Role of Neuraminidase-Producing Bacteria in Exposing Cryptic Carbohydrate Receptors for *Streptococcus gordonii* Adherence

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ABSTRACT *Streptococcus gordonii* is an early colonizer of the oral cavity. Although a variety of *S. gordonii* adherence mechanisms have been described, current dogma is that the major receptor for *S. gordonii* is sialic acid. However, as many bacterial species in the oral cavity produce neuraminidase that can cleave terminal sialic acid, it is unclear whether *S. gordonii* relies on sialic acid for adherence to oral surfaces or if this species has developed alternative binding strategies. Previous studies have examined adherence to immobilized glycoconjugates and identified binding to additional glycans, but no prior studies have defined the contribution of these different glycan structures in adherence to oral epithelial cells. We determined that the majority of *S. gordonii* strains tested did not rely on sialic acid for efficient adherence. In fact, adherence of some strains was significantly increased following neuraminidase treatment. Further investigation of representative strains that do not rely on sialic acid for adherence revealed binding not only to sialic acid via the serine-rich repeat protein GspB but also to β -1,4-linked galactose. Adherence to this carbohydrate occurs via an unknown adhesin distinct from those utilized by *Streptococcus oralis* and *Streptococcus pneumoniae*. Demonstrating the potential biological relevance of binding to this cryptic receptor, we established that *S. oralis* increases *S. gordonii* adherence in a neuraminidase-dependent manner. These data suggest that *S. gordonii* has evolved to simultaneously utilize both terminal and cryptic receptors in response to the production of neuraminidase by other species in the oral environment.

KEYWORDS *Streptococcus gordonii*, *Streptococcus oralis*, adherence, cryptic receptor, galactose beta-1,4-linked, interspecies interactions, neuraminidase, serine-rich repeat proteins, sialic acid, sortase

Streptococcus gordonii is a pioneer species within the oral cavity that is found within the supragingival plaque and colonizes many surfaces, including the buccal mucosa and pharynx (1–3). In addition, if *S. gordonii* enters the bloodstream it can cause subacute infective endocarditis (4). In both colonization and disease, adherence to host surfaces is critical; hence, many studies of *S. gordonii* have focused on elucidating adherence mechanisms (5–13). Current dogma defines the major mechanism of *S. gordonii* adherence to host surfaces as serine-rich repeat protein (SRRP)-mediated adherence to sialic acid (11, 12, 14–16). All sequenced *S. gordonii* strains encode one of two SRRPs, Hsa or GspB (11, 12). Both of these SRRPs have specificity for terminal sialic acid (11, 15). GspB interacts with a narrow range of sialoglycans, specifically sialyl T-antigen (NeuAc α -2,3-Gal β -1,3-GalNAc) and closely related structures (14, 17). In contrast, Hsa bound all structures tested with terminal α -2,3-linked sialic acid (14, 17, 18).

S. gordonii binding to sialic acid may be the predominant adherence mechanism

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during endocarditis. However, the situation is likely more complex in the oral cavity, where many bacterial species produce neuraminidase, which cleaves terminal sialic acid from glycoconjugates (19–21). Although *S. gordonii* does not encode a neuraminidase, there are reasons to believe that *S. gordonii* can benefit from the neuraminidase activity of other bacterial species. *S. gordonii* can utilize sialic acid as a sole carbon source and encodes glycosidases for modification of N-linked glycans following removal of sialic acid (20, 22–24). These data suggest that *S. gordonii* exists in an environment where sialic acid receptors are removed. Recently published data demonstrated that *Streptococcus oralis* utilized a novel mechanism of simultaneously binding to both terminal sialic acid and underlying cryptic receptors exposed by neuraminidase activity (25). We propose that this strategy of simultaneously binding terminal and cryptic receptors is utilized by other bacterial species. This includes bacteria that do not produce terminal receptor-cleaving glycosidases but exist in multibacterial environments, such as the oral cavity, with species that produce such glycosidases.

The effect of neuraminidase on adherence of *S. gordonii* to oral surfaces as a result of potential exposure of underlying carbohydrate binding partners on host cells is unknown. However, prior studies have examined the ability of *S. gordonii* to bind immobilized glycoconjugates. While evidence supports the hypothesis that DL1 exclusively binds via sialic acid, the binding specificity of other strains of *S. gordonii* to immobilized glycans varies (26–30). Multiple strains, including the infective endocarditis isolate ATCC 10558, were shown to be unable to bind glycoconjugates with terminal sialic acid. Furthermore, the majority of strains were shown to bind underlying glycoconjugates independently of their ability to bind sialic acid. In some cases these glycoconjugates had terminal GalNAc- β -1,3-Gal and/or Gal- β -1,4-GlcNAc (26–29). Although informative, studying binding to immobilized glycoconjugates does not simultaneously present the broad range of potential glycan receptors on the host epithelial surface and thus is a poor representation of physiological conditions.

In this study, we examined whether *S. gordonii* relies on sialic acid to bind an oral epithelial cell line or if it can use cryptic receptors exposed by neuraminidase cleavage of sialic acid. The effect of neuraminidase on adherence divided these strains into three categories: those decreased, unchanged, or increased in adherence following removal of sialic acid. Representative strains from all three groups required an SRRP to bind sialic acid. However, strains not significantly decreased in adherence by removal of sialic acid were shown to bind underlying β -1,4-linked galactose. While the adhesin(s) for *S. gordonii* to bind β -1,4-linked galactose was not identified in the current study, it appears distinct from both *S. oralis* and *S. pneumoniae*, and our results suggest it is a sortase A-dependent surface protein(s). The presence of *S. oralis* was able to significantly increase adherence of an *S. gordonii* strain to oral epithelial cells in a neuraminidase-dependent manner, demonstrating that binding to β -1,4-linked galactose is likely relevant in the complex oral environment. These findings establish for the first time that the absence of sialic acid does not reduce binding of many *S. gordonii* strains, highlighting that adherence to carbohydrate structures in complex bacterial environments can be influenced by glycosidases produced by other species.

RESULTS

Most *S. gordonii* strains do not require sialic acid for adherence to oral epithelial cells. Although multiple adherence mechanisms have been described for *S. gordonii*, sialic acid is proposed to be a major receptor (10, 31). However, in the oral cavity where *S. gordonii* is a member of the complex multibacterial community, neuraminidase produced by other bacterial species may well remove this receptor. We obtained 10 strains (Table 1) that were verified as *S. gordonii* by multilocus sequence analysis (MLSA). Where possible, strain designations were confirmed by comparison of the MLSA sequences obtained with available genome sequences. To investigate if *S. gordonii* possesses additional mechanisms to maintain interaction with host cells in the absence of sialic acid, we compared the adherence of these strains to oral epithelial cells (TR146) pretreated with either neuraminidase or buffer (Fig. 1). To allow compar-

TABLE 1 Strains and plasmids used in this study

Strain or plasmid	Characteristic(s)/genotype ^c	Source or reference
Strains		
<i>Streptococcus gordonii</i>		
DL1	<i>S. gordonii</i> Challis	66 ^b
DL1 Δ hsa	Δ hsa::aad9, Spc ^r	This study
SK12	<i>S. gordonii</i> oral isolate	67 ^b
38	Human isolate	68 ^b
SK33	<i>S. gordonii</i> oral isolate	67 ^b
PS478	<i>S. gordonii</i> endocarditis isolate	17
PS478 Δ gspB	Δ gspB::aad9, Spc ^r	This study
PS478 Δ bgaA	Δ bgaA::aad9, Spc ^r	This study
PS478 Δ srtA	Δ srtA::aad9, Spc ^r	This study
PS478 Δ bgaA Δ gspB		This study
G9B	<i>S. gordonii</i> oral isolate	69 ^b
M99	<i>S. gordonii</i> endocarditis isolate	70
ATCC 10558	<i>S. gordonii</i> endocarditis isolate	66 ^b
SK186 ^a	<i>S. gordonii</i> oral isolate	71 ^b
SK186 Δ gspB	Δ gspB::aad9, Spc ^r	This study
SK186 Δ bgaA	Δ bgaA::aad9, Spc ^r	This study
SK186 Δ srtA	Δ srtA::aad9, Spc ^r	This study
SK186 Δ bgaA Δ gspB		This study
72-40		72 ^b
<i>Streptococcus oralis</i>		
ATCC 10557	<i>S. oralis</i> endocarditis isolate	73 ^b
ATCC 10557 Δ bgaA	Δ bgaA::kan-rpsL ⁺ , Kan ^r	This study
ATCC 10557 Δ bgaA Δ nanA	Δ bgaA::kan-rpsL ⁺ Δ nanA::erm, Erm ^r Kan ^r	This study
<i>Escherichia coli</i> strain		
Stellar	Cloning host	Clontech
Plasmids		
pDrive	Cloning vector, Amp ^r Kan ^r	Qiagen
pDrive Δ gspB	pDrive Δ gspB::aad9, Spc ^r Kan ^r	This study
pDrive Δ bgaA	pDrive Δ bgaA::aad9, Spc ^r Amp ^r Kan ^r	This study
pDrive Δ srtA	pDrive Δ srtA::aad9, Spc ^r Kan ^r	This study
pDrive Δ nanA	pDrive Δ nanA::erm, Erm ^r Amp ^r Kan ^r	25
pJET1.2/blunt	Cloning vector, Amp ^r	Thermo Fisher Scientific
pJETSobgaA	pJET1.2 containing a fragment of ATCC 10557 <i>bgaA</i>	This study
pJETSo Δ bgaA	pJETSobgaA with Janus cassette, Amp ^r Kan ^r	This study
pJETgspB	pJET1.2 containing a fragment of 7240 <i>gspB</i>	This study
pJET Δ gspB	pJETgspB with Janus cassette, Amp ^r Kan ^r	This study

^aPreviously named LGR2.^bThese strains were initially identified as different streptococcal species but were reclassified into *S. gordonii*.^cKan^r, kanamycin resistant; Amp^r, ampicillin resistant; Spc^r, spectinomycin resistant; Erm^r, erythromycin resistant.

ison of the adherence levels of the different strains, data were plotted as absolute adherence. Other data in the manuscript are presented as relative adherence. This allows studies to focus on the differences due to mutations or treatments without the distraction of variability in baseline adherence between experiments; this strategy is often used when studying streptococcal adherence to epithelial cells (32–35).

The different *S. gordonii* strains showed substantial diversity in the level of adherence to epithelial cells (Fig. 1). In addition, the effect of neuraminidase was variable and could be divided into three phenotypes. The first group was reduced in adherence following neuraminidase treatment. As expected based on previous data, this group included DL1 (10, 18, 30, 31, 36). Neuraminidase pretreatment reduced adherence of DL1 more than 6-fold, confirming sialic acid as the major receptor for this strain. However, a small amount of residual adherence was observed that could be the result of nonspecific binding, the presence of additional receptors, incomplete removal of sialic acid, or, as neuraminidase treatment occurs before the adherence assay, resialy-

FIG 1 *S. gordonii* strains differ in adherence following neuraminidase pretreatment. The graph shows adherence of *S. gordonii* strains over 1 h to oral epithelial cell line TR146 pretreated with either purified neuraminidase (N) or medium alone for 30 min. Adherence is expressed as a percentage of the inoculum. Values are the means for at least three independent experiments, each performed in triplicate, $\pm$ SD. Asterisks indicate a significant difference in adherence of the *S. gordonii* strain to neuraminidase-treated cells compared to the tissue culture medium control. Statistical significance was tested by two-tailed Student's *t* test. *, $P < 0.05$.

lation during the assay. The second group of strains was not significantly altered in adherence following neuraminidase treatment. Based on these data alone, it is unclear whether these strains bind similarly to sialic acid and underlying receptors or if they utilize a sialic acid-independent adherence mechanism. The third group was significantly increased in adherence following neuraminidase treatment. This increased adherence is likely due to binding to carbohydrates exposed by the removal of sialic acid. However, these data leave it unclear whether this group of strains utilizes sialic acid as a receptor when present or if adherence is mediated via a sialic acid-independent mechanism. Overall, these data demonstrate for the first time that many *S. gordonii* strains bind oral epithelial cells equally or more effectively in the absence of sialic acid.

A role for SRRP binding to sialic acid is maintained across adherence phenotypes. Binding of *S. gordonii* to sialic acid is mediated by the SRRP encoded by individual strains (11, 17, 18, 37). Interestingly, PCR amplification demonstrated that the two strains that are reduced in adherence to neuraminidase-treated cells both encode Hsa, whereas the eight strains that have equal or increased adherence to neuraminidase-treated cells all encode GspB. To determine the role of sialic acid in adherence, we generated insertion-deletion mutations within the SRRP encoded by representatives of each group. As expected, mutation of *hsa* in DL1 reduced adherence more than 98%, and the level of adherence was not significantly different from that of the parental strain to neuraminidase-treated cells (Fig. 2A). These data are consistent with previous work demonstrating that Hsa binding to α -2,3-linked sialic acid is responsible for DL1 adherence to sialoglycconjugates, saliva, platelets, and human cells (10, 11, 18, 30, 31, 36, 38). Furthermore, these data indicate that sialic acid removal is efficient under our experimental conditions. Mutation of *gspB* in PS478 and SK186 also significantly reduced their adherence, demonstrating a role for GspB and its sialic acid binding activity in adherence (Fig. 2B and C). These data establish that representative strains of all three groups can utilize sialic acid as a receptor. Because adherence of the *gspB* mutants to neuraminidase-treated cells was not significantly different from that of the parental strain, these results suggest that an adhesin other than GspB is responsible for binding to a second receptor revealed by removal of sialic acid (Fig. 2B and C).

FIG 2 Serine-rich repeat proteins Hsa and GspB are required for binding to sialic acid but not to underlying carbohydrates. The graphs show adherence of *S. gordonii* strains DL1 (A), PS478 (B), and SK186 (C) and isogenic serine-rich repeat protein mutants to oral epithelial cell line TR146 pretreated with either neuraminidase (N) or medium alone for 30 min. Adherence after an hour of incubation was expressed as a percentage relative to the untreated parental strain. Values are the means for at least three independent experiments, each performed in triplicate, $\pm$ SD. Statistical significance was tested by ANOVA with an LSD *post hoc* test. *, $P < 0.05$. NS, not significant.

***S. gordonii* strains not requiring sialic acid for adherence bind β -1,4-linked galactose.**

Although studies have not examined binding of *S. gordonii* to underlying carbohydrates on cell surfaces, several studies have demonstrated adherence to glycoconjugates, including those with terminal GalNAc- β -1,3-Gal and/or Gal- β -1,4-GlcNAc moieties (26–29). Related streptococcal species *S. oralis* and *S. pneumoniae* have been shown to bind β -1,4-linked galactose exposed by removal of sialic acid (25, 35, 39). To determine if *S. gordonii* strains that retain substantial levels of adherence when sialic acid is removed utilize β -1,4-linked galactose as an underlying receptor, we compared adherence of DL1, PS478, and SK186 in the presence and absence of SpBgaA146-990. This recombinant polypeptide is derived from the *S. pneumoniae* β -galactosidase BgaA and is specific for terminal galactose that is β -1,4 linked to glucose and *N*-acetylglucosamine (lactose or *N*-acetyllactosamine [LacNAc], respectively) (35, 40). As expected given the apparent reliance of DL1 on Hsa and sialic acid for adherence, SpBgaA146-990 did not significantly reduce binding of DL1 to neuraminidase-treated TR146 cells (Fig. 3A). In comparison, adherence of PS478 and SK186 was significantly reduced in the presence of SpBgaA146-990, suggesting that most of the adherence of these strains in the absence of sialic acid is to β -1,4-linked galactose (Fig. 3B and C). As expected, SpBgaA146-990 had no effect in the absence of neuraminidase treatment, as the enzyme cannot act upon glycan structures with terminal sialic acid.

Although we only addressed binding of PS478 and SK186 to β -1,4-linked galactose on TR146 cells, we have demonstrated that 72-40, G9B, and M99 bind β -1,4-linked galactose on the pharyngeal cell line D562 (Fig. 4). These data suggest that adherence to this broadly distributed glycan structure is conserved among *S. gordonii* strains that do not rely on sialic acid for adherence.

S. oralis and *S. pneumoniae* bind β -1,4-linked galactose by different mechanisms. Adherence of *S. oralis* to this carbohydrate structure requires the SRRP Fap1 (25). However, data presented in Fig. 2 demonstrated that adherence of PS478 and SK186 to the underlying receptor(s) was independent of the SRRP in these strains (Fig. 2). Adherence of *S. pneumoniae* to β -1,4-linked galactose is mediated by the β -galactosidase

FIG 3 Some *S. gordonii* strains bind β -1,4-linked galactose on oral epithelial cells. The graphs show adherence of *S. gordonii* DL1 (A), PS478 (B), and SK186 (C), in the presence of SpBgaA146-990 (B) or PBS control, to oral epithelial cell line TR146 pretreated with either neuraminidase (N) or medium alone. Adherence after an hour of incubation was expressed as a percentage relative to the untreated strain. Values are the means for at least three independent experiments, each performed in triplicate, $\pm$ SD. Statistical significance was tested by ANOVA with an LSD *post hoc* test. *, $P < 0.05$. NS, not significant.

BgaA (35). BgaA of *S. gordonii* is predicted to encode carbohydrate-binding modules that confer binding of *S. pneumoniae*. Furthermore, a pneumococcal *bgaA* mutant could be complemented by introduction of *S. gordonii bgaA*. Together these data suggest a role for *S. gordonii* BgaA in adherence (35). However, *bgaA* mutants in PS478 and SK186 were not reduced in adherence (Fig. 5A). Furthermore, we confirmed there was no redundancy in these two known mechanisms of binding to β -1,4-linked galactose by constructing *bgaA gspB* double mutants which were not reduced in adherence to neuraminidase-treated epithelial cells (Fig. 5B). These data suggest that *S. gordonii* uses a previously undescribed adhesin to bind β -1,4-linked galactose on host cells once sialic acid is removed. Although BgaA plays a role in adherence of *S. pneumoniae*, it should be noted that the recombinant SpBgaA146-990 used in our experiments to cleave terminal β -1,4-linked galactose includes only the enzymatic domain of the protein and not the domains mediating adherence (35).

Identification of potential β -1,4-linked galactose adhesins. Many streptococcal adhesins are surface associated in a sortase A-dependent manner (25, 35, 41–44). Proteins surface associated in a sortase A-dependent manner contain an LPXTG motif (where X is any amino acid), which is typically followed by a hydrophobic domain and a positively charged tail (45, 46). Sortase A is a membrane-localized cysteine protease that cleaves between the threonine and glycine of LPXTG motifs and catalyzes the covalent linkage of the cleaved protein to peptidoglycan (47). Mutation of *srtA* in both PS478 and SK186 significantly reduced their adherence to neuraminidase-treated

FIG 4 Some *S. gordonii* strains bind β -1,4-linked galactose on pharyngeal epithelial cells. The graphs show adherence of *S. gordonii* to D562 cells pretreated with neuraminidase in the presence or absence of *SpBgaA146-990* (B). Adherence after an hour was expressed as a percentage relative to the untreated strain. Values are the means for at least three independent experiments, each performed in triplicate, $\pm$ SD. Statistical significance was tested by two-tailed Student's *t* test. *, $P < 0.05$. NS, not significant.

epithelial cells (Fig. 6). For PS478, this residual adherence was comparable to the adherence of the parental strain to cells treated with both neuraminidase and *SpBgaA146-990*. This adherence was not further reduced by addition of *SpBgaA146-990*, suggesting that adherence of PS478 to β -1,4-linked galactose is mediated by, or at least associated with, a sortase A-dependent surface protein. Mutation of *srtA* in SK186 reduced adherence by $\sim 75\%$, but addition of *SpBgaA146-990* further reduced adherence. These data suggest that while the major mechanism of binding to β -1,4-linked galactose depends on the presence of sortase A, an additional mechanism independent of sortase A also contributes to adherence of SK186. In addition, there was a small but significant reduction of the sortase A mutant compared to the level of parental strain when cells were treated with both neuraminidase and *SpBgaA146-990*. These data suggest the existence of a sortase A-dependent adhesin that binds a receptor present following removal of sialic acid and β -1,4-linked galactose. Together these data support the hypothesis that a major mechanism of *S. gordonii* adherence to β -1,4-linked galactose requires a sortase A-dependent surface protein. However, it should be noted that inactivation of *srtA* likely affects 20 to 30 surface proteins and hence could have broader effects on the cell surface that lead to altered adherence.

We hypothesized that *S. gordonii* encodes an additional sortase A-dependent surface adhesin that is required for binding to β -1,4-linked galactose. Of the strains included in this study, complete or partial genome sequences were available for M99 (GCA_001588955.1), G9B (GCA_000959925.1), SK12 (GCA_001589065.1), and Challis (GCA_000017005.1), different substocks of which are called DL1 and CH1 (22, 48, 49). Thus, sequences were available for two of the strains reliant on sialic acid for efficient binding to epithelial cells (DL1 and SK12) and also for two of the strains that retained binding in the absence of sialic acid (M99 and G9B).

Of the 28 putative sortase A-dependent surface proteins encoded within the contigs of G9B, all except two shared high sequence identity with at least DL1 or SK12. Of the

FIG 5 *S. gordonii* binding to β -1,4 galactose is not mediated by BgaA. The graphs show adherence of *S. gordonii* strains PS478 and SK186 and isogenic *bgaA* (A) and *bgaA gspB* (B) mutants to neuraminidase-treated (N) oral epithelial cell line TR146. Adherence after an hour of incubation was expressed as a percentage relative to the untreated parental strain. Values are the means for at least three independent experiments, each performed in triplicate, $\pm$ SD. Statistical significance was tested by ANOVA with an LSD *post hoc* test *, $P < 0.05$. NS, not significant.

remaining two open reading frames, only TZ86_00548 was also present in M99. This locus is predicted to encode a 2,172-amino-acid surface protein found in a range of *S. gordonii* and *S. oralis* isolates. The function of this protein is unknown; however, structural predictions indicate similarity to some domains of other bacterial adhesins (50). These include a fibronectin-binding domain from *S. gordonii* CshA and bacterial Ig-like folds seen in many bacterial adhesins. The presence of this locus in the genome sequences of the two strains not reliant on sialic acid for adherence, and the absence of this locus in the genome sequences of the two strains that are dependent on sialic acid for adherence, caused us to speculate that the protein encoded by TZ86_00548 contributes to recognition of β -1,4-linked galactose moieties by certain strains of *S. gordonii*. Amplification with primers designed to bind conserved regions of the gene confirmed the distribution in genome-sequenced strains but did not result in a product for PS478 or SK186. The absence of TZ86_00548 within these isolates was confirmed by Southern blotting (data not shown). We cannot rule out a role for this gene product in adherence of M99 and G9B; however, it is not contributing to adherence of SK186 and PS478 to β -1,4-linked galactose, the mechanism of which remains to be defined. This

FIG 6 *S. gordonii* binding to β -1,4 galactose is primarily sortase A mediated. The graphs show adherence of *S. gordonii* strains PS478 (A) and SK186 (B) and isogenic serine-rich repeat protein mutants, in the presence of SpBgaA146-990 (B) or PBS control, to oral epithelial cell line TR146 pretreated with either neuraminidase (N) or medium alone. Adherence after an hour of incubation was expressed as a percentage relative to the untreated parental strain. Values are the means for at least three independent experiments, each performed in triplicate, $\pm$ SD. Statistical significance was tested by ANOVA with an LSD *post hoc* test. *, $P < 0.05$. NS, not significant.

adherence may be mediated by a yet-to-be-identified adhesin. Alternatively, the difference in ability of strains to bind β -1,4-linked galactose may be due to allelic variation or differences in production of a surface protein.

The presence of *S. oralis* during adherence assays can increase *S. gordonii* adherence. The studies described thus far were performed using purified neuraminidase. As proof of principle that neuraminidase-producing bacterial species can reveal cryptic receptors for *S. gordonii* adherence, we tested adherence of *S. gordonii* strain SK186 in the presence of a neuraminidase-producing oral bacteria, *S. oralis* strain ATCC 10557. SK186 was selected for this experiment, as this strain's binding is increased by neuraminidase treatment, thus any effect of *S. oralis* on adherence was measurable. In order to distinguish the two species, strains resistant to different antibiotics were generated. As the β -galactosidase BgaA is not required for effective adherence of either *S. oralis* or *S. gordonii*, the markers were inserted at this genomic location (Fig. 5) (25). Addition of *S. oralis* and *S. gordonii* at a seven-to-one ratio increased binding of *S. gordonii* strain SK186 to a level not significantly different from that to cells treated with neuraminidase (Fig. 7). We generated a strain mutated in the gene encoding *S. oralis* neuraminidase, *nana*. This strain did not increase adherence of SK186, demonstrating that neuraminidase is required for the effect of *S. oralis* on *S. gordonii* adherence. These data indicate that neuraminidase-producing bacteria that colonize the oral cavity can influence the adherence of *S. gordonii* to epithelial cells.

FIG 7 *S. oralis* can increase adherence of *S. gordonii* SK186 in a neuraminidase-dependent manner. The graph shows adherence of the *S. gordonii* SK186 $\Delta bgaA$ strain to the oral epithelial cell line TR146 in the presence of neuraminidase (N), the *S. oralis* ATCC 10557 $\Delta bgaA$ (So $\Delta bgaA$) or *S. oralis* ATCC 10557 $\Delta bgaA \Delta nanA$ (So $\Delta bgaA \Delta nanA$) strain, or medium alone. Adherence after an hour of incubation was expressed as a percentage relative to the untreated parental strain. Values are the means for at least three independent experiments, each performed in triplicate, $\pm$ SD. Statistical significance was tested by ANOVA with an LSD *post hoc* test. *, $P < 0.05$. NS, not significant.

DISCUSSION

These experiments expand our understanding of how *S. gordonii* adheres to host surfaces in the multibacterial environment of the oral cavity. Previous studies have reported that the major receptor for *S. gordonii* is sialic acid; however, other bacterial species in the oral cavity produce neuraminidase that can cleave this usually terminal carbohydrate from host glycoconjugates. Our study reveals that many *S. gordonii* strains do not rely on sialic acid for efficient adherence to oral epithelial cells. Further investigations revealed that the majority of binding in the absence of sialic acid, at least for the two strains tested, was to β -1,4-linked galactose. These data suggest that at least some *S. gordonii* strains bind multiple carbohydrates on the epithelial cell surface. Furthermore, as β -1,4-linked galactose is exposed by neuraminidase cleavage of sialic acid, these studies suggest that *S. gordonii* can simultaneously use both terminal and cryptic receptors in the oral cavity. Simultaneous binding to both terminal sialic acid and cryptic receptors exposed by neuraminidase was previously reported for *S. oralis*, which produces a surface-associated neuraminidase (25, 51). In fact, here we demonstrate that *S. oralis* can increase adherence of *S. gordonii* in a neuraminidase-dependent manner, illustrating that binding terminal and cryptic receptors may be utilized by bacterial species that dwell in the same environment as species that can cleave terminal receptors.

β -1,4-Linked galactose acts as a receptor for some other streptococcal species, including *S. oralis* and some isolates of *S. pneumoniae*. The conservation of this receptor may be due to the broad distribution of this carbohydrate structure in cell surface glycosylation and the fact that these species either produce neuraminidase or live in the same environment as species that produce neuraminidase. Despite the conserva-

tion of the receptor, the mechanisms utilized to bind these carbohydrates are distinct (25, 35, 39). The adhesin mediating adherence of *S. gordonii* to β -1,4-linked galactose is yet to be identified; however, our data suggest that this mechanism involves a sortase A-dependent surface protein that serves directly as an adhesin or alternatively as a tether for another surface-associated adhesin.

While *S. gordonii* binds β -1,4-linked galactose, the precise nature of the receptor remains unknown and could be in the context of β -1,4-linked galactose to *N*-acetylglucosamine (LacNAc) or glucose (lactose). LacNAc is common within the N- and O-linked glycans on the epithelial cell surface, while both LacNAc and lactose are common within glycosphingolipids. Here, we demonstrated that M99 and G9B bind to β -1,4-linked galactose on pharyngeal epithelial cells (Fig. 4). It was previously reported that binding of these strains to fetuin was dependent on sialic acid (38, 52–55). The fact that fetuin is decorated with LacNAc-containing glycans suggests that M99 and G9B bind lactose, not LacNAc. Alternatively, it may highlight the need to complete these studies in the context of a host cell.

Even following removal of β -1,4-linked galactose there is still some residual adherence of SK186 and PS478 to oral epithelial cells (Fig. 3). As it has previously been reported that some *S. gordonii* strains can bind immobilized GalNAc- β -1,3-Gal, it would be interesting to determine if the remaining adherence of SK186 and PS478 was due to binding to this carbohydrate structure. However, we could not identify a glycosidase that was specific for this linkage.

This study focuses on adherence to the immortalized oral epithelial cell line TR146. There are many different surfaces in the oral cavity, and we have not investigated if the same adherence mechanism is used in binding to other surfaces, including the teeth and tongue. However, glycan structures containing the carbohydrates discussed in the manuscript are contained within N-linked glycans and many glycolipids and thus are widely distributed on host surfaces. Hence, we expect this mechanism of adherence would be relevant on different host cells. It is less clear if this mechanism is relevant to saliva binding, as a previously published paper demonstrated that binding of several *S. gordonii* strains to low-weight salivary mucin was dependent on sialic acid (28). These strains included 38, which in our study does not require sialic acid for efficient binding to oral epithelial cells.

We observed extensive variation in the adherence of different strains to untreated epithelial cells (Fig. 1). Few studies have compared adherence of different *S. gordonii* strains to that of different host surfaces, so it is not clear if these patterns of adherence would be maintained across surfaces. However, a prior study showed there was no significant difference in the ability of M99 and DL1 to bind platelets, which is consistent with the findings reported here (Fig. 1) (17). Diversity in the adherence efficiency of various streptococcal strains and species to different immobilized carbohydrate receptors has been reported previously, and these findings may help explain the different levels of adherence observed among *S. gordonii* strains in our current study (27, 28).

Although the majority of *S. gordonii* strains tested in this study did not rely on sialic acid for binding, adherence of DL1 and SK12 was reduced by neuraminidase. These strains were the only two to encode Hsa and also showed the highest adherence in the presence of sialic acid (Fig. 1). There are many possible reasons for the higher adherence of these strains, but it is tempting to speculate that the broader binding specificity of Hsa, which has been reported to bind to all tested carbohydrates with terminal α -2,3-linked sialic acid, is a contributing factor (56).

In summary, the manuscript establishes that many *S. gordonii* strains do not rely solely or primarily on sialic acid for adherence to host cells. Furthermore, it demonstrates that bacteria in the same environment as bacteria producing glycosidases that cleave terminal receptors have evolved to simultaneously bind terminal and cryptic receptors.

MATERIALS AND METHODS

Bacterial strains and reagents. The strains used in this study are described in Table 1. *S. gordonii* and *S. oralis* were grown on tryptic soy agar plates supplemented with 5% sheep's blood (Becton

Dickinson and Company) or tryptic soy agar plates (Becton Dickinson and Company) spread with 5,000 U of catalase (Worthington Biochemical Corporation) prior to plating. Plates were incubated overnight at 37°C in 5% CO₂. Broth cultures were grown statically at 37°C in Todd-Hewitt broth (Becton, Dickinson and Company) supplemented with 0.2% (wt/vol) yeast extract (Becton, Dickinson and Company) (THY). Transformations of *S. gordonii* were performed in competence medium supplemented with 10% fetal bovine serum (Corning) and 0.2% glucose as described previously (57). As appropriate, *S. gordonii* transformants were selected on medium containing spectinomycin (200 µg ml⁻¹). *S. oralis* transformations were conducted as previously described and selected on kanamycin (500 µg ml⁻¹) or erythromycin (1 µg ml⁻¹) (25).

Escherichia coli strains were grown at 37°C at 200 rpm in Luria-Bertani (LB) broth or statically on LB agar plates. The medium was supplemented with spectinomycin (50 µg ml⁻¹) or kanamycin (50 µg ml⁻¹) as appropriate.

Unless otherwise specified, all chemicals, substrates, and enzymes were purchased from Sigma-Aldrich (St. Louis, MO).

MLSA. This multilocus sequence analysis (MLSA) scheme utilizes phylogenetic analysis of concatenated sequences from seven housekeeping genes to identify the species of many streptococci, including those within the Mitis group, which includes *S. gordonii* (58). Concatenated sequences from strains in this study were added to M. Kilian's database of concatenated sequences from Mitis group isolates included in the NCBI database, and species were assigned using MEGA6.06 software as previously described (59, 60). When genome sequences of strains included in the study were available, the sequences used for MLSA were identified and compared to the sequences we obtained. In each case they were identical.

Adherence assays. The adherence of *S. gordonii* to TR146 cells, a human squamous epithelial carcinoma cell line isolated from the buccal mucosa, was determined essentially as previously described (39, 61). Briefly, *S. gordonii* strains, with the exception of SK186, were grown in THY to an optical density at 600 nm (OD₆₀₀) of 0.6 ± 0.005 and diluted in antibiotic-free F12 tissue culture medium. The exponential growth phase of SK186 ended earlier than for the other *S. gordonii* strains, hence adherence assays with this strain were performed using bacteria grown to an OD₆₀₀ of 0.55 ± 0.05 . Where appropriate, TR146 cells were pretreated for 30 min at 37°C with *Clostridium perfringens* sialidase or a medium-alone control. The wells in initial experiments represented in Fig. 1 were pretreated with 0.079 µM neuraminidase. However, as the neuraminidase activity decreased during storage, the concentration was doubled to 0.158 µM in order to maintain the same enzymatic activity in further experiments. Wells were inoculated with approximately 2×10^5 bacteria per well, and plates were incubated at 37°C for 60 min. When determining the role of β-1,4-linked galactose in adherence, 0.102 µM rSpBgaA146-990 or an equal volume of phosphate-buffered saline (PBS) was added to the adherence assay (35). Nonadherent bacteria were removed by three washes in PBS. Bacteria adherent to epithelial cells were lifted with 0.25% trypsin–1 mM EDTA and enumerated by serial dilution and plating. Adherence to the human pharyngeal carcinoma cell line Detroit 562 (D562; ATCC CCL-138) was conducted using the same conditions, with the exception that half the concentration of both neuraminidase (0.079 µM) and rSpBgaA146-990 (0.051 µM) was used. All adherence assays were conducted in triplicate on at least three independent occasions.

In adherence assays to define the effect of *S. oralis* ATCC 10557 on *S. gordonii* SK186 adherence, we differentiated the strains using differentially marked *bgaA* mutants, as they were shown here or in previously published studies not to affect adherence (25). *S. gordonii* SK186 was grown as described above, and *S. oralis* ATCC 10557 was grown in THY to an OD₆₀₀ of 0.6 ± 0.005 . We confirmed that coinoculation of ATCC 10557 and *S. gordonii* SK186 in tissue culture medium did not affect viability over a 1-h period. An approximately seven-to-one ratio of *S. oralis* (2×10^7) to *S. gordonii* (3×10^6) was used in the adherence assays, as *S. oralis* did not increase adherence of *S. gordonii* at a one-to-one ratio. Where appropriate, TR146 cells were pretreated with 0.158 µM neuraminidase as described above. Controls confirmed that neither the presence of *S. gordonii* nor the mutation of *nanaA* significantly affected *S. oralis* adherence (data not shown).

To allow comparison of the adherence levels of the different *S. gordonii* strains and the effects of neuraminidase pretreatment (Fig. 1), adherence was reported as the percentage of inoculum bound. Although trends in adherence were maintained between biological replicates, the absolute adherence was variable, so data from the other experiments were reported as relative adherence. For these assays, adherence of the control was adjusted to 100% and the adherence of the test condition(s) was produced relative to that of the control under the same experimental conditions. Data are presented as means $\pm$ standard deviations (SD). For data where comparisons were made only between two different conditions for each strain, statistical significance was determined using two-tailed Student's *t* test, and data points with a *P* value of ≤ 0.05 were considered significant (Fig. 1 and 4). Statistical significance for all other experiments was assessed using an analysis of variance (ANOVA) and least-significant-difference (LSD) *post hoc* test (*P* value of ≥ 0.05). To ensure that neither neuraminidase nor rSpBgaA146-990 was toxic to the epithelial cells at the concentrations used, trypan blue staining was performed as previously described, and no difference in viability was identified (35).

Distribution of Hsa, GspB, and sortase A-dependent surface proteins in *S. gordonii* strains. The distribution of *hsa* and *gspB* was determined by PCR using standard conditions (primers SRRP.1 and SRRP.7 [for *hsa*] as well as SRRP.1 and SRRP.8 [for *gspB*]). *S. gordonii* has been reported to contain one or the other of these two SRRPs, and amplification from all strains resulted in a product with one of the two primer pairs. Screening of *S. gordonii* strains for the predicted sortase A-dependent surface protein present in the G9B (TZ86_00548) and M99 genomes (within accession number [LAWL01000008](#)) and absent from DL1 and SK12 was first conducted by PCR using primers TZ86_00548F and TZ86_00548R.

TABLE 2 Primers used in this study

Group/primer	Primer sequence 5' to 3'	Location (accession no.)
<i>gspB</i> or <i>hsa</i>		
SRRP.1	<u>TCGGATCCAGAATTCGAAGCAATGCCAAAGTGTAGG</u> ^a	648–668 (AY028381.5)
SRRP.2	<u>CACGAACGAAATCGTCCAAATTGAGATGTAGCCGC</u> ^b	1244–1224 (AY028381.5)
SRRP.3	<u>ATAAACCCCTTGATAGGAGCTTTGGGATTGGCGTT</u> ^b	10299–10318 (AY028381.5)
SRRP.4	<u>CTTGTCGACGAATTCACGATGGACTTAATGGTGTT</u> ^a	10804–10784 (AY028381.5)
GspB.5	CTCATTTACGGAGCAAGAAAAG	507–528 (AY028381.5)
GspB.6	TATGCAAGGGTTTATTGTTTTT	1156–1135 (M69221.1)
GspB.i	TTGGAATCTGTATACTGGTG	333807–333826 (JYGL01000001.1)
GspB.ii	TACTTGAGTCACAGAATACTGC	334212–334191 (JYGL01000001.1)
GspB.iii	TCAATAAATTTACTTCCGTC	334622–334603 (JYGL01000001.1)
GspB.iv	GAAATGTAATGTAACGCG	334307–334326 (JYGL01000001.1)
GspB.v	TAGTATCTATCAGTGGTAATC	333716–333736 (JYGL01000001.1)
GspB.vi	GGGCCCTTTCCTTATGCTT	2284–2265 (MF927926.1)
Hsa.5	CGATTTTCGTTTCGTGAATAC	3–22 (M69221.1)
Hsa.6	AATAGCGCAGGAAAACGTAT	1018861–1018841 (CP000725.1)
SRRP.7	TGTTTGACTAGAAGCTTCTGTGC	1847–1825 (AY028381.5)
SRRP.8	TACTAATCTGAAGATCTTAACTAC	1013044–1013020 (CP000725.1)
<i>bgaA</i>		
B.1	<u>TCGGATCCAGAATTCATTAAACAGGTACAGAGCTTAG</u> ^a	1543379–1543358 (CP000725.1)
B.2	<u>CACGAACGAAATCGTCCGCATAAACAGGATTTCTCTG</u> ^b	1542873–1542894 (CP000725.1)
B.3	<u>ATAAACCCCTTGATAGAAGCAGTGCAGCTTCAAGCAG</u> ^b	1536115–1536094 (CP000725.1)
B.4	<u>CTTGTCGACGAATTCAGTAATATTGACTAGAGGCACAG</u> ^a	1535560–1535582 (CP000725.1)
B.5	ATAAAGATCCTAAAGATCCAGG	1543511–1543490 (CP000725.1)
B.6	TATGCAAGGGTTTATTGTTTTT	1156–1135 (M69221.1)
B.i	CTGCGACAAATGAAGAAG	410–427 (KX792092.1)
B.ii	GTTTGGATGTTTCGCACGAAG	1914–1894 (KX792092.1)
B.iii	ATGGACAGCAATCACATTC	837–819 (KX792092.1)
B.iv	CGTCTCAAACAAATGAAG	1402–1419 (KX792092.1)
B.v	GGAATCGTAAAGAGTCTAC	17–36 (KX792092.1)
B.vi	GGGCCCTTTCCTTATGCTT	2284–2265 (MF927926.1)
<i>srtA</i>		
S.1	<u>TCGGATCCAGAATTCCTGATTAAATGTTTTCTAACTGAT</u> ^a	1276706–1276683 (CP000725.1)
S.2	<u>CACGAACGAAATCGTTTCTTCTGGCTCTTCGTGA</u> ^b	1276155–1276175 (CP000725.1)
S.3	<u>ATAAACCCCTTGATATACTTCAATAAATCATACAATCAA</u> ^b	1275467–1275444 (CP000725.1)
S.4	<u>CTTGTCGACGAATTCGTTCAAATAAAGTGCAACCAAATG</u> ^a	1274955–1274978 (CP000725.1)
S.5	CGATTTTCGTTTCGTGAATAC	3–22 (M69221.1)
S.6	CCATCATTTTCGCAGGCTTCTT	1274747–1274767 (CP000725.1)
<i>nanA</i>		
N.5	GGTGAGGTCAAGATGATTACG	505512–505532 (FR720602.1)
N.6	AACGGCCGCCAGTGTGCTG	319–336 (EU233623)
<i>TZ86_00548</i>		
TZ86_00548F	TGGCTGCTCAAGATTTTCWATG ^c	560435–560415 (JYGL01000001.1)
TZ86_00548F2	CTYCGTAATGAAGCAAAAG ^d	558420–558402 (JYGL01000001.1)
TZ86_00548R	AACAGATGTAAAGGTTGGAG	558018–558038 (JYGL01000001.1)
<i>aad9</i>		
S.F	CGATTTTCGTTTCGTGAATAC	3–22 (M69221.1)
S.R	TATGCAAGGGTTTATTGTTTTT	1156–1135 (M69221.1)
<i>Janus</i>		
J.F	CCGTTTGATTTTTAATGGATAATG	773–796 (AF411920.1)
J.R	GGGCCCTTTCCTTATGCTT	2105–2086 (AF411920.1)
<i>pDrive</i>		
T7 promoter	GTAATACGACTCACTATAG	239–258 (DQ996013)
M13F	GTAAACGACGGCCAGT	431–447 (DQ996013)

^aUnderlining indicates nucleotides introduced to allow in-fusion cloning into the pDrive vector.^bUnderlining indicates nucleotides introduced to allow in-fusion cloning with *aad9* (spectinomycin cassette).^cW = A or T.^dY = C or T.

The absence of TZ86_00548 from PCR-negative strains was confirmed by Southern blotting using EcoRV-digested DNA and standard protocols. Membranes were probed with a conserved digoxigenin (DIG)-labeled 402-bp internal fragment of TZ86_00548 (primers TZ86_00548F2 and TZ86_00548R; PCR DIG probe synthesis kit; Roche Diagnostics). Following hybridization at 65°C, washes were performed at 68°C, decreasing from 2× to 0.2× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate). Bound probe was detected using the DIG nucleic acid detection kit.

Generation of streptococcal mutants. Insertion-deletion mutants were generated using allelic exchange methods as previously described (25). All genomic DNA samples were prepared as previously described, with the addition of 100 U/ml⁻¹ mutanolysin to the resuspension buffer (62). The *S. gordonii* mutants were generated using fragments of approximately 500 bp from the 5' and 3' flanking regions of the target gene amplified from genomic DNA using the appropriate primer pairs 1 and 2 as well as 3 and 4, respectively (Table 2). The spectinomycin was amplified (primers S.F and S.R) and cloned in EcoRI-digested pDrive with appropriate flanking fragments (Qiagen, Germantown, MD) using the In-Fusion EcoDry HD cloning kit (Clontech, Mountain View, CA) per the manufacturer's protocol and transformed into *E. coli* Stellar (Clontech). Plasmid constructs were confirmed by PCR with M13F and T7 promoter primers and sequencing.

In order to generate the constructs used to make the *S. oralis* *bgaA* mutant and the *gspB* mutation for generation of the *S. gordonii* *bgaA gspB* double mutants, we used an inverse PCR strategy. Fragments of the target gene were amplified from genomic DNA using the appropriate primer pair (i and ii) and cloned into pJET1.2/Blunt PCR cloning vector (Thermo Fisher Scientific, Waltham, MA). An inverse PCR product of primers iii and iv was then generated and blunt-end ligated to the Janus cassette (primers J.F and J.R), resulting in the final constructs. The plasmids were confirmed by PCR and sequencing. The ATCC 10557 $\Delta bgaA \Delta nanA$ strain was generated using the previously described construct (25).

S. gordonii strains were grown to an OD₆₀₀ of 0.11 to 0.14 in *S. gordonii* competence medium supplemented with 10% fetal bovine serum and 0.2% glucose (57). Approximately 100 to 300 ng of plasmid was added to 500 μ l of culture, and the transformation reaction mixtures were incubated at 37°C for 2 h. Transformants were selected on TS agar plates supplemented with spectinomycin. *S. oralis* transformations were conducted as previously described (25). The resulting colonies were screened for the targeted insertion-deletion by PCR using one primer within the antibiotic cassette and one within the region flanking the construct (primers 5 and 6 or v and vi). Mutations were also shown not to have any generalized growth defect during growth on THY (63). Mutagenesis of *bgaA* and *nanA* was also confirmed by enzymatic activity assays as previously reported (33, 64). The genetic background of all mutants was confirmed to be the same as that of the parental strain using repetitive extragenic palindromic PCR, essentially as previously described but using commercially available PCR buffer (Denville Scientific, Inc.) (65).

Accession number(s). The MLSA sequences were submitted to GenBank and have the accession numbers [MG587046](#) to [MG587080](#).

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REFERENCES

- Frandsen EV, Pedrazzoli V, Kilian M. 1991. Ecology of viridans streptococci in the oral cavity and pharynx. *Oral Microbiol Immunol* 6:129–133. <https://doi.org/10.1111/j.1399-302X.1991.tb00466.x>.
- Mager DL, Ximenez-Fyvie LA, Haffajee AD, Socransky SS. 2003. Distribution of selected bacterial species on intraoral surfaces. *J Clin Periodontol* 30:644–654. <https://doi.org/10.1034/j.1600-051X.2003.00376.x>.
- Nyvad B, Kilian M. 1990. Comparison of the initial streptococcal microflora on dental enamel in caries-active and in caries-inactive individuals. *Caries Res* 24:267–272. <https://doi.org/10.1159/000261281>.
- Douglas CW, Heath J, Hampton KK, Preston FE. 1993. Identity of viridans streptococci isolated from cases of infective endocarditis. *J Med Microbiol* 39:179–182. <https://doi.org/10.1099/00222615-39-3-179>.
- Jenkinson HF, Terry SD, McNab R, Tannock GW. 1993. Inactivation of the gene encoding surface protein SspA in *Streptococcus gordonii* DL1 affects cell interactions with human salivary agglutinin and oral actinomyces. *Infect Immun* 61:3199–3208.
- Petersen HJ, Keane C, Jenkinson HF, Vickerman MM, Jesionowski A, Waterhouse JC, Cox D, Kerrigan SW. 2010. Human platelets recognize a novel surface protein, PadA, on *Streptococcus gordonii* through a unique interaction involving fibrinogen receptor GPIIb/IIIa. *Infect Immun* 78:413–422. <https://doi.org/10.1128/IAI.00664-09>.
- McNab R, Holmes AR, Clarke JM, Tannock GW, Jenkinson HF. 1996. Cell surface polypeptide CshA mediates binding of *Streptococcus gordonii* to other oral bacteria and to immobilized fibronectin. *Infect Immun* 64:4204–4210.
- McNab R, Forbes H, Handley PS, Loach DM, Tannock GW, Jenkinson HF. 1999. Cell wall-anchored CshA polypeptide (259 kilodaltons) in *Streptococcus gordonii* forms surface fibrils that confer hydrophobic and adhesive properties. *J Bacteriol* 181:3087–3095.
- Jakubovics NS, Brittan JL, Dutton LC, Jenkinson HF. 2009. Multiple adhesin proteins on the cell surface of *Streptococcus gordonii* are involved in adhesion to human fibronectin. *Microbiology* 155(Part 11):3572–3580. <https://doi.org/10.1099/mic.0.032078-0>.
- Jakubovics NS, Kerrigan SW, Nobbs AH, Stromberg N, van Dolleweerd CJ, Cox DM, Kelly CG, Jenkinson HF. 2005. Functions of cell surface-anchored antigen I/II family and Hsa polypeptides in interactions of *Streptococcus gordonii* with host receptors. *Infect Immun* 73:6629–6638. <https://doi.org/10.1128/IAI.73.10.6629-6638.2005>.

11. Takahashi Y, Konishi K, Cisar JO, Yoshikawa M. 2002. Identification and characterization of *hsa*, the gene encoding the sialic acid-binding adhesin of *Streptococcus gordonii* DL1. Infect Immun 70:1209–1218. <https://doi.org/10.1128/IAI.70.3.1209-1218.2002>.
12. Bensing BA, Sullam PM. 2002. An accessory *sec* locus of *Streptococcus gordonii* is required for export of the surface protein GspB and for normal levels of binding to human platelets. Mol Microbiol 44:1081–1094. <https://doi.org/10.1046/j.1365-2958.2002.02949.x>.
13. Tanzer JM, Grant L, Thompson A, Li L, Rogers JD, Haase EM, Scannapieco FA. 2003. Amylase-binding proteins A (AbpA) and B (AbpB) differentially affect colonization of rats' teeth by *Streptococcus gordonii*. Microbiology 149(Part 9):2653–2660. <https://doi.org/10.1099/mic.0.26022-0>.
14. Takamatsu D, Bensing BA, Cheng H, Jarvis GA, Siboo IR, Lopez JA, Griffiss JM, Sullam PM. 2005. Binding of the *Streptococcus gordonii* surface glycoproteins GspB and Hsa to specific carbohydrate structures on platelet membrane glycoprotein Ib α . Mol Microbiol 58:380–392. <https://doi.org/10.1111/j.1365-2958.2005.04830.x>.
15. Bensing BA, Gibson BW, Sullam PM. 2004. The *Streptococcus gordonii* platelet binding protein GspB undergoes glycosylation independently of export. J Bacteriol 186:638–645. <https://doi.org/10.1128/JB.186.3.638-645.2004>.
16. Pyburn TM, Bensing BA, Xiong YQ, Melancon BJ, Tomasiak TM, Ward NJ, Yankovskaya V, Oliver KM, Cecchini G, Sulikowski GA, Tyska MJ, Sullam PM, Iverson TM. 2011. A structural model for binding of the serine-rich repeat adhesin GspB to host carbohydrate receptors. PLoS Pathog 7:e1002112. <https://doi.org/10.1371/journal.ppat.1002112>.
17. Deng L, Bensing BA, Thamadilok S, Yu H, Lau K, Chen X, Ruhl S, Sullam PM, Varki A. 2014. Oral streptococci utilize a Siglec-like domain of serine-rich repeat adhesins to preferentially target platelet sialoglycans in human blood. PLoS Pathog 10:e1004540. <https://doi.org/10.1371/journal.ppat.1004540>.
18. Takahashi Y, Sandberg AL, Ruhl S, Muller J, Cisar JO. 1997. A specific cell surface antigen of *Streptococcus gordonii* is associated with bacterial hemagglutination and adhesion to alpha2-3-linked sialic acid-containing receptors. Infect Immun 65:5042–5051.
19. Perlitz MJ, Glickman I. 1966. Salivary neuraminidase. II. Its source in human whole saliva. J Dent Res 45:1239.
20. Beighton D, Whaley RA. 1990. Sialidase activity of the "*Streptococcus milleri* group" and other viridans group streptococci. J Clin Microbiol 28:1431–1433.
21. van der Hoeven JS, van den Kieboom CW, Camp PJ. 1990. Utilization of mucin by oral *Streptococcus* species. Antonie Van Leeuwenhoek 57:165–172. <https://doi.org/10.1007/BF00403951>.
22. Vickerman MM, Iobst S, Jesionowski AM, Gill SR. 2007. Genome-wide transcriptional changes in *Streptococcus gordonii* in response to competence signaling peptide. J Bacteriol 189:7799–7807. <https://doi.org/10.1128/JB.01023-07>.
23. Byers HL, Homer KA, Beighton D. 1996. Utilization of sialic acid by viridans streptococci. J Dent Res 75:1564–1571. <https://doi.org/10.1177/00220345960750080701>.
24. Lombard V, Golaconda Ramulu H, Drula E, Coutinho PM, Henrissat B. 2014. The carbohydrate-active enzymes database (CAZy) in 2013. Nucleic Acids Res 42:D490–D495. <https://doi.org/10.1093/nar/gkt1178>.
25. Singh AK, Woodiga SA, Grau MA, King SJ. 2017. *Streptococcus oralis* neuraminidase modulates adherence to multiple carbohydrates on platelets. Infect Immun 85:e00774. <https://doi.org/10.1128/IAI.00774-16>.
26. Ruhl S, Sandberg AL, Cole MF, Cisar JO. 1996. Recognition of immunoglobulin A1 by oral actinomyces and streptococcal lectins. Infect Immun 64:5421–5424.
27. Murray PA, Prakobphol A, Lee T, Hoover CI, Fisher SJ. 1992. Adherence of oral streptococci to salivary glycoproteins. Infect Immun 60:31–38.
28. Takahashi Y, Ruhl S, Yoon JW, Sandberg AL, Cisar JO. 2002. Adhesion of viridans group streptococci to sialic acid-, galactose- and N-acetylglucosamine-containing receptors. Oral Microbiol Immunol 17:257–262. <https://doi.org/10.1034/j.1399-302X.2002.170409.x>.
29. Hsu SD CJ, Sandberg AL, Kilian M. 1994. Adhesive properties of viridans streptococcal species. Microb Ecol Health Dis 7:127–137.
30. Thamadilok S, Roche-Hakansson H, Hakansson AP, Ruhl S. 2016. Absence of capsule reveals glycan-mediated binding and recognition of salivary mucin MUC7 by *Streptococcus pneumoniae*. Mol Oral Microbiol 31:175–188. <https://doi.org/10.1111/omi.12113>.
31. Takahashi Y, Yajima A, Cisar JO, Konishi K. 2004. Functional analysis of the *Streptococcus gordonii* DL1 sialic acid-binding adhesin and its essential role in bacterial binding to platelets. Infect Immun 72:3876–3882. <https://doi.org/10.1128/IAI.72.7.3876-3882.2004>.
32. Feng L, Zhu J, Chang H, Gao X, Gao C, Wei X, Yuan F, Bei W. 2016. The CodY regulator is essential for virulence in *Streptococcus suis* serotype 2. Sci Rep 6:21241. <https://doi.org/10.1038/srep21241>.
33. King SJ, Hippe KR, Gould JM, Bae D, Peterson S, Cline RT, Fasching C, Janoff EN, Weiser JN. 2004. Phase variable desialylation of host proteins that bind to *Streptococcus pneumoniae* in vivo and protect the airway. Mol Microbiol 54:159–171. <https://doi.org/10.1111/j.1365-2958.2004.04252.x>.
34. Konto-Ghiorgi Y, Mairey E, Mallet A, Dumenil G, Caliot E, Trieu-Cuot P, Dramsi S. 2009. Dual role for pilus in adherence to epithelial cells and biofilm formation in *Streptococcus agalactiae*. PLoS Pathog 5:e1000422. <https://doi.org/10.1371/journal.ppat.1000422>.
35. Singh AK, Pluvinaige B, Higgins MA, Dalia AB, Woodiga SA, Flynn M, Lloyd AR, Weiser JN, Stubbs KA, Boraston AB, King SJ. 2014. Unravelling the multiple functions of the architecturally intricate *Streptococcus pneumoniae* beta-galactosidase, BgaA. PLoS Pathog 10:e1004364. <https://doi.org/10.1371/journal.ppat.1004364>.
36. Urano-Tashiro Y, Yajima A, Takashima E, Takahashi Y, Konishi K. 2008. Binding of the *Streptococcus gordonii* DL1 surface protein Hsa to the host cell membrane glycoproteins CD11b, CD43, and CD50. Infect Immun 76:4686–4691. <https://doi.org/10.1128/IAI.00238-08>.
37. Bensing BA, Lopez JA, Sullam PM. 2004. The *Streptococcus gordonii* surface proteins GspB and Hsa mediate binding to sialylated carbohydrate epitopes on the platelet membrane glycoprotein Ib α . Infect Immun 72:6528–6537. <https://doi.org/10.1128/IAI.72.11.6528-6537.2004>.
38. Takamatsu D, Bensing BA, Prakobphol A, Fisher SJ, Sullam PM. 2006. Binding of the streptococcal surface glycoproteins GspB and Hsa to human salivary proteins. Infect Immun 74:1933–1940. <https://doi.org/10.1128/IAI.74.3.1933-1940.2006>.
39. Limoli DH, Sladek JA, Fuller LA, Singh AK, King SJ. 2011. BgaA acts as an adhesin to mediate attachment of some pneumococcal strains to human epithelial cells. Microbiology 157:2369–2381. <https://doi.org/10.1099/mic.0.045609-0>.
40. Zeleny R, Altmann F, Praznik W. 1997. A capillary electrophoretic study on the specificity of beta-galactosidases from *Aspergillus oryzae*, *Escherichia coli*, *Streptococcus pneumoniae*, and *Canavalia ensiformis* (jack bean). Anal Biochem 246:96–101. <https://doi.org/10.1006/abio.1996.9973>.
41. Bolken TC, Franke CA, Jones KF, Zeller GO, Jones CH, Dutton EK, Hruby DE. 2001. Inactivation of the *srtA* gene in *Streptococcus gordonii* inhibits cell wall anchoring of surface proteins and decreases in vitro and in vivo adhesion. Infect Immun 69:75–80. <https://doi.org/10.1128/IAI.69.1.75-80.2001>.
42. Kharat AS, Tomasz A. 2003. Inactivation of the *srtA* gene affects localization of surface proteins and decreases adhesion of *Streptococcus pneumoniae* to human pharyngeal cells in vitro. Infect Immun 71:2758–2765. <https://doi.org/10.1128/IAI.71.5.2758-2765.2003>.
43. Lee SF, Boran TL. 2003. Roles of sortase in surface expression of the major protein adhesin P1, saliva-induced aggregation and adherence, and cariogenicity of *Streptococcus mutans*. Infect Immun 71:676–681. <https://doi.org/10.1128/IAI.71.2.676-681.2003>.
44. Buscetta M, Papasergi S, Firon A, Pietrocola G, Biondo C, Mancuso G, Midiri A, Romeo L, Teti G, Speziale P, Trieu-Cuot P, Beninati C. 2014. FbsC, a novel fibrinogen-binding protein, promotes *Streptococcus agalactiae*-host cell interactions. J Biol Chem 289:21003–21015. <https://doi.org/10.1074/jbc.M114.553073>.
45. Navarre WW, Schneewind O. 1999. Surface proteins of gram-positive bacteria and mechanisms of their targeting to the cell wall envelope. Microbiol Mol Biol Rev 63:174–229.
46. Schneewind O, Model P, Fischetti VA. 1992. Sorting of protein A to the staphylococcal cell wall. Cell 70:267–281. [https://doi.org/10.1016/0092-8674\(92\)90101-H](https://doi.org/10.1016/0092-8674(92)90101-H).
47. Ton-That H, Faull KF, Schneewind O. 1997. Anchor structure of staphylococcal surface proteins. A branched peptide that links the carboxyl terminus of proteins to the cell wall. J Biol Chem 272:22285–22292.
48. NCBI Resource Coordinators. 2017. Database resources of the National Center for Biotechnology Information. Nucleic Acids Res 45(D1):D12–D17. <https://doi.org/10.1093/nar/gkw1071>.
49. Sabharwal A, Liao YC, Lin HH, Haase EM, Scannapieco FA. 2015. Draft genome sequences of 18 oral *Streptococcus* strains that encode amylase-binding proteins. Genome Announc 3:e00510-15. <https://doi.org/10.1128/genomeA.00510-15>.
50. Soding J, Biegert A, Lupas AN. 2005. The HHpred interactive server for

- protein homology detection and structure prediction. *Nucleic Acids Res* 33:W244–W248. <https://doi.org/10.1093/nar/gki408>.
51. Byers HL, Tarelli E, Homer KA, Beighton D. 2000. Isolation and characterisation of sialidase from a strain of *Streptococcus oralis*. *J Med Microbiol* 49:235–244. <https://doi.org/10.1099/0022-1317-49-3-235>.
 52. Spiro RG. 1962. Studies on the monosaccharide sequence of the serum glycoprotein fetuin. *J Biol Chem* 237:646–652.
 53. Spiro RG. 1962. Studies on fetuin, a glycoprotein of fetal serum. II. Nature of the carbohydrate units. *J Biol Chem* 237:382–388.
 54. Spiro RG. 1964. Periodate oxidation of the glycoprotein fetuin. *J Biol Chem* 239:567–573.
 55. Spiro RG, Bhoyroo VD. 1974. Structure of the O-glycosidically linked carbohydrate units of fetuin. *J Biol Chem* 249:5704–5717.
 56. Bensing BA, Loukachevitch LV, McCulloch KM, Yu H, Vann KR, Wawrzak Z, Anderson S, Chen X, Sullam PM, Iverson TM. 2016. Structural basis for sialoglycan binding by the *Streptococcus sanguinis* SrpA adhesin. *J Biol Chem* 291:7230–7240. <https://doi.org/10.1074/jbc.M115.701425>.
 57. LeBlanc DJ, Hassell FP. 1976. Transformation of *Streptococcus sanguis* Challis by plasmid deoxyribonucleic acid from *Streptococcus faecalis*. *J Bacteriol* 128:347–355.
 58. Bishop CJ, Aanensen DM, Jordan GE, Kilian M, Hanage WP, Spratt BG. 2009. Assigning strains to bacterial species via the internet. *BMC Biol* 7:3. <https://doi.org/10.1186/1741-7007-7-3>.
 59. Jensen A, Scholz CF, Kilian M. 2016. Reevaluation of the taxonomy of the Mitis group of the genus *Streptococcus* based on whole genome phylogenetic analyses, and proposed reclassification of *Streptococcus dentisani* as *Streptococcus oralis* subsp. *dentisani* comb. nov., *Streptococcus tigurinus* as *Streptococcus oralis* subsp. *tigurinus* comb. nov., and *Streptococcus oligofermentans* as a later synonym of *Streptococcus cristatus*. *Int J Syst Evol Microbiol* 66:4803–4820. <https://doi.org/10.1099/ijsem.0.001433>.
 60. Tamura K, Stecher G, Peterson D, Filipinski A, Kumar S. 2013. MEGA6: molecular evolutionary genetics analysis version 6.0. *Mol Biol Evol* 30:2725–2729. <https://doi.org/10.1093/molbev/mst197>.
 61. Gould JM, Weiser JN. 2002. The inhibitory effect of C-reactive protein on bacterial phosphorylcholine platelet-activating factor receptor-mediated adherence is blocked by surfactant. *J Infect Dis* 186:361–371. <https://doi.org/10.1086/341658>.
 62. Whatmore AM, Barcus VA, Dowson CG. 1999. Genetic diversity of the streptococcal competence (*com*) gene locus. *J Bacteriol* 181:3144–3154.
 63. Kloosterman TG, Bijlsma JJ, Kok J, Kuipers OP. 2006. To have neighbour's fare: extending the molecular toolbox for *Streptococcus pneumoniae*. *Microbiology* 152(Part 2):351–359. <https://doi.org/10.1099/mic.0.28521-0>.
 64. King SJ, Hippe KR, Weiser JN. 2006. Deglycosylation of human glycoconjugates by the sequential activities of exoglycosidases expressed by *Streptococcus pneumoniae*. *Mol Microbiol* 59:961–974. <https://doi.org/10.1111/j.1365-2958.2005.04984.x>.
 65. Alam S, Brailsford SR, Whitley RA, Beighton D. 1999. PCR-based methods for genotyping viridans group streptococci. *J Clin Microbiol* 37:2772–2776.
 66. Hare R. 1935. The classification of hemolytic streptococci nose and throat of normal human beings by means of precipitin and biochemical tests. *J Pathol Bacteriol* 41:499–512.
 67. Kilian M, Mikkelsen L, Henriksen J. 1989. Taxonomic study of viridans streptococci: description of *Streptococcus gordonii* sp. nov. and amended descriptions of *Streptococcus sanguis* (White and Niven 1946), *Streptococcus oralis* (Bridge and Sneath 1982), and *Streptococcus mitis* (Andrews and Horder (1906). *Int J Syst Bacteriol* 39:471–484. <https://doi.org/10.1099/00207713-39-4-471>.
 68. Appelbaum B, Golub E, Holt SC, Rosan B. 1979. In vitro studies of dental plaque formation: adsorption of oral streptococci to hydroxyapatite. *Infect Immun* 25:717–728.
 69. Rosan B. 1976. Relationship of the cell wall composition of group H streptococci and *Streptococcus sanguis* to their serological properties. *Infect Immun* 13:1144–1153.
 70. Sullam PM, Valone FH, Mills J. 1987. Mechanisms of platelet aggregation by viridans group streptococci. *Infect Immun* 55:1743–1750.
 71. Handley PS, Carter PL, Wyatt JE, Hesketh LM. 1985. Surface structures (peritrichous fibrils and tufts of fibrils) found on *Streptococcus sanguis* strains may be related to their ability to coaggregate with other oral genera. *Infect Immun* 47:217–227.
 72. Nugent KM, Cole RM. 1977. Characterization of group H streptococcal temperate bacteriophage phi 227. *J Virol* 21:1061–1073.
 73. Washburn MR, White JC, Niven CF, Jr. 1946 *Streptococcus* S.B.E.: immunological characteristics. *J Bacteriol* 51:723–729.