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Characterization of Recombinant and Native Forms of a Cell Surface Antigen of *Porphyromonas* (*Bacteroides*) *gingivalis*

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The cloning of genes encoding putative cell surface antigens of *Porphyromonas gingivalis* ATCC 33277 has been reported previously (B. C. McBride, A. Joe, and U. Singh, Arch. Oral Biol. 55:59S–68S, 1990). This study characterizes the recombinant protein rPgAg1, which is highly expressed in clone BA3, and the corresponding 51-kDa native antigen PgAg1. Cellular localization studies with monospecific antibodies to rPgAg1 in a Western immunoblot assay of a *P. gingivalis* membrane fraction and immunogold labeling of intact *P. gingivalis* cells confirmed the cell surface location of the native PgAg1 molecule. The *pgag1* gene was found to be present in all four strains of *P. gingivalis* examined, and the gene product was expressed. Highly homologous DNA sequences and immunologically related proteins, however, were not detected in related species in the group formerly known as black-pigmented *Bacteroides*. This suggests that PgAg1 is specific to *P. gingivalis* and is highly conserved within this species. A protein data base search with the NH_2-terminal amino acid sequence of rPgAg1 did not identify any significantly similar protein sequences. The high level of expression of rPgAg1 was not dependent on the insertional orientation of the cloned fragment. It therefore appears that a *P. gingivalis* promoter is present which is well recognized by the transcriptional apparatus of the *Escherichia coli* cloning host. The promoter element and structural gene for a specific cell surface antigen of *P. gingivalis* have been cloned.

Porphyromonas (*Bacteroides*) *gingivalis* is frequently isolated from the subgingival microflora of patients with periodontal disease (6, 51). This anaerobic, black-pigmented, coccobacillary rod has been suggested to play an important role in destructive periodontitis. Clinical studies demonstrate that in patients with periodontitis, *P. gingivalis* constitutes a major proportion of the organisms at the diseased site, but it is not commonly found in the microflora at healthy sites. Eradication of *P. gingivalis* from the subgingival microbial population correlates with resolution of the disease (35, 56).

In the establishment and progression of a disease, a pathogen must express virulence determinants which are crucial to bacterium-host interactions. These play a role in microbial colonization, host tissue destruction, evasion of host defense mechanisms, and deleterious modification of host cells (13). Specific cell surface components of *P. gingivalis* have been identified as potential virulence factors. These include the capsule (18, 40, 57), fimbriae (15, 20, 24, 31, 63), lipopolysaccharide (3, 19, 41), hemagglutinins (23, 38, 42), and membrane-associated proteases (17, 54, 62). In addition, cell surface molecules of *P. gingivalis* appear to bind to human fibrinogen (30) and fibronectin (29) and to mediate adherence to basement membrane (39) and other oral microorganisms (26, 32, 53). The exact identity and nature of these adhesins are still under examination.

Specific cell surface antigens of an infecting organism may also evoke a strong immunological response in the host. Studies of the composition of the outer membrane of *P. gingivalis* (9, 25, 59) have identified many membrane-associated proteins which may act as immunogens. Deslauriers et al. (9) detected a series of different-sized polypeptides which are associated with outer membrane fractions of *P. gingivalis* ATCC 33277, including two proteins of 48 and 52 kDa. Kennell and Holt (25) have also identified a number of proteins, including three molecules ranging from 47 to 54 kDa, in outer membrane samples from the same strain.

To determine which specific bacterial antigens are strongly immunogenic, several researchers have undertaken immunological studies with sera from patients with periodontal disease. Watanabe et al. (59) probed whole-cell sonicate extracts and outer membrane protein samples of *P. gingivalis* W50 with human sera. They found a strong antibody reaction with a 46-kDa *P. gingivalis* outer membrane antigen which occurred significantly more frequently with sera from patients with severe periodontitis than with sera from adults with only mild or no clinical evidence of periodontitis. *P. gingivalis* W83 was examined by Duncan et al. (12) for the presence of antigens reactive with antibodies in sera from patients with a history of periodontal disease. A protein of 47 kDa in outer membrane preparations of this strain was recognized by all sera tested from patients with past advanced periodontitis but only by 10% of sera from adults with little or no history of periodontal disease. A subsequent study (8) confirmed that an immunoreactive molecule of 47 kDa, as well as one at 55 kDa, exists in *P. gingivalis* W83. The presence of antibody to immunogenic bacterial antigens may serve as a marker of past or current infection with *P. gingivalis*. It may be of interest to investigate immunodominant cell surface proteins as potential vaccine components.

We have previously reported the cloning of genes encoding putative cell surface proteins of *P. gingivalis* ATCC 33277 (37). The *Escherichia coli* clone BA3 has been selected for further study. The relative molecular weight of the recombinant protein (designated rPgAg1) expressed in BA3 appears to be 48,000 (corrected from 44,000, as given previ-

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ously [37]). In this study, we have characterized the *P. gingivalis* antigen (designated PgAg1) and the *pgag1* gene. Of interest was whether this particular antigen may be a species-specific marker or virulence factor expressed by *P. gingivalis*.

MATERIALS AND METHODS

Bacterial strains and culture conditions. The anaerobic strains *Porphyromonas* (*Bacteroides*) *gingivalis* ATCC 33277, W12, W50 (ATCC 53978), and W83; *Porphyromonas asaccharolytica* (*Bacteroides asaccharolyticus*) ATCC 25260; *Prevotella* (*Bacteroides*) *corporis* ATCC 33547; *Prevotella* (*Bacteroides*) *denticola* ATCC 33185; *Prevotella intermedia* (*Bacteroides intermedius*) ATCC 15032; *Bacteroides levii* ATCC 29147; *Prevotella* (*Bacteroides*) *loeschii* ATCC 15930; and *Prevotella melaninogenica* (*Bacteroides melaninogenicus*) ATCC 25845 were used in the present study. These are members of a group known formerly as black-pigmented *Bacteroides* (BPB). In this report, these organisms will be referred to in their former grouping. All anaerobes were grown in brain heart infusion (BHI) broth (Difco Laboratories, Detroit, Mich.) supplemented with hemin (5 μg/ml) and menadione (1 μg/ml). Laked blood and agar were added to final concentrations of 5% (vol/vol) and 1.5% (wt/vol), respectively, to prepare BHI-blood agar plates. Organisms were grown at 37°C in an anaerobic chamber (Coy Manufacturing, Ann Arbor, Mich.) containing an atmosphere of N_2-H_2-CO_2 (85:10:5). Culture purity was assessed by phase-contrast microscopy and colony isolation on BHI-blood agar plates.

E. coli JM83 was used as the recipient for the plasmid vector pUC18 (61) and the recombinant plasmids pBA3 and pBA3R. Cultures were grown in Luria-Bertani (LB) broth (48) at 37°C under aerobic conditions. Agar was added (to 1.5%) to prepare LB agar plates. Ampicillin (Sigma Chemical Co., St. Louis, Mo.) was used in the medium at 100 μg/ml when required.

P. gingivalis W50 was grown in vivo in Hartley guinea pigs in infectivity assays as described by Grenier and McBride (16).

Isolation of membranes from *P. gingivalis*. Membrane fractions from *P. gingivalis* were prepared by French pressure cell disruption of whole cells and differential centrifugation. Briefly, cells were harvested and washed once with 0.85% (wt/vol) NaCl and once with 20 mM HEPES (*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid, pH 7.4). Cells were resuspended in lysis buffer (20 mM HEPES [pH 7.4], 10 mM EDTA, 10 mM $MgCl_2$, 20 μg of RNase A per ml, 20 μg of DNase I per ml, 20 mM *N*-α-tosyl-L-lysine chloromethyl ketone [TLCK], 200 μM phenylmethylsulfonyl fluoride) and broken by two rounds of French pressure cell disruption at 20,000 lb/in^2 in a cold Aminco French pressure cell (SLM Instruments Inc., Urbana, Ill.). The broken cell suspension was subjected to low-speed centrifugation (6,000 × *g*, 15 min, 4°C) to pellet whole cells and cell debris. A membrane fraction was recovered from the supernatant by centrifugation at 100,000 × *g* for 2 h at 4°C. The membrane pellet was washed once in 20 mM HEPES (pH 7.4), resuspended in membrane resuspension buffer (20 mM HEPES [pH 7.4], 10 mM EDTA, 20 mM TLCK, 200 μM phenylmethylsulfonyl fluoride), and stored frozen at −20°C.

Recombinant DNA methods. Plasmid DNA was isolated by the alkali lysis method and further purified by equilibrium centrifugation in cesium chloride-ethidium bromide gradients (48). Chromosomal DNA was extracted from bacterial cells by the method of Silhavy et al. (50).

DNA was digested with restriction endonucleases according to the recommendations of the manufacturer for each enzyme used. DNA fragments were analyzed by agarose gel electrophoresis (48). Briefly, digested samples were run on 0.7 to 1.0% (wt/vol) agarose gels in a Tris-borate-EDTA buffer. Following electrophoresis, gels were stained in a solution of 0.5 μg of ethidium bromide per ml to visualize the DNA bands. DNA fragments in a *Hin*dIII digest of bacteriophage lambda (Bethesda Research Laboratories [BRL], Inc., Gaithersburg, Md.) were used as DNA molecular weight standards.

Subcloning was done with DNA fragments separated in agarose gels which were prepared and run in a Tris-acetate-EDTA buffer (48). The required fragment was excised from the agarose gel and recovered by the phenol extraction technique of Silhavy et al. (50). Ligations were performed by standard procedures (48). Transformations were done by the method of Chung et al. (7).

The 3.7-kb *Pst*I-*Pst*I fragment of pBA3 was isolated and used as the probe in Southern hybridization studies. Southern hybridization analysis was performed with the BluGENE nonradioactive nucleic acid detection system (BRL), with slight modifications of the recommended protocol. Briefly, the probe was labeled with biotin by using the BRL nick translation system. Samples of pBA3 and chromosomal DNA from BPB species were digested to completion with *Pst*I, electrophoresed on a 0.8% agarose gel, and transferred to nitrocellulose by capillary transfer with 20× SSC (3 M NaCl, 0.3 M sodium citrate [pH 7.0]) as described by Sambrook et al. (48). Hybridization was done overnight at 65°C with the probe in hybridization buffer (6× SSC, 50 mM Tris [pH 7.5], heat-denatured salmon sperm DNA [100 μg/ml], 0.5% [wt/vol] sodium dodecyl sulfate [SDS], 1% Denhardt's solution). The blot was washed two times in 2× SSC containing 0.1% SDS at room temperature, two times in 0.2× SSC containing 0.1% SDS at room temperature, and twice in 0.16× SSC containing 0.1% SDS at 55°C. After a brief rinse in 2× SSC at room temperature, reactive bands were detected by incubation of the blot with streptavidin-alkaline phosphatase and then with dye solution containing nitroblue tetrazolium (NBT) and 5-bromo-4-chloro-3-indolylphosphate (BCIP).

Electrophoretic techniques. SDS-polyacrylamide gel electrophoresis (SDS-PAGE) was carried out with the buffer system of Laemmli (28). For SDS-PAGE, *E. coli* cells and *P. gingivalis* membrane samples were solubilized in sample buffer (62.5 mM Tris-HCl [pH 6.8], 5% [vol/vol] β-mercaptoethanol, 1% SDS, 10% [vol/vol] glycerol, 0.012% [wt/vol] bromophenol blue) at 95°C for 10 min unless otherwise stated. Cell lysate samples of BPB species were prepared as follows. BPB cells were harvested, washed twice with 0.85% NaCl, and incubated with 20 mM TLCK for 10 min on ice. Sample buffer was added, and the preparation was heated to 95°C for 10 min unless otherwise stated.

Gels were stained for protein with Coomassie brilliant blue R-250. Alternatively, proteins were transferred electrophoretically (55) to nitrocellulose for the Western blot immunoassay. Proteins which reacted with specific antibodies were visualized with a goat anti-rabbit immunoglobulin G (IgG)-alkaline phosphatase conjugate (BRL) and then with a dye solution containing NBT and BCIP. Prestained protein standards (BRL) were used to calibrate molecular weights in protein gels and Western blots. The molecular weight markers were myosin (H chain) (224,000), phosphorylase *b*

(109,000), bovine serum albumin (BSA) (71,800), ovalbumin (45,800), carbonic anhydrase (28,500), and β-lactoglobulin (18,400).

Antiserum. Polyclonal antiserum to the recombinant protein (rPgAg1) was generated by immunizing a female New Zealand White rabbit with antigen in an SDS-polyacrylamide gel mixture (21). Briefly, *E. coli* BA3 cells were solubilized in sample buffer at 37°C for 30 min, and proteins were separated electrophoretically in an SDS–7.5% polyacrylamide gel. The gel was washed with several changes of deionized water, stained briefly in Coomassie brilliant blue R-250, and further washed with numerous changes of deionized water over a period of several hours. A horizontal strip containing the 48-kDa rPgAg1 protein was excised from the gel and macerated with a minimal amount of 50 mM Tris (pH 7.2) in a mortar. The gel fragment mixture was passed repeatedly through a 22-gauge needle until it became a slurry. Intramuscular injections with this antigen-gel mixture were given with complete Freund's adjuvant on day 1 (150 μg of protein) and with incomplete Freund's adjuvant on day 14. The rabbit was bled 8 days after the second injection. Monospecific antibodies to rPgAg1 were isolated by affinity purification with antigen bound to nitrocellulose. The protocol used was modified from the method described by Smith and Fisher (52). Briefly, *E. coli* BA3 cells were solubilized in sample buffer at 37°C for 30 min, and proteins were resolved in an SDS–7.5% polyacrylamide gel. Separated proteins were transferred electrophoretically to nitrocellulose. Vertical strips on either side of the blot were removed and stained for proteins with Coomassie brilliant blue R-250. These were then realigned with the central section of the blot and used as templates to excise a horizontal strip containing immobilized 48-kDa rPgAg1. The antigen strip was treated with blocking buffer (3% [wt/vol] BSA in Tris-buffered saline [TBS; 50 mM Tris, 150 mM NaCl, pH 8.1] containing 0.02% [wt/vol] sodium azide) and then incubated for 2 h at room temperature with 1.5 ml of serum diluted 1:5 in blocking buffer. After several washes of the antigen strip to remove unbound material, monospecific antibodies were eluted from the strip by incubation with 1 ml of elution buffer (50 mM glycine, 0.5 M NaCl, 0.5% [vol/vol] Tween 20, 100 μg of BSA per ml [pH 2.3]). The eluted antibody solution was immediately neutralized by the addition of an equal volume of 100 mM Na_2HPO_4. Elution and neutralization were repeated, and the eluates were combined and added to 0.5 volume of blocking buffer. The components of the final affinity-purified antibody preparation were diluted 1:20 relative to whole serum. Both preimmune and immune serum were subjected to the affinity purification procedure with immobilized rPgAg1 as the adsorbent. For use in Western blot immunoassays and immunogold labeling analyses, affinity-purified antibody preparations were diluted 1:50 and 1:10, respectively.

Immunogold labeling of *P. gingivalis*. *P. gingivalis* cells in an early-log-phase culture were harvested and resuspended in phosphate-buffered saline (PBS, pH 7.2) to an optical density at 600 nm of 0.85. The cell suspension was placed on 200-mesh nickel grids coated with Formvar. Excess liquid was removed, and the cell labeling assay was performed by floating the grids (specimen side down) successively on the surface of a drop each of 3% (wt/vol) BSA in TBS (pH 7.5) (20 mM Tris, 0.5 M NaCl), affinity-purified antibodies diluted in 1% BSA–TBS, and goat anti-rabbit IgG–10-nm gold bead conjugate (Sigma Chemical Co.) diluted in 1% BSA–TBS. Between each solution, the grids were washed thoroughly with TBS. Cells were negatively stained with 0.5% (wt/vol) ammonium molybdate and viewed with a Zeiss EM 10C transmission electron microscope.

Partial purification of rPgAg1. Cells from overnight cultures of *E. coli* BA3 were harvested and washed once with 20 mM Tris (pH 7.4). The cells were resuspended in 20 mM Tris (pH 7.4) containing 10 mM $MgCl_2$ and 20 μg each of RNase A and DNase I per ml and passed two times through a French pressure cell at 20,000 lb/in^2. The broken cell suspension was subjected to high-speed centrifugation (100,000 × *g*, 60 min, 4°C), and the supernatant containing soluble proteins was recovered for further processing. The protein sample was concentrated by lyophilization and then dialyzed against 20 mM Tris (pH 7.4). The dialyzed sample was applied to a MonoQ anion-exchange column (Pharmacia FPLC [fast protein liquid chromatography] System; Pharmacia, Uppsala, Sweden), and proteins were eluted with a 0 to 1 M NaCl gradient. Fractions were monitored spectrophotometrically at 280 nm, and those containing protein were analyzed further by SDS-PAGE for the presence and purity of rPgAg1. The bulk of the recombinant protein eluted from the column at 0.124 to 0.128 M NaCl.

Determination of NH_2-terminal amino acid sequences. The primary structure of the NH_2-terminal portions of the 48- and 51-kDa rPgAg1 proteins was determined by automated protein sequencing. The recombinant proteins were separated by SDS-PAGE and transferred electrophoretically to a ProBlott membrane (Applied Biosystems, Inc., Foster City, Calif.). The membrane was stained with Coomassie brilliant blue R-250 to localize the proteins of interest. Immobilized rPgAg1 at 48 and 51 kDa was excised from the blot, and the NH_2-terminal amino acid sequence for each molecule was determined by automated Edman degradation with an Applied Biosystems model 475A gas-phase sequenator. Phenylthiohydantoin amino acid residues were analyzed by an on-line reverse-phase high-pressure liquid chromatography unit (Applied Biosystems model 120). The National Biomedical Research Foundation protein sequence data base was searched for homologous amino acid sequences with the FASTA computer program (44). Percent amino acid homology scores were also evaluated with this program. The automated amino acid sequence determination and data base analysis were carried out at the University of Victoria (Victoria, Canada) protein sequencing facility.

RESULTS

Restriction map. A restriction map of the insert DNA in the recombinant plasmid pBA3 was generated (Fig. 1) for preliminary characterization of the cloned fragment and future localization of the *pgag1* gene. The 3.7-kb (corrected from 4.0 kb, as given in reference 37) *Pst*I-*Pst*I DNA insert contained a single cleavage site each for *Bam*HI, *Bgl*II, *Cla*I, *Eco*RI, *Sca*I, and *Ssp*I.

Localization of the native PgAg1 antigen. Affinity-purified antibodies specific to rPgAg1 were used to probe *P. gingivalis* ATCC 33277 whole-cell lysate and membrane preparations for the presence of the native antigen. *E. coli* BA3 and *P. gingivalis* samples were electrophoresed, and proteins were transferred to nitrocellulose and incubated with affinity-purified antibodies to rPgAg1 in a Western blot immunoassay (Fig. 2). In the BA3 whole-cell lysate sample, an immunoreactive protein was observed at 48 kDa, with an apparent degradation product of 25 kDa. A single immunoreactive band (the presumptive native molecule) was detected in both the whole-cell lysate and membrane preparations of *P. gingivalis*. The apparent molecular mass of the

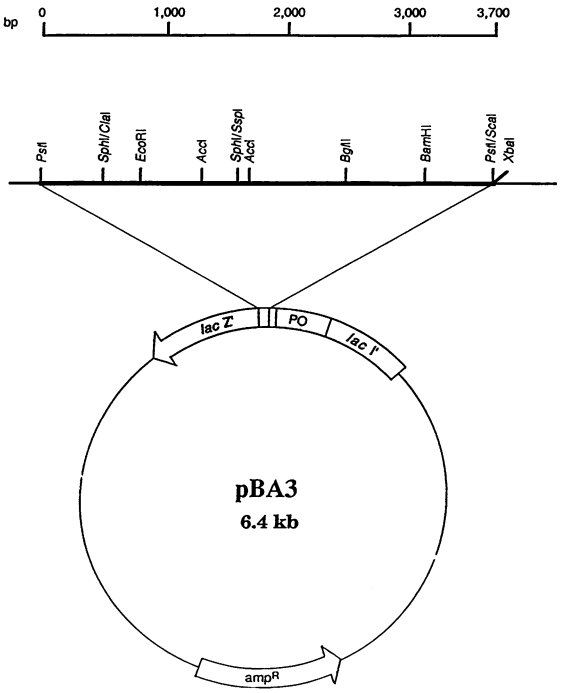

FIG. 1. Restriction map of recombinant plasmid pBA3. pBA3 was constructed by insertion of a 3.7-kb (corrected from 4.0 kb as given in reference 37) fragment of *P. gingivalis* ATCC 33277 chromosomal DNA into the multiple cloning site of pUC18. The *Xba*I site indicated is in the multiple cloning site of the cloning vector. In pBA3, the orientation of the cloned fragment with respect to the *lac* promoter has been arbitrarily defined as being in the forward direction. The direction of transcription of the *bla* (Amp^r) and *lacZ'* genes is indicated by the arrows.

native protein in the *P. gingivalis* cell lysate sample was calculated to be 51 kDa. This suggests that the native PgAg1 antigen exists in a larger form than the recombinant molecule expressed in BA3. The native PgAg1 molecule in the *P. gingivalis* membrane preparation appeared to be slightly larger than the native molecule detected in the cell lysate sample (Fig. 2B, lanes 4 and 5). It is possible that the membrane-associated form of PgAg1 is processed to generate a larger molecule or complex. However, given the differences in preparation and intrinsic composition of the *P. gingivalis* cell lysate and membrane preparations, it is likely that the difference in migration distances of the native molecule in each of these samples is an artifact of the gel or the result of proteolytic degradation occurring in the cell lysate preparation. The immunoreactivity of a component in the membrane fraction of the parent organism with antibodies specific to rPgAg1 does indicate that the native PgAg1 antigen is associated with the membrane of *P. gingivalis*. BA3 and *P. gingivalis* whole cells which were solubilized in SDS-PAGE sample buffer at 37°C for 30 min instead of at 95°C for 10 min exhibited immunoreactive bands similar in size to those observed when samples were solubilized at the higher temperature (result not shown). It appears that the recombinant and native forms of PgAg1 are not heat modifiable with respect to their mobility in SDS-polyacrylamide gels.

To provide further evidence for the cellular location of PgAg1 in *P. gingivalis*, antibodies to rPgAg1 were used in immunogold labeling analysis of intact cells. Incubation of *P.*

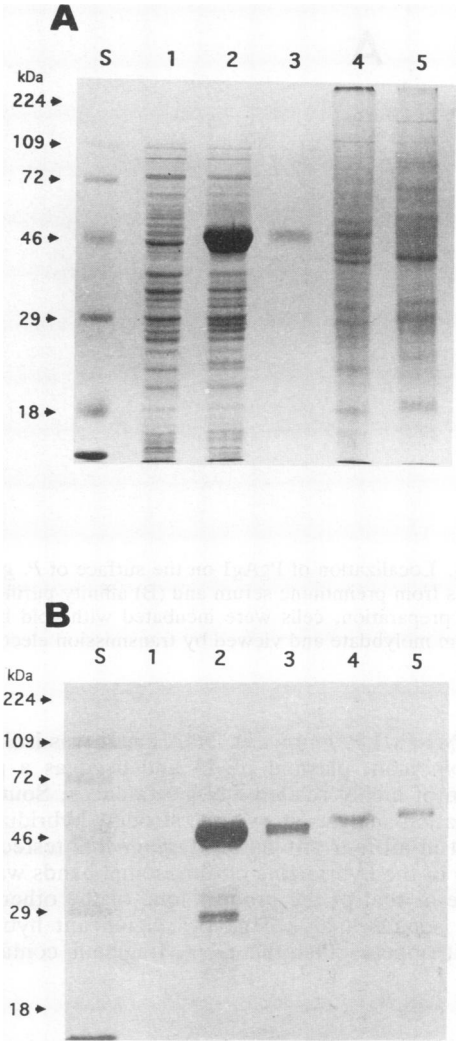

FIG. 2. Identification of the native PgAg1 antigen by Western blot immunoassay analysis. (A) SDS–10% polyacrylamide gel stained with Coomassie brilliant blue R-250; (B) Western blot of the gel reacted with affinity-purified antibodies to rPgAg1. Lanes: S, protein standards; 1, cell lysate of *E. coli* JM83/pUC18; 2, cell lysate of *E. coli* BA3; 3, 1/50 of the amount loaded in lane 2, necessary because of the large amount of recombinant antigen produced in BA3; 4, cell lysate of *P. gingivalis* ATCC 33277; 5, membrane fraction of *P. gingivalis* ATCC 33277. Numbers on the left indicate molecular mass in kilodaltons.

gingivalis ATCC 33277 with monospecific antibodies to rPgAg1 resulted in greater cell surface labeling of these cells with colloidal gold beads than of cells treated with affinity-purified preimmune antibodies (Fig. 3). The Western blot immunoassay and immunogold labeling results indicate that native PgAg1 is a cell surface antigen of *P. gingivalis*.

Southern blot hybridization analysis of BPB. In addition to *P. gingivalis*, other members of the group known formerly as BPB have also been isolated from the oral cavity. It was of interest to analyze several oral isolates (in particular, *Prevotella denticola*, *Prevotella intermedia*, and *Prevotella loeschii*) and nonoral isolates of BPB for the presence of genes homologous to *pgag1*. Genomic DNA was isolated from 11 BPB species and digested to completion with *Pst*I. The

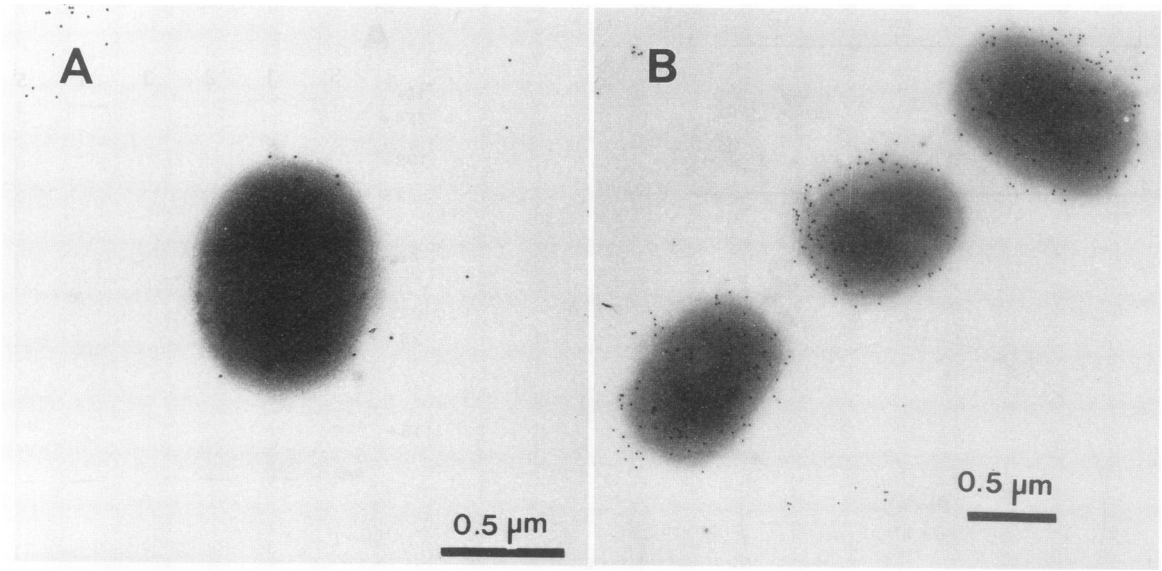

FIG. 3. Localization of PgAg1 on the surface of *P. gingivalis*. *P. gingivalis* ATCC 33277 cells were incubated with (A) affinity-purified antibodies from preimmune serum and (B) affinity-purified antibodies from antiserum to rPgAg1. Following treatment with the appropriate antibody preparation, cells were incubated with gold beads conjugated to goat anti-rabbit IgG. The cells were negatively stained with ammonium molybdate and viewed by transmission electron microscopy.

3.7-kb *Pst*I-*Pst*I *P. gingivalis* DNA insert was isolated from the recombinant plasmid pBA3 and used as a probe for detection of highly related DNA sequences. Southern blot hybridization analysis shows one strongly hybridizing DNA fragment in all four strains of *P. gingivalis* tested (Fig. 4). The size of the hybridizing chromosomal bands was 3.7 kb, the same as that of the probe. None of the other oral and nonoral isolates analyzed gave a significant hybridization signal. It appears that the DNA fragment containing the *pgag1* gene is specific to *P. gingivalis* and exists as a single copy in the bacterial genome.

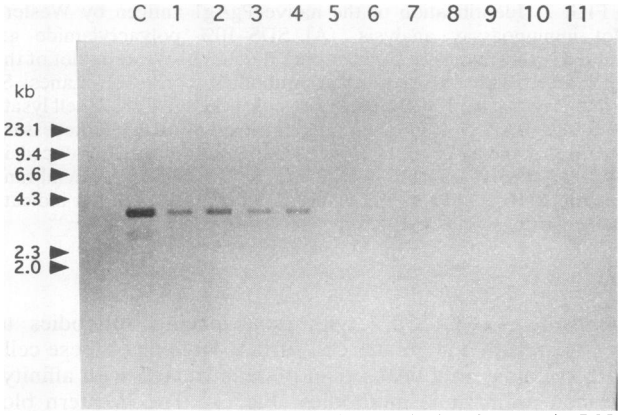

FIG. 4. Southern blot hybridization analysis of genomic DNA from BPB strains. The 3.7-kb *Pst*I-*Pst*I fragment from pBA3 was used as the probe. Lane C, *Pst*I-digested pBA3. Numbered lanes contain *Pst*I-digested chromosomal DNA from strains as follows: 1, *P. gingivalis* ATCC 33277; 2, *P. gingivalis* W12; 3, *P. gingivalis* W50; 4, *P. gingivalis* W83; 5, *P. asaccharolytica* ATCC 25260; 6, *Prevotella corporis* ATCC 33547; 7, *Prevotella denticola* ATCC 33185; 8, *Prevotella intermedia* ATCC 15032; 9, *Bacteroides levii* ATCC 29147; 10, *Prevotella loeschii* ATCC 15930; 11, *Prevotella melaninogenica* ATCC 25845. Numbers on the left indicate molecular size in kilobases.

Western blot immunoassay analysis of BPB. To determine whether PgAg1 or immunologically related proteins are expressed by selected members of the BPB group, Western blot immunoassay analysis was performed with affinity-purified antibodies to rPgAg1. Whole-cell lysates for SDS-PAGE were prepared for 11 BPB species. All strains of *P. gingivalis* screened by Western blot immunoassay expressed a 51-kDa immunoreactive antigen (Fig. 5). None of the other BPB species examined exhibited proteins reactive with affinity-purified antibodies to rPgAg1. With the Southern hybridization results, this indicates that the PgAg1 surface antigen is expressed from the *pgag1* gene in all strains of *P. gingivalis* tested. The PgAg1 molecule does not seem to have epitopes which are immunologically similar to those of proteins expressed by the other BPB species examined.

Although the results presented here were obtained with *P. gingivalis* samples cultured in vitro, there is preliminary evidence that this organism expresses PgAg1 when grown in vivo. Infectious exudate samples were taken from guinea pigs inoculated with *P. gingivalis* W50 that had been grown in vivo and passaged twice. Bacterial cells from this sample were analyzed by Western blot immunoassay with affinity-purified antibodies to rPgAg1. *P. gingivalis* W50 cells cultured in vivo and recovered from the exudate exhibited a single immunoreactive antigen similar in size to that seen in strains grown in vitro (result not shown).

Subclone with insert DNA in reverse orientation. The orientation of the *P. gingivalis* DNA insert with respect to the *lac* promoter in the recombinant plasmid pBA3 was arbitrarily defined as being in the forward direction (Fig. 1). A subclone (designated *E. coli* BA3R) which harbors the recombinant plasmid pBA3R, containing the 3.7-kb insert in the reverse direction to the insert in pBA3, was generated. The orientation of the insert in pBA3R was confirmed by migration of DNA bands following *Xba*I (unique site in

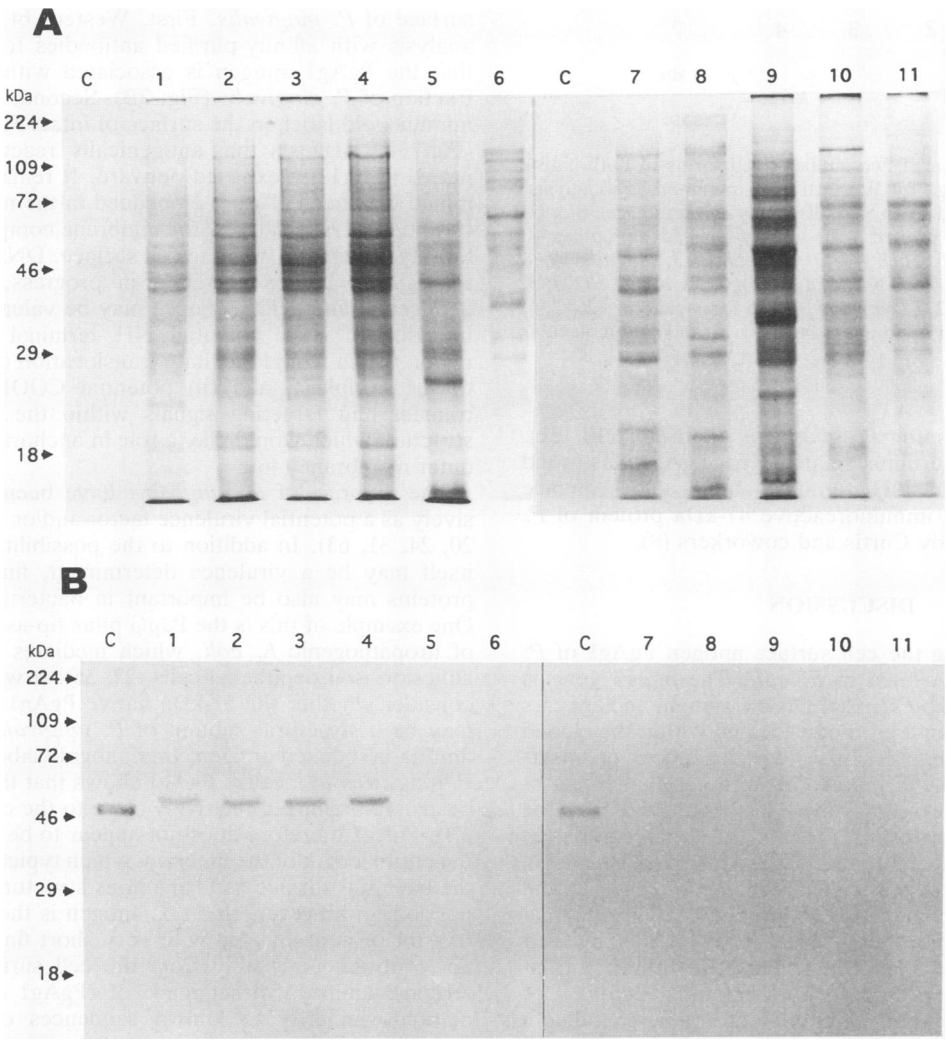

FIG. 5. Western blot immunoassay analysis of BPB. (A) SDS–10% polyacrylamide gel stained with Coomassie brilliant blue R-250; (B) Western blot of the gel reacted with affinity-purified antibodies to rPgAg1. Lanes contained cell lysates from the indicated strains: C, *E. coli* BA3, sample diluted 1:50; 1, *P. gingivalis* ATCC 33277; 2, *P. gingivalis* W12; 3, *P. gingivalis* W50; 4, *P. gingivalis* W83; 5, *P. asaccharolytica* ATCC 25260; 6, *Prevotella corporis* ATCC 33547; 7, *Prevotella denticola* ATCC 33185; 8, *Prevotella intermedia* ATCC 15032; 9, *B. levii* ATCC 29147; 10, *Prevotella loeschii* ATCC 15930; 11, *Prevotella melaninogenica* ATCC 25845. Numbers on the left indicate molecular mass in kilodaltons.

pUC18) and *Bgl*II (unique site in the cloned DNA) double restriction enzyme digestion. Strain *E. coli* BA3R was found to overexpress rPgAg1 to a level similar to that produced by the original clone *E. coli* BA3. This suggests that transcription of the *pgag1* gene is initiated at a *P. gingivalis* promoter which is well recognized by *E. coli* RNA polymerase.

Two different forms of PgAg1 very similar in size. The cloned protein rPgAg1 was partially purified from *E. coli* BA3. The partially purified recombinant protein sample was analyzed by Western blot immunoassay with monospecific antibodies to the 48-kDa rPgAg1 antigen. Two immunoreactive antigens were observed in the preparation containing partially purified recombinant protein (Fig. 6, lane 4). The larger immunoreactive protein appears to be similar in size to the 51-kDa mature native antigen present in *P. gingivalis* ATCC 33277 (Fig. 6, lane 2).

Freshly prepared *P. gingivalis* whole-cell lysates exhibit a single reactive protein of 51 kDa when probed with affinity-purified antibodies to rPgAg1 (Fig. 5; Fig. 6, lane 2). However, cell lysates stored at −20°C for several months show one immunoreactive *P. gingivalis* protein (Fig. 6, lane 3) similar in size to the 48-kDa dominant form of rPgAg1 observed in *E. coli* BA3. It appears that there are two different forms of the surface antigen PgAg1 which are very similar in size. The results from the *P. gingivalis* samples suggest that the 48-kDa species is probably a degradation product of the 51-kDa molecule.

NH_2-terminal amino acid sequence of rPgAg1. The NH_2-terminal amino acid sequences of the 48- and 51-kDa recombinant PgAg1 molecules were determined. Both molecules were found to have the same NH_2-terminal amino acid sequence, M-K-T-Q-E-I-M-T-M-L-E-A-K-H-P-G-E-S-E-F-L-Q-A-V-K-E-V-L-L. No significantly homologous sequences in other proteins were identified in a search of the National Biomedical Research Foundation protein data base. This sequence does not show marked similarity to

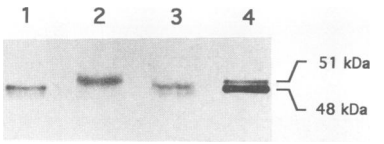

FIG. 6. Identification of two similar-sized forms of both native and recombinant PgAg1 by Western blot immunoassay analysis. Proteins were separated on an SDS–10% polyacrylamide gel, blotted to nitrocellulose, and probed with affinity-purified antibodies to 48-kDa rPgAg1. Lanes: 1, cell lysate of *E. coli* BA3, sample diluted 1:50; 2, freshly prepared cell lysate of *P. gingivalis* ATCC 33277; 3, *P. gingivalis* ATCC 33277 cell lysate stored for 1 year at −20°C; 4, partially purified rPgAg1. Numbers on the right indicate molecular mass.

published sequences for *P. gingivalis* fimbrillin (10, 31). However, the NH_2-terminal sequence for PgAg1 was found to be identical to the NH_2-terminal amino acid sequence presented (4) for an immunoreactive 47-kDa protein of *P. gingivalis* identified by Curtis and coworkers (8).

DISCUSSION

The gene encoding the cell surface antigen PgAg1 of *P. gingivalis* has been cloned in *E. coli*. The *pgag1* gene is located within a 3.7-kb *Pst*I-*Pst*I DNA fragment and appears to be transcribed from a promoter located within the cloned DNA fragment, as expression is not dependent on insertional orientation. The *P. gingivalis* promoter element associated with this gene seems to be well recognized by the transcriptional apparatus of *E. coli*, as the recombinant protein is expressed at a high level (Fig. 2). This is in contrast to promoters associated with a protease (1) and hemagglutinins (45) cloned from *P. gingivalis*. It appears that in *E. coli*, expression of these cloned genes initiated by their own promoters may be less efficient than readthrough transcription initiated by promoters in the cloning vector.

Choi et al. (5) reported the cloning of a gene encoding a superoxide dismutase enzyme from *P. gingivalis*. Although potential promoter sequences were identified in the insert fragment, expression of the cloned gene was initiated from the *lacZ* promoter in the cloning vector. Klimpel and Clark (27) assayed for reactivity between *P. gingivalis* RNA polymerase and antiserum to (i) the *E. coli* core enzyme and (ii) *E. coli* sigma factors. No reactivity was observed between proteins in *P. gingivalis* whole-cell extracts and these antisera in a Western blot immunoassay. They concluded that *E. coli* RNA polymerase may not be sufficiently similar to *P. gingivalis* RNA polymerase to recognize *P. gingivalis* promoters. Recent reports indicate that a second protease gene from P. gingivalis has been cloned with a promoter which appears to be recognized in *E. coli* (2, 43).

In this study, we show evidence that the *P. gingivalis* promoter for our cloned *pgag1* gene is functional and generates a highly expressed gene product in *E. coli*. Little is known about the genetics of *P. gingivalis*, and it would be of interest to examine further the promoters from this gram-negative anaerobe and compare them with well-characterized promoter structures in other organisms. The *P. gingivalis pgag1* promoter may also be of use in efficient expression of other cloned proteins in *E. coli*, a factor which may be important in obtaining large amounts of recombinant protein for study.

Two observations in the cellular localization studies of the native PgAg1 molecule indicate that it is present on the outer surface of *P. gingivalis*. First, Western blot immunoassay analysis with affinity-purified antibodies to rPgAg1 shows that the PgAg1 antigen is associated with the membrane fraction of *P. gingivalis* (Fig. 2B). Second, the detection of immunogold label on the surface of intact *P. gingivalis* cells (Fig. 3B) suggests that antigenically reactive epitopes of native PgAg1 are exposed outward. It remains to be determined whether PgAg1 is embedded in the membrane structure, tightly associated with membrane components, or only loosely associated with the cell surface. DNA sequencing of the *pgag1* gene is currently in progress. A subsequent deduced amino acid sequence may be valuable in the identification of (i) a potential NH_2-terminal leader peptide region which would facilitate translocation from the cytosol to the periplasm and (ii) potential COOH-terminal stop transfer and targeting signals within the mature protein structure which would play a role in anchoring PgAg1 to the outer membrane (46).

The fimbriae of *P. gingivalis* have been studied extensively as a potential virulence factor and/or immunogen (15, 20, 24, 31, 63). In addition to the possibility that fimbrillin itself may be a virulence determinant, fimbria-associated proteins may also be important in bacterial pathogenesis. One example of this is the PapG pilus tip-associated protein of uropathogenic *E. coli*, which mediates this organism's adhesion to uroepithelial cells (22, 36). It was of interest to consider whether the 51-kDa native PgAg1 surface antigen may be a structural subunit of *P. gingivalis* fimbriae or a fimbria-associated antigen. Immunogold labeling analysis of *P. gingivalis* to localize PgAg1 shows that the colloidal gold beads are organized relatively close to the cell surface (Fig. 3B). PgAg1 therefore does not appear to be associated with the entire length of the fimbriae, which typically extend from the bacterial surface as filamentous structures. There is the possibility, however, that this antigen is the structural protein for or is associated with very short fimbriae which do not protrude noticeably from the cell surface. The NH_2-terminal amino acid sequence of rPgAg1 did not show a marked similarity to known sequences of *P. gingivalis* fimbrillin (10, 31).

There is an apparent size discrepancy between the presumptive native PgAg1 protein (51 kDa) and the recombinant molecule (48 kDa) detected in whole-cell lysate samples (Fig. 2B). One possible explanation is that an intact *pgag1* gene has not been cloned, resulting in expression of a truncated rPgAg1 protein. Alternatively, an intact gene may be present and the mature protein may be 48 kDa. In *P. gingivalis*, this 48-kDa protein may undergo subsequent posttranslational modification or associate with another *P. gingivalis* component, so that the final native form of the antigen (or antigen complex) is 51 kDa. Two observations provide evidence that the entire *pgag1* gene has been isolated and that the size of the mature protein generated directly from the gene is 51 kDa. First, in preliminary attempts to purify the recombinant protein by ion-exchange chromatography, a lesser amount of a 51-kDa molecule copurified with the 48-kDa form of rPgAg1. Unlike the other proteins in the partially purified rPgAg1 fraction, this 51-kDa molecule was reactive with affinity-purified antibodies to the 48-kDa rPgAg1 in a Western blot immunoassay (Fig. 6). This indicated that it was not of host origin. Storage of the partially purified rPgAg1 sample at −20°C for 3 months resulted in a noticeable loss of the immunoreactive 51-kDa species relative to the other proteins in the mixture. This suggests that an unstable 51-kDa form of rPgAg1 is expressed in clone BA3. It appears to be rapidly degraded to a

48-kDa molecule, which accumulates in large amounts in the cell. Second, fresh samples of *P. gingivalis* whole-cell lysate probed with affinity-purified antibodies to rPgAg1 in a Western blot immunoassay show that the native antigen is 51 kDa (Fig. 2B). However, cell lysates stored at −20°C for several months exhibit a single immunoreactive *P. gingivalis* protein migrating at 48 kDa (Fig. 6). Therefore, it seems that the 51-kDa native antigen also undergoes degradation to a 48-kDa form when *P. gingivalis* cells are disrupted and conditions are no longer conducive to maintaining the integrity of cellular proteins. The consistent size of the degradation product suggests the presence of a particularly susceptible or specific proteolytic cleavage site and the action of a contaminating proteolytic enzyme(s). It is not clear at present whether cleavage occurs at a site which may be crucial to PgAg1 structure or function. What is known, however, is that the 48- and 51-kDa rPgAg1 molecules possess the same NH_2-terminal amino acid sequence. This indicates that degradation occurs at the COOH terminus of the mature 51-kDa protein. Determination of the COOH-terminal amino acid sequence of the 48- and 51-kDa molecules may assist in clarifying the relationship between these two forms of PgAg1.

In addition to *P. gingivalis*, other members of the group known formerly as BPB are inhabitants of the oral cavity. In particular, *Prevotella intermedia* has also been implicated as an etiologic agent in periodontal disease (51). Knowledge of the specificity of the *pgag1* gene and its gene product may help elucidate whether the protein is either a phenotypic marker, a species- or strain-specific antigen, or possibly associated with the organism's ecological niche or virulence. All *P. gingivalis* strains tested appeared to contain a single copy of the *pgag1* gene in the chromosome. The other BPB species examined (including *Prevotella intermedia*) did not show specific reactivity with the DNA fragment containing the *pgag1* gene, indicating that highly similar DNA sequences are not present.

The gene for the PgAg1 cell surface antigen appears to be species specific, that is, limited to *P. gingivalis*. The use of molecular biological methods for the diagnosis of periodontal disease is being increasingly favored over traditional anaerobic culture procedures (33, 34, 49). DNA methods for detecting specific periodontal pathogens accurately within a highly mixed bacterial sample are very dependent on the specificity of the DNA probes. Whole genomic DNA probes (14, 47) and oligonucleotide probes complementary to the hypervariable regions of 16S rRNA (11) have been developed for the identification of *P. gingivalis*. In this report, we show evidence which suggests that the cloned fragment containing the *pgag1* gene is highly conserved and specific to the periodontopathogen *P. gingivalis*. A more comprehensive test of our probe against other oral microoganisms is required; however, our cloned DNA fragment may also be a good candidate for use as a species-specific probe for the detection of *P. gingivalis* in clinical specimens.

To further characterize the PgAg1 antigen at the protein level, Western blot immunoassay analysis was used to probe for immunologically related proteins expressed by oral and nonoral members of the BPB group. Whole-cell lysates of 11 BPB species were tested with affinity-purified antibodies to rPgAg1 (Fig. 5B). In addition to *P. gingivalis* ATCC 33277, three other strains (W12, W50, and W83) of this species all demonstrated the presence of a 51-kDa immunoreactive antigen. Examination of other oral and nonoral isolates of BPB showed no detectable proteins in these species which have epitopes immunologically related to those of PgAg1. It appears that the PgAg1 cell surface antigen is unique to *P. gingivalis*. In the four *P. gingivalis* strains examined by Southern blot hybridization and Western blot immunoassay, the *pgag1* gene seems to have been conserved, and the gene product was expressed. This suggests that the PgAg1 antigen may be important to the survival of this species, either as a structural protein, for nutritional purposes, or for colonization in the appropriate ecological niche.

Other investigators (8, 12, 59) have identified *P. gingivalis* immunogenic molecules similar in size to the surface protein in our study. In particular, Curtis et al. (8) reported high reactivity between a 47-kDa protein antigen and serum IgG antibody from patients with a history of periodontal disease. It is significant that the NH_2-terminal amino acid sequence of rPgAg1 is identical to the NH_2-terminal amino acid sequence recently presented (4) for the 47-kDa immunoreactive molecule identified by this group of researchers. Closer investigation of the *pgag1* gene and its gene product is clearly warranted. A bacterial surface molecule which evokes a strong immunological response in the host may serve as a useful vaccine component.

The molecular cloning and expression in *E. coli* of the genes for two other surface components of *P. gingivalis* have recently been reported (58, 60). The gene for a cell surface protein of 75 kDa was cloned into pUC19, but the gene product was not expressed from the recombinant plasmid (60). Use of the bacteriophage T7 RNA polymerase-promoter expression vector system was necessary for identification of the cloned protein. The gene encoding a 42-kDa fimbrillin from *P. gingivalis* has also been isolated (58). A small amount of recombinant protein was produced from the gene cloned in pET11b in the absence of induction with isopropyl-β-D-thiogalactopyranoside (IPTG). Recombinant fimbrillin was expressed at a significantly higher level in *E. coli* cells after induction with IPTG. This suggests that expression of the cloned fimbrillin gene is under the control of an IPTG-inducible promoter on the cloning vector.

In conclusion, we have cloned the promoter element and structural gene for a cell surface antigen of *P. gingivalis*. The gene product was expressed by all strains of *P. gingivalis* tested. No highly homologous DNA sequences or immunologically related proteins were detected in other selected species in the group of related organisms known formerly as BPB. Studies are currently in progress to determine the function and possible in vivo significance of this unique cell surface protein.

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