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Streptococcus salivarius Fimbriae Are Composed of a Glycoprotein Containing a Repeated Motif Assembled into a Filamentous Nondissociable Structure

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***Streptococcus salivarius*, a gram-positive bacterium found in the human oral cavity, expresses flexible peritrichous fimbriae. In this paper, we report purification and partial characterization of *S. salivarius* fimbriae. Fimbriae were extracted by shearing the cell surface of hyperfimbriated mutant A37 (a spontaneous mutant of *S. salivarius* ATCC 25975) with glass beads. Preliminary experiments showed that *S. salivarius* fimbriae did not dissociate when they were incubated at 100°C in the presence of sodium dodecyl sulfate. This characteristic was used to separate them from other cell surface components by successive gel filtration chromatography procedures. Fimbriae with molecular masses ranging from 20×10^6 to 40×10^6 Da were purified. Examination of purified fimbriae by electron microscopy revealed the presence of filamentous structures up to 1 μm long and 3 to 4 nm in diameter. Biochemical studies of purified fimbriae and an amino acid sequence analysis of a fimbrial internal peptide revealed that *S. salivarius* fimbriae were composed of a glycoprotein assembled into a filamentous structure resistant to dissociation. The internal amino acid sequence was composed of a repeated motif of two amino acids alternating with two modified residues: A/X/T-E-Q-M/ϕ, where X represents a modified amino acid residue and ϕ represents a blank cycle. Immunolocalization experiments also revealed that the fimbriae were associated with a wheat germ agglutinin-reactive carbohydrate. Immunolabeling experiments with antifimbria polyclonal antibodies showed that antigenically related fimbria-like structures were expressed in two other human oral streptococcal species, *Streptococcus mitis* and *Streptococcus constellatus*.**

Bacterial surface molecules are involved in adhesion to host surfaces, invasion of host cells, bacterial cell-cell contact and communication, and protection from host immune defenses (24). Fimbriae are proteinaceous hair-like appendages found on the bacterial cell surface. They allow bacteria to adhere specifically to a large number of targets, including mammalian cells, host proteins, and other microbial cells (28). They also stimulate elements of the immune system, such as macrophages and spleen cells (21). The fimbriae of gram-negative bacteria have been extensively studied, particularly those of *Escherichia coli* and *Salmonella* spp. They are composed of major protein subunits with molecular masses of 14 to 30 kDa, have diameters ranging from 2 to 8 nm, and usually extend 1 to 2 μm from the bacterial surface (32). The structure, assembly machinery, and relevant genes of gram-negative bacterial fimbriae are well characterized (12). However, very little information has been published on the biogenesis, structure, and genetics of fimbriae of gram-positive bacteria; in this group fimbriae have been reported mainly in oral streptococci, including *Streptococcus salivarius* (22), *Streptococcus parasanguinis* (15) (formerly *Streptococcus parasanguis* [45]), *Streptococcus mutans* (16), and *Streptococcus oralis* (23). *S. parasanguinis* FW213 type 1 fimbriae have been the most extensively characterized. These fimbriae mediate attachment of the bacteria to teeth and have a 36-kDa fimbrial adhesin on their tips. This protein, designated FimA, was identified as a member of the LraI family of streptococcal lipoproteins (11) and as a major virulence factor in *S. parasanguinis*-associated endocarditis (4). Wu et al. (53) have demonstrated that a protein called Fap1 is essential for fimbria formation in *S. parasanguinis* FW213. They concluded that Fap1 is probably the structural subunit of one type of fimbriae produced by this organism. Fap1 contains 2,552 amino acid residues, including a 50-amino-acid N-terminal leader peptide, a cell wall anchorage sequence at the C terminus, and 1,000 repeats of the sequence E/V/I-S (52).

S. salivarius is an early colonizer of the human oral cavity. Its main habitat is the tongue dorsum and buccal epithelium (34). *S. salivarius* is divided into two serological subgroups that carry either fibrils or fimbriae: Lancefield groups K^+ and K^- (22, 49). The surfaces of K^+ strains are coated with dense, short, peritrichous fibrils. Fibrils 76 to 209 nm long have been purified from K^+ strains and have been shown to possess distinct adhesive functions; 91-nm-long fibrils carry antigen B, a 320-kDa glycoprotein involved in coaggregation of *S. salivarius* and *Veillonella* species. Antigen C, a 220- to 280-kDa glycoprotein located on short 72-nm-long fibrils, is involved in salivary agglutination, hemagglutination, and adherence to buccal epithelial cells (23, 48). K^- strains possess flexible peritrichous fimbriae 3 to 4 nm wide and up to 1.0 μm long (22). Unlike the fibrils of K^+ strains, the fimbriae on the surfaces of K^- strains have not been purified, and no information concerning their biochemical composition is available.

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TABLE 1. Immunoreactivity of PAbs HL-72 against various oral streptococcal species

Species group[a]	Strain[b]	Reactivity[c]
Salivarius	*S. salivarius* ATCC 25975 (K^-)[d]	+
	S. salivarius ATCC 7073 (K^+)[d,e]	+
	S. salivarius ATCC 27945 (K^+)[d,e]	+
	S. vestibularis ATCC 49124	−
Mitis	*S. mitis* ATCC 33399	+
	S. parasanguinis ATCC 15912	−
	S. gordonii ATCC 10558	−
	S. oralis ATCC 35037	−
Anginosus	*S. anginosus* ATCC 33397	−
	S. constellatus ATCC 27823	+
	S. intermedius ATCC 27335	−
Mutans	*S. mutans* ATCC 10449	(+/−)
	S. sobrinus ATCC 33478	(+/−)
	S. ferus ATCC 33477	(+/−)
	S. cricetus ATCC 19642	−
	S. downei ATCC 33478	(+/−)
	S. ratti LG-1	(+/−)

[a] Species groups as described by Whiley and Beighton (50).
[b] Most strains were obtained from the American Type Culture Collection; the only exception was *S. ratti* LG-1, which was kindly provided by D. Grenier (Groupe de Recherche en Écologie Buccale, Université Laval, Quebec City, Quebec, Canada).
[c] +, positive reaction; (+/−), weak reaction; −, negative reaction.
[d] The information in parentheses is the Lancefield group K antigen reactivity for *S. salivarius* species.
[e] In *S. salivarius* K^+ strains, fibrils instead of fimbriae were labeled by the antibodies.

In this paper, we report purification and partial characterization of *S. salivarius* fimbriae.

(Some of the results were presented at the 99th General Meeting of the American Society for Microbiology, Chicago, Ill., 1999.)

MATERIALS AND METHODS

Bacterial strains and growth conditions. The wild-type strains used in this study are listed in Table 1. They were cultured in brain heart infusion broth and on brain heart infusion agar (Difco Laboratories, Detroit, Mich.). Hyperfimbriated *S. salivarius* mutant A37 was isolated from Lancefield group K^- strain *S. salivarius* ATCC 25975 by positive selection for resistance to 0.5 mM 2-deoxyglucose (3, 18). For purification of fimbriae, mutant A37 was grown in TYE medium containing (per liter) 10 g of tryptone (Difco), 5 g of yeast extract (Difco), 2.5 g of NaCl, and 2.5 g of Na_2HPO_4 in the presence of 0.2% (wt/vol) lactose and 0.5 mM 2-deoxyglucose. D37 is a fimbria-negative mutant of *S. salivarius* ATCC 25975 obtained by Tn*917* mutagenesis (C. Lévesque, C. Vadeboncoeur, and M. Frenette, Abstr. 99th Gen. Meet. Am. Soc. Microbiol., abstr. J16, 1999). It was cultured in TYE medium in the presence of 0.2% lactose and 10 μg of erythromycin per ml. All organisms were grown at 37°C for 24 h without agitation. *Streptococcus intermedius* ATCC 27335, *Streptococcus sobrinus* ATCC 33478, and *Streptococcus cricetus* ATCC 19642 were grown under anaerobic conditions (80% N_2, 10% H_2, 10% CO_2).

Purification of fimbriae. Cells from 8 liters of an overnight culture of mutant A37 were harvested by centrifugation (10,000 × *g*, 20 min, 4°C) and washed twice with sterile phosphate-buffered saline (PBS) (pH 7.2). The bacterial pellet was then resuspended in 100 ml of 50 mM Tris-HCl (pH 8.6) containing 5 mM EDTA, and 20 g of washed and sterilized glass beads (diameter, 150 to 212 μm; Sigma) was added. The suspension was vigorously shaken for 42 h at 4°C on a horizontal rotator (200 rpm; Junior Orbit Shaker; Lab-Line Instruments, Inc.) and centrifuged twice (10,000 × *g*, 20 min, 4°C) to remove cells and the glass beads (41). The supernatant was recovered, dialyzed overnight at 4°C against 4 liters of 5 mM Tris-HCl (pH 8.0) (buffer A), and lyophilized. The lyophilisate, designated the crude glass bead extract (GBE), was resuspended in 5 ml of sterile distilled water. The GBE was boiled for 15 min in the presence of 1% (wt/vol) sodium dodecyl sulfate (SDS). Insoluble material was removed by centrifugation (10,000 × *g*, 2 min), and the supernatant (1-ml samples) was applied to a Sepharose CL-4B column (1.5 by 25 cm; fractionation range, 60×10^3 to 20×10^6 Da; Sigma) at room temperature equilibrated with 20 mM Tris-HCl (pH 8.0)–0.2 M NaCl (buffer B) containing 0.1% SDS. Proteins were eluted at a flow rate of 20 ml/h, and 1-ml fractions were collected. Proteins were detected in collected fractions by measuring the absorbance at 280 nm. The fimbriae were detected by electron microscopy. Fractions containing fimbriae were pooled, dialyzed against buffer A, concentrated by lyophilization, and resuspended in 1 ml of sterile distilled water. Further purification was performed by separation on a Sepharose CL-2B column (1.5 by 45 cm; fractionation range, 70×10^3 to 40×10^6 Da; Sigma) at room temperature equilibrated with buffer B. The proteins were eluted at a flow rate of 15 ml/h, and 1-ml fractions were collected. The fractions containing the fimbriae were pooled, dialyzed against buffer A, lyophilized, and resuspended in 100 μl of sterile distilled water. The purified fimbriae were conserved at −20°C until they were used.

Production of antibodies. Polyclonal antibodies (PAbs) HL-72 against purified fimbriae were produced by immunizing two female New Zealand White rabbits. Fimbriae (200 μg [dry weight] resuspended in PBS [pH 7.2]) were emulsified with an equal volume of TiterMax adjuvant (Cederlane Laboratories), and the mixture was injected intramuscularly into each hind limb of the animals. Two weeks later, the rabbits received a second injection (as described above), and blood samples were taken 4 weeks after the final injection.

SDS-PAGE. SDS-polyacrylamide gel electrophoresis (PAGE) was performed using the buffer system of Laemmli (30) at a constant voltage (200 V) with gels containing 7.5% polyacrylamide in the separating gel and 4.5% polyacrylamide in the stacking gel. Prior to electrophoresis, the samples were heated at 100°C for 10 min in dissociating buffer containing 2% SDS and 5% 2-mercaptoethanol.

Dissociating treatments. *S. salivarius* fimbriae were treated with various denaturing chemical agents and proteases and analyzed by SDS-PAGE. The fimbriae (100 μg [dry weight]) were subjected to the following treatments: 8.6 M guanidine hydrochloride (Gnd-HCl) at 37°C for 2 h (9); 88% formic acid at 100°C for 10 min (7); 10% trichloroacetic acid (2); 2 and 4% SDS at 100°C for 15 min (26); 6 and 8 M urea at 37°C for 2 h (29); HCl (pH 1.8) at 100°C for 30 min; NaOH (pH 12) at 100°C for 30 min; 6 M Gnd-HCl–dithiothreitol (30 mg/ml) at 50°C for 2 h and 80 mM iodoacetamide at 37°C for 30 min (19); 1.5 and 3 U of V8 protease at 37°C for 1 h; and 1 mg of trypsin and chymotrypsin per ml at 37°C for 1 h (38).

N-terminal amino acid and peptide sequencing. N-terminal sequencing of the purified fimbria preparation was performed by Edman degradation with a model 473A pulsed liquid-phase sequencer from Applied Biosystems. Samples (150 μg [dry weight] of fimbriae) were applied to a trifluoroacetic acid-treated cartridge filter coated with 1.5 mg of Polybrene and 0.1 mg of NaCl. The phenylthiohydantoin (PTH) amino acid derivatives were identified by comparison with standards (PTH Analyser standards; ABI) analyzed online prior to the sequence analysis. The purified preparation of fimbriae (150 μg [dry weight]) was also subjected to cyanogen bromide (CNBr) proteolysis with or without a prior reduction-alkylation treatment. The amino acid sequence of an internal fragment was also determined by Edman degradation as described above. The amino acid sequence analyses, as well as the reduction-alkylation and CNBr treatments, were performed by the Service de Séquences des Peptides de l'Est du Québec.

Glycan detection. Creatinase (2 and 10 μg), transferrin (2 and 10 μg), and purified fimbriae (2 and 10 μg [dry weight]) were blotted onto a nitrocellulose membrane (Schleicher & Schuell, Keene, N.H.) and allowed to air dry. Creatinase and transferrin were used as nonglycosylated and glycoprotein controls, respectively. Carbohydrates were detected with a DIG Glycan Detection Kit from Roche Diagnostics used as recommended by the manufacturer.

Lectin-binding activity. Five horseradish peroxidase-coupled lectins (HRP-lectins) (Sigma) were used: LOTUS A (*Tetragonolobus purpureas*; specific for fucose residues), Con A (*Canavalia ensiformis*; specific for mannose residues), RCA-I (*Ricinus communis*; specific for galactose residues), PNA (*Arachis hypogaea*; specific for galactose residues), and WGA (*Triticum vulgaris*; specific for *N*-acetyl-D-glucosamine and *N*-acetylneuraminic acid residues). The lectins were dissolved in PBS (pH 6.8) at a concentration of 1 mg/ml and stored at −20°C until they were used. Aliquots (50 μl) of an *S. salivarius* ATCC 25975 suspension (optical density at 660 nm in PBS [pH 7.2], 0.2) and a purified fimbria preparation (10 μg [dry weight]) were applied to a nitrocellulose membrane and allowed to air dry. The membrane was incubated in 20 mM Tris-HCl (pH 7.5)–0.5 M NaCl (TBS) containing 3% bovine serum albumin (BSA) for 1 h. All procedures were carried out at room temperature with gentle agitation. The nitrocellulose membrane was then transferred to TBS containing 1.5% BSA and the HRP lectin being investigated (final concentration, 1 μg/ml) (20). The membrane was incubated for 2 h and then washed twice (15 min each) in TBS containing 0.05%

Tween 20 (TTBS) and once in TBS (15 min). The nitrocellulose membrane was stained by using an HRP color development kit (Bio-Rad, Richmond, Calif.) according to the manufacturer's instructions.

Electron microscopy. (i) Negative staining. Ten-microliter samples were applied to carbon-coated Formvar copper grids (Canemco Inc., St-Laurent, Quebec, Canada) and negatively stained with 1% (wt/vol) phosphotungstic acid (PTA) (pH 7.0) for 10 s. The grids were air dried prior to examination with a JEOL 2000 transmission electron microscope (TEM) operating at 80 kV.

(ii) Immunogold labeling with PAbs HL-72. Ten-microliter samples of bacterial suspensions (about 8×10^7 cells/ml) were applied to carbon-coated Formvar copper grids. After 30 min, the grids were blocked with PBS (pH 7.2) containing 1% immunoglobulin G (IgG)-free and protease-free BSA (Jackson Immunoresearch Laboratories) for 30 min. The grids were then incubated with PAbs HL-72 (diluted 1:50 in blocking buffer) for 1 h and rinsed in PBS (pH 7.2) and distilled water. The grids were incubated for 1 h with 12-nm colloidal gold-labeled donkey anti-rabbit IgG (Jackson Immunoresearch Laboratories) diluted 1:40 in blocking buffer and rinsed with PBS (pH 7.2) and distilled water. The samples were negatively stained and examined as described above.

(iii) Immunogold labeling with WGA. Ten-microliter samples of bacterial suspensions (about 8×10^7 cells/ml) were applied to carbon-coated Formvar copper grids. After 30 min, the grids were blocked with PBS (pH 7.2) containing 1% IgG-free BSA for 30 min. The grids were then incubated with 10-nm colloidal gold-labeled WGA lectin (Sigma) diluted 1:50 in blocking buffer for 1 h and rinsed in PBS (pH 7.2) and distilled water. The samples were negatively stained and examined as described above.

(iv) Competition assays. Ten-microliter samples of bacterial suspensions (about 8×10^7 cells/ml) were applied to carbon-coated Formvar copper grids. After 30 min, the grids were blocked with PBS (pH 7.2) containing 1% IgG-free BSA for 30 min. The grids were then incubated with undiluted antifimbria PAbs for 1 h and rinsed in PBS (pH 7.2) and distilled water. The grids were incubated with 10-nm colloidal gold-labeled WGA lectin diluted 1:50 in blocking buffer for 1 h and rinsed in PBS (pH 7.2) and distilled water. The samples were negatively stained and examined as described above.

Dot blot assays with antifimbria PAbs. Aliquots (50 μl) of bacterial suspensions (optical density at 660 nm in PBS [pH 7.2], 0.2) were applied to a nitrocellulose membrane and allowed to air dry. The membrane was incubated in TBS containing 3% BSA for 1 h. All procedures were carried out at room temperature with gentle agitation. The membrane was then transferred to TBS–3% BSA containing antifimbria PAbs HL-72 diluted 1:500 and incubated for 1 h. The membrane was washed twice (15 min each) in TTBS and then incubated with alkaline phosphatase-conjugated goat anti-rabbit IgG (Jackson Immunoresearch Laboratories) diluted 1:5,000 in TBS–3% BSA for 1 h. Finally, the membrane was washed with TTBS twice (15 min each) and with TBS once (15 min), and the antibody conjugate was detected with nitroblue tetrazolium–5-bromo-4-chloro-3-indolyl phosphate as the substrate. The reaction was stopped by washing the membrane in distilled water. Negative controls were prepared by replacing the antifimbria PAbs with the same dilution of normal rabbit serum.

Protein determination. GBE protein assays were performed by the method of Lowry et al. (33) with BSA (Sigma) as the standard. The amount of fimbrial material obtained with the purification protocol described above was evaluated by weighing the purified preparation.

RESULTS

Purification of fimbriae. TEM observations demonstrated that group K^- strain *S. salivarius* ATCC 25975 (wild type) expresses fimbriae up to 1 μm long, but in very small amounts. A spontaneous 2-deoxyglucose-resistant mutant of *S. salivarius* ATCC 25975 that overexpressed these fimbriae was isolated in a previous study (3, 18). This hyperfimbriated mutant, designated A37, was used as a source of fimbriae in the purification protocol. The first step was to extract the fimbriae from the cell surface. We first tried the extraction procedures described in the purification protocols used for fimbriae of the gram-positive bacteria *S. parasanguinis* and *Actinomyces* spp. In the *S. parasanguinis* purification protocol, the fimbriae were extracted from cells by high-speed pulse blending in a homogenizer in the presence of potassium iodide, a chaotropic agent (10). In the protocol for *Actinomyces* spp., the fimbriae were extracted from cells by sonic treatment in Tris-buffered saline (6). However, the yield obtained for *S. salivarius* fimbriae with these extraction procedures was too low to allow further purification of the fimbriae (data not shown). Therefore, a novel extraction procedure was developed.

The cell surface of mutant A37 was sheared with glass beads to obtain a preparation of surface components called the GBE. TEM observations of mutant A37 cells after the glass bead treatment showed that significant amounts of fimbriae could be extracted from cells and that cell lysis was minimal. To identify the major protein subunit of the *S. salivarius* fimbriae, we extracted surface components of wild-type cells and compared the protein compositions of this preparation and that obtained from mutant A37 by SDS-PAGE. Surprisingly, no difference was observed despite the fact that the mutant produced many more fimbriae than the parental strain. These results suggested that *S. salivarius* fimbriae were resistant to dissociation even after boiling in the presence of SDS-PAGE dissociating buffer. To test this hypothesis, the GBE from the hyperfimbriated mutant strain was subjected to boiling in the presence of 2% SDS prior to TEM observation. We observed that this treatment did not alter the fimbrial structure, confirming that fimbriae from *S. salivarius* did not dissociate into subunits when they were treated with SDS and heated at 100°C. Therefore, this property was used to purify the fimbriae.

The GBE of A37 was boiled in the presence of SDS and applied to a column of Sepharose CL-4B equilibrated in the presence of SDS (Fig. 1A). The fimbriae, as verified by TEM, eluted in the void volume of the column (exclusion limit, 20×10^6 Da). This step separated the fimbriae from all the other cell components in the GBE with molecular masses less than 20×10^6 Da. The fimbriae were further purified by fractionation on a Sepharose CL-2B column, which had an exclusion limit of 40×10^6 Da. The fimbriae migrated as a protein with a molecular mass ranging from 20×10^6 to 40×10^6 Da on Sepharose CL-2B (Fig. 1B). SDS-PAGE of the purified fimbriae followed by silver nitrate staining showed that no proteins penetrated the gel, indicating that contaminating proteins were not present (data not shown). With this method, 90 to 150 μg of fimbrial material per g (wet weight) of A37 cells was obtained.

Ultrastructure of fimbriae as determined by electron microscopy. As observed by TEM, the purified fimbriae obtained from hyperfimbriated mutant A37 appeared as a tangled mass of appendages (Fig. 2A) and large bundles of aggregates consisting of multiple thin filaments less than 5 nm wide and up to 1 μm long (Fig. 2B). Immunolabeling with rabbit antisera raised against the purified preparation of fimbriae (PAbs HL-72) stained the fuzzy coat of *S. salivarius* cells, revealing appendages extending from the surfaces of wild-type cells (Fig. 3A) and mutant A37 cells (Fig. 3B). As Fig. 3 shows, the hyperfimbriated mutant cells showed more abundant labeling of fimbriae than the wild-type cells. The immunogold beads did not bind to the controls in the absence of antifimbria PAbs, and no binding was observed when normal rabbit serum was used (data not shown), confirming the specificity of PAbs HL-72 for the fimbriae.

Characterization of fimbriae. (i) Effects of dissociating treatments. In an attempt to determine the protein composition of *S. salivarius* fimbriae, we tried to dissociate the fimbriae

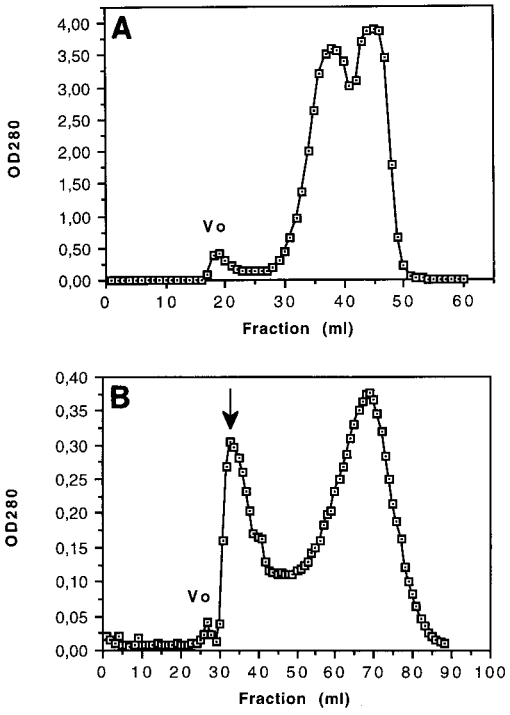

FIG. 1. Gel filtration chromatography. (A) Sepharose CL-4B gel filtration of mutant A37 GBE. The void volume (V_0) containing the fimbriae is indicated. (B) The fractions eluted in the void volume on the Sepharose CL-4B column were pooled and fractionated on a Sepharose CL-2B column. As observed by TEM, the second peak (indicated by the arrow) contained the fimbriae. OD280, optical density at 280 nm.

by using various denaturing chemical agents and proteases. The fimbriae were subjected to treatments previously used to dissociate *E. coli* type 1 pili (8.6 M Gnd-HCl) (9), *Salmonella enterica* serovar Enteritidis fimbriae and *E. coli* curli (88% formic acid) (7), *Proteus mirabilis* MR/P fimbriae (10% trichloroacetic acid) (2), *E. coli* O7:K1:H6 fimbriae (2 and 4% SDS) (26), and enterobacterial fimbriae (6 and 8 M urea) (29). These treatments, which have been shown to be effective in dissociating fimbriae resistant to conventional treatments, were ineffective with *S. salivarius* fimbriae. The fimbriae also remained unaltered following acid (HCl [pH 1.8]) and alkaline (NaOH [pH 12]) treatments. The fimbriae were exposed to a mixture of Gnd-HCl, dithiothreitol, and iodoacetamide, a procedure used to disaggregate structural and cytoskeletal proteins (19). The *S. salivarius* fimbriae were also found to be resistant to this treatment. Protease treatments, including V8 protease, trypsin, and chymotrypsin treatments, which have been used to hydrolyze *Streptococcus sanguinis* (formerly *Streptococcus sanguis* [45]) fibrillar glycoproteins (38), were also ineffective.

(ii) Amino acid analysis. When we attempted to determine the N-terminal amino acid sequence, the Edman degradation reaction was blocked at the third residue, after Ala and Lys. We subsequently treated the fimbriae with CNBr, and using Edman degradation, we obtained the following unique sequence of 30 residues: A-X-T-ϕ-A-X-E-ϕ-A-X-T-ϕ-A-X-Q/M-ϕ-A-X-T-ϕ-A-X-E-ϕ-A-X-T-ϕ-A-X, where X represents a modified amino acid residue and ϕ represents a blank cycle. The signal for each residue was unique and strong, confirming that the sequenced peptide was present in significant amounts. However, we could not definitively determine whether only one site in the fimbriae was hydrolyzed by CNBr, generating the observed sequence, or whether different CNBr-cleavable sites were followed by the same repeated sequence. The sequence consisted of a repeated motif of four residues composed of two known amino acid residues alternating with two unidentified residues: A/X/T-E-Q-M/ϕ, where X represents a modified amino acid residue that migrated during high-performance liquid chromatography at a position close to that of asparagine and ϕ represents a blank cycle. The blank cycle could have been caused by the presence of a glycosylated residue or a cysteine involved in a disulfide bond (35). However, as reduction and alkylation of the fimbriae before CNBr hydrolysis generated the same sequence, disulfide bonds could not account for the presence of blank cycles in this case. Consequently, the blank cycle residues corresponded to modified amino acids.

(iii) Glycan detection and lectin-binding activity. The purified fimbriae were analyzed for the presence of carbohydrates by using a DIG Glycan Detection Kit. Periodate oxidation and subsequent digoxigenin-succinyl amidocaproic acid hydrazide labeling revealed that *S. salivarius* fimbriae contained carbohydrates (data not shown). Whole cells were also assayed for lectin-binding activity. A positive reaction was observed with WGA but not with LOTUS A, Con A, RCA-I, or PNA (data not shown). When purified fimbriae were tested for WGA-binding activity, a positive reaction was also observed (data not shown). As the positive reaction observed with WGA might have resulted from an interaction with *N*-acetyl-D-glucosamine residues of the peptidoglycan, immunolabeling experiments with colloidal gold-conjugated WGA lectin were conducted by using whole cells of wild-type *S. salivarius* and mutant D37, a fimbria-negative mutant. Immunolabeling of the cell surface of the wild-type strain resulted in accumulation of gold particles on organized structures resembling fimbriae (Fig. 4A) similar to those observed with PAbs HL-72 (Fig. 3). No labeling was observed with mutant D37 (Fig. 4B). A competition assay between PAbs HL-72 and colloidal gold-conjugated WGA lectin was performed to determine whether WGA and the PAbs bound to the same epitopes. The fact that no labeling was observed by TEM when wild-type cells were successively incubated with antifimbria PAbs and colloidal gold-conjugated WGA lectin indicated that the antifimbria PAbs completely inhibited WGA binding to wild-type cells (data not shown). These results confirmed that the WGA-binding activity of *S. salivarius* was associated with the fimbriae and was not due to an interaction with peptidoglycan.

Distribution of antigenically related fimbriae in oral streptococci. The presence of *S. salivarius*-like fimbriae in various oral streptococcal species was tested by immunoblotting by using the antifimbria PAbs (Table 1). The salivarius group comprises two closely related species found in the human oral cavity: *S. salivarius* and *Streptococcus vestibularis* (50). Dot blot experiments conducted with three strains of *S. salivarius* and one strain of *S. vestibularis* indicated that all the *S. salivarius* strains tested reacted with the antifimbria PAbs, while the *S. vestibularis* strain did not. Four species of the mitis group found

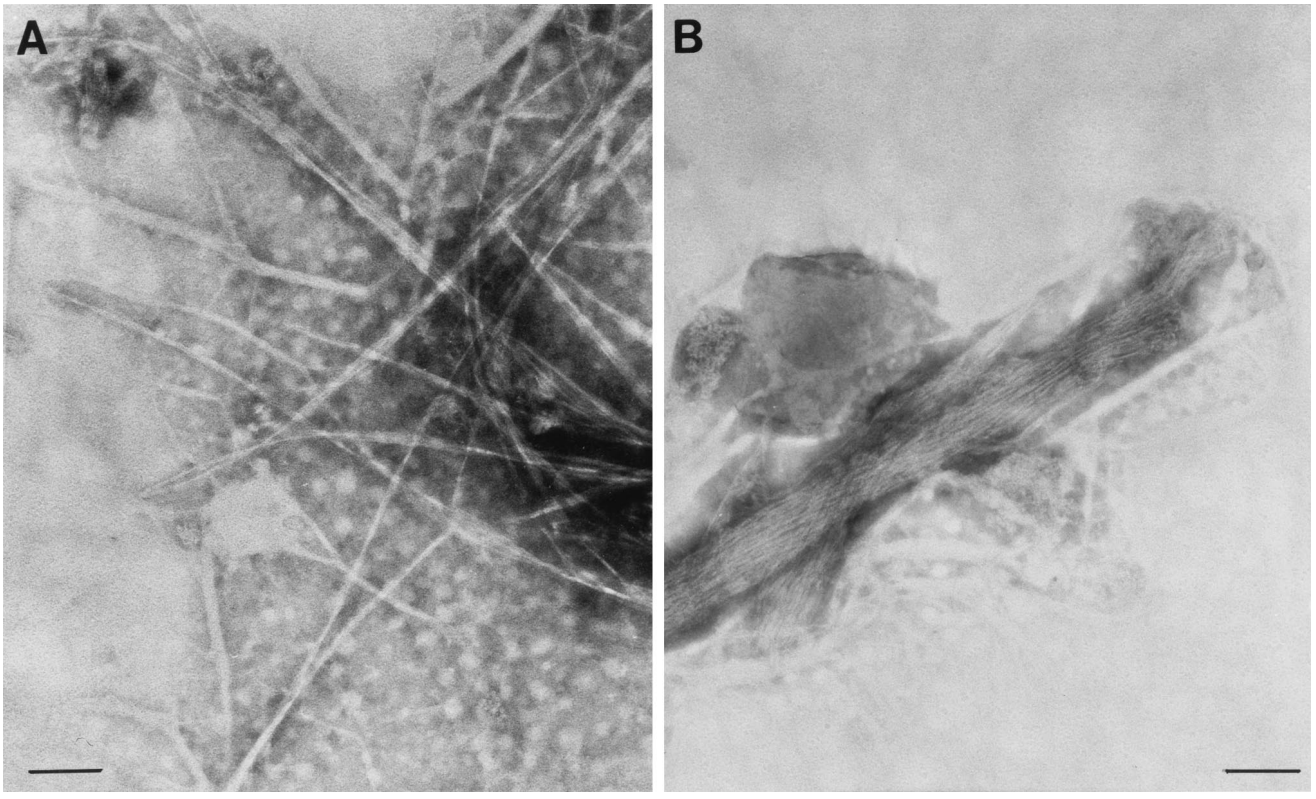

FIG. 2. Electron micrographs of a purified preparation of *S. salivarius* mutant A37 fimbriae negatively stained with 1% PTA (pH 7.0). Large bundles of aggregates consisting of multiple thin filaments can be seen in panel B. Bars, 100 nm.

in the human mouth (50) were also tested: *Streptococcus mitis, S. parasanguinis, Streptococcus gordonii*, and *S. oralis*. The dot blot results revealed that only the *S. mitis* strain reacted with the antifimbria PAbs. We also looked for the presence of *S. salivarius*-like fimbriae in the anginosus group. Three human oral species make up this group: *Streptococcus anginosus, Streptococcus constellatus*, and *S. intermedius* (50). A strong positive reaction was found only with *S. constellatus*. Six species in the mutans group were also tested: *S. mutans, S. sobrinus, Streptococcus ferus, S. cricetus, Streptococcus downei*, and *Streptococcus ratti* (formerly *Streptococcus rattus* [45]). In this group, only *S. ferus* and *S. downei* are not found in the human oral cavity. *S. ferus* is found in oral cavities of wild rats, and *S. downei* is found in oral cavities of monkeys (50). A weak reaction was observed for all the mutans strains tested except the *S. cricetus* strain.

Streptococcal species that reacted positively in the immunoblot assays were further tested by using immunogold labeling and TEM to ensure that the antifimbria PAbs reacted with filamentous structures. All strains that reacted positively with the antifimbria PAbs in dot blot experiments possessed filamentous structures that were labeled by the PAbs (data not shown). *S. salivarius* ATCC 7073 and ATCC 27945, two Lancefield group K^+ strains, did not possess fimbriae but possessed fibrils that were labeled by the antifimbria PAbs. *S. mitis* and *S. constellatus* possessed fimbria-like structures protruding from the cell surface that were also labeled by the *S. salivarius* antifimbria PAbs. Because of the low level of reactivity of PAbs HL-72 in the dot blot assays, immunogold labeling experiments were not performed with the species in the mutans group.

DISCUSSION

In the oral cavity, bacterial adherence to the buccal mucosa, the glycoprotein pellicle of the teeth, and other bacteria is often mediated by fimbriae (21, 23, 31). These structures are found on the majority of oral streptococci. However, there is scant information on their structure, function, and composition (21). In the study described here, we purified and partially characterized *S. salivarius* fimbriae. In the purification process, the first notable observation was that the fimbriae were firmly attached to the cells, as indicated by the fact that the approaches used to extract fimbriae from gram-positive bacteria did not efficiently extract fimbriae from *S. salivarius*. To circumvent this problem, we used a glass bead extraction procedure to shear the cell surface of an *S. salivarius* mutant (A37) that overproduced fimbriae.

Studies to determine the protein composition of *S. salivarius* fimbriae have been handicapped by the resistance of the fimbriae to dissociation by various methods that have been successful with other bacterial fimbriae. These results suggest that the structural subunits of *S. salivarius* fimbriae are linked by covalent bonds rather than by hydrogen and noncovalent hydrophobic interactions, as is the case with fimbriae of gram-negative organisms (39). An inability to dissociate intact fimbriae into monomeric subunits by standard methods has also

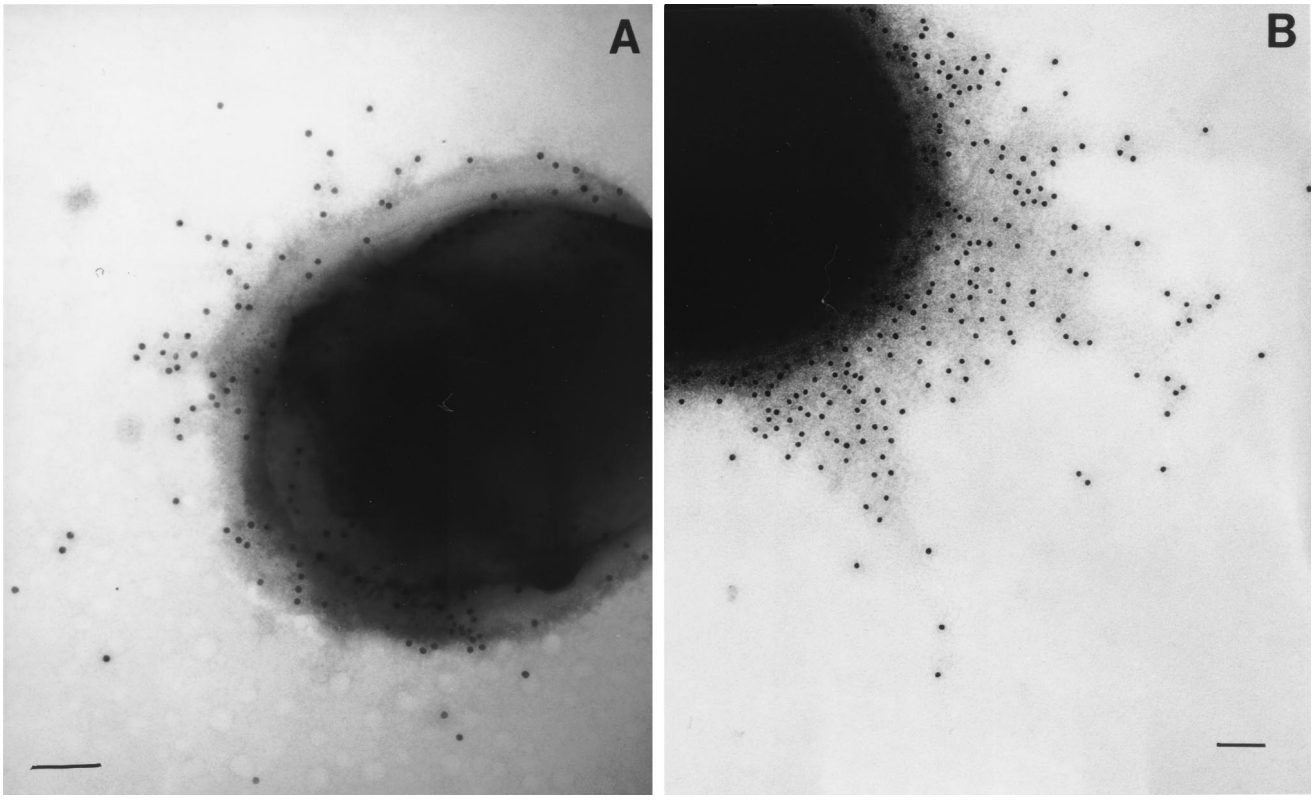

FIG. 3. Immunolocalization of fimbriae on *S. salivarius* ATCC 25975 (A) and mutant A37 (B) cell surfaces by electron microscopy. Bacterial cells were successively incubated with antifimbria PAbs and 12-nm colloidal gold-conjugated donkey anti-rabbit IgG and then negatively stained with 1% PTA (pH 7.0). Bars, 100 nm.

been reported for other gram-positive microorganisms, such as *S. parasanguinis* (14) and *Actinomyces* spp. (54).

Although *S. salivarius* fimbriae were resistant to dissociation, their biochemical composition was studied by amino acid sequence analysis. Attempts to obtain an N-terminal amino acid sequence by Edman degradation failed as the reaction stopped at the third residue. A similar situation has been reported for the conjugative pili of *E. coli* EDP208, in which the blocking group, an acetyl, has been identified as the N-terminal modification by nuclear magnetic resonance (17). The N-terminal residue of the *Candida albicans* fimbrial subunit is also modified and prevents the Edman degradation reaction. However, in this case the modification has not been determined yet (55). The sequence of an internal peptide obtained by CNBr cleavage revealed interesting details concerning the primary structure of *S. salivarius* fimbriae. The sequence of the first 30 amino acid residues consisted of a repeated motif. Several streptococcal cell surface-associated proteins involved in adhesion, colonization, and immunity contain a number of tandem repeated domains that can vary in size from a few to several hundred amino acids (25). One of the best examples of this is the streptococcal M protein family, in which approximately 80% of the entire molecule is composed of amino acid repeat blocks (13). Other examples are the family of clostridial and streptococcal ligand-binding proteins (51) and the antigenic surface proteins C alpha and Ribs of group B streptococci (40). However, to our knowledge, the presence of such repeated sequences has not been reported for any other bacterial fimbriae, except *S. parasanguinis* FW213 fimbriae (52).

The most unusual feature of *S. salivarius* fimbriae was the presence of modified residues in the repeated motif. These residues (X and/or ϕ) might be glycosylated amino acids since our results clearly demonstrated the presence of carbohydrates in the pure preparation of fimbriae. Glycosylation is a post-translational modification process commonly encountered with surface proteins of eucaryotic cells. Although less common in procaryotes, glycosylation has been reported in archaebacteria and eubacteria (36). The best-characterized procaryotic glycoproteins are surface layer proteins (37). Other surface-associated glycoproteins unrelated to the surface layer proteins have also been reported in eubacteria. Glycosylated flagellins have been described for the gram-negative species *Azospirillum brasilense* and *Campylobacter coli* (36) and the gram-positive species *Clostridium tyrobutyricum* (1). Glycosylation of type 4 pili of *Neisseria meningitidis, Neisseria gonorrhoeae*, and *Pseudomonas aeruginosa* has also been demonstrated (5, 46, 47). There is little information available concerning the biological function of the glycan portion of bacterial glycoproteins. In general, it is inferred that bacterial glycans have protective functions similar to those that have been suggested for eucaryotic glycoconjugates (36). Indeed, glycosylation of bacterial cellulases is known to increase resistance to proteolytic degradation of these proteins and to maintain their conformational stability (44). Recent studies have demonstrated that the

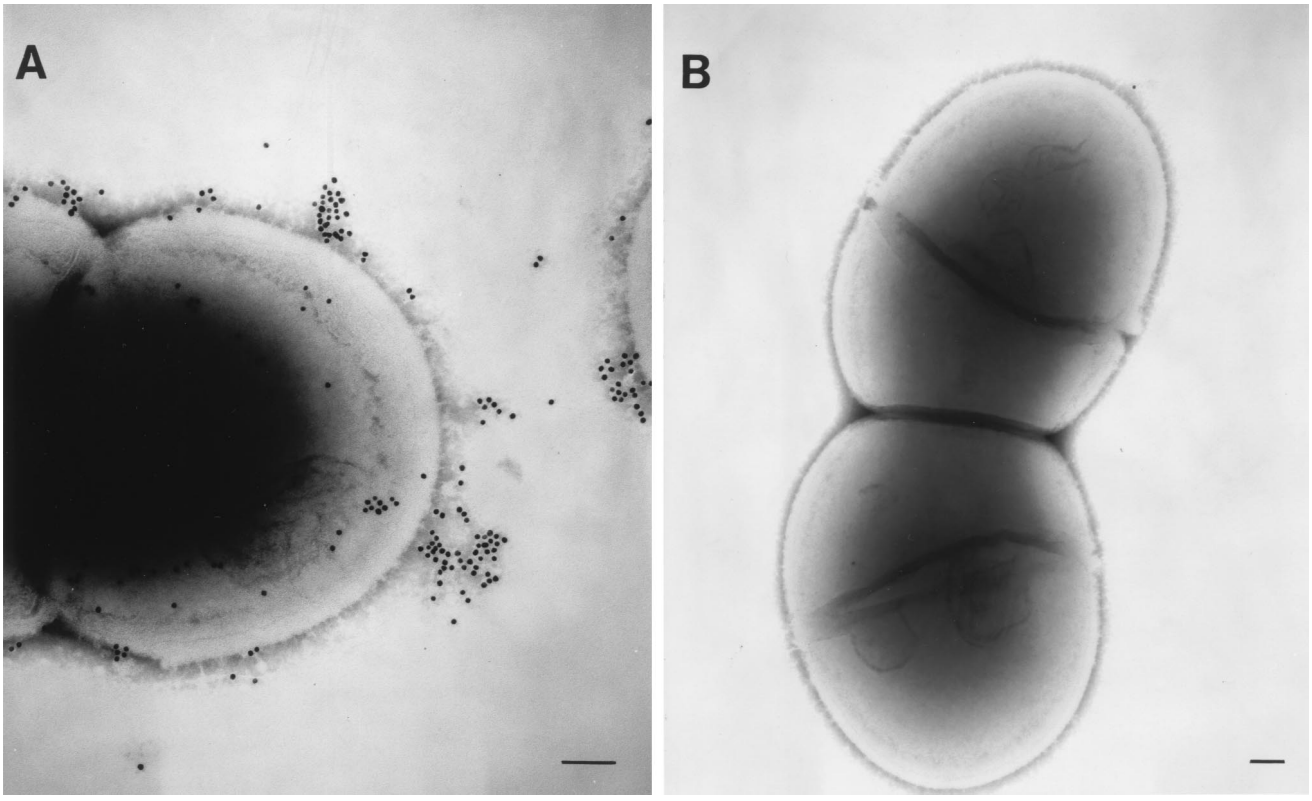

FIG. 4. Immunolocalization of the WGA lectin-binding activity on *S. salivarius* ATCC 25975 (A) and fimbria-negative mutant D37 (B) cell surfaces by electron microscopy. Bacterial cells were incubated with 10-nm colloidal gold-conjugated WGA lectin and negatively stained with 1% PTA (pH 7.0). Bars, 100 nm.

N-linked glycosylation of streptokinase improves the resistance of this protein to proteases (42). The presence of carbohydrates in *S. salivarius* fimbriae could account for their resistance to digestion by proteolytic enzymes. Nevertheless, while the X and ϕ residues may be glycosylated, we cannot rule out the possibility that other modifications of these residues may also occur.

Antifimbria PAbs were also used to study the distribution of antigenically related *S. salivarius* fimbriae among other oral streptococcal species. *S. salivarius* fimbria-like structures were detected in only two other species, *S. mitis* and *S. constellatus*, which belong to the mitis and anginosus groups, respectively. The mitis group contains the largest number of human oral streptococcal species. Most of these organisms are pioneer plaque colonization species and often cause endocarditis (43). Phylogenetic relationships determined by rRNA gene sequence analysis have shown that *S. salivarius* and *S. mitis* are not closely related (27). However, it has been demonstrated that an oral flora containing *S. salivarius* and *S. mitis* is rapidly established soon after birth (34). Since *S. salivarius* and *S. mitis* colonize the mucosal surfaces and dorsum of the tongue, it is possible that an antigenically related filamentous structure is involved in the adherence process. *S. salivarius* and *S. constellatus* are also not closely related (27). Members of the anginosus group are found principally in the gingival crevice and have been associated with abscesses (43). Moreover, studies of organisms involved in periodontal diseases have demonstrated that *S. constellatus* is found at higher levels in patients with refractory periodontitis (8). The conditions in periodontal pockets are very different from those on mucosal surfaces. However, antigenically related filamentous structures could be involved in coaggregation with periodontal disease-associated species. Another possibility is that antifimbria PAbs reacted with a common antigen located in the core structure of the fimbriae that is unrelated to the function of the filamentous structure. Interestingly, TEM observations revealed that fibrils from the K^+ strains of *S. salivarius* were also labeled by Pabs HL-72. This cross-reactivity suggests that the fibrils are antigenically related and possibly structurally related to the fimbriae of *S. salivarius*.

This study represents the most extensive work done to date on *S. salivarius* fimbriae. Taken together, the results suggest that the structure of *S. salivarius* fimbriae is different from the structures of previously described bacterial fimbriae. To obtain more information on the structure and function of *S. salivarius* fimbriae, we are currently working on isolating the genes that code for the structural subunit and fimbria-associated protein(s) using transposon mutagenesis.

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