScaA (16). While genes for these adhesin proteins may contribute to colonization in vivo, none are well established at this time. Recently described genes associated with S. gordonii biofilm formation encode putative proteins involved in signal transduction, peptidoglycan biosynthesis, manganese homeostasis, and fructose PTS (18–20). Furthermore, S. gordonii cells forming biofilms in vivo on heart valves (15) or in vitro on sHA (17) induced genes associated with beta-glucoside metabolism at unexpected frequencies.

To survive in vivo and colonize as a biofilm, S. gordonii must seek a carbon source for growth. Available nutrients, especially sugars, will differ from those found in laboratory growth media. Due to variations in available sugars in nature, bacteria have developed highly controlled sugar metabolism systems. If multiple sugars exist in the environment, bacteria would first consume primary sugars, such as glucose. When primary sugars are exhausted or unavailable, bacteria induce genes for metabolizing secondary sugars (29), including beta-glucosides. Expression of these genes is often subject to carbon catabolite repression by primary sugars (32). Exceptions exist. For example, S. mutans has two systems to metabolize beta-glucoside sugars (3–5). One system is inhibited by glucose, while the other is not.

Induced in vivo during experimental endocarditis (15) and in vitro during biofilm formation on sHA (17), and also required for biofilm formation on plastic microtiter plates (44), four novel transport and metabolism systems for beta-glucosides have been identified. Three are PTS, and the fourth is a binding protein-dependent sugar uptake system for metabolizing multiple sugars including beta-glucosides. Because each of these systems consists of multiple transcriptional units, they appear to organize and function as regulons, rather than operons.

The first putative regulon, bgl, has five genes in two transcriptional groups. The first group encodes two putative transcriptional regulators. The second group encodes three units of a PTS enzyme II permease. Since the bgl locus does not have a beta-glucosidase gene, this gene may be at a separate locus. The bfr locus has a gene encoding a beta-glucosidase (BglF). Whether this gene is related to the bgl regulon is
might have polar effects on their downstream genes (Fig. 1). gomF, which may have proportionally affected their adhesion and/or biofilm formation. The phenotypes of three mutants, MG2026 (escB1-amy-cat), KG205 (iviE-amy-cat), and KG208 (iviH-amy-cat), were also induced by the same two sugars. Although repressed by glucose (Fig. 2B), induction was attributed to the specific presence of esculin rather than the absence of glucose in the esculin medium, because other beta-glucosides, cellbiose, salicin, and arbutin did not induce genes in this regulon. Hence, the sensor system shows considerable specificity for esculin and mammalian cognate sugars.

unknown. The two different regulators may be responsible for controlling the two separate loci. bglC is induced in vivo during experimental endocarditis (15), but it is not induced by any of the commercially available beta-glucoside sugars in vitro. Yet, inactivation of bglC reduced the S. gordonii growth rate (Table 2) and its ability to hydrolyze arbutin and salicin. Nonetheless, the reduction in adhesion to sHA and biofilm formation on plastic might be associated with its defective growth.

The second putative regulon, esc, which is induced by the beta-glucoside esculin and oligochitosaccharides, includes six genes. Three genes encode proteins for transport (EscP), metabolism (EscA), and regulation (EscR) of a PTS. Although the functions of the remaining three genes (EscB, EscC, and EscD) are unknown, EscB may encode a capsule (polyglutamate) synthase, because it is homologous to the capA gene of other bacteria, especially group B streptococci. Three genes of the regulon, escA, escR, and escB, are induced in vivo in the heart during experimental endocarditis (15) and also were induced in vitro by esculin and oligochitosaccharides (Fig. 2A). Although repressed by glucose (Fig. 2B), induction was attributable to the specific presence of esculin rather than the absence of glucose in the esculin medium, because other beta-glucosides, cellbiose, salicin, and arbutin did not induce genes in this regulon. Hence, the sensor system shows considerable specificity for esculin and mammalian cognate sugars.

When the wild-type S. gordonii strain grew in a glucose-containing medium, IPS accumulated (data not shown). Endogenous amylose was induced when the cells were grown in a medium containing both glucose and starch, and IPS was not seen (Fig. 2C). When mutants defective in esculin-inducible genes were grown in the presence of glucose and starch, however, intracellular alpha-amylase appeared to be repressed (35) and IPS accumulated in the cells (Fig. 2B and C). How these genes affect the accumulation of IPS in S. gordonii is unknown. In S. mutans, the accumulation of IPS is regulated by the cell wall synthesis-associated dll operon (36). Similarly, esculin-inducible genes may play a role in S. gordonii cell wall and/or surface polysaccharide synthesis, because mutation in these genes caused defects in in vitro adhesion to sHA, biofilm formation on plastic, and/or the growth rate (Table 2).

The esc regulon may be highly conserved, because esc-like regulons exist in many bacteria, including S. mutans (3). In comparison with the S. mutans bgl (5), the S. gordonii esc also has a RAT site upstream of escP, but it does not have a licT-like gene nearby. Moreover, escB, the gene immediately downstream of escP, is not homologous to S. mutans bglB. Although both S. gordonii esc and S. mutans bgl are induced by esculin, the former is repressed by glucose while the latter is not (4). It is unknown why esculin and oligochitosaccharides also induce several other genes, including msrA. Interestingly, when S. gordonii is shifted from the oral pH (slightly acidic) to blood pH (neutral), both msrA and escR (previous name, Sgp1224) are induced (42).

The third putative regulon, bfb, encodes proteins apparently involved in biofilm-associated beta-glucoside metabolism. It includes seven ORFs, which encode four subunits (BfbA, -B, -C, and -D) of a PTS enzyme II permease, a phospho-beta-glucosidase, and a bglG-like antiterminator. Although unin-
duced in vivo during experimental endocarditis, this regulon was induced during in vitro *S. gordonii* biofilm formation on sHA. As expected, mutants with mutations in several *fbf* genes showed reduced biofilm formation on plastic in vitro. Likewise, a fructose PTS operon has been recently linked to *S. gordonii* biofilm formation (20).

The fourth putative regulon, *gom*, is for glycoprotein-derived oligosaccharide metabolism. It includes 15 genes encoding a binding protein-dependent sugar transport module, a two-component system, a regulator, and five sugar-degrading enzymes (a fucosidase, two mannosidases, an N-acetyl-beta-hexosaminidase, and a beta-glucosidase). The regulon may encode a binding-protein-dependent system for transport and the metabolism of multiple sugars, like the *msm* system in *S. mutans* (31, 39). However, an *msmK*-like gene encoding an ATP-binding protein is absent in the *gom* locus. It may be located elsewhere, as is the case in *Streptococcus equisimilis* (24). Since the substrate sugars, such as fucosides, mannosides, N-acetyl-beta-hexosamines, and beta-glucosides, are commonly found in mammalian glycoprotein-derived oligosaccharides, it is not surprising that this regulon was induced only in vivo in the rabbit and not under in vitro growth conditions. Due to the presence of a two-component regulatory system, *gomH* and *gomI*, expression of the *gom* regulon may also be more sensitive to environmental changes than other binding protein-dependent sugar metabolism systems, such as the *S. mutans msm* operon (39). The *gomA* gene was expressed in vivo and was an insertion site of the IVET plasmid pAK36, but its function is currently unknown. Mutants defective in *manB*, *gomH*, or *gomR* showed reduced or negative fermentation of GlcNAc-1-6-D-mannobiose, which may be a two-component system. Both the in vivo and in vitro selections were done by induction (KG201 to -213) displayed glucose-mediated inhibition of esculin hydrolysis, while strains isolated due to defects in biofilm formation (MG1006 and MG1007) did not, probably because the regulator *amy* gene in these mutants was inserted off frame and thus was not expressed (Table 2). Since wild-type *S. gordonii* showed esculin hydrolysis in the presence of glucose, at least one regulon, *gom*, is not subject to catabolite repression by glucose. Internally released glucose also appears to be a potent catabolite repressor. It can repress multiple regulons for beta-glucoside metabolism. Arbutin and esculin were not fermented by the *amy-cat* reporter gene-fusion strains, MG1015 (*bfr*) and CG532 (*merA*), in the presence or absence of glucose. In wild-type cells, therefore, conditions favoring hydrolysis of stored polysaccharides may promote strong catabolite repression of several beta-glucoside regulons as an important mechanism for survival during sugar starvation.

Based on currently available genomic data published by TIGR, *S. gordonii* has four different beta-glucosidases. Phylogenetic analysis showed that the *S. gordonii* beta-glucosidases of the three PTS are related, but the one in the *gom* regulon, *bgI*, is relatively different. The four different beta-glucosidases do differ more in amino acid sequences from one another, however, than from their respective homologues in other species, reflecting different conserved functions and substrate specificities. Because mammalian-derived beta-glucosides are unknown, we tested multiple commercially available beta-glucoside sugars, cellobiose, arbutin, salicin, esculin, and oligochitosaccharides, to identify possible preferred substrates for each system. Only the *esc* regulon was induced by esculin and oligochitosaccharides. The several beta-glucoside-related genes expressed specifically in vivo may require natural substrates that are embedded in the mammalian oligosaccharides on the heart valve. Interestingly, the *fbf* genes were not induced in vivo but were induced during biofilm formation on sHA. This suggested that *fbf* may have a different function from the three other regulons. Since *Streptococcus exopolysaccharides* have beta-linked repeating disaccharides (7, 43) and its capsule resembles a typical GAG, hyaluronan (34), the expression of these genes in *S. gordonii* may be needed for the synthesis, remodeling, and/or recycling of cell surface adhesins (glycoproteins) and/or capsule-like polysaccharides.

The *amy* gene as a reporter might have introduced artifact due to possible amylase degradation of intracellular glycogen. There might be still-other-undetected artifacts, but the isolation of beta-glucoside genes could not be attributed to one of them because both the in vivo and in vitro selections were done by the induction of *cat* gene expression (chloramphenicol resistance). Moreover, Gahan and Hill (10), using lysterolysin as an IVET reporter, have also identified an in vivo-induced gene encoding beta-glucoside permease in *Listeria*.

In summary, four different beta-glucoside metabolic systems have been identified in *S. gordonii*. These systems are induced in vivo and/or contribute to in vitro adhesion and biofilm formation. Not only could they metabolize sugars to provide en-
ergy and a carbon source for *S. gordonii* survival in the host, but also they may be involved in other physiological processes, such as the synthesis of cell surface glycoproteins and/or polysaccharides involved in adhesion and biofilm formation. To understand their preferred substrates and potential roles in streptococcal virulence, further studies of these systems will be necessary.

**ACKNOWLEDGMENTS**

This work was supported by U.S. Public Health Service grants DE08590 and DE11400. We thank TIGR for its publication of the partially completed *S. gordonii* genomic sequence.

**REFERENCES**

1. Baddour, L. M. 1994. Virulence factors among gram-positive bacteria in experimental endocarditis. Infect. Immun. 62:2143–2148.
2. Caetano-Anollés, G. 1993. Amplifying DNA with arbitrary oligonucleotide primers. PCR Methods Appl. 3:55–94.
3. Cote, C. K., D. Cvitkovich, A. S. Blewes, and A. L. Honeyman. 2000. A novel beta-glucoside-specific PTS locus form *Streptococcus mutans* that is not inhibited by glucose. Microbiology 146:1555–1563.
4. Cote, C. K., and A. L. Honeyman. 2002. The transcriptional regulation of the *Streptococcus mutans* bgl regulon. Oral Microbiol. Immunol. 17:1–8.
5. Cote, C. K., and A. L. Honeyman. 2003. The LiCT protein acts as both a positive and a negative regulator of loci within the bgl regulon of *Streptococcus mutans*. Microbiology 149:1333–1340.
6. Demuth, D. R., Y. Duan, W. Brooks, A. R. Holmes, R. McNab, and H. F. Jenkinson. 1996. Tandem genes encode cell-surface polypeptides SpxA and SpB which mediate adhesion of the oral bacterium *Streptococcus gordonii* to human and bacterial receptors. Mol. Microbiol. 20:403–413.
7. Deng, L., D. L. Kasper, T. P. Krick, and M. R. Wessels. 2000. Characterization of the linkage between the type III capsular polysaccharide and the bacterial cell wall of *B streptococcus*. J. Biol. Chem. 275:7497–7504.
8. Durack, D. T. 1995. Prevention of infective endocarditis. N. Engl. J. Med. 332:38–44.
9. Fernandez-Herrero, L. A., M. A. Badet-Denisot, B. Badet, and J. Berenguer. 1995. glmS of *Thermus thermophilus* HB8: an essential gene for cell-wall synthesis identified immediately upstream of the S-gene. Mol. Microbiol. 17:1–12.
10. Gahan, C. G., and C. Hill. 2000. The use of listeriolysin to identify in vivo induced genes in the gram-positive intracellular pathogen *Listeria monocytogenes*. Mol. Microbiol. 36:498–507.
11. Gong, K., L. Mailloux, and M. C. Herzberg. 2000. Salivary film expresses a complex, macromolecular binding site for *Streptococcus sanguis*. J. Biol. Chem. 275:8970–8974.
12. Herzberg, M. C. 1996. Platelet-streptococcal interactions in endocarditis. Crit. Rev. Oral Biol. Med. 7:222–236.
13. Jordan, H. W., R. J. Fitzgerald, and A. E. Bowler. 1960. Inhibition of experimental caries by sodium metaphosphate and its effect on the growth and metabolism of selected bacteria. J. Dent. Res. 39:116–123.
14. Keyhani, N. O., and S. Rosenmann. 1997. Wild-type *Escherichia coli* grows on the chitin disaccharide, N,N’-diacetylchitobiose, by expressing the chd operon. Proc. Natl. Acad. Sci. USA 94:14367–14371.
15. Kilic, A. O., M. C. Herzberg, M. W. Meyer, X. Zhao, and L. Tao. 1999. Streptococcal reporter gene-fusion vector for identification of in vivo expressed genes. Plasmid 42:67–72.
16. Kolenbrander, P. E., R. N. Andersen, and N. Ganeshkumar. 1994. Nucleotide sequence of the *Streptococcus gordonii* PK488 coaggregation adhesin gene, *sad*, and ATP-binding cassette. Infec. Immun. 62:4469–4480.
17. Lei, Y., Y. Zhang, A. O. Kilic, and M. C. Herzberg. 2002. *Streptococcus gordonii* gene expression during adhesion and biofilm formation in an in vitro model. J. Dent. Res. 81(Spec. Iss. Iss. A117).
18. Luo, C. Y., D. A. Corliss, and N. Ganeshkumar. 2000. *Streptococcus gordonii* biofilm formation: identification of genes that code for biofilm phenotypes. J. Bacteriol. 182:1374–1382.
19. Luo, C. Y., K. Mitraikul, I. B. Voss, C. V. Hughes, and N. Ganeshkumar. 2003. Involvement of the *adl* operon and manganese homeostasis in *Streptococcus gordonii* biofilm formation. J. Bacteriol. 185:2887–2900.
20. Luo, C. Y., K. Mitraikul, I. B. Voss, C. V. Hughes, and N. Ganeshkumar.