JOURNAL OF BACTERIOLOGY, Feb. 1994, p. 1052–1059
0021-9193/94/$04.00+0

Vol. 176, No. 4

Inactivation of the *Porphyromonas gingivalis fimA* Gene Blocks Periodontal Damage in Gnotobiotic Rats

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Received 12 May 1993/Accepted 2 December 1993

Fimbrial production by *Porphyromonas gingivalis* was inactivated by insertion-duplication mutagenesis, using the cloned gene for the *P. gingivalis* major fimbrial subunit protein, *fimA*. By several criteria, this insertion mutation rendered *P. gingivalis* unable to produce fimbrilin or an intact fimbrial structure. A nonfimbriated mutant, DPG3, hemagglutinated sheep erythrocytes normally and was unimpaired in the ability to coaggregate with *Streptococcus gordonii* G9B. The cell surface hydrophobicity of DPG3 was also unaffected by the loss of fimbriae. However, DPG3 was significantly less able to bind to saliva-coated hydroxyapatite than wild-type *P. gingivalis* 381. This suggested that *P. gingivalis* fimbriae are important for adherence of the organism to saliva-coated oral surfaces. Further, DPG3 was significantly less able to cause periodontal bone loss in a gnotobiotic rat model of periodontal disease. These observations are consistent with other data suggesting that *P. gingivalis* fimbriae play an important role in the pathogenesis of human periodontal disease.

Porphyromonas gingivalis is a gram-negative anaerobe that is believed to cause certain forms of adult periodontitis (19, 28, 31, 42). Several reports have indicated that *P. gingivalis* fimbriae are important for mediating adherence to host tissues and other oral bacteria, and fimbriae are probably an important virulence determinant of this organism. Isogai et al. (8) showed that an antifimbrial monoclonal antibody blocked adhesion of *P. gingivalis* to human buccal epithelial cells. Watanabe et al. (39) demonstrated that certain strains of *P. gingivalis* adhered to human gingival and periodontal ligament fibroblasts and to human epithelial cells and suggested that fimbriae were involved in this process. Watanabe et al. (39) further suggested that *P. gingivalis* fimbriae may affect cell surface hydrophobicity, as *P. gingivalis* strains capable of adhering to human cells were fimbriated and more highly hydrophobic than nonadherent *P. gingivalis* strains. *P. gingivalis* was shown to bind to saliva-coated hydroxyapatite (sHAP) beads in a concentration-dependent manner, and this binding was competitively inhibited by purified fimbriae (12). Synthetic peptides derived from the predicted amino acid sequence of the FimA protein of *P. gingivalis* 381 also blocked *P. gingivalis* adherence to sHAP (12). Immunization with purified *P. gingivalis* fimbriae protected against periodontal destruction in a gnotobiotic rat model of periodontal disease (4). These studies suggested that fimbriae are important for adherence to saliva-coated host oral surfaces and may therefore be important for initial interactions of *P. gingivalis* with the oral cavity. Some early studies associated fimbriae with *P. gingivalis* hemagglutination (22, 29), but Yoshimura et al. (41) suggested that fimbriae were not involved in hemagglutination. Most other studies have indicated that fimbriae are not involved in hemagglutination, and this adherence mechanism is dependent upon other outer membrane structures, possibly cell surface proteases or hemagglutinins that are closely associated with proteases on the surface of the organism. In addition to adherence to host structures, bacterial coadherence may also be important for colonization of the oral cavity by periodontopathogens (1, 5, 22, 29). *P. gingivalis* can coadhere with several gram-positive bacteria, including *Actinomyces viscosus*, *Actinomyces naeslundii*, and *Actinomyces israelii* (6, 13, 26) and *Streptococcus mitis* (21). Coadherence of *P. gingivalis* with *A. viscosus* was inhibited by purified rabbit antifimbrial immunoglobulin G (6), suggesting that fimbriae are involved in this process. Stinson et al. (33) demonstrated coadherence of *P. gingivalis* with *Streptococcus gordonii* (formerly *Streptococcus sanguis*), but the role of fimbriae in mediating this adherence was not studied.

In this study, we used targeted insertional mutagenesis (7, 30) to inactivate the *P. gingivalis fimA* gene. Internal coding sequence from the *fimA* gene (2) of *P. gingivalis* 381 was cloned into pVAL-7 and conjugally mobilized into *P. gingivalis* 381, disrupting the *fimA* gene. We found that the resulting insertion-duplication mutants did not produce fimbrillin or intact fimbriae. Further analysis of a nonfimbriated mutant indicated that the bacteria were unable to adhere to sHAP and unable to induce bone loss in gnotobiotic rats.

MATERIALS AND METHODS

Bacterial strains, growth conditions, and plasmids used. The strains and plasmids used are shown in Table 1. *Escherichia coli* strains were grown at 37°C in Luria-Bertani (LB) broth (17) or on LB agar. Stocks were maintained at −70°C in LB plus 15% glycerol, with appropriate antibiotics when necessary (see below). *P. gingivalis* strains were routinely grown on blood agar (BA), which consisted of tryptic soy agar (Scott Laboratories, W. Warwick, R.I.) supplemented with 1 mg of yeast extract (Difco Laboratories, Detroit, Mich.) per ml), 5% defibrinated sheep blood (Crane Laboratories, Syracuse, N.Y.), 0.2 μg of menadione per ml, and 15 μg of hemin per ml. *P. gingivalis* broth cultures were grown in HK broth, which consisted of half-strength brain heart infusion (Difco) containing the same concentrations of menadione and hemin as in BA

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TABLE 1. Strains and plasmids used in this study[a]

Bacterial strain or plasmid	Relevant genotype	Source or reference
E. coli strains		
HB101	Gm^s Ap^s Tp^s Str^r	SUNYAB collection
J53	Ap^s Tp^s Str^s	A. A. Salyers
P. gingivalis strains		
381	Em^s Gm^r	SUNYAB collection
DPG3	Em^r Gm^r (insertion of pDTH2 into *fimA* gene with 3′ duplication of *Hin*cII fragment)	This study
DPG5	Em^r Gm^r (insertion of pDTH2 into *fimA* gene with 5′ duplication of *Hin*cII fragment)	This study
S. gordonii G9B		M. Stinson
Plasmids		
pUC13Bgl2.1	Ap^r *fimA*	2
pVAL-7	Ap^r (*E. coli*), Em^r (*P. gingivalis*), Mob^+ Rep^-	7
R751	Tp^r	7
pDTH2	Like pVAL-7	This study

[a] Abbreviations: Gm^r, gentamicin resistant; Ap^r, ampicillin resistant; Tp^r, trimethoprim resistant; Mob, mobilizable; Rep, replication in *P. gingivalis*; SUNYAB, State University of New York at Buffalo.

plates. HK agar plates were supplemented with 15% agar (Difco). *P. gingivalis* strains were grown at 37°C in an anaerobe chamber (Forma Scientific, Marietta, Ohio) with an atmosphere of 5% CO_2, 10% H_2, and 85% N_2. *P. gingivalis* strains were stored frozen in sheep blood at −70°C. *S. gordonii* G9B (formerly *S. sanguis* G9B) was grown either aerobically in HK broth at 37°C or anaerobically in a chemically defined medium (34) at 34°C. Plasmids were maintained in culture by selection with the following antibiotics: pUCBg12.1, pVAL-7, and pDTH2 in *E. coli*, 50 μg of ampicillin per ml; pDTH2 in *P. gingivalis*, 5 μg of erythromycin per ml; and R751 in *E. coli*, 100 μg of trimethoprim per ml. Chemicals were obtained from Sigma Chemical Co. (St. Louis, Mo.) unless otherwise noted.

Isolation of plasmid and genomic DNAs. Genomic DNA was isolated from *P. gingivalis* according to the method of Wilson (40), with the following additional step. The DNA was treated with 100 μg of RNase A per ml for 30 min at 37°C, precipitated with isopropanol, and suspended at 100 μg/ml of TE (10 mM Tris-HCl [pH 7.4], 1 mM EDTA).

Plasmids pVAL-7 and pUCBg12.1 were purified from *E. coli* by a modification of the alkaline lysis method described by Maniatis et al. (17). Changes included incubation of the cell pellet in solution I for 30 min, followed by addition of only 10 ml of solution II and 7.5 ml of solution III. The DNA pellet was redissolved in 8 ml of TE, and the plasmid DNA was further purified by CsCl-ethidium bromide density gradient centrifugation (17).

Plasmid DNA from *E. coli* transformants was isolated by the alkaline lysis miniprep method described by Maniatis et al. (17). Transformants were grown overnight in LB broth plus ampicillin. Digestion of the cell wall was facilitated by the addition of lysozyme to a final concentration of 5 mg/ml and incubation on ice for 30 min.

Construction of pDTH2. All restriction and DNA-modifying enzymes were purchased from Promega (Madison, Wis.) except *Cla*I, which was obtained from New England Biolabs (Beverly, Mass.). Plasmid pUC13Bgl2.1 was digested with *Hin*cII to isolate the 850-bp internal coding sequence fragment of the fimbrillin gene. *Hin*cII cleavage of pUCBg12.1 also generated a second, 950-bp fragment that contained sequences 5′ to the *fimA* gene. To allow for easier purification of the desired 850-bp fragment, the *Hin*cII digest was ethanol precipitated and subsequently digested with *Taq*I, which cuts the 950-bp *Hin*cII fragment. This rendered the 850-bp fragment easily isolated by agarose gel electrophoresis. The digestions were performed for 2 h in buffers supplied by the manufacturer. The doubly digested plasmid was electrophoresed on a 1.2% agarose gel for 3 h at 5 V/cm in TBE buffer (89 mM Tris, 89 mM boric acid, 2 mM EDTA). The DNA was visualized by soaking the gel in ethidium bromide, and the 850-bp *Hin*cII fragment was excised with a clean razor blade and electroeluted from the gel slice into a Spectra/Por 2 dialysis bag (Spectrum Medical Industries, Los Angeles, Calif.) (17). The DNA was then purified over a NACS PREPAC Convertible column according to the protocol supplied by the manufacturer (BRL-Life Technologies, Gaithersburg, Md.). After elution from the column, the DNA was precipitated with 2 volumes of cold ethanol and suspended in 50 μl of TE.

Plasmid pVAL-7 was digested with *Eco*RV for 2 h, and the enzyme was inactivated by heating at 70°C for 10 min. The plasmid was dephosphorylated by using calf alkaline intestinal phosphatase (17), ethanol precipitated, and suspended in 10 μl of TE. The purified 850-bp *Hin*cII *fimA* fragment was ligated with *Eco*RV- and calf alkaline intestinal phosphatase-treated pVAL-7, using conditions specific for blunt-end ligation at 15°C (17). The ligation mixture was ethanol precipitated at −70°C and suspended in distilled water for electroporation into *E. coli* HB101. Mini-plasmid preparations of transformants were digested with *Pst*I, *Hin*cII, or *Pvu*II to examine the recombinant plasmids.

Electroporation and conjugation. DNA was introduced into *E. coli* HB101 by using the Bio-Rad Gene Pulser as described by the manufacturer. Conjugal transfer of plasmids between *E. coli* strains and between *E. coli* and *P. gingivalis* were performed as described previously (3). *P. gingivalis* transconjugants were selected on BA containing 1 μg of erythromycin and 25 μg of gentamicin per ml. Transconjugants were routinely maintained on BA containing 5 μg of erythromycin and 25 μg of gentamicin per ml. Two *P. gingivalis* transconjugants, designated DPG3 and DPG5, were chosen for further study on the basis of the results of Southern hybridizations (see below).

Southern hybridizations. Hybridizations were performed on *Cla*I-digested genomic DNA from *P. gingivalis* 381 and several transconjugants. *Cla*I was chosen because the enzyme cuts pDTH2 at a single site. Approximately 25 μg of digested DNA was loaded per lane on a 0.8% agarose gel and electrophoresed at 0.7 V/cm for 15 h. DNA was transferred onto Nitroplus membranes (MSI, Westboro, Mass.) by capillary action, and hybridization was performed as previously described (3). Plasmid pVAL-7 was radiolabelled with [α-^{32}P]dCTP (NEN Research Products, Boston, Mass.) by using the nick translation system from Promega Biotech according to manufacturer's instructions. The ^{32}P-labelled plasmid was added to the hybridization mixture at a concentration of 1.3×10^6 cpm/ml. After hybridization and washing, Nitroplus membranes were exposed to Kodak XAR film (Kodak, Rochester, N.Y.) at −70°C, using an intensifying screen.

Hemagglutination. Hemagglutination titers were determined in microtiter plates as previously described (27).

Contact angle measurements. Fifty milliliters of HK broth was inoculated with *P. gingivalis* 381, DPG3, or DPG5; DPG3 and DPG5 cultures also contained 5 μg of erythromycin per ml. These cultures were grown anaerobically overnight at 37°C, and the bacteria were collected by filtration onto microporous

silver membranes (37). The bacteria were air dried, and contact angles between the bacteria and several solvents were measured as described previously (38); these measurements were repeated by additional drying of the cells, until the contact values reached a plateau value. The solvents used were diiodomethane, α-bromonaphthalene, glycerol, and water. From these data, the apolar surface tension component of the bacteria (γ_i^{LW}) and the polar surface tension parameters (γ_i^+ [electron acceptor] and γ_i^- [electron donor]) were calculated, using Young's equation (37).

sHAP binding. sHAP beads were prepared as previously described (12). For the binding assay, 2.5×10^7 to 2.0×10^8 [^{3}H]thymidine-labelled *P. gingivalis* cells (strain 381 or DPG3) were added to tubes containing either HAP or sHAP beads, and the final volume was adjusted to 400 μl. To measure binding, bacteria bound to HAP or sHAP beads were separated from unbound bacteria by Percoll density gradient centrifugation (12), and the amount of bound bacteria was quantitated by liquid scintillation spectrometry. Binding assays were done in duplicate and were repeated at least once or twice on different days for each set of conditions.

Isolation of fimbrial and total membrane preparations. Crude fimbrial preparations from strains 381, DPG3, and DPG5 were isolated by a modification of the mild sonication procedure of Lee et al. (11). Total membrane preparations were obtained from 500-ml *P. gingivalis* cultures grown in HK broth. Cells were harvested by centrifugation in a Sorvall RC5B refrigerated centrifuge at 4,000 × *g* for 20 min at 4°C and suspended in 10 ml of cold water. This suspension was passed once through a French press (SLM Instruments, Urbana, Ill.) at 16,000 lb/in^2. This mixture was centrifuged at 2,000 × *g* for 20 min at 4°C to remove unbroken cells, and the supernatant was centrifuged at 100,000 × *g* for 2 h at 4°C to collect membranes. Membrane pellets were suspended in 300 μl of cold water and stored at −70°C until needed. The concentration of membrane proteins was determined by the method of Markwell et al. (18).

SDS-PAGE and Western blot analysis. Crude fimbrial preparations were examined by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), using the discontinuous system of Laemmli (9). Proteins were separated on a 10% resolving gel in a MINI-PROTEAN II Cell (Bio-Rad). Crude fimbriae were treated either at 100°C for 10 min in the presence of β-mercaptoethanol to completely dissociate the fimbrial proteins or at 80°C for 5 min in the absence of β-mercaptoethanol to partially dissociate the fimbriae. Crude fimbrial proteins were visualized by staining with Coomassie brilliant blue R-250 (25). For Western blot (immunoblot) analysis, proteins from the crude fimbrial preparations were transferred from the SDS-PAGE gels to nitrocellulose membranes, and the Western blots were analyzed with rabbit antifimbrillin antiserum as previously described (11). Western blot analysis of membrane proteins was performed similarly.

Electron microscopy. *P. gingivalis* 381, DPG3, and DPG5 were grown in HK broth without erythromycin for 48 h and harvested by centrifugation. Cell pellets were fixed for 3 h with 2.5% glutaraldehyde in 0.1 M cacodylate buffer, pH 7.2. After being washed in the same buffer, the cells were postfixed in 1.0% OsO_4 in *s*-collidine buffer, pH 7.2, for 1.5 h. En bloc staining was carried out with 1.0% uranyl acetate in 0.1 M maleate buffer, pH 6.5, for 1.5 h. The cells were dehydrated in a graduated series of cold ethanol and propylene oxide prior to infiltration with Epon for 4 h. Cells were then placed on flat embedding molds containing fresh Epon, which was allowed to polymerize at 60°C for 48 h. Ultrathin sections were prepared with an Ultracut-E ultramicrotome equipped with a diamond knife. The sections were double stained with 1% uranyl acetate and lead acetate. The sections were then examined and photographed with a Hitachi H-600 electron microscope.

For negative staining, 1 μl of either wild-type or mutant bacteria in water was applied to Formvar- and carbon-coated grids and air dried. The cells were then negatively stained with 2.0% uranyl acetate in distilled water.

Streptococcal coadherence. Tritium labelling of *P. gingivalis* and preparation of streptococcus-coated CNBr-Sepharose 4B beads (strep-beads) were performed as described by Stinson et al. (33). The assay was carried out by mixing 500 μl of [*methyl*-^{3}H]thymidine-labelled *P. gingivalis* with 50 μl of the strep-bead suspension and incubating at 22°C for 1 h. Adherent and nonadherent *P. gingivalis* cells were separated by differential filtration, and the number of [^{3}H]-labelled *P. gingivalis* cells that adhered to the strep-beads was then estimated by liquid scintillation spectrometry.

Infection of gnotobiotic rats. Male germfree Sprague-Dawley rats (Taconic Farms, Germantown, N.Y.) were kept under gnotobiotic conditions and infected with *P. gingivalis* strains as previously described (4). Briefly, bacteria used for infection were suspended in 5% carboxymethyl cellulose; each rat received 0.5 ml (1.5×10^{12} cells per ml) by gavage (three times) at 48 h intervals. Sham-infected animals received carboxymethyl cellulose only. Forty-two days after the last infection, the rats were sacrificed and exsanguinated under anesthesia, the jaws were removed and defleshed, and the periodontal bone level was determined by morphometric measurement as described previously (4). The average bone loss per site was calculated and expressed in millimeters as the mean distance per site from the cementoenamel junction (CEJ) to the alveolar bony crest (ABC).

RESULTS

Construction of pDTH2. Dickinson et al. (2) reported that in Northern (RNA) blots the cloned *fimA* gene hybridized to a single transcript of ca. 1,450 nucleotides, suggesting that *fimA* is monocistronic. However, the region 5′ to the *fimA* gene was not characterized, and the transcriptional start site was not identified (2). Therefore, we chose to insert pVAL-7 into the *fimA* locus by using only internal *fimA* coding sequence, represented by an 850-bp *Hin*cII fragment from pUC13Bgl2.1 (Fig. 1). Several attempts to insert this *Hin*cII fragment within the unique *Eco*RV site of pVAL-7 (36) repeatedly yielded plasmids suffering significant deletions or rearrangements. We did not further characterize the deleted or rearranged plasmids, but these data suggested that the presence of the *Hin*cII *fimA* fragment might be deleterious to *E. coli* when placed in the *Eco*RV site of pVAL-7, perhaps by expression from an endogenous *Bacteroides* promoter on pVAL-7. However, we fortuitously identified a recombinant plasmid that contained a tandem head-to-tail duplication of the *Hin*cII fragment, as determined by restriction enzyme analysis (data not shown). This plasmid was designated pDTH2 and was used for insertion-duplication mutagenesis. The reason(s) for the stability conferred by the tandem duplication of this fragment in pVAL-7 is currently unknown.

Conjugal transfer and orientation of pDTH2 in *P. gingivalis*. By using R751 to provide conjugal transfer functions, pDTH2 was mobilized into *P. gingivalis* 381. Erythromycin-resistant (Em^r) transconjugants were isolated, at a frequency of approximately 10^{-10} per recipient. Plasmid pDTH2 does not contain a complete replicon that functions in *Bacteroides* spp. (30), suggesting that the plasmid should not autonomously replicate in *Porphyromonas* spp. Southern hybridization analysis of

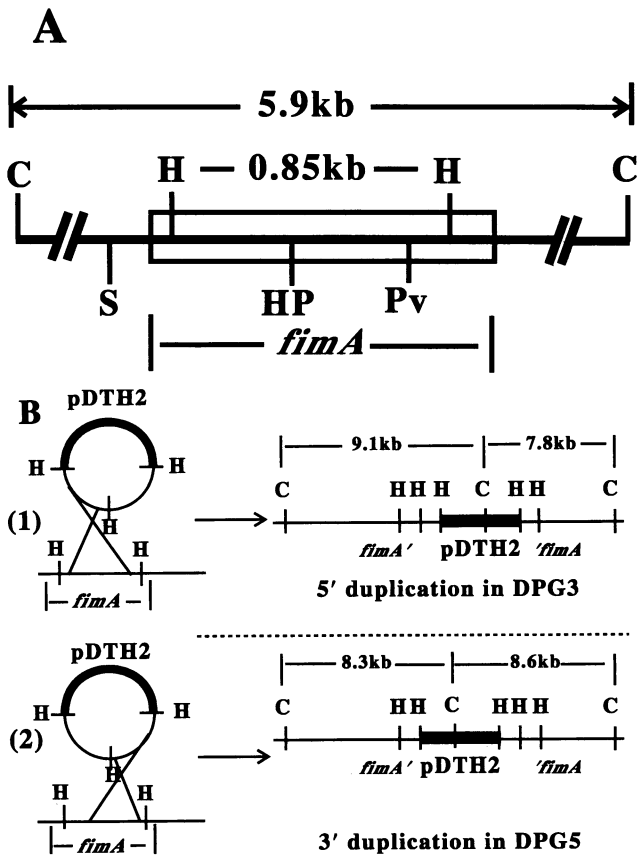

FIG. 1. Two possible outcomes of recombination between pDTH2 and the *P. gingivalis* chromosome. (A) Restriction map of the *fimA* region (from reference 2). Abbreviations for restriction endonuclease cleavage sites: HP, *Hin*PI; S, *Ssp*I; H, *Hin*cII; Pv, *Pvu*II. (B) Recombination events leading to insertion mutations in strains DPG3 and DPG5. (1), the 5′ *Hin*cII *fimA* fragment of pDTH2 recombined with the homologous region of the *fimA* locus, yielding an insertion mutation containing a duplication of the *Hin*cII fragment on the 5′ side of the plasmid insertion. This strain was designated DPG3. (2), recombination between the 3′ *Hin*cII *fimA* fragment of pDTH2 and the *fimA* locus produced an insertion with a duplication of the *Hin*cII fragment on the 3′ side of the inserted plasmid; this strain was designated DPG5.

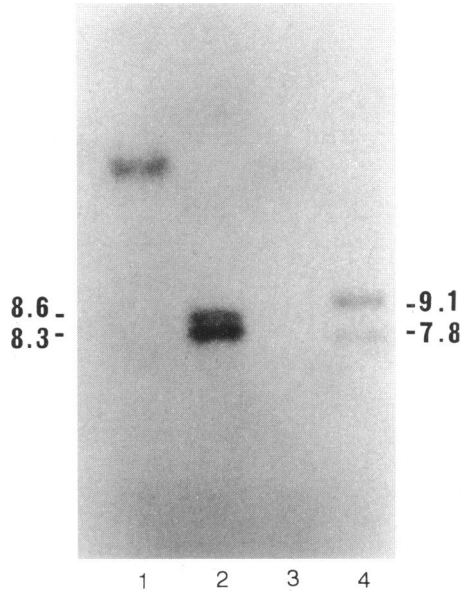

FIG. 2. Southern hybridizations of Em^r transconjugants DPG3 and DPG5. Genomic DNAs isolated from DPG5 (lanes 1 and 2) and DPG3 (lanes 3 and 4) were hybridized with ^{32}P-labelled pVAL-7; lanes 1 and 3 contained uncut genomic DNA, while lanes 2 and 4 contained *Cla*I-cut DNA. Numbers indicate sizes in kilobases.

uncut and *Cla*I-digested genomic DNA of *P. gingivalis* transconjugants was performed. Plasmid pVAL-7 hybridized to uncut genomic DNA from strain DPG3 (Fig. 2, lane 1) and strain DPG5 (faintly) (Fig. 2, lane 3), rather than hybridizing to a band or bands suggestive of an autonomous plasmid. In *Cla*I-digested DNA, ^{32}P-labelled pVAL-7 did not identify a 10.6-kb fragment representing linear pDTH2, as would be expected if pDTH2 were an autonomous replicon in these transconjugants. Rather, each transconjugant harbored two *Cla*I fragments that hybridized with pVAL-7. By comparison of the restriction map of pDTH2 with that of the *fimA* locus (5), an insertion of pDTH2 generating a 5′ duplication of the *Hin*cII fragment (strain DPG3) should yield two fragments (see Fig. 1) of 9.1 and 7.8 kb, as seen in Fig. 2 (lane 4). Similarly, a 3′ tandem duplication (strain DPG5) should yield *Cla*I fragments migrating at 8.3 and 8.6 kb (Fig. 2, lane 2). Control experiments showed that these *Cla*I fragments in DPG3 and DPG5 also hybridized with a *fimA* probe from pUC13Bgl2.1; ^{32}P-labelled pVAL-7 did not hybridize with wild-type DNA (data not shown).

SDS-PAGE and Western immunoblot analysis. Total membrane preparations of strains 381, DPG3, and DPG5 were analyzed by SDS-PAGE for alterations in membrane protein profile and to determine whether the mutants lacked the ability to produce the 43-kDa fimbrillin monomer. Western immunoblot analysis indicated that membrane preparations of both DPG3 and DPG5 lacked the 43-kDa fimbrillin protein present in strain 381 (Fig. 3). We did not observe any truncated proteins that reacted with the antifimbrillin antibody. However, we observed minor differences between wild-type and mutant membrane protein profiles (Fig. 4). Membrane preparations of both insertion mutants (Fig. 4, lanes 2 and 3) showed a slight decrease in the amount of a 29-kDa protein compared with that of wild-type strain 381 (Fig. 4, lane 1). Conversely, membrane protein preparations of both mutant strains contained increased amounts of a 32-kDa protein not observed in membranes from strain 381 (Fig. 4). However, this 32-kDa protein did not react with antifimbrillin or antifimbria antiserum (Fig. 3).

We also prepared crude fimbrial sonicates from mutant and wild-type *P. gingivalis*, as these preparations are highly enriched for fimbriae (11). We did not observe fimbrillin monomers in SDS-PAGE gels of sonicates of DPG3 or DPG5. By contrast, large amounts of the 43-kDa fimbrillin monomer were observed in sonicates of strain 381 (data not shown). We also did not observe any protein present on Western blots of whole-cell lysates of strains DPG3 and DPG5 that reacted with antifimbrillin antiserum (data not shown). Thus, we concluded that the insertions of pDTH2 into *P. gingivalis* 381 interrupted the *fimA* gene and blocked production of the FimA fimbrillin.

Hydrophobicity measurements. From the contact angle measurements obtained, we calculated the apolar and polar surface tension components of strains 381, DPG3, and DPG5, using Young's equation, as described previously (37). The data

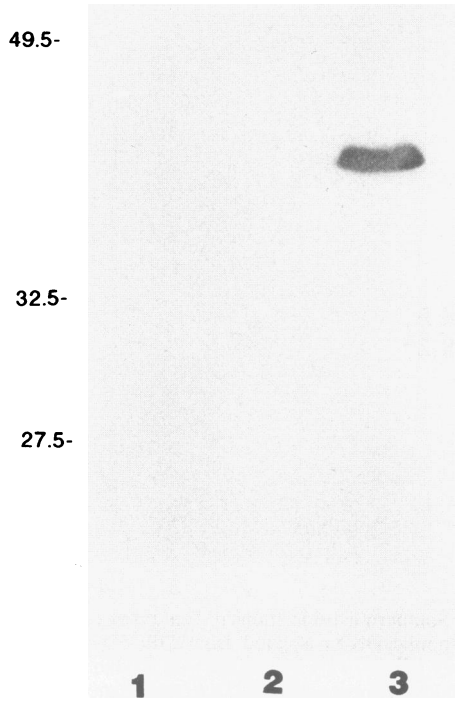

FIG. 3. Western blot analysis of membranes of *P. gingivalis* 381, DPG3, and DPG5. Membrane proteins of *P. gingivalis* DPG3 (lane 1), DPG5 (lane 2), and 381 (lane 3) were separated by SDS-PAGE, transferred to Nitroplus membranes, and incubated with antifimbrillin antiserum to identify the 43-kDa fimbrial component, found only in lane 3. Numbers indicate sizes in kilodaltons.

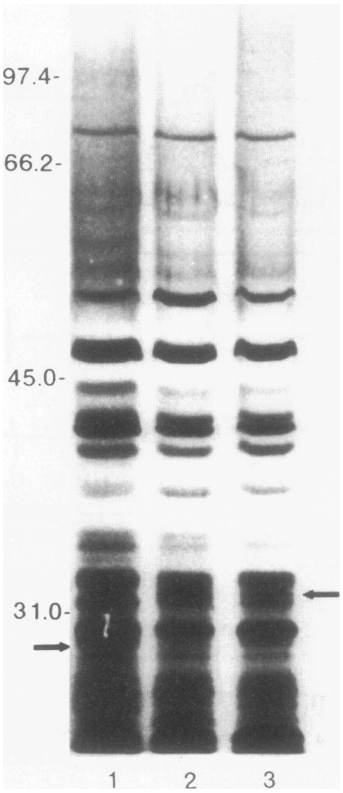

FIG. 4. Comparison of membrane proteins isolated from *P. gingivalis* 381, DPG3, and DPG5. Membranes were isolated from French press-disrupted bacteria and separated by SDS-PAGE. Lanes: 1, membrane proteins from strain 381; 2, membrane proteins from strain DPG3; 3, membrane proteins from strain DPG5. The new 29-kDa protein found in DPG3 and DPG5 is identified by an arrow to the right of lane 3; the protein found in strain 381 that was absent or reduced in the mutants is identified by the arrow at left. The migrations of molecular mass markers (sizes shown in kilodaltons) are at the left.

are presented in Table 2. We did not observe any appreciable difference between *P. gingivalis* 381 and insertion mutant DPG3 in either the apolar surface tension parameter (γ_i^{LW}) or the polar surface tension parameters (γ_i^+ and γ_i^-). Therefore, we concluded that the surface hydrophobicity of insertion mutant DPG3 was not significantly altered by the loss of fimbrial structure. Contact angle measurements suggested that insertion mutant DPG5 had a slightly lowered apolar surface tension parameter and an elevated electron donor parameter (Table 2), suggesting that strain DPG5 was more hydrophilic than strains 381 and DPG3.

Electron microscopy. As seen previously (32), strain 381 bacteria were typically electron dense and had long, slender (ca. 15-nm diameter) fimbriae easily seen in negatively stained preparations (data not shown). By contrast, we were consistently unable to demonstrate any fimbrial structure on the surface of strain DPG3 (data not shown), suggesting that the insertion mutation in DPG3 blocked fimbrial production. Negatively stained preparations of DPG5 had an altered appearance under the electron microscope. Although we did not observe any thin fimbrillar filaments similar to those seen in negatively stained preparations of strain 381, we commonly saw electron-translucent ghosts in several preparations of DPG5 (data not shown). These ghosts represented as many as 30% of the organisms found in some microscopic fields. Similar to the contact angle measurements, these electron microscopic observations suggested that the insertion mutation in DPG5 may have caused significant alterations to the surface of DPG5, in addition to the loss of fimbrial production.

Electron micrographs of cross sections of strain DPG3 indicated that cell envelopes of the mutant appeared typical for a gram-negative bacterium and were not significantly different from the ultrastructure of wild-type strain 381 (data not shown). Taken together, these data suggested that the surface characteristics of DPG3 were minimally altered in comparison with those of 381. In contrast to DPG5, the most significant alteration in the surface or ultrastructure of DPG3 was a complete lack of fimbriae. Thus, we chose not to examine the adherence characteristics of DPG5 further and to concentrate our analysis on strain DPG3.

Hemagglutination. The hemagglutination of sheep erythrocytes by 381 and DPG3 was identical (data not shown), suggesting that strain DPG3 was not impaired in the ability to bind to sheep erythrocytes. As DPG3 lacks recognizable fimbrial structures, this indicated that intact fimbriae are not

TABLE 2. Surface tension components of *P. gingivalis* strains

Strain	Mean[a] mJ/m^2 (SD)		
	Apolar surface tension parameter (γ_i^{LW})	Polar surface tension parameter	
		Acceptor (γ_i^+)	Donor (γ_i^-)
381	33.1(1.2)	0.065 (0.05)	42.26 (9.3)
DPG3	33.9(0.4)	0.225 (0.25)	43.05 (2.1)
DPG5	31.8(<0.01)	0.030 (0.04)	56.40 (4.1)

[a] At least two measurements.

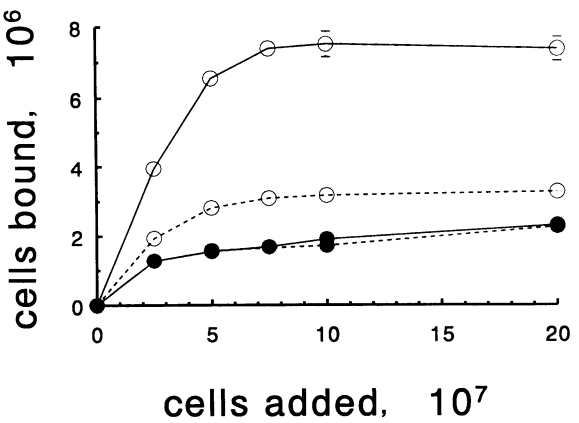

FIG. 5. Binding of *P. gingivalis* 381 and DPG3 to sHAP and HAP. Binding of ^{3}H-labelled *P. gingivalis* 381 (○) and DPG3 (●) to sHAP (——) and HAP (– – –) were measured as described in Materials and Methods. Binding of DPG3 to either substrate was significantly ($P > 0.05$) less than binding of wild-type strain 381 to sHAP or HAP.

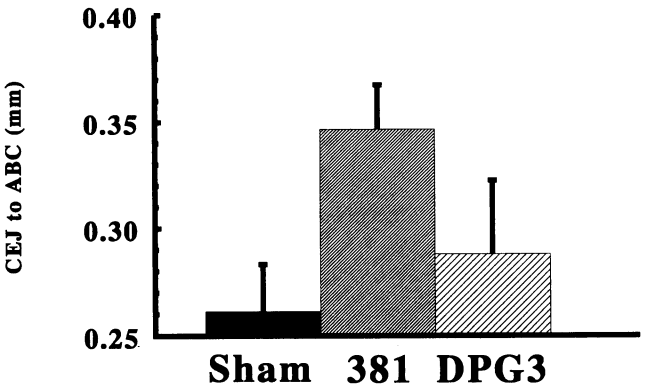

FIG. 6. Periodontal bone loss induced by *P. gingivalis* 381 and DPG3. Gnotobiotic rats were infected with strain 381 or DPG3, and periodontal bone loss was measured 42 days postinfection. Bone loss was estimated as the increase in the mean distance (millimeters) between the CEJ and the ABC compared with that of sham-infected control animals.

required for binding to the erythrocyte surface, consistent with other observations (20, 39).

Streptococcal coadherence. *P. gingivalis* 381 bound efficiently to *S. gordonii* G9B covalently attached to agarose beads ($5.79 \times 10^7 \pm 1.44 \times 10^7$ cells bound). Nonfimbriated DPG3 bound slightly less well to *S. gordonii* ($4.53 \times 10^7 \pm 0.15 \times 10^7$ cells bound), but these differences were not statistically significant ($P = 0.39$ by Student's t test). Thus, an intact fimbrial structure was not required for cohesion between *P. gingivalis* and *S. gordonii*.

Binding to sHAP. As shown previously (12), *P. gingivalis* 381 bound to both HAP and sHAP in a concentration-dependent manner and reached saturation at 10^8 cells added to the binding assay mixture (Fig. 5). At saturation, binding of strain 381 to sHAP beads was fourfold higher than binding to HAP beads, indicating that salivary components enhanced attachment of *P. gingivalis* to this artificial substrate. This difference was statistically significant ($P > 0.05$ by a one-factor analysis of variance Fisher analysis).

Binding of DPG3 to sHAP beads was identical to binding of the mutant to HAP beads alone (Fig. 5). Further, binding of DPG3 to sHAP or HAP was significantly reduced ($P > 0.05$) from the binding of wild-type bacteria to sHAP or HAP (Fig. 5). These data indicated that the fimbrial structure was important for the adherence of *P. gingivalis* to this artificial saliva-coated substrate and suggested that fimbriae are important for adherence to saliva-coated oral surfaces.

Infection of gnotobiotic rats. As shown previously (4), *P. gingivalis* 381 was able to induce significant periodontal bone loss, as measured by an increase in the distance from the CEJ to the ABC compared with that of control, sham-infected rats (Fig. 6). The mean CEJ-to-ABC distance of rats infected with strain DPG3 was significantly reduced from that of rats infected with wild-type bacteria ($P > 0.05$), suggesting that strain DPG3 was severely impaired in the ability to induce periodontal bone loss in these gnotobiotic animals.

DISCUSSION

These data demonstrate that insertion-duplication mutagenesis is a useful tool for generating mutations at a desired locus in *P. gingivalis*. In preliminary experiments, we have examined the stabilities of the insertion mutations in DPG3 and DPG5 and have found that the Em^r phenotype of these strains is not lost at the 10^{-2} level. This suggests that such insertions are stable in the absence of antibiotic selection, as was seen in *Bacteroides* spp. (30). We did not pursue characterization of mutant DPG5, since electron microscopic observations indicated that this mutant may have sustained abnormal morphologic alterations as a result of the mutation. Similar observations were recently made by Lawson (10), who inactivated the *fimA* gene by using a slightly different insertion strategy. Thus, we do not believe that these morphological abnormalities were an allele-specific consequence of the mutation that we generated in DPG5, but they may instead be a result of interruption of the *fimA* gene. The insertion-duplication mutation in DPG5 may have resulted in the expression of a truncated or abnormal fimbrillin protein, which could have led to the morphologically abnormal cells observed under the electron microscope. We could not demonstrate by SDS-PAGE or immunoblot analysis the production of significant amounts of abnormal fimbrillin protein by DPG5. However, low levels of such an abnormal fimbrillin protein may have somehow led to the electron-translucent cells that we observed in DPG5 cultures. Such electron-translucent cells were also observed by Lawson (10). Conversely, we are not aware of the reasons why DPG3 appears to be unaffected by the insertion mutation, other than lacking fimbrial production. Much further work is necessary to understand the region of the *P. gingivalis* genome surrounding the *fimA* locus before the differences between DPG3 and DPG5 will be understood. For instance, complementation assays would be very useful for determining whether the loss of FimA specifically results in the nonfimbriated phenotype observed in these mutants or whether other genes whose expression may have been disrupted by the pDTH2 insertion were affected. However, we currently do not have a second antibiotic resistance marker that could be used to select for transfer of a plasmid carrying an intact *fimA* allele. We have attempted to use pNFD13-2 (24), expressing tetracycline resistance (Tc^r), for such a complementation assay, but this has not yet been successful. Preliminary results suggest that the Tc^r determinant on this plasmid is not expressed efficiently in *P. gingivalis* 381 (data not shown).

Despite these complications, insertional inactivation of the *fimA* gene in DPG3 allowed us to begin to examine the role of the fimbriae in *P. gingivalis* adherence. In the present study, *P.*

gingivalis DPG3 bound poorly to sHAP beads, and this residual binding was equivalent to binding of DPG3 to HAP alone. Lee et al. (12) showed that synthetic peptides derived from the carboxy-terminal one-third of the fimbrillin protein blocked binding to sHAP, as did purified fimbriae. Collectively, these data suggest that the major fimbrial subunit is directly responsible for binding to saliva-coated oral surfaces. Fimbriae are thought to contribute significantly to the cell surface hydrophobic character of *P. gingivalis* (5), as in other bacteria (16, 23). However, the loss of fimbriae by mutation did not significantly alter the hydrophobicity of *P. gingivalis* DPG3, as the nonfimbriated mutant appeared to have the same hydrophobic character as did strain 381. Therefore, the lack of binding of DPG3 to sHAP does not appear to be the result of any alteration in the overall hydrophobicity of the surface of DPG3. Further, strain DPG3 was unable to induce the extent of periodontal bone loss in gnotobiotic rats that was observed when these rats were infected by wild-type *P. gingivalis*. These observations suggest that the ability to produce fimbriae is important for the ability of *P. gingivalis* to engage in the periodontal disease process. The data in the present study and those of Lee et al. (12) suggest that the inability of DPG3 to cause periodontal damage in these gnotobiotic rats may be due to the inability of the organism to adhere to saliva-coated oral surfaces in these animals. Further work is necessary to confirm this suggestion, however.

Although the relationship of fimbrial production and sHAP adherence is clear, the relationship of fimbriae to intergeneric bacterial cohesion and hemagglutination is not presently certain. Mutant DPG3 hemagglutinated sheep erythrocytes as well as did strain 381, suggesting that *P. gingivalis* fimbriae are not responsible for adherence to erythrocytes; this is consistent with many other data suggesting that surface-exposed outer membrane proteins, possible proteases, are responsible for hemagglutination (20, 39). We also observed no difference in the cohesion of strains 381 and DPG3 with *S. gordonii* G9B, suggesting that fimbrial production is not essential for either of these adherence mechanisms. However, most fimbriae examined to date are composed of several proteins, and adherence ligands are often minor components of these structures. The lack of FimA production could have abolished the fimbrial structure without affecting the production of other fimbria-associated adhesins. For example, Pap pilus expression by *E. coli* is genetically separable from *papG* expression; *papG* is the digalactoside-specific adhesin of the Pap pilus (14, 15, 35). Adhesins responsible for *P. gingivalis* hemagglutination or cohesion with streptococci may be part of the fimbrial structure but, in the absence of a functional FimA protein, could be deposited in the outer membrane of *P. gingivalis*. It is possible that the new 32-kDa protein found in membrane preparations of DPG3 may be a fimbria-associated protein, perhaps an adhesin that, while ordinarily contained within the fimbrial structure, is deposited in the outer membrane when fimbriae are not produced. A equally likely yet trivial alternative is that the 32-kDa protein is a product of plasmid pVAL-7, which is inserted into the genome of strain DPG3. Although our data suggest that fimbriae are not involved in hemagglutination or streptococcal cohesion, further work is necessary to substantiate this possibility and to examine other adherence mechanisms in which fimbriae may participate.

ACKNOWLEDGMENTS

This study was supported by U.S.P.H.S. grants DE00158 and DE08240 from the National Institute of Dental Research. Support for the gnotobiotic rat infection assays was provided by the Procter and Gamble Co.

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