Interactions of Streptococcus mutans Fimbria-Associated Surface Proteins with Salivary Components

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Streptococcus mutans has been implicated as the major causative agent of human dental caries. S. mutans binds to saliva-coated tooth surfaces, and previous studies suggested that fimbriae may play a role in the initial bacterial adherence to salivary components. The objectives of this study were to establish the ability of an S. mutans fimbria preparation to bind to salivary components and to determine the specific salivary components that facilitate binding with fimbriae. Enzyme-linked immunosorbent assay (ELISA) established that the S. mutans fimbria preparation bound to components of whole saliva. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and Western blot techniques were used to separate components of whole saliva and determine fimbria binding. SDS-PAGE separated 15 major protein bands from saliva samples, and Western blot analysis indicated significant binding of the S. mutans fimbria preparation to a 52-kDa salivary protein. The major fimbria-binding salivary protein was isolated by preparative electrophoresis. The ability of the S. mutans fimbria preparation to bind to the purified salivary protein was confirmed by Western blot analysis and ELISA. Incubation of the purified salivary protein with the S. mutans fimbria preparation significantly neutralized binding of the salivary protein-fimbria complex to saliva-coated surfaces. The salivary protein, whole saliva, and commercial amylase reacted similarly with antiamylase antibody in immunoblots. A purified 65-kDa fimbrial protein was demonstrated to bind to both saliva and amylase. These data indicated that the S. mutans fimbria preparation and a purified fimbrial protein bound to whole-saliva-coated surfaces and that amylase is the major salivary component involved in the binding.

The mechanism of Streptococcus mutans attachment to saliva-coated tooth surfaces has generated considerable interest, because blocking of attachment may lead to the prevention of dental caries. However, other than studies of salivary proline-rich polypeptides (PRP) (11, 12), little attention has been devoted to the specific salivary components responsible for the initial S. mutans adherence to saliva-coated tooth surfaces. S. mutans antigen I/II has been strongly implicated in the initial adherence to saliva-coated surfaces (13, 21). It is also well established that the later secondary attachment of S. mutans to tooth surfaces occurs with production of water-insoluble glucans by cell-associated glucosyltransferases (GTF) (21). Previously, members of our group characterized fimbrial surface components on S. mutans cells (7). Recently, Viscount et al. demonstrated Streptococcus parasanguinis fimA fimbrial gene homologs in S. mutans by hybridization (32). Because bacterial fimbriae play a significant role in the colonization of many pathogens, the function of S. mutans fimbriae may be to provide an additional mechanism for initial attachment to tooth surfaces. S. mutans strains from caries-active patients have significantly more fimbrial material on their surfaces than strains from caries-free subjects or a laboratory strain (27). In addition, our laboratory has generated data that indirectly suggest that S. mutans strains containing the most fimbriae may also induce the highest numbers of carious lesions (reference 27 and data not published).

Fimbriae have a particular tropism for certain tissues and, more specifically, carbohydrate moieties of glycoproteins associated with that tissue (2, 4, 19, 21). Many gram-negative bacterial fimbriae, including those from the oral microflora, have been well characterized (15). The interactions between Porphyromonas gingivalis recombinant fimbriae and individual salivary components have been examined (1). The greatest binding occurred with acidic PRP. It was also determined that statherin enhanced the binding of P. gingivalis fimbriae to hydroxyapatite (HA) beads. Understanding of the biology of gram-positive fimbriae is not as complete, and relatively little is known regarding gram-positive oral bacterial fimbriae. However, fimbriae from S. parasanguinis (3, 5, 6, 25), Streptococcus sanguinis (23), Streptococcus salivarius (33), and Actinomyces naeslundii (2) have been characterized. Studies conducted on S. parasanguinis FW213 (a member of the group of microorganisms formerly classified as S. sanguinis [S. sanguinis]), which is one of the primary colonizers of dental plaque, have been extensive and demonstrated that attachment to saliva-coated HA is mediated by a 36-kDa adhesive protein, FimA. FimA is found on the fimbrial tips and is able to displace bound FW213 cells (5, 25). S. parasanguinis fimbriae are essential for the microorganism to attach, since wild-type fimbriated FW213 mutants do not (6). Incubation of FimA with HA blocked the binding of 85% of whole cells added to saliva-coated HA (5, 6). The gene that encodes FimA has been cloned (25). Insertional and deletional FimA mutants produce fimbriae, suggesting that FimA is not the structural subunit. In addition, FimA mutants caused significantly less disease in an animal endocarditis model than did bacteria containing the fimbrial protein (3).

Binding of oral streptococci to specific salivary components such as amylase has been described. Several reports described the complex interactions between Streptococcus gordonii whole cells and human salivary amylase (28–30). In this regard, S.
gordonii binding to amylase-coated HA was improved in the presence of maltotriose; however, S. sanguinis adhesion to amylase-coated HA was not enhanced by the presence of maltotriose (28). S. mutans cells have not been shown to bind to amylase.

It is clear that the fimbriae of certain oral bacteria have specific interactions with glycoproteins in the salivary pellicle that coats the tooth surface (26). The purpose of this study was to characterize the interactions between saliva and a preparation of S. mutans fimbiae. In Western blot analysis, a 52-kDa salivary component cross-reacted significantly with the S. mutans fimbia preparation. We chose to isolate and identify the salivary protein and determine the characteristics of binding to the S. mutans fimbia preparation.

MATERIALS AND METHODS

Bacteria. An S. mutans isolate from the saliva of a 7-year-old caries-active child (defined as having ≥5 unrestored surfaces) designated strain A32-2 was used in all experiments; it was maintained in 5% CO₂ and 95% air at 37°C. This strain had previously been described to be heavily fimbrinated (designated CS2 in reference 27).

Fimbrial preparation. A modification (7, 27) of the technique of Morris and colleagues (23) for isolating fimbiae from S. sanguinis whole cells was used for the removal of S. mutans fimbiae. The procedure utilized alternating high- and low-speed centrifugations. S. mutans was grown in 9 liters of Todd-Hewitt broth for 18 h at 37°C in 5% CO₂ and 95% air. Cells were pelleted and washed once using a method described elsewhere (2) and was incubated for 1 h at 25°C. Following a wash step, 100 μl of the A32-2 fimbral preparation (33.0 μg/ml) of saliva and purified 65-kDa fimbrial protein (18 μg/ml) were incubated with S. mutans (no-fimbria control) were added, and the mixture was incubated for 3 h at 37°C and washed three times. Rat antibody to the A32-2 fimbral preparation or rat antibody to the 65-kDa fimbrial protein (both diluted 1:4,000 in Tween-saline) was added (100 μl) and the mixture was incubated for 3 h at 37°C. After a wash step, the substrate (10 μg of orthophenylendiamine dihydrochloride and 14 μl of 30% H₂O₂ in 20 ml of 0.5% citrate buffer [pH 5.0]) was added (100 μl), color development was monitored for 30 min, and the reaction was read at 490 nm with a microplate spectrophotometer (Molecular Devices Corp., Menlo Park, Calif.). In addition, a modification of the ELISA described above was used to determine the efficacy of the purified 52-kDa salivary protein in inhibiting the binding of S. mutans A32-2 fimbiae to a 1:10 dilution of whole saliva. Mixtures of the S. mutans fimbia preparation (33.0 μg/ml) and serially diluted 52-kDa salivary protein (0.5 to 65.0 μg/ml) were incubated for 30 min, and the mixture was used in the untreated fimbra preparation. Controls included whole saliva and BSA.

Immunoblot analysis of binding of fimbiae to salivary components and amylase detection. In order to determine which components in whole saliva bound S. mutans fimbiae, reducing SDS-PAGE was used to separate salivary proteins and immunoblotting was performed to determine if an S. mutans fimbriae to a 1:10 dilution of whole saliva. Mixtures of the S. mutans fimbia preparation (33.0 μg/ml) and serially diluted 52-kDa salivary protein (0.5 to 65.0 μg/ml) were incubated for 30 min, and the mixture was used in the untreated fimbra preparation. Controls included whole saliva and BSA.

RESULTS

Fimbria binding assays. ELISA and immunoblotting were used to establish that the S. mutans fimbia preparation bound to saliva-coated surfaces. An ELISA was performed to determine if an S. mutans A32-2 fimbral preparation bound to human whole saliva. Fimbiae from S. mutans A32-2, a strain isolated from a caries-active subject, demonstrated significant binding compared with the corresponding Tween-saline control (i.e., with no fimbiae) (Fig. 1). The binding of fimbral components to saliva was reduced when either the saliva or fimbiae were diluted. These data provided the first indication that S. mutans fimbiae had binding activity with saliva-coated surfaces. BSA-coated wells did not bind fimbiae (data not shown).

Immunoblot analysis of human whole saliva probed with S. mutans A32-2 fimbiae. The binding of the S. mutans A32-2 fimbral preparation to separated salivary proteins was analyzed by immunoblotting. Human whole-saliva samples were collected from seven healthy subjects. Each saliva sample was electrophoresed, transferred to nitrocellulose paper, and incubated with 100 μl of the S. mutans fimbral preparation. Following an overnight incubation, the nitrocellulose paper was washed with WBT three times for 10 min each, 2 ml of S. mutans fimbrial preparation (33 μg/ml) in WBT was added, and the paper was incubated for 1 h at room temperature. Goat antibody to IgG (Fc specific)-alkaline phosphatase conjugate (1:1000 in WBT; 100 μl) was added and the membrane was incubated for 1 h. Binding of the antibody was detected by addition of alkaline phosphatase substrate (p-nitroblue tetrazolium chloride and 5-bromo-4-chloro-3-indolylphosphate; Bio-Rad) dissolved in 100 mM Tris HCl (pH 9.5). In order to determine whether the 52-kDa salivary protein was amylase, the isolated salivary protein (65.0 μg/ml), commercial purified amylase (10.0 μg/ml), and undiluted whole saliva were electrophoresed by SDS-PAGE, transferred to nitrocellulose, and probed with rabbit anti-human α-amylase (Sigma) followed by alkaline phosphatase-labeled goat anti-rabbit IgG (Sigma) and a substrate, similar to the method described above.

Statistical analysis. The data were reduced by computing the means and standard errors of the means (SEM) of the absorbances of each sample, determined in triplicate. The data were analyzed by Student’s t test, and differences were defined as significant when P was ≤0.05.

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probed with the *S. mutans* fimbria preparation. Fimbriae from the A32-2 strain bound strongly to a 52-kDa salivary protein in all seven saliva samples (Fig. 2). Controls with no fimbriae did not reveal any bands.

Isolation of a 52-kDa salivary protein with *S. mutans* fimbria-binding activity. In order to better understand the interaction between the 52-kDa salivary protein and *S. mutans* fimbriae, the salivary protein was isolated by preparative gel electrophoresis. Following elution, the fractions were analyzed by gel electrophoresis, and fractions that contained only one band were identified (Fig. 3).

ELISA for binding of *S. mutans* fimbriae and purified 65-kDa fimbrial protein to isolated salivary protein, amylase, and whole saliva. In order to ascertain that both the salivary protein and amylase have fimbria-binding characteristics, an ELISA was employed to measure binding. Amylase was chosen because its molecular mass is near 52 kDa and because several oral streptococci have demonstrated the ability to bind to amylase (26–28). In this assay, amylase (10.0 μg/ml) had significantly greater fimbria-binding activity than the no-fimbria Tween-saline control (Fig. 4). Amylase also had an absorbance significantly greater than that of diluted whole saliva (0.5 μg/ml). The isolated salivary protein (65.0 μg/ml) had a lower absorbance than either amylase or whole saliva, but the absorbance was significantly higher than that of the no-fimbria control. Purified 65-kDa fimbrial protein bound similarly to amylase (optical density at 490 nm [OD], 0.250 ± 0.026 [mean ± SEM]) as to a 1:2 dilution of saliva (OD, 0.260 ± 0.030) but not to a Tween-saline negative control (OD, 0.070 ± 0.012).
Inhibition of binding of _S. mutans_ fimbriae to whole-saliva-coated surfaces. In binding assays, an important feature is the ability to inhibit the interaction. The ability to inhibit binding suggests that the interaction is specific. In this system, the purified salivary protein was incubated with the fimbria preparation from _S. mutans_ A32-2. Following incubation with the salivary protein, the mixture was added to whole saliva. The data indicated an inverse relationship between the concentration of the salivary protein and the extent of binding of the _S. mutans_ fimbria preparation to whole saliva (Fig. 5). Whole saliva and BSA controls yielded complete and no inhibition, respectively.

Immunoblot analysis of the purified salivary protein probed with anti-human α-amylase antibody. The purified salivary protein, human amylase, and whole saliva were assayed for reactivity with rabbit antibody to human α-amylase. The results indicated that all three salivary preparations contained components that were recognized by the antiamylase antibody (Fig. 6).

**Discussion**

It is generally accepted that pathogenic bacteria must first attach to a host surface to cause infection. The structures that provide attachment are referred to as adhesins. It is of great importance to characterize not only the bacterial adhesin but also the host ligand. Understanding the mechanism of attachment may aid in prevention of the disease. Several investigators have examined salivary components as potential receptors for _S. mutans_ and other oral bacteria (4, 11, 13, 14, 17, 18, 24). Gibbons et al. (10–12) documented that PRP attach with great affinity to HA and _S. mutans_ whole cells attach to PRP-coated HA beads. The majority of research has focused on the attachment of _S. mutans_ whole cells to saliva-coated surfaces. Our laboratory was interested in determining the ability of the fimbrial preparation to bind to saliva.

Our data provided evidence of binding between the fimbrial preparation and whole saliva. _S. mutans_ A32-2 fimbriae demonstrated a significant increase in binding as indicated by ELISA absorbance, depending on saliva and fimbria concentrations, compared to the no-fimbria control. After determining that a component of saliva bound to _S. mutans_ fimbriae, we separated saliva by electrophoresis and identified the component(s) which bound fimbriae. Immunoblots of _S. mutans_ A32-2 fimbriae demonstrated significant activity with a salivary component at about 52 kDa. Perhaps the best-characterized salivary protein with this molecular mass is amylase. Amylase is a receptor for several oral streptococci (28–30) and has a reported molecular mass of approximately 55 kDa.

In order to determine if the 52-kDa protein was amylase, whole saliva was subjected to preparative gel electrophoresis to separate the salivary protein from other salivary components. Isolation of the salivary protein was successful; however, the separation technique, which used SDS and boiling, denatured the protein and inactivated amylase enzymatic activity (data not shown). Thus, confirmation required utilization of antibodies specific for human α-amylase to detect specific epitopes within the molecule. The purified salivary protein had epitopes that were recognized by antibody to human salivary α-amylase. These data suggest that _S. mutans_ may bind to amylase-coated surfaces. These data are contradictory to published reports that other oral streptococci, such as _S. gordonii_ but not _S. mutans_, bind salivary amylase (28). There are several possible explanations for this finding. The first explanation is that most investigators have analyzed _S. mutans_ whole-cell, but not fimbria, binding activity with salivary components (12, 28). The second reason may be that different systems of measurement provide different results (20, 30). The most commonly utilized techniques for binding determinations are systems that include radionuclides incorporated into whole cells or the receptor. Generally, HA beads have served as the surface for binding amylase or whole saliva. In our studies, although amylase binding to a nitrocellulose membrane or an ELISA plate may not be representative of dental plaque, the binding surfaces used may expose a binding site that is not exposed by attachment to HA. The A32-2 strain was isolated from a caries-active subject, so the fimbriae may have increased the pathogenic potential of this strain by acquiring amylase-binding capabilities. In this regard, Mintz and Fives-Taylor (22) reported that strain variants of _Actinobacillus actinomycetemcomitans_ demonstrated...
differences in adhesion to a human oral carcinoma cell line, suggesting that such alterations may occur in the oral cavity. However, our earlier data indicated that the amylase-binding 65-kDa fimbrial protein was present in various levels in isolates from both caries-active and caries-free subjects and in a laboratory strain (27), suggesting that amylase-binding activity resides in many strains of _S. mutans_.

An important characteristic of binding is the ability to inhibit the interaction. We were able to inhibit the binding of _S. mutans_ fimbriae to whole saliva with competitive inhibition by incubating the isolated salivary protein with the _S. mutans_ A32-2 fimbria preparation. The highest concentration of the purified salivary protein (65.0 µg/ml) caused more than a two-fold decrease in ELISA absorbance compared to the negative control (i.e., with no salivary protein added).

Other studies in this laboratory have demonstrated that protective mucosal immune responses to the fimbriae are able to reduce _S. mutans_ colonization and caries in experimental animals following intranasal immunization with a fimbria-cholera toxin conjugate (8). Furthermore, antibodies to the fimbriae or the 65-kDa fimbrial protein inhibited caries formation and _S. mutans_ colonization in an in vitro caries model study (9). The 65-kDa fimbrial protein that binds amylase was present in fimbrial preparations from all _S. mutans_ strains examined which did not react with specific antibodies to either antigen I/II or GTF (27), suggesting that all _S. mutans_ strains carry an amylase-binding fimbrial protein distinct from either antigen I/II or GTF.

Future studies are planned to investigate the genomic strain variations between various _S. mutans_ isolates carrying different levels of fimbriae by utilizing pulsed-field gel electrophoresis and restriction fragment length polymorphism. We have raised antibody specific for fimbrial proteins, so the screening of cDNA libraries may allow the detection of the gene(s) of interest. Once that is accomplished, the gene can be cloned and a pure polypeptide can be analyzed for binding activity with amylase. Nevertheless, it is clear that a surface fimbrial component of _S. mutans_ A32-2 has binding reactivity primarily with salivary amylase.

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**REFERENCES**


