INFECTION AND IMMUNITY, Dec. 1993, p. 5181–5189
0019-9567/93/125181-09$02.00/0

Vol. 61, No. 12

Proteins with Molecular Masses of 50 and 80 Kilodaltons Encoded by Genes Downstream from the Fimbrilin Gene (*fimA*) Are Components Associated with Fimbriae in the Oral Anaerobe *Porphyromonas gingivalis*

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Received 17 May 1993/Returned for modification 20 August 1993/Accepted 22 September 1993

Flanking DNA regions of the fimbrilin gene (designated *fimA*), which encodes the major subunit protein of *Porphyromonas* (*Bacteroides*) *gingivalis* fimbriae, were cloned in several manners from the *P. gingivalis* chromosome into *Escherichia coli* by screening with probes derived from a 2.5-kb *Sac*I DNA fragment previously cloned. A total of 10.4 kb of DNA fragments from the *P. gingivalis* genome was cloned in the pUC plasmid. Expression of the *fimA* gene and possible flanking genes in the fragments cloned was examined in a pUC plasmid vector system and in a bacteriophage T7 RNA polymerase-promoter expression vector system. The results show that in the pUC plasmid system, a 45-kDa protein, a product of *fimA*, was only poorly expressed as a precursor of the fimbrilin protein (FimA) and could be detected from cell extracts in Western blotting (immunoblotting) analysis as a sharp band but not in colony immunoblotting analysis. On the other hand, in the T7 RNA polymerase-promoter system, the product of *fimA* and products of the possible flanking genes responsible for fimbriation were overproduced as thick bands of the 45-kDa protein and as 63-, 50-, and 80-kDa proteins, respectively, in stained electrophoresis gels. All of the recombinant proteins were insoluble and seemed to be expressed as precursors with leader peptides. The 63-kDa, 45-k*Da (a truncated protein of the 50-kDa protein), and 80-kDa proteins were purified after solubilization with sodium dodecyl sulfate. N-terminal amino acid sequences of the 45-k*Da and 80-kDa proteins were analyzed up to the first 35 residues with a gas-phase sequencer. Monospecific antibodies directed to the recombinant proteins, i.e., the 63-kDa, 45-k*Da, and 80-kDa proteins, were raised in rabbits. By using the antibodies, localization of their matured proteins in *P. gingivalis* was investigated by Western blotting analysis. Immunoblotting analysis suggests that at least the 50- and 80-kDa proteins, encoded by genes downstream from the *fimA* gene, are minor components associated with fimbriae.

Porphyromonas (*Bacteroides*) *gingivalis* is a gram-negative oral anaerobe thought to be one of the most prominent periodontopathogens (19, 35, 36). It has several characteristics of an overtly pathogenic bacterium (9); for example, the organism has unique fimbriae and hemagglutinin, regarded as host attachment factors.

Although the adhesive function of *P. gingivalis* fimbriae has yet to be established, the fimbriae (51, 52) and hemagglutinin (11, 26, 30) reported to date are considered to be physically distinct (22, 25). The majority of *P. gingivalis* strains are fimbriate (38), and almost all of the strains have the *fimA* gene encoding the fimbrilin (FimA), the major fimbrial subunit protein of fimbriae (40). Circumstantial evidence (7, 27, 28, 49) has accumulated to indicate that *P. gingivalis* fimbriae are important as an antigen in host immunoresponses during periodontal disease. Taken together, these data suggest that *P. gingivalis* fimbriae may have an important role in host-parasite interactions in periodontal disease. To elucidate their function and to utilize them efficiently in clinical studies, molecular genetic studies on fimbriation are needed.

Little is known about the nature and molecular organization of genes involved in fimbrial morphogenesis and function in *P. gingivalis*. Unfortunately, there are presently no established cloning systems or shuttle vectors available for *P. gingivalis* that would allow a direct analysis of the genes involved in fimbriation. Although the *fimA* gene has been cloned in *Escherichia coli* and sequenced (6, 43), the 2.5-kb *Sac*I DNA fragment carrying *fimA* does not express detectable levels of fimbrilin protein or mRNA from an endogenous promoter in *Escherichia coli* (47a).

Studies on several types of *E. coli* fimbriae have shown that the fimbrial subunit and adhesin are genetically separable and that the adhesive function is likely to be carried on a protein distinct from the major fimbrial subunit (1, 32, 42). Analysis of fimbriation in several organisms has indicated that genes involved in fimbrial morphogenesis, including the subunit and adhesin, are genetically organized into clusters (2, 3, 12, 13, 21, 31). Since no data prove FimA to be an adhesin, there may be a minor proteinaceous adhesin on the

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TABLE 1. Bacterial strains and plasmids used in this study

Strain or plasmid	Genotype or relevant phenotype[a]	Reference or source
Strains		
E. coli		
JM83	*ara* Δ(*lac-proAB*) *rpsL*(=*strA*) φ80 *lacZ*ΔM15	45
JM109	*recA1 endA1 gyrA96 thi hsdR17 supE44 relA1* λ⁻ Δ(*lac-proAB*) [F′ *traD36 proAB lacI*^{q}ZΔM15]	45
K38	HfrC(λ); a host strain of pGP1-2	45
HMS174	*recA1 hsdR*; a host strain of pT7 plasmids	H. Nikaido[b]
P. gingivalis 381	A heavily fimbriate strain	38, 51
Plasmids		
pUC19	Ap^r	45
pT7-5	Ap^r; contains φ10 promoter for bacteriophage T7 RNA polymerase	H. Nikaido
pT7-6	Essentially identical to pT7-5, except that polycloning sites are arranged in the opposite orientation	H. Nikaido
pGP1-2	Km^r, contains T7 RNA polymerase, p_L, and *c*I857	H. Nikaido; 39
pUC13Bg12.1	Ap^r; carrying a 2.5-kb *Sac*I *P. gingivalis* DNA fragment constituting *fimA*	6

[a] Abbreviations: Ap^r, ampicillin resistance; Km^r, kanamycin resistance, p_L, lambda promoter. The exact orientations of insert DNA fragments with respect to the promoters are shown in Fig. 1 and 3.
[b] A gift from H. Nikaido.

tip of the fimbria. To identify the other fimbrial genes and their products, including a possible adhesin, we have sought to isolate recombinant clones carrying regions upstream and downstream from *fimA* and to test the expression of genes spanning *fimA* carried by the clones. First, we used pUC plasmid vectors to clone the DNA fragments and to test them for the expression of *fimA* and possible associated genes in *E. coli* from endogenous promoters. Second, we applied the cloned DNA fragments to a more efficient expression vector system (39), featuring a bacteriophage T7 RNA polymerase and its unique promoter, to further analyze their products. The overexpression of encoded protein by a coupled T7 RNA polymerase-promoter system has been used in the analysis of gene products from cloned sequences of a number of bacterial species (8, 15, 24, 37, 44). Overexpression of encoded proteins also allows the cellular localization to be examined (24, 44). Recently, Müller et al. (23) used transcription driven by a T7 promoter to analyze flanking genes involved in the production of mature fimbrial subunit protein from *Salmonella enteritidis*.

Research in our laboratories has been directed to the elucidation of the mechanisms and the whole gene structure responsible for fimbriation in *P. gingivalis*. In this article, we show that the T7 RNA polymerase-promoter system can be used to overexpress *P. gingivalis* genes and discuss at least two genes downstream from *fimA* and localization of their products as an initial step. Part of this work has been reviewed recently (47, 50).

MATERIALS AND METHODS

Bacterial strains, plasmids, and growth conditions. Bacterial strains and plasmids used and recombinants constructed in this study are listed in Tables 1 and 2 (33, 45). *E. coli* strains were maintained on L medium supplemented, when necessary, with ampicillin (50 μg/ml), kanamycin (50 μg/ml), or both. Liquid cultures of *E. coli* were grown in M9 medium, L broth, or an enriched medium consisting of 20 g of tryptone, 10 g of yeast extract, 5 g of NaCl, and 2 g of glycerol per liter in 50 mM potassium phosphate buffer (pH 7.2). Induction of the phage T7 expression vector (a gift from H. Nikaido) was carried out by following a protocol provided by Stanley Tabor (38a). Briefly, cells containing the two plasmids were grown in the enriched medium with the antibiotics at 30°C. At the time the growth reached an A_{600} of 1.5, the temperature was raised to 42°C and kept there for 25 min. Rifampin (final concentration, 100 μg/ml) was then added, and the temperature was shifted to 37°C and kept there for an additional 2 h. Manipulations of *E. coli*-containing plasmids were performed as described by Maniatis and colleagues (18, 34). The *P. gingivalis* strains were maintained in a semisolid general anaerobic medium (Nissui, Tokyo, Japan) and grown in a general anaerobic medium broth (48).

DNA isolation, construction of the gene bank, and subcloning. Chromosomal DNA was prepared from *P. gingivalis* 381 essentially as described by Hull et al. (10) except that sodium dodecyl sulfate (SDS) was used to obtain cell lysis and RNase was included to degrade RNA. The lysates were extracted several times with an equal volume of phenol-chloroform (1:1) and once with chloroform. Chromosomal DNA in the aqueous phase was extensively dialyzed against TE (10 mM Tris-HCl [pH 8.0], 1 mM EDTA) buffer and stored at 4°C. Plasmid DNA was isolated by the method of Birnboim and Doly (4) and purified by centrifugation to equilibrium in a CsCl-ethidium bromide gradient by the method of Maniatis and colleagues (18, 34). The chromosomal DNA was completely digested with *Hin*dIII or *Pst*I, and the DNA fragments were ligated into the *Hin*dIII or *Pst*I site, respectively, of the appropriately restricted pUC19 plasmid vector. The ligation mixtures were transformed into *E. coli* JM83, and recombinant clones (white colonies) were selected on L agar plates containing ampicillin, 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-Gal), and isopropyl-β-D-thiogalactoside (IPTG). White colonies were saved individually in 96-well microculture plates filled with L broth containing 10% glycerol at −80°C. For identification of *fimA*-associated clones in the plasmid libraries, recombinant clones were patched onto L agar plates with 50 μg of ampicillin per ml. The plates were incubated overnight at 30°C, and colonies on the plates were adsorbed onto nitrocellulose membrane filters (catalog no. A045 A082C; Advantec Toyo) by placing the filters on the surfaces of the plates. Cells on the surface of each filter were lysed in situ

TABLE 2. Plasmids constructed in this study

Plasmid	Vector	Insert			
		Size (kb)	Terminal of restriction site	*fimA* region overlapped	Orientation[a]
pUC19Bg676	pUC19	4.4	*Pst*I-*Pst*I	5′	Reverse
pUC19Bg889	pUC19	4.4	*Pst*I-*Pst*I	5′	Forward
pUC19Bg708	pUC19	6	*Pst*I-*Pst*I	3′	Reverse
pUC19Bg151	pUC19	5.8	*Hin*dIII-*Hin*dIII	Whole	Reverse
pUC19Bg154	pUC19	5.8	*Hin*dIII-*Hin*dIII	Whole	Forward
pT7-6Bg01	pT7-6	5.8	*Hin*dIII-*Hin*dIII	Whole	Reverse
pT7-6Bg05	pT7-6	5.8	*Hin*dIII-*Hin*dIII	Whole	Forward
pT7-6Bg51	pT7-6	2.2	*Hin*dIII-*Pst*I	5′	Forward
pT7-5Bg52	pT7-5	3.6	*Pst*I-*Hin*dIII	3′	Forward
pT7-6Bg61	pT7-6	3.4	*Pst*I-*Sac*I		Forward
pT7-5Bg62	pT7-5	3.4	*Pst*I-*Sac*I		Reverse
pT7-5Bg71	pT7-5	2.5	*Sac*I-*Sac*I	Whole	Forward
pT7-5Bg72	pT7-5	2.5	*Sac*I-*Sac*I	Whole	Reverse
pT7-5Bg27	pT7-5	1.8	*Sac*I-*Hin*dIII		Forward
pT7-6Bg13	pT7-6	4.4	*Pst*I-*Bam*HI	3′	Forward
pT7-5Bg12	pT7-5	4.4	*Pst*I-*Bam*HI	3′	Reverse
pT7-6Bg82	pT7-6	2.7	*Hin*dIII-*Pst*I		Forward
pT7-5Bg81	pT7-5	2.7	*Hin*dIII-*Pst*I		Reverse

[a] The forward or reverse orientation indicates the normal direction of the transcript of the gene from its endogenous promoter or against the direction of transcription with respect to the vector promoter.

with an alkaline solution, and the filters were subjected to colony hybridization with DNA probes. Other procedures for DNA manipulations were carried out as described by Maniatis and colleagues (18, 34).

Colony immunoblotting. Colony immunoblotting was carried out by a method similar to that used for colony hybridization (34). Colonies were transferred onto membrane filters by placing the membranes on the colonies and lysed in situ on the filters with chloroform vapor. The filters were then washed overnight with TBS (20 mM Tris-HCl [pH 7.4], 0.5 M NaCl) buffer containing 3% bovine serum albumin and lysozyme (40 μg/ml) to remove unbound cell debris. The filters were washed three times with TBS buffer containing 0.05% Tween 20 and immunochemically stained by using enzyme-linked antibody as described below in the section on Western blotting (immunoblotting) analysis.

Preparation of bacterial extracts and separation of soluble and insoluble protein fractions from the extracts. Bacterial cells, gently washed once with 10 mM Tris-HCl (pH 7.4) and 0.15 M NaCl, were suspended in 5 mM Tris-HCl (pH 8.0), and the cell suspension was sonicated until it became transparent by using the probe of a Tomy Seiko (Tokyo, Japan) UD-200 sonicator with 5 to 10 1-min bursts at 0°C. Whole-cell extracts were obtained as supernatant after unbroken cells were removed by centrifugation at 1,000 × *g* for 10 min. The cell extracts were separated to soluble (supernatant) and envelope (pellet) fractions by ultracentrifugation at 143,000 × *g* for 60 min.

Purification of gene products, amino-terminal amino acid sequencing, and preparation of antibodies against them. Whole envelopes were obtained as sediments after centrifugation of homogenate at 143,000 × *g* for 60 min. The envelopes were extracted with 10 mM Tris-HCl (pH 7.4) containing 1% Triton X-100 and then centrifuged at 143,000 × *g* for 60 min. The resulting sediment was extracted with 10 mM Tris-HCl (pH 8.0) containing 1% SDS and 1 mM EDTA at room temperature and then centrifuged at 143,000 × *g* for 60 min. The gene products were fractionated on a gel filtration column, and the fractionated proteins, which were concentrated with Ficoll 400, were purified by preparative SDS-polyacrylamide gel electrophoresis (SDS-PAGE) as described previously (5). An Applied Biosystems 470A sequencer equipped with a 130A separation system as a PTH-amino acid analyzer was used for the automatic amino-terminal amino acid sequencing. Antibodies were raised in rabbits against the purified gene products as described previously (51).

SDS-PAGE and Western blotting analysis. SDS-PAGE was performed in a 1.0-mm-thick slab gel as described by Lutenberg et al. (17). Western immunoblotting analysis was carried out essentially as described earlier (41) except that a TransBlot cell with a cooling coil (Bio-Rad Laboratories) was used at 6 V/cm overnight (38). The samples were heated with SDS at 100°C for 5 min in the presence of 2-mercaptoethanol unless otherwise specified.

Analytical methods. The amount of protein was determined by the method of Lowry et al. (16), with bovine serum albumin as the standard. The protein content of induced proteins in bacterial fractions was estimated by the scanning of stained SDS-polyacrylamide gels with a Shimadzu CS-930 dual-wavelength scanner.

RESULTS

Cloning of a *Hin*dIII DNA fragment carrying *fimA* and *Pst*I fragments carrying upstream and downstream regions of the gene. As described previously, we have cloned the *fimA* gene from *P. gingivalis*, which encodes fimbrilin, a major subunit protein of *P. gingivalis* fimbriae, as a 2.5-kb *Sac*I genomic DNA fragment in pUC13 (6). Although the gene has been sequenced and characterized, the clone carrying a recombinant plasmid (designated pUC13Bg12.1) did not produce any immunoreactive protein detectable with a specific antifimbrilin serum (unpublished data). To facilitate studies on the process of fimbriation in *P. gingivalis*, we aimed to clone upstream and downstream DNA regions of *fimA* as well as to obtain recombinants expressing gene products. The middle of the *Sac*I fragment has two closely spaced *Pst*I sites (see Fig. 1). Therefore, we used the 1-kb *Sac*I-*Pst*I fragment containing the N-terminal region of *fimA* and the 1.5-kb

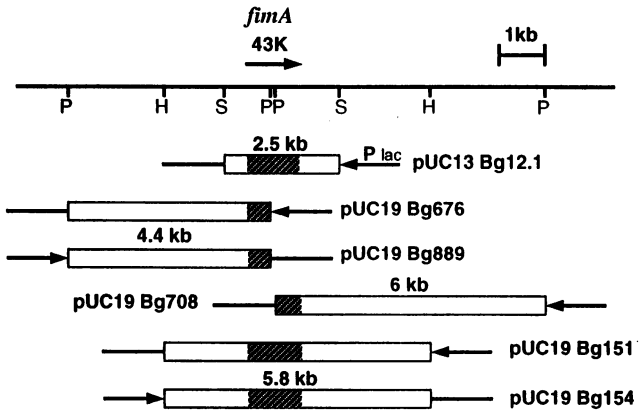

FIG. 1. Recombinant pUC plasmids carrying *fimA* and a part of the *fimA* gene. Relevant restriction sites of the region containing *fimA* in the *P. gingivalis* genome are shown above. The position and direction of transcription of *fimA* and the direction of transcription from the *lac* promoter (P_{lac}) are indicated by the arrows. Symbols: top thin line, chromosomal *P. gingivalis* DNA; thin lines below, vector DNA; bars, corresponding *P. gingivalis* DNA fragments cloned; hatched area in the bars, *fimA*. Abbreviations: H, *Hin*dIII; P, *Pst*I; S, *Sac*I; *fimA* 43K, 43,000-Da fimbrilin of strain 381.

*Pst*I-*Sac*I fragment containing the C-terminal region as probes to screen libraries for clones carrying upstream and downstream regions, respectively, of the *fimA* gene. The upstream probe identified two clones in a *Pst*I library, pUC19Bg676 and pUC19Bg889. Each carried a 4.4-kb *Pst*I fragment containing the upstream region of the *fimA* gene in either the reverse (pUC19Bg676) or the forward (pUC19Bg889) orientation with respect to the *lacZ* promoter on the vector. Similarly, the downstream probe identified one clone, pUC19Bg708, which carried a 6.0-kb *Pst*I fragment containing the downstream region of the *fimA* gene in reverse orientation with respect to the vector promoter. A screen of a *Hin*dIII fragment library yielded two clones, each carrying a 5.8-kb *Hin*dIII fragment with the *fimA* gene located in the center, in either the forward (pUC19Bg154) or the reverse (pUC19Bg151) orientation with respect to the *lacZ* gene. Consequently, a total of 10.4 kb of DNA fragments from the *P. gingivalis* genome was cloned in the pUC19 plasmid as three overlapping fragments as shown in Fig. 1 and Table 2.

Characterization of expression of *fimA* and adjacent genes in *E. coli*. Since the 5.8-kb *Hin*dIII fragment is long enough to carry at least one more coding region in addition to *fimA*, and since *fimA* is situated in the middle of the fragment, we tested for the expression of *fimA* and any other *P. gingivalis* genes in the transformants by using SDS-PAGE and Western blotting analysis. Initially, expression of the genes under various growth conditions was examined by growing the transformants in M9 medium supplemented with glucose, glycerol, or glycerol plus IPTG. Three major conditions of expression were tested, i.e., orientation (endogenous versus vector promoter) with or without IPTG (induction of *lacZ* promoter) and glucose (full suppression of vector promoter). Expression in *E. coli* JM83 and JM109, a strain with a $lacI^q$ mutation, carrying pUC191Bg151, pUC19Bg154, and pUC19, was examined under various growth conditions. The results in JM83 and JM109 were essentially the same. The results with JM109 are shown in Fig. 2. Although we were not able to differentiate protein patterns of cells in SDS-polyacrylamide gels stained with a dye, bands immunoreactive to an antifimbrilin serum in Western blotting analysis were clearly detected in whole-cell extracts and envelopes. The bands appeared at the position where a protein with an apparent molecular weight of 45,000 (45-kDa protein) would migrate. This is slightly larger than fimbrilin, the mature FimA protein in *P. gingivalis*, which has an apparent molecular weight of 43,000 (43-kDa protein; Fig. 2a, lane 10). This result would be consistent with the band in *E. coli* representing fimbrilin's precursor protein with a leader peptide. Although expression of the 45-kDa protein does not appear to be dependent on the *lac* promoter on the plasmid, *E. coli* carrying pUC19Bg154, in which the direction of transcription of *fimA* is the same as

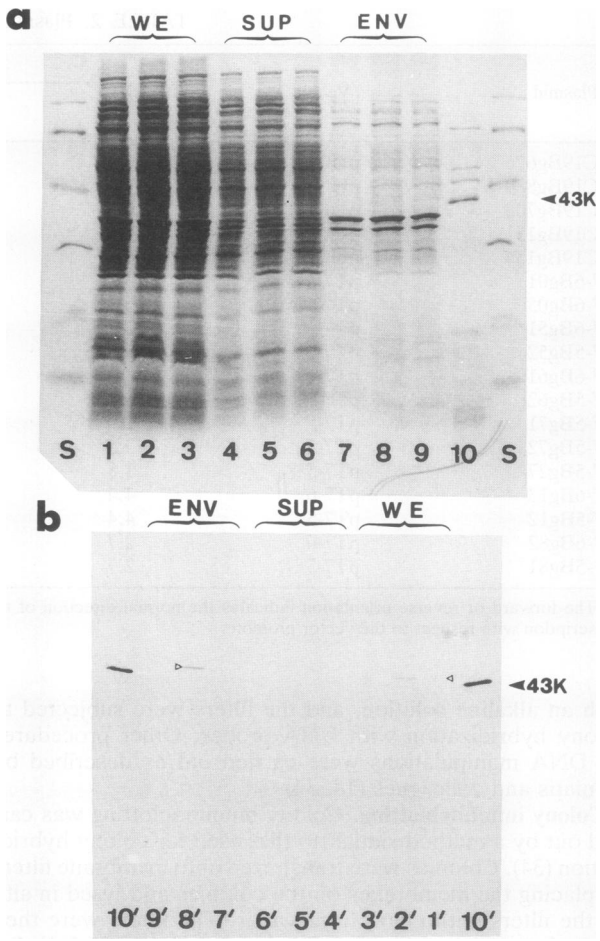

FIG. 2. Expression of the precursor of FimA in a pUC plasmid vector. (a) The electrophoretogram of the protein patterns of fractions from *E. coli* JM109 carrying pUC19Bg151 (lanes 1, 4, and 7), pUC19Bg154 (lanes 2, 5, and 8), and pUC19 (lanes 3, 6, and 9) grown in M9 medium supplemented with glycerol and IPTG. Whole extracts of cells (WE; lanes 1 to 3) were separated into soluble (SUP; lanes 4 to 6) and whole envelope (ENV; lanes 7 to 9) fractions. Whole extract of *P. gingivalis* 381 cells (lane 10) containing fimbriae was used as a control (43K). The same amount of protein (50 μg) of various cell lysates was subjected to SDS-PAGE. Lane S shows a pattern of standard proteins, i.e., from top to bottom, 93-, 66-, 45-, 31-, 21-, and 14-kDa proteins. (b) Identification of protein immunoreactive to the antifimbrilin serum. Lane numbers followed by a prime are identical to the corresponding lane numbers in panel a except that in lane 10', the amount of protein applied was reduced because of the presence of excess FimA for immunological detection. Empty triangles indicate the position of the 45-kDa protein prefimbrilin.

that from the *lac* promoter, gave the most intense band when grown in the presence of IPTG. The band, however, corresponded to less than 0.1 μg of protein, as judged from a band of a given amount of purified fimbriae applied to the gel. Therefore, the level of expression of the *fimA* gene was less than 0.2% of the loaded amount of protein. Because the fimbrilin protein can be expressed from a single copy of its gene as a major protein of *P. gingivalis* 381 detected in dye-stained SDS-polyacrylamide gels, the gene seemed to be inefficiently expressed in *E. coli*.

The expressed 45-kDa protein, the precursor of the FimA protein, was insoluble in cell extracts, and almost all immunoreactive protein was fractionated to a whole envelope fraction (Fig. 2b). When the *E. coli* strains were grown under conditions where the *lac* promoter was suppressed in the presence of glucose, the cells carrying pUC19Bg151 expressed slightly more immunoreactive 45-kDa protein than the cells carrying pUC19Bg154 did (data not shown). In M9 medium in the presence of glycerol, both cells produced similar but lesser amounts of 45-kDa protein. No other proteins encoded by *P. gingivalis* sequences were detected in this system.

Overproduction of the precursor of the FimA protein and possible gene products for fimbriation. Because only a small amount of the 45-kDa protein was expressed in the pUC plasmid system, and because it was impractical to purify the protein from the cells, we sought to use a more efficient expression system, the bacteriophage T7 RNA polymerase-promoter vector system (39), to isolate the protein as well as to identify unknown gene products on the *P. gingivalis* DNA fragments. The expression system used consisted of two compatible plasmids, pGP1-2 and pT7-5 or pT7-6 (pT7 plasmids are essentially identical except that they contain polycloning sites arranged in opposite orientations). To verify whether the expression system was applicable to *P. gingivalis* genes, we first cloned the 5.8-kb *Hin*dIII fragment from pUC19Bg151 in both orientations into the *Hin*dIII of the polycloning site of the pT7-6 plasmid, downstream from the ϕ10 promoter for T7 RNA polymerase. As shown in Fig. 3 and Table 2, the direction of transcription of *fimA* was forward with respect to the ϕ10 promoter in clone pT7-6Bg05 and reverse in clone pT7-6Bg01. These recombinant plasmids were transformed into *E. coli* K38 carrying pGP1-2, a plasmid that provides for expression of phage T7 RNA polymerase. The pGP1-2 plasmid consists of the gene for T7 RNA polymerase under the control of the inducible lambda p_L promoter and of the gene for the heat-sensitive lambda repressor, *c*I857. Clones with both the pT7 recombinant and pGP1-2 were screened on L agar plates in the presence of both ampicillin and kanamycin, which select the recombinant and pGP1-2, respectively. The clones were grown in enriched medium in the presence of the antibiotics, heat induction and the addition of rifampin followed, and then protein patterns in the extracts of these cells were examined by SDS-PAGE and Western blotting analysis. In *E. coli* K38 carrying pT7-6Bg05 and pGP1-2, a protein with an apparent molecular weight of 63,000 (63-kDa protein) and a 45-kDa protein similar to that expressed with pUC plasmids were induced, judging from the protein pattern of whole-cell extracts of K38 carrying pT7-6Bg01 and pGP1-2. In Western blotting with the antifimbrilin serum, the 45-kDa protein was an immunoreactive band. The band was thick but relatively pale when compared with authentic fimbrilin bands. The band appeared only in the recombinant cells with induced proteins, suggesting that expression of the 45-kDa protein was dependent on the T7 RNA polymerase-promoter system

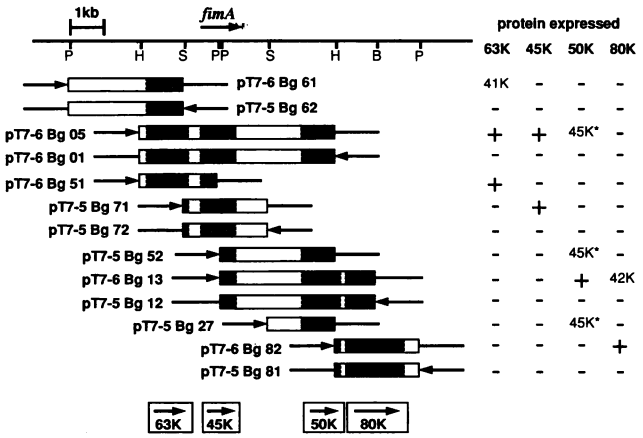

FIG. 3. Identification of locations, directions, and sizes of genes on the 10.4-kb DNA fragment by using various recombinant pT7 plasmids. DNA fragments and induced proteins derived from the fragments in the expression vector system are shown. The arrow in the top line indicates the position of *fimA* and the direction of transcription. Bars show corresponding cloned *P. gingivalis* DNA fragments. Results of expression are shown as the sizes of induced proteins, such as 63-kDa, 45-kDa, and 45-k*Da proteins, to the right. The expressed proteins are indicated by a plus or by size. A minus indicates that no protein was expressed. Related proteins are vertically aligned in columns because the 45-k*Da protein, for instance, is a truncated protein of the 50-kDa protein. Similarly, because the 42-kDa protein, derived from pT7-6Bg13, is a truncated protein of the 80-kDa protein, they are aligned in the same column. Open bars at the bottom of the figure indicate the location and size of the corresponding gene. Preliminary DNA sequencing indicates that there is an open reading frame between the *fimA* and 50-kDa gene which could produce a 20-kDa protein. B, *Bam*HI. Other symbols and abbreviations are the same as those described in the legend to Fig. 1.

and that this protein was overproduced as the precursor of fimbrilin. The corresponding, stained band of the induced 45-kDa protein from the clone seemed to be a doublet (see below). As shown previously in expression of the pUC plasmid system, it was confirmed that the *fimA* gene was expressed as the 45-kDa protein, the precursor form. Moreover, a new protein, the 63-kDa protein, was detected as a gene product from the *Hin*dIII fragment. Interestingly, neither of the induced proteins was stained in Western blotting analysis by using an antiserum against whole *P. gingivalis* cells. Since we easily recognized the induced proteins among numerous stained protein bands of whole-cell extracts in gels, both gene products appeared to be overproduced (data not shown) (see reference 47 and Fig. 4 in reference 50).

In this context, to determine whether *E. coli* cells carrying recombinants with *fimA* could be detected by colony immunoblotting screening, bacterial cells carrying pUC recombinants were grown in the presence or absence of IPTG, and then colony immunoblotting was carried out. None of the cells was detected as a positive colony (data not shown), giving a possible reason why our initial attempts to clone *fimA* by this method were unsuccessful (53). On the other hand, *E. coli* colonies carrying *fimA* in the T7 expression system showed clear, positive reactions when the colonies were induced by heat (data not shown).

Localization of the genes on the DNA fragment and localization of their products in *E. coli*. To identify the region for the 63-kDa protein gene and to ascertain that the 45-kDa protein was a doublet, the 5.8-kb *Hin*dIII fragment was

divided into *Hin*dIII-*Pst*I and *Pst*I-*Hin*dIII fragments, and they were cloned into pT7-6 and pT7-5, respectively, so that the orientation of the inserts was the same as that of pT7-6Bg05. These subclones were designated pT7-6Bg51 and pT7-5Bg52, respectively (Fig. 3). The 63-kDa protein was detected in the clone carrying pT7-6Bg51 as the sole induced protein, and a similar 45-kDa protein in the clone carrying pT7-5Bg52 was stained again as the sole induced protein. This 45-kDa protein, however, was not immunoreactive to the antifimbrilin serum (data not shown). Neither of the subclones expressed any protein bands reactive with the antiserum, suggesting that both clones were not able to produce any truncated protein of fimbrilin. Hereafter, we will call this new protein 45-k*Da protein. To confirm that the 45-kDa and 45-k*Da proteins were different from each other, first, the 2.5-kb *Sac*I fragment carrying the *fimA* gene was cloned in both orientations into pT7-5, and second, a 4.4-kb *Pst*I-*Bam*HI fragment, an extended, longer DNA fragment toward the C terminus from the above *Pst*I-*Hin*dIII fragment encoding the 45-k*Da protein, was cloned into pT7-6 and pT7-5 plasmids. The clone carrying pT7-5Bg71 (forward; Fig. 3 and Table 2), but not the clone carrying pT7-5Bg72 (reverse), produced the immunoreactive, 45-kDa protein as a heavy, thick band as expected. The clone carrying pT7-6Bg13 (forward), but not the clone carrying pT7-5Bg12 (reverse), expressed a 50-kDa protein instead of the 45-k*Da protein. Therefore, the regions of two new structural genes encoding the 63-kDa and 45-k*Da proteins were identified as being upstream and downstream from *fimA*, respectively. The direction of transcription of both genes was the same as that of *fimA*. To confirm these results, shown in Fig. 3 and Table 2, additional recombinants were constructed to identify gene products and to localize genes on the 10.4-kb *Pst*I fragment. Taken together, the 45-k*Da protein was determined to be a truncated 50-kDa protein, and another 80-kDa protein was discovered as a gene product of the region further downstream in the clone carrying pT7-6Bg82 (data not shown).

When *E. coli* cells carrying pT7 recombinants were grown and induced, the induced, overproduced proteins were insoluble in the extracts. Almost all of them were localized in the whole envelope fraction, representing about 40% of the total membrane protein in the case of the clone with pT7-6Bg05, producing the 63-kDa, 45-kDa (the precursor of fimbrilin), and 45-k*Da proteins. The recombinants varied in the amount of induced proteins. It is not clear whether these gene products were located in the membranes or in inclusion bodies as an insoluble form in cytoplasm.

Purification of gene products, amino-terminal amino acid sequencing, and rabbit antisera. The gene products in the membrane fraction were solubilized with a harsh detergent such as SDS but not with a mild detergent, Triton X-100. The 63-kDa, 45-k*Da, and 80-kDa proteins were purified from clones with pT7-6Bg51, pT7-5Bg27, and pT7-6Bg82, respectively, and antibodies against them were raised in rabbits as described in Materials and Methods. Because none of the clones expressing only this fully synthesized 50-kDa protein was available and to avoid potential contamination by other gene products, the 45-k*Da protein from the clone carrying pT7-5Bg52 instead of the 50-kDa protein from the clone carrying pT7-6Bg13 was purified. The purified proteins are shown in Fig. 4.

The amino-terminal amino acid sequences of the 45-k*Da and 80-kDa proteins were determined up to the first 35 residues. The consecutive 28-residue sequence of the 45-k*Da protein was completely in agreement with that deduced from the DNA sequence. In the sequence of the 80-kDa protein, 29 of the first 35 residues matched those predicted from the DNA sequence. The consecutive 10-residue sequences of these proteins which exactly matched those predicted from the preliminary DNA sequence of the downstream region of the *fimA* gene are shown in Fig. 5. The sequence of the 63-kDa protein unfortunately remains undetermined.

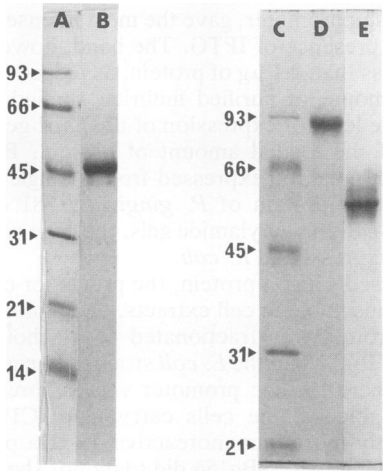

FIG. 4. Purification of the gene products. SDS-PAGE patterns of the purified proteins are shown. Lanes: A and C, standard 93-, 66-, 45-, 31-, 21-, and 14-kDa proteins; B, 45-k*Da protein; D, 80-kDa protein; E, 63-kDa protein. Panels A and B and panels C, D, and E were from separate experiments with slightly different gel concentrations.

The antisera were monospecific to each protein. Antiserum against the 45-k*Da protein also recognized the 50-kDa protein, and antisera against the 63- and 80-kDa proteins also reacted to their truncated 41- and 42-kDa proteins, respectively (data not shown). Because antisera against the gene products had some natural *E. coli* antibodies and because the proteins degraded to some extent in *E. coli* or during the sample preparation, immunoblotting analysis using the antisera sometimes showed the target proteins as well as many protein bands with lower molecular weights than those of the proteins. However, because rabbit antisera do not seem to have natural antibodies against *P. gingivalis* and because *E. coli* and *P. gingivalis* are serologically different from each other, antisera without any specific treatment did not show any cross-reactivity to unrelated proteins in *P. gingivalis* (see the section below).

45-K* Da protein

1	2	3	4	5	6	7	8	9	10
Met	Lys	Met	Lys	Tyr	Phe	His	His	Pro	Ser

80-K Da protein

12	13	14	15	16	17	18	19	20	21
Pro	Pro	Ile	Leu	Phe	Leu	Leu	Val	Gly	Phe

FIG. 5. Amino-terminal amino acid sequences of the 45-k*Da and 80-kDa proteins. These sequences were completely in agreement with the DNA sequence.

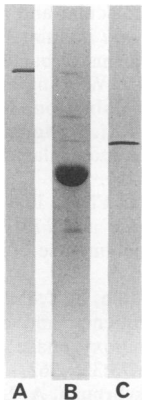

FIG. 6. Association of gene products downstream from *fimA* with fimbriae. The same amount (32 μg) of pure fimbriae was applied to several lanes of an SDS-polyacrylamide gel. After electrophoresis, the gel was cut into two pieces: one was stained with a dye (B) and proteins of the other piece of the gel with two lanes were transferred to a membrane. The transferred membrane was blocked with a buffer containing 1% bovine serum albumin, and then the membrane was cut again into two pieces so that one piece corresponded to one lane; the pieces were separately incubated with antisera against the 80-kDa (A) and 45-k*Da (C) proteins. The thickest band shows the 43-kDa protein fimbrilin.

Localization of gene products in *P. gingivalis*. Antibodies against the gene products, i.e., 63-kDa, 45-k*Da (a truncated protein of 50-kDa), and 80-kDa proteins, were used to localize them in *P. gingivalis*. Since each antiserum was specific to each corresponding gene product, the antisera confirmed the relationship between mature and truncated gene products such as that between the 63-kDa (fully synthesized) protein and a 41-kDa (truncated) protein, as shown in Fig. 3. Although the antisera did not show any detectable bands from *P. gingivalis* whole-cell lysates in immunoblot analysis, each of the antibodies against the 45-k*Da and 80-kDa proteins bound to a minor band with almost the same migration rate as the corresponding gene product when a large amount of pure fimbriae was applied to the gel, as shown in Fig. 6.

Pure fimbrial preparations had several minor proteins constituting less than 1% of the fimbrilin band (43-kDa). These minor proteins always appeared in gels at the positions where 80-, 60-, 50-, 31-, and 25-kDa proteins would migrate. They were not removed from fimbrial preparations by repeated ion-exchange column chromatography and were detectable in gels only when a large amount of fimbriae was dissociated to monomers in SDS by heat treatment (at 80°C for more than 20 min or at 100°C for more than 5 min). Therefore, these minor proteins are likely to be physically associated with fimbriae. Immunoblotting analysis revealed that the antibodies against the 45-k*Da and 80-kDa proteins clearly bound to corresponding minor proteins, the 50-kDa and 80-kDa proteins, respectively, in fimbriae. Interestingly, a minor 60-kDa protein in fimbriae did not react to the antibody against the 63-kDa protein.

DISCUSSION

In this study, we have shown the flanking regions of the *fimA* gene and examined these regions for expression of genes in pUC plasmid and the bacteriophage T7 RNA polymerase-promoter expression systems. Although the pUC plasmid is a good cloning and expression vector and it has been widely used for cloning genes from various organisms, including *P. gingivalis*, the *fimA* gene was poorly expressed, and the 63-kDa, 45-k*Da, and other proteins were not detected. Although genes for a surface protein from *Streptococcus mutans* (29) and for type 2 fimbriae from *Actinomyces naeslundii* (46) have been cloned in pUC plasmids by screening with antibodies, the results obtained from expression in pUC plasmid and T7 RNA polymerase-promoter systems indicate that *E. coli* RNA polymerase is not able to efficiently synthesize RNA from the *P. gingivalis* DNA fragments inserted in plasmid pUC19 and that transcription of the gene is hampered as described recently (14). Likely reasons for the failure to obtain efficient expression of *P. gingivalis* proteins in *E. coli* are as follows. (i) Endogenous *P. gingivalis* promoters do not function in *E. coli*. (ii) There is a transcription termination site recognized by *E. coli* RNA polymerase preceding *fimA* so that a transcript cannot be obtained through the gene. The T7 RNA polymerase may not recognize this site, allowing transcripts to accumulate. (iii) Alternatively, the gene may be transcribed by *E. coli*, but the mRNA may be highly unstable so that it is not possible to accumulate enough to give a significant amount of protein. The T7 system may be so efficient that it produces enough transcript to give a significant amount of protein. (iv) We have shown that the codon usage in the fimbrilin gene is different from that for abundant *E. coli* proteins and that the translation site does not have the optimal Shine-Dalgarno sequence-to-ATG spacing (6). Therefore, the fimbrilin message would be produced in *E. coli*, but translation would be inefficient. The T7 system could simply produce so much message that even inefficient translation is enough. This notion seems to be justified because in the T7 RNA polymerase-promoter system, T7 RNA polymerase synthesizes RNA efficiently from a strong, unique T7 RNA promoter by a rapid and progressive mechanism (39). In fact, it is known that in vitro, transcription by T7 RNA polymerase from a T7 promoter on a plasmid results in transcripts several times the plasmid length (20). A recent report (43) showed that the *fimA* gene was expressed in an expression vector, pET11b, although the expressed protein, 42-kDa protein, seems to be devoid of the leader peptide because it has the same molecular weight as mature fimbrilin. As mentioned above, our data for both pUC and T7 plasmids always suggested that the *fimA* gene was expressed as the 45-kDa precursor protein, which is slightly larger than fimbrilin (43 kDa in our case).

Thirteen recombinants were constructed in the T7 RNA polymerase-promoter system, and 63-, 45-, 50-, and 80-kDa proteins from upstream to downstream were shown to be overexpressed in this system. The 50- and 80-kDa proteins were identified as minor components associated with fimbriae. If these proteins are involved in adhesion, then antibodies directed to them might block binding. To obtain information on the function of these proteins, sequencing of all the genes is also in progress.

Preliminary DNA sequencing of the region downstream from *fimA* revealed that there were indeed open reading frames for the gene products, showing that the nucleotide sequences are in agreement with the data of amino-terminal amino acid sequences of the 50- and 80-kDa proteins and that there is another open reading frame for a 20-kDa protein between *fimA* and the gene encoding the 50-kDa protein (43a).

Immunoelectron microscopic observations of pure fimbriae and whole cells with the antisera against the 80-kDa

and 45-k*Da proteins showed dispersed immunogold particles, indicating a pattern quite different from that obtained with the antifimbrial serum; to date, we have not observed any result that would strongly indicate the association of these proteins to fimbriae. This type of experiment seems to be technically difficult presumably because the fimbriae are thin and single stranded (1). However, the fact that proteins immunoreactive to the antisera were not detected in whole-cell extract from *P. gingivalis* reinforced the idea that these proteins are not contaminants from cells but are minor components associated with fimbriae. We are trying to prove it by a genetic approach.

ACKNOWLEDGMENTS

We thank M. Nishikata and T. Ikeda for critical review, T. Ishikawa, K. Sagisaka, and A. Uchida for carrying out some studies, and M. Yoshikawa and C. Sasakawa for their valuable suggestions. This work would not have been possible without the generous help of R. J. Genco. We also thank K. Barrymore for helpful advice and discussion of the manuscript and H. Nikaido for sending strains of the bacteriophage T7 RNA polymerase-promoter vector system.

This work was supported by grants from the Akiyama Foundation, the Ciba-Geigy Foundation (Japan) for the Promotion of Science, and the Naito Foundation and by Grants-in-Aid for Scientific Research and Co-operative Research (62304036 and 62304049) from the Ministry of Education, Science and Culture of Japan.

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