Hemolysins have been proven to be important virulence factors in many medically relevant pathogenic organisms. Their production has also been implicated in the etiology of periodontal disease. Hemolytic strain 361B of Prevotella melaninogenica, a putative etiologic agent of periodontal disease, was used in this study. The cloning, sequencing, and characterization of phyA, the structural gene for a P. melaninogenica hemolysin, is described. No extensive sequence homology could be identified between phyA and any reported sequence at either the nucleotide or amino acid level. As predicted from sequence analysis, this gene produces a 39-kDa protein which has hemolytic activity as measured by zymogram analysis. Unlike many Ca\(^{2+}\)-dependent bacterial hemolysins, both the cloned and native phyA proteins were enhanced by the presence of EDTA in a dose-dependent fashion with 40 mM EDTA allowing maximum activity. Ca\(^{2+}\) and Mg\(^{2+}\) were found to be inhibitory. The hemolytic activity also was found to have a dose-dependent end point. Through recovery of hemolytic activity from a spent reaction, this endpoint was shown to be the result of end product inhibition.

This is the first report describing the cloning and sequencing of a gene from P. melaninogenica.

**Cloning and Characterization of a Prevotella melaninogenica Hemolysin**

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**Materials and Methods**

**Bacterial strains and growth conditions.** A strongly hemolytic strain (produces easily visible zones of hemolysis after 2 to 3 days of growth) of P. melaninogenica, 361B, was isolated from subgingival plaque taken from an active disease site (>2 mm of bone loss in less than 3 months) in a patient with chronic adult periodontitis. It was routinely cultured on blood agar plates (BAPs) consisting of Trypticase soy agar (BBL, Cockeysville, Md.) and 5.0% blood supplemented with 50-µg/ml menadione or in Trypticase soy broth supplemented with 50-µg/ml menadione. Blood was obtained from sheep, horse, rat (Lampire Biologicals, Pipeville, Pa.), and human sources and preserved by dilution with an equal volume of Alsever’s medium (Lampire Biologicals). Sheep blood was used unless otherwise indicated. Cultures were incubated at 37°C in an aerobic atmosphere of 5% N\(_2\), 10% H\(_2\), and 5% CO\(_2\). Escherichia coli DH5α, MC1061, and HB101 were cultured aerobically in Luria-Bertani broth or on BAPs. When appropriate, ampicillin (50 µg/ml) and isopropyl-β-D-thiogalactopyranoside (IPTG; 40 µg/ml; Sigma Chemical Co., St. Louis, Mo.) were added to the media.

**Genetic methods.** DNA from P. melaninogenica was obtained from a 500-ml overnight culture by modification of the methods of Kado and Liu (19). Briefly, cells were harvested by centrifugation (4,500 × g for 15 min at 4°C) and lysed with lysozyme and sodium dodecyl sulfate (SDS). The DNA was isolated following dye-buoyant density centrifugation and subjected to proteinase K digestion (0.1 mg/ml for 30 min at 37°C). Following phenol-chloroform-isooamyl alcohol extraction (23), dye-buoyant density centrifugation and proteinase K digestion were repeated. A final phenol-chloroform-isooamyl alcohol extraction and ethanol precipitation (23) step yielded the final purified product.

Small amounts of plasmid DNA were isolated from E. coli as described by Holmes and Quigley (16). Large-scale plasmid preparations were made by using the Wizard Maxiprep DNA Purification System (Promega, Madison, Wis.). The library was transformed into E. coli MC1061. Ampicillin-resistant transformants were isolated on BAPs and screened for a hemolytic phenotype after incubation overnight at 37°C.

Total cellular DNA was obtained for Southern blot analysis by using Qiangen columns (Qiagen Inc., Chatsworth, Calif.) in accordance with the manufacturer’s directions. Southern blot analysis was performed by using the Enhanced Chemiluminescence Gene Detection System (Amersham International PLC, Amersham, England). The supplier’s protocols were used without modification for hybridization and for DNA probe preparation. Amersham Hyperbond-N+ membranes were exposed to Hyperfilm-ECL (Amersham International PLC).

PCR was performed using 50 ng of chromosomal DNA as the template, and the primers used were JH18 (CTTGATGCCTCTCTCAGG) and JH28 (GAAAG TTCCGAATGC). The reaction used Vent polymerase (New England Biolabs, Beverly, Mass.) with an initial melting step (94°C, 1 min) followed by 30 cycles of melting (94°C, 1 min) and annealing (54°C, 2 min), and extension (72°C, 3 min). The product was held at 4°C.

**Genetic analysis.** Mapping of insert DNA was performed with restriction endonucleases used in accordance with the manufacturer’s (New England Biolabs) directions. DNA primers were synthesized by the DNA Synthesis Core Laboratory of the University of Florida. Double-stranded DNA sequencing was performed by the DNA Sequencing Core Laboratory of the University of Florida Interdisciplinary Center for Biotechnology Research. Sequence analysis was performed by using the Genetics Computer Group Sequence Analysis Software and MacVector (BII, New Haven, Conn.). Database searches were conducted with BLAST, National Center for Biotechnology Information Database Search (7).
SDS-polyacrylamide gel electrophoresis (PAGE) analysis. SDS-PAGE gels were prepared by the methods of Laemmli (22). Insertion bodies were isolated from overnight IPTG-induced cultures of DHS-α PHYAph by detergent-induced lysis and differential centrifugation (13). Zymogram analysis was performed following standard SDS-PAGE. One of two duplicate 12.5% gels was stained with Coomassie blue R250. The other was renatured by two 10-min washes with water, two 10-min washes with NCN buffer (3 mM sodium citrate, 0.9% sodium chloride, pH 6.8) containing 0.1% Triton X-100, and five 10-min washes with NCN buffer. The renatured separating gel was then placed on a 2-mm-thick agarose gel slab containing 5.0% NCN-washed sheep blood cells and covered with Saran wrap. The gel was incubated overnight at 37°C. Sequencing of an internal fragment of PHYA was performed by excising the PhyA protein band identified by zymogram analysis from an SDS-polyacrylamide gel. It was rinsed in water and macerated in the presence of extraction buffer (0.1% SDS, 10 mM Tris [pH 8.0], 0.1 mM EDTA, 0.02 M NH₄HCO₃). The extract was treated (6) by digestion overnight at 37°C with endoLysC (0.003 U/mg of protein; Promega). The acrylamide gel fragments were removed by centrifugation (14,000 × g for 10 min at 4°C). The supernatant was recovered, vacuum dried, and dissolved in SDS-PAGE loading buffer. Fragment peptides were separated on a Tris-tricine polyacrylamide gel (28). The peptides were electrotransferred to an Immobilon P membrane (Bio-Rad, Richmond, Calif.) by using the Bio-Rad Trans-Blot SD semidry electrophoretic transfer cell in accordance with the manufacturer's directions. The membrane was stained with Coomassie blue R250 (1%, w/v). A large predicted peptide band was excised from the blot and subjected to Edman degradation by the University of Florida Protein Core Laboratory using an Applied Biosystems 473A protein sequencer.

Production of anti-PhyA. Three New Zealand White rabbits were immunized four times (6) with recombinant PhyA purified by SDS-PAGE. PhyA was excised from the gel and macerated in the presence of RIBI adjutant (Sigma Chemical Co.) (13). Serum extracts were pooled, and monospecific anti-PhyA serum was obtained after multiple adsorptions against whole DHS-α/pUC18 cells and agarose beads with covalently attached proteins from DHS-α/pUC18 lysates (12).

Western blot analysis. One of three SDS-PAGE gels was stained with Coomassie blue R250. The other gels were transferred to a nitrocellulose membrane by using a semidyey transfer apparatus in accordance with the manufacturer's (Bio-Rad) directions. The blots were rinsed in acetate buffer (30 mM acetate, pH 4.5). One blot was incubated in acetate buffer alone, and the other was incubated in sodium m-periodate (5 mM sodium m-periodate in acetate buffer) for 1 h at room temperature (25). Both blots were then incubated in 50 mM sodium borohydride for 30 min at room temperature. The blots were washed twice in blocking buffer (1.0% skim milk in phosphate-buffered saline; pH 7.5) for 1 h (1). Both blots were probed with rabbit anti-PhyA serum at a 1:500 dilution overnight at room temperature. Goat anti-rabbit IgG conjugated to horseradish peroxidase (Sigma Chemical Co.) was added at a 1:10,000 dilution, the mixture was incubated for 1 h at room temperature, and reactive proteins were immunodetected by standard methods by using 0.3% 4-chloro-1-napthol (Sigma Chemical Co.) in methanol with 0.018% H₂O₂ in TBS (100 mM Tris-Cl, pH 7.5, 0.9% NaCl) as the substrate (1).

Hemolysis activity assays. The hemolysis activity assay of Chu et al. (5) was used. The assay was performed by combining 1 ml of NCN-washed sheep blood with 0.1 ml of test sample. The final volume was adjusted to 2.5 ml with NCN buffer. Test samples consisted of whole lysates of P. melaninogenica MC1061, and screened for hemolytic clones. Four such clones were identified by zymogram analysis of plasmid DNA regions of identity in the cloned inserts of all four strains. One of these, HA1001, was chosen for further study.

Sequence analysis of the cloned insert in pHA1001 showed that it was 3,033 bp long. Computer analysis identified two open reading frames in the insert (Fig. 2A). One of these, glnA, was incomplete and possessed strong homology to the amino-terminal half of the Bacteroides fragilis gene for glutamine synthetase. The second open reading frame, phyA, had no extensive homology to any reported sequence in the computer databases at either the nucleotide or the amino acid level.

Southern analysis using a 5′-end fragment (Fig. 2A) from pHA1001 as a probe and EcoRI-digested chromosomal DNA indicated a single copy of the phyA gene in the chromosome of 361B (Fig. 3). The probe did not hybridize to the chromosomal DNA of E. coli MC1061.

The XmnI-AseI fragment from pHA1001 containing phyA (Fig. 2A) was subcloned into the multiple cloning site of pUC18 in both orientations. Hemolysis was observed only when the transcriptional sense of phyA placed it under control of the vector’s lacZ promoter. The active subclone, pPHAp (Fig. 1B), also conferred a hemolytic phenotype on two other E. coli hosts, DHS-α and HB101.

Sequence analysis of the 1,394-bp insert in pPHAp (Fig. 2B) identified a consensus Shine-Dalgarno sequence beginning 20 bp 5′ to the translation start codon. Although there is no complementarity on the 16S rRNA of P. melaninogenica to this Shine-Dalgarno sequence, a region (GATTITGGA) 9 to 15 bp 5′ to phyA is largely complementary to the CAAAAC T sequence at the 3′ terminus of the P. melaninogenica 16S rRNA (27). Two putative rho-independent transcription terminators were identified by the Terminator program (3) in the region 33 to 57 bp 3′ to the translation stop codon. Computer analysis of the deduced amino acid sequence indicated that the gene product is an acidic protein with a pI of 5.065 and has a calculated molecular mass of 39,354 Da.

**SDS-PAGE studies.** A 39-kDa protein was observed by SDS-PAGE analysis of E. coli strains carrying phyA (Fig. 4A). The molecular mass of this band was equivalent to the predicted molecular mass of the phyA gene product. Insertion bodies were isolated from DHS-α containing pPHAp grown in the presence of IPTG. This preliminary purification step resulted in an enriched preparation of the 39-kDa protein. Zymogram analysis (Fig. 4B) indicated that the 39-kDa band had hemolytic activity. A somewhat higher-molecular-mass protein (~45 kDa) appeared to have hemolytic activity in the 361B lysate.

No amino-terminal sequence could be obtained from the SDS-PAGE-purified 39-kDa protein. Consequently, an internal peptide fragment was generated by endoLysC digestion and Tris-Tricine gel purification. The amino acid sequence obtained from this fragment, SIFALDNLWD, matched residues 156 to 165 of the PhyA amino acid sequence (Fig. 2B).

**Western blot analysis.** Western blot analysis was performed with lysates of the E. coli subclone, the vector control, and 361B (Fig. 5A) by using rabbit antisera raised to PhyA. A 39-kDa band was recognized in the E. coli subclone (Fig. 5B). However, a 45-kDa band was recognized in the 361B lysates only after m-periodate treatment (Fig. 5C). No bands were visible with the E. coli/pUC18 control with or without m-periodate treatment.

**Activity analysis.** The zones of hemolysis surrounding colonies of P. melaninogenica 361B and the E. coli clones carrying phyA were largest (twice the diameter of the colony) on BAPs made with human blood and smallest (barely visible) on BAPs made with rat blood. Sheep and horse blood gave intermedi-

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ate-sized zones (equal to the diameter of the colony) (data not shown).

When whole 361B cells were tested in an in vitro hemolysin activity assay, the activity was shown to be enhanced in a dose-dependent fashion by the divalent cation chelator EDTA (Fig. 6A). Maximum activity was found in the presence of 40 mM EDTA. Higher concentrations (100 mM) caused spontaneous lysis of the blood cells. At a concentration of 40 mM EDTA, spontaneous lysis of erythrocytes was less than or equal to the lysis observed in the absence of EDTA following overnight incubation at 37°C (data not shown). Fractionation of strain 361B by the methods of Chu et al. (5) revealed hemolytic activity in vesicle, cytoplasmic, and outer membrane-enriched samples, as well as culture supernatants. Hemolytic activity in the E. coli cells harboring pHAphy could only be measured when the bacterial cells were lysed. Unlike the reported calcium-dependent activity of the α-hemolysin produced by some strains of E. coli (2), DH5-α harboring pHApHy displayed an EDTA-enhanced hemolytic activity resembling the native activity in strain 361B (Fig. 6B).

The native activity showed a dose-dependent endpoint. In the case of whole 361B cells, the lysis of sheep erythrocytes, present in excess, ceased after about 3 h, regardless of the initial bacterial cell concentration (Fig. 7A). The amount of lysis observed at this point did depend on the initial bacterial cell concentration. This result indicated that the hemolytic activity of 361B was either consumed in the reaction or subject to some form of end product inhibition. This same phenomenon was observed when lysates of DH5-α harboring pHApHy were used as the test sample, although the endpoint was reached after only 1 h of incubation (Fig. 7B). When 361B cells were rescued from a spent reaction and reassayed, not only did they demonstrate restored hemolytic activity, but the activity was increased relative to that exhibited by fresh (previously unassayed) cells (Fig. 8).

**DISCUSSION**

The importance of iron for P. melaninogenica and related species has been well established (9). In an iron-limited environment such as crevicular fluid (29), production of a hemolysin may allow scavenging of this essential nutrient from host cells. It was shown that production of hemolysin by P. melaninogenica 361B in vitro occurred only under iron-limiting conditions (14). Other work has demonstrated an increase in virulence when the related organism Porphyromonas gingivalis was grown in the presence of excess hemin (24). Since crevicular fluid is an iron-limited environment (29), production of PhyA may generate an increase in crevicular fluid iron levels and therefore may be responsible for increased virulence of P. melaninogenica and/or other potential periodontal pathogens sharing its niche. This could explain the reported correlation between the presence of hemolytic bacteria in subgingival plaque and the progression of periodontitis (14).

In this study, we have cloned a DNA fragment from P. melaninogenica in E. coli by using an expression library. Sequence analysis indicated the presence of two distinct genes, glnA and phyA, in one of the parent clones. The gene glnA showed homology to a glutamine synthetase gene of the related organism B. fragilis. The biological significance, if any, of
the close proximity of the **glnA** and **phyA** genes has not been determined. There is no extensive homology between **phyA** and any reported sequence at either the nucleotide or the amino acid level. Southern analysis indicated that **phyA** originated from and exists as a single-copy gene in 361B, although it has not been determined whether it is present on chromosomal or large episomal DNA.

Although sequences resembling putative promoter consensus sequence (RBS), a sequence complementary to the 3' termini of *P. melaninogenica* 16S rRNA (crRNA), a probable translation start site (TS), the confirmed amino acid sequence from Edman degradation (underlined), and two putative p-independent transcription termination sites (term.).

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**FIG. 2.** (A) Physical map of the 3,033-bp DNA insert of the parent clone, pHA1001. The two open reading frames, **phyA** and **glnA**, are noted by the wide arrows. The restriction cleavage sites **XmnI** (**X**), **StyI** (**S**), **EcoRI** (**E**), **AseI** (**A**), and **PstI** (**P**) are indicated. The probe used for Southern analysis is indicated between the two **StyI** sites. (B) Nucleotide sequence of **phyA** and the predicted amino acid sequence of its product, PhyA. Underlined areas indicate a putative ribosomal binding site (RBS), a sequence complementary to the 3' termini of *P. melaninogenica* 16S rRNA (crRNA), a probable translation start site (TS), the confirmed amino acid sequence from Edman degradation (underlined), and two putative p-independent transcription termination sites (term.).
sus sequences from a related organism, *P. gingivalis* (8, 17), were identified 139 to 145 bp and 88 to 94 bp 5' to the translational start of *phyA*, there is no evidence in this study to suggest that they function as the promoter. However, it was shown that if the *phyA* promoter was cloned, it was not functional in *E. coli*, as indicated by the observation that the hemolytic phenotype was lost when the *phyA* gene was placed in the orientation opposite to the *lacZ* promoter of pUC18. A putative ribosomal binding site was identified based upon similarity to the Shine-Dalgarno sequence (8). However, because this sequence lacked any complementarity to the 3' end of the 16S rRNA from *P. melaninogenica*, it is doubtful that it is involved in the translation of PhyA in 361B, but it may function in the *E. coli* clone. An area just upstream of *phyA* was found to have complementarity with the 3' end of the 16S rRNA (27) from two different American Type Culture Collection *P. melaninogenica* strains. This may actually be the ribosome binding site for 361B. Experimental testing of this hypothesis is required for its verification. Two putative transcription termination signals were identified by computer sequence analysis.

SDS-PAGE analysis of the *E. coli* clone and subclone lysates identified a band with the same molecular weight as that predicted by the *phyA* sequence. This band was not found in the pUC18 control. Zymogram analysis indicated that this band had hemolytic activity. Inclusion bodies were isolated from the subclone and found to contain the predicted 39-kDa protein. This protein also had hemolytic activity as indicated by zymogram analysis. The internal amino acid sequence was obtained because the amino terminus of PhyA appeared to be blocked. The sequence data obtained from the internal fragment of the 39-kDa protein in the inclusion body preparation agreed with the amino acid sequence deduced from the *phyA* DNA sequence. These results provided preliminary evidence that *phyA* is the structural gene for the 361B hemolysin. In accord with this hypothesis, the *phyA* gene was able to confer a hemolytic phenotype on three different strains of *E. coli*, suggesting that the hemolytic phenotype is not due to activation of a cryptic hemolysin gene by *phyA* (10).

A protein with hemolytic activity but with a slightly greater apparent molecular weight was seen by SDS-PAGE in the *P. melaninogenica* lysate. Rabbit antiserum raised against the 39-kDa protein isolated from inclusion bodies was shown to specifically react to this larger protein from *P. melaninogenica*.
liberation of the cytoplasmic activity by spontaneous bacterial differences in cellular localization of the native and recombinant hemolysin in protein transport systems. Posttranslational modification of the native protein than the recombinant protein could therefore be explained by differences in posttranslational modifications such as glycosylation.

Whole cells of *P. melaninogenica* possessed hemolytic activity in an in vitro assay, indicating that the hemolysin is exposed on the surface of the bacterial cell. Hemolytic activity could also be found associated with various subcellular fractions, including cytoplasmic, outer membrane, and vesicle fractions, indicating that the protein is exported to the surface of the cell, where it most likely becomes localized in the outer membrane. However, computer analysis could detect no apparent transport signals in the deduced amino acid sequence. In fact, the deduced amino acid sequence possessed an acidic amino terminus, in contrast to the expected hydrophobic domain. The hemolytic activity of the *E. coli* clones and subclone was localized to the cytoplasmic compartment, indicating differences in protein transport systems. Posttranslational modification of the hemolysin in *P. melaninogenica* may also explain the observed differences in cellular localization of the native and recombinant proteins. The zones of hemolysis observed on culture plates of the *E. coli* clones and subclone were probably due to liberation of the cytoplasmic activity by spontaneous bacterial cell lysis during normal aging of the culture.

Hemolytic activities are functionally characterized as either enzymatic or pore forming. The largest known class of pore-forming cytolysins is the RTX group, of which the *E. coli* α-hemolysin serves as the standard model (2). These hemolysins function in a fashion similar to that of complement by their ability to form pores in the target cell membrane with a characteristic calcium dependency. In contrast, the *P. melaninogenica* hemolysin activity was enhanced by the divalent cation chelator EDTA. Both magnesium and calcium were found to inhibit the reaction. This EDTA enhancement was also observed in the *E. coli* subclone. A *P. gingivalis* strain has been reported (20) to possess a hemolysin which was inhibited by calcium and magnesium; however, in this case, no EDTA enhancement was reported.

Both the native and recombinant hemolysin activities demonstrated an unusual reaction endpoint that was dependent on the initial test sample concentration. This phenomenon could be due either to the consumption of a pore-forming activity or to end product inhibition of an enzymatic activity. Because the hemolytic activity could be recovered from a spent assay still associated with 361B cells, the latter explanation appears to be correct. An identical species-specific activity was also demonstrated between native and recombinant hemolytic activities by using blood from different animal sources. These data demonstrating the similarity of the native and recombinant hemolytic activities with regard to several unusual properties, including the inhibitory effects of divalent cations, the dose-dependent endpoint, and the spectrum of target cell activity, provides additional strong evidence that the structural gene for a *P. melaninogenica* hemolysin has been cloned.

Hemolysins have been proven to be important virulence factors in the pathogenic personalities of many medically important microorganisms (2). In fact, it has been shown that the pathogenic potential of several hemolytic organisms can be drastically reduced by elimination of the hemolytic phenotype (2). Hemolysins have been implicated in periodontal disease (14), but the importance of PhyA to *P. melaninogenica* in vivo has not been established. Preliminary attempts to construct an isogenic mutant of 361B via self-recombination of a heterodiploid intermediate have been unsuccessful, suggesting that PhyA provides an essential function for *P. melaninogenica*. Attempts are under way to isolate a temperature-sensitive mutator in *phyA* to test this hypothesis and to provide definitive proof that *phyA* is the structural gene for the 361B hemolysin.

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