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Porphyromonas gingivalis Genes Isolated by Screening for Epithelial Cell Attachment

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***Porphyromonas gingivalis* is associated with chronic and severe periodontitis in adults. *P. gingivalis* and the other periodontal pathogens colonize and interact with gingival epithelial cells, but the genes and molecular mechanisms involved are unknown. To dissect the first steps in these interactions, a *P. gingivalis* expression library was screened for clones which bound human oral epithelial cells. Insert DNA from the recombinant clones did not contain homology to the *P. gingivalis fimA* gene, encoding fimbrillin, the subunit protein of fimbriae, but showed various degrees of homology to certain cysteine protease-hemagglutinin genes. The DNA sequence of one insert revealed three putative open reading frames which appeared to be in an operon. The relationship between *P. gingivalis* attachment to epithelial cells and the activities identified by the screen is discussed.**

The diseases collectively known as periodontitis are bacterial infections which begin with inflammation of the periodontium and progress to loss of periodontal attachment. Untreated infections can lead to alveolar bone resorption and may result in tooth loss. The change from health to disease is associated with the displacement of a predominantly gram-positive bacterial flora in the gingival sulcus by gram-negative anaerobic bacteria. A small number of gram-negative species have been consistently linked with specific forms of periodontal disease; *Porphyromonas gingivalis* is associated with chronic and severe disease in adults. The organism colonizes the surface of gingival epithelial cells, but to infiltrate and compromise the underlying tissue, it must cross the epithelial cell barrier. The mechanisms used to penetrate tissue are unknown, although a possible pathway was suggested by previous demonstrations that *P. gingivalis* attached to and invaded oral epithelial cells (9, 18, 29, 33, 34). It has been demonstrated that *P. gingivalis* fimbriae mediate attachment to saliva-coated hydroxyapatite (20, 21). In addition, monoclonal antibodies raised against purified fimbriae of *P. gingivalis* 381 inhibit adhesion to buccal epithelial cells (16). The gene encoding fimbrillin (*fimA*), the protein subunit of fimbriae, was cloned and sequenced (8), and two independently isolated *fimA* insertion mutants show a loss of ability to adhere not only to saliva-coated hydroxyapatite (25) but also to human epithelial cells (12). However, in the latter case, it is not known whether fimbrillin itself is the adhesin, fimbriae form the supporting structure for associated adhesins, or fimbria-mediated adhesion is the only mechanism. We wish to dissect the first steps in the interaction between *P. gingivalis* and epithelial cells. In this report, we describe a screening procedure for the isolation of clones containing *P. gingivalis* genes involved in epithelial cell attachment and preliminary characterization of the clones. Insert DNA from one of the clones was analyzed in detail.

MATERIALS AND METHODS

Chemicals, media, bacterial strains, and plasmids. Chemicals and antibiotics were obtained from Sigma Chemical Co., St. Louis, Mo. *Escherichia coli* was grown aerobically at 37°C in Luria-Bertani broth (LB) or on plates of the same medium containing 1.5% agar. As required, ampicillin was added to broth or plates at 50 μg/ml (LBAmp). *P. gingivalis* ATCC 33277 was grown as described previously (9). Plasmid pUC13Bgl2.1 containing the *P. gingivalis fimA* gene was obtained from D. Dickinson, University of Texas, Houston. Clones containing probes for hemagglutinin genes *hagA*, *-B*, *-C*, and *-D* were kindly provided by A. Progulske-Fox, University of Florida, Gainesville.

Recombinant DNA methods. Unless otherwise stated, standard procedures for DNA manipulation were as described by Sambrook et al. (32). Enzyme digests were carried out as recommended by the suppliers. Chromosomal DNA was extracted from *P. gingivalis* ATCC 33277 by the lysis procedure described by Kado and Liu (17). DNA was precipitated from the lysate with ethanol, phenol-chloroform extracted, reprecipitated with ethanol, and dissolved in Tris-EDTA. DNA fragments from restriction enzyme digests were excised from 0.7% separating gels, and purified using Geneclean II (Bio101, La Jolla, Calif.).

Construction of a *P. gingivalis* ATCC 33277 expression library. Chromosomal DNA was partially digested with *Sau*3AI and fractionated by agarose gel electrophoresis. The portion of the gel containing 5- to 10-kb DNA fragments was excised and purified as described above. Phagemid vector pBlueScriptII SK (pBS; Stratagene, La Jolla, Calif.) was digested to completion with *Bam*HI, treated with phosphatase, and gel purified. Chromosomal fragments and vector DNA were mixed in a 2:1 ratio and ligated overnight with T4 DNA ligase. The ligation mix was used to transform calcium-competent cells of *E. coli* NM522 (Stratagene), and ampicillin-resistant transformant clones were selected by overnight growth at 37°C on LBAmp plates. The transformation frequency was 6×10^5 transformants per μg of vector DNA. More than 80% of the transformants contained inserts as calculated from the number of white transformant clones on LBAmp containing isopropyl-β-D-thiogalactopyranoside (IPTG) and 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-Gal).

Southern hybridization. DNA fragments were transferred to either Gene Screen hybridization transfer membranes (NEN Research Products, Boston, Mass.) or nitrocellulose. The enhanced chemiluminescence (ECL) gene detection system (Amersham PLC, Little Chalfont, England) was used without modification of the protocol supplied by the manufacturer. For chemiluminescent-signal detection, filters were exposed to Hyperfilm-ECL (Amersham).

Screening the *P. gingivalis* library for clones which bound epithelial cells. *E. coli* recombinant clones containing the *P. gingivalis* library were replicated to nitrocellulose filters and lysed with lysozyme and sodium dodecyl sulfate (9), since it was not anticipated that expressed proteins would be transported to the *E. coli* surface. Protein from the clones was UV cross-linked to the filters (Stratalinker; Stratagene). The blocking of filters and incubation with and detection of epithelial cells were as described previously (9).

DNA sequencing. Plasmid DNA for sequencing was prepared by the modified alkali lysis/polyethylene glycol precipitation procedure for use with the *Taq* DyePrimer (no. 401386) and *Taq* DyeDeoxyTerminator (no. 401388) cycle-sequencing protocols developed by Applied Biosystems (Perkin-Elmer Corp., Foster City, Calif.). The labelled extension products were analyzed on an Applied Biosystems model 373A DNA sequencer (Perkin-Elmer Corp.). Oligonucleotide primers were synthesized at the DNA Synthesis Core Laboratory, University of Florida, Gainesville. Nucleotide sequences were aligned and assembled with programs in the Sequencher software package (Gene Codes Corp., Ann Arbor, Mich.).

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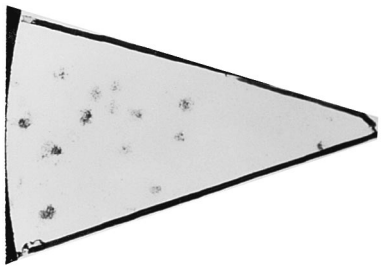


FIG. 1. Nitrocellulose filter replicate of purified *E. coli* recombinant clone isolated in the screen. Dark aggregates are alkaline phosphatase-stained KB epithelial cells attached to single colonies.

DNA and protein analysis. DNA and protein sequences were analyzed with DNAsis and Prosis (Hitachi) software. Hydropathy analysis was performed by the method of Hopp and Wood (14). Homology searches of databases were carried out with the BLAST alogrithm (2).

Nucleotide sequence accession number. The DNA sequences in this study have been deposited with GenBank under accession number U60208.

RESULTS

Quality of the *P. gingivalis* library. The quality of the *P. gingivalis* library was assessed by determining the frequency of *fimA* DNA. One thousand eighty *E. coli* clones were replicated to nitrocellulose filters and treated by standard procedures to release and denature DNA, which was cross-linked to the filters with UV light. A *Sac*I fragment containing the *fimA* gene was isolated from plasmid pUC13Bgl2.1 (8) and used as a probe. Of the 1,080 clones tested, 4 (1 in 270) hybridized to the *fimA* probe (data not shown), demonstrating that this DNA was represented with good frequency in the library.

Isolation of clones expressing *P. gingivalis* epithelial cell binding proteins. We used a modified version of a qualitative filter-binding assay (9, 22) to screen for *E. coli* clones expressing *P. gingivalis* genes for epithelial cell-binding proteins. Clones which bound epithelial cells were purified from the master plate and retested. After screening approximately 150,000 *E. coli* clones from two independent libraries, we identified 15 which bound KB epithelial cells. Clones from the first library were designated by numbers, and those from the second were designated by letters. Figure 1 shows dark-stained aggregates of epithelial cell binding to single colonies of one of the purified clones. Of the 15 clones, 2 failed to grow after subculture on LBAmp plates and several others segregated blue colonies during growth on LBAmp plates containing IPTG and X-Gal, suggesting that potentially toxic protein products were expressed. Intact cells of the *E. coli* clones did not attach to epithelial cell monolayers at levels significantly higher than those of the host strain NM522 containing pBluescript. This was not unexpected, since no attempts were made to direct expressed gene products to the bacterial cell surface.

Southern hybridization analysis of clones. Plasmid DNA from the clones was digested with *Pvu*II, fractionated by agarose gel electrophoresis, and transferred to membranes for Southern hybridization. All plasmids contained 5- to 9-kb inserts displaying different *Pvu*II restriction patterns (Fig. 2A), an indication that they were derived from independent recombinant clones. All the inserts hybridized to whole genomic DNA from *P. gingivalis* (data not shown). None of the inserts hybridized to the *Sac*I fragment probe containing the *fimA* gene, although this probe hybridized to control *P. gingivalis* genomic DNA and the *fimA* clone, which were run on the same gel (data not shown), and clones carrying this gene were detected in the library (see above).

Because adhesins often possess hemagglutinating activity (35, 36), the same blot was probed with fragments of the hemagglutinin genes *hagA*, *-B*, *-C*, and *-D* isolated from *P. gingivalis* 381 (23, 24, 31). Figure 2B and C show the *hagA* and *-D* fragments (solid lines) used as probes and the hybridizing fragments from the inserts. The *hagA* and *-D* genes contain homology not only to each other (23) but also to cysteine protease genes (3). Cross-hybridization was also observed between the internal fragments of *hagA* and pBluescript. No homology to probes from *hagB* or *-C* could be detected.

By restriction fragment length polymorphism (RFLP) analysis, the clones fell into two classes. As shown in Fig. 3, the inserts of one class, represented by clones C and E, hybridized to single fragments of *Pst*I-digested chromosomal DNA from strains ATCC 33277 and 381. The other class of inserts, represented by clones D and F, hybridized to multiple genomic fragments, indicating that they contained either repetitive sequences or multicopy genes. Strain ATCC 33277 possesses two arg-gingipain genes (26), and repetitive sequences are found both within the cysteine protease genes themselves and at flanking chromosomal sites (reviewed in reference 3).

Restriction map and RFLP analysis of p24, *hagA*, and *hagD*. A restriction map was derived for clone p24, which contained the smallest insert and was a representative of clones which hybridized to single *Pst*I genomic fragments. The map of the p24 insert was different from those of *hagA* and *-D* (Fig. 4A) and indicated that it contained a unique DNA fragment. RFLP analysis was used to define the genomic fragment of the p24 insert and to determine whether *P. gingivalis* ATCC 33277 and 381 both contained the gene(s) on this fragment, in addition to *hagA* and *-D*. Genomic DNA (5 μg) was digested with *Pst*I, fractionated on an agarose gel, and transferred to nitrocellulose. The blot was hybridized with (i) the 1.3-kb *Kpn*I repeat fragments from *hagA* (Fig. 2B); (ii) the 1.9-kb internal *Pst*I fragment from *hagD* (Fig. 2C), which also has homology to the *hagA Kpn*I fragments; and (iii) the 2.5-kb *Pst*I fragment of p24 (Fig. 4A). The results are shown in Fig. 4B. First, all the probes hybridized to identically sized *Pst*I fragments in both ATCC 33277 and 381 genomic digests, establishing that the chromosomes of the two strains are similar with respect to these three loci. Second, the *hagA* and *-D* probes hybridized to the same genomic fragments. The 1.9-kb *Pst*I probe from *hagD* hybridized to the 1.9-kb *Pst*I chromosomal fragment, as expected, and also to a 13.6-kb fragment which was presumed to contain the chromosomal copy of *hagA*, since the gene does not have an internal *Pst*I site. The 1.3-kb *Kpn*I fragment from *hagA* hybridized to the same *Pst*I genomic fragments as the *hagD* probe, as predicted by their homology (data not shown). The 2.9-kb *Pst*I fragment from p24 again hybridized to the *Pst*I fragments of *hagA* and *-D* and also to a new 4.2-kb band present in both ATCC 33277 and 381. The p24 insert has a single *Pst*I site, and the probe was generated from this site and the *Pst*I site in the pBluescript vector. Therefore, the 4.2-kb genomic fragment was generated from this internal *Pst*I site and another in the flanking genome sequence. These results confirm that the p24 insert contains a different gene(s); however, under the conditions used, the *hagA* and *-D* probes did not hybridize to the p24 genomic fragment. The interpretation of this result is that the homology between the genes was too weak for detectable quantities of the *hagA* and *-D* probes to hybridize with a single genomic copy of the p24 gene, but when p24 DNA was in excess as the probe, detectable quantities hybridized to the genomic copies of *hagA* and *-D*.

DNA sequence analysis. The DNA sequence of the p24 insert (Fig. 5) confirmed the restriction map. Also shown in Fig. 5 are the deduced amino acid sequences of the putative

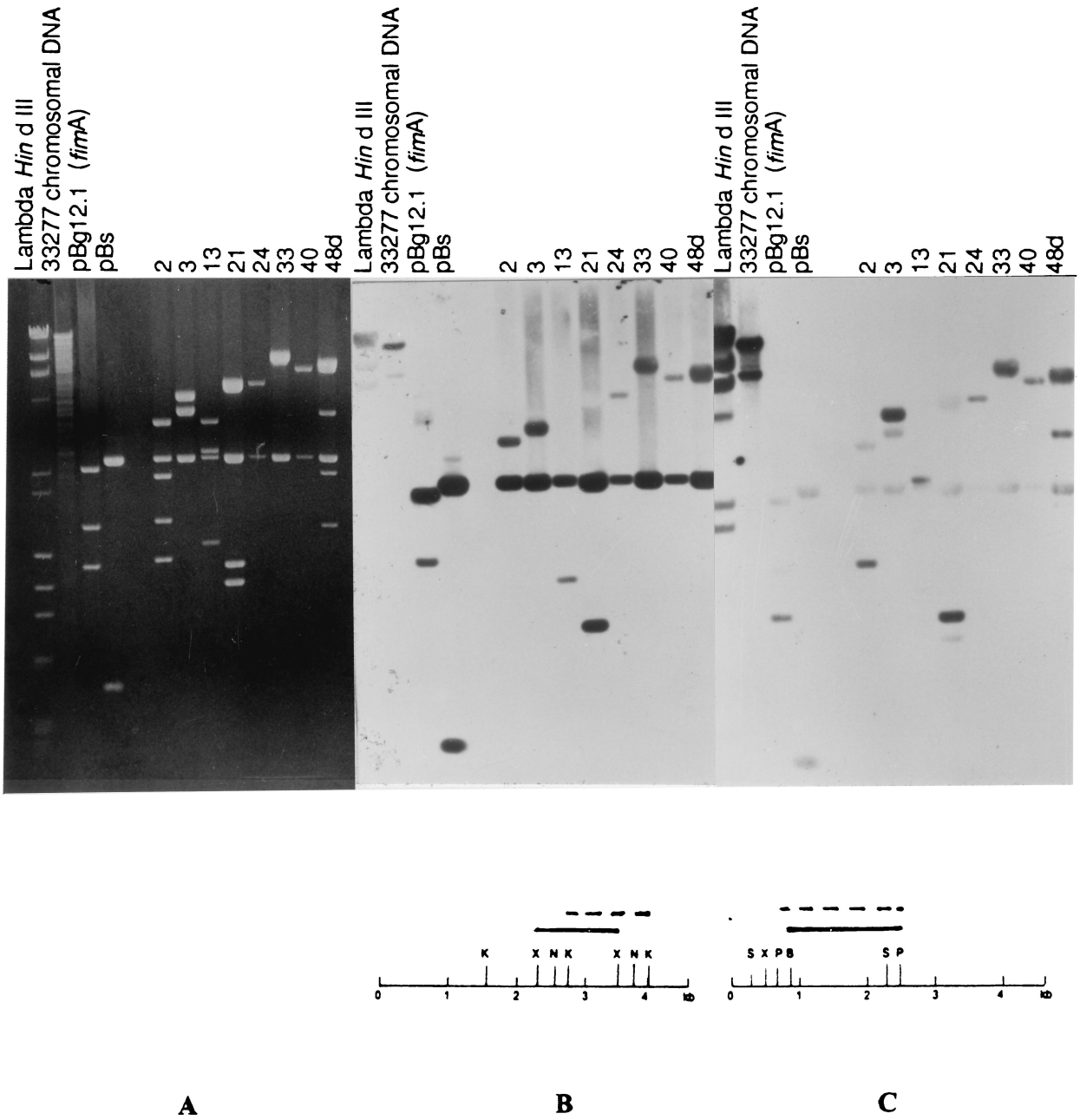


FIG. 2. Hybridization of insert DNA with *P. gingivalis hagA* and *-D* probes. (A) Ethidium bromide-stained *Pvu*II digests of recombinant (lanes 2 to 48d) and control plasmids. (B) Southern hybridization with *Xmn*I-digested *hagA* probe (solid overline). (C) Hybridization with *Bam*HI-*Pst*I-digested *hagD* probe (solid overline). P, *Pst*I; B, *Bam*HI; N, *Nco*I; S, *Ssp*I; K, *Kpn*I; X, *Xmn*I. Broken overlines are probes used in RFLP analyses depicted in Fig. 4.

proteins encoded by the three most significant open reading frames (ORFs), which were 36.8, 21, and 80 kDa. Upstream from the 124-ATG translational start codon of the ORF encoding the 36.8-kDa protein were candidate −35 and −10 *E. coli* promoter consensus sequences, together with a putative ribosome-binding site (37). Similar consensus promoter sequences were not found upstream (5′) of the ORFs encoding the 21- and 80-kDa proteins, although possible ribosome-binding sites were identified. Terminator regions (4) were not detected in the 3′ regions of the ORFs encoding the putative 36.8- and 21-kDa proteins. These observations suggest that the p24-encoding insert may contain a sequence of genes in an operon. In the filter assay, p24 was able to bind epithelial cells without IPTG induction, indicating that binding entities may be expressed from a *P. gingivalis* promoter. Weak DNA homology (72% over 57 bp) was found between the p24 gene and *hagA* (3). However, in the p24 gene, the homology was localized to the noncoding region 5′ of the ORF encoding the 80-kDa protein, while in *hagA*, the homology was within a sequence which was repeated several times (3), and a similar region is present in *hagD*. Thus, the p24 probe could detect the multicopy sequences in *hagA*, but under the hybridization conditions used, the *hagA* probe could not detect the single-copy sequence in the p24 clone. With the BLASTN (2) search al-

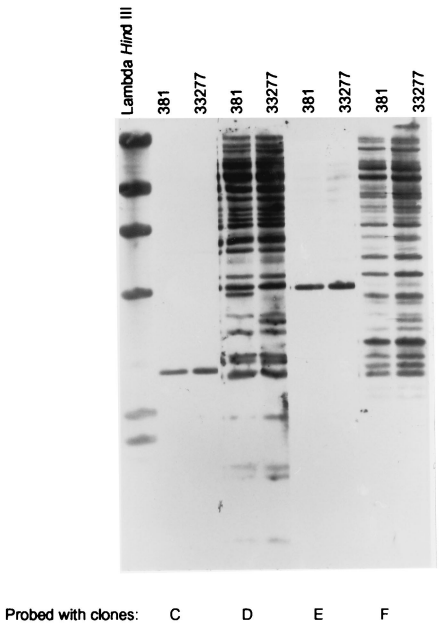


FIG. 3. RFLP analysis showing two classes of clones. Chromosomal DNA (5 μg) from strains 381 and ATCC 33277 was digested with *Pst*I, subjected to electrophoresis, and transferred to nitrocellulose. Inserts from clones C, D, E, and F (from the second library) were used as probes.

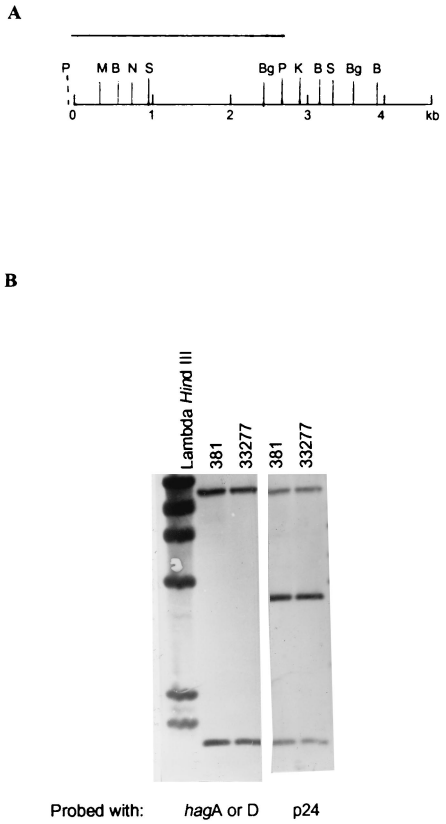


FIG. 4. Restriction map of p24 and RFLP analysis. (A) Restriction map showing the *Pst*I probe (solid overline) used in RFLP analysis. M, *Mlu*I; Bg, *Bgl*II; other enzymes are as described in the legend to Fig. 2. (B) Strain 381 and ATCC 33277 DNA (5 μg) was digested with *Pst*I for RFLP analysis. The *hagA* and *hagD* probes are depicted as broken overlines in Fig. 2B and C.

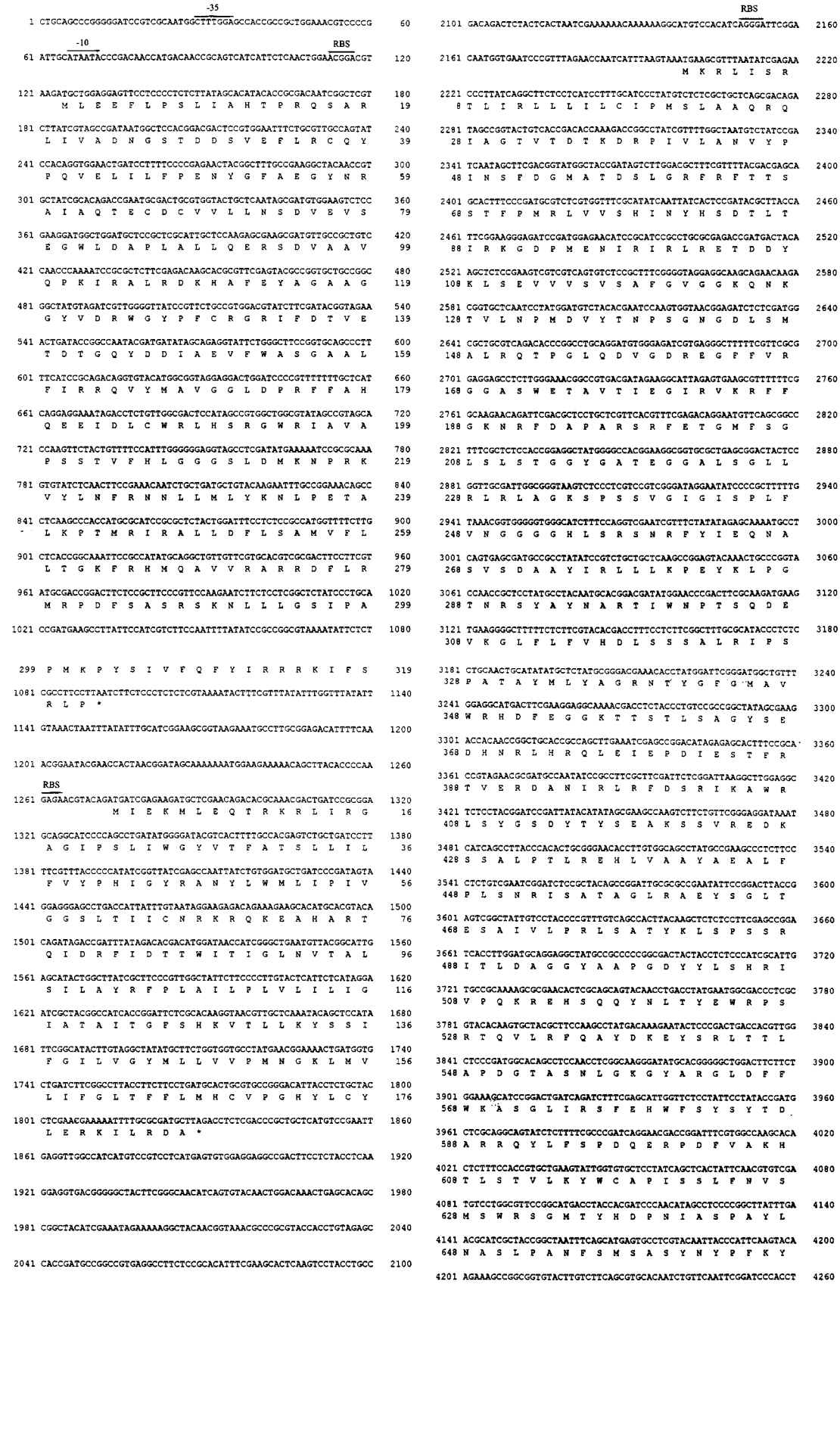
gorithm, no significant homology between the p24-encoding DNA sequence and sequences deposited in the databases was observed.

Protein sequence analysis. Plots of antigenic sites (14) of the 36.8- and 80-kDa ORFs revealed N-terminal sequences containing cores of hydrophobic amino acids, and potential signal peptidase cleavage sites (37) were identified predicting signal sequences of 23 amino acids for both putative proteins (Fig. 6). The hydrophobicity profile of the 21-kDa protein was consistent with that of a cytoplasmic membrane protein, and six membrane-spanning domains in its ORF were predicted by the TopPred II program (6). BLASTP homology searches gave highest scores for positive identity between large stretches of the 36.8-kDa protein ORF and a transmembrane protein encoded by the *whiB* region of *Streptomyces coelicolor* (7). Lower similarities were observed between three regions of the 38.6-kDa protein sequence and that of a hypothetical protein encoded by the *E. coli rfb* region involved in O-specific lipopolysaccharide synthesis (40); the *rfbC* gene of *Myxococcus xanthus*, also involved in O-antigen biosynthesis (11); and an ORF in a multicistronic locus of *Sphingomonas* strain S88, which contains genes required for polysaccharide capsule biosynthesis (39). No significantly high scores were obtained for the other two ORFs.

DISCUSSION

Periodontal pathogens possess a variety of virulence factors, but relatively little is known about their interactions with gingival epithelial cells and tissue at the molecular level. After the initial contact between the bacterium and the epithelial cell, stable attachments involving specific bacterial ligands and epithelial cell receptors are formed. Bacterial surface structures such as pili, fimbriae, and outer membrane proteins with adhesin functions are known in other systems to mediate these specific attachments. This study describes a strategy to identify *P. gingivalis* genes which participate in this interaction. A qualitative filter-binding assay (9, 22) was modified and used to screen a *P. gingivalis* library for recombinant clones expressing *P. gingivalis* genes involved in attachment. The screen depends on the expression in *E. coli* of a *P. gingivalis* gene or the part of the gene which contains a binding motif for an epithelial cell receptor. Since the clones were lysed before being probed with epithelial cells, the screen does not depend on the localization of the expressed protein on the *E. coli* outer surface.

Surprisingly, none of the clones hybridized to the *P. gingivalis fimA* gene, even though we knew that this DNA was well represented, since it was used as a probe to assess library quality. A possible explanation for this result is that *fimA* is expressed poorly, if at all, in *E. coli* from the vector used in this study (41), although there are reports of detectable levels of recombinant protein expressed from similar vectors (10). Alternatively, *fimA* may have been expressed in a biologically inactive form and thus escaped detection in the screen. It is not known whether fimbrillin itself is the adhesive protein which binds to the epithelial cell surface or forms the supporting structure for an associated adhesin, as in other gram-negative bacteria (15). The protein heterogeneity of *P. gingivalis* fimbrial preparations (19, 41) suggests that they are composite structures. There is immunological evidence that the hemagglutinating adhesin HA-Ag2 is distributed along the length of fimbriae in strain ATCC 33277 (5). Thus, the fimbrillin itself may not participate directly in the ligand-receptor interaction. Lastly, it is likely that *P. gingivalis* does not depend solely on


-35
-10
RBS
RBS

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 668 K  K  A  G  G  V  L  V  F  S  V  H  N  L  F  N  S  D  P  T

4261 ACGGCTATCGCTTCGGGGGACAAAACCCATCGGAGGCGTATATCCATCGGTTAGAATCTC   4320
 688 Y  G  Y  R  F  G  G  Q  N  P  S  E  A  Y  I  H  R  L  E  S

4321 TACCCCTTACCGACAGTTTTATATGGTAGGTCTGTTCATCAACTTCGGAATAGACCGCCG   4380
 708 L  P  L  T  D  S  F  I  W  *

4381 ACAAGAAATCATGAACACCGATATAAAAATCAAATCNAGATGAAAACTTTCAAAAGAATC   4440

4441 GCCCTGCTCCTCGTCGCAGGCTTTGCAGGGCTGTGTGCAACATCCGCACAAGGGNCGGCT   4500

4501 TATGCCGAAGTGATGAATCGAAAAGTAGCTGCTCTGGACAGTGTGCCACCGACGGAATAT   4560

4561 GCCACACTGGCTGCGGACTTTTCCCGGATAACAGCCGTGGAAGGATCC               4608
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FIG. 5. DNA sequence analysis of clone p24. Putative promoter sequences and ribosome-binding sites (RBS) are overlined. Nucleotides are numbered at both ends of each line; amino acids are numbered at one end only.

fimbria-mediated binding to epithelial cells and that other surface molecules will participate in this function.

All the clones isolated in the screen showed various levels of DNA homology to two known *P. gingivalis* hemagglutinin genes, *hagA* and *-D*, which contain homology not only to each other (23) but also to a cysteine protease gene (3, 31). Cell-bound *P. gingivalis* hemagglutinins are associated with cysteine protease activity (27), and evidence that these enzymes are also able to interact with epithelial cells comes from separate work on their effect on *P. gingivalis* attachment (8a). In the present study, one class of cloned inserts hybridized to multiple *Pst*I genomic fragments from ATCC 33277 and 381, indicating that they contain or are located close to multicopy sequences in the genome. Numerous reports demonstrate the association of repetitive sequences with cysteine proteases. Genetic analysis established that *P. gingivalis* ATCC 33277 contained two arg-gingipain genes, *rgpA* and *-B* (26). Sequence analysis of the arg-gingipain cysteine protease genes from several strains established that they contain an active site and multiple repeat sequences designated adhesin domains (3, 28, 30). Using the *P. gingivalis* W50 arg-gingipain homolog as a probe, Aduse-Opoku et al. (1) showed that the ATCC 33277 and 381 genomes contained multiple cross-hybridizing fragments. More recently, a repeat region within the porphypain coding sequence was shown to hybridize to multiple genomic fragments, and furthermore, the gene was located upstream from a sequence related to the *P. gingivalis* insertion element IS*1126* (3). Cloned inserts which hybridized to multiple genomic fragments are being analyzed to define and characterize the repeat regions and to determine the extent of their homologies to the genes encoding cysteine proteases.

Clone p24, by contrast, was a representative of the unique, single-genomic-copy insert class. Its restriction map was not

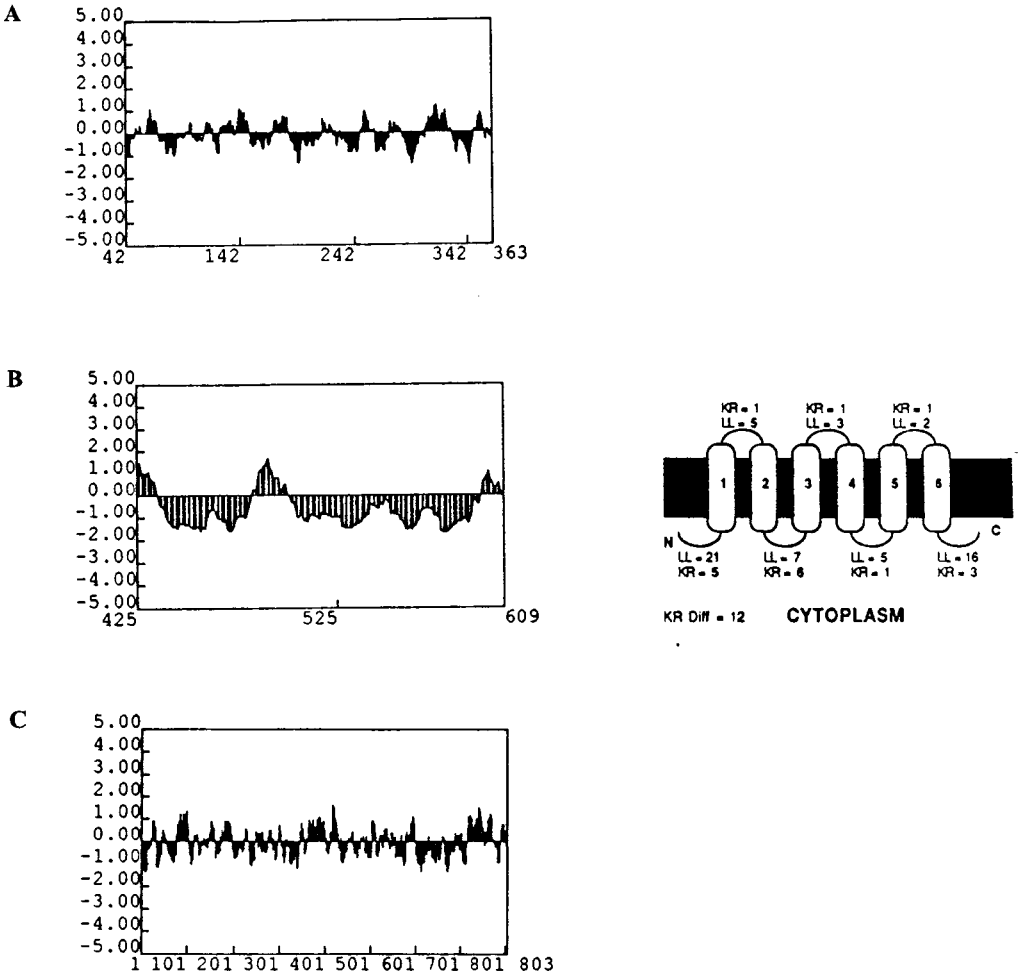

FIG. 6. Hopp and Wood plots of antigenic sites showing the hydropathy ORFs contained in the clone p24 DNA. (A) The ORF encoding the 36.8-kDa protein. (B) The ORF encoding the 21-kDa protein, including the cytoplasmic membrane domain plot. (C) The ORF encoding the 80-kDa protein.

obviously similar to that of *hagA* or *-D*, and RFLP analysis showed that it contained a different DNA fragment. A small region of DNA homology between a p24-noncoding region and repeat 2 of the *hagA* and porphypain-coding sequence (3) was responsible for the cross-hybridization observed. The DNA insert of clone p24 contained sequences for three potential ORFs coding for proteins with molecular masses of 36.8, 21, and 80 kDa. At this time, there are no known bona fide *P. gingivalis* promoter and ribosome-binding sequences. However, a sequence with *E. coli* promoter characteristics was identified upstream from the ORF encoding the 36.8-kDa protein. Thus, one possibility is that the three ORFs are part of an operon. In anticipation of potential virulence being clustered and possibly coregulated within an operon, the library was made to contain relatively large DNA inserts. Hydropathy plots of the deduced amino acid sequences indicated that the putative 36.8- and 80-kDa proteins contained N-terminal signal peptides, suggesting they are exported out of the cytoplasm. Only one ORF (that encoding the 36.8-kDa protein) showed significant homology to those encoding other proteins and lower but perhaps more meaningful homology to those encoding proteins from other gram-negative species which are involved in oligosaccharide synthesis. These proteins are transcribed from operons which in the case of *M. xanthus* and *Sphingomonas* strain S88 also encode ABC transporters (11, 39). Interestingly, the hydrophobicity profile of the next ORF (that encoding the 21-kDa protein) predicted a protein with six cytoplasmic membrane-spanning domains, reminiscent of the integral membrane domains of transporters (13). ATP-binding motifs (38) were not found within the sequence encoding the 21-kDa protein, although they may be contained in related ORFs flanking the p24 insert. The total size of the *P. gingivalis* transcript containing the p24-encoding ORFs is being determined by Northern (RNA) analysis.

The screening procedure was expected to identify a spectrum of *P. gingivalis* genes whose products could potentially interact with host cells and proteins, among which a subset might be involved in colonization and pathogenesis. As yet, only a small number of *P. gingivalis* activities have been identified at the gene level; therefore, the putative proteins encoded in clone p24 are unique, and their role in epithelial cell attachment remains to be determined.

ACKNOWLEDGMENT

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