

Hemoglobinase Activity of the Lysine Gingipain Protease (Kgp) of *Porphyromonas gingivalis* W83

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Porphyromonas gingivalis, an important periodontal disease pathogen, forms black-pigmented colonies on blood agar. Pigmentation is believed to result from accumulation of iron protoporphyrin IX (FePPIX) derived from erythrocytic hemoglobin. The Lys-X (Lys-gingipain) and Arg-X (Arg-gingipain) cysteine proteases of *P. gingivalis* bind and degrade erythrocytes. We have observed that mutations abolishing activity of the Lys-X-specific cysteine protease, Kgp, resulted in loss of black pigmentation of *P. gingivalis* W83. Because the hemagglutinating and hemolytic potentials of mutant strains were reduced but not eliminated, we hypothesized that this protease played a role in acquisition of FePPIX from hemoglobin. In contrast to Arg-gingipain, Lys-gingipain was not inhibited by hemin, suggesting that this protease played a role near the cell surface where high concentrations of hemin confer the black pigmentation. Human hemoglobin contains 11 Lys residues in the α chain and 10 Lys residues in the β chain. In contrast, there are only three Arg residues in each of the α and β chains. These observations are consistent with human hemoglobin being a preferred substrate for Lys-gingipain but not Arg-gingipain. The ability of the Lys-gingipain to cleave human hemoglobin at Lys residues was confirmed by electrospray ionization Fourier transform ion cyclotron resonance mass spectrometry of hemoglobin fragments resulting from digestion with the purified protease. We were able to detect several of the predicted hemoglobin fragments rendered by digestion with purified Lys-gingipain. Thus, we postulate that the Lys-gingipain of *P. gingivalis* is a hemoglobinase which plays a role in heme and iron uptake by effecting the accumulation of FePPIX on the bacterial cell surface.

Iron is an indispensable nutrient for growth of most living organisms. In humans, most of the iron is present intracellularly as hemoglobin (76%) and ferritin (23%). Extracellular iron is rapidly bound by transferrin in serum and lactoferrin at mucosal surfaces (38). Because the in vivo concentration of free iron is too low (10^{-18} M) to support the growth of microorganisms (20), specialized iron acquisition mechanisms are needed for bacterial colonization of the human host. Many pathogenic bacteria produce siderophores capable of chelating iron from transferrin and lactoferrin (8). Also, in *Neisseria gonorrhoeae* siderophore-independent acquisition of iron from transferrin and lactoferrin through direct binding to a specific receptor has been observed (33).

Hemoglobin, the largest reservoir of iron in the human adult (38), is a tetramer composed of two α and two β polypeptide chains. Each polypeptide chain has a noncovalently bound heme group consisting of a porphyrin ring and a ferrous atom. The heme group is another nutrient used by bacteria as a cofactor for cytochromes and catalase (52, 56). However, heme and heme-associated iron are not normally available to pathogenic organisms. Hemoglobin released by lysis of erythrocytes is rapidly bound by haptoglobin and transported to the liver. Also, heme released from hemoglobin is bound by hemopexin. Although several gram-negative bacteria such as *Neisseria* species, *Haemophilus influenzae*, *Vibrio* species, *Bacteroides fragilis*, and *Escherichia coli* are known to use heme and heme iron from hemoglobin (38, 39), the mechanisms of heme extraction from hemoglobin remain poorly understood.

Porphyromonas gingivalis, an obligately anaerobic bacterium, is recognized as an etiologic agent of adult periodontitis (29, 46, 47). It forms black-pigmented colonies resulting from accumulation of hemin (oxidized form of heme) on the cell surface and within the bacterial cell when grown on blood agar (44, 50). Hemin has been shown to be an important supplement for *P. gingivalis* growth (19, 44). Since this organism is unable to synthesize protoporphyrin IX (42) and siderophores have not been reported in this organism (2, 16), hemin may serve a dual nutritional function as an iron and protoporphyrin IX source. At the site of infection, hemoglobin derived from lysed erythrocytes abundant in periodontal pockets is the most probable source of this compound. Although *P. gingivalis* is capable of utilizing various hemin-containing compounds (2, 16), Shizukuishi et al. have shown that this organism utilizes hemoglobin more efficiently than other iron-containing compounds (45).

The mechanism of hemin acquisition from erythrocytes involves hemagglutination, hemolysis, binding, and degradation of the hemoglobin molecule. *P. gingivalis* produces large quantities of cysteine proteases with either arginine (Arg-gingipain; *rgpA* and *rgpB* genes) or lysine (Lys-gingipain; *kgp* gene) specificity. The hemagglutinin/adhesin domain of these enzymes, which is similar to the *P. gingivalis* HagA hemagglutinin (21), has been shown to possess hemagglutinin activities (9, 40). *P. gingivalis* is also able to lyse erythrocytes (5). Although Arg-gingipain has been observed to possess hemolytic activity (43), the role of Lys-gingipain in hemolysis remains unknown. Recently, a hemoglobin binding protein called HbR, intragenically encoded by the *rgpA*, *kgp*, and *hagA* genes, has been identified (35). In addition, the Lys-gingipain has shown hemoglobin binding activity (28). Last, although the hemoglobinolytic potential of *P. gingivalis* has been demonstrated (14,

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TABLE 1. Strains of *P. gingivalis* used in this study

Strain	Characteristics	Comment
W83	RgpA ⁺ Kgp ⁺	Wild type
V2383	RgpA ⁻ Kgp ⁻	Allelic exchange mutant ^a in <i>rgpA</i> , IS195 insertional mutant in <i>kgp</i> (30) (unpublished data)
V2448	RgpA ⁺ Kgp ⁺ HagA ⁻	Allelic exchange mutant ^a in <i>hagA</i> (unpublished data)
V2543	RgpA ⁺ Kgp ⁻	IS195 insertional mutant in <i>kgp</i> (30)
V2546	RgpA ⁻ Kgp ⁺	Allelic exchange mutant ^a in <i>rgpA</i> (unpublished data)
V2577	RgpA ⁺ Kgp ⁻	Allelic exchange mutant in <i>kgp</i> (30)
V2602	RgpA ⁺ Kgp ⁺	V2543 with restored <i>kgp</i> gene constructed in this study

^a Constructed by using the *ermF-ermAM* cassette described and used as previously published (13).

45), the molecular mechanisms of acquiring heme and iron from hemoglobin have not been elucidated.

We have constructed mutants in Lys-gingipain gene, *kgp*, of *P. gingivalis* W83 (30). Using the mouse abscess model, we demonstrated that Kgp played a role in virulence of *P. gingivalis* (30). We have since observed that when grown on blood agar plates these mutants appear white, suggesting a major role of this protease in acquisition of heme from erythrocytes. Because neither the hemagglutinating nor the hemolytic potentials of the mutant strains were totally eliminated, we reasoned that degradation of hemoglobin resulting in release of heme might be involved. To test this hypothesis, we have compared the hemoglobinolytic activities of the mutant and parent strains. These results have been confirmed in studies using a *P. gingivalis* strain with restored Kgp activity and purified Kgp protein. Our results indicate that the Lys-gingipain is

a hemoglobinase. This observation suggests that this enzyme plays a role in heme and iron acquisition by efficient extraction of iron protoporphyrin from hemoglobin that in turn is accumulated on the bacterial cell surface.

MATERIALS AND METHODS

Bacterial strains and plasmids. *P. gingivalis* strains used in this study are described in Table 1. W83 was obtained from H. A. Schenkein, VCU Clinical Research Center for Periodontal Diseases. *P. gingivalis* V2543 was a naturally occurring variant of *P. gingivalis* W83 carrying an insertion sequence (IS)-like element, IS195 inserted into *prtP* (30). However, we now have abandoned this gene designation in favor of *kgp*, indicating the conserved lysine-specific cysteine protease (Lys-gingipain) typically found in *P. gingivalis* (10). We use that genotypic designation hereafter in this report, with Kgp used to designate the Lys-gingipain protease. *P. gingivalis* V2577 was an allelic exchange mutant of W83 carrying the *ermF-ermAM* cassette inserted into the prepropeptide domain of the *kgp* gene (30). *P. gingivalis* V2602 was constructed in this study by restoration of the V2543 mutation via recombination (Fig. 1). V2383 was a double mutant

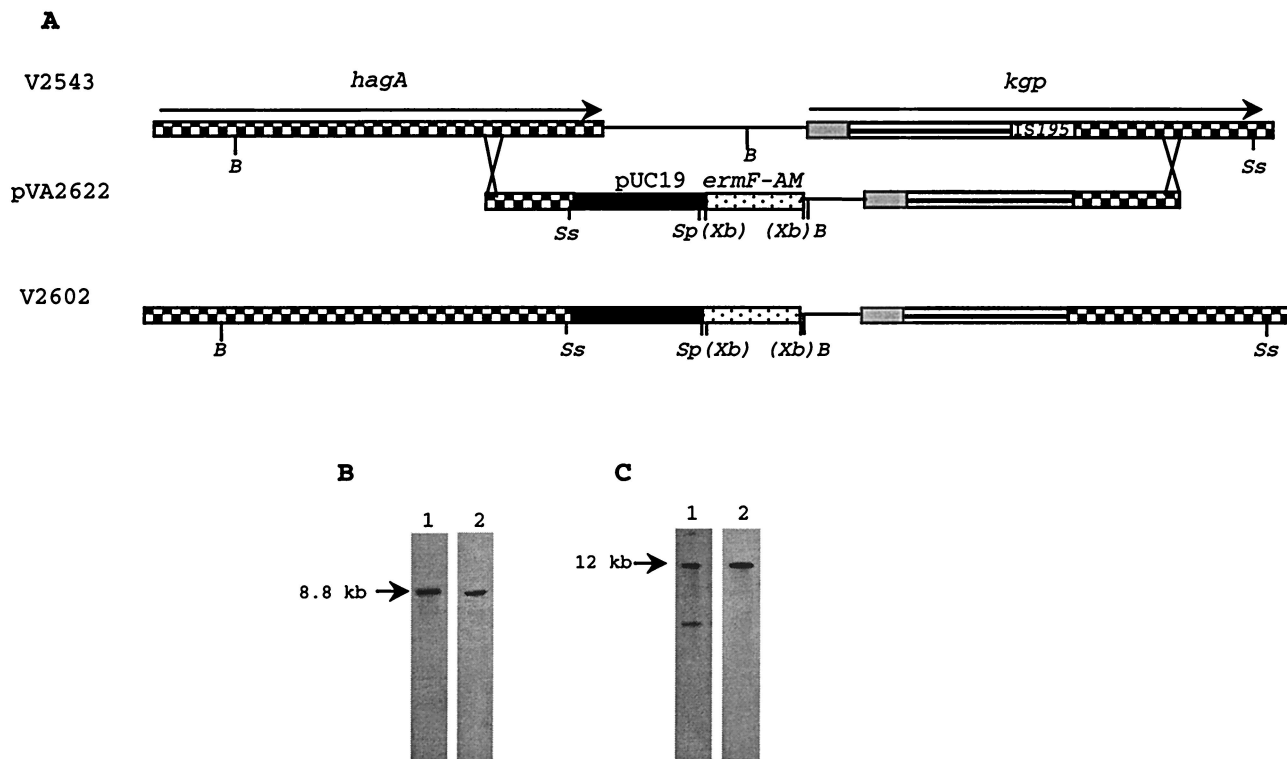


FIG. 1. Restoration of the *kgp* gene in *P. gingivalis* V2543. (A) Maps of *hagA* and *kgp* on the chromosome of V2543. The *hagA* and *kgp* open reading frames are indicated by rightward arrows. Different regions of the *kgp* gene encoding different domains are indicated by various patterns: prepropeptide (grey), protease domain (parallel lines), and hemagglutinin domain (checkerboard). Shadings and patterns indicate similar sequences. Relevant restriction sites: *B*, *Bam*HI; *Xb*, inactive *Xba*I; *Sp*, *Sph*I; *Ss*, *Sst*I. (B) Southern blot analysis of *Sst*I/*Sph*I-digested chromosomal DNA of V2602, probed with the *kgp* fragment encoding the prepropeptide domain (lane 1) and *ermF-ermAM* cassette (lane 2). (C) Southern blot analysis of *Bam*HI-digested chromosomal DNA of V2602 probed with a 1.2-kb fragment encoding hemagglutinin (lane 1) and *ermF-ermAM* cassette (lane 2).

(RgpA and Kgp deficient), V2448 was a HagA mutant, and V2546 was an RgpA mutant.

E. coli TB1 and pUC18 were used in all cloning work. pVA2622 was a suicide vector constructed by cloning of the *ermF-ermAM* cassette into *Xba*I site of a recombinant plasmid consisting of pUC18 and 6.8-kb fragment carrying the entire *kgp* gene from *P. gingivalis* W12 (obtained from A. Progulsk-Fox, Department of Oral Biology, University of Florida, Gainesville) (Fig. 1). The *kgp* gene from *P. gingivalis* W12 was 99.9% identical to the *kgp* homolog from *P. gingivalis* W83 (28).

Growth media and conditions. *P. gingivalis* was grown in brain heart infusion broth (Difco Laboratories, Detroit, Mich.) supplemented with hemin (5 µg/ml), vitamin K₂ (0.5 µg/ml), and cysteine (1%). Agar (2% [wt/vol]) was added when solid medium was required. Cultures were incubated at 37°C in an anaerobic chamber (Coy Manufacturing, Ann Arbor, Mich.) in 80% N₂-10% CO₂-10% H₂. To evaluate black pigment formation, *P. gingivalis* strains were inoculated onto blood agar plates and incubated for 4 days anaerobically at 37°C and then for 10 days anaerobically at room temperature. *E. coli* strains were cultivated aerobically at 37°C in Luria-Bertani (LB) broth (Gibco, BRL Inc., Gaithersburg, Md.). Solid LB medium was obtained by addition of 2% (wt/vol) agar. Antibiotic concentrations used for selection were 0.5 µg/ml for clindamycin (*P. gingivalis*) and 300 µg/ml for erythromycin (*E. coli*).

Restoration of Kgp activity in Kgp mutant. The genetic strategy for restoration of the *kgp* defective gene is depicted in Fig. 1. Briefly, recombinant pUC18 carrying the *ermF-ermAM* cassette and the entire *kgp* gene was electroporated into *P. gingivalis* V2543 (carrying the inactivated *kgp* gene due to insertion of IS195) (30) according to the protocol of Fletcher et al. (13). Clindamycin-resistant colonies testing positive for Lys-gingipain activity were analyzed by Southern blotting to verify the presence and correct chromosomal location of the intact *kgp* gene.

Protein preparation. Extracellular membrane vesicles were prepared as described previously (30). Partially purified Kgp protease from V2546 was prepared by a combination of ammonium sulfate saturation followed by a hemoglobin affinity column with the buffer system of Fujimura et al. (15). Bacteria (2 liters) were grown for 72 h and harvested by centrifugation (10,000 × g, 30 min, 4°C). Extracellular vesicles and proteins present in the culture supernatant were precipitated by addition of ammonium sulfate (40 to 60% saturation), harvested by centrifugation (20,000 × g, 40 min, 4°C), suspended in 120 ml of Tris-HCl buffer (50 mM, pH 9.5) and dialyzed overnight against the same buffer. Vesicular proteins were harvested by centrifugation (15,000 × g, 40 min, 4°C) and stored in 20 ml of sodium acetate buffer (50 mM, pH 5.5). Vesicular proteins were solubilized by addition of octylthioglucoiside (OTG) (54) to a final concentration of 0.6% followed by overnight gentle mixing at 4°C. The mixture was centrifuged at 150,000 × g for 1 h to remove insoluble material, and the supernatant was applied to hemoglobin-conjugated agarose (Sigma Chemical Co., St. Louis, Mo.) column (1 by 5 cm) equilibrated with 50 mM sodium acetate buffer (pH 5.5) containing 0.6% OTG. The column was washed with 3 volumes of the same buffer followed by a wash with 50 mM sodium phosphate buffer (pH 7.2) containing 300 mM sodium chloride and 0.6% OTG. Hemoglobin-bound proteins were eluted with 50 mM Tris-HCl buffer (pH 9.0) containing 0.6% OTG. Affinity column fractions (5 ml each) were analyzed for protein content by monitoring absorbance at 280 nm (*A*₂₈₀). Protease activity was determined with the chromogenic substrates 1 mM *N*-α-benzoyl-DL-arginine-*p*-nitroanilide (BAPNA) (Sigma) and 1 mM *N*-α-benzoyloxycarbonyl-L-lysine-*p*-nitroanilide (*Z*-Lys-*p*NA) (Novabiochem, La Jolla, Calif.) in 50 mM Tris-HCl buffer-10 mM L-cysteine-1 mM CaCl₂ (for Arg-X activity) and pH 7.5 (for Arg-gingipain activity) or 8.0 (for Lys-gingipain activity); 20 µl of eluted fractions and 10 µl of starting material were used in enzyme activity assays (Fig. 5).

Protease activity. Extracellular vesicular protease activity was assayed as described previously (30). Reaction mixtures of 500 µl containing 36 µg of extracellular vesicular proteins were incubated at 37°C in either 50 mM Tris-HCl (pH 7.2)-1 mM BAPNA in the presence of 2 mM dithiothreitol (DTT) or 60 mM Tris-HCl (pH 8.0)-1 mM *Z*-Lys-*p*NA in the presence of 2 mM DTT. Hydrolysis was measured by monitoring *A*₄₀₅. The effect of hemin and imidazole on protease activity was determined by using 10 µg of extracellular vesicular proteins in reaction mixture as described above supplemented with either 15 µM hemin or 20 mM imidazole.

Lysis of erythrocytes. Sheep defibrinated blood obtained from BBL (Becton Dickinson Microbiology Systems, Cockeysville, Md.) was washed in phosphate-buffered saline (PBS) until the supernatant was free of red pigment. Bacterial cells were also washed in PBS and adjusted to an optical density at 660 nm (OD₆₆₀) of 1. Vesicles were prepared as described above. Reaction mixtures were prepared by mixing 1 ml of 2.5% (vol/vol) in PBS sheep erythrocytes containing 2 mM DTT with 50 µl of bacterial cells (OD = 1) or 36 µg of vesicular proteins. After 8 and 15 h of incubation with gentle mixing at 37°C, intact erythrocytes were removed by centrifugation 10,000 × g for 5 min. The hemolytic activity was determined by monitoring absorbance for red pigment released from lysed cells at 520 nm.

Hemagglutination assays. Hemagglutination activity was determined by using bacterial cells (50 µl, OD = 1) and extracellular vesicles (18 µg) as described previously (4).

Hemoglobinase activity. For assay of hemoglobinase activity of vesicular proteins, 500-µl reaction mixtures containing 72 µg of extracellular proteins and 4

µg of bovine hemoglobin (Sigma) per µl (2 mg) in 50 mM Tris-HCl (pH 8.0) were incubated 15 h at 37°C in the presence of 2 mM DTT; 20 µl of this mixture was added to an equal volume of reducing sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) sample buffer (Laemmli sample buffer; Bio-Rad, Hercules, Calif.), boiled for 10 min, and electrophoresed on a 15% polyacrylamide gel. Hemoglobin fragments were visualized by staining with Coomassie blue (0.2% Coomassie blue R-250 in destaining solution) and destaining in destaining solution (45% ethanol, 10% acetic acid). For assay of hemoglobinase activity of purified Kgp protease, a 200-µl reaction mixture containing 150 µg of pure Kgp protease and 600 µg of human hemoglobin (Sigma) in 50 mM Tris-HCl (pH 8.0) was incubated for 15 h at 37°C in the presence of 2 mM DTT; 15 µl of this mixture was analyzed on a 15% polyacrylamide gel. Proteolysis of hemoglobin was visualized as described above.

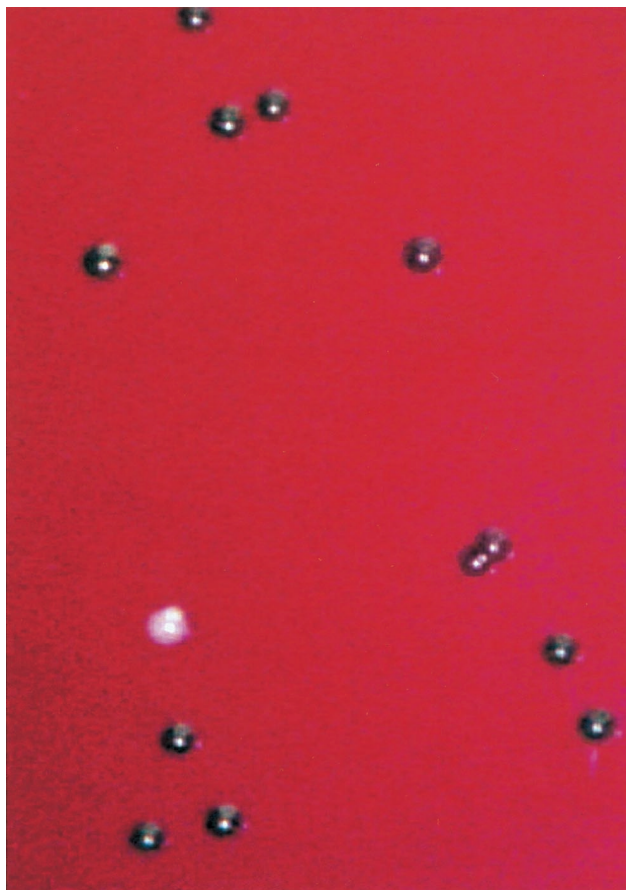
Microdialysis. The development of a microdialysis approach to prepare oligonucleotides, peptides, and proteins has been reported elsewhere in detail (23) and is described here briefly. The protein digests contained >10 mM Tris-HCl as well as additional nonvolatile, monatomic ionic species (e.g., Na⁺). To ensure that the sample was compatible with the electrospray ionization (ESI) process, a rapid (<3 min), single-step microdialysis approach was used. The microdialysis system employed a 13,000-molecular-weight-cutoff (MWCO) conical cellulose acetate dialysis fiber with a counter-current buffer of 10 mM ammonium acetate with a gravity-induced flow rate of 1.2 ml/min (23). Typically, 5 µl of sample was injected into the microdialysis system at a flow rate of 2 µl/min; desalting efficiencies greater than 99.9% were routinely achieved with the dialysate collected in a microcentrifuge tube (23).

Mass spectrometry. Mass spectrometric data for hemoglobin fragments rendered by digestion with purified Kgp were obtained using an ESI (12)-Fourier transform ion cyclotron resonance (FTICR) mass spectrometer (7) modified to accept micro- and nanoelectrospray sources. Electrospray emitters were 50-µm fused-silica capillaries pulled to ca. 10 to 20 µm as previously reported (22). Electrosprayed ions entered into the dielectric capillary of the electrospray ionization source (Analytica of Branford, Inc., Branford, Conn.) coupled to an ESI-FTICR mass spectrometer (Ionspec, Irvine, Calif.). The Ionspec Omega 586 data station was used for processing and signal generation, and the broad-band pulse sequence was employed. The instrument utilized a 4.7-T horizontal bore superconducting magnet with a 128-mm bore (Cryomagnetics, Inc., Oak Ridge, Tenn.). Mass calibration of the FTICR was accomplished by using the isotopically resolved multiply charged ions of bovine ubiquitin as the external standard. The calibration parameters were subsequently verified by analysis of the 5+ charge state of bovine insulin, which resulted in a mass error of less than 10 ppm (0.001%). The dialyzed samples were microsprayed from a solvent of 49/49/2 (vol %) methanol-10 mM ammonium acetate-acetic acid with a flow rate of 0.5 µl/min. Ions were injected for 2 s, and all spectra obtained were single acquisitions.

RESULTS

Restoration of the defective *kgp* gene. To determine whether inability to both accumulate black pigment and degrade hemoglobin resulted from inactivation of the *kgp* gene, we restored the Kgp activity in Kgp-deficient mutant. Two types of Kgp mutants (V2543 and V2577) used in this study were described and characterized previously (28). While V2543 was a naturally occurring mutant resulting from insertion of the IS-like element IS195 into the *kgp* gene, V2577 was an allelic exchange mutant of the *kgp* gene of *P. gingivalis* W83 constructed by insertion of the *ermF-ermAM* gene cassette into the prepropeptide region of *kgp*. The genetic structure of V2543 mutant is shown in Fig. 1A. We chose the Kgp mutant V2543 as a starting point for the restoration of *kgp* gene because it contained neither suicide vector nor resistance cassette integrated into the chromosome.

Following electroporation of *P. gingivalis* V2543 with a suicide vector containing an intact copy of the *kgp* gene and *ermF-ermAM* gene cassette, pVA2622, six clindamycin-resistant colonies were detected after a 10-day incubation period on brain heart infusion plates containing clindamycin. Only one of the six colonies exhibited Kgp activity. This colony was characterized by Southern blot analysis, and the hybridization pattern of *Sph*I-*Sst*I-digested genomic DNA probed with a fragment of *kgp* encoding for the propeptide sequence revealed the presence of a complete copy of the *kgp* gene (Fig. 1A and B, lane 1). These results were confirmed by probing the same blot with the *ermF-ermAM* cassette (Fig. 1A and B, lane 2) (13). Further analyses using V2602 *Bam*HI-digested genomic DNA



probed with a 1.2-kb fragment internal to *hagA* and the *ermF-ermAM* cassette indicated that the insertion of the suicide vector pVA2622 occurred by a double crossover between the hemagglutinin-encoding portion of the *kgp* gene present in the suicide vector and the homologous sequences present in *kgp* and *hagA* genes resident on the chromosome of V2543 (Fig. 1A and C). The recombination that regenerated the *kgp* gene in V2602 was evidently accompanied by a 3-kb deletion upstream of the *kgp* sequence (Fig. 1A). This deletion was replaced by *ermF-ermAM* gene and pUC18 sequence present in the suicide vector. This event may explain the decreased Lys-X proteolytic activity of the strain with restored *kgp* gene compared to the wild-type strain.

Growth of *P. gingivalis* strains on blood agar plates. *P. gingivalis* grown on blood agar plates forms black colonies after several days of incubation. This black colony pigmentation has been shown to be a result of accumulation of hemin derived from erythrocytes on a surface and within bacterial cells (44, 50). All strains tested were able to grow on blood agar plates; however, mutants containing a disrupted *kgp* gene lost the ability to accumulate black pigment (Fig. 2). This ability was restored in V2602 strain containing the restored *kgp* gene (Fig. 2).

Protease activity. Both cells and extracellular vesicles were assayed for protease activity. Examination of extracellular vesicles of *P. gingivalis* strains for protease activity revealed that Lys-gingipain activity was sixfold lower in both V2543 and V2577 than in the wild-type strain (Table 2). This activity was largely restored (70% of wild-type activity) in the strain V2602 containing the restored *kgp* gene. The Lys-gingipain activity of

Strain	Characteristics	Colony
W83	Wild type	Black
V2543	IS195 insertional mutant in <i>kgp</i> (30)	White
V2577	Allelic exchange mutant in <i>kgp</i> (30)	White
V2602	V2543 with restored <i>kgp</i> gene	Black

FIG. 2. Accumulation of black pigment by *P. gingivalis* strains grown on blood agar. *P. gingivalis* strains were inoculated onto blood agar medium and incubated for 4 days anaerobically at 37°C followed by 10 days anaerobically at room temperature. Examples of the two colonial phenotypes are shown. To obtain the photograph, *P. gingivalis* cultures of W83 and V2577 were mixed, diluted, and spread on blood agar plates. Well-isolated colonies thus allowed the simultaneous viewing of both pigment phenotypes. Black-pigmented colonies were formed by the wild-type strain (W83). The mutation abolishing the Lys-gingipain activity (V2577) resulted in loss of pigmentation (nonpigmented colony in the photograph). Strains examined in this study are described at the right.

cells of the Kgp-deficient strains (V2543 and V2577) was fivefold lower than that of the parental W83 and V2602 strains (Table 2). While Arg-gingipain activities were similar for all strains tested, the activity of extracellular vesicles was reduced in the insertion mutant (V2543) and in the strain with restored Lys-gingipain activity (V2602) (results not shown).

Effect of hemin and imidazole on proteolytic activity. Production of white colonies by Kgp-deficient strains indicated that there was a link between Kgp and accumulation of hemin from erythrocytes present on blood agar plates. This prompted our interest in the effect of hemin on Kgp activity. We believed that there may be some hemin present in vesicular preparations and so used imidazole, which interacts with the iron atom present in the hemin molecule, thus distorting the hemin structure (1, 57) and resulting in loss of its ability to bind protein. Arg-gingipain activity was lower in the presence of hemin and higher in the presence of imidazole compared to the control reaction (Table 3). These results indicated the inhibitory effect of hemin on Arg-gingipain and confirmed the results of U et al. (55). Lys-X activity was not affected by the presence of either hemin or imidazole (Table 3).

Hemagglutination activity. Since spontaneous mutants deficient in the ability to attach to erythrocytes also have shown loss of pigmentation (4), we wondered whether similar results would be obtained for our Kgp-deficient strains. *P. gingivalis*

TABLE 2. Lys-gingipain proteolytic activities of extracellular vesicles and cells of *P. gingivalis* strains

Strain	A_{405}^a	
	Vesicles	Cells
W83	2.8, 3.0	1.0, 1.0
V2543	0.5, 0.5	0.2, 0.2
V2577	0.5, 0.5	0.2, 0.2
V2602	2.1, 2.2	0.9, 0.9

^a Proteolytic activity was measured spectrophotometrically by monitoring the release of *p*-nitroanilide resulting from hydrolysis of Z-Lys-*p*NA detectable at A_{405} . Results for two obtained in two independently performed assays are presented.

TABLE 3. Effects of hemin and imidazole on protease activity of extracellular vesicles of *P. gingivalis* W83

Addition to reaction	A_{405}^a					
	Arg-gingipain activity			Lys-gingipain activity		
	15 min	30 min	60 min	15 min	30 min	60 min
None	0.4	0.6	0.9	0.5	0.8	1.2
Hemin	0.1	0.1	0.1	0.5	0.7	1.2
Imidazole	1.7	2.2	2.6	0.5	0.8	1.2

^a Protease activity was monitored by change of A_{405} resulting from release of *p*-nitroanilide from chromogenic substrates.

W83, V2543, V2577, and V2602 were assessed for hemagglutination ability. The hemagglutinating activity of cells of the strain containing the restored *kgp* gene, V2602, was comparable to that of the wild type. However, the Kgp-deficient strains showed significantly reduced hemagglutination activity (eightfold for V2543 and fourfold for V2577 [Fig. 3A]). The activity of extracellular vesicles of the wild-type strain exceeded that of the insertion mutant strain V2543 (fourfold) and the complemented strain V2602 (fourfold) but was similar to that of the allelic exchange mutant V2577 (Fig. 3B).

Hemolytic activity. Hemolytic activities of extracellular vesicles and cells of the Kgp-deficient mutants V2543 and V2577 was lower than hemolytic activities of vesicles and cells of the wild-type strain (Table 4). Hemolytic activity was partially restored in cells of the *P. gingivalis* strain with a restored *kgp* gene (V2602) (Table 4).

Hemoglobinase activity. To assess the hemoglobinase activity of Kgp, we examined bovine hemoglobin degradation by extracellular vesicular proteins from *P. gingivalis* W83, V2543, V2577, V2602, V2383, V2448, and V2546. Hydrolytic activities of Kgp-deficient strains V2543 and V2577 were significantly lower than that of wild-type strain W83 (Fig. 4, lanes 3 to 6). While in the case of the wild-type strain almost all hemoglobin used for the reaction was degraded into fragments that were not detectable on an SDS-15% polyacrylamide gel (Fig. 4, lanes 3 and 4), Kgp mutants degraded only part of the hemoglobin used for the reaction and created at least one detectable fragment (approximately 6 to 8 kDa) (Fig. 4, lanes 5, 6, and 8).

TABLE 4. Hemolytic activities of extracellular vesicles and cells of *P. gingivalis* strains

Strain	A_{520}^a			
	Vesicles		Cells	
	8 h	15 h	8 h	15 h
W83	0.70	0.97	0.40	0.62
V2543	0.27	0.58	0.24	0.33
V2577	0.24	0.67	0.22	0.32
V2602	0.30	0.51	0.28	0.54

^a Hemolysis of erythrocytes was monitored by the change of absorbance for red pigment resulting from release of hemoglobin from the lysed cells at 520 nm in assays using 36 μ g of extracellular vesicles or 50 μ l of cells (OD = 1).

The ability to degrade hemoglobin into fragments no longer detectable on SDS-polyacrylamide gels was restored in the strain containing the restored *kgp* gene, V2602 (Fig. 4, lane 7). The hemoglobin degradation pattern by RgpA⁻ (V2546 [Fig. 4, lane 10]) and HagA⁻ (V2448 [Fig. 4, lane 9]) strains was similar to that of the wild-type strain. The double mutant V2383 (RgpA⁻ Kgp⁻) displayed a digestion pattern similar to that of the Kgp-deficient strains (Fig. 4, lanes 5, 6, and 8).

Purification of Kgp from *P. gingivalis*. Hemoglobinolytic activities for vesicles indicated that although both Arg-gingipain and Lys-gingipain were capable of degrading hemoglobin, the latter protease was far more efficient because it degraded hemoglobin into many fragments undetectable by SDS-PAGE. For further analysis of hemoglobin fragments rendered by Lys-gingipain, we separated the Arg-gingipain and Lys-gingipain activities. Since Lys-gingipain, but not Arg-gingipain, has been shown to bind hemoglobin (28), we chose hemoglobin affinity chromatography to isolate the Lys-gingipain. We obtained one fraction exhibiting Lys-gingipain activity but not Arg-gingipain activity (Fig. 5, fraction 14) and considered it a partially purified Kgp. The hemoglobinase activity of partially purified Kgp was comparable to that obtained with extracellular vesicles from wild-type strain W83 (Fig. 6).

Hemoglobinase activity of Kgp. We used mass spectrometry to demonstrate fragments of the hemoglobin α and β chains predicted to occur following Kgp digestion. Figure 7B shows a

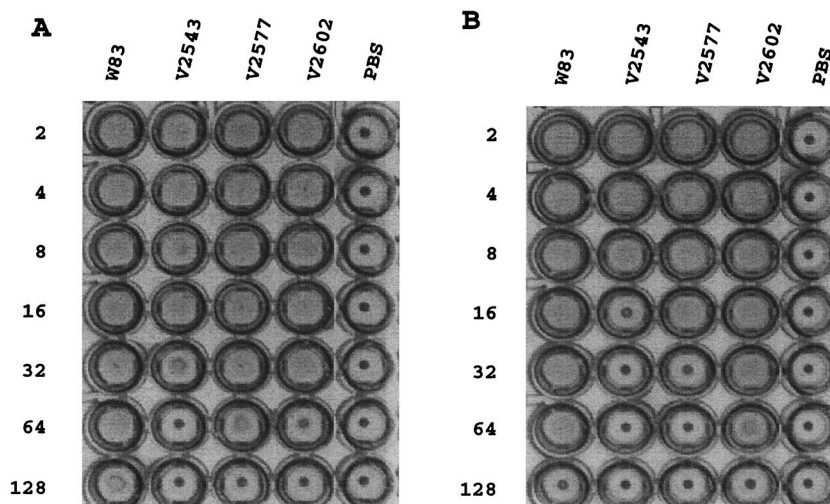


FIG. 3. Hemagglutinating activities of *P. gingivalis* strains. Hemagglutinating activities of 18 μ g of extracellular vesicles (A) and 50 μ l of cells (B) were analyzed as described in Materials and Methods. The reciprocals of serial twofold dilutions are shown at the left; the strains tested are given at the top. PBS was used as a control.

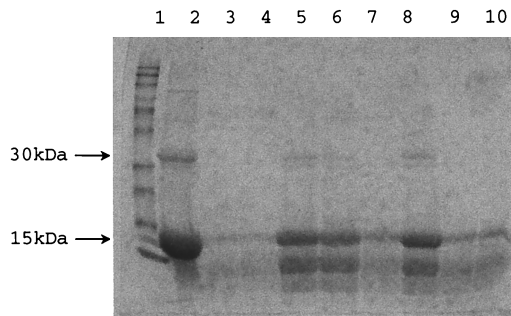


FIG. 4. Hemoglobinase activities of extracellular proteins of *P. gingivalis* strains. Samples of 500 μ l containing 72 μ g of extracellular vesicular protein and 4 μ g of bovine hemoglobin (Sigma) per μ l in 50 mM Tris-HCl (pH 8.0) were incubated 15 h at 37°C in the presence of 2 mM DTT; 20 μ l of this mixture was added to equal volume of reducing SDS-PAGE sample buffer (Laemmli sample buffer; Bio-Rad), boiled for 10 min, and electrophoresed on a 15% polyacrylamide gel. Proteolysis of hemoglobin was visualized as described in Materials and Methods. Lanes: 1, BenchMark prestained protein ladder (Gibco, BRL); 2, hemoglobin only; 3, hemoglobin, W83, and DTT; 4, hemoglobin and W83; 5, hemoglobin, V2577, and DTT; 6, hemoglobin, V2543, and DTT; 7, hemoglobin, V2602, and DTT; 8, hemoglobin, V2383 (RgpA⁻ Kgp⁻), and DTT; 9, hemoglobin, V2448 (HagA⁻), and DTT; 10, hemoglobin, V2546 (RgpA⁻), and DTT.

typical positive-ion ESI-FTICR mass spectrum of the hemoglobin fragments rendered by Kgp. The fragments in the protease digest were observed as protonated species, $(M + nH)^{n+}$, where n is the number of protons. Direct charge state determination was facilitated by the isotopic resolution obtained over the wide m/z range where the fragments reside which is unique to FTICR; the charge (z) was determined as the reciprocal of the carbon-13 isotopic spacing (24). The two insets in Fig. 7B illustrate the isotopic resolution that was achieved for two different fragments varying in intensity. Mass accuracy for the observed digestion fragments were determined from the monoisotopic peak, with errors typically being less than 60 ppm (0.006%) compared to the theoretical masses. Figure 7A illustrates the sequences of the Kgp peptides demonstrated by ESI-FTICR.

Table 5 summarizes the observed Kgp digestion fragments observed in the ESI-FTICR mass spectrum (Fig. 7) in addition to the theoretical monoisotopic masses. Digest fragments of less than 700 Da, which account for 9 of the 23 possible digestion fragments, were not observed or expected due to the essential usage of the microdialysis purification step. Microdi-

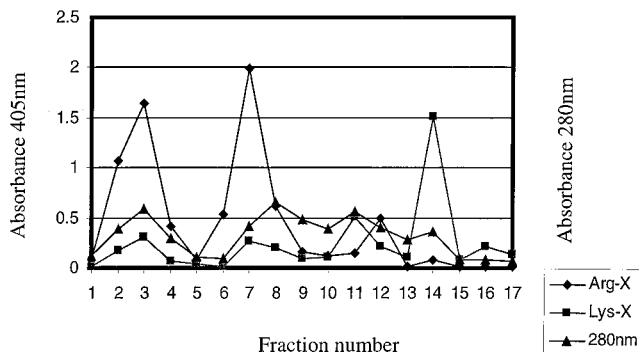


FIG. 5. Hemoglobin-agarose chromatography. The 40 to 60% ammonium sulfate fraction from *P. gingivalis* culture supernatant solubilized with 0.6% (wt/wt) OTG was subjected to hemoglobin-agarose chromatography. Fractions (5 ml) were collected and assayed for protein content (A_{280}) and protease activity (A_{405}) as described in Materials and Methods.

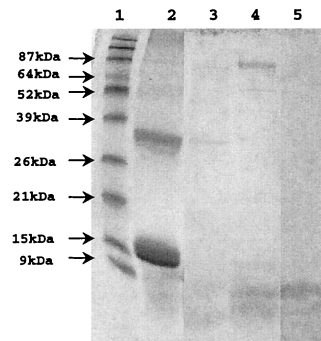


FIG. 6. SDS-PAGE analysis of hemoglobinase activity of purified Kgp. A 200- μ l reaction mixture containing 600 μ g of human hemoglobin and 2 mM DTT in 50 mM Tris-HCl (pH 8.0) was incubated 15 h at 37°C in the presence of either 150 μ g of pure Kgp protease or 30 μ g of vesicular proteins from W83; 15 μ l of this mixture was analyzed on a 15% polyacrylamide gel. Protein fragments were visualized by staining with Coomassie blue R-250. Lanes: 1, BenchMark prestained protein ladder; 2, hemoglobin; 3, hemoglobin plus Kgp; 4, Kgp; 5, hemoglobin plus W83 vesicles.

alysis of protein standards of various molecular masses (1.2 to 29 kDa) using the same 13,000-MWCO dialysis fiber has been shown to filter out low-molecular-weight proteins in addition to the buffer and salt (23). The observance of proteins less than 13,000 Da is not uncommon, and it is also speculated to be related to the initial concentration of the peptide or protein (23). The observed hemoglobin fragments verify Kgp digestion at 5 of the 10 expected cleavage sites for the α chain and 6 of the 9 for the β chain. The demonstration of several of the predicted Kgp-generated peptides argues strongly against the possibility of hemoglobin degradation by other proteases or peptidases that might be present in our partially purified enzyme preparation. Additional peaks in the spectrum not related to hemoglobin suggests autodigestion of Kgp.

DISCUSSION

The existence of nonpigmented variants of *P. gingivalis* has been reported, but the mechanism of pigment accumulation from erythrocytes has not been previously explained (18, 25, 37). Although other work (35) has suggested that pigmentation may be related to Kgp activity, we have demonstrated here that the black pigmentation of *P. gingivalis* colonies observed on blood agar plates is dependent on Kgp activity. We have shown

TABLE 5. Comparison of theoretical and experimental monoisotopic masses of human hemoglobin fragments

Fragment	Monoisotopic mass (Da)	
	Theoretical	Measured
α chain		
VLSPADK	728.4066	728.4255
TYFPFDLSHGSAQVK	1,832.8844	1,833.0992
VADALTNAVAVHDDMP-NALSALSDLHAHK	2,995.4820	2,995.6590
β chain		
SAVTALWGK	931.5100	931.5587
VHLTPEEK	951.5600	951.5587
VVAGVANALAHK	1,148.6665	1,148.7779 ^a
VLGAFSDGLAHLNLK	1,668.8830	1,668.9468

^a Charge-state determination is tentative due to observation of overlapping isotopic distributions.

A Human Hemoglobin Alpha Chain

VLSPADK**K**TNVKAAW**GK**VGAHAGEYGAELERMFLSFPTTK**I**YFPFDLSHGSAQ**VK**GHG**KK**VADAL**TNA**
 VAHVDDMPNALSALSDLHA**HK**LRVDPVNF**K**LLSHCLLVTLAAHLPAEFTPVHASLD**K**FLASVSTVL**TSKYR**

Human Hemoglobin Beta Chain

VH**L**TPEEK**S**AVTALW**GK**VNVDEVGGEALGRLLVVYPWTQRFFESFGDLSTPDAVMGNPK**V**KAHG**KK**VLGAFSD
 GLAHL**DNLK**GT**F**ATLSELHCD**K**LHVDPENFRLLGNVLVCVLAHHFGKEFTPPVQAA**YQK**VVAGVANALAH**KYH**

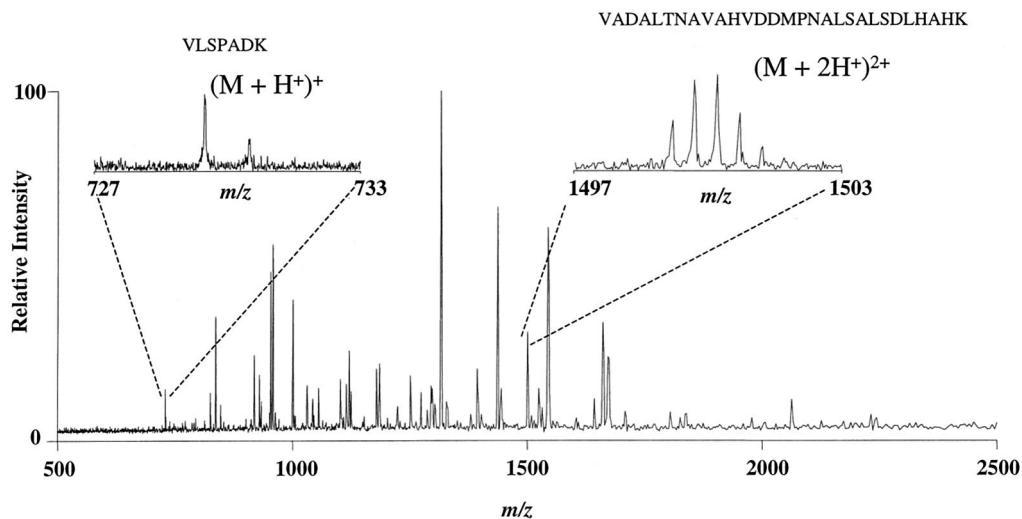
B

FIG. 7. Mass spectrometric analysis of Kgp-digested hemoglobin fragments. (A) Amino acid sequence of human α and β hemoglobin chains. Lysine residues are indicated in boldface; amino acid sequences of peptides demonstrated by ESI-FTICR spectroscopy are shaded. (B) ESI-FTICR mass spectrum of a human hemoglobin sample digested by the lysine-specific cysteine protease Kgp. The hemoglobinolytic potential of Kgp is verified by the observance of expected hemoglobin digestion fragments. The resolved carbon-13 isotopic distribution is evident in the two insets for two of the hemoglobin fragments selected as examples. Additional peaks in the spectrum not related to hemoglobin are suggestive of autodigestion of the protease.

that *kgp* mutants obtained naturally or by allelic exchange were nonpigmented. We also have restored by recombination the *kgp* gene of a strain that contained an insertionally inactivated allele of this gene. This strain expressed Kgp activity and formed pigmented colonies when grown on blood agar plates. There also were striking differences between the interactions of our *P. gingivalis* strains and sheep erythrocytes. *P. gingivalis* W83 was able to mediate hemagglutination and to effect hemolysis (Table 4; Fig. 3). Both of these properties were greatly reduced in *kgp* mutants of this strain (Table 4; Fig. 3). Finally, our biochemical and biophysical data clearly supported the notion that hemoglobin was a preferred substrate for Kgp (Table 5; Fig. 4, 6, and 7). These observations lead us to conclude that Kgp production is necessary for the provision of large amounts of hemin from hemoglobin. This hemin accumulates on the cell surface, ultimately leading to pigment formation (44). However, other factors, including surface binding components, may be involved in the pigmentation process. For example, hemin binding proteins as well as the hemin binding capability of lipopolysaccharide may be important (3, 11, 27).

Kgp is a multidomain polypeptide composed of a signal peptide, prepropeptide, protease domain, and hemagglutinin/adhesin domain. The hemagglutinin/adhesin domain, also associated with the Arg-gingipain protease RgpA and the hemagglutinin HagA (9, 21, 41, 53), has been shown to contain determinants which bind to erythrocytes (9). To determine the involvement of Kgp in hemin acquisition, we analyzed our mutants for the ability to hemagglutinate and lyse erythrocytes,

and for the ability to degrade hemoglobin. Our results with cell fractions indicated that Kgp contributes to hemagglutination of *P. gingivalis* (Fig. 3). However, as previously reported (30), its role was less significant in the vesicle fraction. The reason for this is unknown. Our general lack of knowledge about the formation of vesicles precludes a ready answer to this question, but several possibilities, including the uneven partitioning of membrane associated proteins during vesicle formation, exist.

Although, the ability of Rgp to lyse erythrocytes has been observed (43), our study provides the first evidence that Kgp also contributes to this process (Table 4). Kgp-deficient strains were still capable of degrading erythrocytes but not to the same extent as the wild type.

Our analysis of hemoglobinase activities for wild-type, mutant, and *kgp*-restored variants of W83 was instructive. We found that the Kgp-deficient strain had reduced ability to degrade both α and β chains of hemoglobin. Because hemoglobin contains numerous Lys residues (i.e., human hemoglobin contains 11 Lys residues in the α chain and 10 Lys residues in the β chain [Fig. 7]), it was reasonable to postulate that Kgp specificity qualified it as a hemoglobinase, degrading hemoglobin into small peptides and effecting the efficient release of hemin. Although the hemoglobinolytic activity of Kgp explains the generation of high amounts of hemin from hemoglobin, the mechanism of storage of hemin on a surface of *P. gingivalis* remains to be elucidated. However, the reports of hemin binding surface components (3, 11, 27) are in keeping with our

recent preliminary observation that the Lys-gingipain protease was able to bind heme (data not shown).

Although Fujimura et al. have reported that Arg-gingipain is also capable of degrading hemoglobin (14), its role is likely to be minor on both theoretical and practical grounds (Fig. 4). First, there are only three Arg residues in either α or β chain of human hemoglobin molecule (Fig. 7). Therefore, this protease presumably degrades hemoglobin into three (α chain) or four (β chain) fragments. Second, the role of Arg-gingipain may be insignificant since its activity is inhibited by heme (55), and so the degradation of hemoglobin and subsequent release of heme by the action of the enzyme would be self-limiting. On the other hand, our results have shown that Lys-gingipain activity is not affected by the presence of heme (Table 3). Because high concentrations of heme favor binding of both heme and hemoglobin by *P. gingivalis* (17, 49) Lys-gingipain activity in high concentrations of heme is an important strategy in initiating the process of iron acquisition. The phenotypic manifestation of this activity is the accumulation of black pigment when cells are grown on blood agar. Our observed Kgp-related hemoglobinase activity is in contrast to the report of Fujimura et al. (14), who failed to see substantial degradation of hemoglobin by this activity. The reason for this inconsistency is not known but may be attributable to differences in strain origin, enzyme purification methods, or other technical factors.

In light of the results obtained by other laboratories and results presented here, a plausible explanation for the contribution of Kgp to provision of heme from erythrocytes abundant in the periodontal pocket (34) can be proposed. First, Kgp degradation of fibrinogen deregulates the clotting cascade, maximizing the availability of free erythrocytes (6, 26, 37, 40, 53). Second, Kgp binds erythrocytes and degrades them, releasing hemoglobin. Third, the 51-kDa protein encompassing the catalytic domain of Kgp binds hemoglobin (28). Taken together, our results suggest that Kgp is a principal protein involved in acquisition of heme from hemoglobin.

We recently showed that Kgp-deficient strains had reduced virulence in a mouse abscess model (30). Multiple possibilities may explain this. Kgp has been shown to degrade fibrinogen (26, 37, 40, 53). We have observed that this activity also correlates with the number of Lys residues present in this protein. The activity was shown to be very high for the γ chain, which contains 32 Lys residues and 10 Arg residues. The α and β chains, which have been shown to be equally degraded by Arg-gingipains and Lys-gingipains (35), contain a similar number of Arg and Lys residues. The fibrinolytic activity may serve to diminish the clotting process followed by prolonged bleeding providing the bacteria with erythrocytes. The erythrocytes, in turn, may be degraded by Lys-gingipain, resulting in release of hemoglobin. Hemoglobin is then bound and degraded by Lys-gingipain, resulting in the provision of the essential nutrient, heme.

Heme has a profound effect on virulence of *P. gingivalis* (31, 32). Bacteria grown under heme limitation conditions were less virulent than their counterparts grown in heme excess. Heme can contribute to virulence in several ways. First, heme can serve as an iron source. Second, the oxidation-reduction potential of heme, required as a prosthetic group of cytochrome *b*, allows it to mediate electron transfer with generation of cellular energy that is required for growth and propagation of *P. gingivalis*. Third, heme has a regulatory effect on expression of protease activity in *P. gingivalis* (31, 48). In addition, heme could be detrimental to mammalian cells because of the combined lipophilic and oxidative nature of the molecule (51), resulting in lipid peroxidative catalysis of cellular

membranes and constituents. The black pigmentation of *P. gingivalis* cells may be a form of sequestering and detoxifying of heme that in high concentrations (10 to 20 $\mu\text{g/ml}$) has been shown to have antibacterial effects on *P. gingivalis* as well as other gram-positive cocci and gram-negative rods. Heme has been proposed to inactivate bacterial cells through an oxidation-reduction process related to peroxide (36). The deteriorating effect of heme on bacteria can be abrogated in the presence of thiol reagents. An alternative way is formation of black pigmentation composed of μ -oxo dimer, $[\text{Fe(III)}\text{PPIX}]_2\text{O}$ (50), serving not only as a scavenger of heme but also binding free oxygen and thereby reducing the heme-mediated oxygen radical cell damage as well as protecting from reactive oxidants generated by neutrophils (50). Therefore, interference with mechanisms involved in accumulation of black pigmentation may be significant in controlling the pathogenic potential of *P. gingivalis*.

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