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# Generation and Purification of Recombinant Fimbrillin from *Porphyromonas (Bacteroides) gingivalis* 381

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**Fimbrillin is the major subunit protein of fimbriae from the human periodontal pathogen *Porphyromonas (Bacteroides) gingivalis*. We describe here the generation and initial characterization of recombinant fimbrillin (r-fimbrillin) isolated from *P. gingivalis* 381. A fragment of DNA encoding the gene for fimbrillin was generated by polymerase chain reaction and cloned into the expression vector pET11b. Plasmids containing the recombinant gene were transfected into *Escherichia coli*. Clones were selected on plates for ampicillin resistance and individually screened by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) for protein production after activation with IPTG (isopropyl-β-D-thiogalactopyranoside). One clone, OW0.2, produced significant amounts of a 42-kDa protein after induction with IPTG. This clone contained the pET11b plasmid with a 1-kb insert that had sequence homology to the gene encoding fimbrillin. The majority of recombinant protein from clone OW0.2 was found in the cytoplasm within inclusion bodies. Protein aggregates were solubilized in 8 M urea, and SDS-PAGE analysis showed two major protein bands, one at 42 kDa and the other at 17 kDa. These two proteins coeluted from a DEAE-Sepharose column at 0.15 M NaCl and were reactive to rabbit antiserum to fimbrillin in a Western blot (immunoblot). A preparation giving a single protein band at 42 kDa in SDS-PAGE was obtained by size fractionation by using continuous-elution electrophoresis. Lymph node cells from animals immunized with either fimbrillin from *P. gingivalis* or r-fimbrillin showed antigen-specific proliferation to both *P. gingivalis* fimbrillin and r-fimbrillin in an in vitro recall assay. Therefore, it appears that r-fimbrillin is chemically, antigenically, and serologically identical to fimbrillin isolated from *P. gingivalis* 381.**

Fimbriae, filamentous fibrils on the surface of bacteria, have been implicated to play a major role in bacterial pathogenesis by serving as both an adhesin for bacterial colonization (18) and as a target for antibody responses which protect the host against infections of these potentially harmful organisms (19, 26). *Porphyromonas (Bacteroides) gingivalis* is a black-pigmented gram-negative bacterium which has been implicated as a major causative agent in the pathogenesis of adult periodontal disease (22). Colonization of the subgingival area has been shown to be initiated by the attachment of *P. gingivalis* bacteria to host cells and to the preexisting microbial flora (24). Fimbriae are thought to play a major role in the colonization of *P. gingivalis* in the periodontal pocket through their affinity for human crevicular epithelial cells (7), human buccal epithelial cells (8), and oral gram-positive bacteria (6). A recent study showed that *P. gingivalis* fimbriae could also be involved in colonization by promoting coaggregation with *Actinomyces viscosus* (6). *P. gingivalis* cells have also been shown to adhere to erythrocytes (14), although these results are more controversial. While several studies claim that fimbriae or synthetic peptides derived from their amino acid sequence were responsible for hemagglutination (14, 16), experiments by Yoshimura et al. (30) and others (3, 8, 12, 17) question the role of purified fimbriae in hemagglutination. Therefore, the functional role of fimbriae and purified fimbrillin in colonization and hemagglutination is still in question.

While fimbriae may play an important role as an adhesin in the pathogenesis of periodontal disease, they may also play a major role in the immune response against *P. gingivalis*. Fimbriae may have been shown to be a major target for antibody responses in patients with advanced periodontal disease (13, 27). In addition, immunization with purified 42-kDa protein but not 70-kDa protein from *P. gingivalis* prevents the onset of periodontal disease in hamsters infected with *P. gingivalis* (5). These results suggest that fimbriae are an important component of both the infectious capability of *P. gingivalis* as well as in the efforts of the host to eliminate that infection.

The fimbriae of *P. gingivalis* 381 have been purified and characterized (29, 30). Fimbriae from *P. gingivalis* 381 were found to be single-stranded filaments composed of helical polymers of regularly spaced protein subunits. The constitutive protein, the fimbrillin, has an apparent molecular mass of approximately 42 kDa, as determined by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (30), consisting of a high content of β-pleated sheets, with very little evidence for extensive posttranslational glycosylation (30). The gene encoding the fimbrillin molecule from *P. gingivalis* 381 has now been cloned and sequenced (4). This sequence reveals a 347-amino-acid molecule with a predicted molecular mass of 37.6 kDa (4). Recently, the fimbrial gene was cloned in the bacteriophage T7 polymerase-promoter vector system (28). Western blot (immunoblot) analysis showed that a 45-kDa protein, presumably corresponding to the fimbrillin, was induced. However, no purification of recombinant fimbrillin (r-fimbrillin) was reported.

The aim of our studies is to characterize the immunologic properties of fimbrillin from *P. gingivalis* 381. Toward this end, we have isolated, cloned, and expressed the gene for

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fimbrillin from *P. gingivalis* 381 in an inducible prokaryotic expression vector and purified the induced protein. This article describes the initial biochemical and immunologic characterization of this r-fimbrillin.

## MATERIALS AND METHODS

**Bacterial strains and plasmid.** *P. gingivalis* 381 was obtained from Ernest Newbrun (University of California at San Francisco). This strain served as the source of fimbriae and fimbrillin for all chemical studies and as the source of DNA for the isolation, construction, and expression of the gene encoding for fimbrillin in *Escherichia coli*. These cells were grown in Trypticase-yeast extract media enriched with hemin (10 μg/ml) and vitamin K1 (5 μg/ml) in an anaerobic atmosphere (80% $N_2$, 10% $H_2$, 10% $CO_2$). *E. coli* BL21(DE3) cells, genotype $F^-$ *ompT* $r_B^-$ $m_B^-$ (DE3), which served as the host cells for the plasmid, were cultured in LB broth or plates. Plasmid pET11b (Novagen, Madison, Wis.) was used as the vector for cloning and sequence analysis.

**Rabbit antiserum.** A rabbit antiserum to fimbrillin, a kind gift of F. Yoshimura, was prepared as previously described (30).

**Construction of the pET11b plasmid containing the recombinant fimbrillin gene.** DNA was obtained from *P. gingivalis* 381 by lysing the cells in the presence of proteinase K. Briefly, 100 μg (wet weight) of pelleted cells in the logarithmic phase of cell growth was lysed by suspension in 1 ml of distilled water and then incubated at 95°C for 10 min. Ten milligrams of proteinase K was added to the 1-ml suspension, which was then incubated at 55°C for 30 min. The treated cell suspension was then centrifuged, and the supernatant was used in graded amounts as a template for polymerase chain reaction (PCR).

The primers we used for PCR were constructed on the basis of the published sequence of fimbrillin of Dickinson et al. (4) and are shown in Fig. 1A. Both forward and reverse primers contained 18 to 21 bases corresponding to the 5′ and 3′ terminal sequences of the desired coding segment. The forward primer incorporated the *Nde*I restriction site immediately before the coding region since the final three bases of the *Nde*I site, ATG, code for the initiation methionine residue. Immediately before the *Nde*I site, a *Bam*HI site was added, which would have allowed for cloning the PCR product into a pUC18 vector for amplification, should that have been necessary. The reverse primer amplifies a PCR product with a TAG stop codon immediately after the coding segments, which is followed by the *Bam*HI site. PCR amplification was performed with 2.5 U of *Taq* polymerase (Promega), 100 mM each of deoxynucleoside triphosphate (dNTP) (Promega), 500 ng of each primer, and 1 to 10 μl of template DNA, in a total volume of 100 μl. The reaction cycled 40 times with denaturing, annealing, and extension temperatures of 94, 60, and 72°C, respectively. The cycles were made progressively longer during the amplification procedure, with 20 cycles of 1 min, 10 cycles of 1.5 min, and 10 cycles of 2 min. The PCR product was precipitated and digested with *Nde*I and *Bam*HI, isolated by using the low-melting-temperature agarose technique of Seelan and Grossman (21), and then ligated to the plasmid pET11b (Novagen), a plasmid containing the T7 *lac* promoter, which also expresses the *lac*I gene repressor for efficient repression of basal transcription levels. In the pET11b plasmid, recombinant protein expression is induced by IPTG (isopropyl-β-D-thiogalactopyranoside). The DNA segments chosen for expression were cloned unidirectionally into the *Nde*I and *Bam*HI sites of pET11b. Transformation of *E. coli* cells with the pET11b plasmid containing the fimbrillin gene and selection on plates of LB medium containing 50 μg of ampicillin per ml (LB-AMP) were performed by using a modification of protocol outlined by FMC. Since pET11b does not make β-galactosidase, we could not screen for recombinant clones by using X-Gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside)–IPTG induction. Instead, we screened for recombinant clones by IPTG induction and protein production as described below.

**DNA sequence analysis.** Sequencing of the r-fimbrillin gene in the OW0.2 plasmid was accomplished by the method of dideoxy chain termination described by Sanger et al. (20). Double-stranded templates were denatured with NaOH-EDTA, neutralized, and ethanol precipitated. Sequencing reactions were performed by the Sequenase (U.S. Biochemicals) protocol.

**Screening of the recombinant clones for protein production.** The transformed *E. coli* cells were plated on LB-AMP plates, and single colonies were randomly selected to test for protein expression. A 3-ml culture was grown in LB-AMP media, and, when cultures were visibly turbid but before saturation, IPTG was added to 0.4 mM to one-half of the culture, and the other half was allowed to continue growing without induction. After 3 h of growth, 1 ml of each culture was pelleted in a microcentrifuge (13,000 rpm, 5 min), and the pellets were resuspended in 0.1 ml of SDS sample buffer and analyzed by SDS-PAGE. Successful induction of the protein showed a band of the expected size that was far more prominent than any bacterial protein. Cultures were grown from the chosen colonies, mixed with glycerol to 15%, and frozen at −70°C. Future cultures were inoculated from these frozen stocks.

**SDS-PAGE and Western blot.** SDS-PAGE was performed by the method of Laemmli (9) with a 12% separating gel. Samples were boiled for 5 min in sample buffer (0.125 M Tris-Cl [pH 6.8], 8% SDS, 40% glycerol, 20% β-mercaptoethanol). Proteins were either stained with Coomassie blue or silver nitrate (1) or electrotransferred to a polyvinylidene difluoride membrane in glycine-methanol buffer (25). The unoccupied sites on the membrane were blocked by incubation for 30 min in 20 mM Tris–0.5 M NaCl (pH 7.4) (TBS) containing 0.5% Tween 20 and 10% fish gelatin. The membrane was then incubated with a rabbit antiserum to fimbrillin diluted 1:500 in TBS containing Tween 20 and 10% fish gelatin for 1 h. The membrane was washed in TBS-Tween 20 and incubated with a goat anti-rabbit immunoglobulin G conjugated to alkaline phosphatase (Zymed Laboratories). After being washed, the bound antibodies were visualized by the addition of the chromogenic substrate BCIP-NBT (5-bromo-4-chloro-3-indolylphosphate toluidinium–nitroblue tetrazolium). The reaction was stopped by immersing the membrane in distilled water.

**Purification of *P. gingivalis* fimbriae.** *P. gingivalis* fimbriae were prepared by a modification of the procedure described by Yoshimura et al. (30). Briefly, *P. gingivalis* 381 cells were harvested by centrifugation at 10,000 × *g* for 20 min, resuspended in 20 mM Tris-Cl (pH 7.4) containing 10 mM $MgCl_2$ and 0.15 M NaCl, and stirred with a magnetic bar. A supernatant was obtained by centrifugation at 10,000 × *g* for 15 min. Proteins were precipitated by adding $(NH_4)_2SO_4$ to achieve 40% saturation and were recovered by centrifugation at 10,000 × *g* for 25 min. The pellet was resuspended in a minimal volume of 20 mM Tris-Cl (pH 8.0) and dialyzed against the same buffer. The dialyzed sample was subjected to further purification on a DEAE-Sepharose CL-6B column

equilibrated with 20 mM Tris-Cl (pH 8.0). The proteins were eluted with a gradient of 0 to 1.0 M NaCl. Fractions were analyzed by SDS-PAGE, and those containing fimbriae were pooled.

**Cell lysis and solubilization of the inclusion bodies.** Fifty milliliters of LB-AMP was infected with clone OW0.2 cells and grown overnight. The bacteria were centrifuged and resuspended in 50 ml of fresh LB-AMP (to eliminate the β-lactamase), and this culture was added to 1 liter of media. The bacteria were grown under agitation at 37°C for 3 h, and then IPTG was added at a final concentration of 0.4 mM. After 4 h of incubation under agitation with IPTG, cells were centrifuged and 3 g (wet weight) of cells was resuspended in 12 ml of lysis buffer consisting of 50 mM Tris (pH 8.0), 1 mM EDTA, and 0.1 M NaCl. Lysozyme was added at 0.2 mg/ml, and cells were incubated for 30 min at room temperature, under agitation. Triton X-100 was then added to 1%, phenylmethylsulfonyl fluoride (PMSF) was added to 0.1 mM, and agitation was continued for a second 30-min period. This resulted in partial lysis of the cells. The sample was frozen overnight and, upon thawing, exhibited significant viscosity indicating complete lysis. DNase I was added to 10 μg/ml along with $MgCl_2$ to 10 mM and an additional 0.1 mM PMSF. The lysate was incubated under agitation until it was no longer viscous. The sample was then centrifuged at 5,000 × *g* for 15 min, and both supernatant and pellet were tested by SDS-PAGE to determine the presence of the r-fimbrillin in the soluble or insoluble material. The insoluble material was then washed with a solution of 100 mM Tris (pH 8.5) containing 1 M urea (freshly prepared) and centrifuged at 5,000 × *g* for 10 min. The pellet was first resuspended in a minimal volume of buffer containing 50 mM Tris (pH 8.5), 1 mM EDTA, and 100 mM NaCl and then slowly added to a solution of 8 M urea freshly prepared in the same buffer. PMSF was added to 0.1 mM, and this solution was then incubated for 1 h at room temperature. This solution was then added slowly to 9 volumes of 50 mM $KH_2PO_4$ (pH 10.7) containing 1 mM EDTA and 50 mM NaCl, maintaining the pH at 10.7 with KOH during the dilution, and incubated at room temperature for 30 min. The pH was then adjusted to 8.0 with HCl. The solution was again incubated at room temperature for at least 30 min and centrifuged at 10,000 × *g* for 30 min to remove the remaining insoluble material. The supernatant was then concentrated and dialyzed against 20 mM Tris (pH 8.0).

**Purification of the recombinant protein.** The solubilized material was subjected to further purification by chromatography on DEAE-Sepharose, by using the same protocol described above for *P. gingivalis* 381 fimbriae. Alternatively, the crude recombinant material was separated by continuous-elution electrophoresis by using a preparative electrophoresis system (Bio-Rad, model 491 Prep Cell) with an 8% acrylamide gel as described in the manufacturer's instructions.

**Induction of proliferation by lymph node cells in vitro.** CBA/J female mice (6 to 8 weeks old; Jackson Laboratory, Bar Harbor, Maine) were immunized in the hind footpads with purified fimbrillin from *P. gingivalis* 381 or r-fimbrillin with 20 μg of antigen emulsified in complete Freund's adjuvant containing H37Ra mycobacteria (GIBCO Laboratories, Grand Island, N.Y.). Eight to 10 days after immunization, draining lymph nodes were removed, and $3.5 \times 10^5$ cells along with 20 or 4 μg of antigen per ml was added to each well of a 96-well microtiter plate (Costar, Cambridge, Mass.). Cells and antigen were cultured in RPMI 1640 medium with 2 mM L-glutamine, 50 μM 2-mercaptoethanol, 50 μg of gentamicin per ml, and 5% fetal bovine serum (KC Biologicals, Kansas City, Mo.). Cultures were incubated for 5 days, pulsed with 1 μCi of [$^3$H]thymidine (New England Nuclear, Boston, Mass.), harvested after 3 h, and counted in a liquid scintillation counter.

## RESULTS

**Generation and screening of recombinant fimbrillin gene from *P. gingivalis*.** DNA was obtained from *P. gingivalis* 381 as described in Materials and Methods. The fimbrillin gene from this DNA preparation was amplified by PCR. The primers used are shown in Fig. 1A. Bands are clearly visible after PCR amplification at approximately 1 kb, the predicted size of the amplified fimbrillin gene from *P. gingivalis* 381 as shown in Fig. 1B.

The amplified DNA was purified from primers, dNTPs, and smaller fragments and ligated into pET11b. A genetic map of the pET11b-fimbrillin gene construct is shown in Fig. 1C. The ligated pET11b plasmid was then used to transform competent cells of *E. coli* BL21(DE3).

The transformed cells were screened on LB-AMP plates, since only those cells expressing the ampicillin resistance gene after transformation with plasmid would grow on these plates. Several colonies were picked at random and tested by SDS-PAGE for protein expression after induction with IPTG. Transformed cells induced with IPTG produced a protein migrating at 42 kDa at a significantly elevated level compared with that produced by uninduced cells. Figure 2 shows representative patterns of induction for clone OW0.2 (Fig. 2, lanes 2 and 3). Western blot analysis of these clone products showed that the broad band at 42 kDa was reactive with rabbit antifimbrillin serum, and the relative amount of protein produced per milliliter of media was highest for clone OW0.2 as determined by enzyme-linked immunosorbent assay. Therefore, clone OW0.2 was selected for further study because of its efficient production of r-fimbrillin.

Our initial characterization of clone OW0.2 involved isolating and sequencing the fimbrillin gene insert. The insert was sequenced in both directions by the dideoxy sequencing method of Sanger et al. (20). The nucleotide sequence of this construct confirmed its fidelity to the sequence of the fimbrillin gene from *P. gingivalis* 381 published by Dickinson et al. (4) (data not shown).

**Chemical characterization of r-fimbrillin protein from clone OW0.2.** We then sought to identify the appropriate conditions which would allow the purification of the recombinant protein generated by clone OW0.2 after induction with IPTG but which would not destroy its chemical and antigenic properties. While induction of clone OW0.2 with IPTG leads to the production of a protein with a molecular mass of 42 kDa (Fig. 2, lanes 2 and 3), these preparations are heavily contaminated with cellular proteins from *E. coli*. Cells were first subjected to lysis treatment with lysozyme and Triton X-100 (Fig. 2, lane 3). After treatment, cell lysates were centrifuged at 5,000 × *g*, and both the supernatant and pellet were subjected to SDS-PAGE. The results shown in Fig. 3 indicate that the recombinant material was recovered in the insoluble fraction (lane 5) but not in the soluble fraction (lane 4). This indicated that the r-fimbrillin accumulates as insoluble inclusion bodies in the cytoplasm. A procedure for the isolation and solubilization of inclusion bodies was developed so that a maximum of contaminants could be separated from the r-fimbrillin.

Figure 3 shows the fractions obtained during the washing and solubilization steps. Washing the insoluble pellet of the

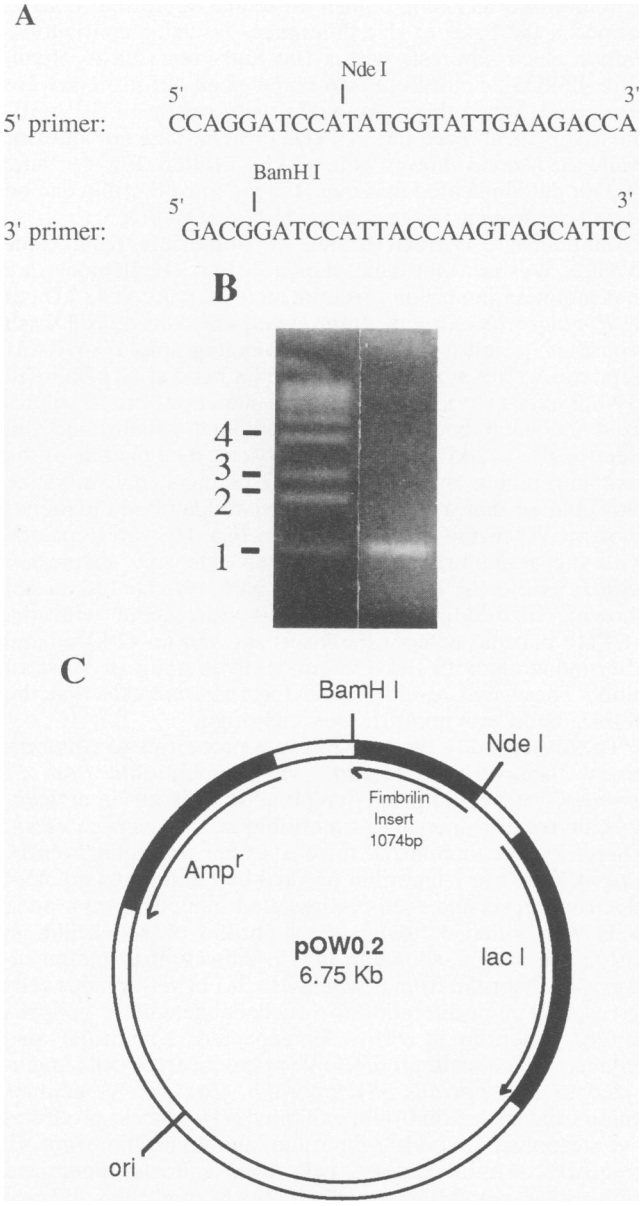


FIG. 1. Generation of fimbrillin gene construct in expression vector. (A) Primers used to amplify the fimbrillin gene for cloning. The 5′ PCR primer corresponding to positions 228 to 254 (4) of the genomic fimbrillin sequence has an *Nde*I site engineered into the indicated site to allow cloning of the PCR product directly into the ATG start codon so that no heterologous leader sequence is expressed. The 3′ PCR primer has a *Bam*HI site engineered into the indicated site to allow cloning into the *Bam*HI site of the pET11b plasmid. (B) Product of PCR obtained by using the indicated primers and genomic DNA from *P. gingivalis* 381. The product was run on a 1% agarose gel. Molecular size markers are labeled in the left margin (in kilobases). The apparent size of this product is 1 kb, corresponding to the predicted size of 1,074 bp of the full-length fimbrillin gene. This PCR product was purified, digested with *Nde*I and *Bam*HI, and ligated into the pET11b expression vector at the *Nde*I and *Bam*HI cloning sites. Ligated plasmids were transfected into *E. coli*, selected on LB-AMP plates, cloned, and screened by protein production after induction by IPTG. (C) Restriction and genetic map of the expression vectors pET11b-OW. The locations of the inserted PCR product, ampicillin resistance gene, and the *lacI* gene are shown by heavy lines. The thin arrows inside the circle indicate the directions of transcription of the indicated genes.

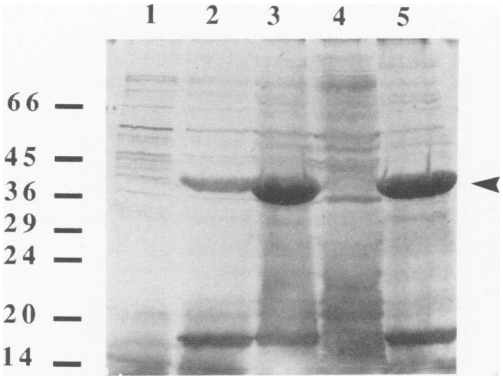


FIG. 2. Localization of the 42-kDa protein in the *E. coli* cells. Lanes: 1, uninduced OW0.2 cells; 2, OW0.2 cells after induction with IPTG for 4 h; 3, IPTG-induced OW0.2 cell lysate obtained by treatment with lysozyme and Triton X-100; 4, supernatant (soluble fraction) obtained after centrifugation of the induced OW0.2 cell lysate; 5, pellet (insoluble material) obtained after centrifugation of the induced OW0.2 cell lysate. All samples were boiled for 5 min in sample buffer and subjected to SDS-PAGE. The gel was then stained with silver nitrate. Molecular size markers are labeled in the left margin (in kilodaltons). The arrowhead indicates the 42-kDa band corresponding to the r-fimbrillin.

lysate with 1 M urea proved to be effective in removing contaminating material with minimal solubilization of recombinant material (lane 1). Further separation was performed with 8 M urea under alkaline conditions and by centrifugation at 10,000 × *g* for 30 min. Under these conditions, most of the recombinant material was solubilized (lane 3), while the insoluble material still containing the majority of contaminants was recovered as a small pellet (lane 2). The solubilized material contained two major proteins, one at 42 kDa and another at 17 kDa.

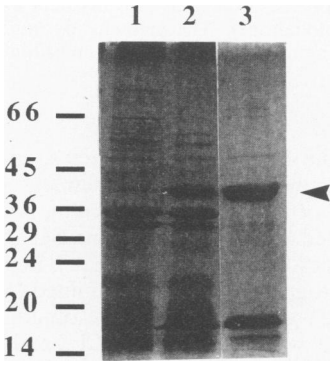


FIG. 3. Solubilization of the inclusion bodies. Insoluble material of the cell lysate was washed once with 100 mM Tris (pH 8.5) containing 1 M urea, centrifuged, and successively solubilized with 50 mM Tris (pH 8.5)–1 mM EDTA–100 mM NaCl and 50 mM $KH_2PO_4$ (pH 10.7)–1 mM EDTA–50 mM NaCl. Samples were boiled in sample buffer for 5 min and subjected to SDS-PAGE. The gel was stained with silver nitrate. Lanes: 1, supernatant obtained after washing the insoluble material of the cell lysate with 1 M urea (this step was effective in removing contaminant material with minimal solubilization of recombinant material); 2, final pellet obtained after solubilization of the inclusion bodies; 3, solubilized inclusion bodies. Molecular size markers are labeled in the left margin (in kilodaltons). The arrowhead indicates the 42-kDa band corresponding to the r-fimbrillin.

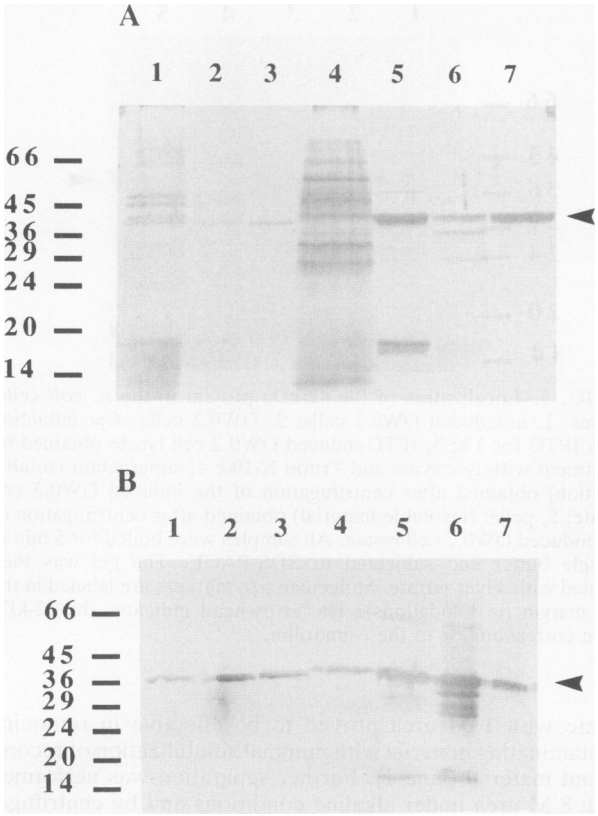


FIG. 4. SDS-PAGE and immunoblot analysis. *P. gingivalis* and clone OW0.2 cell extracts were subjected to SDS-PAGE. After electrophoresis, proteins were either silver stained (A) or transferred to polyvinylidene difluoride and reacted with rabbit antiserum to *P. gingivalis* fimbriae (B). Lanes: 1, *P. gingivalis* outer membranes; 2, *P. gingivalis* cell wash with $MgCl_2$; 3, purified *P. gingivalis* fimbriae on DEAE-Sepharose column; 4, uninduced OW0.2 cells; 5, crude solubilized inclusion bodies; 6, chromatographed r-fimbrillin; 7, pure 42-kDa protein obtained by continuous-elution electrophoresis. Molecular size markers are labeled in the left margin (in kilodaltons). The arrowheads indicate the 42-kDa band corresponding to both *P. gingivalis* fimbrillin and r-fimbrillin.

To separate the 42- and 17-kDa species, and to determine if the recombinant protein had chemical characteristics similar to those of *P. gingivalis* fimbrillin, solubilized material was submitted to further separation. Separation based on charge differences determined by DEAE-Sepharose CL-6B column chromatography was first examined for this purpose. Fimbriae from *P. gingivalis* 381 were obtained by chromatography of cell washings with $MgCl_2$, and fimbrillin was purified on a DEAE-Sepharose CL-6B column and then compared with the recombinant protein by SDS-PAGE (Fig. 4A) and Western blot (Fig. 4B). The *P. gingivalis* fimbriae fraction obtained by chromatography on DEAE-Sepharose appeared, after SDS-PAGE, as a major band at 42 kDa and a doublet of minor proteins at 60 and 58.5 kDa (Fig. 4A, lane 3). When solubilized r-fimbrillin was subjected to chromatography on a DEAE-Sepharose CL-6B column, the 42-kDa protein eluted at the predicted point, i.e., with 0.15 M NaCl (30), but was still contaminated with the 17-kDa protein (Fig. 4A, lane 6). Interestingly, there was no doublet contaminant in preparations of r-fimbrillin obtained from the OW0.2 clone.

Solubilized inclusion bodies were then subjected to separation on the basis of size differences by using continuous-elution electrophoresis with a Bio-Rad Prep Cell to obtain pure 42-kDa r-fimbrillin. Two major peaks of protein were recovered. When these two peaks were run on SDS-PAGE, the first peak showed pure 17-kDa protein (data not shown), while the second showed pure 42-kDa protein (Fig. 4A, lane 7). Our data indicated that over 100 mg of r-fimbrillin can be recovered from a 1-liter culture of clone OW0.2.

**Immunologic characterization of r-fimbrillin from clone OW0.2.** Western blot data, shown in Fig. 4B, demonstrate that rabbit antifimbrillin serum detected a band at 42 kDa in all *P. gingivalis* extracts: outer membranes, crude cell wash with $MgCl_2$, and fimbriae after chromatography on DEAE-Sepharose. This serum also detected a band at 42 kDa in all OW0.2 extracts: cell lysate before induction, crude solubilized inclusion bodies, chromatographed r-fimbrillin, and electroeluted 42-kDa protein. We observed no binding of the goat anti-rabbit immunoglobulin G conjugated to alkaline phosphatase that was used as the second antibody (data not shown). When the results shown in Fig. 4B were repeated with the antifimbrillin antisera after extensive absorption with *E. coli* cells, identical results were obtained (data not shown). In addition, *E. coli* cells transfected with the pET11b plasmid without the insert showed no 42-kDa band after induction with IPTG in either silver stains or Western blots. These two results, taken together, indicate that the 42-kDa band was not of *E. coli* cell origin.

To further study the immunologic properties of r-fimbrillin, we sought to determine whether fimbrillin from *P. gingivalis* 381 could prime lymph node cells for an antigen-specific recall response to r-fimbrillin in vitro or vice versa. Therefore, we immunized mice to either fimbrillin from *P. gingivalis* 381 or r-fimbrillin purified by continuous-gradient electrophoresis and then restimulated immune lymph node cells with either *P. gingivalis* fimbrillin or r-fimbrillin in vitro. The results, shown in Fig. 5, indicate that immunization with fimbrillin from *P. gingivalis* 381 in vivo primes cells to respond by proliferation to a rechallenge with *P. gingivalis* 381 fimbrillin in vitro. As expected, r-fimbrillin also induced a significant proliferative response from cells immunized to *P. gingivalis* 381 fimbrillin. Conversely, animals immunized with r-fimbrillin exhibited a significant proliferative response to both r-fimbrillin and fimbrillin from *P. gingivalis* 381. In contrast, cells from animals immunized with either r-fimbrillin or *P. gingivalis* fimbrillin did not respond to bovine serum albumin (BSA), demonstrating the antigenic specificity of the reaction to both *P. gingivalis* 381 fimbrillin and r-fimbrillin.

## DISCUSSION

These results demonstrate that we have successfully isolated, cloned, and expressed the gene for fimbrillin, the fimbrial subunit protein of *P. gingivalis* 381. The gene was cloned into the pET11b expression vector and is expressed at high quantities in *E. coli* cells after induction with IPTG. Sequence analysis of the gene construct transfected into clone OW0.2 cells, as well as biochemical and serological analysis of the recombinant protein produced by these cells, indicates that a 42-kDa protein produced by clone OW0.2 after induction with IPTG is r-fimbrillin, which retains the same chemical and antigenic properties of the fimbrillin molecule from the parental strain *P. gingivalis* 381.

To facilitate the identification of r-fimbrillin during purification procedures, we utilized antiserum against fimbrillin

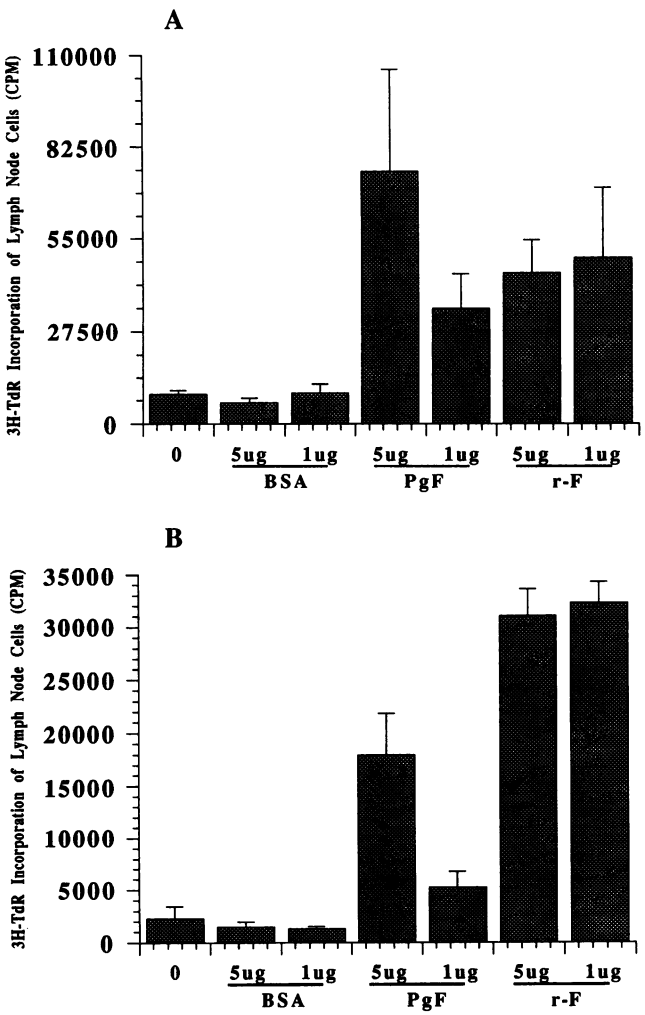


FIG. 5. Proliferative response of mice lymph node cells to *P. gingivalis* fimbriae or r-fimbrillin. CBA/J mice were immunized with 100 μg of either purified fimbriae from *P. gingivalis* 381 cells (A) or purified r-fimbrillin (B). Draining lymph node cells were removed and restimulated in culture with no addition (control), BSA, purified fimbriae from *P. gingivalis* 381 (PgF), or purified r-fimbrillin (r-F). Cells were pulsed with [$^3$H]thymidine (3H-TdR) after 3 days and harvested, and radioactivity was measured in a scintillation counter. Values represent the average of triplicate cultures ± standard error of the mean. The response of lymph node cells of *P. gingivalis* fimbriae-immunized animals to *P. gingivalis* fimbriae and r-fimbrillin is significantly different than the response to BSA ($P < 0.01$). Likewise, the response of lymph node cells of r-fimbrillin-immunized animals to *P. gingivalis* fimbriae and r-fimbrillin is significantly different than the response to BSA ($P < 0.001$).

from *P. gingivalis* 381 obtained from F. Yoshimura (30). While a little recombinant protein is produced from the pET11b vector prior to induction with IPTG, the appearance of a single major 42-kDa protein species in OW0.2 cells after induction with IPTG most likely represents the r-fimbrillin protein produced in these cells. Further confirmation that this protein is r-fimbrillin comes from the fact that this 42-kDa protein is the identical size of fimbrillin isolated from *P. gingivalis* 381 and that it strongly reacts in Western blot to antiserum against *P. gingivalis* 381 fimbrillin.

Fimbrillin molecules are normally expressed in fimbriae of *P. gingivalis* and can be obtained either from the supernatant of *P. gingivalis* cultures, from shedding from the cell membrane, or from a rather mild shearing and cell wash of the outer cell membrane of *P. gingivalis* cells. In contrast, the majority of r-fimbrillin produced by *E. coli* cells was found in the cytoplasmic fraction and therefore did not appear to be incorporated in the fimbriae of the host *E. coli* cells. While small amounts of r-fimbrillin could be detected in cell lysates of *E. coli* cells after solubilization with 1% Triton X-100, most of the recombinant protein remained insoluble. This suggested that the majority of r-fimbrillin produced was found within inclusion bodies (11). Therefore, a new procedure for isolating fimbrillin had to be developed to isolate this protein. Our initial attempts at purification took advantage of the fact that even after solubilization of whole-cell lysate with nonionic detergent, and then treatment with 1 M urea in 100 mM Tris (pH 8.5), the r-fimbrillin protein remained insoluble. After solubilization of the inclusion bodies by treatment with 8 M urea, the majority of contaminating proteins have been removed. This procedure left us with a major protein band at 42 kDa, a single major contaminant at 17 kDa when this preparation was subjected to SDS-PAGE, and several minor contamination bands with molecular masses of 25 to 40 kDa. Fortunately, this treatment did not appear to significantly alter the biochemical and serologic properties of the molecule, since the r-fimbrillin isolated by this method still ran as expected at 42 kDa on SDS-PAGE and still reacted with fimbrillin-specific antisera in Western blot.

The purification of the 42-kDa protein was complicated by the presence of several major contaminants. The first was a series of bands at 25 to 40 kDa and the second was a major band of protein at 17 kDa in SDS-polyacrylamide gels. The nature of these contaminating proteins is at present unknown. In SDS-polyacrylamide gels, there is no major protein band at 17 or 25 to 40 kDa from *E. coli* cell lysates containing the OW0.2 plasmid prior to induction with IPTG. Interestingly, these contaminating proteins also do not appear during the purification of fimbrillin from *P. gingivalis* 381, suggesting they are related to the r-fimbrillin expressed in *E. coli* cells, either as a breakdown product resulting from the rather harsh conditions of purification or as a host protein which associates with r-fimbrillin during purification. However, these proteins react very strongly to rabbit anti-fimbrillin serum, suggesting it may be antigenically related to r-fimbrillin. Interestingly, these background bands were observed in Western blots from IPTG-induced OW0.2 cells prior to and even after DEAE chromatography of r-fimbrillin. We therefore performed several different experiments to ensure that these bands were not contamination from *E. coli* cells. First, our antisera was absorbed on uninduced *E. coli* cells. When this antiserum was used to develop Western blots from chromatographed r-fimbrillin, the same background bands were observed. Second, when we analyzed *E. coli* cells transfected with pET11b plasmid lacking the OW0.2 insert, no such bands were seen with uninduced or induced *E. coli* cells. Therefore, it is quite likely that these background bands represent incomplete or enzymatically digested fimbrillin molecules, which can easily be removed by continuous-gradient electrophoresis, yielding a pure 42-kDa band.

After solubilization, we first used the purification scheme normally used to isolate fimbrillin from *P. gingivalis* 381 for separating r-fimbrillin from these contaminating proteins (30). When cell washings from *P. gingivalis* 381 were subjected to DEAE-Sepharose column chromatography, we routinely observed a relatively pure band of fimbrillin at 42

kDa on SDS-PAGE. However, when this procedure was used to purify the r-fimbrillin, the anion-exchange column failed to separate the major 42-kDa protein from the contaminating proteins, both of which eluted at 0.15 M NaCl. It was therefore necessary to carry out a different purification step to separate the 42-kDa species from the contaminating species, and this was done rather easily by continuous-elution electrophoresis on the basis of the size differences between these two species by using a Bio-Rad Prep Cell, which yielded essentially pure 42-kDa r-fimbrillin. The relationship of these contaminating proteins to r-fimbrillin is currently under investigation.

Fimbrillin and fimbriae have also been found to be major antigenic structures on the surface of *P. gingivalis* cells. Patients with adult periodontitis have elevated serum antibody levels to fimbriae from *P. gingivalis* (27), which originate from plasma cells located in the inflamed gingiva of adult periodontitis patients (13). Interestingly, administration of purified fimbriae from *P. gingivalis* 381 to BALB/c mice generates a significant antibody response of the immunoglobulin G and A subclasses (15). In addition, immunization of rats with purified fimbriae from *P. gingivalis* 381 protected periodontal tissue against destruction mediated by a periodontal *P. gingivalis* infection in gnotobiotic rats (5). These results suggest that fimbriae can serve as an important immunologic rejection antigen for *P. gingivalis* during chronic periodontal disease.

The antigenic relationship of fimbriae with fimbrillin is still under investigation. While several monoclonal antibodies raised against fimbriae have been reported to react to partially and totally denatured fimbrillin (2), suggesting many epitopes in common between fimbriae and fimbrillin, other investigators report that monoclonal antibodies against fimbriae do not react to denatured fimbrillin (8, 10, 27). In addition, antiserum raised in rabbits to fimbriae from *P. gingivalis* 381 showed limited cross-reactivity to purified 42-kDa fimbrillin and vice versa (10, 23, 27), suggesting that some antigenic determinants which exist on fimbriae do not exist on purified denatured fimbrillin. Our results with Yoshimura's rabbit antiserum show that our r-fimbrillin has serologic epitopes similar or identical to those expressed on fimbrillin directly isolated from *P. gingivalis* 381 cells. In addition, fimbrillin from *P. gingivalis* 381 and r-fimbrillin show antigenic cross-reactivity at the level of T-cell proliferation as well, in that fimbrillin from *P. gingivalis* 381 could prime T cells to proliferate to r-fimbrillin, and vice versa, but not to BSA. These results demonstrate that r-fimbrillin is antigenically identical to or very cross-reactive with fimbrillin isolated from *P. gingivalis* 381 at both the antibody and T-cell responsiveness levels. Therefore, we suggest that r-fimbrillin can now be used to measure the nature of immunogenic determinants in native fimbriae as well as a source of protein for protective immunizations and vaccine development.

We described here a clone of *E. coli* cells containing the gene for fimbrillin from *P. gingivalis* 381 in a prokaryotic expression vector which can be induced with IPTG to generate recombinant protein at high quantities. This r-fimbrillin can be purified with relative ease from a complex mixture of proteins in the host expression system. The generation of genetically engineered r-fimbrillin will allow us to study in detail all functional, biochemical, and immunological properties of that major antigen of *P. gingivalis*.

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