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Hemolytic Activity in the Periodontopathogen *Porphyromonas gingivalis*: Kinetics of Enzyme Release and Localization

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Porphyromonas gingivalis **W50, W83, A7A1-28, and ATCC 33277 were investigated for their abilities to lyse sheep, human, and rabbit erythrocytes. All of the *P. gingivalis* strains studied produced an active hemolytic activity during growth, with maximum activity occurring in late-exponential–early-stationary growth phase. The enzyme was cell bound and associated with the outer membrane. Fractionation of *P. gingivalis* W50 localized the putative hemolysin almost exclusively in the outer membrane fraction, with significant hemolytic activity concentrated in the outer membrane vesicles. Ca^{2+} and Mg^{2+} ions significantly increased the expression of hemolytic activity. Hemolytic activity was inhibited by proteinase K, trypsin, the proteinase inhibitors Na-P-tosyl-L-lysine chloromethyl ketone and benzamidine, the metabolic inhibitor *M*-chlorophenylhydrazone, and iodoacetate. KCN and sodium azide (NaN_3) only partially inhibited *P. gingivalis* hemolytic activity, while antiserum to whole cells of each of the *P. gingivalis* strains had a significant inhibitory effect on hemolytic activity. The *P. gingivalis* W50 hemolysin was inhibited by cysteine, dithiothreitol, and glutathione at concentrations of at least 10 mM; at low concentrations (i.e., 2 mM), dithiothreitol did not completely inhibit hemolytic activity. Heating to temperatures above 55°C resulted in an almost complete inhibition of hemolytic activity. The effect of heme limitation (i.e., iron) on hemolysin production indicated that either limitation or starvation for heme resulted in significantly increased hemolysin production compared with that of *P. gingivalis* grown in the presence of excess heme.**

A large number of observations implicate dental plaque in the subgingival environment as a major factor in the progression of periodontal disease (38). A very small number of species, maybe as few as 12, in this microbiota are positively associated with the progression of periodontal disease. These include selected black-pigmented *Bacteroides* spp., *Porphyromonas gingivalis*, *Prevotella intermedia*, *Bacteroides melaninogenicus*, *Bacteroides loeschei*, and *Bacteroides denticola*. These species elaborate a variety of virulence factors which could affect the integrity of connective tissue and alveolar bone (8, 35, 45, 48). *P. gingivalis* is considered of major importance in the pathogenesis of periodontitis (14, 18, 22, 35). One of the principal mechanisms by which *P. gingivalis* obtains its metabolic requirements is the elaboration of potent proteolytic enzymes into the extracellular environment (22). Several of these proteases are capable of degrading connective-tissue proteins into small peptides for use in growth and metabolism (47, 48). Almost 60 years ago, Burdon (7) demonstrated that black-pigmented *Bacteroides* spp. had an obligate requirement for heme-iron and that iron was a major requirement for infection (50). Since heme released as the result of hemolysin activity is rapidly sequestered from bacteria by the host heme-binding protein hemopexin, it remains to be determined how these heme-requiring anaerobes obtain this molecule from the host. Important to the growth and metabolism of *P. gingivalis* in the restricted ecological niche of the periodontal environment is its strict nutritional requirement for iron (i.e., heme). In fact, the emergence of *P. gingivalis* in a periodontal lesion may be a de facto result of increased localized inflammation and bleeding. The results of McKee et al. (36), Marsh et al. (34), and, recently, Bramanti and Holt (4) as well as Shah and Gharbia (45) clearly demonstrate that heme modulates *P. gingivalis* virulence. Kay et al. (23) have demonstrated that their *P. gingivalis* W50 strain also possessed hemolytic activity, which appeared to be concentrated in the extracellular vesicles. The report presented here describes a putative hemolysin which is sensitive to -SH-containing molecules and appears to have a close association with the outer membrane and outer membrane vesicles.

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MATERIALS AND METHODS

Culture conditions. *P. gingivalis* W50, W83, ATCC 33277, and A7A1-28 were used in this study. For growth, the cells were plated to enriched Trypticase soy agar or were grown in 2.1% (wt/vol) Mycoplasma broth (BBL, Becton Dickinson, Cockeysville, Md.) supplemented with 5 μg (wt/vol) of heme per ml. All cultures were incubated in a Coy anaerobic chamber (85% N_2, 10% H_2, and 5% CO_2) maintained at 37°C. Cultures were incubated for 24 h or for times appropriate for the experimental design and were harvested and fractionated as described below. *Escherichia coli* HB101 was used as a hemolysin-negative control.

Cell fractionation. For localization of the *P. gingivalis* hemolysin, cultures were grown, harvested by centrifugation, and washed twice with 3 mM sodium citrate–0.9% NaCl buffer (pH 6.8) (NCN buffer). Cell envelope, outer membrane vesicles, spent growth supernatant, and soluble-vesicle supernatant were isolated as described in Fig. 1.

Cell pellets and spent growth supernatant. Bacterial pellets were separated from spent growth supernatant by centrifugation (16,000 × *g*, 15 min), and the cells were suspended in NCN buffer, washed once in this buffer, and stored frozen at −20°C until used.

Outer membrane vesicles. Outer membrane vesicles were separated from the spent growth supernatant by ultracentrif-

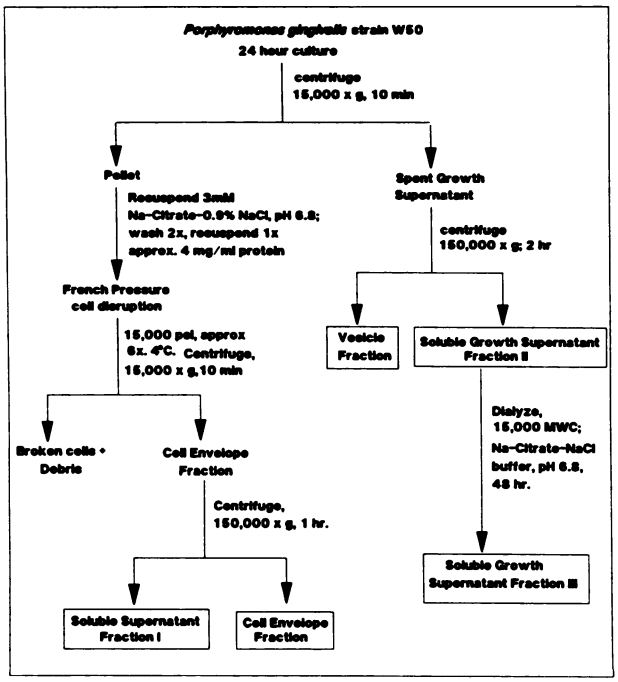

FIG. 1. Flow diagram for the separation of cell envelope, outer membrane vesicles, and soluble supernatant fractions from *P. gingivalis* W50. The fractions indicated were separated and either used immediately in the hemolysin assay or stored frozen at −20°C until used.

ugation at 150,000 × *g* for 2 h, and the pellets were suspended in NCN buffer and dialyzed against this buffer for at least 48 h. In some experiments, the spent growth supernatant was precipitated with $(NH_4)_2SO_4$ (70% final concentration) and the pellet was dialyzed against buffer.

Outer membrane-enriched fraction. An outer membrane-enriched fraction was obtained from *P. gingivalis* W50 by French pressure cell disruption according to the procedure of Kennell and Holt (25) outlined in Fig. 1. Cells (see above) were adjusted to 6×10^{10} cells per ml, thoroughly mixed, and broken in a cold French pressure cell at 15,000 lb/in^2 at least six times (99% breakage). Whole cells and debris were separated at 5,000 × *g* for 10 min, and the cell envelope-enriched fraction was collected (150,000 × *g*, 2 h). This fraction was either used immediately or stored frozen. The fractions were maintained at 4°C. In some experiments, a protease inhibitor cocktail identical to that described by Kennell and Holt (25) was added prior to cell fractionation.

Effect of growth on bacterial lysis and hemolysin release. For determination of the release of cytoplasmic material as a function of growth and of the effect of NCN buffer on *P. gingivalis* W50 hemolytic activity, cells were radiolabeled with [^{3}H]thymidine. [^{3}H]thymidine (2 μCi/ml) was incorporated into the Mycoplasma broth medium at zero time, and the culture was incubated anaerobically for 120 h as described above. Approximately 6 ml of the radiolabeled culture was removed at various times, centrifuged at 15,000 × *g* for 10 min, washed once, and frozen at −20°C until analyzed. The distribution of radioactivity between whole cells and the growth medium as a function of time was determined after centrifugation. To determine whether the release of *P. gingivalis* hemolysin was due to cell lysis or was an active process, the radiolabeled cells were washed twice with NCN buffer, suspended to a known cell number in the buffer, and used in the hemolysin assay (see below). Incorporation of [^{3}H]thymidine was measured in a Beckman LS 1701 scintillation counter.

Effect of calcium and magnesium. The effect of Mg^{2+} and Ca^{2+} ions on hemolytic activity of whole cells and outer membrane vesicles was determined. $MgCl_2$ and/or $CaCl_2$ (10 mM) was mixed with 2×10^{10} *P. gingivalis* organisms per ml or 0.6 mg of vesicle protein per ml and 2×10^9 sheep erythrocytes per ml. Hemolytic activity was determined as described below.

Protein determination. Protein concentration was determined by the BCA procedure (Pierce Chemical Co., IL) as modified by Kennell and Holt (25).

Hemolysis assay. Sheep erythrocytes were washed with NCN buffer until the supernatant was visually free of hemoglobin pigment. In some experiments, fresh human and rabbit erythrocytes were compared with sheep erythrocytes; however, sheep erythrocytes were used routinely. The washed erythrocytes were suspended in NCN buffer to a concentration of 2×10^9 erythrocytes per ml. For the hemolysin assay, *P. gingivalis* cells were routinely suspended to a final concentration of 2×10^{10} bacteria per ml in buffer and mixed with an equal volume of erythrocytes. This mixture was then slowly mixed in a water bath at 37°C. Samples (0.1 ml) were withdrawn at intervals from 0.5 to 20 h and immediately diluted with 0.9 ml of buffer. The released hemoglobin was separated from the lysed and whole erythrocytes by centrifugation at 3,500 rpm (approximately 2,000 × *g*) for 5 min, the resulting supernatants were diluted another 10-fold with NCN buffer, and the A_{405} was determined. Control erythrocytes were incubated as described above but with no test material.

Hemolysis inhibition assays. (i) Effect of heat. *P. gingivalis* strains were suspended in buffer to a concentration of 4×10^{10} cells per ml and heated at temperatures between 37 and 80°C for 5, 10, and 15 min in a temperature-controlled water bath. The cells were cooled in ice and used immediately in the hemolysin assay.

(ii) Effects of proteinase K, trypsin, and protease inhibitors. The effects of Proteinase K and trypsin (Sigma Chemical Co., St. Louis, Mo.) on hemolysin function were determined. Proteinase K (1 mg/ml) and trypsin (0.5 to 2.5 mg/ml) were mixed with an equal volume of 2×10^{10} *P. gingivalis* cells per ml, incubated at 37°C for 2 h, and then washed twice with buffer. The protease inhibitors TLCK (Na-P-tosyl-L-lysine chloromethyl ketone) and benzamidine were also examined. Concentrations of between 2 and 10 mM were mixed with 2×10^{10} *P. gingivalis* cells per ml, either singly or together, and incubated and washed as described above. The treated cells were immediately used for the determination of hemolytic activity.

(iii) Effect of *P. gingivalis* antibody. The effect of polyvalent antibody against *P. gingivalis* W50 whole cells on *P. gingivalis* W50 hemolytic activity was determined. Antibody was prepared in BALB/c mice by subcutaneously injecting 0.2 ml of solution containing 10^{10} whole cells per ml emulsified with an equal volume of incomplete Freund's adjuvant into the nape of the neck. Antisera from all animals were pooled. The effect of the polyvalent antibody on hemolysin production was determined by mixing 2×10^{10} *P. gingivalis* W50 cells per ml with complement-inactivated (56°C, 30 min) antiserum. The cells were incubated with the antibody for 1 h at 25°C, washed with NCN buffer, and then assayed for hemo-

lytic activity. Preimmune serum was used to determine nonspecific binding of antibody to *P. gingivalis* whole cells.

(iv) Effect of cysteine, DTT, and glutathione. The effects of the sulfur-containing compounds cysteine, dithiothreitol (DTT), and glutathione on *P. gingivalis* W50 hemolysin were tested. These were mixed with NCN buffer to a final concentration of 200 mM, and 4×10^{10} *P. gingivalis* W50 cells per ml were added. The cells were incubated for 2 to 4 h, centrifuged, washed once with buffer, and assayed for their effect on hemolytic activity.

Hemagglutination assay. Hemagglutination activities of whole *P. gingivalis* W50 cells as well as several of the cell fractions (Fig. 1) were also determined. Whole cells were suspended in buffer to 2×10^{10}/ml, while cell fractions were suspended in buffer to a protein concentration of 1 mg/ml. Whole cells and cell fractions were serially diluted (twofold) in buffer, and the dilutions were mixed with an equal volume of washed sheep erythrocytes in buffer in 96-well microtiter plates. The plates were shaken at 120 rpm for 3 min, and the resulting mixture was incubated at room temperature for approximately 3 h. A positive hemagglutination reaction was taken as the reciprocal of the last dilution showing complete hemagglutination.

Effects of antimetabolites on hemolytic activity. *P. gingivalis* W50 was grown into late exponential phase (20 to 24 h), and then NaN_3 (20 to 50 mM), carbonyl cyanide *M*-chlorophenylhydrazone (CCCP; 500 μM), KCN (500 μM), dinitrophenol (500 μM), or iodoacetate (20 to 40 mM) was added. The cell suspensions were incubated anaerobically for an additional 3 h, harvested, and washed three times in buffer. The cell pellets were assayed for hemolytic activity.

Effect of heme limitation on hemolytic activity. *P. gingivalis* W50 was grown for 24 h in the absence of added heme and then transferred at least four additional times in heme-free Mycoplasma broth (4). The effect of a limiting heme concentration (0 to 0.5 μg/ml) and inorganic iron (200 mM $FeCl_3$) on hemolytic activity was then examined by growing *P. gingivalis* W50 in the presence of these compounds and using the cells in the hemolytic activity assay (see above).

RESULTS

Lysis of erythrocytes. The relationship between *P. gingivalis* W50 growth phase and hemolysin production is seen in Fig. 2. The increase in *P. gingivalis* W50 hemolysin activity in whole cells occurred as a function of cell growth, with maximum activity occurring at approximately 15 h. There was a slight decrease in intracellular hemolysin in late stationary phase (i.e., at 35 h). The hemolytic activity of the vesicle fraction also increased as a function of cell growth. Note in Fig. 2 that separation of the vesicles from the soluble supernatant separated a vesicle-associated hemolytic activity. On a protein basis, the vesicles contained significant hemolytic activity compared even with whole cells (see Fig. 4). The soluble supernatant contained some hemolytic ability, but this ability was lower than that of the whole cells and vesicles and probably was a result of cell lysis.

Ca^{2+} and Mg^{2+} ions also had a significant effect on *P. gingivalis* W50 hemolysis (Fig. 3). The addition of Mg^{2+} or Ca^{2+} ions either separately or together at a final concentration of 10 mM resulted in maximum hemolysis occurring within 3 h instead of the approximately 5 h required in the absence of these cations (compare Fig. 3 with Fig. 2). These cations had identical effects on hemolytic activity from isolated outer membrane vesicles (data not shown).

The distribution of hemolytic activity in the putative *P. gingivalis* pathogenic strains W83, ATCC 33277, and A7A1-28 was also determined (data not shown). All strains showed significant hemolytic activity between 4 and 8 h, as did *P. gingivalis* W50 (Fig. 2). *E. coli* HB101 was devoid of hemolytic activity.

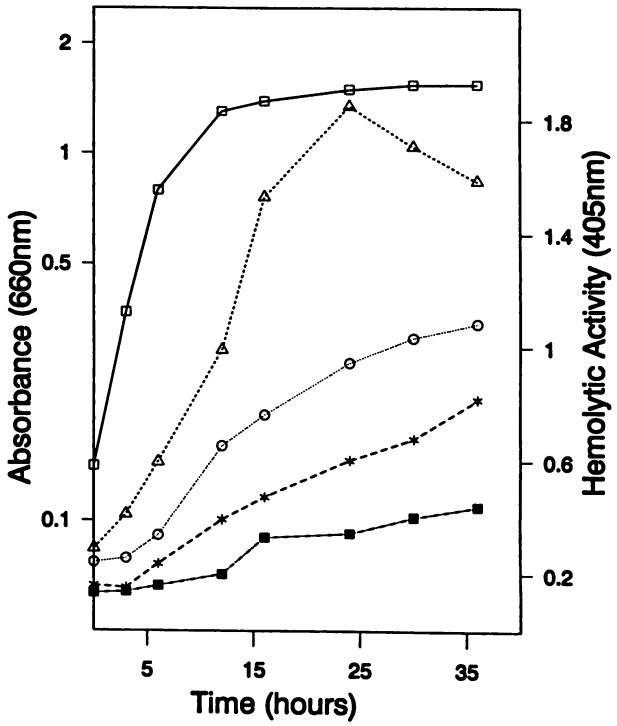

FIG. 2. Kinetics of growth (□) and hemolytic activity (△) of whole cells of *P. gingivalis* W50 as a function of time. Hemolytic activity in the supernatant fraction (vesicles plus soluble components) (○) and outer membrane vesicles (*) and that released as soluble hemolytic activity (■) followed cell growth and whole-cell-associated hemolysin formation.

Localization of *P. gingivalis* W50 hemolytic activity. The distribution of hemolytic activity in the various cell fractions described in Fig. 1 was determined for *P. gingivalis* W50 (Fig. 4). On a total-protein basis, the *P. gingivalis* W50 vesicle fraction contained higher hemolytic activity than any other fractions. Concentrations of vesicle protein of between 0.03 and 0.3 mg/ml resulted in an almost dose-dependent lysis of sheep erythrocytes, with significant hemolysis occurring in the vesicles at 0.3 mg of vesicle protein per ml (data not shown). Interestingly, while the cell envelope fraction contained hemolytic activity, it was significantly lower than that found in the other fractions. Addition of the soluble-component fraction to the cell envelope fraction had only a slight additive effect on the increase in hemolytic activity. The enriched cell envelope fraction or spent supernatant fraction never approached the hemolytic values obtained for the vesicle fraction even at 1 mg of protein per ml (data not shown).

[^{3}H]thymidine release was used to determine whether the release of hemolysin into the extracellular environment was the result of cell lysis (Fig. 5). [^{3}H]thymidine uptake, while low, occurred coincident with cell growth and hemolytic activity. While there was some release of [^{3}H]thymidine from late-exponential growth phase (i.e., 20 h), probably due to cell lysis, this initial lysis remained stable for up to 120 h

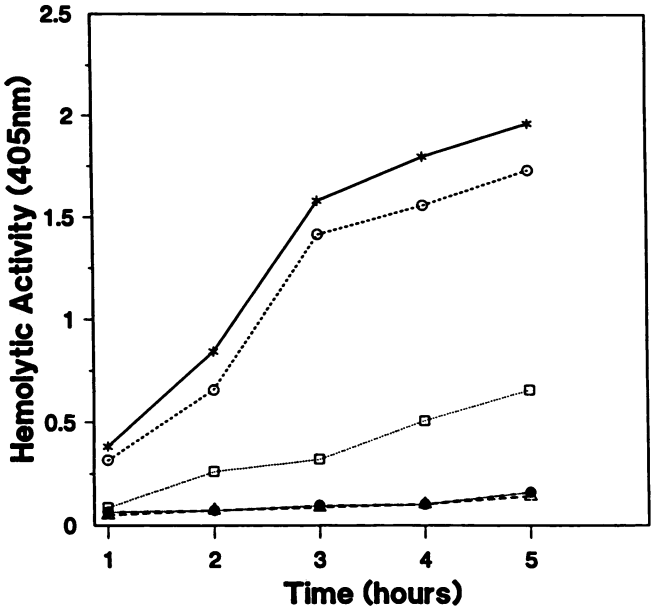

FIG. 3. Effect of Mg^{2+} ($MgCl_2$) (*) and Ca^{2+} ($CaCl_2$) (○) on hemolytic activity of whole cells of *P. gingivalis* W50. Cells were grown, pelleted, and assayed for hemolytic activity as described in the text. In the absence of these cations (□), there was low hemolytic activity (compare the comparable assay fraction in Fig. 2), while neither cation had an effect on the erythrocytes in the assay in the absence of whole cells. Mg^{2+} (△) and Ca^{2+} (●) controls (plus erythrocytes alone) are also indicated.

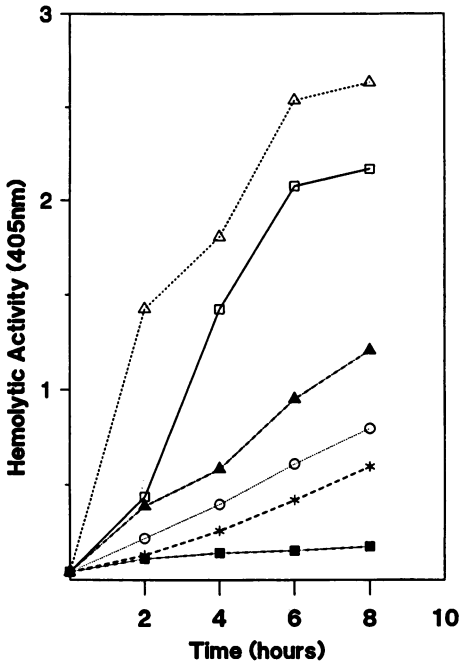

FIG. 4. Hemolytic activity of *P. gingivalis* W50 fractions (Fig. 1). Outer membrane vesicles (△) had a significantly higher hemolytic activity than whole cells (□), cell envelope fraction (*), or soluble supernatant fraction II or III (○). The addition of these fractions to the cell envelope fraction (▲) did not result in a significant increase in hemolytic activity. Also shown is the negative control (■) (incubation of erythrocytes in buffer).

of the experiment. *P. gingivalis* W50 whole cells suspended in 10 mM EDTA and 100 mM sucrose did not result in any significant DNA release (as measured by [^{3}H]thymidine release; data not shown). However, the hemolysin which was released was recovered from the enriched outer membrane fraction after ultracentrifugation of the growth supernatant (data not shown). Therefore, it is more than likely that the hemolysin of *P. gingivalis* W50 is located close to the cell surface, most probably in the outer membrane, where it is concentrated into outer membrane vesicles.

Effects of proteinase K, trypsin, and proteinase inhibitors on hemolytic activity. The effects of proteinase K, trypsin, and protease inhibitors on *P. gingivalis* W50 hemolytic activity are shown in Fig. 6. Both proteinase K (0.5 mg/ml) and trypsin (1 mg/ml) had significant inhibitory effects on hemolytic activity. Proteinase K reduced the hemolytic activity in whole cells by at least 80%, while trypsin reduced it by approximately 50 to 60%. TLCK and benzamidine at concentrations as low as 500 μM were very effective in inhibiting all hemolysin activity (Fig. 6). In the absence of trypsin or the proteinase inhibitors, there was no erythrocyte lysis.

Effect of storage on hemolytic activity. Storage of *P. gingivalis* W50 whole cells at either 0 or 4°C aerobically or anaerobically was not inhibitory to hemolytic activity during the 7 days of storage (data not shown). Storage at 20 or 37°C resulted in significant loss of hemolytic activity, with almost all of the hemolytic activity being lost at 3 and 7 days, respectively.

Effect of heating on hemolytic and hemagglutination activity. Heating whole cells of *P. gingivalis* W50 affected hemolytic activity and hemagglutination capacity as shown in Fig. 7. Temperatures between 37 and 42°C resulted in a significant spike in hemolytic activity. At 42°C and above, there was a significant decrease in hemolytic activity, with essentially all of the activity being lost between 55 and 80°C. Increased temperature (42°C) also resulted in increased hemagglutination activity (Fig. 7), while temperatures above 42°C resulted in only a small increase in hemagglutination, which was probably a result of bacterial aggregation. This hemagglutination activity was rapidly destroyed at about 70°C. Since hemagglutination activity was significantly more heat stable than hemolytic activity, the moiety causing hemolytic activity might be either a heat-stable macromolecule or a glycoprotein protected from heat by the saccharide component. The enriched cell envelop and vesicle-associated hemolytic activity were also heat sensitive, with almost 95% of the activity being lost at 55°C (data not shown).

Effects of metabolic inhibitors on *P. gingivalis* W50 hemolytic activity. The effects of the metabolic inhibitors KCN, NaN_3, CCCP, dinitrophenol, and iodoacetate on *P. gingivalis* W50 hemolytic activity is seen in Fig. 8. All of these inhibitors interfere with energy production or proton motive force and function to uncouple electron flow from energy formation. CCCP, which also functions to depolarize the cytoplasmic membrane, had a significant effect on hemolytic activity (Fig. 8). Since CCCP functions as a proton ionophore, it is likely that the release of the hemolysin across the cytoplasmic membrane requires proton motive surface potential. Iodoacetate at concentrations of between 20 and 40 mM had an inhibitory effect on hemolytic activity identical to that of CCCP, supporting a role for proton motive force in *P. gingivalis* hemolytic activity. KCN, even at high concentrations (i.e., 200 mM) had little inhibitory effect on the activity of the *P. gingivalis* W50 intracellular hemolysin,

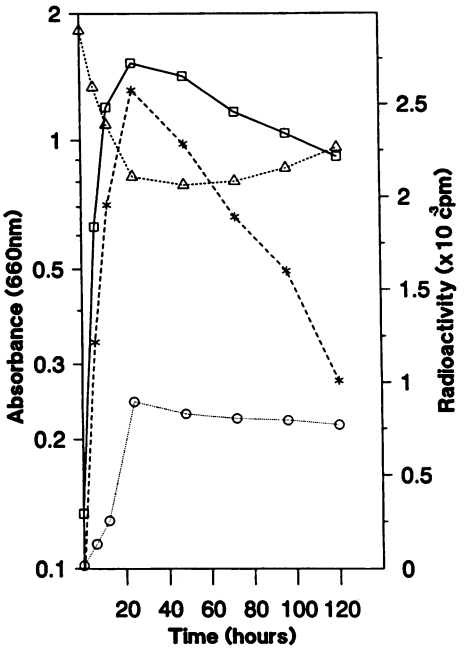

FIG. 5. Relationship between growth (□), hemolytic activity (*), and release of [^{3}H]thymidine into the growth supernatant (△) in *P. gingivalis* W50. Cell growth and whole-cell-associated hemolytic activity were maximum at approximately 20 to 24 h. Some cell lysis (as measured by [^{3}H]thymidine release) occurred over the course of the experiment, but it was negligible compared with that associated with the outer membrane vesicles and whole cells (○) themselves.

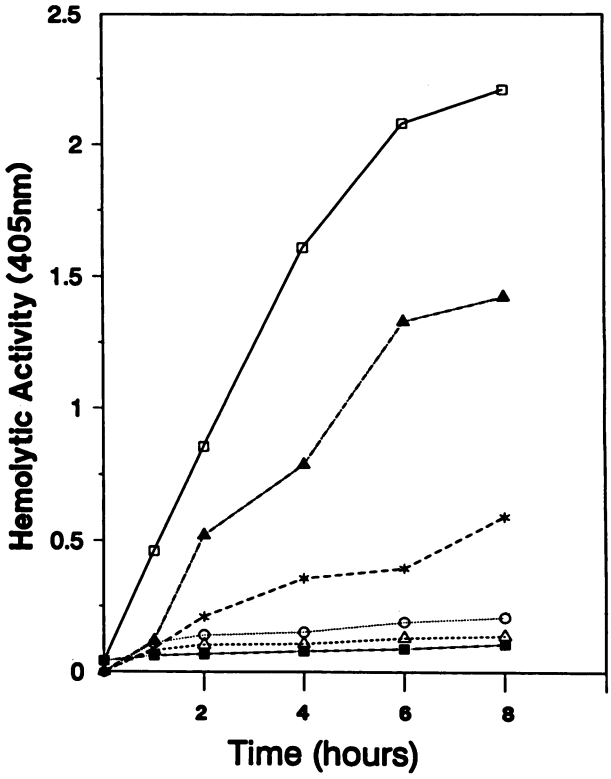

FIG. 6. Effects of trypsin (▲), proteinase K (*), and protease inhibitors TLCK (△) and benzamidine (●) on hemolytic activity of *P. gingivalis* W50 whole cells compared with control cells (□). ■, negative control.

while NaN_3 and dinitrophenol reduced hemolytic activity by approximately 20%. It is more than likely, then, that the major inhibitory activity of these antimetabolites was through membrane depolarization rather than direct interference with electron flow.

Effects of cysteine, DTT, and glutathione on *P. gingivalis* W50 hemolytic activity. Since Shah and Gharbia (45) showed that *P. gingivalis* W83 produced a presumptive cysteine proteinase which appeared in the culture supernatant after cell growth, we examined the effects of the SH-containing compounds cysteine, DTT, and glutathione on the hemolytic activity in *P. gingivalis* W50 (Fig. 9). Both cysteine and DTT at concentrations of 10 and 100 mM resulted in a significant reduction in hemolysin production. Cysteine at both 10 and 100 mM and DTT at 100 mM resulted in the complete inhibition of *P. gingivalis* W50 hemolysin activity, while DTT at 10 mM inhibited hemolytic activity during a 4-h period, after which the inhibition was overcome such that at 10 h, approximately 75% of the control activity was restored. At 2 mM DTT, there was essentially no inhibition of *P. gingivalis* W50 hemolytic activity (data not shown). Identical results were obtained with the other *P. gingivalis* strains used in these studies (data not shown). Glutathione at 10 mM also completely inhibited hemolytic activity of whole cells of *P. gingivalis* W50.

Effect of *P. gingivalis* W50 antibody on *P. gingivalis* W50 hemolytic activity. Polyvalent antibody to *P. gingivalis* W50 whole cells was prepared in at least 20 BALB/c mice, and the effect of $(NH_4)_2SO_4$-precipitated immunoglobulin G antiserum on *P. gingivalis* W50 hemolysin was tested. At the antibody titers tested (1/1,600 and 1/6,400), there was an almost complete inhibition of hemolytic activity over the entire period (6 h) of the hemolysis assay (data not shown). Heterologous antiserum prepared against *P. gingivalis* W83 (whole cells) as well as the various cell fractions (Fig. 1) also resulted in almost complete inhibition of hemolytic activity (data not shown) in both homologous and heterologous strains.

Effect of heme limitation on hemolytic activity. Bramanti and Holt (4) have demonstrated that *P. gingivalis* W50, W83, 381, and ATCC 33277 modulate several outer membrane proteins as a function of heme concentration. Thus, cells grown with excess heme (5 μg/ml) repressed these heme-responsive proteins, while cells grown in the presence of low heme (0.5 μg/ml) or starved for heme synthesized at least 8 to 12 heme-associated outer membrane proteins. Since erythrocytes can serve as a source of heme in vivo, it is more than likely that hemolytic activity (hemolysin expression) will vary in a manner similar to that observed by Bramanti and Holt (4) for the expression of heme-binding proteins. The results of growth of *P. gingivalis* W50 under heme excess, heme limitation, and heme starvation and in the presence of the inorganic iron source $FeCl_3$ on the expression of hemolytic activity in whole cells of *P. gingivalis* W50 are seen in Fig. 10. Growth under heme limitation or starvation or in the presence of the iron-chelating compound 2,2′-bipyridyl resulted in *P. gingivalis* W50 producing almost twice as much hemolysin at 6 h as cells grown in excess heme (5 μg/ml). While *P. gingivalis* W50 was capable of growth in the presence of $FeCl_3$, there was repression of hemolytic activity almost identical to that of cells grown in excess heme.

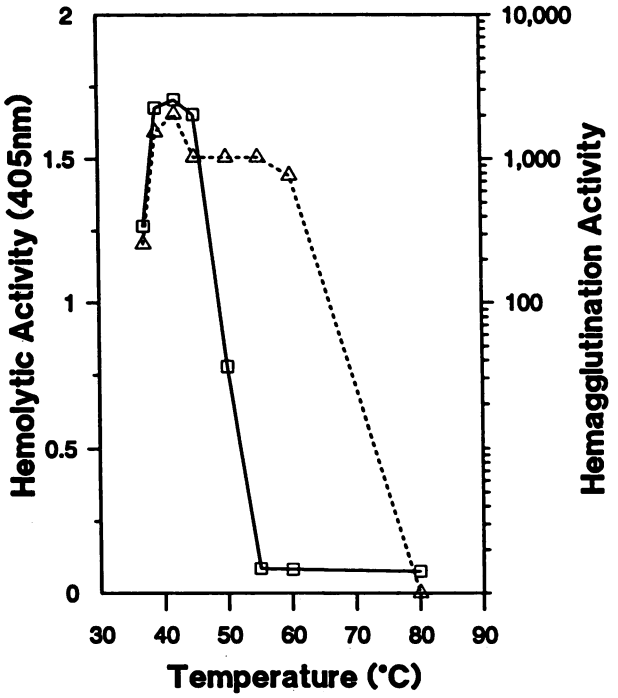

FIG. 7. Effect of incubation temperature of *P. gingivalis* W50 whole cells on hemolytic activity (□) and hemagglutination (△). Cells were incubated for 10 min at temperatures between 37 and 42°C, immediately cooled in an ice bath, and then assayed for hemolytic activity. Between 37 and 42°C, there was an increase in both hemolytic and hemagglutination activities, while temperatures above 42°C resulted in the destruction of these activities.

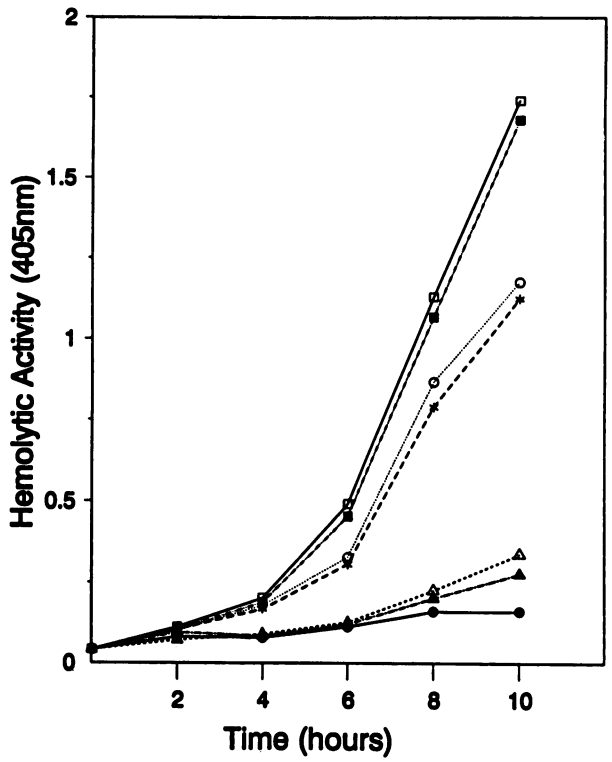

FIG. 8. Effects of the metabolic inhibitors KCN (■), NaN_3 (○), dinitrophenol (*), CCCP (△), and iodoacetate (▲) on hemolytic activity of whole cells of *P. gingivalis* W50. □ and ●, positive and negative controls, respectively.

DISCUSSION

The oral bacteria which inhabit the subgingival environment of the periodontium survive in a highly reduced and protein-rich ecological niche (38). An important characteristic of this microbiota, therefore, is their almost obligatory requirement to utilize nitrogenous substrates as sources of energy. With very few exceptions, the members of this microbiota elaborate a large number of proteolytic and hydrolytic enzymes into the pocket, where they are able to function in the destruction of host tissue and supply low-molecular-weight substrates for metabolism. The proteases, for example, have been shown to function in the degradation of host plasma proteins, including activation of the fibrinolytic and hemolytic systems.

P. gingivalis strains elaborate a larger number of proteolytic, fibrinolytic, and hydrolytic enzymes in vitro (see reference 22 for a review of the literature). *P. gingivalis* also produces a hemolysin(s) which is active in the release of heme from erythrocytes (23, 45). These enzymes probably function to degrade host tissues and secretions for metabolism. *P. gingivalis* strains are also capable of degrading components of the complement system and inactivating specific host protease inhibitors. Interestingly, Falkler et al. (15) reported that their *P. gingivalis* strains were not hemolytic. However, it appears from their report that they stored their cells or used cultures which were devoid of hemolytic activity because of culture manipulation. We have reported here and Kay et al. (23) have reported that *P. gingivalis* hemolytic activity is sensitive to a variety of environmental parameters. Kay et al. (23) showed that freeze-dried cells of *P. gingivalis* W50 effected erythrocyte lysis only 2% above control levels, clearly an indication of destruction of the *P. gingivalis* hemolysin.

The requirement of *P. gingivalis* for heme (which is utilized as an iron source; 4, 34, 36) and the fact that these bacteria appear devoid of any siderophore systems (4) dictate the necessity for an active hemolytic system. Therefore, the release of heme from the hemoglobin molecule during the in vivo course of inflammatory periodontal disease may be a major consequence in this disease process and may in fact be a controlling event for the growth and emergence of *P. gingivalis* in the subgingival environment. Previous studies (8) have demonstrated that the *P. gingivalis* hemolysin is capable of degrading the hemoglobin molecule and eventually transporting it into the cell (4). While free hemoglobin and heme are rapidly bound by haptoglobin and hemopexin, making them unavailable for use as a source of iron, *P. gingivalis* is able to degrade both of these iron-binding proteins and is therefore capable of obtaining iron from them. The results reported in the study presented here show that *P. gingivalis* strains may obtain their heme requirement by the action of a putative cell-associated hemolysin. Its release from growing cells occurs through the blebbing of the outer membrane vesicle, with which it appears to be intimately associated.

The hemolytic activity which is elaborated by the *P. gingivalis* strains used in this study appears to be different from that reported by Shah and Gharbia (45). Those investigators observed that their *P. gingivalis* strain (strain W83) produced a "cysteine proteinase" which was found almost exclusively in the growth supernatant and was stimulated by

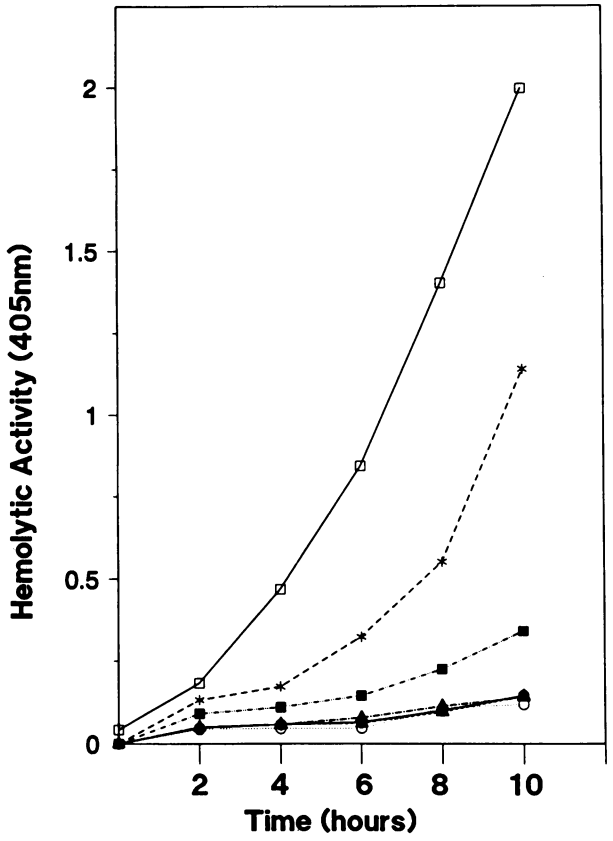

FIG. 9. Effect of -SH-containing compounds DTT, cysteine, and glutatione on hemolytic activity of whole cells of *P. gingivalis* W50. Cysteine at 10 (▲) and 100 (●) mM and glutathione at 100 mM (○) resulted in complete inhibition of hemolytic activity. DTT at 100 mM (■) was also effective in the inhibition of hemolytic activity. It was, however, only partially effective at 10 mM (*). □, positive control.

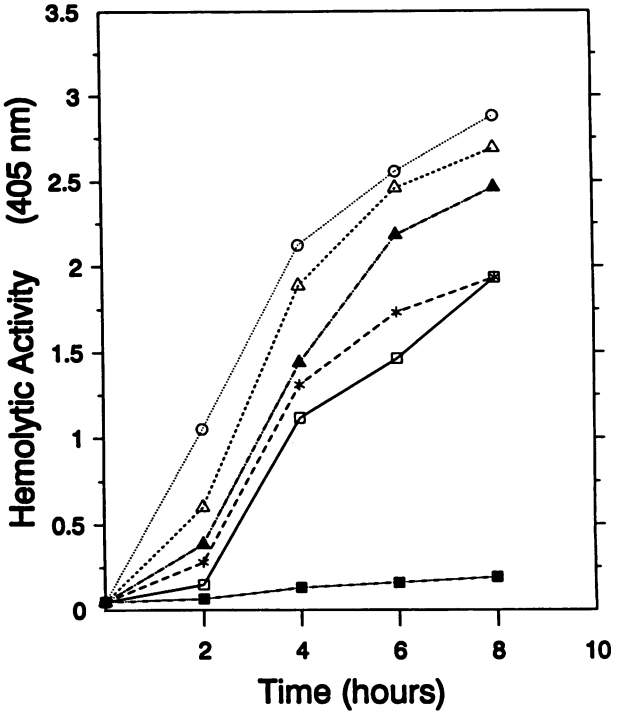

FIG. 10. Effect of iron (heme) on hemolytic activity of whole cells of *P. gingivalis* W50. Compared with incubation in the presence of high concentrations (5 μg/ml) of heme (□) or $FeCl_3$ (*), incubation of *P. gingivalis* whole cells in the presence of the iron-chelating agent 2,2'-bipyridyl (▲) or with low concentrations of heme (0.5 μg/ml) (△) or no heme (○) resulted in a significant increase in hemolytic activity. Control (■) erythrocytes displayed no background hemolytic activity.

cysteine. The thiol blocking agent 2,2'-dipyridyl disulfide inhibited the action of the enzyme as well as its hemagglutination ability. In contrast, we have found that the *P. gingivalis* W50 hemolytic activity was inhibited by low to moderate concentrations of cysteine, glutathione, and DTT. However, DTT inhibition occurred only at a concentration of 10 mM; at 2 mM, DTT was not inhibitory to *P. gingivalis* hemolytic activity. Kay et al. (23) have also demonstrated that *P. gingivalis* W50 retained significant hemolytic activity in extracellular vesicles and in an extracellular soluble protein. Those authors also confirmed the results presented here that show that the *P. gingivalis* hemolytic activity was sensitive to DTT at concentrations of 10 mM.

For the most part, hemolysin-producing prokaryotes (both gram positive and gram negative) all appear to possess similar properties (1, 24, 30, 49). For example, the *Serratia marcescens* hemolysin required active cell metabolism and proton transport for activity, since the addition of dinitrophenol and iodoacetate to the culture significantly reduced hemolytic activity (5). High temperature, proteases such as trypsin and proteinase K, and generalized metabolic inhibitors were all destructive to the hemolytic activity of *S. marcescens*, *E. coli*, *Actinobacillus pleuropneumoniae*, and *Moraxella bovis* and were identical in these activities to that which we report here for the *P. gingivalis* strains. Lian et al. (30) have reported the molecular cloning and expression of a hemolysin from *A. pleuropneumoniae* serotype 1. This hemolysin, while thermolabile, had an apparent molecular size of only 29.5 kDa. It also functioned biologically independent of Ca^{2+} (see below) and showed no immunological cross-reactivity with the 105-kDa hemolysin of other *A. pleuropneumoniae* strains (17). In addition to their similarity in biological activity, the hemolysins of *E. coli* (16, 20), *A. pleuropneumoniae* (13, 17), and *Vibrio cholerae* (43, 46) had similar distributions of molecular size on sodium dodecyl sulfate-polyacrylamide gels between 105 to 110 kDa. *Pseudomonas aeruginosa* produced at least two hemolysins, one heat labile and identical to phospholipase C and the other a heat-stable glycolipid (1). The *Pseudomonas cepacia* hemolysin (39) was similar to the *Pseudomonas pseudomallei* hemolysin, which produces a hemolytic lysolipoid endotoxin (42). Almost 70% of the hemolytic activity of *P. pseudomallei* strains occurred in the growth supernatant, and, interestingly, Ca^{2+}, EDTA, *N*-ethylmaleimide, and *p*-chloromercuribenzoate enhanced or were inhibitory to its activity. DTT or 2-mercaptoethanol also had no effect on the hemolytic activity of the excreted hemolysin. We are in the process of attempting to isolate the putative *P. gingivalis* hemolysin; however, it appears similar in its instability (data not shown) to the hemolysin of *A. pleuropneumoniae* (13) in that there was significant loss of hemolytic activity after ammonium sulfate precipitation and ion-exchange chromatography.

Characteristic of the hemolysins of gram-negative bacteria

(3) and of the *P. gingivalis* strains studied here was the effect of Ca^{2+} and Mg^{2+} on both soluble hemolysin and membrane- or vesicle-associated hemolysin. There was a significant increase in hemolytic activity in the presence of these two molecules, with maximum hemolytic activity occurring within 2 h in the presence of these cations compared with almost 8 h in their absence. In contrast, hemolytic isolates of *Proteus mirabilis*, *Proteus vulgaris*, and *Morganella morganii* displayed two different hemolytic activities, a cell-associated hemolysin and an excreted soluble hemolysin (28). The cell-free hemolytic activity was Ca^{2+} dependent, while the cell-associated hemolytic activity was Ca^{2+} independent. *M. bovis* and *E. coli* (21, 44) also produced a hemolysin with activities which were similar to those of the other gram-negative bacteria. Hemolysin production paralleled growth, with maximum activity occurring in late logarithmic phase. Ca^{2+} significantly increased hemolytic activity, while Mg^{2+} increased the activity only slightly. *A. pleuropneumoniae* also secreted a hemolysin, and it appeared that Ca^{2+} was required for maximum hemolysin production in these actinobacilli (17). However, the addition of Ca^{2+} to the extracellular growth supernatant did not increase hemolytic activity, nor was there any reduction in hemolytic activity when EGTA [ethylene glycol-bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid] was added to the supernatant. Therefore, free Ca^{2+} was required for hemolysin production but not for biological activity in these strains.

Functionally, hemolytic activity can be considered a virulence factor (19, 22, 26, 33, 37). Its virulence function is thought to occur by increasing the availability of iron for the in vivo growth and metabolism of resident or invading bacteria. Since essentially all of the iron which occurs in a host is intracellular and the remaining extracellular iron is usually bound by albumin, haptoglobin, hemopexin, transferrin, or lactoferrin (6, 40, 41), competition for and successful acquisition of available iron are essential for the maintenance of the pathogenic state. Therefore, the establishment of a bacterial infection requires that the invading microorganism be able to successfully compete for the very low concentrations of iron which are found in the host (41). This competition occurs through the action of siderophores or iron-binding proteins which are capable of removing iron from host iron-binding proteins (4, 27). Hemolysins function to raise the level of iron by lysing erythrocytes at local sites of infection and releasing hemoglobin for bacterial acquisition. It is the presence of this available iron (albeit in very small concentrations) which provides the bacteria with their virulence potential (29, 31). Braun et al. (5) and Bramanti and Holt (4) have determined that the level of iron (in most cases occurring as heme) regulates virulence; increasing concentrations result in lower virulence capabilities than limiting iron. McKee et al. (36) have shown that high heme concentrations (>2.5 μg/ml) resulted in lower virulence in a murine lethality model than a low concentration (0.33 μg/ml). However, in recent studies, Holt and Bramanti (22) have determined that the virulence of *P. gingivalis* W50 was significantly enhanced at a limiting heme concentration (0.15 μg/ml). The reasons for the differences in these two observations are unclear. Hemolysin synthesis in *V. cholerae* has also been shown to be regulated by iron concentration and required at least 10 μM iron to reduce hemolysin synthesis. Iron-limited *S. marcescens* also showed a significant increase in hemolytic activity compared with iron-replete cells. The results presented in this study also have demonstrated that iron concentration had a significant effect on the activity of the *P. gingivalis* hemolysin. High concentrations of heme (5.0 μg/ml) resulted in significantly lower hemolytic activity than 0.5 μg of heme per ml.

Mechanistically, bacterial hemolysins appear to act at the level of the erythrocyte membrane, causing membrane damage by intercalating into the erythrocyte membrane and forming a pore through which the protoheme-containing hemoglobin molecule is able to leak out into the environment. Bacterial hemolysin is identical in function to other bacterial cytolysins (2). In addition to its toxicity for erythrocytes (32), the *E. coli* hemolysin has been shown to be cytotoxic for fibroblasts (10, 37), granulocytes (19, 24, 26), and human peripheral leukocytes (9–12). To date, we still have no indication of how the *P. gingivalis* hemolysin functions in its interaction with erythrocytes; however, because its physiological activities are almost identical to those of other prokaryotic hemolysins, there is no reason to believe that it functions any differently from these enzymes.

In order for oral pathogens such as *P. gingivalis* to survive in the complex and highly competitive environment of the periodontium, they must compete metabolically with a myriad of bacteria which inhabit this environment. Not only must they compete for available nutrients, which in most instances are present in limiting amounts, but they must also be able to overcome numerous host defenses in order to establish an infection. To do so, they have evolved mechanisms for eliciting a large number of proteolytic enzymes (22) as well as for extracting small amounts of essential nutrients and cofactors, such as iron, which they extract from heme-containing compounds. This ability to remove heme from hemoglobin in a highly hemorrhagic milieu provides these bacteria with a distinct advantage as they grow and emerge in this complex environment.

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