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MOLECULAR CLONING AND SEQUENCING OF THE FIMBRILIN GENE OF *PORPHYROMONAS GINGIVALIS* STRAINS AND CHARACTERIZATION OF RECOMBINANT PROTEINS

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The *fimA* gene encoding the subunit protein of fimbriae, fimbrilin, from nine strains of *Porphyromonas gingivalis* was cloned using polymerase chain reaction and the nucleotide sequence was determined. Analysis of the nucleotide and the deduced amino acid sequences revealed that the *fimA* gene of the test strains was composed of 1044 to 1083 bp, and the molecular weight of the deduced polypeptides was calculated to be 37,527 to 38,239. All the test strains shared the same or similar sequences, but simultaneously considerable differences in the sequences were found among the strains. Western blot analysis using rabbit antiserum to *P. gingivalis* 381 fimbriae demonstrated that the recombinant fimbrilins of 8 out of 9 strains expressed in *Escherichia coli* reacted with the antiserum exhibiting a 43 to 48 kDa band. Taken together, these results indicate that four genetical clusters are noted among the *fimA* gene of *P. gingivalis* strains.

Porphyromonas gingivalis is a gram negative, strictly anaerobic, black-pigmented rod and is suspected of being a major pathogen in periodontal diseases. The organism possesses peritrichous fimbriae that may mediate cell adhesion to oral tissues, and thus are important in colonization. The fimbriae of *P. gingivalis* strain 381 were isolated and purified (1, 2), providing a useful tool for immunobiological studies (3). The purified fimbriae are composed of a 41 kDa subunit protein named fimbrilin. A gene encoding the fimbrilin of *P. gingivalis* strain 381 (designated *fimA*) was cloned and sequenced (4). Immunochemical studies have suggested that *P. gingivalis* may include two or three subgroups (5, 6). In this study, we cloned the fimbrilin gene from nine strains of *P. gingivalis* and compared the nucleotide and deduced amino acid sequences of the gene and properties of the recombinant (r-) fimbrilins.

MATERIALS AND METHODS

Microorganisms: *P. gingivalis* strains 381, ATCC33277, BH18/10, HW24D1, OMZ314, OMZ409, ATCC49417, 6/26 and HG564 were grown anaerobically in GAM broth (Nissui, Tokyo, Japan) supplemented with hemin and menadione. *P. gingivalis* fimbriae and rabbit antiserum to purified fimbriae were prepared as described previously (5). *Escherichia coli* strains JM109 and MV1184 were purchased from Takara (Kyoto, Japan) and were cultured in Luria-Bertani (LB) media and 2 x YT media, respectively (7). pTrc99 was purchased from Pharmacia LKB Biotechnology, and used as a cloning vector.

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DNA manipulations: Oligonucleotides, M1 (5'GCGCGAATTCGCGCAGCAAGGCCAGCCCGGAGCACAACAC3'), M2 (5'CGCGGAATTCGAGCGAACCCCGCTCCCTGTATTCCGATA3'), M4 (5'AATTGGATCCGCGCAGCAAGGCCAGCCCGG3') and M5 (5'AGAGGGATCCGAGCGAACCCCGCTCCCTGT3') for polymerase chain reaction (PCR) were designed according to the sequence of the *fimA* gene reported by Dickinson et al.(4). Underlined sequences at the 5' end are constructed as substrate for *Eco*R I (M1 and M2) or *Bam*H I (M4 and M5), respectively. Restriction enzymes and ligase were purchased from Toyobo (Osaka, Japan), New England Biolabs (Beverly, MA), or Takara. Treatment of DNA with these enzymes were done as recommended by the manufacturers. All other DNA manipulations were carried out using standard procedures (7).

Organisms of *P. gingivalis* were collected by centrifugation, suspended in 50 mM NaCl, 50 mM Tris-HCl (pH 7.4) and 50 mM EDTA, followed by addition of SDS up to a final concentration of 1.6% to lyse the cells. The lysate was treated with Proteinase K (Merck) and RNaseA (Boehringer Mannheim) at 37°C for 1 h. Thereafter, the DNA was treated with phenol and phenol:chloroform, and collected by ethanol precipitation.

PCR was performed for 30 cycles at 94°C for 1 min, 65°C for 2 min, and 72°C for 2.5 min after denaturation at 94°C for 1.5 min using AmpliTaq DNA polymerase (Takara) by a TSR-300 thermal sequencer (Iwaki Glass, Chiba, Japan). The PCR products were separated by electrophoresis using a 1.2% agarose gel and the amplified DNA was extracted from the gel using QIAEX (DIAGEN, Düsseldorf, FRG). The DNA was digested with *Eco*R I or *Bam*H I and ligated into *Eco*R I- or *Bam*H I- cleaved pTrc99, respectively. The ligation mixture was transformed into *E. coli* JM109 and streaked out onto LB-agar containing 100 μg/ml ampicillin. Recombinant plasmids harboring the fimbrilin gene were screened by colony hybridization with the PCR-amplified *fimA* gene of strain 381 as a probe.

The insert containing the fimbrilin gene was subcloned into pUC118 or 119 (Takara) and transformed into *E. coli* MV1184. Then a series of deletion mutants were generated by using a Kilo-Sequence Deletion Kit (Takara) and the single strand DNA was obtained after infection of helper phage M13KO7 (Takara). Nucleotide sequences were determined by the dideoxy chain termination method, using the Sequenase 2.0 sequencing kit (United States Biochemical Co., Cleveland, Ohio) with [^{35}S]dCTPαS (Amersham) as described previously (8).

SDS-PAGE and Western blot analyses: *E. coli* carrying recombinant plasmid was grown to middle log phase in LB-broth, to which 1 mM isopropyl-β-D-galactopyranoside (IPTG) was added, and further incubated for 3 h. Cells were then collected by centrifugation and suspended in SDS gel-loading buffer (7) and boiled for 5 min. The cellular proteins separated by SDS-PAGE were transferred onto Immobilon membrane (Millipore). The membrane was reacted with rabbit anti-381 fimbriae antibody and the antibody bound to protein band(s) was detected by solid-phase immunoassay using swine anti-rabbit immunoglobulins conjugated with alkaline phosphatase (Dakopatts, Glostrup, Denmark).

Nucleotide sequence accession numbers: The nucleotide sequences for the *fimA* genes of *P. gingivalis* 381, ATCC33277, BH18/10, HW24D1, OMZ314, OMZ409, ATCC49417, 6/26 and HG564 have been entered into DDBJ under accession numbers D17794, D17795, D17796, D17797, D17798, D17799, D17800, D17801 and D17802, respectively.

RESULTS AND DISCUSSION

Cloning of the *fimA* gene and expression of recombinant proteins

A 1.3 kb DNA fragment of nine strains of *P. gingivalis* was amplified by PCR using oligonucleotides M1 and M2 as primers. The fragment was then digested with *Eco*R I and electrophoresed. The amplified nucleotide fragments from all strains except strain HG564 had no *Eco*R I cleavage site in the middle of the fimbrilin gene. The *Eco*R I cleaved DNA fragments were cloned into pTrc99 and transformed into *E. coli* JM109. For cloning of the HG564 fimbrilin gene, we used the oligonucleotide primers, M4 and M5. After PCR using M4 and M5 as primers, the amplified nucleotide fragment of HG564 was digested with *Bam*H I and cloned into pTrc99, and transformed into *E. coli* JM109 as described above.

Recombinant fimbrilin (r-fimbrilin) of each strain was expressed by the induction of IPTG. Western blot analysis revealed that all r-fimbrilins except that of HG564 reacted with anti 381 fimbriae serum and their molecular weight was larger than the native 41 kDa fimbrilin of strain 381

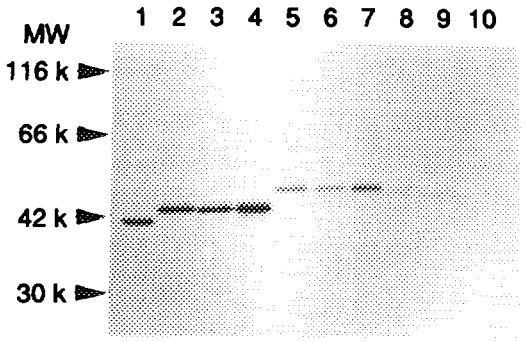

Fig. 1. Western blot analysis of recombinant fimbrilin expressed in *E. coli*. Samples are *P. gingivalis* 381 native fimbriae (lane 1) and *E. coli* extracts containing recombinant fimbrilin of 381, ATCC33277, BH18/10, HW24D1, OMZ314, OMZ409, ATCC49417, 6/26 and HG564 (lanes 2 to 10). After separation by SDS-PAGE, samples were electroblotted onto PVDF membrane and incubated with an antiserum to *P. gingivalis* 381 native fimbriae followed by alkaline phosphatase conjugated swine anti rabbit immunogloblins.

(Fig 1). The molecular mass of the r-fimbrilins of strains 381, ATCC33277 and BH18/10 was calculated to be 43 kDa and reacted strongly with antiserum to 381 fimbriae . In contrast, those of strains HW24D1, OMZ314 and OMZ409, ATCC49417 or 6/26 gave a major band with a molecular mass of 46 to 48 kDa. Of interest was that the r-fimbrilin of strain HG564 did not clearly react with the antiserum. However, the expression of the r-fimbrilin was confirmed by Western blotting using anti HG564 whole cell serum (results not shown).

Nucleotide sequencing analysis of fimbrilin genes

The primary nucleotide sequences of the cloned *fimA* genes from various *P. gingivalis* strains were determined (Fig 2). The sequence of the *fimA* gene of strain 381 was essentially same as that reported by Dickinson et al. (4) except one substituted base. However, when the amino acid sequence was deduced, it was in agreement with their sequence. Sequence of the *fimA* gene from strain ATCC33277 was identical to that of 381. The *fimA* gene of strain BH18/10 was essentially same as that of 381 except one base substitutions. On the other hand, sequences of the *fimA* gene of HW24D1, OMZ314 and OMZ409 were very similar, forming another cluster that is considerably different from those of the first three strains. Strains ATCC49417 and 6/26 were similar to strains HW24D1, OMZ314 and OMZ409 in terms of the sequences of their *fimA* gene; however, significant differences still existed. Comparison of the *fimA* nucleotide sequences between these strains revealed that the similarity of the *fimA* genes between HW24D1 and ATCC49417 was stronger than that between strains HW24D1 and 6/26. In Western blot analysis, r-fimbrilins of ATCC49417 and HW24D1 exhibited a band at an electrophoretically similar position, while the molecular mass of 6/26 r-fimbrilin was smaller than that of the other strains. The *fimA* gene of HG564 was different from that of the other eight strains. It was noted that the site of the initiation codon in the *fimA* nucleotide sequence was different only in this strain.

Furthermore, as shown in Fig 2, the upstream regions of the *fimA* genes from all strains are almost identical. In these regions, putative -35 (5'TTGTTG3') and -10 (5'TATGAC3') sequences were detected, with 3 and 4 out of 6 bases matching those of the consensus promoter in *E. coli*, respectively. The two sequences are separated by 17 bp, an optimal spacing for expression in vivo in *E. coli* (9). A possible Shine-Dalgarno sequence (5'AGAAGGT3') was found 15 bp upstream from the start codon (GTG) of the *fimA* gene of all strains except strain HG564, which is in agreement with the description of Dickinson et al. (4). In the *fimA* gene of HG564, the putative

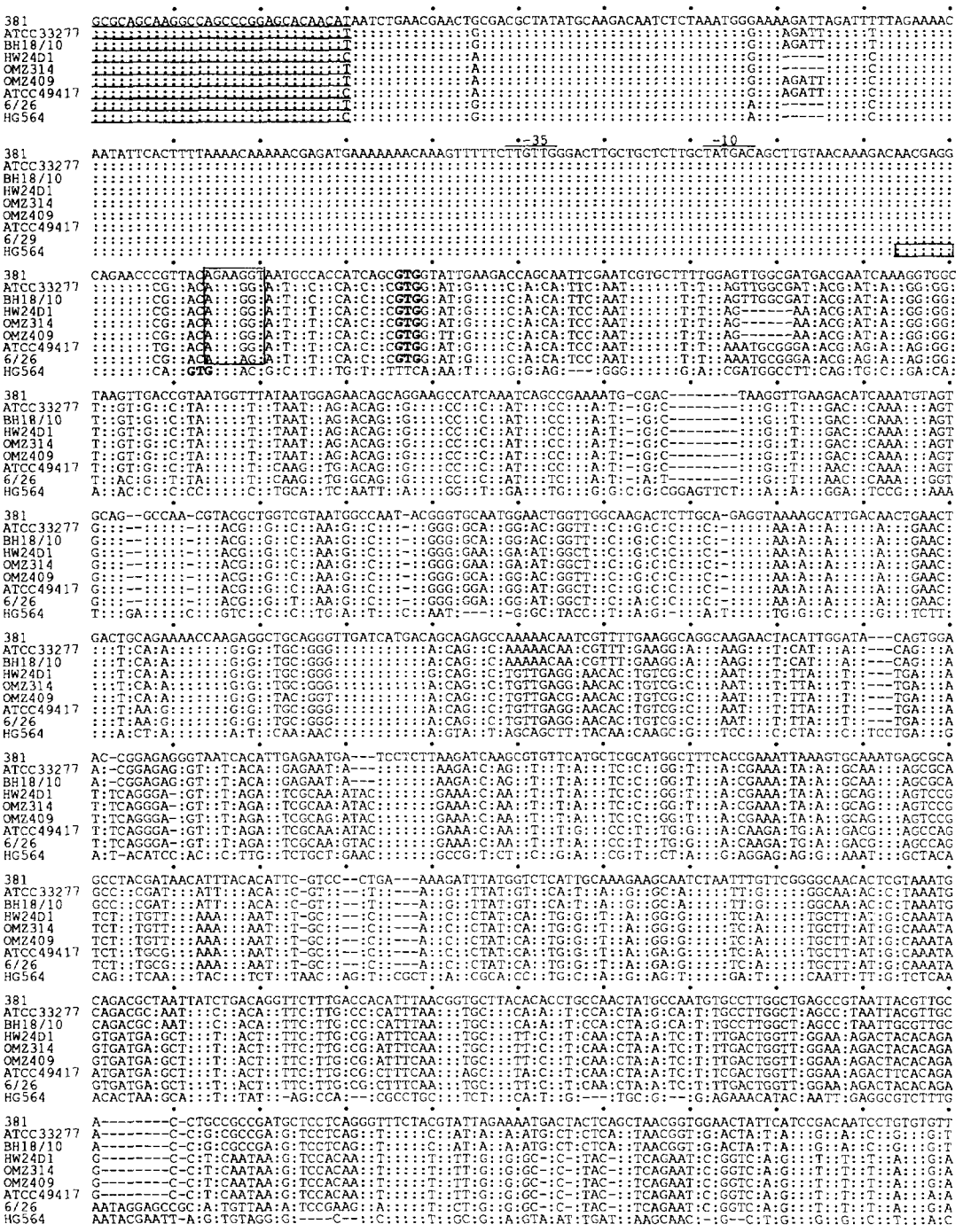

Fig. 2. Nucleotide sequence of fimbrilin genes from nine strains of *P. gingivalis*. The putative start and stop codons are in boldface. The sequcences used as primers for PCR are underlined. Nucleotide identities are indicated by colons. Spaces (-) have been added to one or more of the sequences in order to maintain the best alignment. Putative -35 and -10 sequences are overlined. The putative Shine-Dalgarno sequences are boxed. The position where the 381 *fimA* sequence is different from that of Dickinson et al. (4) (GenBank accession number M19405) is indicated in parentheses. Our sequcence data have been deposited in the DDBJ Data Library under accession numbers D17794 to D17802.

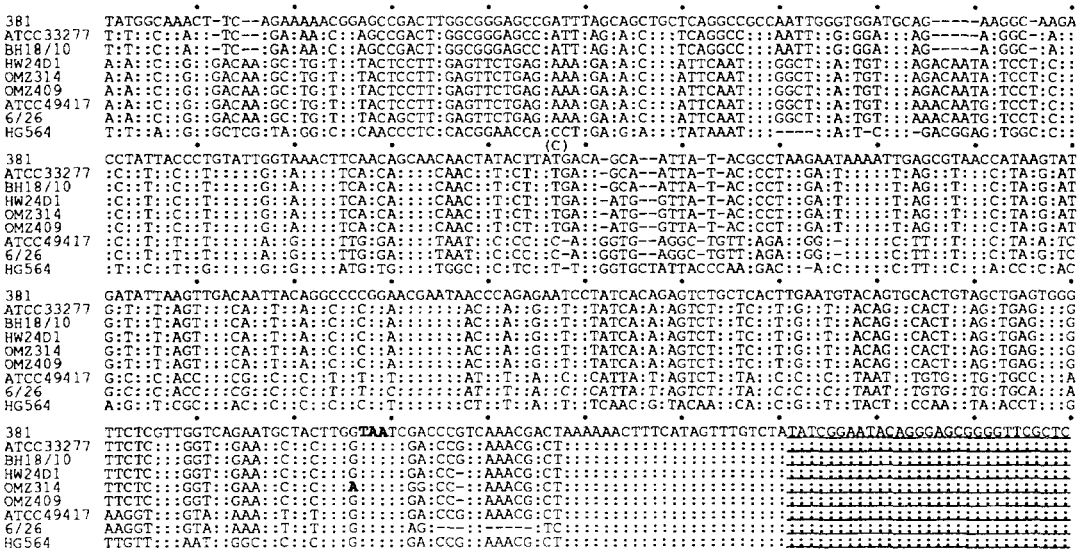

Fig. 2 - Continued

initiation codon (GTG) was mapped to 24 bp upstream of the other *fimA* genes, which may satisfy the necessary condition as a start codon proposed by Stormo et al. (10). The start site is preceded by a putative Shine-Dalgarno sequence (5'AACGAGG3') at a distance of 11 bp. This sequence is very complementary (6 out of 7 bases) to the sequence 3'UUCCUCC5' found at the 3' end of *Bacteroides fragilis* (11) and *Bacteroides nodosus* (12) 16S rRNA. Therefore, the spacings between the start codon and the Shine-Dalgarno sequence in the *fimA* gene of *P. gingivalis* are longer than 9 bp, while the spacings have been reported to be 5 to 9 bp in prokaryotes (13). Similarly, it has been reported that other genes of *P. gingivalis* exhibit longer spacing between these two sequences, e.g. the genes for methylase (14) and collagenase (15). These results may indicate that the longer spacings are a characteristic feature of *P. gingivalis* genes.

Sequences of fimbrilin proteins

The deduced amino acid sequences and calculated molecular mass of the r-fimbrilins of the nine strains are presented in Fig. 3. Assignments of the amino-termini of mature fimbrilins were based on the published protein sequences of *P. gingivalis* fimbrilins (6).

Hydrophilicity plotting of the amino acid sequences was performed to predict possible antigenic segments as described previously (16). In strains 381 and HW24D1, the putative antigenic oligopeptide sequences were very similar, while those of strains 6/26 and HG564 exhibited different antigenic regions. Faint immunochemical reactivity of the HG564 r-fimbrilin against the anti-381 fimbriae serum by Western blot analysis (Fig. 1) may be a reflection of the discrepancies of these amino acid sequences. Ogawa et al. (17) reported that some synthetic oligopeptide segments of strain 381 fimbrilin inhibited ELISA reactions between native fimbrial antigen and rabbit antibody to the fimbriae. The immunodominant peptides, however, did not coincide with the putative antigenic sites obtained by the Hopp and Woods formula (16). During the polymerization of the fimbrilin to generate fimbriae, the putative antigenic epitopes may be folded within, thus unable to exhibit inherent antigenicity. Such antigenic differences between the fimbriae and the fimbrilins were described by others (2). It should be noted that the 381 synthetic peptide segments exhibiting immunobiological activities correspond to the well conserved amino acid sequences (e.g. peptides 71-90, 91-111, and 312-331).

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                .         .         .         .         .         .         .         .         .         .
381     M-------VLKTSNSNRAFGVGDDESKVAKLTVMVYNGEQQEAIKSAENAT---KVEDIKC-SAGQRTLVVMANTGAMELVGKTLAEVKALTTELTAENQ
ATCC33277 M-------VLKT:NSN:F:VGDDES:VA:::V:::N:EQ::A::SA:N:T---:::D:K:-SAGQ:T:::M::TGAME:V::T:A::K:::E::AE::
BH18/10 M-------VLKT:NSN:F:VGDDES:VA:::V:::N:EQ::A::SA:N:T---:::D:K:-SAGQ:T:::M::TGAME:V::T:A::K:::E::AE::
HW24D1  M-------VLKT:NPN:F:E--DES:VA:::V:::N:EQ::A::SA:N:T---:::D:K:-SAGQ:T:::M::TGEMK:A::T:A::K:::E::AE::
OMZ314  M-------VLKT:NPN:F:E--DES:VA:::V:::N:EQ::A::SA:N:T---:::D:K:-SAGQ:T:::M::TGEMK:A::T:A::K:::E::AE::
OMZ409  M-------VLKT:NPN:F:E--DES:VA:::V:::N:EQ::A::SA:N:T---:::D:K:-SAGQ:T:::M::TGAME:V::T:A::K:::E::AE::
ATCC49417 M-------VLKT:NPN:F:NAGDEA:VA:::V:::K:EQ::A::SA:N:T---:::N:K:-SAGQ:T:::M::TGGME:A::T:A::K:::E::EG::
6/26    M-------VLKT:NPN:F:NAGDEA:VA:::V:::K:EQ::A::SV:N:I---:::N:K:-GAGQ:T:::M::TGGME:A::T:A::K:::E::EG::
HG564   METDATVSFIIK:GEG:V:DGLADA:IT:::A:::A:QI::G::TV:E:DGVL:::G:P:KSGAN:V:::V::H-NYE:T::S:N::E::::S::AE::
                .         .         .         .         .         .         .         .         .         .
381     EAAGLIMTAEPKTIVLKAGKNYIGY-SGTGEGNHIEND-PLKIKRVHARMAFTEIKVQMSAAYDNIYTFVP--EKIYGLIAKKQSNLFGATLVNADANYL
ATCC33277 E:AG::::AEPKTIVLKA:K:YI::-S:TGEG:HIEND-::KIK::H:RMA:TEIK:Q:SAA:D:I:T:V:--EK:YG:I:::Q:NL::AT:VNADAN::
BH18/10 E:AG::::AEPKTIVLKA:K:YI::-S:TGEG:HIEND-::KIK::H:RMA:TEIK:Q:SAA:D:I:T:V:--EK:YG:I:::Q:NL::AT:VNADAN::
HW24D1  E:AG::::AEPVEVTLVA:N:YY::-D:SQGG:QISQDT::EIK::H:RMA:TEIK:Q:SPS:V:K:N:A:--EN:YA:V:::E:NL::AS:ANSDDA::
OMZ314  E:AG::::AEPVEVTLVA:N:YY::-D:SQGG:QISQDT::EIK::H:RMA:TEIK:Q:SPS:V:K:N:A:--EN:YA:V:::E:NL::AS:ANSDDA::
OMZ409  E:TG::::AEPVDVTLVA:N:YY::-D:SQGG:QISQDT::EIK::H:RMA:TEIK:Q:SPS:V:K:N:A:--EN:YA:V:::E:NL::AS:ANSDDA::
ATCC49417 E:AG::::AEPVEVTLVA:N:YY::-D:SQGG:QISQDT::EIK::R:RIA:TKIE:T:SQS:A:K:N:A:--EN:YA:V:::K:NL::AS:ANNDDA::
6/26    E:AG::::AEPVEVTLVA:N:YY::-D:SQGG:QISQGT::EIK::H:RIA:TKIE:T:SQS:A:K:N:A:--EN:YA:V:::K:NL::AS:ANSDDA::
HG564   N:KN::::GKSAAFTIKP:S:HY::PD:TTSD:LVSAGT::AVT::H:GIS:AGVE:N:ATQ:Q:Y:S:N:ADAK:AA:V:::D:KI::NS:VSNTNA::
                .         .         .         .         .         .         .         .         .         .
381     TGSLTTFNGAYTPANYANVPWLSRNYVA---PAADAPQGFYVLENDYSANGGTIHPTILCVYGKLQKN-GADLAGADLAAAQAANWV--DAEGKTYYPVL
ATCC33277 T:SL:TFN:A:T:ANYANVPW:SRNYVA---PAADAPQ::::::ND:SANGGTIH::::VY:::QKN-:AD:AGADLAA:QA:NWV--DAEGK::::::
BH18/10 T:SL:TFN:A:T:ANYANVPW:SRNCVA---PAADAPQ::::::ND:SANGGTIH::::VY:::QKN-:AD:AGADLAA:QA:NWV--DAEGK::::::
HW24D1  T:SL:NFN:A:S:ANYTHVDW:GRDYTE---PSNNAPQ::::::ST:AQNAG-LR::::VK:::TKHD:TP:SSEEMTA:FN:GWIVADNNPT::::::
OMZ314  T:SL:NFN:A:S:ANYTHVDW:GRDYTE---PSNNAPQ::::::ST:AQNAG-LR::::VK:::TKHD:TP:SSEEMTA:FN:GWIVADNNPT::::::
OMZ409  T:SL:NFN:A:S:ANYTHVDW:GRDYTE---PSNNAPQ::::::ST:AQNAG-LR::::VK:::TKHD:TP:SSEEMTA:FN:GWIVADNNPT::::::
ATCC49417 T:SL:TFN:A:T:ANYTHVDW:GRDFTE---PSNNAPQ::::::ST:AQNAG-LR::::IK:::TKHD:TP:SSEEMTA:FN:GWIVANNDPT::::::
6/26    T:SL:TFN:A:S:ANYTHVDW:GRDYTEIGAATVNTPK::::::ST:AQNAG-LR::::VK:::TKHD:TA:SSEEMTA:FN:GWIVANNDPT::::::
HG564   Y:VQ:P-A:L:T:DAAGETYE:EASLN----TNYAVGA::::::SK:DASNE-LR::::IY:::LDKD:NP:TEPALTD:IN:GFC--DGDGT::::::
                .         .         .         .         .         .         .         .         .         .
381     VNFNSNNYTYDSNYTPKN-KIERNHKYDIKLTITGPGTNNPENPITESAHLNVQCTVAEWVLVGQNATW    (347, 37587)
ATCC33277 ::FNS:N:T:DSNYTPKN-::E::HKYD:K:T:::::::N::::ITES:H:::Q:T:AE:VL:G:NATW    (347, 37587)
BH18/10 ::FNS:N:T:DSNYTPKN-::E::HKYD:K:T:::::::N::::ITES:H:::Q:T:AE:VL:G:NATW    (347, 37527)
HW24D1  ::FNS:N:T:DNGYTPKN-::E::HKYD:K:T:::::::N::::ITES:H:::Q:T:AE:VL:G:NATW    (348, 38089)
OMZ314  ::FNS:N:T:DNGYTPKN-::E::HKYD:K:T:::::::N::::ITES:H:::Q:T:AE:VL:G:NAT-    (347, 37903)
OMZ409  ::FNS:N:T:DNGYTPKN-::E::HKYD:K:T:::::::N::::ITES:H:::Q:T:AE:VL:G:NATW    (348, 38076)
ATCC49417 ::FES:N:T:TGEAVEKG-::V::HKFD:N:T:::::::N::::ITES:N:::N:V:AA:KG:V:NVIW    (350, 37911)
6/26    ::FES:N:T:TGEAVEKG-::V::HKFD:N:T:::::::N::::ITES:N:::N:V:AA:KG:V:NVIW    (353, 38023)
HG564   ::YDG:G:I:SGAITQGQN::V::NHYK:T:N:::::::T::::QPVQ:N:::T:Q:TP:VV:N:AATW    (360, 38239)
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Fig.3. Deduced amino acid sequences for the fimbrilin encoded by *fimA* genes of various *P. gingivalis* strains. Amino acid identities are shown by colon. Spaces (-) have been added to one or more of the sequences in order to maintain the best alignment. The putative signal peptides are underlined. The number of composed amino acid and molecular weight of the fimbrilin of each strain are given in parentheses.

Lee et al. (18) have classified *P. gingivalis* fimbriae into four types on the basis of the sequences of the first 20 amino-terminal amino acids. Comparison with the complete deduced amino acid sequences obtained here has indicated that both sequences are basically in agreement with each other. The sequences of strains 381, ATCC33277 and BH18/10, strains HW24D1, OMZ314 and OMZ409, strains ATCC49417 and 6/26, and strain HG564 can be separated into type I, II, III, and IV, respectively (6). The secondary structure prediction by the Chou-Fasman method (19) and the phylogenetic tree constructed by the neighbor-joining method (20) supported this classification (results not shown). On the other hand, our previous studies on the antigenicity of lipopolysaccharide (LPS) of *P. gingivalis* strains indicated that the organisms could be classified into at least two LPS serogroups (21). Very recently, Loos and Dyer (22) examined restriction fragment length polymorphisms (RFLPs) of the *fimA* gene of various *P. gingivalis* strains and divided 39 strains into 9 RFLP groups. In that study, it was found that the *fimA* locus of strain HG564 diverged from that of strain 381, confirming our present results.

In summary, the present study clearly indicates that the *fimA* genes of *P. gingivalis* retained conserved regions as well as individually variable nucleotide sequences among strains belonging to this species. The relationship between amino acid sequences encoded by specific loci of the *fimA* gene and specific immunobiological and/or pathological traits remains to be elucidated.

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