Cell Surface Glycoside Hydrolases of *Streptococcus gordonii* Promote Growth in Saliva

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**ABSTRACT**

The growth of the oral commensal *Streptococcus gordonii* in saliva may depend on a number of glycoside hydrolases (GHs), including three cell wall-anchored proteins that are homologs of pneumococcal β-galactosidase (BgaA), β-N-acetylglucosaminidase (StrH), and endo-β-N-acetylglucosaminidase D (EndoD). In the present study, we introduced unmarked in-frame deletions into the corresponding genes of *S. gordonii* DL1, verified the presence (or absence) of the encoded proteins on the resulting mutant strains, and compared these strains with wild-type strain DL1 for growth and glucan foraging in saliva. The overnight growth of wild-type DL1 was reduced 3- to 10-fold by the deletion of any one or two genes and approximately 20-fold by the deletion of all three genes. The only notable change in the salivary proteome associated with this reduction of growth was a downward shift in the apparent molecular masses of basic proline-rich glycoproteins (PRG), which was accompanied by the loss of lectin binding sites for galactose-specific *Erythrina cristagalli* agglutinin (ECA) and mannose-specific *Galanthus nivalis* agglutinin (GNA). The binding of ECA to PRG was also abolished in saliva cultures of mutants that expressed cell surface BgaA alone or together with either StrH or EndoD. However, the subsequent loss of GNA binding was seen only in saliva cocultures of different mutants that together expressed all three cell surface GHs. The findings indicate that the growth of *S. gordonii* DL1 in saliva depends to a significant extent on the sequential actions of first BgaA and then StrH and EndoD on N-linked glycans of PRG.

**IMPORTANCE**

The ability of oral bacteria to grow on salivary glycoproteins is critical for dental plaque biofilm development. Little is known, however, about how specific salivary components are attacked and utilized by different members of the biofilm community, such as *Streptococcus gordonii*. *Streptococcus gordonii* DL1 has three cell wall-anchored glycoside hydrolases that are predicted to act on host glycans. In the present study, we introduced unmarked in-frame deletions in the corresponding genes, verified the presence (or absence) of encoded proteins on the resulting mutant strains, and compared these strains with wild-type DL1 for growth and glucan foraging in saliva. The results indicate that the growth of *S. gordonii* DL1 depends to a significant extent on sequential action of these cell surface GHs on N-linked glycans of basic proline-rich salivary glycoproteins, which appears to be an essential first step in salivary glucan foraging.

The development of dental plaque in the absence of exogenous nutrients has long been thought to depend on bacterial foraging of host salivary glycans (1, 2). Little is known, however, about how different oral species attack and utilize salivary macromolecules as substrates for growth and whether cross-feeding between species influences dental plaque biofilm development. Recently, we compared the growth of different oral bacteria in filter-sterilized saliva that was first heat treated to inactivate endogenous glycoside hydrolases and then dialyzed to remove possible low-molecular-mass growth substrates. Growth through two transfers in this model system was observed elsewhere for strains of *Streptococcus gordonii*, *Streptococcus oralis*, and *Streptococcus mitis* but not for strains of certain other species, including *Actinomyces* spp., *Abiotrophia defectiva*, *Rothia* spp., *Streptococcus salivarius*, *Streptococcus sanguinis*, *Streptococcus sobrinus*, and *Streptococcus vestibularis*, which grew poorly or not at all (3). Importantly, the growth of the first three streptococcal species mentioned above was accompanied by degradation of basic proline-rich glycoproteins (PRG), whereas species that failed to grow did not alter the electrophoretic mobility or lectin reactivity of PRG nor any other salivary component.

*S. gordonii* strain DL1 (i.e., Challis) was one of the streptococci that grew well in saliva and appeared to utilize PRG as a growth substrate. Annotation of this strain in the Carbohydrate-Active Enzymes database (CAZY; www.cazy.org) revealed 31 putative glycoside hydrolases (GHs) distributed among 16 GH families. Based on specific GH activities associated with these families, we surmised that host glucan foraging could involve at least 17 putative GHs of *S. gordonii*. Of these, three were identified as cell wall-anchored proteins based on the predicted presence of N-terminal signal peptides and C-terminal sorting motifs (4). Moreover, these three proteins were homologues of *Streptococcus pneumoniae* β-galactosidase (BgaA), β-N-acetylglucosaminidase that grew well in saliva and appeared to utilize PRG as a growth substrate. Annotation of this strain in the Carbohydrate-Active Enzymes database (CAZY; www.cazy.org) revealed 31 putative glycoside hydrolases (GHs) distributed among 16 GH families. Based on specific GH activities associated with these families, we surmised that host glucan foraging could involve at least 17 putative GHs of *S. gordonii*. Of these, three were identified as cell wall-anchored proteins based on the predicted presence of N-terminal signal peptides and C-terminal sorting motifs (4). Moreover, these three proteins were homologues of *Streptococcus pneumoniae* β-galactosidase (BgaA), β-N-acetylglucosaminidase...
(StrH), and endo-β-N-acetylglucosaminidase D (EndoD), which are known to act on host glycans (5, 6) and, in the case of BgaA and StrH, to promote the growth of S. pneumoniae on the human serum component α-1-acid glycoprotein (7).

Based on the information summarized above, we hypothesized that BgaA, StrH, and EndoD of S. gordonii are critical for salivary glycan foraging. To test this hypothesis, we deleted the genes for these proteins in different combinations from S. gordonii DL1 and then compared the growth of the wild-type and mutant strains in saliva. The results indicate that the growth of S. gordonii DL1 depends on the following steps.

**MATERIALS AND METHODS**

**Plasmids and bacterial strains.** The plasmids listed in Table 1 were purified by using QIAprep spin miniprep kits (Qiagen) from Escherichia coli DH5α, grown in LB broth containing either ampicillin (75 μg/ml) or erythromycin (500 μg/ml) to maintain antibiotic resistance. Wild-type S. gordonii DL1 and glycoside hydrolase deletion mutant (ΔGH mutant) strains (Table 1) were maintained at ~70°C as frozen stock cultures in Todd-Hewitt broth (THB; Oxoid) containing 40% glycerol and were transferred to THB or another complex medium, as indicated below, at the beginning of each experiment.

**Plasmid construction.** Unmarked in-frame deletions were introduced into S. gordonii DL1 using a nonrepressible allelic exchange vector similar to pCWU3, which was developed for use in Actinomyces oris (11). We found that the kanamycin resistance and mCherry fluorescence markers of pCWU3 were not optimal for allelic exchange in S. gordonii DL1, and we therefore prepared a similar plasmid, designated pEG (Fig. 1 and Table 1), that contained the ermAM cassette and expressed green fluorescent protein (GFP) from the constitutive S. gordonii promoter, Prpsj. The ermAM cassette was amplified by PCR from pKSerm2 using KOD hot start DNA polymerase (Novagen) and primers ermAM-F/ermAM-R (Table 2). The resulting PCR product was cloned into the AclI site of dephosphorylated pUC19c to obtain pUC19c-1. The 198-bp sequence of Prpsj, which is located immediately upstream from rpsj (i.e., SGO_1186), was amplified from S. gordonii DL1 genomic DNA with primer pair rpsj-F/rpsj-R. The 720-bp sequence of gfp (including two TAA stop codons) was codon optimized for expression in S. gordonii and synthesized by Blue Heron Biotechnology, Inc. (Bothell, WA). The optimized sequence was amplified from a plasmid containing derivative of pBR322 using primer pair gfp-F/gfp-R. The sequences of Prpsj and gfp were linked by overlap extension PCR performed with primer pair rpsj-F/gfp-R and using the corresponding PCR products as the template. The resulting 0.9-kb PCR product (Prpsj-gfp) was cloned into the EcoRI site of ermAM-containing pUC19c-1 to obtain pEG.

Gene exchange cassettes containing deletion constructs of S. gordonii DL1 bgaA, strH, or endoD (i.e., SGO_1486, SGO_0405, or SGO_0208, respectively) flanked by approximately 1-kb adjacent gene-targeting sequences were prepared from overlapping PCR products amplified from genomic DNA with primer pairs (Table 1) GH2-1/GH2-2 and GH2-3/GH2-4, GH20-1/GH20-2 and GH20-3/GH20-4, and GH85-1/GH85-2 and GH85-3/GH85-4. The deletions in the gene exchange cassettes removed residues 32 to 2303 from the 2,350-amino-acid-coding sequence of bgaA, residues 27 to 1129 from the 1,142-amino-acid-coding sequence of strH, and residues 25 to 1532 from the 1,345-amino-acid-coding sequence of endoD. Each cassette was cloned into pEG as indicated in Table 1 to obtain pEGΔbgaA, pEGΔstrH, or pEGΔendoD respectively.

**Isolation of ΔGH mutants.** Transformation of S. gordonii DL1 was performed as previously described (12) by incubating early-log-phase cells with 30 to 50 ng of pEGΔbgaA, pEGΔstrH, or pEGΔendoD. Reaction mixtures were plated on brain heart infusion (BHI) agar (Oxoid) containing 10 μg/ml erythromycin to select for Em<sup>+</sup> transformants that also expressed GFP from the integrated plasmid. Integration of each plasmid at the target site was confirmed by PCR performed with primers designed from adjacent plasmid and S. gordonii chromosomal sequences in Table 2, primers UC19-F with GH2-6, GH20-6, or GH85-6 and UC19-R with GH2-5, GH20-5, or GH85-5. Plasmid-bearing clones were then transferred and characterized by PCR, colony PCR, and sequencing. The strain for pEGΔbgaA was used for the remaining experiments.

**TABLE 1** Bacterial strains and plasmids used in this study

<table>
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<th>Plasmids or strain</th>
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<tr>
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<td>pEG with bgaA deletion cassette in KpnI site</td>
<td>This study</td>
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<td>pEG with strH deletion cassette in SalI and HindIII sites</td>
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passaged several times in antibiotic-free medium to allow excision of integrated plasmids (accompanied by loss of Em' and GFP fluorescence) prior to plating. Plasmid-free (nonfluorescent) colonies were typically seen at frequencies of from 10⁻⁴ to 10⁻³ and were distinguished from plasmid-bearing (fluorescent) colonies using a filter paper reader. The nonfluorescent colonies were screened by PCR with primer pairs that amplified across deletions (Table 2, GH2-F/GH2-R, GH20-F/GH20-R, or GH85-F/GH85-R) prior to plating. Plasmid-free (nonfluorescent) colonies were typically integrated plasmids (accompanied by loss of Em' and GFP fluorescence) before repeating the procedure described above. As summarized in Table 1, ΔβgaA ΔstrH, ΔβgaA ΔendoD, and ΔstrH ΔendoD double mutants were derived from ΔstrH or ΔendoD single mutants (i.e., strains ΔH/D or ΔH/D, respectively), and the ΔβgaA ΔstrH ΔendoD triple mutant (i.e., strain ΔA/H/D) was derived from the ΔβgaA ΔendoD double mutant strain (Table 1).

**Enzyme assays.** Bacteria cultured in THB, heart infusion broth (HIB; Becton, Dickinson), or whole saliva were washed in saliva-salts buffer (3) and homogenized in 2.5 volumes of buffer, and disrupted by sonication on ice. The culture was collected by centrifugation (13,000 g, 1 h at 24°C) and applied to small columns of Ni²⁺-nitrilotriacetic acid (NTA)-agarose. The columns were rinsed with wash buffer (50 mM Tris-HCl, 0.5 M NaCl, and 20 mM imidazole, pH 8.0) to remove unbound material and eluted with 0.5 M imidazole (in wash buffer) to obtain recombinant proteins containing the putative catalytic region of each cell surface GH were prepared for use as immunogens. The nucleotide sequences for amino acid residues 80 to 609 of BgaA (13, 14), 136 to 507 of StrH, or 6 to 396 of EndoD were synthesized, and cloned into pTrcHisB (Invitrogen) by Blue Heron Biotechnology, Inc. Plasmid-bearing E. coli EC100 bacteria were grown at 37°C with aeration in Luria-Bertani broth supplemented with ampicillin (150 μg/ml). At an A₆₀₀ of 0.4, the cultures were rapidly chilled in ice water, and isopropyl thigalactopyranoside (IPTG) was added to a final concentration of 1 mM. Cultures were then incubated for an additional 6 h at 24°C (to permit the formation of inclusion bodies). Cells were harvested by centrifugation (13,000 × g for 15 min at 5°C), washed by resuspension and centrifugation in 25 mM Tris-HCl (pH 7.5) buffer, homogenized in 2.5 volumes of buffer, and disrupted by sonication on ice. The extracts were clarified by high-speed centrifugation (180,000 × g for 1 h at 5°C) and applied to small columns of Ni²⁺-nitrilotriacetic acid (NTA)-agarose. The columns were rinsed with wash buffer (50 mM Tris-HCl, 0.5 M NaCl, and 20 mM imidazole, pH 8.0) to remove unbound material and eluted with 0.5 M imidazole (in wash buffer) to obtain 6×His-BgaA (60.4 kDa), 6×His-StrH (41.8 kDa), or 6×His-EndoD (73.8 kDa).

Rabbit antisera against keyhole limpet hemocyanin (KLH; Pierce)-conjugated 6×His-BgaA, KLH-conjugated 6×His-StrH, or 6×His-EndoD alone were used in the ELISA.

**Immunological methods.** N-terminally polyhistidine (6×His)-tagged recombinant proteins containing the putative catalytic region of each cell surface GH were prepared for use as immunogens. The nucleotide sequences for amino acid residues 80 to 609 of BgaA (13, 14), 136 to 507 of StrH (15, 16), and 113 to 762 of EndoD (17), including in-frame 5’-BamHI and 3’- HindIII cloning sites and the requisite start (ATG) and stop codons (TAG or TGA), were optimized for expression in E. coli, synthesized, and cloned into pTrcHisB (Invitrogen) by Blue Heron Biotechnology, Inc. Plasmid-bearing E. coli EC100 bacteria were grown at 37°C with aeration in Luria-Bertani broth supplemented with ampicillin (150 μg/ml). At an A₆₀₀ of 0.4, the cultures were rapidly chilled in ice water, and isopropyl thigalactopyranoside (IPTG) was added to a final concentration of 1 mM. Cultures were then incubated for an additional 6 h at 24°C (to permit the formation of inclusion bodies). Cells were harvested by centrifugation (13,000 × g for 15 min at 5°C), washed by resuspension and centrifugation in 25 mM Tris-HCl (pH 7.5) buffer, homogenized in 2.5 volumes of buffer, and disrupted by sonication on ice. The extracts were clarified by high-speed centrifugation (180,000 × g for 1 h at 5°C) and applied to small columns of Ni²⁺-nitrilotriacetic acid (NTA)-agarose. The columns were rinsed with wash buffer (50 mM Tris-HCl, 0.5 M NaCl, and 20 mM imidazole, pH 8.0) to remove unbound material and eluted with 0.5 M imidazole (in wash buffer) to obtain 6×His-BgaA (60.4 kDa), 6×His-StrH (41.8 kDa), or 6×His-EndoD (73.8 kDa).

**Rabbit antisera against keyhole limpet hemocyanin (KLH; Pierce)-conjugated 6×His-BgaA, KLH-conjugated 6×His-StrH, or 6×His-EndoD alone were used in the ELISA.**
were prepared at Covance Research Products (Denver, PA) by subcutaneous injections of the antigen in Freund's adjuvant following standard protocols. Dot immunoblotting was performed with bacteria from overnight HIB cultures that were spotted (~6 x 10^7 bacteria per 3-mm spot) on nitrocellulose membranes with a Bio-Dot microfiltration apparatus (Bio-Rad). Membranes were blocked in Tris-buffered saline containing 0.1% Tween 20 and 2% skim milk and then incubated with 1/1,000 anti-GH antiserum that was first preabsorbed at a dilution of 1/10 with triple mutant strain ΔA/H/D to remove background anti-S. gordonii antibody. Bound anti-GH antibody was detected using horseradish peroxidase-conjugated goat anti-rabbit IgG (Bio-Rad) and a metal-enhanced 3,3’-diaminobenzidine (DAB) substrate kit (Life Technologies).

**Growth of bacteria in saliva.** The protocol used to measure planktonic growth of bacteria in saliva is described elsewhere (3). Briefly, whole saliva from a single donor was periodically collected on ice, reduced with dithiothreitol to facilitate sterile filtration, heat treated (30 min at 70°C) to inactivate endogenous GH activities, and dialyzed against saliva-salt buffer (2% NaCl, 0.1% Tween 20 and 2% skim milk) to facilitate sterile filtration, heat treated (30 min at 70°C) to inactivate endogenous GH activities, and dialyzed against saliva-salt buffer to increase pH stability and remove possible low-molecular-mass growth substrates. Growth studies in saliva were initiated with bacteria from overnight THB cultures of S. gordonii wild-type and mutant strains that were washed and suspended in saliva to an optical density at 640 nm (OD_{640} of 1 (~1 x 10^9/ml). Primary saliva cultures were inoculated by 1/1,000 dilution of these suspensions into fresh saliva and incubated overnight at 37°C. Each primary saliva culture was then diluted 1/1,000 into three aliquots (2 ml each) of fresh saliva, and the resulting secondary saliva cultures were incubated for 24 h at 37°C, which was sufficient for maximum growth. Genomic DNA templates were prepared from bacteria that were pelleted from 1-ml aliquots of each primary or secondary saliva culture by high-speed centrifugation. Quantitative PCR (qPCR) was performed in triplicate on each template.

**Biochemical analysis of saliva.** Saliva from uninoculated control and secondary growth cultures was filtered (0.22-μm pore-size cellulose nitrate membrane) and assayed in triplicate for protein and carbohydrate, using a bicinechonic acid (BCA) protein assay kit (Thermo Fisher Scientific) and the phenolsulfuric acid assay for carbohydrate (19), with glucose as the standard. SDS-PAGE of reduced saliva was performed as described elsewhere (3), using NuPAGE 4 to 12% Bis-Tris minigels in morpholinepropanesulfonic acid (MOPS) buffer, followed by silver staining (SilverQuest silver staining kit; Invitrogen). An ~70-KDa band was excised from a silver-stained gel of control saliva and destained by following the recommendations in the SilverQuest silver staining kit. The excised band was rinsed with 50 mM bicarbonate, reduced with dithiothreitol, and alkylated with iodoacetamide prior to overnight digestion with trypsin (Promega). Tryptic peptides were separated and identified by nanoscale liquid chromatography-coupled to tandem mass spectrometry (NanoLC-MS/MS) performed by Applied Biomics, Inc. (Hayward, CA). The resulting peptide mass and associated fragmentation spectra were submitted to a GPS Explorer workstation equipped with the MASCOT search engine (Matrix Science, London, United Kingdom) to search the Swiss-Prot database. SDS-PAGE-separated salivary proteins were transferred to nitrocellulose membranes for lectin blotting, which was performed as described elsewhere (3), using fluorescein-labeled *Sambucus nigra* agglutinin (SNA; Vector Laboratories) at 3 μg/ml, *Erythrina cristagalli* agglutinin (ECA; Vector Laboratories) at 2 μg/ml, and glycogen-specific rabbit antiserum with bacteria grown in HIB (Fig. 2).

**RESULTS**

**Cell surface phenotypes of wild-type and ΔGH mutant strains.** In previous studies of *S. pneumoniae* (6), deletion of bgaA or strH abolished the hydrolysis of 2-Np-β-Gal or 4-Np-β-GlcNAc, respectively, in assays performed with lysates of this streptococcus. In the present study, deletion of each gene from *S. gordonii* resulted in a threefold reduction in hydrolysis of the corresponding substrate in assays performed with washed HIB-grown cells. Notwithstanding contributions of other cellular enzyme activities, hydrolysis of 2-Np-β-Gal was reduced for all mutants that lacked bgaA (strains ΔA, ΔA/H, ΔA/D, and ΔA/H/D). Likewise, hydrolysis of 4-Np-β-GlcNAc was reduced for all mutants that lacked strH (strains ΔH, ΔA/H, ΔD/H, and ΔA/H/D). However, the activities of certain bgaA- and strH-expressing mutants were not identical to those of the wild type. Most notably, the BgaA activity (2-Np-β-Gal hydrolysis) of mutants that expressed bgaA but lacked endoD (strains ΔD and ΔH/D) was about half that of the wild type, and the StrH activity (4-NP-β-GlcNAc hydrolysis) of mutants that expressed strH but lacked bgaA (strains ΔA and ΔA/D) was about 4 times that of the wild type. While the basis of these observations remains to be explained, it is important to note that comparable differences in cell surface GH expression were also seen between wild-type *S. gordonii* DL1 cells grown in different culture media. Cells grown in HIB (no added glucose) typically had about 3 times more StrH activity than cells grown in THB (0.2% glucose). More importantly, wild-type cells grown in saliva had up to 10 times more StrH activity than cells from HIB (data not shown). Comparable studies of cell surface EndoD activity were not possible because colorimetric or fluorogenic substrates are not commercially available.

The presence of BgaA, StrH, and EndoD on strain DL1, as well as the expected presence or absence of these proteins on different ΔGH mutant strains, was firmly established by the reactions of GH-specific rabbit antisera with bacteria grown in HIB (Fig. 2). Importantly, the deletion of each gene abolished the binding of the corresponding GH-specific antisera. Reduced binding of anti-BgaA antiserum to mutant strains ΔD and ΔH/D was also noted, in accordance with the reduced BgaA activities of these strains mentioned above.

**Cell surface glycoside hydrolases of *S. gordonii*.** Cell surface glycoside hydrolases of *S. gordonii* were analyzed by two-dimensional gel electrophoresis (isoelectric focusing on pI 3 to 10 strips, followed by SDS-PAGE on 4 to 12% Bis-Tris gels) have also been described (3). SDS-PAGE of salivary proteins either stained with a LIVE/DEAD BacLight bacterial viability kit (Thermo Fisher Scientific) and examined with a Meta 510 confocal microscope (Zeiss) to compare biofilm formation. Following microscopy, the staining solution was drained from the flow cells and replaced with 250 μl lysis buffer for extraction and subsequent purification of genomic DNA templates for qPCR.
times, calculated from measurements of culture turbidity during the mid-log phase of growth, were nearly identical (≈54 min for each), as were the overnight cell densities (≈10^9/ml for each). In striking contrast, the endpoint cell density of the triple mutant strain ΔA/H/D in 24-h saliva cultures was approximately 20-fold lower than that of *S. gordonii* DL1 (Fig. 3A). The endpoint cell densities of mutants that lacked one or two cell surface GHs were 3- to 10-fold lower than that of the wild type. Thus, the actions of cell surface BgaA, StrH, and EndoD were required for maximum growth of *S. gordonii* DL1 in saliva.

The growth and biofilm formation of wild-type strain DL1 and mutant strain ΔA/H/D were also compared in parallel tracks of flow cells perfused with saliva (Fig. 4). The biofilm formation by each strain was similar after 4 h of incubation at 37°C but was consistently greater for wild-type strain DL1 than for the triple mutant strain ΔA/H/D after 18 h of incubation. Biovolume measurements after 18 h (Fig. 4) revealed 3.9 times more wild-type than mutant cells, in good agreement with the results from qPCR, which indicated a 3.5-fold difference in genomic DNA extracted from the parallel tracks. The lack of propidium iodide staining (red) in the mutant biofilm (Fig. 4B) is also noteworthy, as it indicates that the deletions in strain ΔA/H/D do not grossly affect membrane potential or permeability.

**Biochemical characterization of saliva from growth cultures.**

The growth of wild-type *S. gordonii* DL1 in secondary saliva cultures (Fig. 3A) was associated with a 23% loss of salivary protein as measured by the BCA assay (Fig. 3B) and a 46% loss of salivary carbohydrate detected by the phenol-sulfuric acid assay (Fig. 3C). The losses of protein and carbohydrate were significantly less in saliva cultures of all ΔGH mutant strains, and for certain mutants, such as the triple mutant strain ΔA/H/D, the concentrations of salivary protein and carbohydrate were the same as in control saliva. The SDS-PAGE profiles of these samples (Fig. 3D) were generally similar, except for the position of a salivary component that migrated as a diffuse 70-kDa band in the electrophoretic pattern of control saliva. This component appeared at the same position in the salivary profiles of mutants that expressed one or no cell surface GHs (strains ΔA, ΔH, and ΔD) but was shifted downward in the profiles of mutants that expressed two cell surface GHs (strains ΔA/H, ΔA/D, and ΔA/H/D). The same band was noticeably absent from the salivary profile of wild-type strain DL1, which instead contained additional bands that migrated be-
between 40 and 60 kDa. NanoLC-MS/MS of the trypsin-digested 70-kDa band, excised from one-dimensional gels of control saliva as indicated in Fig. 3D, identified the specific decapeptide RPQG GNQPR derived from the nonglycosylated N-terminal regions of two salivary proline-rich proteins, PRB3 and PRB4 (20), along with peptides from other salivary components (e.g., α-amylase, IgA heavy chain, and albumin) that migrate below PRB3 and -4 in SDS-PAGE.

Samples of control saliva and saliva from cultures of wild-type strain DL1 and mutant strain ΔA/H/D culture were also compared by two-dimensional (2-D) gel electrophoresis. The most prominent difference between these samples involved migration of basic PRGs, which appeared in the 40- to 60-kDa region of the DL1 pattern, rather than in the 70- to 80-kDa region, as in saliva from the un inoculated control or the culture of triple mutant strain ΔA/H/D (Fig. 5, boxed region). A comparable shift in the apparent molecular mass of PRG isoforms that migrated between pI 8 and 10 was also seen between different samples. Thus, basic PRGs appeared to be major substrates for GH-dependent growth of S. gordonii DL1 in saliva.

To assess changes in PRG glycosylation, we transferred SDS-PAGE-separated saliva samples (Fig. 3D) to nitrocellulose and probed the resulting transfers with fluorescein isothiocyanate (FITC)-labeled lectins that bind different features of N-linked glycans. In these experiments, PRG from control saliva appeared as a prominent ~70-kDa band on blots incubated with Sia-reactive SNA, Gal-reactive ECA, GlcNAc-reactive SWGA, Man-reactive GNA, and Fuc-reactive AAL (Fig. 6A). Other, less-prominent lectin-reactive salivary components were also detected, including one at ~80 kDa and another between 50 and 60 kDa. Unlike PRG from control saliva, PRG from saliva cultures of S. gordonii DL1 appeared in the 40- to 60-kDa region of blots and was labeled by Sia-reactive SNA, GlcNAc-reactive SWGA, and Fuc-reactive AAL but not by either Gal-reactive ECA or Man-reactive GNA. In contrast, PRG from saliva cultures of mutants that lacked bgaA (i.e., strains ΔA, ΔA/H, ΔA/D, and ΔA/H/D) migrated as a diffuse ~70-kDa band and reacted strongly with all five lectins, whereas PRG from cultures of mutants that expressed bgaA (i.e., strains ΔH, ΔD, and ΔH/D) migrated slightly faster than PRG from control saliva and reacted with all lectins except Gal-reactive ECA.

The loss of GNA binding to PRG was only seen in saliva cultures of S. gordonii DL1, which suggested that the actions of BgaA, StrH, and EndoD were all required for this effect. To test this hypothesis, we set up mono- and cocultures of different mutant strains that, in combination, expressed all three enzymes. In these experiments (Fig. 6B), PRG from monocultures of mutant strains ΔH, ΔA/D, ΔA/D, and ΔA/H migrated and were detected (as in Fig. 6A) by binding Man-reactive GNA and other lectins, with the exception of ECA, which failed to bind PRG from saliva cultures of mutants that expressed cell surface BgaA (strains ΔH and ΔD). In contrast, PRG from cocultures of strains ΔH plus ΔA/D or of strains ΔD plus ΔA/H migrated in the 40- to 60-kDa region, like PRG from saliva cultures of wild-type DL1, and bound SNA, SWGA, and AAL but not ECA or GNA. Thus, the loss of GNA reactivity resulted from the action first of BgaA and then those of StrH and EndoD.

**DISCUSSION**

The previous observation that the number of genes for cell wall-anchored GHs was generally greater in oral streptococci that grew well in saliva, such as S. gordonii and S. oralis, than in species that failed to grow suggested an important role for these enzymes in salivary glycan foraging (3). Four putative cell wall-anchored GHs were identified from S. gordonii DL1, of which three were predicted to act on host glycans based on their homology with pneumococcal BgaA (13, 14), StrH (15, 16), and EndoD (17). In the present study, we introduced unmarked, in-frame deletions in the corresponding genes and verified cell surface expression of the encoded proteins on the resulting mutant strains by their reactions with anti-BgaA, anti-StrH, or anti-EndoD antisera (Fig. 2). Deletion of these genes did not affect the growth of S. gordonii DL1 in glucose-containing THB medium but resulted in an ~20-fold reduction of cell densities in 24-h planktonic saliva cultures, as well as an ~4-fold reduction of biofilm formation in flowing saliva (Fig. 3 and 4). The only obvious change in the salivary proteome associated with the growth of S. gordonii DL1 was a reduction in the apparent molecular mass of basic PRGs, which was accompanied by the loss of lectin-binding sites for Gal-specific
ECA and Man-specific GNA. Importantly, these effects were not seen in saliva from cultures of mutant strain A/H9004, which lacked all three cell surface GHs. The removal of ECA binding sites from PRG in saliva cultures of different mutant strains correlated with the cell surface expression of BgaA. However, the subsequent loss of GNA binding sites was seen only in saliva cocultures of A/H9004 GH mutant strains that in combination expressed all three cell surface GHs. Considered together, these findings indicate that the growth of S. gordonii DL1 in saliva depends to a considerable extent on the actions first of BgaA and then of StrH and EndoD on N-linked glycans of basic PRG.

Insight into the structural basis of salivary glycan foraging by S. gordonii was gained from NanoLC-MS/MS of the PRG band from control saliva (Fig. 3D), which associated this component with PRB3, the most abundant of which was a biantennary asialo-oligosaccharide with substitutions of three fucose residues (24). The proposed sequential cleavage of this structure by BgaA, StrH, and EndoD of S. gordonii (Fig. 7) is consistent with previously determined substrate specificities of these enzymes from S. pneumoniae, as summarized by King (5), and was demonstrated in the present study by lectin blotting of PRG from saliva cultures of S. gordonii wild-type and ΔGH mutant strains (Fig. 6). The binding of Sia-reactive SNA to PRG from all saliva cultures, although not expected based on the asialo structure shown in Fig. 7, is readily explained by the presence of terminal α2-6-linked Sia on a similar oligosaccharide previously identified from PRB3 (24). The fact that the growth of S. gordonii DL1 does not affect SNA binding indicates that the presence of termi-
nal sialic acid protects certain N-linked glycan chains of PRG from glycan foraging by this species, which unlike *S. pneumoniae* (6) and other viridans group streptococci (25), lacks sialidase activity.

ECA reacts well with both Galβ1-4GlcNAc and Fuca1-2Galβ1-4GlcNAc termini (26, 27). Thus, the ability of all BgaA-expressing wild-type and ΔGH mutant strains to abolish binding of ECA to PRG (Fig. 6) suggests cleavage of Gal from terminal Galβ1-4GlcNAc to expose terminal GlcNAcβ1-2Manα and cleavage of Fuca1-2Gal from terminal Fuca1-2Galβ1-4GlcNAc to expose terminal Fuca1-3GlcNAcβ1-2Manα (Fig. 7). Terminal GlcNAcβ1-2Manα is a preferred substrate for StrH of *S. pneumoniae* (15), but it remains to be determined whether this enzyme of *S. gordonii* also acts on the same linkage in Fuca1-3GlcNAcβ1-2Manα to expose terminal Manα1-3Manα (Fig. 7). Our ability to detect the action of StrH by lectin blotting of PRG was limited by the specificities of currently available GlcNAc- and Fuca-binding lectin probes. Thus, GlcNAc-specific SWGA (28) has been shown to bind internal GlcNAc residues (29) and Fuca-specific AAL (30) reacts well with both α1-3- and α1-6-linked Fuca branches. Although not revealed by lectin blotting, the action of StrH was clearly required for the subsequent removal of GNA-binding structures from PRG in cocultures of mutants that expressed this enzyme in combination with BgaA and EndoD (Fig. 6B).

The ability of GNA to detect sequential action of BgaA, StrH, and EndoD on PRG undoubtedly depends on the specificity of this lectin for terminal Man residues in branched oligosaccharides (31) like those that decorate PRB3 (Fig. 7). This finding does not, however, imply that all GNA-reactive glycans of PRG are acted upon by EndoD. Indeed, binding of GNA to PRG from control saliva was not abolished by the growth of any mutant that expressed EndoD (i.e., strains ΔA, ΔH, and ΔA/H) (Fig. 6). A possible explanation for this puzzling result comes from the previous identification of complex Manα1-2 and Manα1-3-containing oligosaccharides from PRB3 (24). Such structures may bind GNA but not be substrates of EndoD, which has a strict specificity for the oligomannosyl cores of glycoproteins (32). The removal of these high-mannose-type glycans from PRG during the growth of *S. gordonii* DL1 can be explained by the actions of BgaA and StrH on adjacent branches of the same oligosaccharide to expose α1-3-linked terminal Man, which is essential for the glycan recognition and subsequent cleavage by EndoD of *S. pneumoniae* (33). A previously described mannosidase activity of *S. gordonii* and other viridans group streptococci (34) that acts on Manα glycoforms may also contribute to the removal of high-mannose-type glycans from PRG.

The sequential actions of BgaA, StrH, and EndoD on N-linked glycans of PRG are predicted to yield free Gal and GlcNAc, both of which support the growth of *S. gordonii* DL1 (3). As discussed above, the products of BgaA and EndoD are also likely to include fucosylated oligosaccharides. It is noteworthy that *S. gordonii* DL1, which does not grow on α-fucose, has genes for two putative α-1-fucosidases (i.e., SGO_0150 and SGO_1771) that are predicted to be cytoplasmic proteins. Recombinant proteins encoded by these genes were recently identified, respectively, as α1-2- and α1-3- and α4-specific α-1-fucosidases, based on their abilities to cleave fucosylated oligosaccharides (E. Guthrie, unpublished data). Thus, SGO_0150, which occurs as an isolated gene in *S. gordonii* DL1, may act on Fuca1-2Gal that is cleaved from PRG by the action of BgaA. Moreover, SGO_1771, which occurs in the previously described *gom regulon* (35) along with genes for a structurally characterized N-acetylglucosaminidase (36) and three putative mannosidases, may function in the metabolism of larger, Man-containing branched oligosaccharides that result from the action of EndoD. Indeed, inactivation of certain genes in the *gom locus* was found to reduce the growth of *S. gordonii* on both α1-6-mannobiose and GlcNAcβ1-2Man (35). Further studies are needed to determine whether oligosaccharides released from basic PRG by cell surface GHS during the growth of *S. gordonii* DL1 in saliva are natural substrates of the intracellular glycoside hydrolases encoded by genes in the *gom locus* and at other loci.

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**References**


