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 anti-amylase 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 hydroxypatite (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 adhesin 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 cells bind well to saliva-coated HA in an in vitro tooth model, whereas afimbriated 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. gordonii 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 fimbriae. In Western blot analysis, a 52-kDa salivary amylase displayed significant activity with the S. mutans fimbria preparation. We chose to isolate and identify the salivary protein and determine the characteristics of binding to the S. mutans fimbria 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% CO$_2$ and 95% air at 37°C overnight in Todd-Hewitt broth (Difco Laboratories, Detroit, Mich.) and passages were made at 30°C. This strain has previously been described to be heavily fibrillar (designated CS2 in reference 27).

Fimbria preparation. A modification (7, 27) of the technique of Morris and colleagues (23) for isolating fimbriae from S. gordonii whole cells was used for the removal of S. mutans fimbriae. 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% CO$_2$ and 95% air. Cells were pelleted and washed once with buffered saline, 1 mM CaCl$_2$, and 1 mM phenylmethylsulfonyl fluoride (pH 7.2) stored at −20°C overnight. Phenylmethylsulfonyl fluoride was added to inhibit endogenous proteolytic digestion of fimbrial proteins, and CaCl$_2$ was used to reduce fimbrial aggregation. Frozen cells were thawed and then suspended in fimbria buffer, and 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, 4°C, 10 min) to remove intact cells and cell debris, and the fimbria preparation in the supernatant was isolated by ultracentrifugation (110,000 × g, 4°C, 2 h). The pellet containing the fimbria preparation was resuspended in fimbria buffer and centrifuged (16,274 × g, 4°C, 10 min) to remove cellular debris and aggregated fimbriae, and the supernatant was divided into aliquots and stored at −20°C. The protein concentration was determined by using a micro-protein assay (Bio-Rad Laboratories, Hercules, Calif.).

Preparation of salivary components and the purified fimbrial protein. Saliva was collected from seven healthy individuals (neither caries free [i.e., no decayed, missing, or filled (DMF)-free)], 4°C, 2 h). The pellet containing the fimbria preparation was resuspended in fimbria buffer and centrifuged (16,274 × g, 4°C, 10 min) 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 solubilization of isolated polyacrylamide gel electrophoresis (SDS-PAGE). Samples of 0.1 M carbonate-bicarbonate buffer (pH 9.6) was added for enzyme-linked immunosorbent assay (ELISA). In order to separate salivary protein fractions, preparative gel electrophoresis (Prep cell model 491; Bio-Rad) was utilized. The resolving and stacking gels were composed of 10 and 3% acrylamide, respectively. Saliva samples (50-μl samples in saline) were boiled for 7 min and electrophoresed with a minigel electrophoresis apparatus (Mini-Protean II; Bio-Rad) for 60 min at 150 V. After gel electrophoresis, proteins were stained with Coomassie Brilliant Blue (10 g/ml) in WBT was added, and the paper was incubated for 30 min at 20°C. The membrane was incubated with rat antibody to A32-2 fimbriae (diluted 1:100 in WBT) for 1 h at room temperature. Goat antibody to IgG (Fc-specific)–alkaline phosphatase conjugate (1:1,000 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-nitrophenyl phosphate in 100 mM NaCl containing 0.5% Tween-20 (WBT) for 1 h at 25°C]. The nitrocellulose paper was incubated with undiluted whole saliva were electrophoresed by SDS-PAGE, transferred to 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 Company, Los Angeles, Calif.) diluted in 0.1% TBS (0.9% NaCl containing 0.5% Tween-20) and the nitrocellulose paper was incubated with rat antibody to A32-2 fimbriae (diluted 1:500 in WBT) for 1 h at room temperature. Goat antibody to IgG (Fc-specific)–alkaline phosphatase conjugate (1:1,000 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-nitrophenyl phosphate; 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 (52.0 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 considered significant when P ≤ 0.05.

RESULTS

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

Immunoblot analysis of human whole saliva probed with S. mutans A32-2 fimbriae. The binding of the S. mutans A32-2 fimbria 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
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).

**FIG. 1.** Binding of *S. mutans* fimbriae to whole-saliva-coated ELISA plates. The ability of *S. mutans* fimbriae (0.33 to 33.00 μg/ml) to bind to saliva (undi- luted and diluted 1:2 and 1:10) was determined by ELISA. The negative controls were wells that did not contain fimbriae. The ELISA absorbances (means ± SEM) represent a relative measurement of binding between fimbriae and saliva. ND, not determined.

**FIG. 2.** Representative immunoblot of whole saliva from seven different subjects. Blots were probed with fimbriae from *S. mutans* A32-2, followed by rat antibody to fimbriae of *S. mutans* A32-2, and alkaline phosphatase-labeled goat antibody to rat IgG. Whole-saliva samples from seven subjects (lanes 1 through 7) are shown. The arrow indicates the molecular mass of the major salivary component that bound fimbriae.

**FIG. 3.** Representative dually stained (Coomassie brilliant blue and silver) SDS-PAGE gel containing purified salivary protein. Purified salivary protein was collected by preparative gel electrophoresis and analyzed by SDS-PAGE. The arrow on the right indicates the molecular mass of the isolated salivary component.

**FIG. 4.** *S. mutans* A32-2 fimbria binding to salivary proteins. ELISA plate wells were coated with the purified salivary protein (65.0 μg/ml), whole saliva (diluted 1:10), and amylase (10.0 μg/ml). After blocking with 1% BSA, the *S. mutans* A32-2 fimbria preparation (33.0 μg/ml) was incubated with the various salivary proteins. The controls did not include fimbriae. The ELISA absorbances (means ± SEM) represent a relative measurement of binding between fimbriae and salivary components.
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* fimbriae 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 at about 52 kDa. Perhaps the best-characterized receptor for several oral streptococci (28–30) and has a 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 \( \mu \)g/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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