JOURNAL OF CLINICAL MICROBIOLOGY, May 2000, p. 1909–1914 Vol. 38, No. 5
0095-1137/00/$04.00+0
Copyright © 2000, American Society for Microbiology. All Rights Reserved.

Distribution and Molecular Characterization of *Porphyromonas gingivalis* Carrying a New Type of *fimA* Gene

ICHIRO NAKAGAWA,* ATSUO AMANO, RICHARD K. KIMURA, TAKAYUKI NAKAMURA, SHIGETADA KAWABATA, AND SHIGEYUKI HAMADA

Department of Oral Microbiology, Osaka University Faculty of Dentistry, Suita-Osaka 565-0871, Japan

Received 30 November 1999/Returned for modification 7 February 2000/Accepted 22 February 2000

Fimbriae of *Porphyromonas gingivalis* are filamentous appendages on the cell surface and are thought to be one of the virulence factors. The *fimA* gene encoding the subunit protein of fimbriae, fimbrillin (FimA), was classified into four typeable variants (types I to IV). We previously examined the distribution of *P. gingivalis* in terms of *fimA* genotypes in periodontitis patients using a *fimA* type-specific PCR assay. However, some patients harbored *P. gingivalis* with untypeable *fimA*. In this study, we have cloned a new type (type V) of *fimA* from dental plaque samples. *P. gingivalis* with type V *fimA* was isolated from dental plaque of a periodontitis patient, and the isolate was named HNA-99. The deduced amino acid sequences were compared with those of type I *P. gingivalis* ATCC 33277, type II strain HW24D1, type III strain 6/26, and type IV strain HG564, and the homologies were found to be 45, 44, 43, and 55%, respectively. Southern blot analysis showed that the clinical isolate HNA-99 possessed *P. gingivalis*-specific genes *sod* and *kgp*. However, in terms of serological specificities, type V FimA showed a difference from other types of FimA. In addition, type V *P. gingivalis* bacteria were detected in 16.4% (12 of 73) of the *P. gingivalis*-positive patients with periodontitis by PCR assay using specific primers. Thus, a new type of *fimA* gene is now established, and the *fimA* genotyping could be useful in determining the disease-associated genotypes of *P. gingivalis* involved in the development of adult periodontitis.

Adult periodontitis is a chronic infection of the periodontium that results in periodontal destruction and alveolar bone loss (11, 32). *Porphyromonas gingivalis*, a gram-negative and black-pigmented anaerobe, has been etiologically associated with various types of periodontal diseases including adult periodontitis (1, 2). The organism expresses a number of potential virulence factors, which have been implicated in the pathogenesis of adult-onset periodontitis (18). *P. gingivalis* fimbriae have been reported to exhibit a wide variety of biological and immunological activities and are recognized as a major virulence factor in the infection and pathogenesis of this organism (5, 17, 24).

Several studies have demonstrated a divergence in in vitro pathogenicities among strains of *P. gingivalis* when evaluated by subcutaneous injection of the organism into rodents (15, 16, 25, 33). Other approaches have included serological and genetic typing to examine the relationship of *P. gingivalis* and its periodontal pathogenicity (10, 16, 17, 19, 26, 29, 30). However, no clear relationship between experimental dermal infections and oral infections was demonstrated regarding the virulence capability of *P. gingivalis*.

Recently, we examined the distribution of *P. gingivalis* in periodontitis patients using genotyping of the *fimA* gene, which encodes fimbrillin (FimA), a subunit protein of fimbriae of this organism (6). These results indicated that the occurrence of the organisms with different *fimA* genotype distributions showed a clear relationship with periodontal destruction, and *P. gingivalis* with type II *fimA* was found to be significantly predominant in severe periodontitis patients. The investigation also revealed the existence of a *P. gingivalis* strain(s) untypeable by our PCR assay, which enables us to divide *fimA* genes into four types, and the prevalence of the untypeable strain(s) was 6.3% of the dental plaque samples from periodontitis patients. These data indicate that unknown *fimA* genes could exist within the *P. gingivalis* strains, and those untypeable organisms may affect the development and progression of periodontitis. In this study, we cloned a new type of the *fimA* gene from the untypeable *fimA* specimens, and we isolated a *P. gingivalis* strain carrying a new *fimA* gene.

* Corresponding author. Mailing address: Department of Oral Microbiology, Osaka University Faculty of Dentistry, Suita-Osaka 565-0871, Japan. Phone: 81-6-6879-2879. Fax: 81-6-6878-4755. E-mail: ichiro@dent.osaka-u.ac.jp.

MATERIALS AND METHODS

Bacterial strains. *P. gingivalis* strains ATCC 33277 (*fimA* type I), HW24D1 (*fimA* type II), 6/26 (*fimA* type III), and HG564 (*fimA* type IV) were selected from our culture collections. These organisms were grown in GAM broth (Nissui, Tokyo, Japan) supplemented with 5 μg of hemin per ml and 1 μg of menadione per ml anaerobically (80% N_2, 10% H_2, and 10% CO_2) at 37°C. For the binding assay, these organisms were grown anaerobically in tryptic soy (TS) broth (Difco, Detroit, Mich.) supplemented with hemin and menadione. *Escherichia coli* XL10-Gold (Stratagene, La Jolla, Calif.) was cultured in Luria-Bertani medium or on a Luria-Bertani agar plate supplemented with 100 μg of ampicillin per ml for cloning and sequencing of the cloned gene. For isolation of *P. gingivalis* from clinical specimens, TS agar (Difco) supplemented with 5% rabbit blood, hemin, and menadione (TS blood agar) was used.

Clinical specimens. Subgingival plaque samples were collected from patients with periodontitis. These subjects were enrolled with informed consent (6). The bacterial genomic DNA from plaque samples was isolated with a DNA isolation kit according to the manufacturer's instructions (Puregene; Gentra Systems, Minneapolis, Minn.), and the isolated DNA was dissolved in 100 μl of TE (10 mM Tris HCl [pH 8.0] and 1 mM EDTA) buffer. The clinical parameters of the patients were described in our previous study (6).

Cloning of a new *fimA* gene by PCR. Among 73 samples which were positive for the *P. gingivalis* 16S rRNA gene in our previous study (6), five clinical specimens were found to contain an untypeable *fimA* gene(s). Thus, these five samples were used as templates for a new *fimA* gene. Oligonucleotides M11 (AATCTGAAC GAACTGCGACGCTAT) and M12 (CTCCCTGTATTCCGAATATAGAC) were designed for amplification of the *fimA* gene with the open reading frame (ORF) and promoter region according to the sequences of the *fimA* genes reported previously (14). The PCR amplification was performed in a total volume of 50 μl consisting of 0.2 μM (each) primer, 5 μl of template DNA, and 2.5 U of ExTaq (Takara Shuzo, Otsu, Japan) according to the manufacturer's instructions. The amplification reaction was performed in a model 9700 thermal cycler (PE Applied Biosystems, Branchburg, N.J.) with the following cycling parameters: an initial denaturation at 95°C for 5 min; 30 cycles consisting of 94°C for 30 s, 55°C for 30 s, and 72°C for 1 min; and a final extension at 72°C for 7 min. The PCR products were separated by electrophoresis using a 1% agarose gel, and the amplified DNA (about 1.3 kb) was extracted using QIAEX (Qiagen,

Düsseldorf, Germany). The DNA was directly cloned into pGEM-T vector (Promega, Madison, Wis.). The nucleotide sequence of the cloned gene was determined by using a dye-terminator reaction with a model 310 Genetic Analyzer (PE Applied Biosystems).

Data analysis of nucleotide sequence and amino acid sequence. Data analyses of nucleotide sequences and deduced amino acid sequences were performed with GeneWorks software (IntelliGenetics, Mountain View, Calif.). Multiple alignment analysis and construction of a phylogenetic tree were performed with CLUSTAL W in the DNA Data Bank of Japan (DDBJ; Mishima, Japan) (28). The sequence data for the *fimA* genes of *P. gingivalis* 381 (type I *fimA*), ATCC 33277 (type I), BH18/10 (type I), HW24D1 (type II), OMZ314 (type II), OMZ409 (type II), ATCC 49417 (type II), 6/26 (type III), and HG564 (type IV) were obtained from DDBJ under accession no. D17794, D17795, D17796, D17797, D17798, D17799, D17800, D17801, and D17802, respectively.

Prevalence of *fimA* type-specific *P. gingivalis* in periodontitis patients. Supra- and subgingival plaque samples were taken from a total of 93 periodontitis patients in our previous study (6). The plaque samples were then processed to isolate bacterial DNA as described previously (6). The isolated DNA was dissolved in 100 μl of TE buffer at 65°C for 10 min and then stored at −20°C until use. The detection of *P. gingivalis* and *fimA* typing were performed as described previously (6). The specificities and sensitivities of the *fimA* genotype-specific sets (types I to IV) for the primers have been previously demonstrated (6). A ubiquitous primer set that matches almost all bacterial 16S rRNA genes was used as a positive control, and the *P. gingivalis* species-specific primers (16S rRNA) were used for *fimA* typing. All primers were purchased from Amersham Pharmacia Biotech (Tokyo, Japan). PCR was performed as described previously (6). To demonstrate the presence of *P. gingivalis* with a new, genotype V *fimA* gene, type-specific primers were constructed as follows; fimV-f, AACAACAGTCTC CTTGACAGTG; fimV-r, TATTGGGGGTCGAACGTTACTGTC. The specificities of the prospective primers were tested by the program Amplify (21), and no amplification was detected for any of the strains listed in our previous report (6) other than the prospective positive samples (data not shown).

Isolation of *P. gingivalis* with a new type of *fimA* gene. Subgingival plaque samples containing *P. gingivalis* with the type V *fimA* gene were spread onto TS blood agar following dilution with phosphate-buffered saline and cultured anaerobically for 5 days at 37°C. Black-pigmented colonies were directly screened by using PCR with the specific primers (6). The clinical isolates were further examined by Gram staining and for anaerobic growth, the inability to ferment glucose, and the production of indole. The obtained isolate was maintained in GDO medium (group of difficult organisms medium; Nissui).

Analysis of genes of *P. gingivalis* strains. Southern blot analysis was performed as described by Ausubel et al. (7). Total genomic DNAs from lysozyme lysates of the organisms were digested with *Eco*RI, *Bam*HI, or *Hin*dIII (New England Biolabs, Beverly, Mass.), and the DNA fragments separated by 1% agarose gel electrophoresis were transferred to a nylon membrane (GeneScreen; NEN, Boston, Mass.). The *fimA* gene (1.3 kb) was amplified from genomic DNA of *P. gingivalis* ATCC 33277 with the M11 and M12 primers. The *sod* (superoxide dismutase; 575-bp) and *kgp* (Lys-gingipain; 560-bp) genes were amplified from the genomic DNA of the organism. These genes were then radiolabeled with [α-^{32}P]dCTP (Amersham Pharmacia Biotech) by using the BcaBest DNA labeling kit (Takara Shuzo). The blotted membranes were prehybridized and hybridized according to the protocol described by the manufacturer (NEN). After hybridization, the membranes were washed in 1× SSPE (180 mM NaCl, 10 mM $NaPO_4$, 1 mM EDTA, pH 7.4) at room temperature twice and washed in 0.1× SSPE for 20 min at 65°C. The washed membranes were exposed to Kodak BioMax MR films for 2 h at −70°C with an intensifying screen.

Immunoreactivity of FimA. Bacterial cells were harvested by centrifugation at 5,000 × *g* for 10 min and washed twice with ice-cold phosphate-buffered saline. The cells were resuspended in sodium dodecyl sulfate (SDS) gel loading buffer (7) and boiled for 5 min. The cellular proteins were separated by SDS–12% polyacrylamide gel electrophoresis and were transferred onto a polyvinylidene difluoride membrane (Immobilon; Millipore, Bedford, Mass.) at 24 mA for 1.5 h. The transferred protein bands were reacted with rabbit antibodies to recombinant FimA (rFimA) derived from strain 381 (type I *fimA*) and strain HG564 (type IV *fimA*) antibodies, respectively. The reactions were visualized using an alkaline phosphatase-conjugated anti-rabbit immunoglobulin G antibody (New England Biolabs) and 5-bromo-4-chloro-3-indolylphosphate–nitroblue tetrazolium substrate (Moss Inc., Pasadena, Md.).

Assay for binding of *P. gingivalis* cells to saliva-coated hydroxyapatite (sHA) beads. Abilities of *P. gingivalis* cells to bind sHA beads were examined as described previously (3). Briefly, HA beads (3 mg) were incubated with 300 μl of clarified human whole saliva in silicon-coated borosilicate tubes at 25°C for 18 h. For determination of specific binding of salivary proteins, HA beads (3 mg) were incubated with buffered KCl (pH 6.8) for the same periods. The sHA and uncoated HA beads were washed twice with buffered KCl and incubated with 10^6 to 10^8 ^{3}H-labeled cells at room temperature for 1 h. After incubation, the reaction mixture was layered on 1.5 ml of 100% Percoll (Amersham Pharmacia Biotech) in a new siliconized borosilicate tube to separate unbound cells. The radioactivities of the cell-bound beads were determined in a liquid scintillation counter (model 1401; LKB, Uppsala, Sweden). The assay was performed in triplicate and repeated three times. One-way analysis of variance and the Tukey-Kramer test were used for statistical analysis of comparison of binding abilities ($P < 0.01$).

Nucleotide sequence accession number. The type V *fimA* gene sequence is available from GenBank (accession no. AB027294).

RESULTS

Cloning of a new type of *fimA*. Cloning of a new *fimA* gene was carried out using a pair of PCR primers, M11 and M12. Five out of 73 plaque samples containing *P. gingivalis* that possessed untypeable *fimA* genes were used as templates. An expected-size DNA fragment was amplified by PCR from three of these samples. After cloning of PCR products into pGEM-T vector, the nucleotide sequences were determined for at least five individual clones from each sample. The sequences of all the clones were found to be identical (data not shown). The multiple alignment analysis showed that this gene fragment has sequence homology with type I *fimA* (strain ATCC 33277) (49%), type II *fimA* (strain HW24D1) (49%), type III *fimA* (strain 6/26) (52%), and type IV *fimA* (strain HG564) (59%). This gene fragment contained a −10 region (−35 to −40) and a −35 region (−58 to −63) upstream of the putative initiation codon, which are essential for expression of the *fimA* gene of *P. gingivalis* (34). This promoter sequence was found to be conserved among all *fimA* types. The comparison of the deduced amino acid sequences of type V *fimA* and other types of *fimA* is shown in Fig. 1. Type V FimA has an 18-amino-acid putative signal sequence, and this region was very similar to that of type IV FimA (strain HG564). However, the sequence of type V FimA was considerably different from those of other strains. The percent homologies of type V FimA with type I FimA (strain ATCC 33277), type II (HW24D1), type III (6/26), and type IV (strain HG564) were 42, 41, 40, and 52%, respectively. The phylogenetic tree showed that type IV *fimA* (strain HG564) and type V *fimA* made a cluster, but the branch length between type IV and type V (0.466) was longer than those between type I and type II (0.231) and type I and type III (0.270) (Fig. 2).

Isolation and characterization of *P. gingivalis* carrying the type V *fimA* gene. The distribution of *P. gingivalis* with type V *fimA* was examined in plaque samples from 73 periodontitis patients carrying *P. gingivalis* by PCR using the type V *fimA*-specific primers and the four previous *fimA* type-specific primers (6). Type V *fimA P. gingivalis* was detected, solely or in combination with other types, in 16.4% of the samples. The total incidence of type V *fimA* was higher than that of type I but was almost the same as that of type IV (Table 1).

We next attempted to isolate *P. gingivalis* carrying the type V *fimA* gene from clinical specimens. A positive PCR gave a single band with the expected size (462 bp) as assessed by electrophoresis (data not shown). Several isolates were obtained by colony PCR assay with *P. gingivalis* 16S rRNA-specific primers and type V *fimA*-specific primers. One of these isolates was designated strain HNA-99.

Southern hybridization analysis of digested genomic DNAs from various *P. gingivalis* strains representing types I to V of *fimA* was performed (Fig. 3). The intensity of hybridization obtained was similar among all the tested strains, except for strain HNA-99, when probed with the ^{32}P-labeled *fimA* gene of ATCC 33277 (type I). The genomic DNA from HNA-99 showed a weak hybridization with this probe but was reactive with the *sod* and *kgp* gene fragments from ATCC 33277 with a signal intensity similar to those of other strains.

Western blot analysis revealed that the 41-kDa FimA of strain HNA-99 did not react with anti-rFimA of strain 381 (type I). On the other hand, FimA of strain HNA-99 was faintly reactive against anti-rFimA of strain HG564 (type IV)

```
381         --------MVLKTSNSNRAFGVG---DDESKVAKLTVMVYNGEQQEAIKSAENAT---KVEDIKCSAG-QRTLVVMANTG------AMEL   69
ATCC33277   --------MVLKTSNSNRAFGVG---DDESKVAKLTVMVYNGEQQEAIKSAENAT---KVEDIKCSAG-QRTLVVMANTG------AMEL   69
BH18/10     --------MVLKTSNSNRAFGVG---DDESKVAKLTVMVYNGEQQEAIKSAENAT---KVEDIKCSAG-QRTLVVMANTG------AMEL   69
HW24D1      --------MVLKTSNPNRAFG-----EDESKVAKLTVMVYNGEQQEAIKSAENAT---KVEDIKCSAG-QRTLVVMANTG------EMKL   67
OMZ314      --------MVLKTSNPNRAFG-----EDESKVAKLTVMVYNGEQQEAIKSAENAT---KVEDIKCSAG-QRTLVVMANTG------EMKL   67
OMZ409      --------MVLKTSNPNRAFG-----EDESKVAKLTVMVYNGEQQEAIKSAENAT---KVEDIKCSAG-QRTLVVMANTG------AMEL   67
ATCC49417   --------MVLKTSNPNRAFGNA---GDEAKVAKLTVMVYKGEQQEAIKSAENAT---KVENIKCSAG-QRTLVVMANTG------GMEL   69
6/26        --------MVLKTSNPNRAFGNA---GDEAKVAKLTVMVYKGEQQEAIKSVENAI---KVENIKCGAG-QRTLVVMANTG------GMEL   69
HG564       METDATVSFIIKSGEGRAVGDG----LADAKITKLTAMVYAGQIQEGIKTVEEADGVLKVEGIPCKSGANRVLVVVANHN-------YEL   79
HNA-99(V)   METDATVSFIIKAGSPQRETEPNSLLDSDAKITKLTAMVYAGQVQEGIKTVEDADNVLKVEGIKCKSGANKVLVVVANYDKNAGGDAIDF   90
                    *                   *  *** *** *  ** **  * *   *** * *  *    *** **

381         PKTIVLKAGKNYIGYSGTG---EGNHIEND-PLKIKRVHARMAFTEIKVQMSAAYDNIYTVGKTLAEVKALTTELTAENQEAAGLIMTAE  155
ATCC33277   PKTIVLKAGKNYIGYSGTG---EGNHIEND-PLKIKRVHARMAFTEIKVQMSAAYDNIYTVGKTLAEVKALTTELTAENQEAAGLIMTAE  155
BH18/10     PKTIVLKAGKNYIGYSGTG---EGNHIEND-PLKIKRVHARMAFTEIKVQMSAAYDNIYTVGKTLAEVKALTTELTAENQEAAGLIMTAE  155
HW24D1      PVEVTLVAGNNYYGYDGSQ---GGNQISQDTPLEIKRVHARMAFTEIKVQMSPSYVNKYNAGKTLAEVKALTTELTAENQEAAGLIMTAE  154
OMZ314      PVEVTLVAGNNYYGYDGSQ---GGNQISQDTPLEIKRVHARMAFTEIKVQMSPSYVNKYNAGKTLAEVKALTTELTAENQEAAGLIMTAE  154
OMZ409      PVDVTLVAGNNYYGYDGSQ---GGNQISQDTPLEIKRVHARMAFTEIKVQMSPSYVNKYNVGKTLAEVKALTTELTAENQEATGLIMTAE  154
ATCC49417   PVEVTLVAGNNYYGYDGSQ---GGNQISQDTPLEIKRVRARIAFTKIEVTMSQSYANKYNAGKTLAEVKALTTELTEGNQEAAGLIMTAE  156
6/26        PVEVTLVAGNNYYGYDGSQ---GGNQISQGTPLEIKRVHARIAFTKIEVTMSQSYANKYNAGKTLAEVKALTTELTEGNQEAAGLIMTAE  156
HG564       SAAFTIKPGSNHYGYPDGT--TSDNLVSAGTPLAVTRVHAGISFAGVEVNMATQYQNYYSTGKSLNEVEALTTSLTAENQNAKNLIMTGK  167
HNA-99(V)   SNAFTIKPGTNYYGYPAGTGTTQDNLIETGNALKVTRVHAAMSIQNVTVTFDPQYSSNYYTGKTLDQVKAMTIQLTQDNQSAKFLIMTGE  180
                  * *  **        *       *   ** *         *     *   *  ** *  * * *  **  ** *  ****

381         FVPEK--IYGLIAKKQSNLFGATLVNADANYLTGSLTTFNGAYTPANYANVPWLSRNYVAP---AADAPQGFYVLENDYSANGGTIHPTI  240
ATCC33277   FVPEK--IYGLIAKKQSNLFGATLVNADANYLTGSLTTFNGAYTPANYANVPWLSRNYVAP---AADAPQGFYVLENDYSANGGTIHPTI  240
BH18/10     FVPEK--IYGLIAKKQSNLFGATLVNADANYLTGSLTTFNGAYTPANYANVPWLSRNCVAP---AADAPQGFYVLENDYSANGGTIHPTI  240
HW24D1      FAPEN--IYALVAKKESNLFGASLANSDDAYLTGSLTNFNGAYSPANYTHVDWLGRDYTEP---SNNAPQGFYVLESTYAQNAG-LRPTI  238
OMZ314      FAPEN--IYALVAKKESNLFGASLANSDDAYLTGSLTNFNGAYSPANYTHVDWLGRDYTEP---SNNAPQGFYVLESTYAQNAG-LRPTI  238
OMZ409      FAPEN--IYALVAKKESNLFGASLANSDDAYLTGSLTNFNGAYSPANYTHVDWLGRDYTEP---SNNAPQGFYVLESTYAQNAG-LRPTI  238
ATCC49417   FAPEN--IYALVAKKKSNLFGASLANNDDAYLTGSLTTFNGAYTPANYTHVDWLGRDFTEP---SNNAPQGFYVLESTYAQNAG-LRPTI  240
6/26        FAPEN--IYALVAKKKSNLFGASLANSDDAYLTGSLTTFNGAYSPANYTHVDWLGRDYTEIGAATVNTPKGFYVLESTYAQNAG-LRPTI  243
HG564       FNPADAKIAALVAKKDSKIFGNSLVSNTNAYLYGVQTPAG-LYTPDAAGETYELEASLNTN----YAVGAGFYVLESKYDASNE-LRPTI  251
HNA-99(V)   FKPQ--NVAGLICKKQSKIFGASLDFGT-DYLGGVATTAA-AYTPTSYDNSVSWLTKP-------YAAKAGFYIMESVYQVGNN-LRPTI  258
            * *      *  ** *  **  *      ** *  *     * *                              ***  *  *     ***

381         LCVYGKLQKNG-ADLAGADLAAAQAANWVDAEG--KTYYPVLVNFNSNNYTYDSNYT-PKNKIERNHKYDIKLTITGPGTNNPENPITES  326
ATCC33277   LCVYGKLQKNG-ADLAGADLAAAQAANWVDAEG--KTYYPVLVNFNSNNYTYDSNYT-PKNKIERNHKYDIKLTITGPGTNNPENPITES  326
BH18/10     LCVYGKLQKNG-ADLAGADLAAAQAANWVDAEG--KTYYPVLVNFNSNNYTYDSNYT-PKNKIERNHKYDIKLTITGPGTNNPENPITES  326
HW24D1      LCVKGKLTKHDGTPLSSEEMTAAFNAGWIVADNNPTTYYPVLVNFNSNNYTYDNGYT-PKNKIERNHKYDIKLTITGPGTNNPENPITES  327
OMZ314      LCVKGKLTKHDGTPLSSEEMTAAFNAGWIVADNNPTTYYPVLVNFNSNNYTYDNGYT-PKNKIERNHKYDIKLTITGPGTNNPENPITES  327
OMZ409      LCVKGKLTKHDGTPLSSEEMTAAFNAGWIVADNNPTTYYPVLVNFNSNNYTYDNGYT-PKNKIERNHKYDIKLTITGPGTNNPENPITES  327
ATCC49417   LCIKGKLTKHDGTPLSSEEMTAAFNAGWIVANNDPTTYYPVLVNFESNNYTYTGEAV-EKGKIVRNHKFDINLTITGPGTNNPENPITES  329
6/26        LCVKGKLTKHDGTALSSEEMTAAFNAGWIVANNDPTTYYPVLVNFESNNYTYTGEAV-EKGKIVRNHKFDINLTITGPGTNNPENPITES  332
HG564       LCIYGKLLDKDGNPLTEPALTDAINAGFCDGDG--TTYYPVLVNYDGNGYIYSGAITQGQNKIVRNNHYKITLNITGPGTNTPENPQPVQ  339
HNA-99(V)   LCVYGKLKKTETQDFSQEELDAAVAAGYCDGNA--ITYYPVLVNYNGYGYTYTGENT-GLNKILRNNHYKISLTVKGPGTNTPEGPLPEE  345
            **  ***                  *  *         ********     * *          ** **    * *   ***** ** *

381         AHLNVQCTVAEWVLVGQNATW-       347        (mol wt: 37587)
ATCC33277   AHLNVQCTVAEWVLVGQNATW-       347        (mol wt: 37587)
BH18/10     AHLNVQCTVAEWVLVGQNATW-       347        (mol wt: 37527)
HW24D1      AHLNVQCTVAEWVLVGQNATW-       348        (mol wt: 38089)
OMZ314      AHLNVQCTVAEWVLVGQNAT--       347        (mol wt: 37903)
OMZ409      AHLNVQCTVAEWVLVGQNATW-       348        (mol wt: 38076)
ATCC49417   ANLNVNCVVAAWKGVVQNVIW-       350        (mol wt: 37911)
6/26        ANLNVNCVVAAWKGVVQNVIW-       353        (mol wt: 38023)
HG564       ANLNVTCQVTPWVVVNQAATW-       360        (mol wt: 38239)
HNA-99(V)   ANLNVNCEVVSWNVVNQSAIWN       367        (mol wt: 39776)
            * *** * *   *  * *
```

FIG. 1. Comparison of predicted amino acid sequences for FimAs encoded by the *fimA* genes of various *P. gingivalis* strains and type V *fimA* gene. Amino acid identities are shown by asterisks. Hyphens are used to indicate the positions of gaps in the multiple alignment. The putative signal peptides are underlined. The number of amino acids and the molecular weight of the FimA of each strain are given. The alignment of the deduced amino acid sequences was performed with the CLUSTAL W program of the DNA Data Bank of Japan.

(Fig. 4). The immunogenicity of the HNA-99 FimA was clearly different from those of type I, type II, and type III FimAs, although a weak cross-reaction was found between type IV and type V FimAs.

Binding of *P. gingivalis* cells to salivary components. To determine whether the variation of FimA types could be related to functional ability, levels of binding of *P. gingivalis* organisms to HA beads coated with whole saliva were compared. As shown in Fig. 5, *P. gingivalis* strains ATCC 33277 (type I) and HW24D1 (type II) strongly bound to sHA beads, while strains 6/26 (type III), HG564 (type IV), and HNA-99 (type V) showed weak binding capabilities.

DISCUSSION

Since fimbriae appear to be important for attachment and invasion by *P. gingivalis*, characterization of fimbriae and elucidation of the attachment mechanisms have been extensively attempted (17). Here, we have successfully cloned a type V *fimA* gene, a new genotype of the fimbrillin gene of *P. gingivalis*, and have isolated the organism carrying this gene. *P. gingivalis* FimAs were classified into four types on the basis of the first 20 N-terminal amino acids (20) and variations in the nucleotide sequences of the *fimA* gene (14). As shown in Results, the multiple alignment analysis showed that our cloned gene fragment has sequence homology of 49 to 52% with other types of *fimA* genes. The highly homologous sequence strongly suggests that it is a new type of *P. gingivalis fimA* gene, and we designated it type V *fimA*.

P. gingivalis possessing the type V *fimA* gene was detected with a higher frequency (total, 16.4%) than were type I *fimA* organisms (13.7%) in the present cohort. This finding suggest that the present type of *P. gingivalis* is involved in the etiology of periodontitis. A wide variety of studies suggested that *P.*

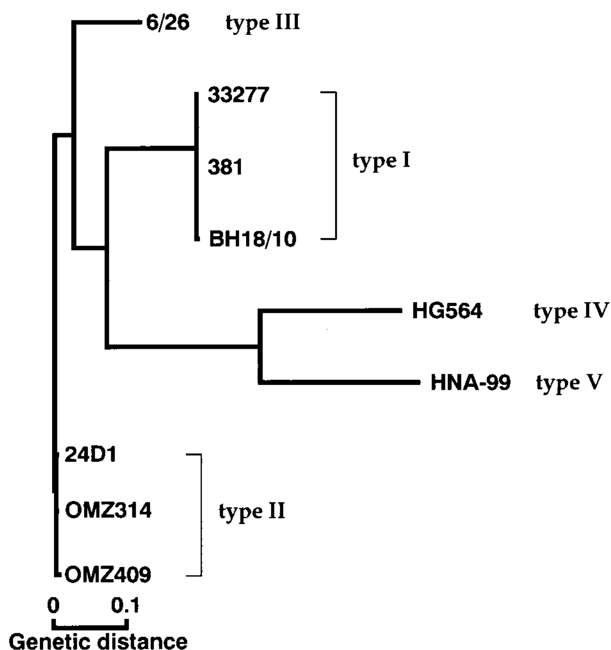

FIG. 2. Evolutionary relationships based on synonymous site variation in the *fimA* gene of *P. gingivalis*. The neighbor-joining method was used to construct the phylogenetic tree, using CLUSTAL W (DNA Data Bank of Japan) and Tree-View software (http://taxonomy.zoology.gla.ac.uk/rod/treeview.html).

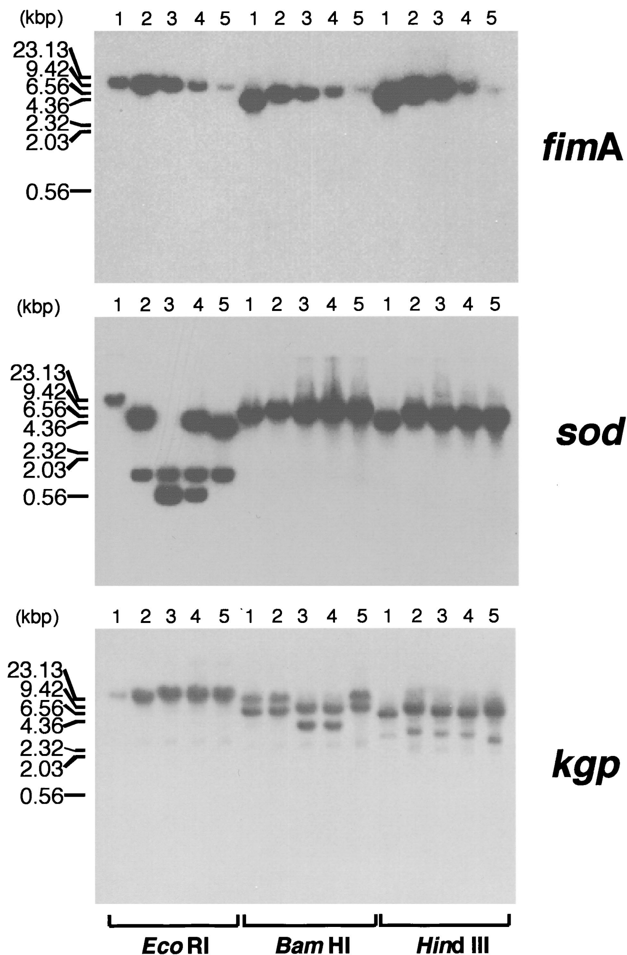

FIG. 3. Southern blot analyses of *P. gingivalis* specific genes. Genomic DNA was isolated from each strain; digested with *Eco*RI, *Bam*HI, and *Hin*dIII; and then separated on a 1% agarose gel. After blotting to a nylon membrane, *P. gingivalis* specific genes were probed with ^{32}P-labeled *fimA*, *sod*, and *kgp* gene fragments from strain ATCC 33277. Lanes: 1, ATCC 33277; 2, HW24D1; 3, 6/26; 4, HG564; 5, HNA-99.

gingivalis fimbriae are major virulence factors and are possible candidates for use in a vaccine (17). Thus, we attempted further characterization of the isolate in this study.

Interestingly, analyzing the promoter region of *fimA* shows that the type V *fimA* gene shares the identical 5′ upstream (promoter region) and 3′ downstream regions of the ORF with the other types of *fimA* genes (data not shown). This promoter region is essential for the expression of FimA (34), and the gene expression is tightly regulated by environmental conditions (4, 33); these observations also support the view that it is a new type of *fimA* gene in *P. gingivalis*. In addition, the phylogenetic tree revealed that the genetic distances of the *fimA* gene among *P. gingivalis* strains were less than 0.5. Boyd et al. (9) reported that the *fimA* gene of *Salmonella enterica* was hypervariable among natural isolates and that the genetic distance between subspecies IV and VII was 0.87, still suggesting a common ancestor. In this context, the genetic distance of 0.5 strongly suggests that the *fimA* gene clusters of *P. gingivalis* strains were derived from a common ancestor, even though the branch lengths between the tested strains are different (Fig. 2).

TABLE 1. Distribution of *P. gingivalis* with type I to V *fimA* in 73 clinical samples from periodontitis patients

fimA type(s)	Frequency of occurrence (% of the total samples)
I	5.5
II	52.1
III	6.8
IV	9.6
V	2.7
I and II	6.8
I and V	1.4
II and IV	2.7
II and V	8.2
IV and V	4.1

Southern blot analysis revealed that the hybridization patterns resembled each other for all strains when probed with the type I *fimA* gene, while the hybridization intensity was weak with strain HG564 (type IV) and only faint bands were observed for strain HNA-99 (type V) (Fig. 3). Loos and Dyer (21) used probe 1 (855-bp fragment of the internal $fimA_{381}$ coding sequence) and probe 2 (2.5-kb fragment including the ORF as well as about 800 bp flanking each side of the ORF) for restriction fragment length polymorphism analyses. The results showed that probe 1 hybridized only rarely with the *fimA* gene of strains W50, W12, 9-14K-1, AJW-1, and HG564, while probe 2 hybridized with all samples. Our *fimA* probe included, in addition to the *fimA* sequence, a short-ended 200-bp flanking sequence, which could have been the cause for the weak hybridization with the samples of strains HG564 and HNA-99. On the other hand, other *P. gingivalis*-specific genes, *sod* and *kgp*, could hybridize with all the test samples with similar signal intensities (Fig. 3). These results suggest that the *fimA* diversity is most likely generated through mutation and genetic exchange within the ORF but not in the promoter region of the

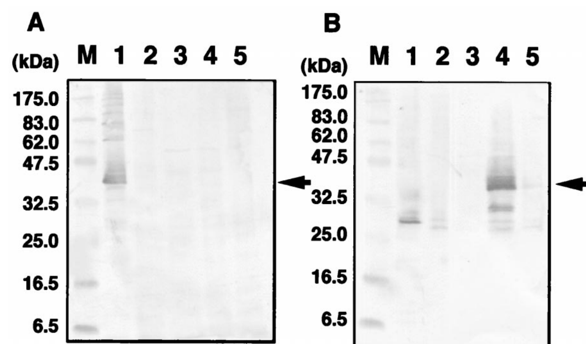

FIG. 4. Western blot analyses of the FimAs in five type-representative strains of *P. gingivalis*. Whole-cell lysates of *P. gingivalis* (2×10^7 cells) were separated by SDS–12% polyacrylamide gel electrophoresis. After electrophoresis, gels were transferred to polyvinylidene difluoride membranes and FimA was detected with antibodies to rFimA (381, type I *fimA* [A], and HG564, type IV *fimA* [B]). Lanes: M, prestained protein marker; 1, *P. gingivalis* ATCC 33277; 2, *P. gingivalis* HW24D1; 3, *P. gingivalis* 6/26; 4, *P. gingivalis* HG564; 5, *P. gingivalis* HNA-99. Arrows indicate FimA.

fimA gene. The process of antigenic variation through gene cassette recombination has been shown for the pathogenic *Neisseria* species (20); however, multiple *fimA* alleles within a single strain were not observed for *P. gingivalis* strains (Fig. 3). Furthermore, the phase variation of type 1 fimbriation in *E. coli* is controlled by the site-specific DNA inversion of recombinases (8), but the site-specific inversion was not found in phase variation of *fimA* gene expression (34). These observations also supported the concept that the genetic variation of *fimA* has occurred within the ORF in *P. gingivalis*; however, the mechanism of genetic exchange of the *fimA* gene is still unknown. Further studies are needed for analysis of the genetic diversity of the *fimA* gene of *P. gingivalis*.

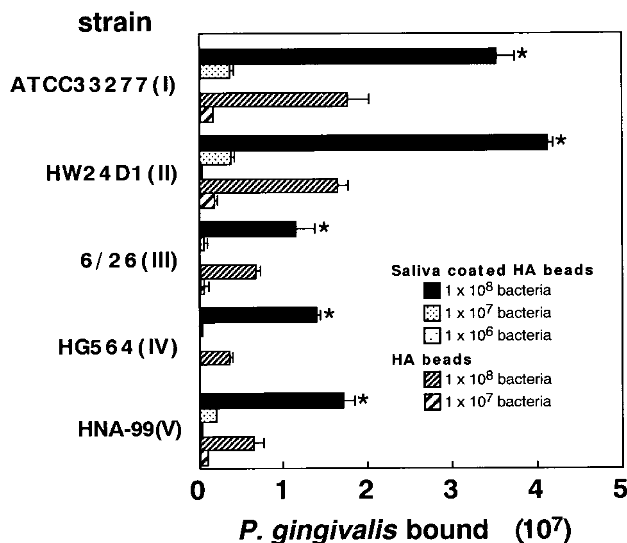

FIG. 5. Binding of *P. gingivalis* representing the five different *fimA* types to HA and sHA beads. Three milligrams of HA beads equilibrated with buffered KCl or clarified whole human saliva was added to a siliconized borosilicate tube and incubated with different numbers of the ^{3}H-labeled *P. gingivalis* (10^6 to 10^8) cells in a total volume of 300 μl with a gentle, oscillating motion for 1 h at room temperature. The mixture was layered on 100% Percoll to separate unbound cells from the bead-bound cells. After washing, radioactivity of the bead-bound cells was quantitated with a liquid scintillation counter. The results are shown as the mean values of triplicate samples from three individual experiments. One-way analysis of variance and the Tukey-Kramer test were used for the comparison of the binding abilities of *P. gingivalis* cells (*, $P < 0.0001$).

Western blot analysis using antisera against rFimA of strains 381 and HG564 indicated that the immunoreactivity of type V FimA was significantly different from those of other strains. It should be noted that a 41-kDa protein from strain HNA-99 was very faintly reactive with the anti-HG564 rFimA antibody. Our previous study revealed that the *fimA* gene of HG564 was considerably different from that of 381 and that the type IV rFimA did not clearly react with anti-rFimA (type I) antibody (14). These results indicate that the antigenic variation of fimbriae of *P. gingivalis* may depend on some specific epitope of FimA. It was reported that the immune response against FimA was enhanced by immunization with the synthetic peptide FP381(201-221) in guinea pigs (26) and that the synthetic peptide PgF-P8 could induce a protective immune response in a chamber infection model using mice (12). These antigenic epitopes are conserved among type I, II, and III *fimA* strains but not in the type IV and V organisms. The differences in immunoreactivity may influence the pathogenicity of *P. gingivalis* strains. In addition, serum antibody responses against type V FimA in periodontitis patients are not yet known. The purification of type V FimA and the antibody responses to type V FimA are now under investigation in our laboratory.

The activity of *P. gingivalis* HNA-99 binding to salivary proteins was almost 50% of that of *P. gingivalis* ATCC 33277 (Fig. 5). The site of active binding of *P. gingivalis* fimbriae to salivary proteins has been examined by using whole saliva-coated HA beads (3). The C-terminal region between amino acids 226 and 337 of FimA of strain 381 was essential for the binding of the organism to salivary components (20), and synthetic peptide FimA (amino acids 266 to 286) could inhibit the *P. gingivalis* whole-cell binding to whole saliva (23). These regions are conserved between type I and type II FimA, but only 10 amino acids are conserved in type IV and type V FimAs. The differences in amino acid compositions of the C-terminal region may reflect the ability of FimA to bind salivary components.

Taking all data into consideration, we conclude that the type V *fimA* is a new type of *P. gingivalis fimA* gene. However, the immunoreactivity of type V FimA is significantly different from those of other known types of FimA. Since the *fimA* genotyping assay enables us to differentiate disease-associated and nonassociated clones of *P. gingivalis*, further studies are needed to establish the association of type V *P. gingivalis* with periodontal diseases.

ACKNOWLEDGMENTS

We thank K. Kataoka and M. Kuboniwa for help in the collection of clinical samples. We also thank S. Morishima for his generous gift of rFimA-specific antibodies.

This work was supported by grant-in-aid C-10671933 from the Ministry of Education, Science and Culture of Japan.

REFERENCES

1. **Albandar, J. M., L. J. Brown, R. J. Genco, and H. Löe.** 1997. Clinical classification of periodontitis in adolescents and young adults. J. Periodontol. **68:**545–555.
2. **Albandar, J. M., L. J. Brown, and H. Löe.** 1997. Putative periodontal pathogens in subgingival plaque of young adults with and without early-onset periodontitis. J. Periodontol. **68:**973–981.
3. **Amano, A., H. T. Sojar, J. Y. Lee, A. Sharma, M. J. Levine, and R. J. Genco.** 1994. Salivary receptors for recombinant fimbrillin of *Porphyromonas gingivalis*. Infect. Immun. **62:**3372–3380.
4. **Amano, A., A. Sharma, H. T. Sojar, H. K. Kuramitsu, and R. J. Genco.** 1994. Effects of temperature stress on expression of fimbriae and superoxide dismutase by *Porphyromonas gingivalis*. Infect. Immun. **62:**4682–4685.
5. **Amano, A., T. Nakamura, S. Kimura, I. Morisaki, I. Nakagawa, S. Kawabata, and S. Hamada.** 1999. Molecular interactions of *Porphyromonas gingivalis* fimbriae with host proteins: kinetic analyses based on surface plasmon

resonance. Infect. Immun. **67:**2399–2405.
6. **Amano, A., I. Nakagawa, K. Kataoka, I. Morisaki, and S. Hamada.** 1999. Distribution of *Porphyromonas gingivalis* strains with *fimA* genotypes in periodontitis patients. J. Clin. Microbiol. **37:**1426–1430.
7. **Ausubel, F. M., R. Brent, R. E. Kingston, D. D. Moore, J. G. Seidman, J. A. Smith, and K. Struhl.** 1994. Current protocols in molecular biology, section 2. John Wiley & Sons, Inc., New York, N.Y.
8. **Blomfield, I. C., D. H. Kulasekara, and B. J. Eisenstein.** 1997. Integration host factor stimulates both FimB- and FimE-mediated site-specific DNA inversion that controls phase variation of type 1 fimbriae expression in *Escherichia coli*. Mol. Microbiol. **23:**705–717.
9. **Boyd, E. F., and D. L. Hartl.** 1999. Analysis of the type 1 pilin gene cluster *fim* in *Salmonella*: its distinct evolutionary histories in the 5′ and 3′ regions. J. Bacteriol. **181:**1301–1308.
10. **Califano, J. V., R. E. Schifferle, J. C. Gunsolley, A. M. Best, H. A. Schenkein, and J. G. Tew.** 1999. Antibody reactive with Porphyromonas gingivalis serotypes K1-6 in adult and generalized early-onset periodontitis. J. Periodontol. **70:**730–735.
11. **Christersson, L. A., C. L. Fransson, R. G. Dunford, and J. J. Zambon.** 1992. Subgingival distribution of periodontal pathogenic microorganisms in adult periodontitis. J. Periodontol. **63:**418–425.
12. **Deslauriers, M., S. Haque, and P. M. Flood.** 1996. Identification of murine protective epitopes on the *Porphyromonas gingivalis* fimbrillin molecule. Infect. Immun. **64:**434–440.
13. **Engels, E. R.** 1993. Contributing software to internet: the Amplify program. Trends Biochem. Sci. **18:**448–450.
14. **Fujiwara, T., S. Morishima, I. Takahashi, and S. Hamada.** 1993. Molecular cloning and sequencing of the fimbrilin gene of Porphyromonas gingivalis strains and characterization of recombinant proteins. Biochem. Biophys. Res. Commun. **197:**241–247.
15. **Genco, C. A., D. R. Kapczynski, C. W. Cutler, R. J. Arko, and R. R. Arnold.** 1992. Influence of immunization on *Porphyromonas gingivalis* colonization and invasion in the mouse chamber model. Infect. Immun. **60:**1447–1454.
16. **Grenier, D., and D. Mayrand.** 1987. Selected characteristics of pathogenic and nonpathogenic strains of *Bacteroides gingivalis*. J. Clin. Microbiol. **25:** 738–740.
17. **Hamada, S., A. Amano, S. Kimura, I. Nakagawa, S. Kawabata, and I. Morisaki.** 1998. The importance of fimbriae in the virulence and ecology of some oral bacteria. Oral Microbiol. Immunol. **13:**129–138.
18. **Holt, S. C., I. Kesavalu, S. Walker, and C. A. Genco.** 1999. Virulence factors of *Porphyromonas gingivalis*. Periodontol. 2000 **20:**168–238.
19. **Laine, M. L., B. J. Appelmelk, and A. J. van Winkelhoff.** 1997. Prevalence and distribution of six capsular serotypes of *Porphyromonas gingivalis* in periodontitis patients. J. Dent. Res. **76:**1840–1844.
20. **Lee, J. Y., H. T. Sojar, G. S. Bedi, and R. J. Genco.** 1991. *Porphyromonas* (*Bacteroides*) *gingivalis* fimbrillin: size, amino-terminal sequence, and antigenic heterogeneity. Infect. Immun. **59:**383–389.
21. **Loos, B. G., and D. W. Dyer.** 1991. Restriction fragment length polymorphism analysis of the fimbrillin locus, *fimA*, of *Porphyromonas gingivalis*. J. Dent. Res. **71:**1173–1181.
22. **Nagata, A., T. Man-yoshi, M. Sato, and R. Nakamura.** 1991. Serological studies of *Porphyromonas* (*Bacteroides*) *gingivalis* and correlation with enzyme activity. J. Periodontal Res. **26:**184–190.
23. **Nagata, H., A. Sharma, H. T. Sojar, A. Amano, M. J. Levine, R. J. Genco.** 1997. Role of the carboxyl-terminal region of *Porphyromonas gingivalis* fimbrillin in binding to salivary proteins. Infect. Immun. **65:**422–427.
24. **Nakamura, T., A. Amano, I. Nakagawa, and S. Hamada.** 1999. Specific interactions between *Porphyromonas gingivalis* fimbriae and human extracellular matrix proteins. FEMS Microbiol. Lett. **175:**267–272.
25. **Neiders, M. E., P. B. Chen, H. Suido, H. S. Reynolds, J. J. Zambon, M. Shlossman, and R. J. Genco.** 1989. Heterogeneity of virulence among strains of Bacteroides gingivalis. J. Periodontal Res. **24:**192–198.
26. **Ogawa, T.** 1994. The potential protective immune responses to synthetic peptides containing conserved epitopes of *Porphyromonas gingivalis* fimbrial protein. J. Med. Microbiol. **41:**349–358.
27. **Rumpf, R. W., A. L. Griffen, B. G. Wen, and E. J. Leys.** 1999. Sequencing of the ribosomal intergenic spacer region for strain identification of *Porphyromonas gingivalis*. J. Clin. Microbiol. **37:**2723–2725.
28. **Thompson, J. D., D. G. Higgins, and T. J. Gibson.** 1994. CLUSTAL W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice. Nucleic Acids Res. **22:**4673–4680.
29. **Tran, S. D., and J. D. Rudney.** 1996. Multiplex PCR using conserved and species-specific 16S rRNA gene primers for simultaneous detection of *Actinobacillus actinomycetemcomitans* and *Porphyromonas gingivalis*. J. Clin. Microbiol. **34:**2674–2678.
30. **Tran, S. D., and J. D. Rudney.** 1999. Improved multiplex PCR using conserved and species-specific 16S rRNA gene primers for simultaneous detection of *Actinobacillus actinomycetemcomitans*, *Bacteroides forsythus*, and *Porphyromonas gingivalis*. J. Clin. Microbiol. **37:**3504–3508.
31. **Van Steenbergen, T. J., F. G. Delemarre, F. Namavar, and J. De Graaff.** 1987. Differences in virulence within the species *Bacteroides gingivalis*. Antonie Leeuwenhoek **53:**233–244.
32. **Williams, R. C.** 1990. Periodontal disease. N. Engl. J. Med. **322:**373–382.
33. **Xie, H., S. Cai, and R. J. Lamont.** 1997. Environmental regulation of fimbrial gene expression in *Porphyromonas gingivalis*. Infect. Immun. **65:**2265–2271.
34. **Xie, H., and R. J. Lamont.** 1999. Promoter architecture of the *Porphyromonas gingivalis* fimbrillin gene. Infect. Immun. **67:**3227–3235.