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# *Streptococcus oralis* Neuraminidase Modulates Adherence to Multiple Carbohydrates on Platelets

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**ABSTRACT** Adherence to host surfaces is often mediated by bacterial binding to surface carbohydrates. Although it is widely appreciated that some bacterial species express glycosidases, previous studies have not considered whether bacteria bind to multiple carbohydrates within host glycans as they are modified by bacterial glycosidases. *Streptococcus oralis* is a leading cause of subacute infective endocarditis. Binding to platelets is a critical step in disease; however, the mechanisms utilized by *S. oralis* remain largely undefined. Studies revealed that *S. oralis*, like *Streptococcus gordonii* and *Streptococcus sanguinis*, binds platelets via terminal sialic acid. However, unlike those organisms, *S. oralis* produces a neuraminidase, NanA, which cleaves terminal sialic acid. Further studies revealed that following NanA-dependent removal of terminal sialic acid, *S. oralis* bound exposed β-1,4-linked galactose. Adherence to both these carbohydrates required Fap1, the *S. oralis* member of the serine-rich repeat protein (SRRP) family of adhesins. Mutation of a conserved residue required for sialic acid binding by other SRRPs significantly reduced platelet binding, supporting the hypothesis that Fap1 binds this carbohydrate. The mechanism by which Fap1 contributes to β-1,4-linked galactose binding remains to be defined; however, binding may occur via additional domains of unknown function within the nonrepeat region, one of which shares some similarity with a carbohydrate binding module. This study is the first demonstration that an SRRP is required to bind β-1,4-linked galactose and the first time that one of these adhesins has been shown to be required for binding of multiple glycan receptors.

**KEYWORDS** endocarditis, neuraminidase, *Streptococcus oralis*, adherence, β-1,4-linked galactose, host glycans, platelets, serine-rich repeat protein, sialic acid

Infective endocarditis (IE) is an infection (usually bacterial) of the endocardium, most often the surfaces of the valves. Despite improvements in diagnosis, therapeutics, and surgical treatments, there has been no substantive improvement in the survival rate of IE patients over the last 30 years (1–4). Vegetative growths on the endocardium that contain bacteria and host factors lead to the clinical effects of IE, including valvular incompetence and congestive heart failure. Treatment requires several weeks of antibiotics, and approximately 50% of patients undergo surgery (4). The morbidity and mortality due to IE are high, with an in-hospital death rate of 17.7% and a 1-year mortality approaching 40% (2–5).

IE cases can be divided into acute and subacute IE. Acute IE, which is most commonly caused by staphylococci, typically affects previously normal valves and has a severe and sudden onset. Subacute IE requires a previously damaged endocardium and is more subtle in presentation, with infections often progressing for several weeks prior to diagnosis. The precise mechanisms by which subacute IE develops are poorly understood. It is proposed that a preexisting sterile platelet-fibrin nidus on the damaged endocardial surface becomes infected with bacteria, leading to IE (6, 7). It is not

clear if bacteria adhere directly to this nidus from the circulation or if bacteria bind to circulating platelets, which then act as "Trojan horses" carrying bacteria to the damaged endothelial surface (7–9). However, it is clear that adherence to platelets is a critical requirement for development of subacute IE. Viridans group streptococci are a common cause of subacute IE that can enter the bloodstream from the oral cavity after dental work and normal brushing activities (4, 10–13). While several species of viridans group streptococci can cause IE, *Streptococcus oralis* is often identified as the most common cause of viridans group streptococcus-associated IE (11, 14).

Despite the importance of *S. oralis* in IE, little is known about the adherence mechanisms utilized by this bacterial species. The majority of studies defining mechanisms of streptococcal adherence during IE have focused on *S. gordonii*. For *Streptococcus sanguinis* and *Streptococcus gordonii*, sialic acid residues on glycoprotein Ibα (GPIbα) have been identified as the major receptors on platelets (15–17). Adherence to sialic acid is mediated via serine-rich repeat proteins (SRRPs) that contain a Siglec-like domain (9, 17–20). Mutagenesis of SRRPs in several streptococcal species reduced pathogenesis in a rat model of IE (21–23). These studies demonstrate the essential nature of glycans in bacterium-platelet interactions. The limited studies performed with *S. oralis* indicate that this organism may also interact with host carbohydrates, including sialic acid (24). Tilley and coworkers recently demonstrated that *S. oralis* can adhere to and induce aggregation of platelets via interaction with host GPIbα (25). However, the bacterial adhesin and the biochemical nature of this interaction remain unknown. *S. oralis* has been shown to bind to glycans on several salivary glycoproteins, and neuraminidase treatment reduced binding of some strains to saliva-coated hydroxyapatite and mucin (24–26). Furthermore, agglutination of red blood cells by *S. oralis* was shown to be inhibited in the presence of some carbohydrates, including galactose and lactose (27).

Here we demonstrate that *S. oralis* binds to sialic acid on platelets and, following cleavage of terminal sialic acid by *S. oralis* neuraminidase, to the underlying carbohydrate β-1,4-linked galactose. Given the ability of many bacteria to both bind to and modify host glycans, this novel adherence strategy is likely employed by other bacterial species. Furthermore, our studies provide the first demonstration that an SRRP is required for binding to β-1,4-linked galactose and for adherence to multiple distinct carbohydrate receptors. These data suggest that domains of unknown function in SRRPs may bind additional carbohydrates.

## RESULTS

***S. oralis* binds to sialic acid on platelets.** *S. gordonii* and *S. sanguinis*, which (like *S. oralis*) cause subacute infective endocarditis, mediate binding to sialic acid on platelets (15–17). To determine if the *S. oralis* endocarditis isolate ATCC 10557 binds to sialic acid, we tested its adherence to platelets pretreated with neuraminidase, which removes terminal sialic acid, or with buffer alone. Removal of sialic acid significantly reduced bacterial adherence, indicating that this carbohydrate is a receptor for *S. oralis* on the platelet surface (Fig. 1). Adherence to platelets was also significantly reduced in the presence of 1 mM free sialic acid, indicating that this carbohydrate can compete with the receptor for binding to the bacterial adhesin (Fig. 1).

Despite the contribution of sialic acid as a receptor for *S. oralis* adherence, significant binding was still observed when this receptor was removed, indicating that an additional mechanism(s) exists.

**β-1,4-Linked galactose on the surface of platelets acts as a receptor for *S. oralis*.** Unlike *S. sanguinis* and *S. gordonii*, *S. oralis* produces a neuraminidase that cleaves terminal sialic acid (28). This enzymatic activity both cleaves the sialic acid receptor and exposes underlying carbohydrates (28, 29). Removal of sialic acid reveals a range of glycan structures, whose terminal carbohydrates may act as an additional receptor(s) (27). Previously published data demonstrated that agglutination of red blood cells by *S. oralis* was inhibited by lactose. Furthermore, it was recently demonstrated that pneumococci bind neuraminidase-exposed β-1,4-linked galactose (30, 31).
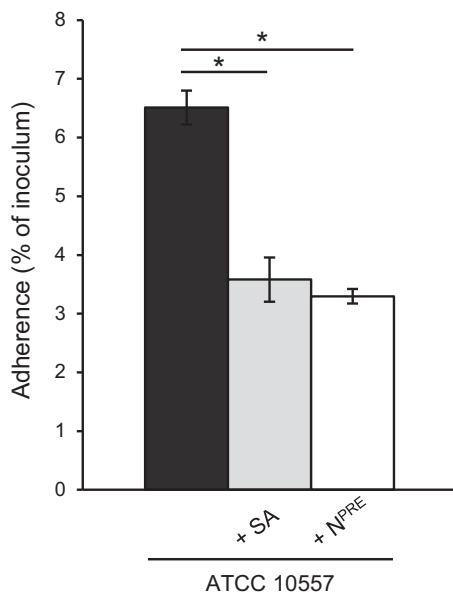
**FIG 1** *S. oralis* binds terminal sialic acid on platelets. The graph shows adherence of *S. oralis* ATCC 10557 to platelets pretreated with *Clostridium perfringens* neuraminidase (N$^{PRE}$; 0.78 $\mu$M) or in the presence of sialic acid (SA; 1 mM). Adherence is expressed as a percentage of the inoculum. Values are the means for at least three independent experiments, each performed in triplicate, $\pm$ SD. Statistical significance was tested by two-tailed Student's *t* test. *, $P \leq 0.001$.

Together, these data led to the hypothesis that $\beta$-1,4-linked galactose may act as a receptor for *S. oralis*.

Pretreatment of platelets with neuraminidase and the recombinantly expressed active site domain of pneumococcal $\beta$-galactosidase BgaA specific for terminal $\beta$-1,4-linked galactose (*Sp*BgaA146-990) reduced *S. oralis* adherence significantly more than treatment with neuraminidase alone did (Fig. 2A). These data indicate that *S. oralis* can bind both sialic acid and $\beta$-1,4-linked galactose. Further supporting the hypothesis that $\beta$-1,4-linked galactose is a receptor for *S. oralis* was the significant reduction in adherence of *S. oralis* to neuraminidase-treated platelets in the presence of 5 mM lactose and a 250 $\mu$M concentration of a recombinantly expressed carbohydrate binding module which specifically binds $\beta$-1,4-linked galactose (CBM71) (Fig. 2B) (30). Pretreatment of platelets with *Sp*BgaA146-990 alone had no significant effect on bacterial adherence (Fig. 2A). This enzyme can act only following removal of terminal sialic acid, so these data are consistent with previous studies showing that platelets are highly sialylated and indicate that neuraminidase activity is required to expose $\beta$-1,4-linked galactose as a receptor on the platelet surface (Fig. 2A).

The studies presented here focus on adherence of *S. oralis* type strain ATCC 10557 to platelets. Adherence to platelets is proposed to be critical to the development of IE; however, adherence to other host factors also likely contributes to development of disease. Binding of ATCC 10557 to endothelial cells was also mediated via adherence to sialic acid and $\beta$-1,4-linked galactose (data not shown). Adherence to sialic acid and $\beta$-1,4-linked galactose is not limited to ATCC 10557. Adherence of an additional *S. oralis* strain, 9A2, was significantly reduced by neuraminidase and *Sp*BgaA146-990 (data not shown).

**The neuraminidase NanA is required for exposure of $\beta$-1,4-linked galactose.** The studies reported thus far demonstrated that *S. oralis* can bind to terminal sialic acid and that *S. oralis* can bind to $\beta$-1,4-linked galactose following pretreatment of platelets with neuraminidase. In order for this mechanism to be relevant during infective endocarditis, *S. oralis* neuraminidase must expose $\beta$-1,4-linked galactose as a receptor. Addition of *Sp*BgaA146-990 to adherence assays significantly reduced adherence to
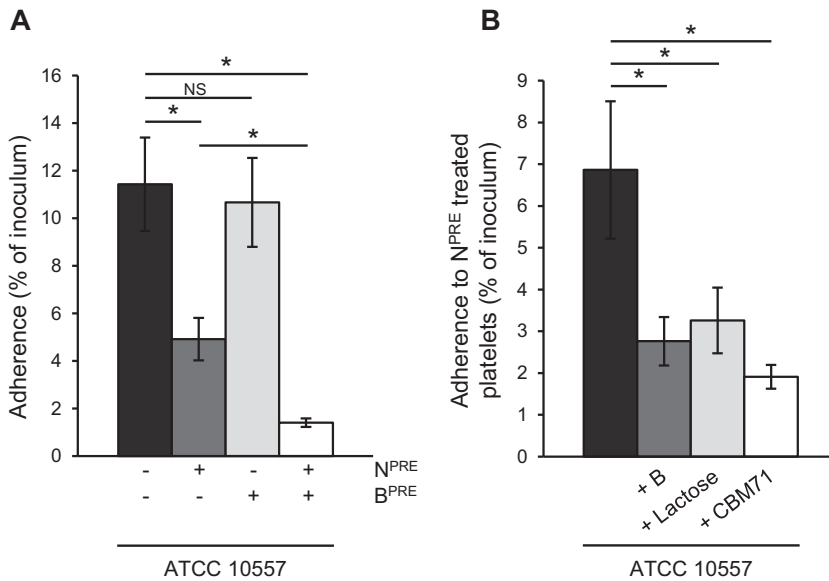
FIG 2 *S. oralis* binds β-1,4-linked galactose following removal of sialic acid. (A) Adherence of *S. oralis* ATCC 10557 to platelets was further reduced following pretreatment with *Clostridium perfringens* neuraminidase (N$^{PRE}$; 0.78 μM) and the β-1,4-linked galactose-specific β-galactosidase *Sp*BgaA146-990 (B$^{PRE}$; 0.054 μM). (B) Further data support the binding of ATCC 10557 to β-1,4-linked galactose. Adherence of *S. oralis* ATCC 10557 to neuraminidase-pretreated platelets was reduced in the presence of *Sp*BgaA146-990 (B; 0.054 μM); CBM71, which is specific for β-1,4-linked galactose (250 μM); or lactose (5 mM). Adherence is expressed as a percentage of the inoculum. Values are the means for three independent experiments, each performed in triplicate, ± SD. Statistical significance was tested by two-tailed Student's *t* test. *, $P \leq 0.03$; NS, not significant.

platelets, demonstrating that *S. oralis* neuraminidase activity is sufficient to reveal the receptor (Fig. 3). Addition of exogenous neuraminidase with *Sp*BgaA146-990 further reduced adherence. This is likely because the purified neuraminidase used is more effective than that produced by *S. oralis* at revealing β-1,4-linked galactose.
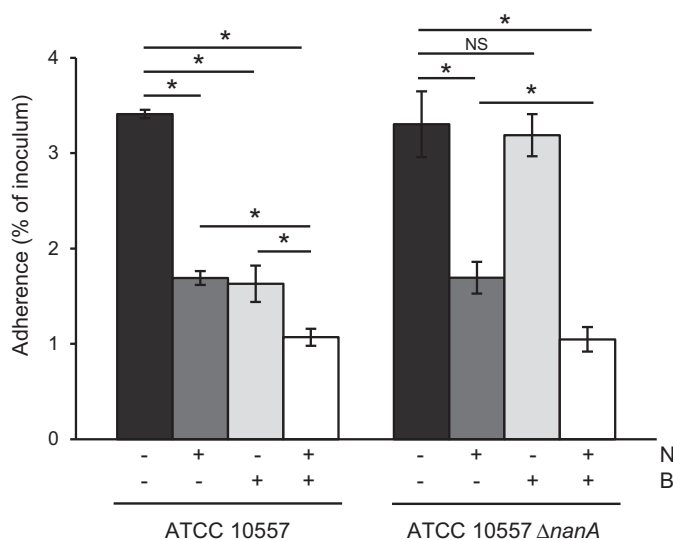


FIG 3 *S. oralis* reveals β-1,4-linked galactose in a NanA-dependent manner. The graph shows adherence of *S. oralis* ATCC 10557 and *S. oralis* ATCC 10557 Δ*nanA* to platelets in the presence of *Clostridium perfringens* neuraminidase (N; 0.78 μM), *Sp*BgaA146-990 (B; 0.054 μM), both enzymes, or a PBS control. Adherence is expressed as a percentage of the inoculum. Values are the means for three independent experiments, each performed in triplicate, ± SD. Statistical significance was tested by two-tailed Student's *t* test. *, $P \leq 0.01$; NS, not significant.

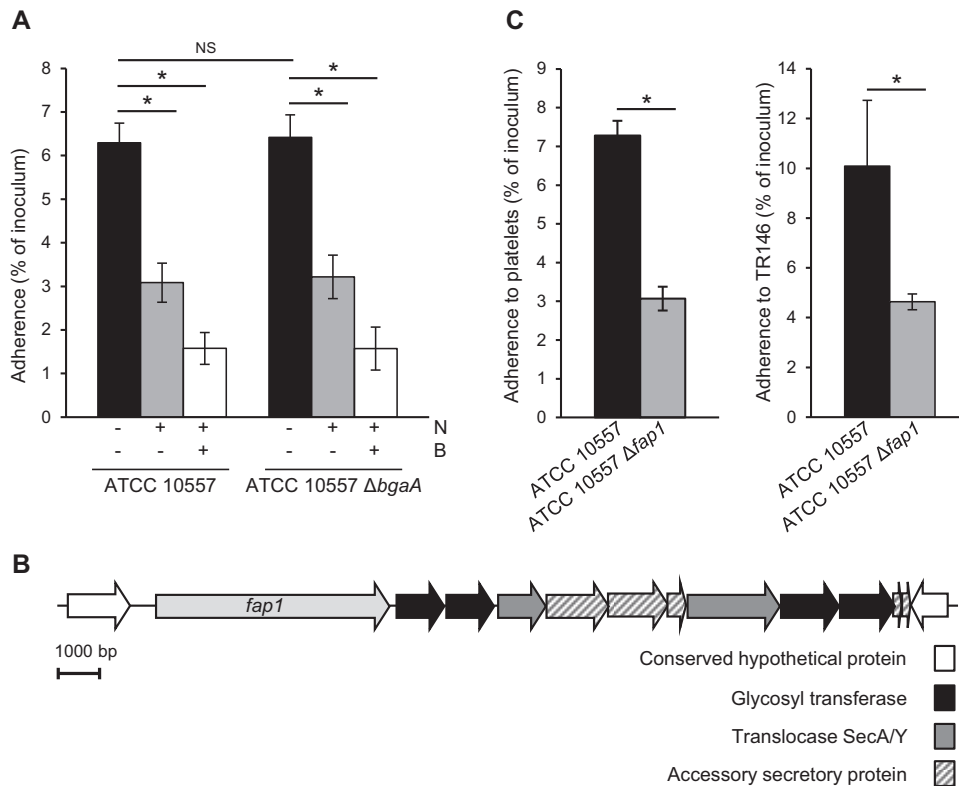**FIG 4** *S. oralis* serine-rich repeat protein Fap1, but not the β-galactosidase BgaA, is required for effective binding to platelets. (A) Mutation of *bgaA* does not significantly alter binding of ATCC 10557 to platelets. (B) Schematic representing the genomic arrangement of the ATCC 10557 *fap1* locus. Open reading frames predicted within the *fap1* locus are shown by arrows. (C) Fap1 contributes to *S. oralis* adherence. Adherence levels of ATCC 10557 and ATCC 10557 Δ*fap1* to platelets and the immortalized oral epithelial cell line TR146 are expressed as percentages of the inoculum. Adherence data are the means for at least three independent experiments, each performed in triplicate, ± SD. Statistical significance was tested by two-tailed Student's *t* test. *, $P \leq 0.03$.

*S. oralis* neuraminidase activity is predicted to be encoded by *nanA* and surface associated in an SrtA-dependent manner (28, 32). Our genome sequence of ATCC 10557 contains a gene predicted to encode a neuraminidase. To determine whether the gene encoding NanA is required to expose β-1,4-linked galactose, a mutant (ATCC 10557 Δ*nanA*) was generated and tested in adherence assays (Fig. 3). Adherence of the *nanA* mutant was not significantly different from that of the parent. We expected the mutant to exhibit increased adherence, as this strain cannot remove sialic acid receptors; however, one possible explanation for the observed data is that receptors exposed by cleavage of sialic acid compensate for the partial removal of this receptor. Addition of *Sp*BgaA146-990 did not reduce adherence of the *nanA* mutant, demonstrating that *nanA* is required for exposure of β-1,4-linked galactose. These data indicate that *S. oralis* can bind to terminal sialic acid and that following cleavage of that receptor by bacterial neuraminidase, *S. oralis* can bind to the underlying β-1,4-linked galactose. Thus, these data strongly suggest that *S. oralis* binds multiple carbohydrates on the platelet surface.

**An ortholog of the *S. gordonii* serine-rich repeat protein GspB contributes to *S. oralis* adherence.** ATCC 10557 contains a gene predicted to encode an ortholog of the pneumococcal adhesin BgaA (30). Carbohydrate binding modules (CBMs) within the C-terminal region of BgaA mediate adherence to β-1,4-linked galactose on host cells (30, 31). Despite conservation of these CBMs in *S. oralis* BgaA, a *bgaA* mutant was not significantly altered in binding to platelets (Fig. 4A). Furthermore, adherence of this mutant was reduced in the presence of glycosidases, similarly to that of the parental strain. These data demonstrate that there is no role for *bgaA* in adherence of ATCC 10557 to platelets in this model system. It should be noted that the recombinant

*Sp*BgaA146-990 used in this study to cleave terminal β-1,4-linked galactose includes only the enzymatic domain of the protein, not the CBMs (30).

Examination of the genome sequence revealed that ATCC 10557, like some *S. gordonii* and *S. sanguinis* strains, encodes a member of the SRRP family of adhesins. This is a family of glycosylated surface proteins expressed by many Gram-positive organisms. Many of these proteins are known to play roles in attachment to a variety of host and bacterial surfaces and in biofilm formation (15, 17, 18, 21, 33–50). The *S. oralis* SRRP is encoded within a typical locus for this protein family, which includes genes predicted to encode proteins for glycosylation and secretion of the SRRP (47) (Fig. 4B). Microscopy suggests that this protein family forms fimbria-like structures on the surfaces of bacteria (33, 34, 51, 52). The SRRP encoded by *S. oralis* has not previously been studied, but it was annotated in a previous genome sequence as fimbria-associated protein 1 (Fap1), based on very limited sequence similarity to the SRRP from *Streptococcus parasanguinis* (34, 38, 53). As a result, we use the same nomenclature here despite there being no direct evidence that this protein is associated with fimbriae in *S. oralis*. To determine if *S. oralis* Fap1 contributes to adherence to platelets, we generated a *fap1* mutant. A significant reduction in adherence of ATCC 10557 Δ*fap1* compared to that of the parent demonstrated a role for this protein in adherence (Fig. 4C). Although we have successfully complemented other *S. oralis* mutations, attempts to generate a stable *fap1* complement were unsuccessful. This is likely due to the highly repetitive nature of *fap1* and likely explains why studies of other SRRPs also lack complementation. However, we confirmed the phenotype with a second, independent *fap1* mutant (data not shown). Although the focus of the present study is *S. oralis* adherence to platelets, it is likely that mechanisms important in that interaction are also important in the oral cavity. This study demonstrated that a *fap1* mutant had reduced adherence to oral epithelial cells, but further studies would be required to define the role of Fap1 in the oral cavity (Fig. 4C).

**Evidence suggests that *S. oralis* Fap1 binds both sialic acid and β-1,4-linked galactose.** SRRPs contain two glycosylated serine-rich repeat (SRR) regions which comprise the majority of the protein, a cell wall anchor, and one or two nonrepeat regions within the N-terminal region (16, 52). The nonrepeat regions of SRRPs mediate adherence, and diversity in the modular structure of these regions accounts for the different receptors for these proteins, including sialic acid, keratins, and fibrinogen (16, 20, 23, 35, 40, 44, 45, 51, 54–56). BLAST searches identified no significant sequence similarity between the nonrepeat region of *S. oralis* SRRP and other available sequences. However, HHpred, a server for remote homolog detection using structural information, discovered 24% identity over 107 amino acids with GspB, the SRRP encoded by some *S. gordonii* strains (15, 57). The region of similarity is within the Siglec domain of GspB, which is required for sialic acid binding. These data suggested that Fap1 may also bind sialic acid. Supporting this hypothesis, neither neuraminidase nor free sialic acid significantly reduced adherence of the *fap1* mutant (Fig. 5A). During completion of this study, Bensing et al. identified a threonine-arginine (Thr-Arg) motif conserved in sialoglycan-binding SRRPs that is required for orienting the sialic acid residue (20, 56). This motif is required for efficient glycan binding, platelet binding, and full virulence in the rat model of infective endocarditis (20, 56). These residues are conserved in ATCC 10557 Fap1, and mutagenesis of the arginine residue significantly reduced adherence, further supporting the hypothesis that Fap1 acts as an adhesin binding sialic acid (Fig. 5B). However, adherence was reduced by the presence of *Sp*BgaA146-990, indicating that despite Fap1 being required for binding to β-1,4-linked galactose, the conserved arginine residue does not contribute to binding of this carbohydrate.

Thus far, there has been no evidence that SRRPs contribute to binding of multiple glycan receptors. However, as the nonrepeat region of Fap1 is larger than that of GspB, it is possible that this region contains additional receptor binding domains. As a result, the contribution of Fap1 to binding of β-1,4-linked galactose was tested. There was no significant reduction in adherence of the *fap1* mutant to neuraminidase-treated plate-
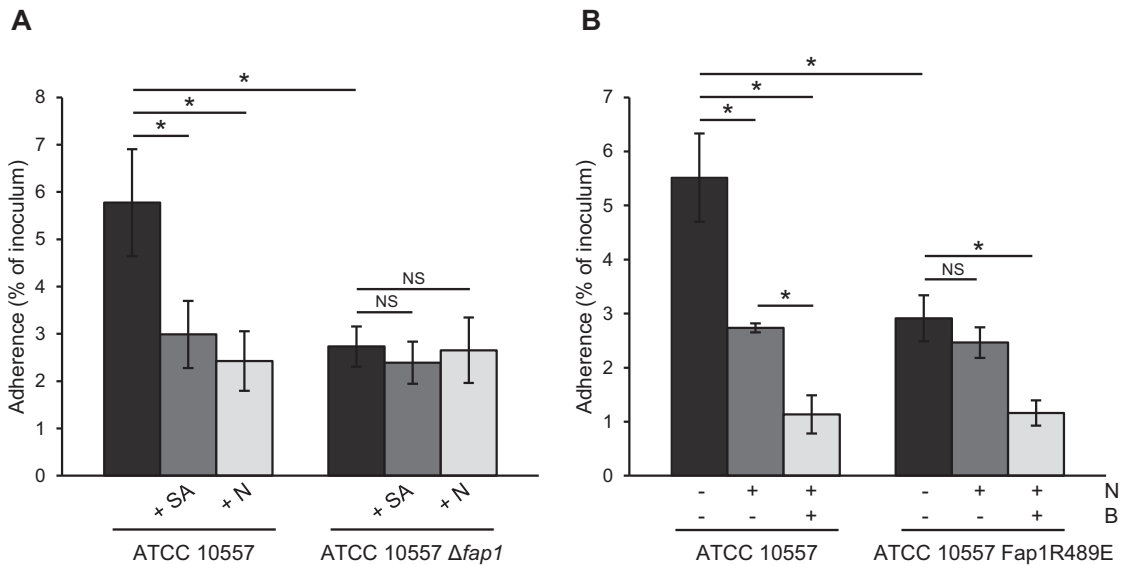
**A** **B**



**FIG 5** Fap1 is required for binding of ATCC 10557 to sialic acid on platelets, and this interaction requires a conserved residue within the Siglec domain. (A) ATCC 10557 Δ*fap1* did not show further reduced binding to platelets in the presence of *Clostridium perfringens* neuraminidase (N; 0.78 μM) or sialic acid (SA; 1 mM). (B) A conserved arginine residue within the nonrepeat region of Fap1 is required for binding of sialic acid but not β-1,4-linked galactose carbohydrates. The graph shows adherence of *S. oralis* ATCC 10557 and ATCC 10557 Fap1R489E to platelets in the presence of neuraminidase, *Sp*BgaA146-990 (B; 0.054 μM), both enzymes, or a PBS control. Adherence is expressed as a percentage of the inoculum. Values are the means for at least three independent experiments, each performed in triplicate, ± SD. Statistical significance was tested by two-tailed Student's *t* test. *, $P \leq 0.02$; NS, not significant.

lets in the presence of *Sp*BgaA146-990, lactose, or CBM71 (Fig. 6A). These data indicate that Fap1 is required for binding to both sialic acid and β-1,4-linked galactose. This is the first demonstration that an SRRP is required for binding of β-1,4-linked galactose.

During completion of this study, a paper was published characterizing the binding specificity of an SRRP from *S. mitis* strain SF100 (55). Comparison of the nonrepeat regions of SF100 and Fap1 revealed high levels of amino acid identity (95%). Structural predictions of the nonrepeat region of SF100 indicate the presence of Siglec and unique domains required for binding of sialic acid (55). The study confirmed that this region binds to sialic acid in the context of the sialyl T antigen (Neu5Ac–α-2,3Gal–β-1,3GalNAc). Interestingly, these sialic acid binding domains are flanked by domains that do not resemble any reported structures (Fig. 6B) (55). We identified the same organization in the Fap1 nonrepeat region. Use of the intensive mode of Phyre2 identified a low level of identity (11%) over 174 amino acids between the C-terminal domain of Fap1, encompassing the unique domain and a C-terminal domain of unknown function, and a region of an endoglucanase encoded by *Ruminiclostridium thermocellum* (58–60). This region of the endoglucanase contains a polycystic kidney disease (PKD) domain found in a wide variety of prokaryotic and eukaryotic proteins and a family 44 CBM with specificity for β-1,4-linked glucose polymers. Despite the high levels of sequence similarity between Fap1 and SF100, Phyre2 did not identify any match between the nonrepeat region of SF100 and the CBM of the endoglucanase (60). These data suggest that there may be an additional carbohydrate binding domain within the nonrepeat region of Fap1. Deletion of the predicted CBM resulted in a strain with an adherence indistinguishable from that of ATCC 10557 Δ*fap1*, which was not further reduced by neuraminidase or *Sp*BgaA146-990 (data not shown). These data indicate that the mutant protein was nonfunctional. Future studies will further address the hypothesis that the nonrepeat region of Fap1 binds a second carbohydrate.

## DISCUSSION

Many bacterial species bind to the host via surface glycans. Furthermore, some bacterial species, including many streptococci, express a range of glycosidases that can modify these host glycans (61). This suggests that some bacterial species bind to
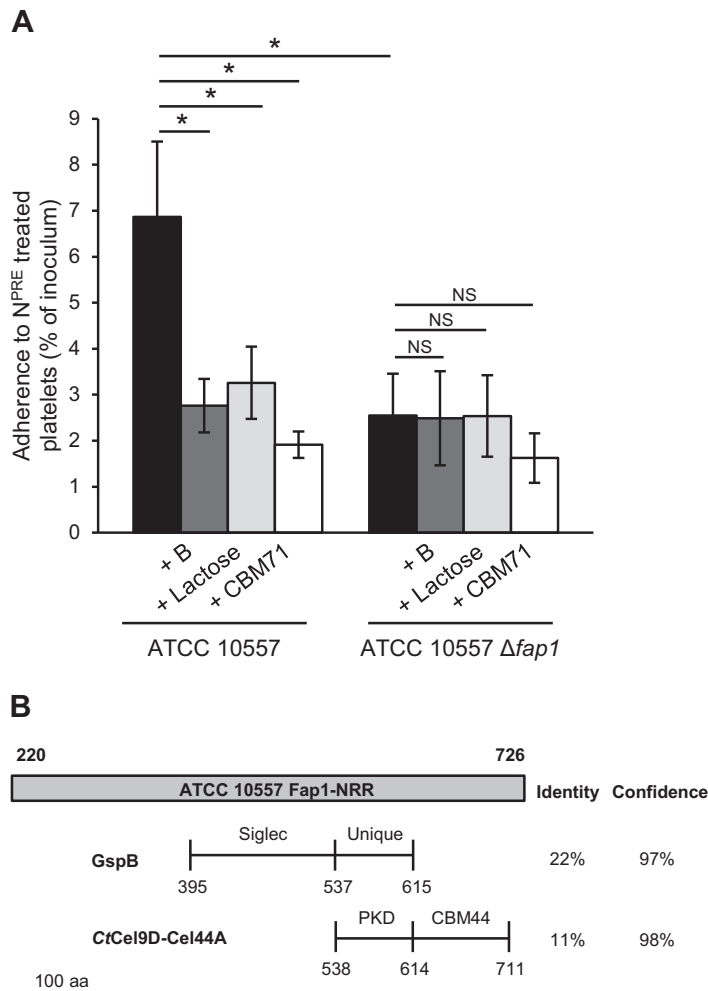
**FIG 6** Fap1 is required for binding of ATCC 10557 to β-1,4-linked galactose on platelets. (A) ATCC 10557 Δ*fap1* did not show further reduced binding to neuraminidase-pretreated (N^PRE; 0.78 μM) platelets in the presence of *Sp*BgaA146-990 (B; 0.054 μM), CBM71 (250 μM), or lactose (5 mM). Adherence is expressed as a percentage of the inoculum. Values are the means for three independent experiments, each performed in triplicate, ± SD. Statistical significance was tested by two-tailed Student's *t* test. *, $P \leq 0.03$; NS, not significant. (B) Schematic representing domains predicted by Phyre2 within the nonrepeat region (NRR) of Fap1. Numbers refer to the amino acid positions within the stated protein. The horizontal lines denote the domains identified within GspB (PDB entry 3QC5) and *Ct*Cel9D-Cel44A (PDB entry 2C26) as sharing structural similarity with the nonrepeat region of Fap1. The vertical lines indicate the boundaries of the different domains. The percent identity and confidence in the Phyre2 prediction are shown on the right.

receptors that the bacterium can also cleave. This was demonstrated for pneumococci, which can both bind to and cleave β-1,4-linked galactose (30). Here we demonstrate that following initial adherence of *S. oralis* to sialic acid, the neuraminidase expressed by this bacterium cleaves this carbohydrate receptor and reveals β-1,4-linked galactose, which acts as an additional receptor (Fig. 7). It is not known whether *S. oralis* can bind other carbohydrate structures revealed by the action of extracellular bacterial glycosidases. Not only can other carbohydrates be revealed by cleavage of sialic acid, but *S. oralis* also encodes additional glycosidases that can deglycosylate host glycans. These include BgaA, which can cleave β-1,4-linked galactose.

Both sialic acid and β-1,4-linked galactose are widely distributed within glycan structures on glycoproteins and glycolipids. The wide distribution of these carbohydrates suggests that this adherence mechanism mediates binding to these carbohydrates in a range of contexts and, as a result, is likely important in adherence to not only platelets and endothelial cells but also other host surfaces, including the oral cavity.
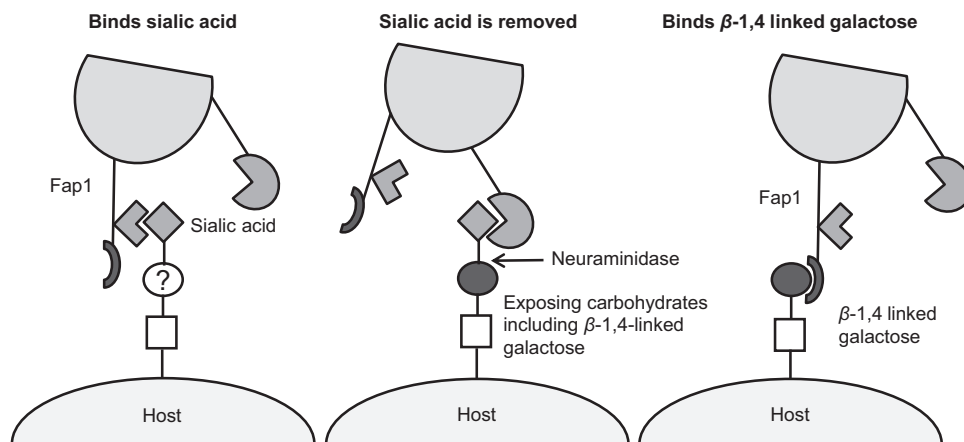
**FIG 7** Schematic of the proposed model of *S. oralis* adherence to sialic acid and β-1,4-linked galactose.

Previous studies have demonstrated a reduction in binding of other SRRP mutants to salivary proteins and *in vitro* models of oral surfaces (41, 62–64). The demonstration that a *fap1* mutant has reduced adherence for an oral epithelial cell line supports the hypothesis that this protein contributes to *S. oralis* binding in the oral cavity; however, further studies will be required to determine the precise contribution.

It is not known whether *S. oralis* binds to carbohydrates decorating a specific glycoprotein(s) on the platelet surface. Sialic acid on GPIbα is the major platelet receptor for SRRPs expressed by *S. sanguinis* and *S. gordonii* (15–17). There are other common glycoproteins on the platelet surface, and the basis for the specificity for GPIbα is currently unclear. It has been shown that *S. oralis* can adhere to and induce aggregation of platelets via interaction with GPIbα, but it is not known if the interaction of *S. oralis* with this protein requires an SRRP or glycans (25). Furthermore, there is still significant binding of platelets to *S. oralis* even in the presence of anti-GPIb.

The sialic acid- and β-1,4-linked galactose-containing glycan structures bound by *S. oralis* remain to be defined. The evidence provided here strongly supports the hypothesis that Fap1 directly binds sialic acid. Given the high level of conservation between the nonrepeat regions of Fap1 and the SF100 SRRP and the specificity of the SF100 nonrepeat region for binding of the sialyl T-antigen structure, it is possible that Fap1 also binds sialic acid in the same context. Interestingly, the most common glycan present on GPIbα is a biantennary O-linked glycan, one branch of which consists of the sialyl T antigen and the other of which contains sialic acid-capped β-1,4-linked galactose (65, 66). Whether or not *S. oralis* specifically binds GPIbα, this highlights that these carbohydrates can be present on the same glycan structure.

This is the first demonstration that an SRRP is required to bind β-1,4-linked galactose. Furthermore, this is the first report of a requirement for an SRRP to bind two distinct carbohydrate receptors, although it has been suggested that the pneumococcal SRRP PsrP binds both keratin, in a glycan-independent manner, and an as yet undefined glycan structure in saliva (63). While at first glance it may be surprising that BgaA does not mediate binding of *S. oralis* ATCC 10557 to β-1,4-linked galactose, it has been reported that BgaA does not contribute to adherence of all pneumococcal isolates (30, 31).

We hypothesize that distinct binding sites within Fap1 directly bind sialic acid and β-1,4-linked galactose (Fig. 7). While the evidence strongly suggests that Fap1 binds sialic acid, no direct evidence thus far supports the direct binding of β-1,4-linked galactose by Fap1. However, additional domains of unknown function, one of which shares some structural similarity with a CBM, are present within the nonrepeat region of Fap1. Unfortunately, deletion of this domain resulted in a nonfunctional Fap1 protein. Glycan arrays utilizing the nonrepeat region of SF100 detected no binding to the three β-1,4-linked galactose carbohydrates on the array (Galβ-1,4Glc, Galβ-

1,4GlcNAc6S, and Galβ-1,4GlcNAc) (55). This does not rule out Fap1 directly binding to β-1,4-linked galactose. It can be difficult to detect the weak binding of some carbohydrate binding domains, including CBMs, by using glycan arrays. It is the avidity of multiple interactions between CBMs and carbohydrates that allows these modules to mediate binding (30). Furthermore, at present, it is not known if SF100 binds β-1,4-linked galactose, and if so, if the SRRP is required for binding to this carbohydrate. Despite high levels of sequence similarity, no structural similarity with CBMs was identified for the SF100 SRRP. Mutation of the conserved arginine residue previously reported to be required for sialic acid binding does not affect binding to β-1,4-linked galactose. Thus, it is clear that the regions of Fap1 required for binding to the two carbohydrates are distinct. If Fap1 does not directly bind β-1,4-linked galactose, then the most likely explanation for a requirement of Fap1 in binding this carbohydrate is that Fap1 interacts with another protein which is the adhesin or forms an adhesin complex with Fap1 on the bacterial surface. Studies are under way to define the role of Fap1 in binding β-1,4-linked galactose.

At this time, it is unclear whether SRRPs of other related streptococci contribute to binding of β-1,4-linked galactose or other carbohydrates. However, it is highly likely that bacterial species that bind host carbohydrates and express glycosidases, or are present in an environment with these enzymes, have strategies to bind multiple carbohydrates. It has been reported that other viridans group streptococci, including *S. gordonii*, can bind carbohydrate structures revealed by removal of sialic acid, although the adhesins that bind these carbohydrates remain to be defined (67, 68). Adherence to these underlying carbohydrates is likely not relevant to the binding of *S. gordonii* to platelets during development of endocarditis, as platelets are highly sialylated and *S. gordonii* does not produce neuraminidase. However, these mechanisms may play an important role in the complex microbial environment of the oral cavity, where there are many neuraminidase-positive species.

In summary, this study increases our understanding of *S. oralis* adherence and SRRPs. The data presented here provide strong evidence that *S. oralis* binds multiple host carbohydrates and that this adherence occurs in a *fap1*-dependent manner. This is the first report to describe an SRRP required to bind two distinct receptors. Further studies are required to determine whether SRRPs of other related streptococci are required to bind multiple carbohydrates. However, mechanisms of adherence to multiple host carbohydrates are likely employed by other carbohydrate-binding bacterial species that are in an environment containing extracellular carbohydrate-modifying enzymes.

## MATERIALS AND METHODS

**Bacterial strains, culture media, and chemicals.** The *S. oralis* strains used in this study are described in Table 1. ATCC 10557 was confirmed as *S. oralis* by use of a previously published multilocus sequence typing scheme (69). *S. oralis* was grown on tryptic soy agar plates supplemented with 5% sheep blood. Plates were incubated overnight at 37°C in 5% $CO_2$. Broth cultures were grown statically at 37°C in Todd-Hewitt broth (Becton, Dickinson and Co., Sparks, MD) supplemented with 0.2% (wt/vol) yeast extract (Becton, Dickinson and Co., Sparks, MD) (THY). As appropriate, broth and agar plates were supplemented with 1 $\mu$g ml$^{-1}$ erythromycin, 200 $\mu$g ml$^{-1}$ spectinomycin, 500 $\mu$g ml$^{-1}$ kanamycin, or 200 $\mu$g ml$^{-1}$ streptomycin (Fisher Scientific, Fair Lawn, NJ). C medium with 5% yeast extract (pH 8; C+Y) was used for *S. oralis* transformations (70).

*Escherichia coli* strains were grown at 37°C and 200 rpm in Luria-Bertani (LB) broth or statically on LB agar plates. As appropriate, the medium was supplemented with 200 $\mu$g ml$^{-1}$ erythromycin, 100 $\mu$g ml$^{-1}$ ampicillin, 50 $\mu$g ml$^{-1}$ kanamycin, or 50 $\mu$g ml$^{-1}$ spectinomycin (Fisher Scientific, Fair Lawn, NJ).

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

**Generation of *S. oralis* mutants.** Insertion-deletion mutants of ATCC 10557 were generated using allelic exchange methods. All genomic DNAs were prepared as previously described, with the addition of 100 U ml$^{-1}$ mutanolysin to the resuspension buffer (71). Fragments of approximately 500 bp from the 5' and 3' regions of the target gene were amplified from *S. oralis* genomic DNA by use of appropriate primer pairs (1/2 and 3/4, respectively) (Table 2). The spectinomycin resistance cassette, amplified by using primers S.F and S.R, and flanking fragments were cloned into EcoRI-digested pDrive (Qiagen, Germantown, MD) by use of an In-Fusion EcoDry HD cloning kit (Clontech, Mountain View, CA) per the manufacturer's protocol and then transformed into *E. coli* Stellar (Clontech). Plasmid constructs were confirmed by PCR with the M13F and T7 promoter primers followed by sequencing. Due to recombi-

**TABLE 1** Strains and plasmids used in the study

| Strain or plasmid | Genotype, phenotype, and/or characteristic[a] | Source or reference |
|---|---|---|
| **Strains** | | |
|   *Streptococcus oralis* strains | | |
|     ATCC 10557 | Endocarditis isolate | 78 |
|     ATCC 10557 Δ*bgaA* | Δ*bgaA*::*aad9* Spc$^r$ | This study |
|     ATCC 10557 Δ*nanA* | Δ*nanA*::*erm* Erm$^r$ | This study |
|     ATCC 10557 Δ*fap1* | Δ*fap1*::*aad9* Spc$^r$ | This study |
|     ATCC 10557 Sm$^r$ | Lys → Thr mutation in RpsL [*rpsL(K56T)*] conferring Sm$^r$ | This study |
|     ATCC 10557 Fap1 Janus | Δ*fap1*::*kan/rpsL$^+$* *rpsL(K56T)* Kan$^r$ Sm$^s$ | This study |
|     ATCC 10557 Fap1R489E | Arg → Glu mutation in Fap1 [*fap1(R489E)*]; *rpsL(K56T)* Sm$^r$ | This study |
|     ATCC 10557 Fap1Δ622-708 Janus | Δ*fap1*::*kan/rpsL$^+$* *rpsL(K56T)* Kan$^r$ Sm$^s$ | This study |
|     ATCC 10557 Fap1Δ622-708 | Deletion of nucleotides encoding amino acid residues 622 to 708 in Fap1; *rpsL(K56T)* Sm$^r$ | This study |
|     9A2 | Human oral cavity isolate | 69 |
|   *Escherichia coli* strain | | |
|     Stellar | Cloning host | Clontech |
| **Plasmids** | | |
|   pEASY-T1*ermB* | pEASY-T1 containing *ermB* cassette; Amp$^r$ Kan$^r$ | This study |
|   pDrive | Cloning vector; Amp$^r$ Km$^r$ | Qiagen |
|   pDriveΔ*bgaA* | pDriveΔ*bgaA*::*aad9* Spc$^r$ Amp$^r$ Kan$^r$ | This study |
|   pDriveΔ*nanA* | pDriveΔ*nanA*::*erm* Erm$^r$ Amp$^r$ Kan$^r$ | This study |
|   pDriveΔ*fap1* | pDriveΔ*fap1*::*aad9* Spc$^r$ Amp$^r$ Kan$^r$ | This study |
|   pGEX-5X-3 | Expression vector; Amp$^r$ | GE Biosciences |
|   pGEX-5X-3 Fap1NRR | pGEX-5X-3 encoding Fap1 amino acids 220 to 726; Amp$^r$ | This study |
|   pGEX-5X-3 Fap1NRR Janus | pGEX-5X-3-Fap1NRR with Janus cassette; Amp$^r$ Kan$^r$ | This study |
|   pGEX-5X-3 Fap1NRRR489E | pGEX-5X-3-Fap1NRR with Arg → Glu mutation in Fap1 [*fap1(R489E)*]; Amp$^r$ | This study |
|   pJET1.2/Blunt | Cloning vector; Amp$^r$ | Thermo Fisher Scientific |
|   pJET Fap1C | pJET1.2/Blunt containing Fap1 sequence corresponding to amino acids 500 to 860 | This study |
|   pJET Fap1C Janus | pJET Fap1C with Janus cassette; Amp$^r$ Kan$^r$ | This study |
|   pJET Fap1Δ622-708 | pJET Fap1C with in-frame deletion of *fap1* region encoding amino acids 622 to 708 | This study |

[a]Sm$^r$, streptomycin resistant; Sm$^s$, streptomycin sensitive; Kan$^r$, kanamycin resistant; Amp$^r$, ampicillin resistant; Spc$^r$, spectinomycin resistant; Erm$^r$, erythromycin resistant.

nation of the spectinomycin resistance cassette at an unexpected site within the *nanA* locus, the spectinomycin resistance cassette was exchanged for an erythromycin resistance cassette. The *ermB* cassette was amplified from pEASY-T1*ermB* (primers E.F and E.R) and was ligated into an inverse PCR product of the original construct amplified using primers 2 and 3.

*S. oralis* cells were grown to an optical density at 600 nm (OD$_{600}$) of 0.12 ± 0.02 diluted in fresh medium (1:20), and competence was induced by the addition of competence-stimulating peptide (2 µg ml$^{-1}$) and CaCl$_2$ (1 mM) (72). Approximately 100 ng of plasmid was added, and the culture was incubated at 37°C for 2 h. Transformants were selected on TS agar plates supplemented with the appropriate antibiotic. The resulting colonies were screened for the targeted deletion by PCR using primers 5 and 6 and were shown not to have any generalized growth defect during growth on rich medium. Mutagenesis of *bgaA* and *nanA* was also confirmed by enzymatic activity assays as previously reported (73, 74). In cases where phenotypes were observed, flanking regions were sequenced to ensure that no spurious changes were introduced.

ATCC 10557 Fap1R489E and ATCC 10557 Fap1Δ622-708 were generated using the two-step Janus cassette system (75). For ATCC 10557 Fap1R489E, a gene fragment encoding the Fap1 nonrepeat region (amino acid residues 220 to 726; primers NRR.F and NRR.R) was cloned into pGEX-5X-3 (GE Healthcare, Chicago, IL) by use of In-Fusion to generate pGEX-5X-3 Fap1NRR. For ATCC 10557 Fap1Δ622-708, a gene fragment encoding amino acid residues 500 to 860 (primers NRRI.R and Δ622-708.R) was cloned into the pJET1.2/Blunt PCR cloning vector (Thermo Fisher Scientific, Waltham, MA) to create pJETFap1C. The constructs for generation of the Janus intermediates were made by blunt-end ligation of the Janus cassette (primers J.F and J.R) into inverse PCR products from pGEX-5X-3 Fap1NRR (primers NRRI.F and NRRI.R) and pJETFap1C (primers Δ622-708I.F and Δ622-708I.R). Following confirmation of the constructs (pGEX-5X-3 Fap1NRRJanus and pJET Fap1C Janus), these plasmids were transformed into ATCC 10557 Sm$^r$. Transformants were selected on kanamycin and confirmed by PCR with primers flanking the construct (R489E.5/R489E.6 and Δ622-708.5/F.6). The second round of transformation replaced the Janus cassette with a fragment of DNA containing the desired mutation. For generation of ATCC 10557 Fap1R489E, this was plasmid pGEX-5X-3 Fap1NRRR489E, in which the codon for Glu 489 was changed to a codon for Arg (primers R489E.F and R489E.R) by use of a QuikChange II site-directed mutagenesis kit (Stratagene, La Jolla, CA). In the case of ATCC 10557 Fap1Δ622-708, plasmid pJETFap1-Δ622-708 was generated by self-ligation of an inverse PCR product from pJETFap1C (primers Δ622-708I.F and Δ622-708I.R), resulting in an in-frame deletion in the predicted CBM (amino acids 622 to 708). Final mutants were selected on streptomycin and confirmed by sequencing.

**TABLE 2** Primers used in this study

| Target or group | Name | Sequence (5′-3′) | Location (accession no.) |
|---|---|---|---|
| *nanA* | N.1 | TCGGATCCAGAATTCAGTCGGTGCAGCATCGGTAG[a] | 506101–506121 (FR720602) |
|  | N.2 | CACGAACGAAAATCGGTTTGAACTCCATGTGAACTG[b] | 506596–506575 (FR720602) |
|  | N.3 | ATAAACCCTTGCATACACCGGTCCATTATCAACAG[b] | 508977–508998 (FR720602) |
|  | N.4 | CTTGTCGACGAATTCCTTTTCTCTTCTTGCCAAATGC[a] | 509436–509415 (FR720602) |
|  | N.5 | GGTGAGGTCAAGATGATTACG | 505512–505532 (FR720602) |
|  | N.6 | ATACCGACTGCTCCACCTTG | 509829–509810 (FR720602) |
| *bgaA* | B.1 | TCGGATCCAGAATTCCAGATGTTCGAGGTCAATAC[a] | 664999–665019 (FR720602) |
|  | B.2 | CACGAACGAAAATCGCCAATGGCCTTTTTCCAT[b] | 665731–665714 (FR720602) |
|  | B.3 | ATAAACCCTTGCATAGAAGTGCAGAAGGTCAAGCAG[b] | 672597–672617 (FR720602) |
|  | B.4 | CTTGTCGACGAATTCTTATACTCTGTCGCATGTAC[a] | 673259–673240 (FR720602) |
|  | B.5 | TCTTGCCAAATGACCAACTC | 664767–664786 (FR720602) |
|  | B.6 | GTTGTTGTATCACCTTCATG | 673397–673378 (FR720602) |
| *fap1* | F.1 | TCGGATCCAGAATTCAGAAACAGACCGTGTAACTC[a] | 1541056–1541037 (FR720602) |
|  | F.2 | CACGAACGAAAATCGGAGCTGACTCACTTGCTTGG[b] | 1540428–1540447 (FR720602) |
|  | F.3 | ATAAACCCTTGCATACAGCTGTGACAGGTATCGGTC[b] | 1535264–1535244 (FR720602) |
|  | F.4 | CTTGTCGACGAATTCGGTACAAGGCTAGGTCTTCGC[a] | 1534668–1534688 (FR720602) |
|  | F.5 | AAGTTACCTCGGCTACCGGC | 1541474–1541455 (FR720602) |
|  | F.6 | ATCTAAGTTTGATCTAACGATGA | 1534633–1534655 (FR720602) |
| Fap1R489E | NRR.F | TCGAAGGTCGTGGGATCCGCTCGGGAGACAGTGAAAGAATC[c] | 1540431–1540409 (FR720602) |
|  | NRR.R | CACGATGCGGCCGCTCGAGATTACTTACTGAAATTGACG[c] | 1538911–1538930 (FR720602) |
|  | NRRI.F | AACCCGTGATTGAAACGGTG | 1539663–1539682 (FR720602) |
|  | NRRI.R | TTAGCGGAAATACTACTGACG | 1539590–1539570 (FR720602) |
|  | R489E.F | CTTCCTGATCATAGGTGAAAACATA**CTC**GGTCCACAAACTATTTACTGCTTGA[d] | 1539597–1539649 (FR720602) |
|  | R489E.R | TCAAGCAGTAAATAGTTTGTGGACC**GAG**TATGTTTTCACCTATGATCAGGAAG[d] | 1539649–1539597 (FR720602) |
|  | R489E.5 | ACTGCTAGTTCAAGTGACTC | 1540536–1540517 (FR720602) |
|  | R489E.6 | ACTAGCACTTGCTGAAGCAGAC | 1538884–1538905 (FR720602) |
| Fap1Δ622-708 | Δ622-708.R | TGACTGAAGCGGATTGGCTCG | 4777–4797 (KX792093) |
|  | Δ622-708I.F | GCGCACAGTAAAGCTCACGTC | 4058–4079 (KX792093) |
|  | Δ622-708I.R | GATGTGTTTAATGGACAAATTAC | 4341–4363 (KX792093) |
|  | Δ622-708.5 | TCTAGCGAAGAATGAGATAG | 1540162–1540143 (FR720602) |
| *aad9* | S.F | CGATTTTCGTTCGTGAATAC | 5418–5399 (KM009065) |
|  | S.R | TATGCAAGGGTTTATTGTTTTC | 4265–4286 (KM009065) |
| *erm* | E.F | CTCGAGCGGCCGCCAGTG[e] | 264–282 (EU233623) |
|  | E.R | AACGGCCGCCAGTGTGCTG[e] | 319–336 (EU233623) |
| Janus | J.F | CCGTTTGATTTTTAATGGATAATG | 7–30 (AY334019) |
|  | J.R | GGGCCCCTTTCCTTATGCTT | 247511–247527 (AE005672) |
| pDrive | T7 promoter | GTAATACGACTCACTATAG | 239–258 (DQ996013) |
|  | M13F | GTAAAACGACGGCCAGT | 431–447 (DQ996013) |

[a]Underlining indicates nucleotides introduced to allow In-Fusion cloning into the pDrive vector (Qiagen).
[b]Underlining indicates nucleotides introduced to allow In-Fusion cloning with *aad9* (spectinomycin cassette).
[c]Underlining indicates nucleotides introduced to allow In-Fusion cloning into the pGEX-5X-3 vector (GE Biosciences).
[d]Bold letters indicate nucleotide changes to alter Arg 489 to Glu in Fap1.
[e]These primers were used to amplify the *erm* cassette from pEASY-T1*ermB*.

**Adherence assays.** Assays to determine adherence to human platelets were adapted from the work of Sullam et al. (76). In brief, blood from healthy donors was collected in 1:5 (vol/vol) acid citrate dextrose solution (56 mM trisodium citrate dehydrate, 65 mM citric acid, 100 mM dextrose). As the blood was drawn from healthy donors and no identifiers were collected, the Institutional Review Board at Nationwide Children's Hospital deemed this study to not involve human subjects. Pooled platelet-rich plasma (PRP) was collected by centrifugation of blood samples at 200 × *g* for 10 min at room temperature and diluted 1:4 (vol/vol) in platelet wash buffer (9.3 mM trisodium citrate dehydrate, 5.3 mM citric acid, 17.3 mM dextrose, 145.5 mM NaCl, pH 6.5). Platelets were collected by centrifugation at 750 × *g* for 10 min and washed twice in platelet wash buffer. Platelets were suspended in platelet wash buffer at 1 × 10⁷ platelets ml⁻¹ and fixed with 1% paraformaldehyde for 10 min at room temperature. Following three washes in phosphate-buffered saline (PBS), platelets were resuspended in PBS. To test bacterial adherence to platelets, each well of a flat-bottomed 96-well microtiter plate was coated with $1 \times 10^6$ fixed platelets for 60 min at 37°C, and unbound platelets were removed by three PBS washes. Control wells were coated with 1% (wt/vol) bovine serum albumin (BSA) in PBS. All wells were blocked with 1% BSA in PBS for 60 min at 37°C prior to adherence assays.

Where appropriate, wells were pretreated for 30 min with 50 μl of PBS containing 0.78 μM purified *Clostridium perfringens* sialidase, a 0.054 μM concentration of the recombinantly expressed *Sp*BgaA146-990, or both. Control wells were incubated with PBS alone, and all wells were washed three times with PBS prior to addition of bacteria. Where stated, glycosidases, sialic acid, lactose, or CBM71 was mixed with bacterial cells at the specified concentration, and adherence was assessed in the same manner. *Sp*BgaA146-990 and CBM71 were recombinantly expressed and purified as previously described (30).

Bacteria were grown to an $OD_{600}$ of 0.6 ± 0.05, and approximately 2 × $10^5$ bacteria in PBS were allowed to adhere at 37°C for 60 min. Nonadherent bacteria were removed by three washes in PBS, and adherent bacteria were lifted with 0.25% trypsin-1 mM EDTA and enumerated by serial dilution. All adherence assays were performed in triplicate on at least three independent occasions. Bacterial adherence was calculated as the percentage of the inoculum adherent to the platelets. Data are presented as means ± standard deviations (SD). The preparation of platelets from pooled blood from different donors and multiple platelet preparations resulted in some differences in adherence of wild-type bacteria between sets of experiments; however, the trends were maintained. Statistical significance was determined using two-tailed Student's *t* test, and data points with *P* values of ≤0.05 were considered significant.

Human umbilical vein endothelial cells (HUVECs) were grown in 24-well plates in EBM-2 medium supplemented with EGM-2 SingleQuats (Lonza, Walkersville, MD). Adherence to HUVECs was assessed in the same way as that described above, except that pretreatment with enzymes was conducted in 200-$\mu$l aliquots of antibiotic-free medium and 5 × $10^5$ bacteria were added per well.

The buccal keratinizing squamous cell line TR146 was grown in 24-well plates in F-12 medium with 10% fetal bovine serum (vol/vol), 2 mM glutamic acid, and 1% penicillin-streptomycin solution (77). Adherence to TR146 cells was assessed as described for other cells, except that 1 × $10^6$ bacteria were added per well.

**Accession number(s).** As there were small sequence differences between our strain and the published genome sequences, the nucleotide sequences of the *nanA*, *bgaA*, and *fap1* regions were submitted to GenBank under accession numbers KX792091, KX792092, and KX792093, respectively.

## ACKNOWLEDGMENTS

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