Purification and characterization of hemolysin from *Porphyromonas gingivalis* A7436

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Abstract

*Porphyromonas gingivalis*, a periodontal pathogen, has the ability to lyse erythrocytes. The hemolytic activity of *P. gingivalis* A7436 was purified as a 45-kDa protein from the culture supernatant of a 3-days old culture using nickel-nitrilotriacetic acid chromatography. Erythrocytes treated with purified *P. gingivalis* hemolysin showed the presence of pores and extracellular debris by scanning electron microscopy. Active immunization of mice with 15 μg hemolysin induced neutralizing antibodies to hemolysin. Heating at 60°C and treatment with trypsin and dithiothreitol abolished hemolytic activity, while incubation with the protease inhibitor Na-p-tosyl-l-lysine chloromethyl ketone caused no effect. We report here for the first time purification of a hemolysin from *P. gingivalis* A7436. The amino acid sequence of an internal peptide of hemolysin showed sequence similarity with fimbrillin from *P. gingivalis* HG564. However, the amino acid composition of purified hemolysin was different from that of *P. gingivalis* fimbrillin. Also, the ability to lyse but not agglutinate erythrocytes and to bind to nickel-nitrilotriacetic acid differentiates *P. gingivalis* hemolysin from fimbrillin. © 1999 Federation of European Microbiological Societies. Published by Elsevier Science B.V. All rights reserved.

Keywords: Hemolysin; Ni-NTA chromatography; *Porphyromonas gingivalis*

1. Introduction

Protoheme is an absolute requirement for growth of the obligate anaerobe *Porphyromonas gingivalis* [1,2]. Heme is probably derived from erythrocytes in the natural niche for the organism, the periodontal pockets. Therefore, it is particularly important for the organism to lyse erythrocytes and acquire heme to survive in vivo. A number of investigators have reported the presence of hemolytic activity in *P. gingivalis* [3–5]. These investigators have studied the hemolytic activity in different strains of *P. gingivalis* and have reported that hemolytic activity was released into the culture supernatant. However, there are discrepancies in the properties of the hemolytic activities reported by different investigators. Also, hemolysin has not yet been purified from *P. gingivalis* despite several reports on its presence in different strains of *P. gingivalis*. The cloning and expression of two hemolysin genes from *P. gingivalis* in *Escherichia coli* has been reported [6]. However, the recombinant hemolytic product(s) has not been purified. This study reports the purification and characterization of hemolysin from *P. gingivalis* A7436.
2. Materials and methods

2.1. Bacterial strains and growth conditions

*P. gingivalis* A7436 was typically maintained on anaerobic blood agar plates at 37°C in an anaerobic chamber (with 85% N₂, 10% CO₂ and 5% H₂) for 3–5 days, inoculated into Schaedler broth and grown for 24 h until the absorbance at 600 nm ($A_{600}$) reached 1.0, unless otherwise specified.

2.2. Isolation of hemolysin from *P. gingivalis*

Hemolysin was isolated from culture supernatant (CS) of *P. gingivalis* A7436 obtained after removal of the cells by centrifugation of a 3-days old culture at 20,000 $\times g$ for 30 min. CS was then subjected to 50% ammonium sulfate precipitation on ice and centrifuged at 20,000 $\times g$ for 90 min. The ammonium sulfate precipitated proteins (ASP) were dissolved in wash buffer (0.5 M sodium phosphate buffer containing 0.5 M NaCl, pH 8.0) and loaded on a nickel-nitrilotriacetic acid (Ni-NTA) agarose column. Ni-NTA chromatography [7] was then used to purify hemolysin under non-denaturing conditions as described earlier [8]. Briefly, Ni-NTA agarose (Qiagen, Chatsworth, CA, USA) was equilibrated with wash buffer and loaded with ASP dissolved in wash buffer. The flow-through (FT) obtained from the column was collected and the column was washed with wash buffer until $A_{280}$ was zero. Bound proteins were eluted with elution buffer (0.1 M potassium phosphate buffer containing 0.75 M NH₄Cl, pH 6.6) in 1-ml fractions and tested for hemolytic activity and in chromogenic and hemagglutination (HAg) assays. The protein content was determined with Bio-Rad protein assay reagent and the fractions showing hemolytic activity were subjected to SDS-PAGE.

2.3. Assays for hemolytic activity, Arg-gingipain (RGP) and Lys-gingipain (KGP) activities and HAg

Assays for hemolytic and HAg activities were conducted with sheep red blood cells (RBCs, 10% suspension) from ICN/Cappel (Aurora, OH, USA). At the time of assay, RBCs were diluted to 1% in NCN buffer (3 mM sodium citrate, 0.9% sodium chloride, pH 6.8). Hemolytic activities of whole cells, ASP, FT, wash and fractions were determined by mixing 1 ml of 1% RBCs with 0.1 ml of each bacterial sample. Mixtures were incubated at 37°C for 18 h. Intact RBCs were removed by centrifugation (8000 $\times g$, 2 min) and the amount of lysis was measured by the quantification of hemoglobin release in the supernatant as determined at $A_{540}$. Control RBCs were incubated in NCN buffer as described above without the addition of a bacterial sample. The effect of elution buffer and wash buffer on RBCs was determined by incubating 0.1 ml of each buffer with RBCs under identical conditions. Complete RBC lysis was achieved by mixing RBCs obtained from 1 ml of 1% RBC suspension with 1 ml distilled water. The percent lysis of the experimental samples (mean value of triplicate readings) was calculated relative to the value for this complete lysis control. The HAg assay was performed as described earlier [9] by incubating 0.1 ml sample with 0.1 ml of 0.2% (v/v) RBCs for 16 h at 4°C in U-bottom microtiter plates. Positive HAg was observed as a red carpet coating the bottom of the well. The RGP and KGP activities were determined in a chromogenic assay by determining the catalytic activity towards benzyloxy carbonyl-Arg-$p$-nitroanilide (BApNA) and benzyloxy carbonyl-Lys-$p$-nitroanilide (BLpNA) using a multi-well microtiter plate reader at $A_{410}$, as described by Gron et al. [10]. A final substrate of 0.5 mM BLpNA (BLpNA for KGP) or 0.25 mM BApNA (BApNA for RGP) in 0.1 M Tris-HCl, 0.1 M NaCl, pH 7.6, and 10 mM L-cysteine was mixed with 0.1 ml sample and incubated at 37°C for 1 h. For assaying enzyme cleavage after lysine, the buffer used was at pH 8.0. Pure RGP and KGP (kindly provided by Dr J. Potempa (University of Georgia, Athens, GA, USA) were used as positive controls.

2.3.1. Amino acid sequence and amino acid composition of hemolysin

The 45-kDa band from the hemolytic fraction was subjected to SDS-PAGE and in-gel digestion with trypsin. The peptides were purified by high performance liquid chromatography and the peptide showing the highest peak was selected for sequencing using Edman degradation. The 45-kDa protein from the hemolytic fraction was also electroblotted on a
PVDF membrane and analyzed for its amino acid content. The amino acid composition and sequencing was performed by Dr J. Pohl (Emory University, Atlanta, GA, USA). The protein BLAST database search program (January 1999) was used to determine sequence homology.

2.4. Production of antibodies to hemolysin

To study the immunogenicity of hemolysin and to raise antibodies to it, 8-weeks old BALB/c mice were injected with hemolysin into subcutaneous (s.c.) chambers [11] (implanted 10 days prior to the start of immunization) on days 1, 7, 14 and 21. Each mouse was injected with hemolysin (5 µg protein of the Ni-NTA fraction per injection) without adjuvant. Chamber fluid (CF) samples were collected on days 0 (pre-immune serum), 12, 20 and 28. Anti-hemolysin antibody titers in CF were determined by an enzyme-linked immunosorbent assay (ELISA) using the hemolytic fraction (50 ng protein per well) as the antigen. The reactive titer was defined as the reciprocal of the dilution consistently showing a 2-fold increase in absorbance over that obtained with the pre-immune CF.

2.5. Scanning electron microscopy (SEM)

Control RBCs and RBCs incubated with 2 µg hemolysin at 37°C for 18 h were fixed in 2.5% (v/v) glutaraldehyde, 0.1 M sodium cacodylate buffer (pH 7.4) for a minimum of 3 h. Prior to dehydration in ethanol, samples were stored in 1% (v/v) osmium tetroxide in 0.1 M sodium cacodylate buffer (pH 7.4) for 2 h. Critical point drying was performed with CO2 and the samples were sputter-coated with 10 nm gold-palladium (60:40, w/w). The samples were observed with a JEOL JSM-820 (Japan) scanning electron microscope.

2.6. Hemolysis inhibition assays

2.6.1. Effect of heat

Aliquots of hemolysin (Ni-NTA fraction) were heated to temperatures between 37 and 80°C for 10 min in a temperature-controlled water bath, cooled on ice and immediately assayed for hemolytic activity.

2.6.2. Effects of trypsin and protease inhibitor Na-p-tosyl-L-lysine chloromethyl ketone (TLCK)

The effects of trypsin (2.5 mg ml⁻¹) and protease inhibitor TLCK (10 mM) on hemolytic activity were examined by incubating each with hemolysin at 37°C for 1 h and assaying the hemolytic activity.

2.6.3. Effect of anti-hemolysin antibody

The effect of polyclonal antibody against *P. gingivalis* A7436 hemolysin was determined by mixing hemolysin with complement-inactivated (56°C, 30 min) anti-hemolysin antiserum. The mixture was incubated at 37°C for 1 h and assayed for hemolytic activity. Pre-immune serum was incubated similarly with hemolysin to determine non-specific binding and inhibition of antibody to hemolysin.

2.6.4. The effect of cysteine and dithiothreitol (DTT)

The effects of the sulfur-containing compounds cysteine and DTT at a 10 mM concentration on hemolysin were tested by incubating each with hemolysin at 37°C for 2 h and then assaying the hemolytic activity.

3. Results

3.1. Hemolytic activity of *P. gingivalis* A7436

Lysis of RBCs by *P. gingivalis* started after 4 h of incubation with the RBCs and increased slowly with time. For this work, an 18-h incubation time was used for the assay. Cells obtained from an 18-h culture of *P. gingivalis* A7436 showed the ability to lyse RBCs (activity = 22.1 ± 1.8%). Control RBCs incubated in NCN buffer showed a very low spontaneous lysis (4.5 ± 0.8%). The cell-associated hemolytic activity decreased with an increasing growth time and was almost negligible after 30 h of growth (activity = 8.2 ± 1.4%). Hemolytic activity was secreted into the CS and the activity gradually increased in the CS, reaching a maximum in a 3-days old culture (30.2 ± 2.1%). Hence, CS of a 3-days old culture was used for the purification of hemolysin. The addition of the iron chelator 2-2’ bipyridyl (BPD) at a 10 µM concentration to Schaedler broth during growth of *P. gingivalis* A7436 did not increase the hemolytic activity. Hemolytic activity of cells and CS of...
1-day old BPD-grown *P. gingivalis* (20.1 ± 1.6 and 27.6 ± 1.1%, respectively) was similar to that observed with cells and CS of 1-day old *P. gingivalis* grown in the absence of BPD (21.1 ± 1.4 and 26.8 ± 1.6%, respectively).

### 3.2. Purification and characterization of hemolysin from *P. gingivalis* A7436

Hemolytic activity could be isolated by 50% ASP of CS. ASP obtained from the CS of *P. gingivalis* A7436 contained hemolytic, HAg, RGP and KGP activities. As shown in Table 1, hemolytic activity from ASP could be separated from the other activities by Ni-NTA chromatography since hemolytic activity eluted to Ni-NTA agarose, while the RGP, KGP and HAg activities did not. The latter activities were recovered in the FT and washes obtained on washing the column with the wash buffer, while the bound hemolysin could be eluted with the elution buffer containing NH₄Cl. When 1 ml ASP (containing approximately 200 μg total protein obtained by precipitation of 50 ml CS) was loaded on Ni-NTA, hemolytic activity eluted from the column in the first 1-ml fraction (33.1 ± 1.2% with 100 μl of the fraction containing approximately 1.0 μg protein) with subsequent fractions showing a negligible activity (6.5 ± 2.1%). Hence, only the first 1-ml fraction was used as hemolysin for subsequent studies. The hemolytic fraction showed no detectable RGP, KGP or HAg activities. No hemolytic activity was observed in the FT or wash (6.8 ± 1.1 and 5.2 ± 0.8%, respectively) obtained from the Ni-NTA column. On subjecting the hemolytic fraction to SDS-PAGE and silver staining, a single band of 45 kDa was observed (Fig. 1). The amino acid sequence of a peptide obtained by digestion of hemolysin was found to be AAFTIKPGSNHYGYPGGT, which showed 94% similarity with the deduced amino acid sequence for fimbrillin from *P. gingivalis* HG564 and 38% similarity with that of fimbrillin from other *P. gingivalis* strains (Fig. 2). Table 3 shows the amino acid composition of purified hemolysin and that of fimbrillin from *P. gingivalis* [12].

### 3.3. Electron microscopy

Electron micrographs show the effect of hemolysin on sheep RBCs (Fig. 3). SEM revealed the difference in the appearance of hemolysin-treated cells (Fig. 3B) as compared to control untreated RBCs (Fig.

<table>
<thead>
<tr>
<th>KGP activity (BpNA assay)</th>
<th>RGP activity (BpNA assay)</th>
<th>HAg activity (RBC agglutination)</th>
<th>Hemolytic activity (RBC lysis)</th>
</tr>
</thead>
<tbody>
<tr>
<td>ASP</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>FT</td>
<td>+</td>
<td>+</td>
<td>−</td>
</tr>
<tr>
<td>Wash</td>
<td>+</td>
<td>+</td>
<td>−</td>
</tr>
<tr>
<td>Fraction</td>
<td>−</td>
<td>−</td>
<td>+</td>
</tr>
</tbody>
</table>

HAg, hemagglutination; ASP, ammonium sulfate precipitate of culture supernatant; FT, flow-through obtained after loading ASP on a Ni-NTA column; Wash, obtained by washing a column after ASP loading and FT removal; Fraction, first 1-ml fraction obtained on elution. Activities are recorded as + or −, depending on the presence or absence of each activity, respectively.
Hemolysin-treated cells were generally smaller and there appeared to be extracellular debris around the RBCs. Close scrutiny of the hemolysin-treated cells showed a few pore-like structures on the surface of some of the hemolysin-treated RBCs (Fig. 3B).

### 3.4. Hemolysis inhibition assays

The effects of different agents on hemolytic activity are shown in Table 2. Trypsin, incubation at 60°C, DTT and anti-hemolysin antibody abolished hemolytic activity. Cysteine and the protease inhibitor TLCK did not affect the hemolytic activity.

### Table 2

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Hemolytic activity (%)b</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nonea</td>
<td>30.1 ± 2.1</td>
</tr>
<tr>
<td>Trypsind (1 mg ml⁻¹)</td>
<td>9.1 ± 2.1</td>
</tr>
<tr>
<td>Heat: 40°C</td>
<td>28.9 ± 0.6</td>
</tr>
<tr>
<td>50°C</td>
<td>16.2 ± 2.4</td>
</tr>
<tr>
<td>60°C</td>
<td>8.1 ± 0.7</td>
</tr>
<tr>
<td>l-Cysteine (10 mM)</td>
<td>28.8 ± 1.2</td>
</tr>
<tr>
<td>DTT (10 mM)</td>
<td>7.4 ± 1.3</td>
</tr>
<tr>
<td>TLCK (1 mM)</td>
<td>29.4 ± 1.4</td>
</tr>
<tr>
<td>Anti-hemolysin antibody</td>
<td>5.8 ± 0.7</td>
</tr>
</tbody>
</table>

Note, spontaneous lysis (lysis of RBCs incubated in NCN buffer for 18 h) was 4.4 ± 1.1.

*aHemolytic Ni-NTA fraction of *P. gingivalis* A7436.

*bAverage of triplicates expressed as a percentage of total RBCs lysed after 18 h.

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**Fig. 2.** Amino acid sequence of an internal peptide obtained from *P. gingivalis* hemolysin (hem) and its comparison with the deduced amino acid sequence for fimbrillin (fim) from different *P. gingivalis* strains. Mismatches in the fimbrillin sequences are indicated by underlining the amino acids. A space (-) has been added to maintain the best alignment. The *P. gingivalis* strains are given in parentheses. The numbers in the square brackets indicate the position of the peptide.

**Fig. 3.** The effect of *P. gingivalis* hemolysin on sheep erythrocytes (RBCs) as examined by scanning electron microscopy. (A) Control RBCs incubated in NCN buffer at 37°C for 18 h (magnification ×12000). (B) RBCs in NCN buffer exposed to 2 μg hemolysin of *P. gingivalis* A7436 at 37°C for 18 h (magnifications ×8000). Pores (indicated by arrows) are visible on some of the hemolysin-treated RBCs along with extracellular debris. The bar on each micrograph is 1 μm in length. A composite image was constructed by Adobe Photoshop 3.0.
3.5. Immunogenicity of hemolysin

Antibodies could be raised in mice in the implanted s.c. chambers without the use of adjuvant. After two injections of hemolysin (5 μg each), the titer to hemolysin in CF as determined by an ELISA was 1:4000 and increased to 1:12800 after the third injection and to 1:16000 after a fourth injection. The A_{492} values ranged from 0.26 to 0.81 with immune CF as compared to 0.05-0.08 for non-immune CF.

4. Discussion

A number of investigators have reported the presence of hemolytic activity in different P. gingivalis strains [3–6,13]. Adherence to and lysis of RBCs by purified RGP has also been reported [14,15]. From our studies, it appears that hemolysin is independent of the catalytic and HAg activities associated with RGP since the hemolytic activity could be separated from the latter two by Ni-NTA chromatography. Hemolysin was unaffected by TLCK and cysteine, which are known to inhibit and activate RGP, respectively [14–17]. These observations point to the fact that hemolytic activity and RGP activity are distinct from each other. The expression of P. gingivalis hemolytic activity has shown to be regulated by iron [3]. Excess iron has shown to depress hemolytic activity [3,6], while addition of the iron-chelator BPD increases hemolytic activity [3,6]. Genco et al. [13] have reported a higher hemolytic activity in a hemin uptake deficient mutant of P. gingivalis as compared to the wild-type strain. However, we did not observe any significant difference between hemolytic activity of a BPD-grown culture and a culture grown in the absence of BPD.

Hemolytic activity from P. gingivalis whole cells and extracellular vesicles has been characterized by several investigators [3–6]. However, there are discrepancies in the sensitivity of hemolytic activity to different agents/treatments. Hoshi et al. [4] have reported P. gingivalis hemolysin to be a thiol-dependent, ‘hot-cold’ toxin, while Chu et al. [3] have reported that it does not exhibit activity in the cold and is inhibited by DTT, TLCK and cysteine. Kay et al. [5] have reported hot-cold hemolytic activity in P. gingivalis which is inhibited by DTT. Karunakaran and Holt [6] have reported that P. gingivalis hemolytic activity is enhanced by DTT, inhibited by cysteine and not affected by TLCK. These discrepancies may be due to the fact that different strains were used by different investigators. Hemolytic activity in all strains, however, was heat- and trypsin-sensitive. We have isolated hemolysin from P. gingivalis A7436 as a single protein whose hemolytic activity was heat- and trypsin-sensitive, was unaffected by cysteine and was inhibited by DTT.

Chu et al. [3] have attempted the purification of P. gingivalis hemolysin but reported a loss of significant activity after ammonium sulfate precipitation and ion-exchange chromatography. Karunakaran and Holt [6] have suggested that a 48- and 18-kDa polypeptide confer hemolytic activity to P. gingivalis but failed to purify hemolysin from E. coli recombinants harboring P. gingivalis hemolysin gene(s). We have purified P. gingivalis hemolysin as a 45-kDa protein that causes pore formation in RBCs leading to leakage of cellular cytoplasm. It appears to be a strong immunogen inducing the production of neutralizing antibodies in mice. The hemolysin of P. gingivalis

<table>
<thead>
<tr>
<th>Amino acids</th>
<th>Hemolysina</th>
<th>Fimbrillinb</th>
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<tbody>
<tr>
<td>% Residues</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Asx</td>
<td>12.2 ± 1.0</td>
<td>13.7</td>
</tr>
<tr>
<td>Thr</td>
<td>8.2 ± 1.1</td>
<td>8.3</td>
</tr>
<tr>
<td>Ser</td>
<td>6.0 ± 0.9</td>
<td>4.4</td>
</tr>
<tr>
<td>Glx</td>
<td>9.5 ± 1.3</td>
<td>11.0</td>
</tr>
<tr>
<td>Pro</td>
<td>6.9 ± 1.2</td>
<td>3.4</td>
</tr>
<tr>
<td>Gly</td>
<td>11.4 ± 1.5</td>
<td>10.0</td>
</tr>
<tr>
<td>Ala</td>
<td>10.3 ± 1.1</td>
<td>12.7</td>
</tr>
<tr>
<td>Val</td>
<td>7.0 ± 0.1</td>
<td>6.0</td>
</tr>
<tr>
<td>Met</td>
<td>1.8 ± 0.1</td>
<td>1.5</td>
</tr>
<tr>
<td>Ile</td>
<td>4.2 ± 0.1</td>
<td>4.1</td>
</tr>
<tr>
<td>Leu</td>
<td>6.5 ± 0.2</td>
<td>7.3</td>
</tr>
<tr>
<td>Tyr</td>
<td>4.3 ± 0.2</td>
<td>3.8</td>
</tr>
<tr>
<td>Phe</td>
<td>3.4 ± 0.2</td>
<td>2.3</td>
</tr>
<tr>
<td>Lys</td>
<td>4.0 ± 0.2</td>
<td>7.2</td>
</tr>
<tr>
<td>His</td>
<td>2.5 ± 0.2</td>
<td>1.6</td>
</tr>
<tr>
<td>Arg</td>
<td>1.7 ± 0.1</td>
<td>1.8</td>
</tr>
<tr>
<td>Cys and Trp</td>
<td>ND</td>
<td>ND</td>
</tr>
</tbody>
</table>

ND, not determined.
aValues represent the mean value obtained from two different batches of purified hemolysin from P. gingivalis A7436.
bFimbrillin from P. gingivalis 2561 [12].
bound strongly to Ni-NTA, similar to the *Mycobacterium avium* hemolysin [8] and could be eluted with a combination of a high NH₄Cl concentration and low pH with no loss of activity. Because Ni-NTA is known for its ability to bind proteins containing surface-exposed neighboring histidine residues [7], it is possible that the *P. gingivalis* hemolysin possesses such residues. Hemolysin did indeed show reactivity with monoclonal antibody raised against polyhistidine (Sigma Chemical) in an ELISA (A⁴⁹₂ = 0.46).

Although two distinct hemolysin genes from *P. gingivalis* have been cloned in *E. coli* [6], the sequence of these genes has not been reported by the investigators [6]. Currently, there is no information on *P. gingivalis* hemolysin in databases. Thus, we were not able to compare our peptide sequence to these hemolysins. However, the peptide sequence of hemolysin isolated by us from *P. gingivalis* was different from that of fimbriolin. Hemolysin contained a higher amount of histidine, proline, serine and phenyl alanine and a lower amount of aspartate/asparagine, alanine and lysine as compared to fimbriolin [12]. Hemolysin also differed from fimbriolin in the following properties. (1) It lacks the ability to agglutinate RBCs, whereas fimbriolin has been reported to possess hemagglutinating activity [19]. (2) It can lyse RBCs, (3) it binds strongly to Ni-NTA and likely possesses surface-exposed polyhistidine residues. (4) It does not show multimeric forms like fimbriolin [12] and shows a single 45-kDa band at 80°C and at 100°C, unlike fimbriolin which shows a 43-kDa band at 100°C and multimeric forms at 80°C [12]. (5) Hemolysin is secreted into the culture supernatant and its concentration in CS increases with time, while that in the cells decreases with an increasing time period. The results show that, except for some sequence homology, *P. gingivalis* hemolysin is distinct from fimbriolin.

It is possible that in vivo, hemolysin, HAg and RGP go hand in hand in agglutinating RBCs and digesting surface proteins of RBCs resulting in the release of hemoglobin. Released hemoglobin may then be captured by the hemoglobin receptor protein (HbR) of *P. gingivalis* [16] and proteolytically degraded to release hemin. Hemin, which is very important in the survival and virulence of this organism [20], could then be stock-piled by *P. gingivalis* cells. Thus, hemolysin and the RGP/HAg/HbR complex probably exist closely in vivo facilitating efficient and effective cooperation for acquiring iron in vivo. This ability to remove heme from hemoglobin in a highly hemorrhagic milieu provides the organism with a distinct advantage to grow and emerge in the complex environment of the periodontium. The effects of hemolysin on different host cells may provide insights into the pathogenesis and virulence of *P. gingivalis*.

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