Identification and characterization of a hemagglutinin gene from *Prevotella intermedia*

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In this study, we describe the cloning of a gene encoding hemagglutinin from *Prevotella intermedia* to characterize hemagglutinin function and elucidate its possible role in the infectious process mediated by the organism. We screened genomic libraries created in *Escherichia coli* JM109, using antibodies against *P. intermedia*, and the positive clones were subsequently tested for their hemagglutinating activity, using rabbit red blood cells. We isolated a clone of strain 0543 in which, a 3.6-kb *P. intermedia* chromosomal DNA fragment was inserted into the pUC18 plasmid. Our deletion analysis-based subcloning procedures identified a hemagglutinin gene—designated *phg*. The open-reading frame of *phg* encoded a 35-kDa protein, and the expression of a recombinant protein of the predicted size was observed in a fusion protein expression system. While the DNA analysis of *phg* revealed no homology to known DNA sequences, the putative promoter, ribosome binding, and transcriptional termination sequence were found in the flanking DNA regions. In the N-terminus of the deduced amino acid sequence, we found a consensus leader peptide sequence for secretory proteins. These findings indicate that hemagglutinin might be expressed on the surface of *P. intermedia* to mediate its function.

Key Words: Hemagglutination; Periodontitis; *Prevotella*; Virulence

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Introduction

Prevotella intermedia is a gram-negative black-pigmented anaerobe, which has been implicated in many types of human periodontal diseases, due in part to its frequent recovery from lesions [1-4] and possession of putative virulence factors [5]. Many virulence factors associated with the pathogenic characteristics of *P. intermedia* have been identified, some of which may be involved in bacterial coaggre-

gation [4,6-8], the stimulation of host immune T-cells [9], or damage to host tissues [10-12]. Among these virulence factors, hemagglutinin has been suggested as a possible adhesin, mediating the attachment of oral microorganisms to host tissues in the oral cavity [13-15]. Previous results in our laboratory showed that *P. intermedia* mediated hemagglutination and that this activity may be associated with fimbriae [16,17].

However, the exact mechanism of how *P. intermedia*

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mediates hemagglutination has not been determined since the bacterial components responsible for hemagglutinating activity have not yet been isolated and characterized. In the present study, the molecular cloning of a hemagglutinin gene of *P. intermedia* was pursued to elucidate the role of hemagglutinin in the infectious process, which may lead to a better understanding of the pathogenesis of periodontal disease mediated by *P. intermedia*.

Materials and Methods

Bacterial strains and growth conditions

P. intermedia was maintained on blood agar plates consisting of 3% Trypticase soy broth (Becton Dickinson Microbiology Systems, Cockeysville, MD, USA), 0.5% yeast extract (Difco Laboratories, Detroit, MI, USA), 5% sheep blood, 5 µg/mL hemin, and 1 µg/mL menadione. For growth in liquid media, *P. intermedia* was cultured in Todd-Hewitt broth (Becton Dickinson) supplemented with 5 µg/mL hemin and 1 µg/mL menadione. The cells were grown and maintained at 37°C in an anaerobic chamber containing an atmosphere of 85% N₂, 10% H₂, and 5% CO₂. *E. coli* strains were grown aerobically at 37°C on Luria-Bertani (LB) medium consisting of 1% Bacto-tryptone (Difco), 1% NaCl, and 0.5% yeast extract. For solid medium, 1.5% agar was added

to LB broth. When indicated, 50 µg/mL ampicillin was added to the media to select recombinant clones of the *E. coli* strains. The bacterial strains used in the present study are shown in Table 1.

Plasmids and recombinant DNA techniques

The plasmid vectors used in the present study are shown in Table 1. All of the restriction enzymes and modifying enzymes used in this study were purchased from Promega Corporation (Madison, WI, USA) unless otherwise indicated, and used according to the manufacturer's directions. Ligations were performed at 16°C using T4 DNA ligase. Ligation mixtures were used to transform *E. coli* JM109 to acquire ampicillin resistance. After overnight incubation, the selected clones were screened by crack preparations, and clones showing plasmids of the appropriate size were further analyzed by restriction mapping of the plasmid DNA isolated using the Wizard Miniprep (Promega) Kit. Chromosomal DNA was isolated using the Wizard Genomic DNA Purification Kit (Promega). Chromosomal and plasmid DNA were separated on 0.8% agarose gels by electrophoresis according to standard procedures. DNA fragments were recovered from agarose gels using the Wizard DNA Clean-up System (Promega) according to the manufacturer's directions.

Table 1. Bacterial strains and plasmids

Strains/Plasmid	Characteristics	Reference
Strains		
17	Clinical isolate of <i>P. intermedia</i>	6, 7
JM109	[F' <i>traD36 proAB lacI^qZΔM15</i>] D(<i>lac-proAB</i>) <i>e14-(mcrA) endA1 recA1 gyrA96 thi-1 hsdRI7 (rk⁻mk⁺) supE44 relA1</i>	Promega
0543	Recombinant clone containing the 3.6-kb <i>Bam</i> HI DNA insert in pUC18	This study
0543-1	Recombinant clone containing the 1.1-kb <i>Bam</i> HI- <i>Bgl</i> II DNA fragment in pUC18	This study
0543-2	Recombinant clone containing the 2.5-kb <i>Bam</i> HI- <i>Bgl</i> II DNA fragment in pUC18	This study
0543-3	Recombinant clone containing the 1.2-kb <i>Ssp</i> I DNA fragment in pUC18	This study
0543-4	Recombinant clone containing the 2.0-kb <i>Eco</i> RV- <i>Bam</i> HI and 0.2-kb <i>Eco</i> RV- <i>Bam</i> HI fragment in pUC18	This study
PH001	Recombinant clone containing the 930-bp ORF of <i>phg</i> in pMAL-c2	This study
Plasmids		
pUC18	<i>lacI^q</i> , <i>lacZa</i> , Amp ^r , 2.7 kb	Pharmacia
pPI0543	The 3.6-kb <i>P. intermedia</i> DNA insert, Amp ^r , 6.3 kb	This study
pMAL-c2	<i>malE</i> , <i>lacI^q</i> , Amp ^r , 6.4 kb	New England Biolab

Construction of genomic library, subcloning, and sequencing

The genomic library of *P. intermedia* 17 was constructed as follows. The 1–5 kb chromosomal DNA fragments partially digested with *Sau*3AI or *Hind*III were ligated into pUC18, and the resultant recombinant plasmids were transformed into JM109. About 3,500 recombinant clones were screened with the antibody produced against whole *P. intermedia* 17 cells. Some of the positive clones were subsequently screened with the antibody produced against *P. intermedia* fimbriae [16]. The resulting positive clones were tested for hemagglutinating activity. The hemagglutinating clones were selected and subcloning procedures were performed by deletion analysis.

Sequencing was performed for both DNA strands at the University of Florida DNA Sequence Core facility using *Taq* Dye Primer and *Taq* Dye Terminator cycle sequencing protocols with fluorescent primers and dideoxynucleotides, respectively.

Polymerase chain reaction

The 930-bp open reading frame (ORF) of the hemagglutinin gene was amplified using polymerase chain reaction (PCR). Primers 5'-GGGGAAGCTTTTATGGCAATACTACTACTCT (KAL 33), and 5'-AAGCGGTACATACTCATTATA (KAL 34) were used as the reverse and forward primers, respectively. The DNA template used for PCR was recombinant plasmid pPI0543 containing the whole 3.6 kb insert in pUC18. A *Hind*III restriction site (underlined above) was engineered into the primer KAL 33 to facilitate the insertion of the PCR product into the *Hind*III cloning site of the expression vector pMAL-c2. Terminal GGGG bases were added to the primer KAL33 to promote cleavage at the *Hind*III restriction sites. Inverse PCR procedures was performed as described previously to amplify the flanking regions to obtain the entire genetic sequence [18].

Hemagglutination assay

The hemagglutination assay was performed using procedures described previously [16]. Briefly, rabbit red blood

cells were washed with phosphate-buffered saline (PBS, pH 7.2) and resuspended in the same buffer to make a 2% (v/v) suspension. The bacterial suspensions were washed three times in PBS and adjusted to an OD₅₅₀ of 2.0. Two-fold serial dilutions of the bacterial suspensions (50 µL) were made in PBS, and 50 µL of the washed erythrocyte suspension was added to microtiter plate wells. The mixture was shaken for 30 minutes on a vibrator and stored overnight at 4°C before visual inspection.

Expression of the recombinant protein

To study the expression of the recombinant protein and further purify a recombinant hemagglutinin, a Protein Fusion & Purification System (New England Biolab, Beverly, MA, USA) was used. The 930-bp ORF of the hemagglutinin gene was amplified by PCR as described above. The amplified product was treated with Klenow enzyme for blunt-ending and then digested with *Hind*III. This DNA fragment was then inserted into the pMAL-c2 vector, which was cut with *Xmn*I and *Hind*III. The resultant recombinant plasmid was transformed into competent JM109 cells, and the recombinant clone was selected by restriction mapping. One of these clones, which contained the correct recombinant plasmid size, was identified, designated as PH001, and used for further studies. Strain PH001 was grown in LB broth containing ampicillin (100 µg/mL) to an OD₆₀₀ of 0.3, and Isopropyl β-d-1-thiogalactopyranoside (IPTG) was then added to the culture to a final concentration of 0.3 mM. The culture was allowed to grow for another two to three hours. For further isolation of the fusion protein, PH001 cells were harvested and centrifuged. The pellets were resuspended in column buffer (20 mM Tris-HCl, 200 mM NaCl, and 1 mM EDTA), and kept at -20°C overnight. The pellets were then thawed and subjected to sonication for preparation of the lysate. The lysate was further processed by centrifugation to obtain crude cell extract for isolation of the fusion protein. The remaining pellet was resuspended in column buffer for analysis by gel electrophoresis. To isolate the fusion protein, the crude extract was mixed with amylose resin and incubated for 15 minutes on ice. After the mixture supernatant was removed by centrifugation, the remaining resin was washed in column buffer and

resuspended in sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) sample buffer for analysis. The proteins were separated on 12% separating gel and visualized by staining with Coomassie blue. This sample represented the fusion protein bound to amylose.

Gene accession number

The gene sequence reported in this study was submitted to GenBank with an accession number AF017417.

Results

Restriction mapping and analysis of subclones

Recombinant clones positively reacting to the anti-*P. intermedia* whole-cell antibody were subsequently screened with an antibody prepared against the partially-purified fimbrial preparation of *P. intermedia*. The resultant positive clones were tested for hemagglutination, of which six clones were found to exhibit hemagglutinating activity. The restriction mapping data suggested that these clones contained 0.8–3.7 kb chromosomal DNA inserts in pUC18 (data not shown). One clone, designated 0543, which contained

a 3.6-kb DNA fragment, was selected for further study. To verify the origin of the DNA insert, Southern blot analysis was performed using a digoxigenin-labeled 3.6-kb DNA fragment as a probe. The results confirmed that the 3.6-kb insert originated from the *P. intermedia* chromosome (data not shown). The 3.6-kb DNA insert was found to possess two distinct ORFs. Subcloning procedures using deletion analysis identified a 930-bp ORF, which conferred hemagglutinating activity to the *E. coli* strain, as shown in Fig. 1. The hemagglutinating activity of the recombinant clones compared with those of *P. intermedia* and the control *E. coli* strain is shown in Fig. 2. Subclone 0543-1 containing a 1.1-kb *Bam*HI-*Bgl*II DNA fragment of the 942-bp ORF was unable to agglutinate rabbit red blood cells. However, subclones 0543-2 and 0543-3, both of which contained a 930-bp ORF, possessed hemagglutinating activity. In contrast, subclone 0543-4, which had an incomplete 930-bp ORF, did not possess hemagglutinating activity.

Sequence analysis of *phg*

The nucleotide sequence analysis of the cloned hemagglutinin gene did not reveal any significant homology to

Fig. 1. Subcloning of the 3.6-kb insert of strain 0543 and the hemagglutinating activity of the subclones. Strain 0543 contains the whole 3.6-kb *Bam*HI insert, 0543-1 contains the 1.1-kb *Bam*HI-*Bgl*II DNA fragment, 0543-2 contains the 2.5-kb *Bam*HI-*Bgl*II DNA fragment, 0543-3 contains the 1.2-kb *Ssp*I DNA fragment, and 0543-4 contains the 2.0-kb *Eco*RV-*Bam*HI and the 0.2-kb *Eco*RV-*Bam*HI fragment in pUC18. The enzyme restriction sites are shown: B, *Bam*HI; E, *Eco*RV; Bg, *Bgl*II; and S, *Ssp*I. The 930-bp open reading frame of the *phg* gene, ORF1, is shown as . The open reading frame homologous to the *hbp* gene, ORF2, is shown as .

Fig. 2. Hemagglutination by the recombinant clones and *P. intermedia*. (A) *P. intermedia* 17, (B) JM109 containing pUC18, (C) Strain 0543-4, (D) Strain 0543, (E) Strain 0543-2, and (F) Strain 0543-3. All bacterial suspensions were washed three times in phosphate-buffered saline and adjusted to an OD₅₅₀ of 2.0. The bacterial suspensions were two-fold serially diluted to test hemagglutinating activity with rabbit red blood cells. Strong agglutinations appeared as clumps of erythrocytes distributed in the wells. When not agglutinated, the erythrocytes settled to the bottom of the wells and formed solid buttons.

known gene sequences. The mol% GC content of the entire 3.6-kb insert was 44.9%. The %GC of *phg* was 45.6%, which was consistent with the known *P. intermedia* chromosomal mol% GC content. Along with the ORF, a putative Pribnow Box (-10 TATA), a ribosomal binding site, and a potential stem-loop structure downstream of the stop codon were found. The consensus signal sequence (KRYILIIIVALSIGSAIAG) is also identified in the amino-terminal region of the deduced amino acid sequence (Fig. 3). The *phg* sequences, with around 97% homology, are consistently found in other clinical isolates of *P. intermedia*, including strains 17-2, KCOM 1944, KCOM 1933, KCOM 2836, KCOM 2734, KCOM 2837, OMA14, and 25611, as determined by the BLAST program (<https://blast.ncbi.nlm.nih.gov/Blast.cgi>). The derived amino acid sequence was 310 residues in length with a size of 35,740 daltons. The

protein was found to be basic and hydrophilic with an estimated isoelectric point of 9.5. A consensus signal peptide for secretory protein was found in the amino-terminal region, and the estimated cleavage site was ideally consistent with the rules of von Heijne (-3 and -1) for gram-negative bacteria [19].

Expression of recombinant hemagglutinin

To study the expression of the recombinant protein, a Protein Fusion & Purification System (New England Biolab, Beverly, MA, USA) was used. The recombinant hemagglutinin was expressed and detected by the fusion protein expression system. The ORF of *phg* was placed under the control of the *tac* promoter and translational signal from the vector pMAL-c2 to produce a fusion protein consisting of maltose-binding protein and recombinant hemagglutinin. The recombinant *E. coli* clone expressing the fusion protein, PH001, was selected and grown as described in the Materials and Methods section. As shown in Fig. 4, the recombinant hemagglutinin was produced as a fusion protein with a maltose-binding protein, resulting in a ca. 77-kD

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1  ATTTACAGCTTTATGCTCTTTCTTTCCTGCTACGAGCAAGTGTCTGCAATAATTTCTGCCACTCCCTTTTCATCTTT  80
81  TTTAAACCTTTGCTTGGTCTTCGGCATAATACCTTATATTTGCAATTCATTCATCTCTAACAAAGTAT  ATG  156
1  157  AAG  CCG  TAC  ATA  CTC  ATT  ATA  GTA  GCA  CTT  GCA  AGT  ATT  GGT  TCG  GCA  ATA  GCT  GGC  216
2  K  R  Y  F  L  I  Y  I  V  A  L  A  S  I  G  S  A  I  A  21
217  CCC  AAA  ATA  CGT  TGG  AAC  CAA  GCC  TAC  CAA  AAC  TAC  TTC  AAC  AAA  TAC  AAG  AAT  ATC  GCA  276
22  P  K  I  R  W  N  Q  A  Y  Q  N  Y  F  N  K  Y  K  N  I  A  41
277  ATC  AGG  GAG  ATG  CAA  CGC  TAC  GGC  ATA  CCC  GCC  AGC  ATT  ACA  CTC  GCA  CAG  GGC  GTT  TTG  336
42  I  R  E  M  Q  R  Y  G  I  P  A  S  I  T  L  A  Q  G  V  L  61
337  GAA  AGC  GGA  GCA  GGC  AAC  AGC  CGC  TTG  GCT  ACC  GTG  GCG  AAC  AAC  CAC  TTC  GGC  ATT  AAG  396
62  E  S  G  A  G  N  S  R  L  A  T  V  A  N  N  H  F  G  I  K  81
397  TGC  CAC  GAT  TGG  ACA  GGC  CCC  TCT  ATC  TCG  CAC  GAC  GAC  GAG  TTT  GGC  GAA  TGT  TTC  456
82  C  H  D  W  T  G  P  S  I  S  H  D  D  D  E  L  G  E  C  F  101
457  CGC  GTT  TAC  AAC  AAT  GCG  GTG  GAA  AGC  TAC  GAA  GAC  CAC  TCT  AAA  TTC  TTG  AGA  GCA  CGC  516
102  R  V  Y  N  N  A  V  E  S  Y  E  D  H  S  K  F  L  R  G  R  121
517  AAG  CGA  TAC  AGC  AAT  CTG  TTC  CTG  CTT  TCA  AAG  ACG  GAC  TAT  CGA  GGC  TGG  GCA  CAC  GGA  576
122  K  R  Y  S  N  L  F  L  L  S  K  T  D  Y  R  G  W  A  H  G  141
577  CTG  AAG  CGT  GCA  GGC  TAT  GGC  ACC  AAC  CCC  GTC  TAC  GCT  TAC  AGC  CTG  ATT  GAT  ATC  ATA  636
142  L  K  R  A  G  Y  A  T  N  P  V  Y  A  Y  S  L  I  D  I  I  161
637  GAG  TTG  TAT  AGA  CTC  TAC  GAA  TAC  GAC  AAT  ATG  GTG  CTT  AGA  AAG  GGG  AAC  AAC  CAC  TGG  696
162  E  L  Y  R  L  Y  E  Y  D  N  M  V  L  R  K  G  N  N  D  W  181
697  GAC  AGC  CCA  ACC  CAA  CCA  CAA  CAG  AAC  AGT  TCG  TAC  CAA  GAT  ATG  CGA  CAG  TTC  CGT  GCG  756
182  D  T  P  T  Q  P  Q  Q  N  S  S  Y  A  D  M  R  Q  F  R  A  201
757  TTC  AAC  GAG  AAC  TGC  TAC  CTT  ACG  GCA  CAC  GCT  GGC  GAA  ACC  TAC  GAC  AAC  ATA  GCA  AGA  816
202  F  N  E  N  C  Y  L  T  A  H  A  G  E  T  Y  D  N  I  A  R  221
817  GAA  ATA  GGA  ATA  TCA  GGC  CAC  AAG  TTA  GCC  AAA  TAC  AAC  GAG  CGT  CCC  GAA  GAT  GCG  CCG  876
222  E  I  G  I  S  A  H  K  L  A  K  Y  N  E  R  P  E  D  A  P  241
877  TTG  AAC  GAG  GGC  GAA  ATA  GTA  TGG  CTG  AGA  AAG  CAA  CGC  CAC  GTG  CCA  GAA  AAC  TTT  936
242  L  N  E  G  E  I  V  W  L  R  K  K  Q  R  H  V  P  E  N  F  261
937  GGC  AAG  CAA  TAC  CAT  ACA  GTA  AGA  AGC  AAC  GAA  AGT  CTA  TAC  GAT  ATT  TCA  CAG  CAG  TAC  996
262  G  K  Q  Y  H  T  V  R  S  N  E  S  L  Y  D  I  S  Q  Q  Y  281
997  GGT  GTG  CGC  CTG  AAG  AAC  ATT  ATG  AAG  GCG  AAC  AGG  AAA  ATT  GTA  AAA  CGA  GGC  ATC  AAG  1056
282  G  V  R  L  K  N  I  M  K  A  N  R  K  I  V  K  R  G  I  K  301
1057  GCA  GGC  GAC  AGA  GTA  GTA  TTG  CCA  TAA  ACGAAAAACACGACGACGCAAGTGGCTTACACAAATATACAGGAG  1127
302  A  G  D  R  V  V  L  P  *  IR  310
1128  CGAAGACACATCTGGTCTCTGATTTTTCACCACTCCCGAATAAGAACAACCTTTATATATATAGGGGAAACAG  1204
IR
1205  ATGATAAAGGTGTAAAT  1221

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Fig. 3. Nucleotide sequence of *phg* and the flanking regions and a deduced amino acid sequence of *phg*. The putative promoter site, -10 region (TATATT) and a potential ribosome binding site (AAAG) are underlined. A potential stem-loop structure found downstream of the stop codon is marked as IR. The consensus signal sequence (KRYILIIIVALSIGSAIAG) is underlined in the amino-terminal region of the deduced amino acid sequence.

Fig. 4. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis of the lysates of strain PH001. The protein profiles of lysates obtained under different induced conditions are shown: Lane 1, uninduced; Lane 2, 1-h IPTG induction; Lane 3, 2-h IPTG induction; Lane 4, the soluble fraction (supernatant) of the IPTG-induced cell lysate; Lane 5, purified fusion protein. For details, refer to the main text.

protein. The expression was significantly increased when it was induced with IPTG (lanes 2 and 3), compared to that of uninduced growth (lane 1). The 77-kD fusion protein was shown as a single band in lane 6, which was isolated by binding to the amylose-bound resin. The fusion protein was highly expressed in the soluble crude extract samples and the insoluble matter of the lysate (lanes 4 and 5).

Discussion

In the present study, a hemagglutinin gene, designated *phg*, of *P. intermedia* was cloned and expressed in *E. coli* to determine the putative roles of hemagglutinin as a virulence factor. Among the recombinant clones selected based upon their reactivity with polyclonal anti-*P. intermedia* whole-cell and fimbrial antibodies, strain 0543 consistently exhibited the strongest activity. The DNA sequence analysis of this clone revealed the presence of ORFs in the 3.6-kb insert. One ORF with a size of 930 bp, ORF1, was identified as the *phg* ORF.

Further, the other ORF, ORF2, with a size of 940 bp was found to be homologous to the heme-binding protein gene (*hbp*) of *Bacteroides fragilis*, a closely related black-pigmented bacterium [20]. To gain more information regarding ORF2, inverse PCR procedures were performed to amplify the flanking regions to obtain the entire genetic sequence of ORF2. It was found that the 1.7-kb putative *hbp* gene of *P. intermedia* was highly homologous (75% similarity) to *hbp* of *B. fragilis* and that this gene was ubiquitous in other oral black-pigmented bacteria. The two *phg* and *hbp* genes were located in tandem within a 548-bp interval. The possible existence of gene clusters of hemagglutinin and heme-binding protein is very interesting since it is known that bacterial genes with related functions are frequently located very close to each other [21,22]. Even though the exact roles of hemagglutinin and the heme-binding protein of *P. intermedia* are not known, it can be postulated that hemagglutinin may be involved in promoting the attachment of the bacteria to red cells and that the heme-binding protein could help the bacteria utilize heme present in red cells to survive and proliferate inside the host. In contrast, sequence analysis of the putative hemagglutinin gene showed no significant homology to the known DNA con-

tained in databases. Nucleotide and amino acid sequence analysis did not reveal the presence of any other potential hemagglutinin genes in the genomes of several clinical isolates of *P. intermedia* as determined by BLAST search (<https://blast.ncbi.nlm.nih.gov/Blast.cgi>).

However, *phg* contained the consensus signal sequence characteristic of secretory proteins, suggesting that this gene may encode a protein that is transported and expressed on the surface of the bacterial cell [23,24]. To mediate hemagglutination, it may be essential for the bacteria to express hemagglutinin on its surface. In the deduced amino acid sequence of the *phg* gene, a region of short basic amino acids followed by long hydrophobic amino acids and short polar amino acids were found in the amino-terminal portion (Fig. 3). Alanines were found at -3 and -1 positions from the putative cleavage site. These are consistent with the consensus signal sequences for secretory proteins of gram-negative bacteria [25,26]. The recombinant hemagglutinin was expressed using a fusion protein system. A single 77-kD band was detected, as a result of the fusion between the 42-kD maltose-binding protein and the 35-kD hemagglutinin. This result suggests that the *phg* gene was successfully expressed in its correct size in *E. coli*.

A separate study found that the hemagglutinating activity of the clinical isolates of *P. intermedia* was closely associated with the presence of the hemagglutinin gene (*phg*) [7] among the strains. This result also supports the hypothesis that *phg* may play a crucial role in the hemagglutinating activity of *P. intermedia*.

Recent biochemical data from our laboratory indicated that a putative hemagglutinin of *P. intermedia* isolated from the fimbriae was a 3.8-kD protein [17]. The present study, however, found that the putative hemagglutinin was a 35-kD protein. This suggests that two different hemagglutinins may function in *P. intermedia*. The presence of multiple hemagglutinins has been shown in *P. gingivalis* [13,14,27,28]. Even though the exact functions of the multiple hemagglutinins have not yet been adequately elucidated, it appears that multiple hemagglutinins could be involved in a variety of functions including the adherence to other host tissues as well as to red cells. It would be interesting to determine whether multiple hemagglutinin genes are also present in *P. intermedia*. While the 3.8-kD

hemagglutinin was isolated from the fimbriae, the origin of the 35-kD hemagglutinin is not known. Immunological studies using antibodies prepared against the purified recombinant hemagglutinin could elucidate the origin of the 35-kD hemagglutinin.

While this study is one of the first reports of gene cloning of the putative virulence factors from *P. intermedia*, there remain technical difficulties for further studies of *P. intermedia* virulence since no gene transfer system is currently available for *P. intermedia*. To ultimately prove that *phg* is a hemagglutination gene, *phg* isogenic mutants deficient in hemagglutinating activity should be constructed. We anticipate that the information on the hemagglutinin gene of *P. intermedia* may lead to further genetic developments for studying *P. intermedia* pathogenicity and a better understanding of the pathogenesis of periodontal disease.

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Conflicts of Interest

The authors declare that they have no competing interests.

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