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Construction and Characterization of a *fimA* Mutant of *Porphyromonas gingivalis*

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Although fimbriae of *Porphyromonas gingivalis* have been implicated as playing a major role in adherence to gingival tissue surfaces, no conclusive genetic evidence has yet been obtained. The *fimA* gene, the determinant for the major fimbrial subunit protein, was cloned and sequenced (D. P. Dickinson, M. A. Kubiniec, F. Yoshimura, and R. J. Genco, J. Bacteriol. 170:1658–1665, 1988). We undertook to inactivate the *fimA* gene by a homologous recombination technique and examined the *fimA* mutant for changes in surface properties, including production of fimbriae, adherence to human gingival fibroblasts and epithelial cells, hemagglutinating activity, and surface hydrophobicity. To inactivate the *fimA* gene, we disrupted a *fimA* clone by insertion of a DNA segment containing an erythromycin resistance (Em^r) gene. This was then delivered into *P. gingivalis* ATCC 33277 from an *Escherichia coli* K-12 strain, SM10 λ *pir*, by using a mobilizable suicide vector, pGP704; recombination at the *fimA* locus led to the isolation of a *fimA* mutant. Disruption of the *fimA* locus and disappearance of FimA production were confirmed by Southern hybridization with a *fimA*-specific DNA probe and Western immunoblotting with a monoclonal antibody against the FimA protein, respectively. The *fimA* mutant constructed failed to express long (0.5- to 1.0-μm) fimbriae from the bacterial surface and had a diminished adhesive capacity to tissue-cultured human gingival fibroblasts and epithelial cells. Observation of the bacteria adhering to human gingival fibroblasts by scanning electron microscopy revealed that the wild-type strain had dramatic local changes in the appearance of the microvilli at the point of contact with large bacterial clumps, whereas the *fimA* mutant did not. In contrast, neither the hemagglutinating activity nor the surface hydrophobicity was changed in the *fimA* mutant. These data thus constitute the first direct genetic evidence demonstrating that the FimA protein of *P. gingivalis* is essential for the interaction of the organism with human gingival tissue cells through a function(s) encoded by the *fimA* gene.

Porphyromonas gingivalis is an obligately anaerobic gram-negative coccobacillus that has been implicated as a causative agent of periodontal disease (31, 35, 36, 38, 43, 47). Although the exact etiology of periodontal disease is still unclear, experimental infection studies have suggested the involvement of *P. gingivalis* as a causative agent (9, 12, 20, 40). This organism possesses virulence-associated factors including fimbriae, hemagglutinin, protease, collagenase, lipopolysaccharides, vesicles, and outer membrane proteins (8, 15, 33, 39, 44, 47). It has been shown that *P. gingivalis* can adhere to oral epithelial cells (3, 25, 34), erythrocytes (13, 26), other bacteria (7), and collagen beads (23). Adherence is an essential step in the pathological process leading to periodontitis (34, 36). This adherence capacity is thought to be mediated by various surface proteins of *P. gingivalis*. Fimbriae in particular have been suggested as playing an important role in facilitating the initial interaction between the bacteria and the host (14). Indeed, afimbriated variants of *P. gingivalis* have been shown to have a diminished capacity for adherence to oral epithelial cells (5, 41).

The fimbriae of *P. gingivalis* were originally purified by Yoshimura et al. (45), who demonstrated that the major subunit (fimbrilin) was a 43-kDa protein by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). By using a synthetic oligonucleotide probe designed on the basis of the amino acid sequence near the N-terminal domain of the 43-kDa protein, Dickinson et al. (4) cloned and sequenced a gene encoding fimbrilin on a 2.5-kb *Sac*I segment. It has been shown that the majority of *P. gingivalis* isolates were fimbriated and possessed the *fimA* gene (18, 37) and that fimbriae of *P. gingivalis* act as a strong antigen in host immunoresponses (11), strongly suggesting that the FimA protein plays a crucial role in bacterial interaction with host gingival tissue.

Nevertheless, because of the absence of genetic exchange systems for *P. gingivalis*, there was no direct genetic evidence that *fimA* is essential for fimbrial expression and for adhesion to the host cells. We therefore decided to establish a versatile gene exchange system in *P. gingivalis* by using a suicide vector, pGP704 (21), and attempted to mutate the *fimA* gene. The resulting *fimA*-inactivated mutant was incapable of producing the 41-kDa (43-kDa) FimA protein, expressing fimbriae, or adhering to tissue-cultured cells, demonstrating that the *fimA* gene is an essential determinant for the adhesive ability of *P. gingivalis*.

MATERIALS AND METHODS

Bacterial strains, plasmids, and cultivation conditions. *P. gingivalis* ATCC 33277 was cultivated anaerobically (5% CO_2, 10% H_2, 85% N_2) in brain heart infusion broth (BHI; Difco Laboratories, Detroit, Mich.) supplemented with yeast extract

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(0.5%), hemin (5 μg/ml), and vitamin K_1 (10 μg/ml). *Escherichia coli* strains were grown in LB broth (10 g of tryptone, 5 g of yeast extract, and 5 g of NaCl per liter) or LB agar. Plasmid pE5-2 was originally constructed by Shoemaker et al. (32). Plasmid pGP704 and *E. coli* SM10 λ *pir* were kindly provided by J. J. Mekalanos (21). For selection or maintenance of the plasmid-containing strains, antibiotics were added to the media at the following concentrations: erythromycin, 10 μg/ml; tetracycline, 5 μg/ml; ampicillin, 250 μg/ml; and gentamicin, 100 μg/ml.

Transfer of pKDH1. The method described by Progulske-Fox et al. (28) was used with slight modifications. Briefly, 1.0 ml of a culture of *E. coli* SM10 λ *pir* harboring pKDH1 grown up to mid-log phase was mixed with 2.0 ml of an anaerobically grown mid-log-phase culture of *P. gingivalis* ATCC 33277 recipient and pelleted by centrifugation. The pellet was spotted onto a BHI blood agar plate without antibiotics and incubated at 37°C in an anaerobic chamber for 24 h. The cells were then transferred to a fresh tube containing 1.0 ml of BHI broth and vortexed for 20 s. The cell suspension was concentrated by centrifugation, and a 0.1-ml portion was plated onto a BHI blood agar plate containing erythromycin and gentamicin and incubated at 37°C in an anaerobic chamber for 7 days.

Isolation and manipulation of plasmid DNA. Plasmid DNA was isolated from *E. coli* strains either by the method of Birnboim and Doly (1) or by CsCl-ethidium bromide density gradient centrifugation (30) or both. Restriction enzymes and T4 DNA ligase were purchased from Takara Shuzo, Kyoto, Japan. Restriction endonuclease digestion and ligation with T4 DNA ligase were performed by standard procedures.

Isolation of chromosomal DNA. Chromosomal DNA was isolated from *P. gingivalis* strains by a modification of the method of Marmur (19). Briefly, bacterial cells grown to late log phase were lysed in 1.0% SDS containing 50 mM NaCl, 50 mM Tris-HCl, and 100 mM EDTA (pH 7.5). RNA and protein were removed by successive treatment with RNase A (1 μg/ml) and pronase (0.5 mg/ml), and then the high-molecular-weight DNA was further purified by repeated phenol extraction.

Southern blot analysis of *fimA* by using the fimbrilin clone. The *fimA* probe and the Em^r probe were prepared as the 2.5-kb *Sac*I fragment from pUC13Bg12.1 and the 3.8-kb *Eco*RI fragment from pE5-2, respectively. The DNA probes were labeled by using the enhanced chemiluminescence (ECL) gene detection system (Amersham Corp., Amersham, United Kingdom). *Sac*I-digested chromosomal DNA resolved in a 0.7% agarose gel was transferred to a Hybond-N^+ membrane (Amersham) and hybridized to the DNA probes by following the protocol described by the manufacturer (Amersham).

SDS-PAGE. SDS-PAGE of whole-cell lysates was performed by the method described by Neville (24). Protein extracts were heated at 100°C for 5 min in loading buffer (62.5 mM Tris-HCl buffer [pH 6.8] containing 2% SDS, 10% [vol/vol] glycerol, 2.5% 2-mercaptoethanol, and 0.1% bromophenol blue). Samples were applied to a 12.5% (wt/vol) polyacrylamide slab gel with a 4% (wt/vol) stacking gel and electrophoresed at a 30-mA constant current for 1 h. The proteins were stained with Coomassie brilliant blue. For molecular weight calibration, a low-molecular-weight marker kit (Daiichi Pure Chemicals, Tokyo, Japan) was used.

Western blotting. Western immunoblotting was performed by the method of Burnette (2). Whole cells were subjected to SDS-PAGE in duplicate on different gels; one was stained with Coomassie brilliant blue, and the other was electroblotted onto a polyvinylidene difluoride membrane (Immobilon; Nihon Millipore Kogyo, Yonezawa, Japan) at 200 mA for 1 h. The membrane was treated with TBS (20 mM Tris-HCl [pH 7.4], 0.5 M NaCl) containing 1% (vol/vol) bovine serum albumin (BSA) to block unoccupied protein-binding sites. It was then incubated with a monoclonal antibody specific for *P. gingivalis* 381 fimbriae provided by H. Isogai, Sapporo Medical College, Sapporo, Japan, at 37°C for 1 h. After this, it was washed with TBS-Tween and incubated for 1 h with peroxidase-conjugated goat anti-mouse immunoglobulin G antibody (Zymed). It was then immersed into a 4-chloro-1-naphthol (Tokyo Chemical Industry, Tokyo, Japan) solution to develop the color, washed, and dried.

Electron microscopy. The fimbriae of *P. gingivalis* cells were observed by electron microscopy by using a modification of the negative-staining techniques of Leung et al. (16). Briefly, bacterial cells from a 16-h anaerobic culture were collected by centrifugation (18,500 × *g* for 1 min), washed, and resuspended in reduced transport fluid (RTF) (17). Copper grids (150-mesh) were covered with a thin film of collodion, which was then coated with carbon. The supported films were made hydrophilic by ion bombardment before use. A drop of cell suspension was applied to the specimen grid and negatively stained with 2% (wt/vol) uranyl acetate for 60 s at room temperature. The specimens were examined with a JEM-200CX electron microscope (Nippon Denshi Co., Tokyo, Japan) operating at 80 kV.

Immunoelectron microscopy. *P. gingivalis* cells were washed with RTF and resuspended to approximately 5×10^8 bacterial cells per ml of RTF. The cells were then immunostained by gold labeling. Briefly, the bacterial cell suspension was deposited on a collodion film-coated grid and incubated at 37°C for 1 h with 10 μl of monoclonal antibody for *P. gingivalis* 381 fimbriae at a 1:100 dilution in phosphate-buffered saline (PBS) containing 1% (wt/vol) BSA. After being rinsed five times with RTF, the grid was incubated at 37°C for 20 min with goat anti-mouse gold-conjugated immunoglobulin G diluted 1:10 with PBS containing BSA (5-nm gold; Bio Cell Research Laboratories). The grid was rinsed three times with RTF and negatively stained with 2% (wt/vol) uranyl acetate for 60 s at room temperature. It was then examined with a JEM-200CX electron microscope operating at 80 kV.

Hemagglutination assay. Hemagglutinating activity in a round-bottom microtiter plate was determined by the method of Inoshita et al. (13). Bacterial suspensions adjusted to an optical density at 550 nm (OD_{550}) of 1.0 were diluted in a twofold series with PBS (pH 7.4). A 50-μl aliquot of this and an equal volume of a 2% suspension of sheep erythrocytes were mixed and incubated for 90 min at room temperature. The hemagglutination titer was expressed as the last dilution showing complete agglutination of the erythrocytes.

Determination of surface hydrophobicity. We determined the relative surface hydrophobicities of *P. gingivalis* ATCC 33277 and its mutants (MPG1 and MPG2) by using the method of Rosenberg et al. (29) to measure their ability to absorb *n*-hexadecane. Briefly, bacterial suspensions in RUM buffer (22.2 g of $K_2HPO_4 \cdot H_2O$, 7.26 g of KH_2PO_4, 1.8 g of urea, and 0.2 g of $MgSO_4 \cdot 7H_2O$ per liter [pH 7.1]) were adjusted to an OD_{550} of 1.0. Then 2 ml of bacterial suspension was placed in tubes (13 by 100 mm), and 400 μl of *n*-hexadecane was added; this mixture was then vigorously mixed on a vortex mixer for 60 s. Each layer was stored for 15 min, the OD_{550} of the aqueous phase was measured, and hydrophobic activity (HP) was calculated from to the formula %HP = [OD (initial) − OD (exp)] × 100/OD (initial), where OD (exp) is the OD_{550} measured after the 15-min storage.

Human gingival fibroblast and epithelial cell preparation. Two kinds of human oral tissue-cultured cells, human gingival fibroblasts and a human epithelial cell line from carcinoma of

the gingiva (Ca9-22), were used in the adherence assay. Ca9-22 cells were kindly provided by Y. Abiko, Nippon University School of Dentistry, Matsudo, Japan. All cells were cultured to confluency in Dulbecco's modified Eagle's medium (Nissui Pharmaceutical, Tokyo, Japan) containing 10% fetal bovine serum (GIBCO Laboratories, Grand Island, N.Y.) in a 24-well plate for 3 days in a 5% CO_2 atmosphere.

Adherence assay. The values for percent adsorption to human gingival fibroblasts and human epithelial cells (Ca9-22) were determined as follows. Strains of *P. gingivalis* were cultured in BHI broth supplemented with 10 μCi of [^{3}H]thymidine (18.2 Ci/mmol; New England Nuclear Corp., Boston, Mass.) per ml, washed twice, and resuspended in buffered KCl (50 mM KCl, 1 mM KH_2PO_4, 1 mM $CaCl_2$, 0.1 mM $MgCl_2$). Radiolabeled cells of *P. gingivalis* strains were adjusted to an OD_{550} of 1.0 in buffered KCl, and 200 μl of each bacterial suspension was added to preparations of fibroblasts or epithelial cells grown in a 24-well plate, washed twice, and replaced with buffered KCl; this was then mixed gently for 90 min. After incubation, the mixture was washed three times with the same buffer to remove nonadherent bacteria and the amounts of attached bacteria were counted with a liquid scintillation counter. The capacity of each bacterial strain to adhere to the target cells was estimated as the percentage of bound bacteria, in terms of (counts per minute of adhered bacteria divided by counts per minute added) × 100. All experiments were performed in triplicate. For scanning electron microscopy, human gingival fibroblast layers cultured on round glass slides were incubated for 60 min with the bacterial suspension. Bacterial cells attached to human gingival fibroblasts were observed with a JSM-820 scanning electron microscope (Nippon Denshi Co.).

RESULTS

Construction of a *fimA*-inactivated mutant of *P. gingivalis*. The procedure for disrupting the *fimA* gene of *P. gingivalis* by using a 3.8-kb *Eco*RI DNA fragment from pE5-2 encoding resistance markers of tetracycline (Tc^r), clindamycin (Cc^r), and erythromycin (Em^r) (Tc^r/Cc^r-Em^r, abbreviated as Em^r hereafter) is shown in Fig. 1. This fragment was inserted by blunt-end ligation into the *Eco*T14I site in the *fimA* gene on pUC13Bg12.1 (pEM1). The 5.4-kb *Pvu*II segment on pEM1 containing *fimA*::Em^r was then subcloned into the *Sma*I site on pGP704, and the resulting pGP704 construct containing *fimA*::Em^r (pKDH1) was used to deliver the *fimA*::Em^r segment into *P. gingivalis*. Note that a ca. 250-bp DNA from the 3′ end of *fimA*::Em^r was lost in subcloning.

Because the *mob* function is required for delivery of pKDH1 into *P. gingivalis*, an *E. coli* K-12 strain, SM10 λ *pir*, containing *mob* as an integrated RP4 was used as the donor. Through this system, pKDH1 could be mobilized from SM10 λ *pir* to *P. gingivalis* ATCC 33277 (Fig. 2). To counterselect against the donor *E. coli* strain, we took advantage of the gentamicin-resistant phenotype of *P. gingivalis*. Consequently, Em^r-Gm^r transconjugants of *P. gingivalis* that had received pKDH1 were isolated at a high (10^{-4} per donor) frequency. One of the transconjugants thus obtained was designated as MPG1 and used for further characterization. By using a similar procedure, another Em^r derivative of the *P. gingivalis* strain (MPG2) was constructed with the use of another DNA segment (derived from one of the genomic libraries of *P. gingivalis*) containing the Em^r segment and used as the control for MPG1 (see Discussion).

Characterization of the *fimA* mutant of *P. gingivalis*. To characterize the locus of the *fimA* gene in MPG1, we performed Southern hybridization analysis with two different probes; one was the 2.5-kb *Sac*I fragment of pUC13Bgl2.1 (*fimA* probe), and the other was the 3.8-kb *Eco*RI fragment of pE5-2 (Em^r probe) (Fig. 1). The *fimA* probe hybridized to the 9.0- and 2.5-kb *Sac*I fragments from MGP1 and to the 2.5-kb fragment from MGP2. The Em^r probe hybridized to MPG1 and MPG2 but not to the wild-type strain (Fig. 3). These results taken together clearly indicated that the *fimA* gene in MPG1 had been disrupted by integration of pKDH1. It probably resulted from a single recombination event between the two *fimA* sites on the chromosome and on pKDH1 (Fig. 2). To confirm the absence of the FimA product in MPG1, whole-cell lysates prepared from MPG1, MPG2, and the wild-type strain were analyzed by SDS-PAGE followed by Coomassie staining, Western blotting, and probing with a monoclonal antibody against *P. gingivalis* fimbriae. This revealed that a protein band of 41 kDa disappeared from MPG1 (Fig. 4A, lane 2) but not from the wild type or MPG2 (Fig. 4A, lanes 1 and 3). Furthermore, the protein band of the wild type and MPG2 was shown to react with monoclonal antibody (Fig. 4B, lanes 1 and 3). This indicated that the FimA protein is not expressed by MPG1 (Fig. 4B, lane 2).

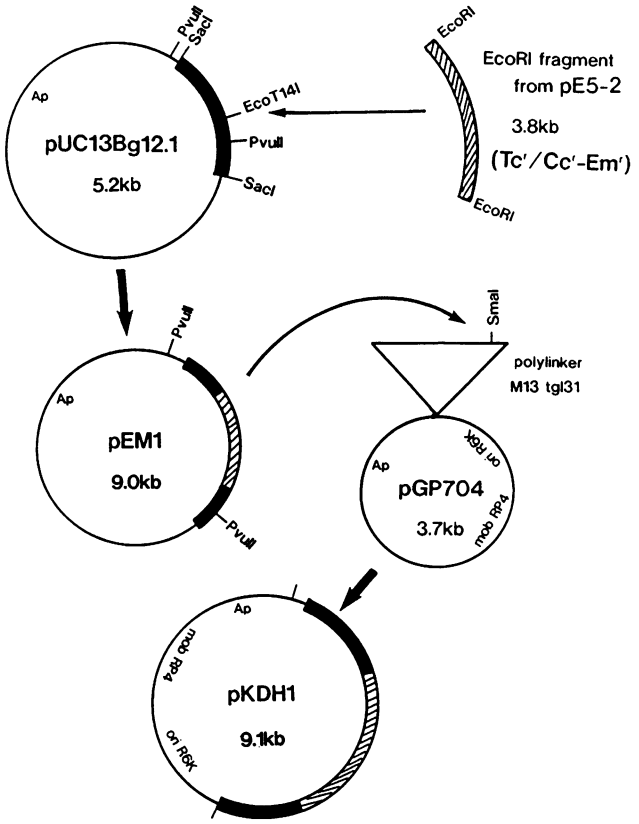

FIG. 1. Construction of pKDH1. A 3.8-kb *Eco*RI fragment (Tc^r/Cc^r-Em^r) from pE5-2 was inserted into the *Eco*T14I site of the *fimA* gene on pUC13Bg12.1 by blunt-end ligation. A *Pvu*II fragment containing the mutated *fimA* gene was then subcloned into the *Sma*I site of pGP704, and the resulting plasmid is designated pKDH1. This plasmid was maintained in *E. coli* K-12 strain SM10 λ *pir*. The thick bar on pUC13Bg12.1 represents the cloned *fimA* region.

Effect of disruption of the *fimA* gene on expression of the fimbriae on the bacterial surface. Expression of fimbriae on the surface of MPG1 and the wild-type strain was investigated

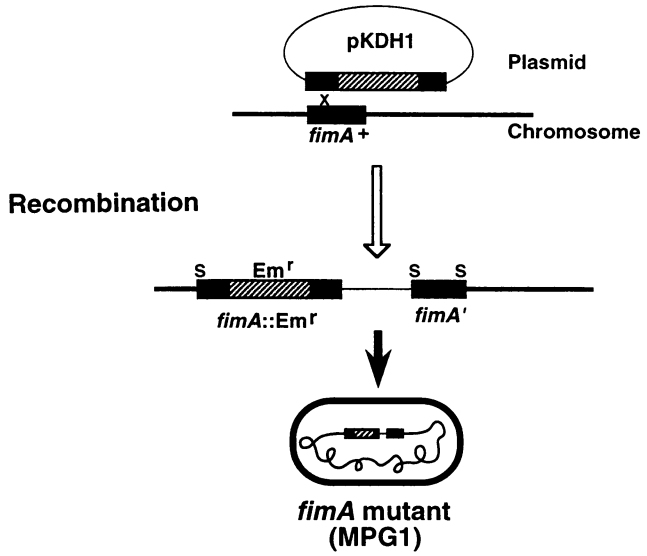

FIG. 2. Disruption of the *fimA* gene in *P. gingivalis*. A detailed description is given in the text.

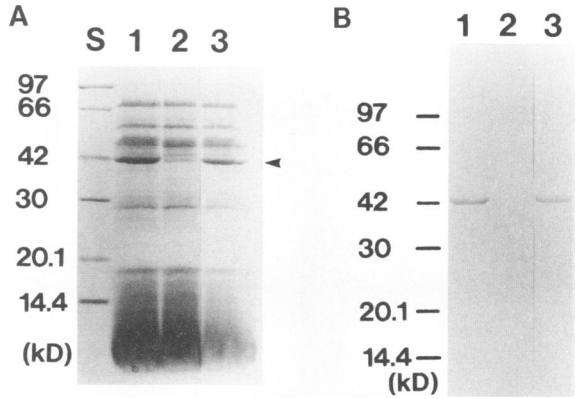

FIG. 4. Expression of the FimA protein from wild-type and *fimA* mutant *P. gingivalis* strains. (A) SDS-PAGE of Coomassie blue-stained whole cell lysates; (B) same as panel A, but Western blot analysis was performed with monoclonal antibody against the fimbrilin subunit (FimA). The arrowhead indicates the position of the 41-kDa protein. Lanes: S, standard proteins; 1, *P. gingivalis* ATCC 33277 (wild-type strain); 2, MPG1 (*fimA* mutant); 3, MPG2 (Em^r derivative of the wild-type strain).

by electron microscopy. The wild-type strain produced long (0.5- to 1.0-μm) fimbrial structures from the surface (Fig. 5A), whereas these structures were not observed on MPG1 (Fig. 5B). Interestingly, very short (0.1- to 0.5-μm) fimbria-like projections were seen on the surface of MPG1 (Fig. 5B). Immunogold electron microscopy showed that a monoclonal antibody against the fimbriae bound only to the long fimbria-like projections of the wild-type strain (Fig. 5C). In contrast, no binding of the immunogold to the surface of MPG1 was observed (Fig. 5D).

Capacity of the *fimA* mutant to adhere to cultured human tissue cells. It is well known that fimbriated *P. gingivalis* strains can bind to epithelial cells from gingival tissue, whereas nonfimbriated strains such as W50 and W83 have reduced binding capacity (5, 41), suggesting that the fimbriae play an important role in the interaction between bacteria and the host tissue surface. Hence, the capacity of MPG1 and the wild-type strain to adhere to human gingival fibroblasts and to Ca9-22 cells were compared. MPG1 was less adherent to both of these cultured cells than were the wild-type strain and MPG2 (see Table 2).

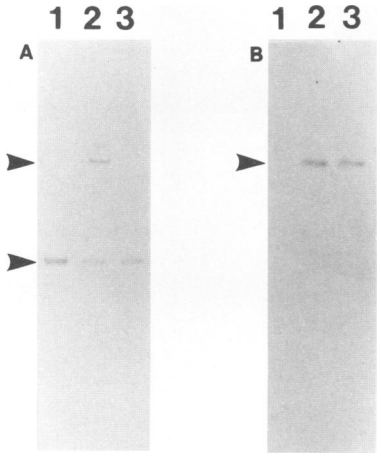

FIG. 3. Southern hybridization analysis of the *fimA* locus in MPG1. Chromosomal DNA samples from the *P. gingivalis* strains were digested with *Sac*I, electrophoresed in an agarose gel, transferred onto a membrane filter, and hybridized with *fimA* probe (A) or Em^r probe (B). Lanes: 1, *P. gingivalis* ATCC 33277 (wild-type strain); 2, MPG1 (*fimA* mutant); 3, MPG2 (Em^r derivative of the wild-type strain). Arrowheads indicate hybridized bands.

Hemagglutinating activity and surface hydrophobicity of the *fimA* mutant. Although it has been suggested that the hemagglutinin of *P. gingivalis* contributes to adhesive ability, the molecular mechanisms underlying hemagglutination are as yet unclear. Thus, we examined the effect of *fimA* mutation on the expression of hemagglutinating activity. The sheep erythrocyte-hemagglutinating activities of the *fimA* mutant (MPG1), the wild type, and MPG2 were same (Fig. 6), indicating that the *fimA* function is not directly involved in expressing the hemagglutinating activity of this organism. It also seems that the *fimA* function does not affect surface hydrophobicity, since no significant difference in surface hydrophobicity was observed between the mutant and wild type (Table 1).

Interaction of the wild type and the *fimA* mutant of *P. gingivalis* with human gingival fibroblasts. Although the *fimA* mutant of *P. gingivalis* was unable to efficiently adhere to two types of cultured cells, human gingival fibroblasts and human epithelial cells (Table 2), it fully expressed hemagglutinating activity and surface hydrophobicity (Table 1; Fig. 6). These results prompted us to examine by scanning electron microscopy the interactions of MPG1 and the wild-type strain of *P. gingivalis* with cultured human gingival fibroblasts. At 60 min after contact of the wild-type strain with human gingival fibroblasts, a dramatic localized change in the appearance of the microvilli at the point of contact was observed (Fig. 7A and C). Interestingly, the wild type that attached to human gingival fibroblasts formed large clumps (Fig. 7C); however, none of these changes were observed when the *fimA* mutant came into contact with the human gingival fibroblasts (Fig. 7B and D).

DISCUSSION

In this study, we have developed a simple procedure for inactivating a gene on the chromosome of *P. gingivalis* and

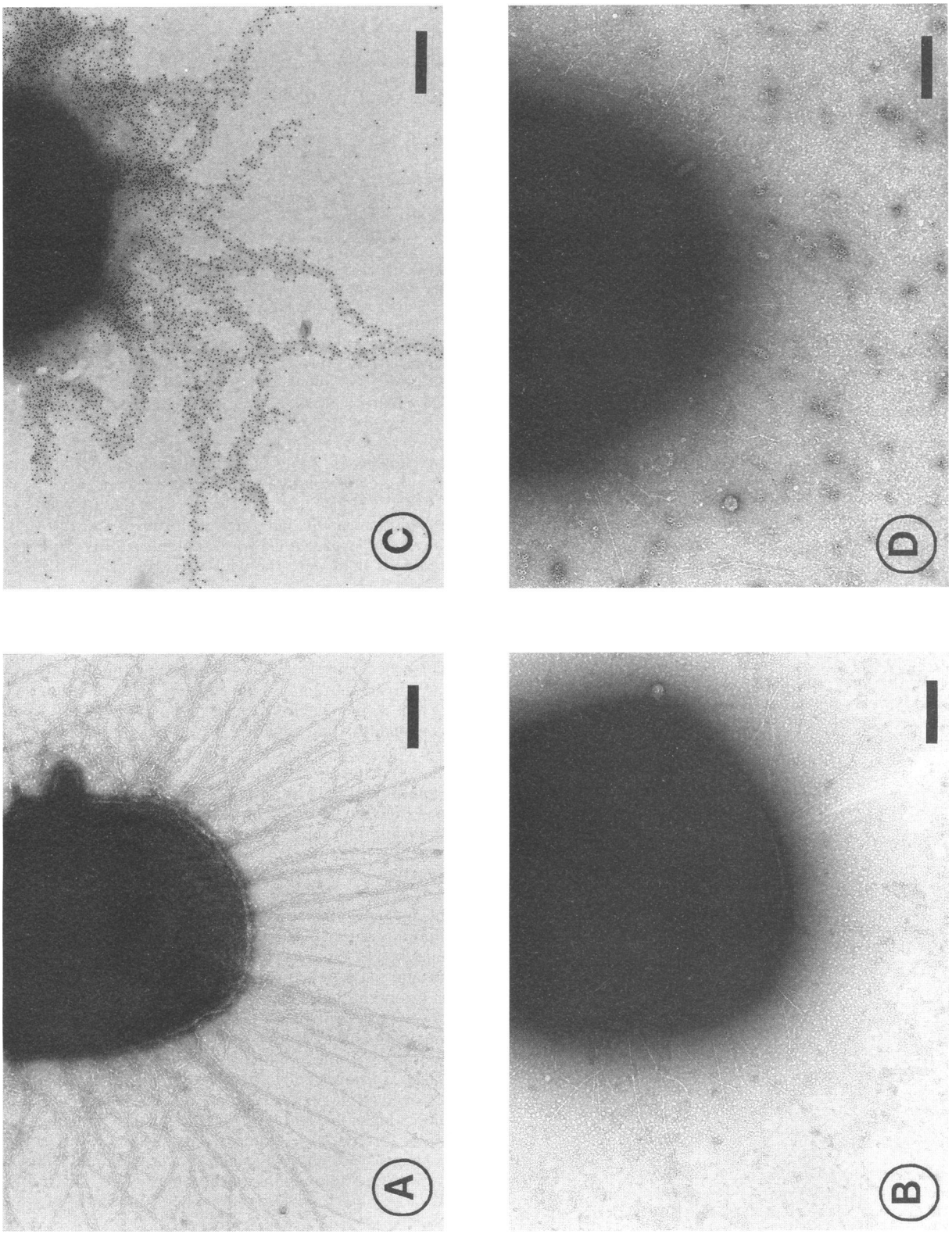

FIG. 5. Examination of fimbriae expressed from the wild-type and *fimA* mutant of *P. gingivalis* by electron microscopy and immunogold electron microscopy. Samples were prepared by negative staining with uranyl acetate. (A) *P. gingivalis* ATCC 33277 possessed numerous thin fimbriae on its surface. (B) MPG1 possessed thin, short fimbrial structures. (C and D) Electron micrographs of the immunogold-labeled fimbriae of *P. gingivalis* ATCC 33277 and MPG1, respectively. Bar, 0.2 μm.

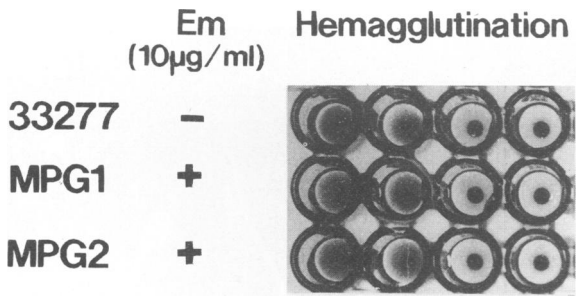

FIG. 6. Hemagglutinating activity of the *fimA* mutant. Hemagglutination by *P. gingivalis* ATCC 33277, MPG1 (*fimA* mutant), and MPG2 (Em^r derivative of the wild-type strain) is shown.

used it to mutate the *fimA* gene encoding the major fimbrilin subunit protein (Fig. 1 and 2). The *fimA* mutant thus constructed failed to express the 41-kDa FimA protein (Fig. 4), resulting in disappearance of fimbriae from the bacterial surface (Fig. 5) and loss of adhesiveness to gingival fibroblasts (Table 2; Fig. 7). The nucleotide sequence of the *fimA* gene that had been cloned in *E. coli* indicated a polypeptide of 35,924 Da, smaller than the estimate of 43 kDa derived from SDS-PAGE (4). Subsequent studies have indicated that the *fimA* gene is located in a 10.4-kb *Pst*I segment of *P. gingivalis*, in which several other genes, encoding 20-, 50-, 63-, and 80-kDa polypeptides, have been assigned as putative fimbria-associated genes (46). Thus, direct genetic evidence linking the role of the *fimA* gene and the other four associated genes in the adhesive phenotype of *P. gingivalis* remains to be established. To inactivate the *fimA* function of *P. gingivalis*, we used the cloned *fimA* gene on pUC13Bg12.1 and disrupted the gene by inserting a 3.8-kb *Eco*RI segment containing resistance markers for Em^r, Cc^r, and Tc^r (Fig. 1). The Em^r and Cc^r markers are expressed in *Bacteroides* spp., whereas the Tc^r marker is expressed in *E. coli* (32). The resulting *fimA*-inactivated clone (*fimA*::Em^r) was subcloned into pGP704, a suicide R6K-borne delivery vector (21). The plasmid derivative of pGP704 containing the *fimA*::Em^r clone (pKDH1) could be maintained only in the donor strain, SM10 λ *pir*, which produces the R6K-specific π protein, an essential replication protein for R6K and plasmids derived therefrom. The delivery of pKDH1 to *P. gingivalis* was mediated through the *mob* function provided by the integrated RP4, which encodes a broad-host-range conjugal transfer function. We thus reasoned that such a delivery system might work in *P. gingivalis*. Analysis of the DNA structure of the *fimA* locus of MPG1 by Southern hybridization indicated that the Em^r transconjugant appeared as the consequence of integration of pKDH1 into the *fimA* gene, by a homologous recombination event between the *fimA* gene on the chromosome and the Em^r-mutated *fimA* gene on pKDH1 (Fig. 2 and 3). This recombination event also gave rise to a truncated *fimA* gene (*fimA'*) downstream of the Em^r-mutated *fimA*. The Em^r marker in MPG1 was found to be unstable during subculturing unless erythromycin was added to the culture medium. Excision of the pKDH1 portion seemed to occur by the second homologous recombination between the tandem repeats of *fimA* on the chromosome (10). To prevent this, we added 10 μg of erythromycin per ml to the medium for cultivation of MPG1 in this study (see Materials and Methods). Thus, MPG2 was constructed (Em^r *P. gingivalis*) as a control to monitor the effect of erythromycin on MPG1.

TABLE 1. Comparative hydrophobicity of *P. gingivalis* ATCC 33277 and its mutants

Strain	OD_{550}[a]		%HP[b]
	Initial	Final	
ATCC 33277	0.82	0.10	87.8
MPG1[c]	0.85	0.07	91.8
MPG2[c]	0.92	0.13	85.9

[a] Values indicate means of triplicate experiments. Final OD_{550} is the same as OD (exp) in text.
[b] For the calculation of %HP (hydrophobicity), see the text.
[c] Cultured in media containing erythromycin (10 μg/ml).

TABLE 2. Adherence of *P. gingivalis* ATCC 33277 and its mutants to human cells

Strain	% of bacteria adherent to[a]:	
	Gingival fibroblasts	Epithelial cells
ATCC 33277	5.29 ± 0.43	5.28 ± 0.38
MPG1[b]	1.65 ± 0.24	1.83 ± 0.07
MPG2[b]	6.15 ± 0.58	5.59 ± 0.06

[a] The percentage of bound bacteria was evaluated from the counts per minute of adherent bacteria and initially added bacteria. The percentages were means of triplicate experiments ± standard deviations.
[b] Cultured in media containing erythromycin (10 μg/ml).

Electron-microscopic observation of the surface of *P. gingivalis* revealed that the *fimA* mutant failed to produce long (0.5- to 1.0-μm) fimbrial structures, possessing instead short (0.1- to 0.5-μm) fimbria-like surface structures, different immunologically as well as morphologically from those expressed by the wild-type strain (Fig. 5A and B). Although we could not exclude the possibility that the short fimbriae appearing on MPG1 were produced together with numerous long fimbriae on the wild-type strain, it would be tempting to speculate that the expression of the short fimbriae is under the control of a different gene-regulatory system from that for the long fimbriae (FimA). Indeed, our preliminary data have suggested that the short fimbriae appear from MPG1 more efficiently at 30°C than at 37°C, whereas the long fimbriae appear from the wild type most efficiently at 37°C (10). Alternatively, truncated fimbriae may arise as a consequence of the *fimA* gene itself or by the affected production of some other fimbria-associated proteins, as mentioned above. Although we could not conclude that either of these two possibilities was the case, we confirmed the absence of normal fimbriae on the surface of MPG1 by using immunogold electron microscopy with a monoclonal antibody against the FimA protein (Fig. 5C and D).

P. gingivalis possesses at least two adhesins, the fimbriae and hemagglutinin. Both of these functions have been shown to be involved in bacterial attachment to human cells, autoaggregation, and aggregation to other organisms (7). Therefore, to elucidate the relationship between hemagglutinating activity and the fimbriated phenotype of *P. gingivalis*, the *fimA* mutant and the wild-type strain were both examined for hemagglutinating activity by using sheep erythrocytes. The results showed that the *fimA* mutant retained a similar level of hemagglutinating activity to that of the wild-type strain, indicating that the *fimA* function does not directly affect the expression of the hemagglutinating activity of *P. gingivalis* (Fig. 6).

Previous studies have suggested that the surface hydrophobicity of oral bacteria plays an important role in their adherence to the host tissues (6, 22, 42). For example, Weiss et al. (42) and Gibbons and Etherden (6) compared surface hydrophobicity in several species of oral bacteria and found an

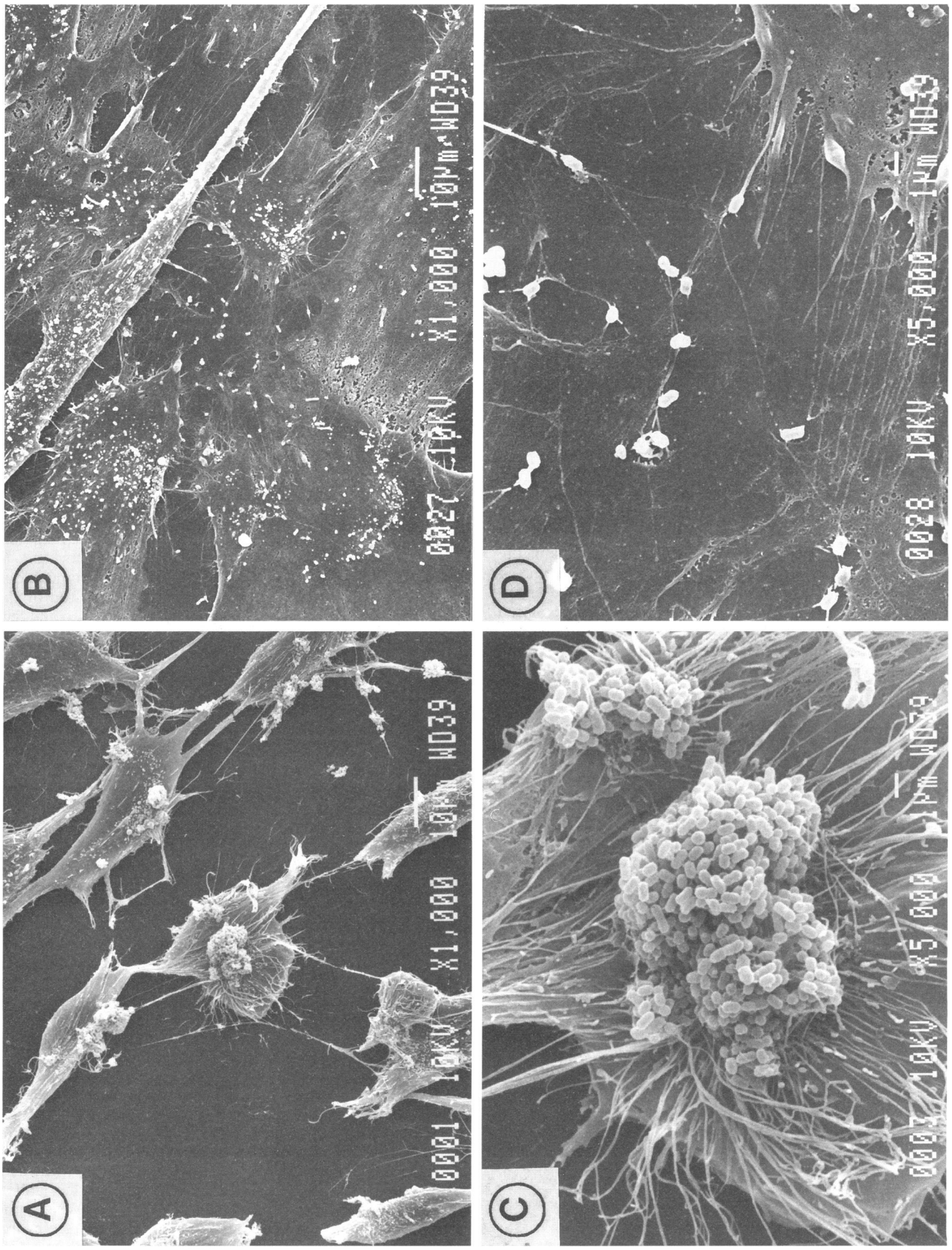
A
0001 10KV X1,000 10μm WD39
B
0027 10KV X1,000 10μm WD39
C
0007 10KV X5,000 1μm WD39
D
0028 10KV X5,000 1μm WD39

FIG. 7. Adherence of *P. gingivalis* to human gingival fibroblasts. Scanning electron micrographs of human gingival fibroblasts incubated with *P. gingivalis* ATCC 33277 (A and C) and MPG1 (B and D). *P. gingivalis* ATCC 33277 adhered with autoaggregation to human gingival fibroblasts. However, MPG1 (*fimA* mutant) showed no autoaggregation and less adherence. Magnifications are shown on the figure.

overall correlation between the adsorption of bacteria to hexadecane and their attachment to experimental substrates such as saliva-treated hydroxyapatite. Peros et al. (27) noted that *P. gingivalis* 381 had fewer fimbriae on the bacterial surface when the bacteria were grown in the presence of tetracycline at a sublethal concentration, under which conditions the surface hydrophobicity of the bacteria was significantly reduced. However, the surface hydrophobicity of the *fimA* mutant was not significantly different from that of the wild-type strain (Table 1), indicating that the observed surface hydrophobicity of *P. gingivalis* does not depend on the presence or absence of fimbriae on the bacterial surface.

The adherence capacity of the *fimA* mutant and the wild-type strain to two different types of tissue-cultured cells, human gingival fibroblasts and human carcinoma gingival epithelial cells, was examined. The adherence capacity of the *fimA* mutant was reduced to one-third of the wild-type level (Table 2). To further investigate the reduced adherence capacity of the *fimA* mutant, we examined bacterial interaction with the human gingival fibroblasts by using scanning electron microscopy. At 60 min after contact, profound changes in the normal architecture of the fibroblast surface occurred with the appearance of long microvilli surrounding large bacterial clumps (Fig. 7A and C). However, no such change was observed with the *fimA* mutant (Fig. 7B and D). Recently, Duncan et al. (5) investigated the interactions of *P. gingivalis* ATCC 33277 and W50, an afimbriated variant of *P. gingivalis*, with human oral epithelial cells (KB cells). The authors noted the appearance of microvilli on KB cells with attached, autoaggregated *P. gingivalis* ATCC 33277 but not with strain W50. These results indicate that upon contact with epithelial cells, wild-type *P. gingivalis* triggers a sequence of events that may lead to the reorganization of cytoskeletal components, giving rise to long microvilli, which facilitate fimbrial contact with human oral tissue. Further investigation of the molecular events occurring during the interactions between *P. gingivalis* and host cells is needed to elucidate the exact role of fimbriae in the pathogenesis of periodontal disease. Finally, the DNA exchange system presented in this study offers a simple way of introducing mutations into genes of interest in *P. gingivalis* and will thus be useful in establishing the identity of putative virulence-associated factors of this organism.

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