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 saliva-coated surfaces and 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 asso-
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 fimbrae. In Western blot analysis, a 52-kDa salivary protein was recognized by the S. mutans fimbrae preparation. We chose to isolate and identify the salivary protein and determine the characteristics of binding to the S. mutans fimbra 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 overnight in Todd-Hewitt broth (Difco Laboratories, Detroit, Mich.) and passages every 2–3 days. The strain has been previously 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 fimbrae from S. gordonii whole cells was used for the removal of S. mutans fimbrae. 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 with 10 mM phosphate-buffered saline, 1 mM CaCl2, and 1 mM phenylmethylsulfonyl fluoride (pH 7.2) and stored as a pellet at −20°C until used.

In some cases, whole saliva was used. After the saliva was centrifuged (16,274 g for 30 min at 2°C), the fimbriae were removed with a Waring blender by using two 1-min cycles at high speed. Following blending, the sample was centrifuged (16,274 g × 15 min at 2°C) to remove cellular debris and aggregated fimbriae, and the supernatant was divided into aliquots and stored at −20°C. Prior to use, the saliva samples were centrifuged (2,800 g × 4°C, 10 min) and protein concentrations were determined. Saliva samples were diluted to 500 μg of protein/ml in physiological saline for solubility and usage. Synthetic polyacrylamide gel electrophoresis (SDS-PAGE) was performed using 12.5% stacking gels and 3% acrylamide resolving gels (110,000 g, 3 h) to block any unbound sites, and the nitrocellulose paper was blocked in a solution of defatted milk (1% milk fat; Carnation instant milk; Carnation Company, Los Angeles, Calif.) diluted in PBS-buffered saline (0.05 NaCl containing 0.5% Tween-20) overnight at 4°C. The unbound salivary components were removed by washing the nitrocellulose paper (Bio-Rad) overnight at 4°C at a constant voltage of 30 V in a mini-transblot electrophoretic transfer cell (Bio-Rad) (31). The nitrocellulose paper was blocked in a solution of defatted milk (1% milk fat; Carnation instant milk; Carnation Company, Los Angeles, Calif.) diluted in PBS-buffered saline (0.05 NaCl containing 0.5% Tween-20) (WBT) for 2 h at 25°C. The nitrocellulose paper was washed with PBS for 30 min and then incubated with rat antibody to A32-2 fimbrae (diluted 1:500 in WBT) for 1 h at room temperature. Goat antibody to rat IgG (Fc specific)–alkaline phosphatase conjugate (1:1,000 in WBT; 100 μl) (Sigma) was added and the membrane was incubated for 1 h. Binding of the antibody was detected by addition of alkaline phosphatase substrate (n-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 saliva protein was amylase, the isolated saliva 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 a-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 = 0.05.

RESULTS

Fimbria binding assays. ELISA and immunoblotting were used to establish that the S. mutans fimbra preparation bound to saliva-coated surfaces. An ELISA was performed to determine if an S. mutans A32-2 fimbra preparation bound to human whole saliva. Fimbriae 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 fimbrae) (Fig. 1). The binding of fimbrial components to saliva was reduced when either the saliva or fimbrae were diluted. These data provided the first indication that S. mutans fimbrae had binding activity with saliva-coated surfaces. BSA-coated wells did not bind fimbrae (data not shown).

Immunoblot analysis of human whole saliva probed with S. mutans A32-2 fimbrae. The binding of the S. mutans A32-2 fimbra 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 M carbonate-bicarbonate buffer (pH 9.6) and incubated for 3 h at 37°C or overnight at 4°C. The unbound salivary components were removed by washing the plates three times with normal saline containing 0.05% Tween 20 (Twee-n-saline). A solution of 1% goat serum albumin (BSA) (Sigma) in carbonate-bicarbonate buffer was added (200 μl) to block any unbound sites, and the mixture 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 salivary protein) and purified 65-kDa fimbrial protein (1 μg of protein/ml) in Twee-saline (no-fimbra control) were added, and the mixture was incubated for 3 h at 37°C and washed three times. Rat antibody to the A32-2 fimbra 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, a horseradish peroxidase (Fc specific) conjugated to horseradish peroxidase (Sigma) was added (100 μl; 1:8,000 dilution) and the mixture was incubated for 3 h at 37°C. After a final wash step, the substrate (10 mg of orthophenylene diamine dihydrochloride and 14 μl of 30% H2O2, in 20 ml of 0.5 M 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 fimbrae to a 1:10 dilution of whole saliva. Mixtures of the S. mutans fimbra preparation (33.0 μg/ml) and serially diluted 52-kDa salivary protein (0.5 μg to 65.0 μg/ml) in saline were incubated with rat antibody to A32-2 fimbrae (diluted 1:500 in WBT) overnight at 4°C at a constant voltage of 30 V in a mini-transblot electrophoretic transfer cell (Bio-Rad) (31). The nitrocellulose paper was blocked in a solution of defatted milk (1% milk fat; Carnation instant milk; Carnation Company, Los Angeles, Calif.) diluted in PBS-buffered saline (0.05 NaCl containing 0.5% Tween-20) (WBT) for 2 h at 25°C. The nitrocellulose paper was washed with WBT three times for 10 min each, 2 ml of S. mutans fimbral preparation (33 μg/ml) in WBT was added, and the paper was incubated for 1 h at room temperature. The membranes were washed three times with 0.1 M carbonate-bicarbonate buffer (pH 9.6) and incubated in PBS-buffered saline at 37°C for 30 min. After a brief wash, 100 μl of the A32-2 fimbral preparation (diluted 1:4,000 in WBT) was added (100 μl; 1:4,000 dilution) and the mixture was incubated for 1 h at room temperature. Goat antibody to rat IgG (Fc specific)–alkaline phosphatase conjugate (1:1,000 in WBT; 100 μl) (Sigma) was added and the membrane was incubated for 1 h. Binding of the antibody was detected by addition of alkaline phosphatase substrate (n-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 saliva protein was amylase, the isolated saliva 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 a-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 = 0.05.
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 significant activity with a salivary 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).

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, 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).

![Graph](http://cvi.asm.org/)
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 amylose-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 amylose-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 fimbiae to whole saliva with competitive inhibition by incubating the isolated salivary protein with the S. mutans A32-2 fimbia 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 fimbiae are able to reduce S. mutans colonization and caries in experimental animals following intranasal immunization with a fimbia-cholera toxin conjugate (8). Furthermore, antibodies to the fimbiae 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 amylose 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 amylose-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 fimbiae by utilizing pulsed-field gel electrophoresis and restriction fragment length polymorphism. We have raised antibody specific for fimbral 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 amylose. Nevertheless, it is clear that a surface fimbral component of S. mutans A32-2 has binding reactivity primarily with salivary amylose.

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REFERENCES