

Degradation of Native Human Hemoglobin following Hemolysis by *Prevotella loescheii*

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***Prevotella loescheii* PK1295 can grow on native hemoglobin as a source of heme. Supernatants of *P. loescheii* cultures hemolysed human erythrocytes and degraded native hemoglobin. These combined activities may provide heme (or iron) for the growth of *P. loescheii* and other dental plaque bacteria.**

Prevotellae and porphyromonas have been associated with periodontal disease (19). These bacteria express adhesive surface proteins that permit attachment to and colonization of oral tissues (8) and proteases that hydrolyze various matrix proteins, with consequent damage to connective tissue and alveolar bone (13).

Several species of prevotellae and porphyromonas have an absolute requirement of heme for growth (6). The capture of heme molecules by the bacteria is critical in the competition between host and pathogen, since freed iron can be sequestered by either host or bacterial chelators (2, 3, 15, 22).

This study is concerned with the oral bacterium *Prevotella loescheii* PK1295 (ATCC 43852), which possesses a lectin-like protein that mediates the attachment of certain gram-positive oral bacteria and hemagglutinates mammalian erythrocytes (10).

To establish that the growth of *P. loescheii* PK1295 was dependent on heme, we serially transferred the organisms into modified Schaedler broth (11) devoid of heme. After four transfers, growth in the heme-free medium was less than 15% that of the hemin-grown control (Table 1). Substitution of freshly prepared and thoroughly dialyzed hemoglobin (17) for hemin (at the same heme concentration, 7.7 μ M) produced essentially identical final cell densities, indicating that hemoglobin served as a source of heme for *P. loescheii* (Table 1).

To attribute to hemoglobin the ability to sustain the growth of *P. loescheii* in the oral cavity, one must demonstrate two facts: the existence of a hemolytic activity that liberates hemoglobin from erythrocytes, as has already been shown for related oral microorganisms (5, 7, 18), and the presence of a proteolytic activity that liberates the prosthetic group. Proteolytic activity in the culture supernatants of prevotellae and porphyromonas has already been demonstrated (13). However, since native proteins are poor substrates for proteases, this activity must be proven effective for native hemoglobin.

To demonstrate hemolytic activity in the culture supernatant of *P. loescheii*, we mixed 1% (vol/vol) washed human erythrocytes in Tris-buffered saline with an equal volume of culture supernatant of *P. loescheii* at different growth intervals and incubated the mixture for 2 h at 37°C. After centrifugation (3,000 $\times g$ for 5 min), the supernatant was removed and the released hemoglobin was measured at 545

nm (1). Control erythrocytes were incubated in Schaedler broth as described above. Figure 1 shows the increase in *P. loescheii* hemolytic activity as a function of cell growth.

Proteolytic activity was assayed in cell-free culture supernatants of *P. loescheii* (harvested at the end of the log phase of growth, 24 h), clarified through a 0.45- μ m-pore-size filter (Millipore) and adjusted to pH 6.5 at 37°C, by mixing of 100 μ l of supernatant with 10 μ l (20 μ g) of native hemoglobin. The reactions were stopped by the addition of sodium dodecyl sulfate (SDS) sample buffer containing 10 mM 2-mercaptoethanol and boiling for 15 min (9). Treated protein (2.5 μ g) was layered on 15% SDS-polyacrylamide slab gels. Protein bands were visualized by Coomassie blue staining (21). The electropherograms (Fig. 2A) were scanned densitometrically (Camag TLC scanner II and Camag SP4270 TLC integrator); the results of these scans (Fig. 2B) demonstrated the complete cleavage of native hemoglobin. No proteolytic activity was observed with a culture super-

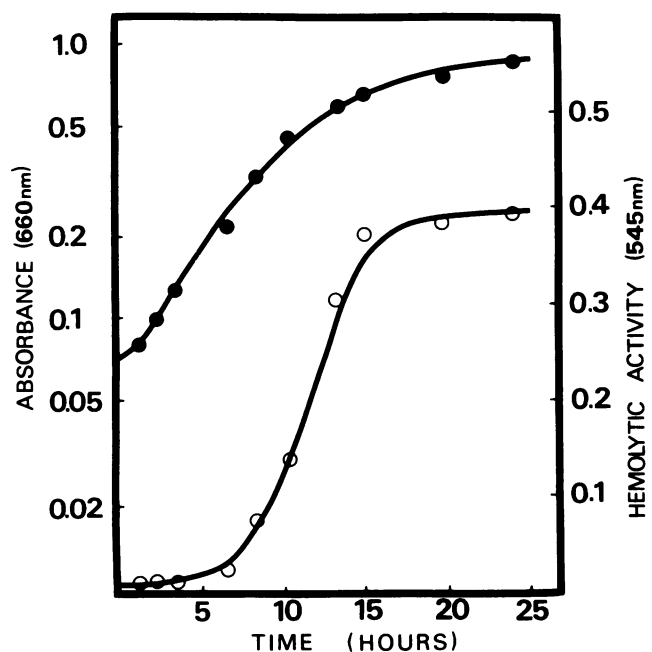


FIG. 1. Kinetics of growth of *P. loescheii* cultures (●) and hemolytic activity of culture supernatants (○) as a function of that growth over time.

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TABLE 1. Effect of heme and hemoglobin on the growth of *P. loescheii*^a

Compound added to growth medium	Optical density (660 nm) during the following transfer:			
	1	2	3	4
None	0.530 ± 0.007	0.355 ± 0.032	0.209 ± 0.007	0.090 ± 0.008
Hemin	0.625 ± 0.078	0.640 ± 0.015	0.580 ± 0.014	0.633 ± 0.018
Hemoglobin	0.660 ± 0.085	0.697 ± 0.049	0.685 ± 0.034	0.640 ± 0.021

^a Screw-cap tubes containing 10 ml of Schaedler broth with and without 7.7 μM hemin were inoculated with 4 × 10⁸ cells per ml and incubated anaerobically (GasPak anaerobic system; BBL) at 37°C. Growth was measured spectrophotometrically, and cultures were transferred after the cells entered the late logarithmic phase of growth. When appropriate, equimolar amounts of hemoglobin were substituted for hemin. Values (means ± standard errors) were from three experiments and represented the averages of triplicate determinations.

nant that had been boiled for 10 min or with fresh Schaedler medium. The activity of the culture supernatant was not reduced by passage through an XM100 filter (Amicon), indicating that it was not associated with particulate matter. In view of the resistance of native proteins to proteases (16), these results suggested the presence of a protease highly specific for native hemoglobin.

To investigate this possibility, we incubated the following native protein solutions (20 μg of each protein) at 37°C with 100 μl of the above-described culture supernatant (Fig. 2) for 24 h: native hemoglobin, freshly prepared from human blood as described above (17), casein, myoglobin (type II), cytochrome *c* (type III), bovine serum albumin (fraction V),

ovalbumin, RNase A, horseradish peroxidase (type VI), superoxide dismutase, collagen (type IV, from human placenta), fibronectin, immunoglobulin A (human), and immunoglobulin G (human) (all purchased from Sigma Chemical Co., St. Louis, Mo.); and catalase (Cooper Biomedical, Freehold, N.J.). Degradation was assayed by the same gel assay as that used above (Fig. 2). Table 2 shows that only hemoglobin and casein were completely cleaved. Catalase was degraded by only 40%, and the other proteins were only slightly affected, if at all.

Reports dealing with the presence of proteases in *P. loescheii* have been contradictory, probably because of differences in both the strains and the substrates tested. *P. loescheii* ATCC 15930 was reported to be devoid of proteolytic activity for collagen (12, 20) and gelatin (20). The same strain degraded hemopexin but not albumin, transferrin, or haptoglobin (4). An undesignated strain of *P. loescheii* isolated from salivary fluid hydrolyzed casein (14). Extracellular vesicles of *Porphyromonas gingivalis* degraded purified human hemoglobin in the presence of dithiothreitol (7). However, this activity was assayed on a commercial preparation of human hemoglobin that was lyophilized, a procedure that considerably denatures this protein.

The specificity of proteolytic activity for native hemoglobin is worth emphasizing, since other native heme-containing proteins, i.e., cytochrome *c*, horseradish peroxidase, and myoglobin, were not attacked; catalase was degraded slowly.

The *P. loescheii* hemoglobin-degrading activity should be contrasted with the proteolytic activities reported for *P.*

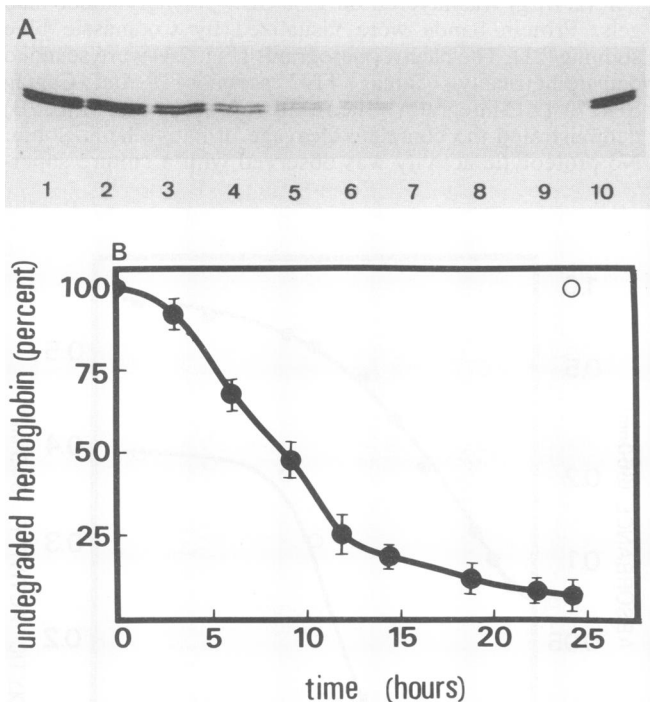


FIG. 2. Effect of *P. loescheii* culture supernatant proteolytic activity on human hemoglobin. (A) SDS-polyacrylamide gel electrophoresis of native human hemoglobin incubated with culture supernatant at different times (lanes 1 to 9) up to 24 h at 37°C; for the control (lane 10), hemoglobin was incubated for 24 h with a culture supernatant that had been boiled for 10 min or with fresh medium. (B) Quantification of the cleavage of hemoglobin as a function of incubation time (●); for the control, hemoglobin was incubated with a culture supernatant that had been boiled for 10 min or with fresh medium (○). The results represent a typical experiment of three. The time points correspond to lanes 1 to 9 in panel A.

TABLE 2. Proteolytic activity of *P. loescheii* culture supernatant

Protein ^a	% of protein remaining after 24 h of incubation ^b
Hemoglobin	4
Casein	5
Myoglobin	86
Cytochrome <i>c</i>	100
Bovine serum albumin	85
Ovalbumin	95
RNase A	100
Horseradish peroxidase	100
Catalase	62
Superoxide dismutase	100
Human immunoglobulin A	99
Human immunoglobulin G	100
Fibronectin	98
Collagen (human placenta)	93

^a The protein concentration was 20 μg.

^b Values were from three experiments and represented the averages of duplicate determinations. Assays were performed as described in the legend to Fig. 2.

gingivalis by Carlsson et al. (4). While the proteolysis of haptoglobin and hemopexin by *P. gingivalis* potentially provides this organism with an indirect means of acquiring heme from plasma proteins, *P. loescheii* PK1295 appears to obtain its heme for growth directly, by combining its ability to adhere to erythrocytes, hemolyse them, and degrade the liberated native hemoglobin.

These pathogenic mechanisms, expressed simultaneously by a single organism, represent an excellent example of bacterial adaptation to a specific environment. Furthermore, since the fimbria-associated adhesins of *P. loescheii* mediate coaggregation with other oral bacteria (23), it is conceivable that exploitation of the hemolytic and proteolytic (for hemoglobin) activities of *P. loescheii* allow other bacteria to contribute to the pathogenicity of periodontal plaque.

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