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Roles of Porphyrins and Host Iron Transport Proteins in Regulation of Growth of *Porphyromonas gingivalis* W50

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***Porphyromonas gingivalis* (*Bacteroides gingivalis*) requires iron in the form of hemin for growth and virulence in vitro, but the contributions of the porphyrin ring structure, porphyrin-associated iron, host hemin-sequestering molecules, and host iron-withholding proteins to its survival are unknown. Therefore, the effects of various porphyrins, host iron transport proteins, and inorganic iron sources on the growth of *P. gingivalis* W50 were examined to delineate the various types of iron molecules used for cellular metabolism. Cell envelope-associated hemin and iron stores contributed to the growth of *P. gingivalis* in hemin-free culture, and depletion of these endogenous reserves required eight serial transfers into hemin-free medium for total suppression of growth. Comparable growth of *P. gingivalis* was observed with 7.7 μM equivalents of hemin as hemoglobin (HGB), methemoglobin, myoglobin, hemin-saturated serum albumin, lactoperoxidase, cytochrome *c*, and catalase. Unrestricted growth was recorded in the presence of haptoglobin-HGB and hemopexin-hemin complexes, indicating that these host defense proteins do not sequester HGB and hemin from *P. gingivalis*. The iron chelator 2,2′-bipyridyl functionally chelated hemin-associated iron, resulting in dose-dependent inhibition of growth in hemin-restricted cultures at 1 to 25 μM 2,2′-bipyridyl concentrations. In the absence of an exogenous iron source, protoporphyrin IX did not support *P. gingivalis* growth. These findings suggest that the iron atom in the hemin molecule is the critical constituent for growth and that the tetrapyrrole porphyrin ring structure may represent an important vehicle for delivery of iron into the *P. gingivalis* cell. *P. gingivalis* does not have a strict requirement for porphyrins, since growth occurred with nonhemin iron sources, including high concentrations (200 μM) of ferric, ferrous, and nitrogenous inorganic iron, and *P. gingivalis* exhibited unrestricted growth in the presence of host transferrin, lactoferrin, and serum albumin. The diversity of iron substrates utilized by *P. gingivalis* and the observation that growth was not affected by the bacteriostatic effects of host iron-withholding proteins, which it may encounter in the periodontal pocket, may explain why *P. gingivalis* is such a formidable pathogen in the periodontal disease process.**

Iron is an essential nutrient for the growth and metabolism of essentially all prokaryotic organisms (52), as well as for the initiation and progression of many infectious diseases (7). This relationship between iron acquisition and microbial pathogenesis is dependent upon the ability of bacterial cells to procure iron from their host at the site of infection (52, 53) and to use it in their own metabolic processes (7, 16). The host defends itself against bacterial iron appropriation by the synthesis of a large number of iron-sequestering (binding) proteins (IBPs), including hemosiderin, ferritin, hemoglobin (HGB), haptoglobin (HPT), and hemopexin (HPX) (24, 32, 53). Additional high-affinity IBPs such as serum albumin (SA), transferrin (TF) in serum, and lactoferrin (LF) in mucosal secretions are also involved in the sequestration and transport of host iron (27, 53). These host iron-withholding defenses deny iron to the bacterium and limit the initiation of bacterium-mediated infectious processes. Therefore, the growth of the invading or opportunistic bacterium is dependent upon its ability to successfully compete with the host IBPs for this essential element. One mechanism by which the invading bacterium competes for available iron in an iron-restricted environment is by the synthesis and expression of a variety of high-affinity iron transport molecules, or siderophores (27, 35, 38). These siderophores chelate iron from host IBPs and then bind to specific bacterial outer membrane receptors for transport directly into the cell (35). In addition to siderophores, several gram-negative species (i.e., *Neisseria gonorrhoeae*, *Haemophilus influenzae*, *Bacteroides fragilis*) (12, 14, 26, 36, 39, 55) also express IBPs in their outer membranes. These IBPs function independently of the siderophores and sequester minute amounts of iron from the environment by interacting directly with the host IBPs (i.e., TF, LF, and HGB). These IBPs represent novel outer membrane proteins which are formed in response to minute levels of iron and may represent an alternate mechanism by which iron-containing molecules are bound and transported into the cell.

There are essentially no observations of the role of iron in the pathogenesis of the oral inflammatory and bone destructive disease periodontitis. In fact, the role of iron in the modulation of oral microbial pathogenesis, especially in the emergence of selected members of the oral microbiota in the restricted environment of the periodontal pocket remains equivocal. Most important to the emergence of selected members of the periodontopathic microbiota are the dynamic environmental transformations which evolve in the resident microbial ecosystem as a result of shifts in bacterial and host-derived nutrients (i.e., iron or hemin concentration) (17, 19, 21, 33). Most notable among the periodontopathogens affected by shifts in iron concentration is *Porphyromonas gingivalis* (formerly *Bacteroides gingivalis*) (4, 5, 30). A relationship may exist between the progression of periodontal disease (i.e., bone destruction), the levels of hemin (ferric-protoporphyrin IX [PPIX]), and the emergence of *P.*

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gingivalis in this highly restricted ecosystem (5, 21). Therefore, *P. gingivalis* must be able to obtain iron from host iron-containing compounds which are found in the periodontal pocket.

Since the environmental iron concentration has emerged as an important attribute in the expression of bacterial virulence, this study examined the effects of different iron sources on the regulation of growth of *P. gingivalis* as a function of various environmental conditions. In addition, we determined whether classical iron-containing molecules most frequently associated with bacterium-host interactions (i.e., TF, LF, and SA) could modulate the growth of *P. gingivalis*.

MATERIALS AND METHODS

Stock cultures and medium preparation. *P. gingivalis* W50 was used in all studies and was maintained as described previously (6). For liquid growth, 4- to 5-day growth of *P. gingivalis* was inoculated into 2.1% (wt/vol) mycoplasma broth base (BBL, Cockeysville, Md.) supplemented with 1 μg (wt/vol) of menadione and 5.0 μg (wt/vol) of (7.7 μM) hemin per ml. All cultures were grown in a Coy anaerobic chamber at 37°C in a 5% CO_2–10% H_2–85% N_2 atmosphere. Stock cultures of *Escherichia coli* MG1655 were grown in Luria broth (42) aerobically at 37°C. Growth rates of *P. gingivalis* were determined by inoculation of a 10% (vol/vol) inoculum of a late-logarithmic-phase culture into anaerobically equilibrated mycoplasma broth. Optical density was determined at 660 nm (OD_{660}) (Spectronic 710; Bausch and Lomb, Inc., Rochester, N.Y.). Culture purity was assessed by Gram stain and anaerobic subculture on enriched Trypticase soy agar (6). Contaminating iron and hemin were removed from all glassware by a Chromerge wash (Manostat, New York, N.Y.) for 24 h and rinsed at least 10 times in deionized distilled H_2O. All cultures were harvested at the late logarithmic growth phase by centrifugation at 12,000 × *g* for 20 min at 4°C. Supernatants were removed and stored at −20°C until they were used in siderophore assays (see below). Whole cells were washed three times in cold phosphate-buffered saline (PBS) (pH 7.2) and then used immediately for the isolation of the cell envelope.

Iron exhaustion and growth conditions. Since *P. gingivalis* is capable of storing iron (hemin) and using it for growth (5, 41), we employed a dual approach to study the effect of hemin- and iron-containing compounds on growth.

(i) Substrate substitution and hemin restriction. The hemin and iron substrates (Table 1) to be tested were added directly to the mycoplasma basal broth. After the cultures were inoculated, growth was recorded, and at the early stationary growth phase, the cultures of *P. gingivalis* were transferred as a 10% (vol/vol) inoculum into a vessel of homologous uninoculated medium containing the respective hemin or iron source. This process was repeated at least 10 times with the same hemin or iron substrate (Fig. 1A).

Hemin restriction was achieved either by 10 successive transfers (of a 10% inoculum after stationary growth was achieved) of the *P. gingivalis* stock culture into mycoplasma basal broth supplemented with 0.8 or 1.5 μM hemin or by the addition of 1 to 50 μM 2,2′-bipyridyl (BPD), the iron-chelating agent, into broth containing 1.5 μM hemin (Table 1; Fig. 1A).

(ii) Iron and hemin exhaustion and hemin repletion. Endogenous stores of iron and hemin in *P. gingivalis* whole cells were exhausted by serial passage of the culture at least six times into hemin-free medium after each culture achieved stationary growth (Fig. 1B). These iron-depleted cells (containing 42 pg of iron and 94 pg of hemin per μg of cell envelope protein) were then inoculated into basal broth plus test substrates for growth studies (Fig. 1B; Table 1). $CaCl_2$ was also used to precipitate iron (13) from the mycoplasma basal broth medium, and the effect of the porphyrin and metal additions (Table 1) on the growth of *P. gingivalis* was studied (Fig. 1B).

In repletion experiments, hemin- and iron-restricted cultures were transferred into hemin-free medium (negative control) or repleted into basal broth with 1.5 or 7.7 μM hemin (Fig. 1B).

The effect of the various hemin-containing compounds on the growth of *E. coli* MG1655 was studied by using an M9 minimal medium (42), which was $CaCl_2$ and Chelex-100 pretreated (13) to reduce contaminating levels of iron (Table 1).

Cell envelope preparation. Cell envelopes were prepared by French pressure cell disruption of freshly prepared whole cells in PBS (pH 7.2) at 15,000 lb/in^2. The cell envelopes were recovered after low-speed (10,000 × *g*, 30 min) and high-speed (200,000 × *g*, 60 min) centrifugation. The envelopes were washed and resuspended in iron-free water. The protein and hemin concentrations of the envelopes were determined after 30 s of sonication. The envelopes were stored at −20°C until used.

Iron and hemin determinations. The iron content of deionized distilled H_2O, mycoplasma basal broth, M9 minimal medium, cell envelope, metal salts, all porphyrins, and heme and iron proteins was determined by either graphite furnace atomic absorption spectrophotometry or by the Ferrozine assay (46). An iron atomic absorption standard solution (Aldrich Chemical Co., Milwaukee, Wis.) was used as a standard. The hemin content of heme and iron proteins was analyzed by a pyridine hemochromagen assay (40) with bovine hemin as the standard.

Protein determination. Total protein content was determined by a modification of the bicinchoninic acid protein assay (Pierce, Rockford, Ill.) (23) with human SA as the standard.

Human serum HPX, HPT, and HSA-SA. HPX was isolated from healthy human donors by heme-agarose affinity chromatography by the methods of Stull (47) and of Tsutusi and Mueller (49). This semipurified HPX was applied to a Sephadex G-100 superfine column (Pharmacia, Inc., Piscataway, N.J.) (45 by 2.5 cm, 112-ml bed volume) in 0.5 M NaCl buffer (pH 7.5), and the purified HPX was eluted at a flow rate of 20 ml h^{-1}.

Human HPT with mixed phenotypes represented by 30% (1:1), 50% (2:1), and 20% (2:2) was purchased from Sigma and Calbiochem (San Diego, Calif.). Hemin-saturated human SA (HSA) was prepared by mixing 300 μM HSA with bovine hemin at a 1:4 stoichiometric ratio in 25 ml of 10 mM HEPES (*N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid) buffer (pH 7.4). This mixture was incubated for 2 h at 25°C with mixing and then dialyzed against three changes (4 liters each) of 10 mM HEPES buffer (pH 7.4). Unbound insoluble hemin was removed by ultracentrifugation at 200,000 × *g* for 1 h at 4°C, and the resulting supernatant was applied to a Sephadex G-100 column in 0.5 M NaCl (pH 7.5), as described above. The HSA-SA complex was eluted at a flow rate of 60 ml h^{-1}. HSA-SA fractions were combined and concentrated in a SpeedVac concentrator (Savant Instruments, Inc., Farmingdale, N.Y.). Purified HSA-SA was suspended in deionized distilled H_2O and then lyophilized. HSA-SA, HPX, and HPT protein purities were assessed by

TABLE 1. Compositions of media with porphyrin and iron compounds and BPD used in growth experiments with *P. gingivalis* W50 and *E. coli* MG1655

Medium	Concn (μM)		
	Supplement	Hemin or porphyrin	Iron
Mycoplasma basal broth (2.1%)	Hemin or porphyrin source		
	None	0	1.1
	Hemin (7.7)	7.7	9.1
	Hemin (1.5)	1.5	2.7
	Hemin (0.8)	0.8	1.9
	PPIX (proto) (7.7)	7.7	1.3
	HGB (1.3)	7.7	9.1
	HGB (1.3) + HPT[a] (2.6)	7.7	9.1
	HPT (2.6)	0	1.1
	Hemin (7.7) + HPX (15.4)	7.7	9.1
	HPX (15.4)	0	1.1
	Human methemoglobin (1.3)	7.7	9.1
	Human myoglobin (5.1)	7.7	9.1
	Bovine lactoperoxidase (1.3)	7.7	36.4
	Equine cytochrome *c* (1.9)	7.7	3.0
	Bovine catalase (10.0)	7.7	25.4
	HSA (hemin saturated) (5)	12.6	36.1
	Organic iron source (human)		
	SA (hemin-free) (5)	0	23.6
	LF (iron saturated) (12.8)	0	24.4
	TF (iron saturated) (25)	0	54.9
	Apo-TF (25)	0	1.5
	Inorganic iron source		
	Ferrous chloride (10–200)	0	11.1–201.1
	Ferric chloride (10–200)	0	11.1–201.1
	Ferric ammonium citrate (10–200)	0	11.1–201.1
	Ferric citrate (10–200)	0	11.1–201.1
	Ferric nitrate (10–200)	0	11.1–201.1
	Iron chelator BPD		
	Hemin (1.5) + BPD (1)	1.5	2.7
	Hemin (1.5) + BPD (10)	1.5	2.7
	Hemin (1.5) + BPD (25)	1.5	2.7
	Hemin (1.5) + BPD (50)	1.5	2.7
	BPD (1–50)	0	1.1
Basal broth pretreated with $CaCl_2$	Porphyrin or metal source		
	None	0	0.5
	Hemin (7.7)	7.7	8.7
	PPIX (7.7)	7.7	0.5
	PPIX-Zn^{2+}[b] (7.7)	7.7	0.5
	PPIX (7.7) + ferric chloride (7.7)	7.7	8.8
	PPIX (7.7) + zinc chloride (7.7)	7.7	0.6
	Hemin (7.7) + zinc chloride (7.7)	7.7	8.9
	Zinc chloride (7.7)	0	0.6
	Ferric chloride (7.7)	0	8.3
M9 minimal medium pretreated with $CaCl_2$ and Chelex-100	Porphyrin or metal source		
	None	0	0.1
	HGB (1.3)	7.7	8.1
	HGB (1.3) + HPT[a] (2.6)	7.7	8.1
	HPT (2.6)	0	0.1
	Hemin (7.7) + HPX (15.4)	7.7	8.1
	HPX (15.4)	0	0.1

[a] HPT with mixed phenotypes of 30% (1:1), 50% (2:1), 20% (2:2) (Sigma and Calbiochem).
[b] Aldrich Chemical Co. Inc.

sodium dodecyl sulfate-polyacrylamide gel electrophoresis (data not shown).

Siderophore assays. Siderophores were determined in culture supernatants from *P. gingivalis* cells grown in basal broth either with or without added hemin or iron sources (Table 1) by the siderophore assay of Schwyn and Neilands (43), the hydroxamate assay of Atkin et al. (3), and the phenolate assay of Arnow (2). All assays were performed in

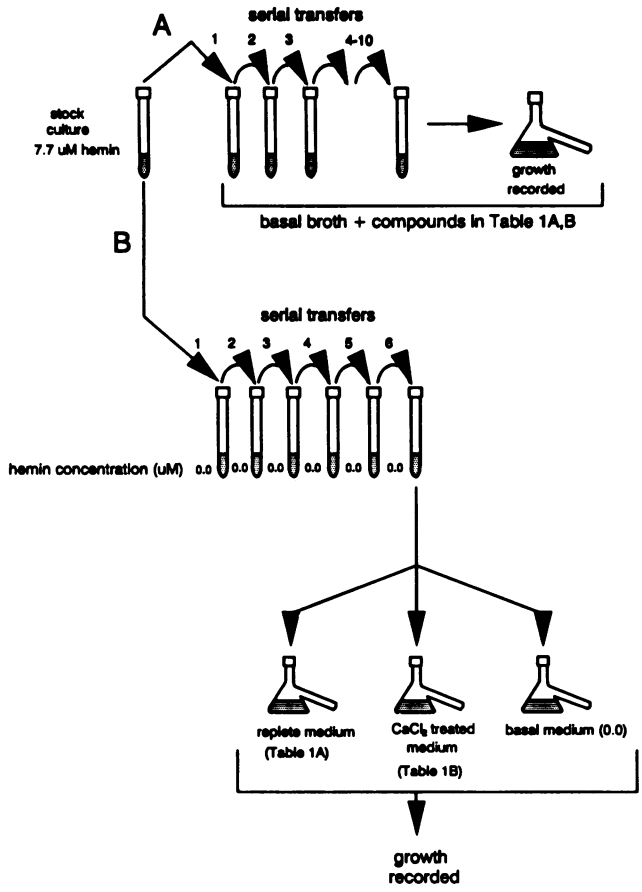

FIG. 1. Scheme for the growth studies of *P. gingivalis* strains. Strains were grown in excess (7.7 μM) hemin and transferred to either basal broth plus the compounds to be studied (Table 1) (A) or basal medium devoid of hemin (B). All transfers were done with 10% (vol/vol) inocula. The depleted cells were then transferred into either replete medium (Table 1), $CaCl_2$-treated basal medium (Table 1), or untreated basal medium, and growth was recorded spectrophotometrically at 660 nm.

triplicate, and uninoculated sterile medium and the appropriate reference standards were employed with each assay.

Chemical reagents. All chemical reagents used in this study were of the highest quality and were purchased from Sigma Chemical Co. (St. Louis, Mo.), unless otherwise indicated. Iron-saturated TF and LF were purchased from Sigma. Iron saturation was determined by graphite furnace atomic absorption spectroscopy.

RESULTS

Effect of hemin (iron) depletion and restriction on the growth of *P. gingivalis*. *P. gingivalis* was grown in a complex peptide medium, mycoplasma basal broth, with an incidental iron content estimated to be 1.1 μM (Table 1). This level of iron did not support the growth of *P. gingivalis* (Table 2). The effects of hemin depletion and of limiting hemin concentrations on the growth of *P. gingivalis* are listed in Table 2. Hemin excess is considered to be 7.7 μM hemin, a concentration beyond which the growth rate of *P. gingivalis* did not increase. Growth in the presence of excess hemin resulted in a mean generation time (MGT) of 3.32 h and a maximum cell yield (OD_{660max}) of 2.02 (Table 2). Exhaustion of endogenous hemin stores in the cells by serial transfer of hemin-excess-grown *P. gingivalis* into hemin-free medium (0.0 hemin) resulted in a significant reduction in the MGT from 3.32 to approximately 30 h at the seventh passage in hemin depletion conditions. At least eight serial transfers were required before growth was completely arrested. Between passages 1 to 3, the cells grew with an MGT similar to that of *P. gingivalis* grown in hemin excess. However, at passage 4, there was a significant increase in MGT, such that the cells displayed an MGT which was 2.5 times slower than that of cells grown under hemin excess, indicating that a critical iron concentration is required for optimal *P. gingivalis* growth.

TABLE 2. Effect of hemin restriction and depletion on the growth of *P. gingivalis* W50

Status of and hemin concn (μM) in growth medium[a]	MGT ± SD[b] (h)	OD_{660max} ± SD[b]
Hemin excess (7.7)	3.32 ± 0.72	2.02 ± 0.27
Hemin depleted (0.0)		
Passage 1	4.41 ± 0.14	1.71 ± 0.07
Passage 2	4.88 ± 0.16	1.68 ± 0.04
Passage 3	4.91 ± 0.18	1.67 ± 0.03
Passage 4	10.04 ± 0.13	0.95 ± 0.06
Passage 5	18.41 ± 0.17	0.51 ± 0.02
Passage 6	25.54 ± 0.21	0.31 ± 0.04
Passage 7	29.73 ± 0.10	0.10 ± 0.01
Passage 8	NG[c]	NG
Hemin restricted[d]		
Hemin (1.5)	8.23 ± 0.15	1.17 ± 0.02
Hemin (0.8)	10.30 ± 0.14	1.14 ± 0.02
Hemin[e] (0.0)	NG	NG
Hemin repleted (0.0→7.7), passage 7	6.20 ± 0.14	1.50 ± 0.01

[a] Growth medium, 2.1% mycoplasma broth plus 1.0 μg of menadione per ml.
[b] Results are the means of 3 to 5 experiments.
[c] NG, no growth.
[d] Bacterial cells were serially transferred at least 10 times in each respective hemin-limited medium (Fig. 1B).
[e] No hemin, no added iron source.

Growth of *P. gingivalis* in hemin-restricted culture (0.8 or 1.5 μM) resulted in *P. gingivalis* growth with an MGT that was 2.5 to 3.5 times slower than that of cells grown in hemin excess (Table 2). At hemin concentrations less than 0.4 μM or in the absence of added hemin, *P. gingivalis* did not grow (data not shown). Importantly, transfer of the hemin-depleted cells (0.0 μM hemin, passage 7; MGT, 29.73 h) into hemin-excess culture resulted in an almost immediate resumption of growth (5). However, when the *P. gingivalis* cells were transferred under these conditions, the MGT was approximately two times longer than that of cells grown under hemin excess without a prior hemin depletion (Table 2); however, after four generations, the cells assumed the MGT of the hemin-excess condition.

Effect of BPD-induced hemin (iron) restriction on the growth of *P. gingivalis*. The deferrating compound BPD added to hemin-excess medium (under anaerobic conditions) functions to selectively chelate the iron molecule in hemin and, thereby, makes it unavailable for use (37, 51). The effects of BPD on the growth of *P. gingivalis* are given in Table 3. The addition of BPD at concentrations between 1 and 100 μM (nontoxic to *P. gingivalis*; data not shown) to

TABLE 3. Effect of BPD-induced iron restriction on the growth and yield of *P. gingivalis* W50

Status of and supplement (μM) in growth medium[a]	MGT ± SD[b] (h)	OD_{660max} ± SD
Hemin (1.5)	8.23 ± 0.15	1.17 ± 0.02
Iron restricted[c]		
Hemin (7.7)[d] + BPD (200)	6.30 ± 0.10	1.42 ± 0.03
Hemin (1.5)[e] + BPD (1)	9.80 ± 0.14	0.93 ± 0.01
Hemin (1.5) + BPD (10)	13.15 ± 0.21	0.77 ± 0.01
Hemin (1.5) + BPD (25)	16.75 ± 0.50	0.55 ± 0.02
Hemin (1.5) + BPD (50)	NG[f]	NG
Iron replete[g]		
Hemin (1.5) + BPD (1) → hemin (1.5)	9.40 ± 0.14	1.16 ± 0.03
Hemin (1.5) + BPD (1) → hemin (7.7)	3.23 ± 0.09	1.85 ± 0.12
Hemin (1.5) + BPD (10) → hemin (1.5)	9.65 ± 0.07	1.07 ± 0.02
Hemin (1.5) + BPD (10) → hemin (7.7)	3.49 ± 0.12	1.89 ± 0.04
Hemin (1.5) + BPD (25) → hemin (1.5)	12.65 ± 0.35	0.95 ± 0.10
Hemin (1.5) + BPD (25) → hemin (7.7)	3.82 ± 0.48	1.87 ± 0.17

[a] Growth medium, 2.1% mycoplasma broth plus 1.0 μg of menadione per ml.
[b] Results are the means of 4 to 9 experiments.
[c] Bacterial cells were serially transferred at least 10 times in each respective hemin-limited medium (Fig. 1B).
[d] 7.7 μM, excess hemin concentration.
[e] 1.5 μM, limiting hemin concentration.
[f] NG, no growth.
[g] Iron-restricted cultures repleted by seven passages with the indicated concentrations (1.5 or 7.7 μM) of hemin.

hemin-excess cultures (7.7 μM) had no effect on the MGT, even after 10 serial transfers (data not shown). However, the addition of BPD to *P. gingivalis* grown with limiting hemin (1.5 μM) resulted in an inhibition of *P. gingivalis* growth (Table 3). By lowering the amount of iron (coordinated to hemin) added to the medium, we could effectively lower the concentration of BPD required to chelate this essential metal. The addition of BPD at concentrations between 1 and 25 μM to the mycoplasma broth-hemin medium (1.5 μM hemin) resulted in a dose-dependent reduction in growth, with an increase in the MGT from 9.80 to 16.75 h compared with that of the BPD-free control culture (MGT, 8.23 h; Table 3). Concentrations of BPD at 50 μM or greater completely arrested growth. In the presence of 1 to 25 μM BPD, *P. gingivalis* growth inhibition (MGT, 9.80 to 16.75 h) was reversible upon transfer into 1.5 μM hemin–mycoplasma broth (compared with MGT of 9.40 to 12.65 h). Transfer of the iron-restricted cells grown in the presence of 1 to 25 μM BPD into excess hemin (7.7 μM)–mycoplasma broth resulted in the complete abrogation of the inhibitory effects of BPD. Since BPD functionally chelates iron, the BPD observations reported here suggest that the iron atom in the hemin molecule is the critical constituent for the growth of *P. gingivalis*. Growth inhibitory effects were not observed with the addition of the iron chelators ethylenediamine-di(*o*-hydroxyphenylacetic acid) (EDDA) and Desferal (deferoxamine; Ciba-Geigy, Summit, N.J.) at 1 to 400 μM concentrations to hemin cultures of *P. gingivalis*. Although EDDA and Desferal have been shown to effectively chelate iron in aerobic systems (26, 39), these iron chelators did not function to deny iron from *P. gingivalis* in an anaerobic environment.

TABLE 4. Growth of *P. gingivalis* with different porphyrin and/or metal sources

$CaCl_2$-treated growth medium supplemented with 7.7 μM (each) of:	MGT ± SD[a] (h)	OD_{660max} ± SD[a]
PPIX	NG[b]	NG
PPIX + $FeCl_3$	2.79 ± 0.35	2.23 ± 0.18
PPIX-Zn^{2+}	NG	NG
PPIX + $ZnCl_2$	NG	NG
Hemin	2.96 ± 0.87	2.23 ± 0.27
Hemin + $ZnCl_2$	3.49 ± 0.84	2.23 ± 0.28
$ZnCl_2$	NG	NG
$FeCl_3$	NG	NG
No porphyrin	NG	NG

[a] Results are the means of 3 to 9 experiments.
[b] NG, no growth.

Role of PPIX in the growth of *P. gingivalis*. While iron sequestration from hemin with BPD revealed *P. gingivalis* dependence on hemin-associated iron, the functional role of the porphyrin ring structure (PPIX) in the growth of *P. gingivalis* still remained unclear. Therefore, we compared the effects of the tetrapyrrole porphyrin ring and PPIX (minus iron) in the presence and absence of iron, as well as PPIX supplemented with zinc, on the growth of *P. gingivalis* in mycoplasma basal broth medium which had been treated with $CaCl_2$ to reduce residual medium levels of iron to below 0.5 μM (Table 4). PPIX (minus coordinated iron), zinc-saturated PPIX, and $ZnCl_2$ plus PPIX were unable to support the growth of *P. gingivalis* (Table 4). Importantly, PPIX plus 7.7 μM ferric iron supported the growth of *P. gingivalis* at a rate comparable to that of the 7.7 μM hemin control (MGT of 2.79 versus 2.96 h; Table 4). Therefore, *P. gingivalis* requires PPIX-coordinated iron for growth under the experimental conditions used in this study.

Iron and hemin concentrations of the cell envelope of *P. gingivalis*. The effect of hemin concentration on the storage of hemin and iron in the *P. gingivalis* cell envelope was determined by growing *P. gingivalis* with excess hemin and comparing the hemin and iron concentrations in the cell envelope under these conditions to cells which were either starved for hemin or grown under hemin restriction (Table 5). Under hemin-excess conditions, *P. gingivalis* cell envelopes contained 274 pg of iron and 1588 pg of hemin per μg of cell envelope protein. Significantly, starvation for hemin or growth of *P. gingivalis* under hemin restriction (either 0.8 or 1.5 μM hemin) resulted in a 33 to 47% decrease in cell envelope-associated iron concentration and a 19 to 70% decline in the hemin concentration compared with those of control cells (Table 5). A progressive depletion of the cell envelope-associated hemin stores by 63% occurred after four serial transfers into hemin-free medium; continued transfer (i.e., passage 6) resulted in essentially complete depletion of cell envelope hemin stores (94% reduction). In comparison, depletion of the *P. gingivalis* cell membrane-associated iron by direct starvation for hemin required at least four serial passages to deplete the cell envelope-associated iron by 42%; six serial passages resulted in an 85% reduction in cell envelope-associated iron (Table 5). Interestingly, the dramatic decrease in cell envelope-associated iron and hemin at passage 4 correlated with the effect of

TABLE 5. Effects of hemin excess and starvation on storage of hemin and iron in the cell envelopes of *P. gingivalis* W50[a]

Status of and supplement (μM) in growth medium[b]	Hemin concn (pg/μg of cell envelope protein) ± SD[c]	Hemin content (%)	Iron concn (pg/μg of cell envelope protein) ± SD	Iron content (%)
Excess hemin (7.7)	1588 ± 52	100	274 ± 3	100
Hemin restricted				
Hemin (1.5)	1280 ± 202	80.6	183 ± 12	66.8
Hemin (0.8)	477 ± 23	30.0	145 ± 2	52.9
Iron restricted				
Hemin (1.5) + BPD (1)	499 ± 151	31.4	138 ± 1	50.4
Hemin (1.5) + BPD (25)	397 ± 59	25.0	105 ± 5	38.3
Hemin depleted (0.0)				
Passage 1	1291 ± 205	81.3	268 ± 22	97.8
Passage 2	1122 ± 50	70.7	224 ± 14	81.8
Passage 3	892 ± 70	56.2	210 ± 2	76.6
Passage 4	579 ± 173	36.5	161 ± 7	58.8
Passage 5	173 ± 41	10.9	46 ± 3	16.8
Passage 6	94 ± 8	5.9	42 ± 5	15.3
Hemin repleted (0.0→7.7)				
Passage 4	ND[d]	ND	250 ± 60	91.2
Passage 5	2326 ± 250	146.5	195 ± 1	71.2
Passage 6	ND	ND	100 ± 4	36.5

[a] Iron concentration was determined by Ferrozine assay and graphite furnace atomic absorption spectrophotometry; hemin content was determined by pyridine hemochromagen assay with human hemin as the standard; protein content was determined by the bicinchoninic acid assay (Pierce) with bovine serum albumin as the standard.
[b] Growth medium was 2.1% mycoplasma broth plus 1.0 μg of menadione per ml.
[c] Results are the means of two experiments performed in triplicate.
[d] ND, not done.

hemin restriction on cell growth which exhibited a significant decline in cellular growth between passages 3 and 4 (Table 2). The addition of hemin to hemin-starved cells resulted in a restoration of membrane-associated iron (passages 4, 5, and 6) and hemin (passage 5) concentrations after only three or four generations of growth to 36 to 92 and 147%, respectively, of hemin-excess control levels (Table 5). Additions of BPD (1 and 25 μM) to hemin-restricted cultures resulted in a 50 to 62% and a 69 to 75% decrease in cell envelope iron and hemin, respectively (Table 5).

Growth of *P. gingivalis* with different hemin sources. Several hemin-containing compounds were tested for their abilities to support the growth of *P. gingivalis* (Table 6). For comparative purposes, the hemin concentrations in these compounds were normalized to 7.7 μM hemin, and their effects on growth were compared with that of *P. gingivalis* in 7.7 μM hemin. All of the hemin-containing substrates tested supported the growth of *P. gingivalis* at an MGT comparable to that of the control cells. While cytochrome *c* and catalase supported the growth of *P. gingivalis*, they did so only at MGTs of 5.50 and 10.36 h, respectively. Comparable cell yields were observed, except for catalase (OD_{660max} of 0.46).

Since hemin or HGB is required for growth of *P. gingivalis* (Tables 2 and 6), and since these compounds are immediately bound by HPX and HPT in the host environment, respectively, the effects of these compounds on the growth of *P. gingivalis*, in both the presence and absence of hemin and HGB, were determined (Table 6). Both HPX and HPT (hemin-free, Table 1) at the concentrations tested did not support *P. gingivalis* growth. However, addition of HPX or HPT to hemin- or HGB-supplemented medium was not inhibitory to the growth of *P. gingivalis* compared with its growth in the mycoplasma basal broth hemin- or HGB-supplemented medium. It is more than likely that these two hemin-binding compounds bound essentially all available hemin and HGB at the HPX and HPT molar concentrations tested (2:1) (see below) and that the *P. gingivalis* growth observed occurred by proteolytic cleavage of these host proteins, resulting in liberation of hemin and HGB (8, 22). Note in Table 6 that even in the presence of these hemin-binding compounds, maximum cell yields occurred comparable to that of *P. gingivalis* growing in 7.7 μM hemin. To determine the functional capability of the purified HPX and HPT to sequester hemin and HGB, the growth inhibitory effects of both HPX and HPT were examined in *E. coli*, which has been shown to be susceptible to the bacteriostatic effects of biologically active host hemoproteins (15). *E. coli* MG1655 could not grow in M9 medium in the absence of an added iron source or in the presence of only HPX or HPT (Table 6). However, with addition of hemin (7.7 μM) or HGB (1.3 μM), the *E. coli* cells grew with comparable MGTs of 1.00 h each; however, their maximum growth yields did vary (OD_{660max}, 1.30 for hemin and 0.94 for HGB). Importantly, inhibition of growth of *E. coli* was observed with the addition of HPX to hemin-supplemented M9 medium and HPT to HGB-supplemented medium (Table 6). These findings indicate that the host hemin- and HGB-sequestering molecules were biologically active and functioned to block bacterial access to both hemin and HGB with *E. coli* but not with *P. gingivalis*, implying a mechanism of hemin acquisition with *P. gingivalis* different from that observed with *E. coli*.

Effects of different iron sources on the growth of *P. gingivalis* W50. While we (5, 21) and others (4, 25, 30, 41) have determined that *P. gingivalis* has a requirement for hemin, it is unknown whether *P. gingivalis* is capable of utilizing

TABLE 6. Effect of hemin sources on growth of *P. gingivalis* W50 and *E. coli* MG1655

Strain and hemin source (μM)	MGT ± SD[a] (h)	OD_{660max} ± SD[a]
P. gingivalis W50[b]		
HGB (1.3)	3.28 ± 0.86	1.98 ± 0.29
Methemoglobin (1.3)	3.45 ± 0.07	2.00 ± 0.01
Myoglobin (5.1)	4.75 ± 0.07	1.87 ± 0.02
SA (hemin saturated) (5.0)	3.68 ± 0.70	1.32 ± 0.10
Lactoperoxidase (1.3)	3.05 ± 0.07	2.03 ± 0.01
Cytochrome *c* (1.9)	5.50 ± 0.14	1.62 ± 0.01
Catalase (10.0)	10.36 ± 0.25	0.46 ± 0.02
Hemin (7.7) + HPX[c] (15.4)	3.50 ± 0.10	1.96 ± 0.13
HPX (15.4)	NG[d]	NG
HGB (1.3) + HPT[e] (2.6)	3.55 ± 0.82	1.99 ± 0.23
HPT (2.6)	NG	NG
E. coli MG1655[f]		
Hemin (7.7)	1.01 ± 0.34	1.30 ± 0.08
Hemin (7.7) + HPX[c] (15.4)	NG	NG
HPX (15.4)	NG	NG
HGB (1.3)	1.16 ± 0.24	0.94 ± 0.02
HGB (1.3) + HPT[e] (2.6)	NG	NG
HPT (2.6)	NG	NG
None[g]	NG	NG

[a] Results are the means of 3 to 9 experiments.
[b] *P. gingivalis* was grown in a basal medium containing 2.1% mycoplasma broth plus 1.0 μg of menadione per ml. All heme compounds were normalized to a 7.7 μM concentration; heme content was determined by a pyridine hemochromagen assay.
[c] HPX was isolated from human serum and purified to homogeneity by heme-agarose affinity chromatography.
[d] NG, no growth.
[e] HPT with mixed phenotypes 30% (1:1), 50% (2:1), 20% (2:2) (Sigma and Calbiochem) isolated from human serum.
[f] *E. coli* was grown in M9 minimal medium pretreated with $CaCl_2$ and Chelex-100.
[g] None, M9 minimal medium alone with no added heme source.

TABLE 7. Growth of *P. gingivalis* with different iron sources

Source and concn (μM) of iron in growth medium	MGT ± SD[a] (h)	OD_{660max} ± SD[a]
Host IBP[b]		
SA (hemin free) (5)	2.78 ± 0.83	2.23 ± 0.25
LF (iron saturated) (12.8)	8.80 ± 0.69	1.31 ± 0.15
TF (iron saturated) (25)	3.40 ± 0.75	2.49 ± 0.05
Apo-TF (iron free) (25)	NG[c]	NG
Inorganic iron		
Ferric chloride (200)	8.05 ± 0.07	1.13 ± 0.04
Ferrous chloride (200)	14.65 ± 0.75	0.37 ± 0.01
Ferric citrate (200)	10.75 ± 0.35	0.77 ± 0.01
Ferric ammonium citrate (200)	30.90 ± 0.42	0.25 ± 0.01
Ferric nitrate (200)	34.40 ± 1.13	0.16 ± 0.05
None[d]	NG	NG

[a] Results are the means of 4 to 9 experiments.
[b] All iron-binding proteins were normalized to the concentration normally found in gingival crevicular fluid.
[c] NG, no growth.
[d] None, no added iron source to the basal medium.

additional organic or inorganic iron sources for growth. The effects of several of the more classical host IBPs, SA, LF, and TF as well as inorganic iron sources on the growth of *P. gingivalis* were determined (Table 7). SA and TF supported the growth of *P. gingivalis* (Table 7) at growth rates comparable to those observed with hemin sources (Table 6), while LF only supported *P. gingivalis* growth at an MGT of 8.8 h. Apo-TF (iron-free TF) did not support *P. gingivalis* growth, suggesting that protein-bound iron was essential for growth. Although inorganic iron supported the growth of *P. gingivalis*, it required the addition of iron at concentrations of 200 μM or greater. *P. gingivalis* growth with ferric iron sources (ferric chloride and ferric citrate) resulted in MGTs of 8 to 11 h; ferrous iron (ferrous chloride) and nitrogenous inorganic iron sources (ferric ammonium citrate and ferric nitrate) were also not utilized efficiently, with resultant lengthy MGTs (Table 7). The fact that such high iron concentrations were required for even very slow growth suggests that *P. gingivalis* is not capable of efficiently processing inorganic forms of iron and favors protein-bound organic iron sources. This was noted for *P. gingivalis* grown with SA or TF and resulted in an almost 15% increase in cell biomass (OD_{660max} of 2.23 to 2.49) compared with the cell yield observed with hemin (OD_{660max} of 2.02) (Table 2). The *P. gingivalis* cell biomass was significantly reduced (OD_{660max} of 0.16 to 1.13) in the presence of inorganic iron.

Siderophore production of *P. gingivalis*. The excretions of siderophores into the growth supernatants of *P. gingivalis* grown under all test conditions (Table 1) were assayed for the presence of siderophores of both the phenolate and hydroxamate classes. No siderophores were detected in any of the culture supernatants whether grown in iron- or hemin-starved, -restricted, or -replete conditions (data not shown), nor did the addition of the siderophore deferoxamine enhance the growth of hemin-starved or iron-restricted *P. gingivalis* (data not shown).

DISCUSSION

The inflamed periodontal pocket provides an ecological niche in which there is the selection of a unique microbiota which has the ability to survive in an otherwise hostile environment. Survival in this "multigeneric commune" requires that these resident genera develop mechanisms for sharing the end products of metabolism, as well as for obtaining nutrients, notably iron, which might be in very short supply. A large number of studies have convincingly demonstrated that the availability of iron plays a critical role in both the emergence and virulence of a large number of pathogenic species (7, 14–16, 20, 27, 38, 52, 53). We (5, 10, 21) and several others (9, 22, 25, 30, 31, 34, 41, 44) have demonstrated that iron is an important regulatory factor both in *P. gingivalis* growth and in its expression of virulence. Therefore, an understanding of the role of iron in the growth and virulence of this pathogen in the subgingival environment is of paramount importance in comprehending the possible control of the progression of the periodontal disease process.

To date, the literature concerned with the growth and physiology of the genus *Porphyromonas* (and *Bacteroides*) has either described an absolute hemin requirement for growth or implied, by inclusion of hemin in the growth medium, this requirement (4, 5, 22, 29–31, 41, 44). Previous studies have reported the storage of protohemin by *P. gingivalis* (5, 41), which results in a dark-pigmentation phenotype, when cultures are grown on blood agar plates (44), and provides its black-pigmented *Bacteroides* character. We have reported previously (5, 21) that hemin could satisfy *P. gingivalis's* entire iron requirement, as well as provide it with the ability to utilize endogenous membrane-stored hemin for growth under periods of hemin deprivation. Ultimately, when all of the cell-associated hemin was de-

pleted, there was a cessation of growth. Interestingly, even after significant hemin deprivation, growth could be restored almost immediately to a rate comparable to that of hemin-excess conditions (5). This feast-famine phenomenon of hemin storage and utilization has also been observed in *Bacteroides melaninogenicus* (41), which can survive for eight generations, and in *H. influenzae*, which can survive for four generations (54) after transfer into hemin-free culture. Ecologically, this ability of *P. gingivalis* to take up hemin very rapidly from the environment and to store and utilize it during periods of iron starvation provides it with an important selective nutritional advantage for long-term survival in its habitat, especially in the iron-limited niche of the healthy periodontal pocket (17, 33, 48). A switch to a hemin-rich environment (the developing inflammatory periodontal pocket) may provide *P. gingivalis* with available hemin (19), which not only satisfies its iron requirement but also allows storage of this iron source for later use in a hemin-limited environment (i.e., resolving periodontal pocket).

In the absence of an added iron source, *P. gingivalis* did not grow, even though the mycoplasma basal broth contained 1.1 μM of endogenous iron. Since hemin is a tetrapyrrole molecule composed of PPIX, which is coordinately linked to iron at its center, it is more than likely that *P. gingivalis* is capable of processing growth-medium-associated iron in only a specific form, that is, coordinated to a porphyrin structure. Our observations with PPIX alone or supplemented with zinc indicate unequivocally that the *P. gingivalis* strain examined in this study required the entire hemin molecule for growth and could not survive with iron-free PPIX. Recently, Barua and coworkers (4) reported that *P. gingivalis* A7A1-28 was capable of utilizing PPIX to support growth. While we have no explanation for the differences in their observations compared with ours, it might be possible that there were levels of iron in their growth medium which were able to support the growth of *P. gingivalis* A7A1-28 in the presence of PPIX alone. *H. influenzae* and *B. fragilis* have been reported to be capable of using PPIX for growth (1, 12, 26, 51, 54). *H. influenzae* possesses an enzyme which coordinates iron to PPIX, resulting in heme and cytochrome formations (54) which are then used for growth. *B. fragilis* may also grow in the presence of PPIX, but its survival may be a result of iron contamination in the growth medium, which together with PPIX is a utilizable substrate (45, 50, 51). The fact that *P. gingivalis* is also capable of using inorganic iron in conjunction with exogenous PPIX for growth suggests that *P. gingivalis* may possess a mechanism of metallization of PPIX to form hemin, which may then be processed for cellular metabolism, similar to that which has been reported to occur in *Bacteroides ruminicola* (28) and in *H. influenzae* (54). Therefore, the tetrapyrrole structure of hemin may represent an important vehicle for delivery and transport of iron into the *P. gingivalis* cell.

Our studies employing the iron chelator BPD, which competitively chelates coordinated iron from within the hemin molecule, showed inhibition of *P. gingivalis* growth, indicating that hemin was required as an iron source. While high concentrations of BPD (125 to 300 μM) have been reported to produce iron-restricted conditions for *P. gingivalis* A7A1-28 (4) and several different enteric *Bacteroides* species (37, 50), our observations with *P. gingivalis* W50 revealed that high concentrations (>100 μM) of BPD in the presence of high hemin concentrations (15.4 μM) were, in fact, inhibitory (data not shown), suggesting that growth inhibition with high concentrations of BPD was a result of interference with cellular metabolism rather than from competitive saturable iron chelation by BPD. Addition of excess PPIX (7.7 μM) to the BPD-treated cultures had no measurable effect on growth (data not shown), supporting our contention that it is the iron atom in the hemin molecule which is essential for the growth of *P. gingivalis*. In the absence of a porphyrin source, *P. gingivalis* may process alternate organic and inorganic iron molecules for growth, showing for the first time that *P. gingivalis* does not have a strict requirement for hemin or porphyrin-related compounds. Substitution of hemin with host IBPs, such as TF, LF, or hemin-free SA, supported the growth of *P. gingivalis*. TF and SA are found primarily in serum (53) as well as in gingival crevicular fluid (11, 33), while LF is derived primarily from mucosal secretions (53) and polymorphonuclear leukocytes (7, 17, 27), which release large amounts into the periodontal milieu in inflammatory conditions (17). Interestingly, our observations also revealed that *P. gingivalis* was capable of growing on both organic and inorganic iron sources in the absence of hemin, indicating that while hemin may be a preferential source of iron, it is not essential for growth. Since *P. gingivalis* is capable of utilizing a large number and wide variety of host hemin and IBP iron sources, it may provide this host-associated species with a selective ecological advantage through circumvention of host iron-withholding defenses.

While we have a fairly in-depth understanding of the role of iron (i.e., hemin) in the in vitro metabolism of *P. gingivalis*, we still have only a vague understanding of its role in vivo. Upon release of free hemin and HGB into tissue fluids as a result of tissue damage and hemolytic activity, HGB is bound immediately by HPT, while hemin is bound by HPX and SA (53), all of which may function in vivo to limit bacterial growth. However, as we have described in this report, *P. gingivalis* was capable of utilizing a broad range of hemin-containing molecules in vitro as iron sources (Table 6), even in the presence of HPT and HPX. Previous studies have shown that *P. gingivalis* is capable of degrading HPT, HPX, SA (8), and HGB (22), all of which are found at very high levels in the periodontal pocket (11, 48). The extensive proteolytic capability of *P. gingivalis* (18) may contribute to the degradation of these host hemin-sequestering proteins, and, in this way, liberate hemin and HGB for binding and uptake into the cell. *H. influenzae* is able to acquire iron from hemin, myoglobin, HGB, HGB-HPT, hemin-SA, and hemin-HPX (39, 47). Unlike *P. gingivalis*, however, *H. influenzae* could not utilize catalase, lactoperoxidase, or cytochrome *c* as hemin sources (47), indicating that *P. gingivalis* is capable of metabolizing a broader range of hemin substrates (Table 6). While several enteric *Bacteroides* spp. are capable of utilizing hemin and HGB for growth (50), it is not known whether HPX or HPT complexed to hemin compounds (as in the studies reported here) are bacteriostatic for these species.

The clinical significance of the observations reported here with regard to hemin and HGB in the natural course of periodontal disease and the contribution made by *P. gingivalis* can only be surmised. It is likely that *P. gingivalis* is capable of releasing HGB from erythrocytes into the developing periodontal pocket through direct proteolytic action or by hemolytic destruction (10, 18). Chu et al. (10) have recently shown that *P. gingivalis* produces a cell-bound hemolysin that is concentrated in extracellular vesicles and that the expression of this hemolytic activity is regulated by the environmental hemin concentration. Importantly, since

the vesicles contained concentrated levels of hemolysin, these structures may be released by *P. gingivalis* into the periodontal milieu and interact with erythrocytes directly, resulting in HGB release. How the hemin molecule is processed by *P. gingivalis* and how it contributes to virulence of this putative periodontal pathogen remain to be determined. Since *P. gingivalis* does not produce siderophores, binding and uptake of host iron may occur through the expression of novel outer membrane proteins (5). The occurrence of hemin-binding proteins in *P. gingivalis* and their expression in response to hemin-limited conditions are currently under investigation.

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REFERENCES

1. **Al-Jalili, T. A. R., and H. N. Shah.** 1988. Protoheme, a dispensable growth factor for *Bacteroides fragilis* grown by batch and continuous culture in a basal medium. Curr. Microbiol. **17**:13–18.
2. **Arnow, L. E.** 1937. Colorimetric determination of the components of 3,4-dihydroxyphenylalanine-tyrosine mixtures. J. Biol. Chem. **118**:531–537.
3. **Atkin, C. L., J. B. Neilands, and H. J. Phaff.** 1970. Rhodotorulic acid from species of *Leucosporidium*, *Rhodosporidium*, *Rhodotorula*, *Sporidiobolus*, and *Sporobolomyces*, and a new alanine-containing ferrichrome from *Cryptococcus melibiosum*. J. Bacteriol. **103**:722–733.
4. **Barua, P. K., D. W. Dyer, and M. E. Neiders.** 1990. Effect of iron limitation on *Bacteroides gingivalis*. Oral Microbiol. Immunol. **5**:263–268.
5. **Bramanti, T. E., and S. C. Holt.** 1990. Iron-regulated outer membrane proteins in the periodontopathic bacterium, *Bacteroides gingivalis*. Biochem. Biophys. Res. Commun. **166**: 1146–1154.
6. **Bramanti, T. E., G. G. Wong, S. Weintraub, and S. C. Holt.** 1989. Chemical characterization and biologic properties of lipopolysaccharide from *Bacteroides gingivalis* strains W50, W83, and ATCC 33277. Oral Microbiol. Immunol. **4**:183–192.
7. **Bullen, J. J.** 1981. The significance of iron in infection. Rev. Infect. Dis. **3**:1127–1138.
8. **Carlsson, J., J. F. Hofling, and G. K. Sundqvist.** 1984. Degradation of albumin, haemopexin, haptoglobin and transferrin by black-pigmented *Bacteroides* species. J. Med. Microbiol. **18**:39–46.
9. **Carman, R. J., M. D. Ramakrishnan, and F. H. Harper.** 1990. Hemin levels in culture medium of *Porphyromonas* (*Bacteroides*) *gingivalis* regulate both hemin binding and trypsinlike protease production. Infect. Immun. **58**:4016–4019.
10. **Chu, L., T. E. Bramanti, J. E. Ebersole, and S. C. Holt.** 1991. Hemolytic activity in the periodontopathogen *Porphyromonas gingivalis*: kinetics of enzyme formation and localization. Infect. Immun. **59**:1932–1940.
11. **Cimasoni, G.** 1983. Crevicular fluid update. Monogr. Oral Sci. **12**:62–68.
12. **Coulton, J. W., and J. C. S. Pang.** 1983. Transport of hemin by *Haemophilus influenzae* type b. Curr. Microbiol. **9**:93–98.
13. **Cowart, R. E., M. P. Marquardt, and B. G. Foster.** 1980. The removal of iron and other trace elements from a complex bacteriological medium. Microbios Lett. **13**:117–122.
14. **Dyer, D. W., E. P. West, and P. F. Sparling.** 1987. Effects of serum carrier proteins on the growth of pathogenic neisseriae with heme-bound iron. Infect. Immun. **55**:2171–2175.
15. **Eaton, J. W., P. Brandt, J. R. Mahoney, and J. T. Lee, Jr.** 1982. Haptoglobin: a natural bacteriostat. Science **215**:691–693.
16. **Finkelstein, R. A., C. V. Sciortino, and M. A. McIntosh.** 1983. Role of iron in microbe-host interactions. Rev. Infect. Dis. **5**:S759–S777.
17. **Friedman, S. A., I. D. Mandel, and M. S. Herrera.** 1983. Lysozyme and lactoferrin quantitation in the crevicular fluid. J. Periodontol. **54**:347–350.
18. **Grenier, D., G. Chao, and B. C. McBride.** 1989. Characterization of sodium dodecyl sulfate-stable *Bacteroides gingivalis* proteases by polyacrylamide gel electrophoresis. Infect. Immun. **57**:95–99.
19. **Hanioka, T., S. Shizukuishi, and A. Tsunemitsu.** 1990. Hemoglobin concentration and oxygen saturation of clinically healthy and inflamed gingiva in human subjects. J. Periodontal Res. **25**:93–98.
20. **Helms, S. D., J. D. Oliver, and J. C. Travis.** 1984. Role of heme compounds and haptoglobin in *Vibrio vulnificus* pathogenicity. Infect. Immun. **45**:345–349.
21. **Holt, S. C., and T. E. Bramanti.** 1991. Factors in virulence expression and their role in periodontal disease pathogenesis. Crit. Rev. Oral Biol. Med. **2**:177–281.
22. **Kay, H. M., A. J. Birss, and J. W. Smalley.** 1990. Haemagglutinating and haemolytic activity of the extracellular vesicles of *Bacteroides gingivalis* W50. Oral Microbiol. Immunol. **5**:269–274.
23. **Kennell, W., and S. C. Holt.** 1990. Comparative studies of the outer membranes of *Bacteroides gingivalis*, strains ATCC 33277, W50, W83, 381. Oral Microbiol. Immunol. **5**:121–130.
24. **Koskelo, P., and U. Muller-Eberhard.** 1977. Interaction of porphyrins with proteins. Semin. Hematol. **14**:221–226.
25. **Lev, M., K. C. Keudell, and A. F. Milford.** 1971. Succinate as a growth factor for *Bacteroides melaninogenicus*. J. Bacteriol. **108**:175–178.
26. **Maciver, I., T. O'Reilly, and M. R. W. Brown.** 1990. Porphyrin ring source can alter the outer membrane protein profile of non-typeable *Haemophilus influenzae*. J. Med. Microbiol. **31**: 163–168.
27. **Martinez, J. L., A. Delgado-Iribarren, and F. Baquero.** 1990. Mechanisms of iron acquisition and bacterial virulence. FEMS Microbiol. Rev. **75**:45–56.
28. **McCall, D. R., and D. R. Caldwell.** 1977. Tetrapyrrole utilization by *Bacteroides ruminicola*. J. Bacteriol. **131**:809–814.
29. **McDermid, A. S., A. S. McKee, and P. D. Marsh.** 1988. Effect of environmental pH on enzyme activity and growth of *Bacteroides gingivalis* W50. Infect. Immun. **56**:1096–1100.
30. **McKee, A. S., A. S. McDermid, A. Baskerville, A. B. Dowsett, D. C. Ellwood, and P. D. Marsh.** 1986. Effect of hemin on the physiology and virulence of *Bacteroides gingivalis* W50. Infect. Immun. **52**:349–355.
31. **Minhas, T., and J. Greenman.** 1989. Production of cell-bound and vesicle-associated trypsin-like protease, alkaline phosphatase and *N*-acetyl-β-glucosaminidase by *Bacteroides gingivalis* strain W50. J. Gen. Microbiol. **135**:557–564.
32. **Mueller-Eberhard, U., and W. T. Morgan.** 1975. Porphyrin-binding proteins in serum. Ann. N.Y. Acad. Sci. **244**:624–649.
33. **Mukherjee, S.** 1985. The role of crevicular fluid iron in periodontal disease. J. Periodontol. **56**(Suppl):22–27.
34. **Mukherjee, S., R. A. Murphy, and E. J. Wawszkiewcz.** 1988. Effect of hemoglobin and of ferric ammonium citrate on the virulence of periodontopathic bacteria. Oral Microbiol. Immunol. **3**:192–195.
35. **Neilands, J. B.** 1982. Microbial envelope proteins related to iron. Annu. Rev. Microbiol. **36**:285–309.
36. **Otto, B. R., M. Sparrius, A. M. J. J. Verweij-van Vught, and D. M. MacLaren.** 1990. Iron-regulated outer membrane protein of *Bacteroides fragilis* involved in heme uptake. Infect. Immun. **58**:3954–3958.
37. **Otto, B. R., A. M. J. J. Verweij-van Vught, J. van Doorn, and D. M. MacLaren.** 1988. Outer membrane proteins of *Bacteroides fragilis* and *Bacteroides vulgatus* in relation to iron uptake and virulence. Microb. Pathog. **4**:279–287.
38. **Payne, S. M.** 1988. Iron and virulence in the family *Enterobacteriaceae*. Crit. Rev. Microbiol. **16**:81–111.
39. **Pidcock, K. A., J. A. Wooten, B. A. Daley, and T. L. Stull.** 1988. Iron acquisition by *Haemophilus influenzae*. Infect. Immun. **56**:721–725.
40. **Riggs, A.** 1981. Preparation of blood hemoglobin of vertebrates.

Methods Enzymol. **76**:1–29.
41. **Rizza, V., P. R. Sinclair, D. C. White, and P. R. Courant.** 1968. Electron transport system of the protoheme-requiring anaerobe *Bacteroides melaninogenicus*. J. Bacteriol. **96**:665–671.
42. **Sambrook, J., E. F. Fritsch, and T. Maniatis.** 1989. Molecular cloning, a laboratory manual, 2nd ed., vol. 3, appendix A, p. 3. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.
43. **Schwyn, B., and J. B. Neilands.** 1987. Universal chemical assay for the detection and determination of siderophores. Anal. Biochem. **160**:47–56.
44. **Shah, H. N., R. Bonnet, B. Matteen, and R. A. Williams.** 1979. The porphyrin pigmentation of subspecies of *Bacteroides melaninogenicus*. Biochem. J. **180**:45–50.
45. **Sperry, J. F., M. D. Appleman, and T. D. Wilkins.** 1977. Requirement of heme for growth of *Bacteroides fragilis*. Appl. Environ. Microbiol. **34**:386–390.
46. **Stookey, L. L.** 1970. Ferrozine—a new spectrophotometric reagent for iron. Anal. Chem. **42**:779–781.
47. **Stull, T. L.** 1987. Protein sources of heme for *Haemophilus influenzae*. Infect. Immun. **55**:148–153.
48. **Tollefsen, T., and E. Saltvedt.** 1980. Comparative analysis of gingival fluid and plasma by crossed immunoelectrophoresis. J. Periodontal Res. **15**:96–106.
49. **Tsutusi, K., and G. C. Mueller.** 1982. Affinity chromatography of heme-binding proteins: an improved method for the synthesis of heme-agarose. Anal. Biochem. **121**:244–250.
50. **Verweij-van Vught, A. M. J. J., B. R. Otto, F. Namavar, M. Sparrius, and D. M. MacLaren.** 1988. Ability of *Bacteroides* species to obtain iron from iron salts, haem-compounds and transferrin. FEMS Microbiol. Lett. **49**:223–228.
51. **Verweij-van Vught, A. M. J. J., M. Sparrius, F. Namavar, W. A. C. Vel, and D. M. MacLaren.** 1986. Nutritional requirements of *Bacteroides fragilis* and *Bacteroides vulgatus* with reference to iron and virulence. FEMS Microbiol. Lett. **36**:47–51.
52. **Weinberg, E. D.** 1978. Iron and infection. Microbiol. Rev. **42**:45–66.
53. **Weinberg, E. D.** 1984. Iron withholding: a defense against infection and neoplasia. Physiol. Rev. **64**:65–102.
54. **White, D. C., and S. Granick.** 1963. Hemin biosynthesis in *Haemophilus*. J. Bacteriol. **85**:842–850.
55. **Williams, P., and M. R. W. Brown.** 1985. Influence of iron restriction on growth and the expression of outer membrane proteins by *Haemophilus influenzae* and *H. parainfluenzae*. FEMS Microbiol. Lett. **33**:153–157.