INFECTION AND IMMUNITY, Jan. 1991, p. 383–389
0019-9567/91/010383-07$02.00/0

Vol. 59, No. 1

Porphyromonas (Bacteroides) gingivalis Fimbrillin: Size, Amino-Terminal Sequence, and Antigenic Heterogeneity

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Bacterial fimbriae mediate cell adhesion and are important in colonization. Fimbrial proteins from strains of *Porphyromonas (Bacteroides) gingivalis* isolated from different individuals were compared for their size, amino-terminal sequence, and antigenic diversity. Two major protein components of the crude fimbrial preparations differed in apparent molecular mass, ranging from 41 to 49 kDa for the fimbrillin monomer and from 61 to 78 kDa for the other major protein. The amino-terminal sequence of the antigenically related group of proteins of the fimbrillin monomer in the 41- to 49-kDa range showed significant homology; however, minor sequence heterogeneity was observed, mainly in residues 4 to 6. One of the observed amino-terminal sequences, AFGVGDDESKVAKLTVMVYNG, resembled the deduced sequence of *P. gingivalis* 381 (D. P. Dickinson, M. K. Kubiniec, F. Yoshimura, and R. J. Genco, J. Bacteriol. 170:1658–1665, 1988). Fimbriae from all the strains of *P. gingivalis* showing this sequence contained a fimbrillin monomer of 43 kDa and showed a strong reaction with both polyclonal and monoclonal antibodies directed to the fimbriae from *P. gingivalis* 2561 (381). Fimbriae from strains showing amino-terminal sequence variations in residues 4 to 6 (i.e., substitution of VGD with either E or NAG) were more diverse in their molecular sizes. Most of these variant fimbriae showed weak reactions with the polyclonal antibodies and no reaction with the monoclonal antibodies induced to the fimbriae of strain 2561. No correlation could be established between the molecular size and immunological reactivity of the fimbrillin monomer of *P. gingivalis* strains. Strains 9-14K-1 and HG 564 not only showed markedly different sequences from the other three amino-terminal sequences but also did not react with either polyclonal or monoclonal antibodies to the fimbriae of strain 2561. Strains W50, W83, and AJW 5 failed to show any immunological reactivity with the antibodies to fimbrillin or fimbriae of strain 2561. Fimbriae from different strains revealed different immunologic reactions with rabbit antisera to each of the synthetic peptides of residues 59-78 (peptide I), 79-100 (peptide J), and 91-108 (peptide E) of strain 381. These results suggest that *P. gingivalis* fimbrillin subunits have size, sequence, and antigenic heterogeneity among the strains and that these differences may be important in the function and immune reactivities of the fimbriae.

Several recent studies have implicated the black-pigmented gram-negative anaerobe *Porphyromonas (Bacteroides) gingivalis* in the pathogenesis of several oral diseases including periodontitis (30), pulpal infections (10, 33), and severe extraoral infections including tonsillar abscesses (3). The first step in microbial colonization of oral surfaces involves the attachment or the adherence of bacteria to the teeth or oral epithelial surfaces. Numerous studies have established that major determinants of the adherence of bacteria to host surfaces are specific interactions mediated by macromolecules on the bacterial surface, called adhesins, that combine with complementary structures on host tissue surfaces (1, 8, 9, 13, 23). Several observations suggest that these bacterial adhesins are frequently present in filamentous surface appendages called pili or fimbriae. Ultrastructural studies of different strains of *P. gingivalis* show the presence of fimbrialike structures (24, 25, 31, 34). Recently, fimbriae have been purified from *P. gingivalis* 381, and it has been found that the fimbriae of this strain are polymers of repeated fimbrillin monomer subunits and that they have an approximate molecular mass of 43 kDa (35, 36). The complete amino acid sequence was predicted from the cloned gene encoding the fimbrillin subunit (5).

Monoclonal antibodies (MAbs) against purified fimbriae from strain 381 block the adherence of *P. gingivalis* to buccal epithelial cells (12). The exact role of the fimbriae in the pathogenesis is not clear; however, adhesion of the bacteria to oral surfaces that is mediated by fimbriae or fimbria-associated adhesins is likely to be important in colonization. Fimbriae from several pathogens are strongly antigenic (6, 7) and have been used to produce vaccines (17, 29). *P. gingivalis* fimbrial adhesins are therefore attractive candidates for a vaccine. If the fimbrial protein is to be a practical *P. gingivalis* vaccine candidate, then at least some of the surface-exposed epitopes of this protein must be common to most of the strains of *P. gingivalis*. Suzuki and coworkers (32) suggested size and antigenic variation of the fimbrillin subunits from different strains of *P. gingivalis*. The present study was undertaken to examine the sizes, amino-terminal sequences, and antigenic diversity of the fimbrillin subunit proteins of *P. gingivalis* strains as a basis of understanding the role of this heterogeneity in fimbrial function and immune reactivity.

MATERIALS AND METHODS

Bacterial strains and culture conditions. The strains of *P. gingivalis* used in this study are shown in Table 1. Most strains are from subgingival plaques of individual patients with periodontal disease, although some strains are from human saliva or infected human root canals. The strains represent a wide geographic distribution, with strains from three cities in the United States and from Germany, Japan, and The Netherlands. All strains of *P. gingivalis* were grown in half-strength (18.5 mg/ml) brain heart infusion broth

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TABLE 1. Strains of *P. gingivalis* used in this study and their sources

Strains	Source	Origin
2561	Plaque	Buffalo, N.Y.
1432	Plaque	Buffalo, N.Y.
1112	Plaque	Buffalo, N.Y.
FAY 19M-1	Plaque	Buffalo, N.Y.
THUR 28BM2	Plaque	Buffalo, N.Y.
9-14K-1	Plaque	Buffalo, N.Y.
EM 3	Root canal	Buffalo, N.Y.
A7A1-28 (ATCC 53977)	Plaque	Arizona
A7A2-10	Plaque	Arizona
JKG 7	Plaque	Ann Arbor, Mich.
W	Oral cavity	Ann Arbor, Mich.
W50 (ATCC 53978)	Clinical specimen	Germany
W83	Clinical specimen	Germany
ESO 75	Plaque	Japan
ESO 101	Plaque	Japan
ESO 127	Plaque	Japan
HG 445	Periodontal abscess	The Netherlands
HG 564	Plaque	The Netherlands
HG 565	Plaque	The Netherlands
AJW 1	Plaque	Buffalo, N.Y.
AJW 2	Plaque	Buffalo, N.Y.
AJW 3	Saliva	Buffalo, N.Y.
AJW 4	Plaque	Buffalo, N.Y.
AJW 5	Plaque	Buffalo, N.Y.

(Difco) supplemented with 5 mg of yeast extract per ml, 5 μg of hemin per ml, and 0.2 μg of menadione per ml and buffered at pH 7.4. The cells were grown at 37°C for 2 days in an anaerobic chamber (85% N_2, 10% H_2, and 5% CO_2).

Fimbria preparations. Fimbria-rich preparations from *P. gingivalis* 2561 (381) were prepared by modifications of the procedure described by Yoshimura and coworkers (35). Briefly, *P. gingivalis* 2561 from 1.5 liters of a brain heart infusion broth culture was harvested by centrifugation at 8,000 × *g* for 25 min at room temperature. The pelleted cells were then suspended in 20 mM Tris buffer, pH 7.4, containing 0.15 M NaCl and 10 mM $MgCl_2$ (TBS-$MgCl_2$). This suspension was agitated on a magnetic stirrer for 4 h at room temperature. The supernatant was obtained by centrifugation at 10,000 × *g* for 30 min at 4°C. Ammonium sulfate was added to the supernatant to make a 40% saturated solution at 4°C. The precipitated proteins were collected by centrifugation at 10,000 × *g* for 25 min at 4°C, suspended in a small volume of 20 mM Tris buffer (TB), pH 8.0, and dialyzed against TB. The dialyzed sample was subjected to further purification on a DEAE Sepharose CL-6B column (2.5 × 25 cm) equilibrated with TB. The column was washed with 200 ml of TB and then eluted with a linear gradient of 0 to 0.5 M NaCl. The fractions were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), and the fimbria-rich fractions were pooled. The partially purified fimbria preparation obtained by 40% ammonium sulfate precipitation was dialyzed against 0.1 M phosphate buffer (pH 7.2) and used as the immunogen for hybridoma production. This preparation contained mainly the 43-kDa component and a trace amount of the 75-kDa component when assessed by SDS-PAGE. A highly purified preparation of fimbriae was obtained by repetitive differential precipitation of the partially purified preparation described above at pH 6.5 in the presence of 1% SDS and 0.2 M $MgCl_2$ at 4°C for 16 to 18 h (unpublished data). This later preparation was used as the immunogen for obtaining polyclonal antiserum.

For Western immunoblot and amino acid sequence analysis, 100-ml cultures of *P. gingivalis* strains were harvested by centrifugation. Each cell pellet was suspended in 30 ml of TBS-$MgCl_2$ and subjected to ultrasonication (Vibra Cell, model VC250; Sonic and Materials Inc.) with a 3-mm microtip at 20 W output on pulse setting with 50% duty cycle for 5 to 10 min in an ice bath. Under these conditions few (<1%) broken cells were observed. The supernatant was obtained by centrifugation at 30,000 × *g* for 30 min at 4°C. The crude fimbriae were precipitated with 40% ammonium sulfate at 4°C followed by dialysis against TB.

Synthesis and characterization of peptides. Three peptides, peptide I (VVMANTGAMELVGKTLAEVK), peptide J (ALTTELTAENQEAAGLIMTAEP), and peptide E (AAG LIMTAEPKTIVLKAG), corresponding to residues 59 to 78, 79 to 100, and 91 to 108, respectively, of the fimbrillin sequence of *P. gingivalis* strain 381 (5), were synthesized. The choice of the peptides for studies of fimbrial antigenicity was based on the secondary structure and hydrophilicity predictions according to the algorithms of Chou and Fasman (4). An additional cysteine residue was placed at the carboxyl terminus of each peptide, so that the peptide could be coupled to the carrier protein with a heterobifunctional cross-linker.

Peptide synthesis was performed by a solid-phase technique (22) by using tertiary butyl oxycarbonyl (t-Boc)-protected amino acids and the *t*-butoxycarbonyl-4-methylbenzyl-L-cysteine phenylacetamidomethyl resin substituted to the extent of 0.22 mmol/g as the solid support. Synthesis was performed in a Beckman model 990 synthesizer, and the peptide chains were assembled by using a double-coupling program. At the end of the program for each amino acid, a sample of resin was removed and tested for completion of coupling by the Kaiser ninhydrin test (14). The peptides were cleaved from the resin with simultaneous removal of the side chain-protecting groups by treatment with hydrogen fluoride for 1 h at 0°C in the presence of anisole. The peptide-resin mixture was washed four times with anhydrous ether to remove anisole, and the peptides were extracted with 5% acetic acid and lyophilized. The crude peptides were purified by semipreparative high-pressure liquid chromatography on an RP-18 column. Amino acid analysis and sequence analysis were performed on each of the peptides synthesized to assess their purity and establish their amino acid sequences.

Preparation and characterization of antisera. Polyclonal rabbit antibodies (PAbs) to fimbriae, fimbrillin, and synthetic peptides were prepared. The highly purified fimbriae of strain 2561 described above were used as the immunogen for the production of antifimbria antibodies. For the preparation of antibodies against the fimbrillin monomer, fimbriae were completely dissociated at 100°C in the presence of 1% SDS and 5% β-mercaptoethanol and then separated on a preparative SDS-polyacrylamide gel as described by Yoshimura and coworkers (36). The fimbrillin band was eluted from the gel and used as the immunogen.

For the production of PAbs to synthetic peptides, each peptide was conjugated to thyroglobulin by using *m*-maleinimido benzoyl-*N*-hydroxysuccinimide ester (MBS) as a cross-linker, as described by Schmidt and coworkers (27). Briefly, 10 mg of thyroglobulin, dissolved in 3 ml of phosphate-buffered saline (PBS) (pH 7.4), was activated by mixing with 5 mg of MBS in 1 ml of *N*,*N*-dimethylformamide. The solution was stirred for 2 h at room temperature, and unreacted MBS was removed by gel filtration on Sephadex G-25 in 0.1 M phosphate buffer, pH 6.0. Synthetic

peptides (5 mg each) were reduced with sodium borohydride, and excess borohydride was destroyed with acetic acid. The neutralized and reduced peptides were combined with MBS-activated thyroglobulin and stirred overnight at room temperature. The resulting peptide-carrier conjugate was subsequently isolated by gel filtration on Sephadex G-25 in 0.1 M ammonium bicarbonate buffer, pH 8.0.

New Zealand White rabbits (3 kg) were injected at multiple sites subcutaneously with 50 μg of the fimbriae or fimbrillin or with 500 μg of the appropriate conjugated peptide-carrier in complete Freund's adjuvant. Starting two weeks after the first injection, the rabbits were boosted weekly for four weeks with the respective immunogens in incomplete Freund's adjuvant. Six weeks after the first injection, each rabbit was bled and the antibodies were tested against the corresponding antigen by an enzyme-linked immunosorbent assay (ELISA) and Western blotting. After the appropriate antibody titer was obtained, the rabbits were bled by heart puncture and the sera were prepared and stored at −20°C.

MAb production. MAbs to *P. gingivalis* 2561 fimbriae were generated by the procedure of Köhler and Milstein (15) as modified by McKearn (20). The fimbriae, partially purified by chromatography on DEAE Sepharose CL-6B as described above, were used as the immunogen. Eight-week-old BALB/c mice were immunized subcutaneously with 100 μg of the fimbriae in complete Freund's adjuvant. The mice were boosted with the same amount of fimbriae in incomplete Freund's adjuvant three times at weekly intervals. Three days before sacrifice, the fimbriae in 0.1 M PBS (pH 7.2) were injected intraperitoneally into the mice. Spleen cells from the immunized mice were hybridized with SP 2/0 Ag-14 mouse myeloma cells by the procedure described previously (2). Briefly, spleen cells and myeloma cells were mixed in a ratio of 1:0.2 and fused with polyethylene glycol 3400 at a final concentration of 50%. Hybrid cells surviving hypoxanthine-aminopteridine-thymidine nutritional selection were tested for antibody secretion by ELISA by using microtiter plates coated with 1 μg of the partially purified fimbriae per well. Positive clones were subcloned by limiting dilution at an average cell density of less than 1 cell per well on normal mouse spleen cells as feeder cells. Only wells showing a single colony on microscopic examination were further expanded. The culture supernatant of each monoclone was tested for immunoreactivity against fimbriae by Western blotting. The confirmed clones producing MAbs against fimbriae were injected into BALB/c mice previously primed with 0.5 ml of Pristane, and ascites fluid was collected. After adding 0.05% sodium azide, the ascites fluid was kept at 4°C. Ascitic fluid from only one of the clones was highly reactive with the fimbriae and was identified as an immunoglobulin G1 (IgG1) isotype by ELISA and double immunodiffusion. This ascitic fluid was used in the subsequent studies.

SDS-PAGE and immunoblot analysis. SDS-PAGE was performed by the method of Laemmli (16) by using a 10% running gel (Mini-PROTEAN II Cell; Bio-Rad). For immunoblot analysis, the proteins separated by SDS-PAGE were transferred to nitrocellulose membranes by using the PolyBlot transfer system (Model SBD-1000; American Bionetics). The unoccupied binding sites on the membranes were blocked by incubating for 1 h with Tris-buffered saline (20 mM Tris base, 0.5 M NaCl [pH 7.5]) containing 1% bovine serum albumin. The membranes were then incubated with predetermined dilutions of either MAbs or rabbit PAbs for 3 h. The membranes were washed and treated with 1:500-diluted goat anti-mouse IgG or anti-rabbit IgG conjugated with horseradish peroxidase (Bio-Rad) for 1 h. After being washed, the bound antibodies were visualized by addition of the 4-chloro-1-naphthol color developing reagent (Bio-Rad). The reaction was stopped by immersing the membranes in distilled water.

Automated Edman degradation. The amino acid sequence of synthetic peptides as well as the fimbrillin monomer separated by SDS-PAGE was determined by automated stepwise sequencing on an Applied Biosystems model 477A gas-phase sequencer with an on-line model 120A PTH analyzer. The chemicals used for both instruments were obtained from Applied Biosystems, Inc. Synthetic peptide samples, containing 200 to 500 pM peptide, were spotted on a glass-fiber disk, previously loaded with Biobrene and precycled three times. PTH amino acid analysis were performed on a PTH-C_{18} cartridge column (2.1 × 220 mm; Applied Biosystems) packed with octylsilyl-type sorbent (5 μM). Separation of PTH amino acids was obtained at 55°C with a linear gradient of 11 to 40% solvent A (5% aqueous tetrahydrofurane, 18 ml of 3 M acetate buffer [pH 3.8], and 2.5 ml of 3 M acetate buffer per liter [pH 4.6]) at a flow rate of 0.2 ml/min. Solvent B was acetonitrile containing 500 nM of DMPTU per liter.

The amino-terminal amino acid sequences of the fimbrillin subunit protein from different strains of *P. gingivalis* were determined in the gas-phase sequencer by the procedure described by Matsudaira (19). Briefly, the crude fimbriae (0.5 mg of protein per gel), obtained by the ultrasonication procedure described above, were separated by preparative SDS-PAGE on the mini-gel system. The proteins were transferred onto polyvinylidene difluoride membranes (Immobilon; Millipore) on the PolyBlot transfer system for 40 min at 2.5 mA/cm^2 of gel. After staining with 0.1% Coomassie brilliant blue for 2 min, the membranes were decolorized with destaining solution (60% methanol, 7% acetic acid). The membranes were washed twice with deionized water on a shaker for 10 min each. The fimbrillin bands were excised with a clean razor blade, dried, and then placed in the sequencer.

RESULTS

Molecular size of fimbriae. During the course of our study of the structure and function of *P. gingivalis* fimbriae, we found that fimbrial preparations from strain 2561 often contained two major protein components, a 43-kDa protein, which constitutes the major component of fimbriae, and a 75-kDa protein, as well as a few minor protein components. In order to compare the sizes and antigenic heterogeneity of the 43-kDa components, crude fimbrial extracts from different strains of *P. gingivalis* were subjected to SDS-PAGE. Figure 1 shows the SDS-polyacrylamide gel of a representative group of these strains, indicating the heterogeneity of size. There are two major bands for each strain; one is in the 41- to 49-kDa range, and another is in the 61- to 78-kDa range. We have successfully separated the 43-kDa fimbrial component from the higher-molecular-mass aggregate of strain 2561 and have produced PAbs and MAbs against this fimbrial component. Components in the 41- to 49-kDa range comprise an antigenically related group of proteins, the fimbrillin monomer, of all strains tested except for strains W50, W83, and AJW 5. Strains W50, W83, and AJW 5 have little detectable material in the 41- to 49-kDa range. We will focus on the 41- to 49-kDa fimbrial components in this report.

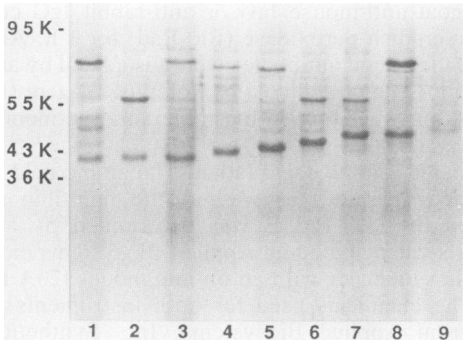

FIG. 1. SDS-PAGE analysis of crude fimbriae from representative strains of *P. gingivalis*. Four micrograms of the fimbriae per lane was treated at 100°C for 7 min in the presence of β-mercaptoethanol to obtain the fimbrillin monomers of completely dissociated fimbriae. The fimbriae were then electrophoresed on an SDS–10% polyacrylamide gel and stained with Coomassie blue R-250. Lanes: 1, JKG 7; 2, 9-14K-1; 3, FAY 19M-1; 4, 2561; 5, EM 3; 6, HG 445; 7, A7A1-28; 8, THUR 28BM2; 9, W50.

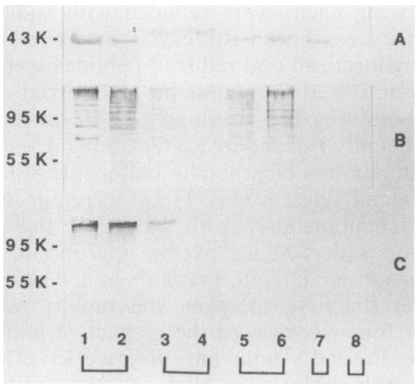

FIG. 2. Immunoblot of completely dissociated fimbriae of the representative strains showing different amino-terminal sequences with PAbs and MAbs. The SDS–10% polyacrylamide gels were electrophoretically transferred to nitrocellulose membranes and incubated with the antibodies at a dilution of 1:200. The second antibody was goat anti-rabbit IgG or mouse IgG-horseradish peroxidase conjugate (1:500). The panels show immunoreactivity of completely dissociated fimbriae (100°C; 7 min in the presence of β-mercaptoethanol) with PAbs raised against the fimbrillin subunit of strain 2561 (A) and immunoreactivity of partially dissociated fimbriae (80°C; 5 min in the absence of β-mercaptoethanol) with PAbs (B) and MAbs (C) raised against intact polymeric fimbriae. Lanes: 1, 2561; 2, ESO 75; 3, A7A1-28; 4, THUR 28BM2; 5, FAY 19M-1; 6, AJW 1; 7, 9-14K-1; 8, W50. Brackets indicate representative strains showing identical amino-terminal sequences.

Antigenic heterogeneity of fimbriae. In order to assess the antigenic heterogeneity of the fimbrillin components from different strains of *P. gingivalis*, PAbs raised against the 43-kDa fimbrillin (monomer) and fimbriae (polymer) and MAbs against the fimbriae of *P. gingivalis* 2561 were used. Antigens for the immunoblot analysis were prepared by heating the crude fimbrial preparations either at 100°C in the presence of β-mercaptoethanol to obtain the monomeric (completely dissociated) fimbrillin or at 80°C in the absence of β-mercaptoethanol to obtain the oligomeric (partially dissociated) fimbriae. The anti-fimbrillin PAbs reacted only with a single protein band appearing in the molecular mass range of 41 to 49 kDa of all strains of *P. gingivalis* tested. However, no immunoreactive band was observed for strains W50, W83, and AJW 5 (Fig. 2A; Table 2). The antifimbrillin PAbs did not react with oligomeric fimbriae. On the other hand, the PAbs to intact fimbriae reacted with oligomeric fimbriae from some but not all strains (Fig. 2B; Table 2). The MAb reacted with the oligomeric fimbriae of strains 2561, W, 1432, 1112, ESO 75, ESO 101, HG 565, AJW 2, A7A1-28, and A7A2-10 (Fig. 2C; Table 2). The MAbs did not show immunological cross-reactivity with other strains tested. These results suggest that considerable antigenic heterogeneity exists for the 41- to 49-kDa polymeric fimbriae of *P. gingivalis*.

Amino-terminal sequence of fimbriae. The sequences of the amino-terminal 20 residues of the immunoreactive 41- to 49-kDa protein bands were determined by solid-phase microsequencing. Proteins were separated on the SDS-polyacrylamide gels and electroblotted onto polyvinylidene difluoride membranes. The membranes were stained with Coomassie blue, and the fimbrillin bands in the 41- to 49-kDa range, previously shown to be immunoreactive with antifimbrillin PAbs, were used for amino acid sequence analysis. For the sake of comparison, different strains with identical amino-terminal sequences have been grouped together in Fig. 3. The amino-terminal sequence of strain 381, obtained from the cloned gene, has been included in Fig. 3 for reference. Recent observation has suggested that strains 381 and 2561 are closely related, as they show similar restriction endonuclease patterns (18). With the exception of strains 9-14K-1 and HG 564, all the strains tested showed sequence identity for the first three amino acids and then from amino acids 7 to 20. However, sequence heterogeneity exists from residues 4 to 6. The amino-terminal sequences of strains 9-14K-1 and HG 564 were more distinct, yet 12 of 20 residues were identical to the other strains (Fig. 3). Although no immunologically reactive protein could be detected for strains W50, W83, and AJW 5, efforts were made to obtain the amino acid sequence of Coomassie blue-stained material in the 41- to 49-kDa range. None of these proteins from strain W50 showed amino-terminal sequence patterns similar to the fimbrial sequences of the other strains.

Reactivity of fimbriae with PAbs to synthetic peptides. PAbs to the synthetic peptides E, I, and J, representing residues in the stretch from 59 to 108 in the predicted fimbrillin sequence of strain 381 (5), were used for immunoblot analysis to determine whether heterogeneity exists in the internal region of the amino-terminal portion of the fimbrillin subunits. Figure 4 shows immunoreactivity of the anti-synthetic peptide antibodies with the 100°C-treated fimbriae from the representative strains of each group. Anti-synthetic peptide E (anti-E) recognized many of the strains tested but did not react with strains JKG 7, 9-14K-1, or HG 564. Anti-J also reacted with many of the strains tested, but not with strains AJW 4 or JKG 7. Anti-I showed negative reactions with strains THUR 28BM2, AJW 3, JKG 7, FAY 19M-1, and AJW 1. A low dilution of the PAbs (1:30 to 1:50) to the peptides was used because the PAbs usually revealed weak positive reactions. None of the PAbs to the peptides reacted with W50, W83, and AJW 5, even at the lowest dilution tested (1:30). Details of the immunoreactivity of the PAbs with different fimbrillin and correlation with other properties are shown in Table 2.

DISCUSSION

This study presents a survey of the structural and antigenic variation in the fimbrillin subunits of different strains of

TABLE 2. Comparison of fimbrial proteins from different strains of *P. gingivalis*

Strain	Mol. size (kDa)[a]	PAb anti-fimbrillin[b]	PAb anti-fimbriae[c]	MAb anti-fimbriae[d]	PAb anti-synthetic peptides[e]		
					E	I	J
2561	43	+	+	+	+	+	+
W	43	+	+	+	+	+	+
1432	43	+	+	+	+	+	+
1112	43	+	+	+	+	+	+
ESO 75	43	+	+	+	+	+	+
ESO 101	43	+	+	+	+	+	+
HG 565	43	+	+	+	+	+	+
AJW 2	43	+	+	+	+	+	+
A7A1-28	49	+	+(w)[f]	+	+	+	+
A7A2-10	49	+	+(w)	+	+	+	+
AJW 4	49	+	−	−	+	+	−
THUR 28BM2	49.5	+	±[g]	−	+	−	+
AJW 3	41	+	+(w)	−	+	−	+
JKG 7	40.5	+	−	−	−	−	−
FAY 19M-1	41.5	+	+	−	+	−	+
AJW 1	42	+	−	−	+	−	+
ESO 127	49	+	±	−	+	+	+
HG 445	46.5	+	±	−	+	+	+
EM 3	45.5	+	+(w)	−	+	+	+
9-14K-1	41	+	−	−	−	+	+
HG 564	41	+	−	−	−	+	+
W50		−	−	−	−	−	−
W83		−	−	−	−	−	−
AJW 5		−	−	−	−	−	−

[a] Molecular (Mol.) size of the fimbrillin band reactive with polyclonal rabbit antiserum to the dissociated monomeric 43-kDa component (fimbrillin) from strain 2561. Strains W50, W83, and AJW 5 did not show any immunologically reactive bands.

[b] Reactivity of the polyclonal rabbit antiserum to the fimbrillin from strain 2561 with completely dissociated fimbriae (100°C; 7 min, in the presence of β-mercaptoethanol).

[c] Reactivity of the polyclonal rabbit antiserum to the purified 43-kDa polymeric fimbriae from strain 2561 with partially dissociated oligomeric fimbriae (80°C; 5 min in the absence of β-mercaptoethanol).

[d] Reactivity of the MAb to the 43-kDa polymeric fimbriae from strain 2561 with partially dissociated oligomeric fimbriae.

[e] The anti-synthetic peptide antibodies were tested against completely dissociated fimbriae from the 24 strains tested.

[f] Weak positive.

[g] Very weak positive.

P. gingivalis obtained from a wide geographic distribution. Most of the strains tested, with the exception of W50, W83, and AJW 5, showed an immunologically cross-reactive protein in the 41-to-49-kDa range when tested by immunoblot analysis using PAbs to the fimbrillin monomer purified from *P. gingivalis* 2561. The size diversity of the components which reacted with antifimbrillin from different strains has been suggested by Suzuki and coworkers (32). They reported that 15 of 63 clinical isolates of *P. gingivalis* had fimbrillin proteins with an apparent molecular mass of 46 kDa, while other isolates had a 43-kDa fimbrillin. In the present study we have found that strain variation in the molecular size of fimbrillin is considerable, with components from various strains appearing as 41, 42, 43, 46, and 49 kDa (Fig. 1).

In addition to the size variation, there appears to be immunological diversity among the fimbriae of *P. gingivalis* strains, as is apparent from the immunoblots using PAbs and

Strains	Sequence
	5 10 15 20
381[a]	AFGVGDDESKVAKLTVMVYNG
2561	AFGVGDDESKVAKLTVMVYNG
W	AFGVGDDESKVAKLTVMVYNG
1432	AFGVGDDESKVAKLTVMVYN
1112	AFGVGDDESKVAKLTVMVYNG
ESO 75	AFGVGDDESKVAKLTVMVYNG
ESO 101	AFGVGDDESKVAKLTVMVYNG
HG 565	AFGVGDDESKVAKLTVMVYN
AJW 2	AFGVGDDESKVAKLTVMVYNGE
A7A1-28	AFG E DESKVAKLTVMVYNGE
A7A2-10	AFG E DESKVAKLTVMVYNGE
AJW 4	AFG E DESKVAKLTVMVYNGE
THUR 28BM2	AFG E DESKVAKLTVMVYNGE
AJW 3	AFG E DESKVAKLTVMVYNGE
JKG 7	AFG E DESKVAKLTVMVYNGE
FAY 19M-1	AFGNAGDESKVAKLTVMVYNG
AJW 1	AFGNAGDESKVAKLTVMVYNG
ESO 127	AFGNAGDEAKVAKLTVMVYNG
HG 445	AFGNAGDEAKVAKLTVMVYNG
EM 3	AFGNAGDEAKVAKLTVMVYNG
9-14K-1	AVGDGLADAKITKLTAMVYA
HG 564	AVGDGLADAKITKLTAMVYAG

FIG. 3. Fimbriae grouped on the basis of the sequences of the first 20 amino-terminal amino acids of the fimbrillin proteins from different strains of *P. gingivalis*. The boxed region indicates major variation in the sequences of the fimbrillin. For sequencing, the crude fimbriae (0.5 mg of protein) were separated on a preparative SDS–10% polyacrylamide gel and then electroblotted onto polyvinylidene difluoride membrane. The membrane was stained with 0.1% Coomassie blue. After destaining, the stained fimbrillin band was excised and placed in the sequencer. [a], Reference sequence from the cloned gene of strain 381 (5).

MAbs to fimbriae. PAbs against native fimbriae from *P. gingivalis* 2561 reacted strongly with oligomeric fimbrial preparations (80°C for 5 min) of most but not all strains (16 of 21) with detectable fimbrillin. PAbs against monomeric fimbrillin (denatured at 100°C) reacted with all 21 strains, demonstrating that although they are distinct, all of the fimbriae also share antigenicity. MAbs to the fimbriae from strain 2561 cross-reacted with those strains which contained a 43-kDa fimbrillin subunit, as well as with strains A7A1-28 and A7A2-10, which have a 49-kDa fimbrillin subunit, but not with the other strains. No immunologically reactive fimbrillin component was observed for strains W50, W83,

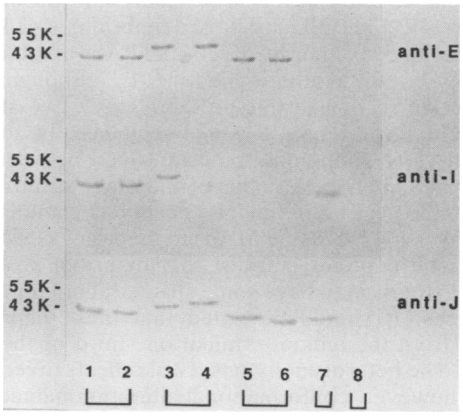

FIG. 4. Immunoreactivity of completely dissociated fimbriae with PAbs to synthetic peptides E, I, and J. Protein-transferred nitrocellulose membranes were incubated with anti-peptide antibodies (1:30 to 1:50) and then treated with goat anti-rabbit IgG-horseradish peroxidase conjugate (1:500). The fimbrial samples and lanes were the same as those presented in Fig. 2.

and AJW 5, even when a threefold excess of antigens or high concentrations of antibodies were used. This result is consistent with the observation by Suzuki and coworkers (32), who could not find any immunologically reactive fimbrial component in strain W83 and several other strains which were not tested in this study. In the case of W50, none of the Coomassie blue-stained bands around the 41- to 49-kDa range showed an amino-terminal sequence similar to the sequence observed for the other strains (data not shown). However, these strains appear to be sparsely fimbriated under an electron microscope (11, 21, 32; unpublished data), and it is not known whether these strains contain totally unrelated fimbrialike structures or whether they are too sparsely fimbriated to be detected by immunological techniques we used.

We have further expanded studies of the size and immunological heterogeneity of the strains of *P. gingivalis* on the basis of the amino acid sequences of the first 20 residues from different strains. Although all strains revealed considerable amino-terminal sequence homology, differences were observed. Fimbrillin subunits with identical molecular size generally showed identical sequences for the first 20 residues (Table 2). All the strains tested, with the exception of 9-14K-1 and HG 564 (Fig. 3), had an Ala-Phe-Gly tripeptide as the first three residues and then either a deletion or a variation at positions 4 to 6, compared with the amino acid sequence of strain 381 (5). The variable region is followed by a conserved segment of residues 7 to 20. Strains 9-14K-1 and HG 564 have a sequence for the first 20 amino acids that varies notably from the other strains. These observations reveal that with the exception of residues 4 to 6, much of the amino-terminal sequence is conserved in most of the strains of *P. gingivalis*. This pattern is similar to the conserved amino-terminal sequence heterogeneity observed for *Neisseria gonorrhoeae* (26, 28), although no homology was found between *P. gingivalis* and the gonococci in the first 20 amino acids. Analysis of the entire amino acid sequence of fimbriae from representative strains of *P. gingivalis* will be necessary to establish whether there are discrete fimbrial types, as is only suggested by the results reported here.

It is interesting to note that all the strains showing amino-terminal sequence identity to strain 2561 not only have identical molecular size of the fimbrillin monomer but also show immunological cross-reactivity with MAbs raised against intact fimbriae of strain 2561. On the other hand, strains 9-14K-1 and HG 564 have a fimbrillin of 41 kDa which is not recognized either by MAbs and PAbs to intact fimbriae or by PAbs to synthetic peptide E, although PAbs to fimbrillin do recognize this protein. These two strains also have an identical amino-terminal sequence. In contrast, no clear-cut correlation could be established between size and immunological characteristics of the other strains.

Immunological differences observed among various strains by using PAbs and MAbs to fimbriae, as well as PAbs to synthetic peptides, suggest that fimbrillin proteins from different strains may have some structural or conformational differences. It should be noted that these peptides were derived from the amino-terminal one-third of the fimbrillin protein. The heterogeneity observed reflects diversity in this region; however, conformational alteration induced by variation at other sites cannot be ruled out from the present data. Additional information is needed to confirm this heterogeneity. In order to avoid any possibility that the apparent lack of cross-reactivity has resulted from conformational artifacts arising from the disaggregation methods employed, precaution was taken to treat fimbriae from all strains under identical conditions. Despite the marked antigenic, size, and sequence heterogeneity, there appear to be common epitopes among the fimbriae of *P. gingivalis*, as demonstrated by extensive cross-reactivity with PAbs to the fimbrillin monomer and with anti-E and anti-J peptide antibodies. Full evaluation of serotypes of *P. gingivalis* fimbriae requires testing a large battery of strains with antisera prepared against representative fimbriae from tentative clusters of strains, such as those identified by the present study. We are developing methods for the purification of intact fimbriae from different strains of *P. gingivalis* for such studies. Peptide mapping of fimbrillin and complete sequence analysis of fimbrillin genes from representative isolates of tentative strain clusters may also help in understanding the nature and basis of variation in fimbrillin monomers from *P. gingivalis*.

P. gingivalis strains show variations in size, amino-terminal sequence, and antigenicity of fimbriae; however, the role of this variability in the function of the fimbriae is as yet unclear. Variation in antigenicity, for example, has been shown to be important in modulating the immune response to fimbriae from other organisms such as *N. gonorrhoeae*, and further experiments will be needed to determine if this is the case for *P. gingivalis*.

ACKNOWLEDGMENTS

This study was supported in part by U.S. Public Health Service grants DE08240, DE07034, DE04898, and DE06514.

The authors thank Darlene Badgett for her excellent technical assistance and Karen Flowers for her help in typing the manuscript.

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