Characterization of a Tn4351-generated hemin uptake mutant of Porphyromonas gingivalis: evidence for the coordinate regulation of virulence factors by hemin

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The ability of Porphyromonas gingivalis to acquire iron in the iron-limited environment of the host is crucial to the colonization of this organism. Here, the isolation and characterization of a transpositional insertion mutant of P. gingivalis A7436 (designated MSM-3) which is defective in the utilization and transport of hemin, is reported. P. gingivalis MSM-3 was initially selected on the basis of its non-pigmented colony phenotype on anaerobic blood agar plates following mutagenesis with the Bacteroides fragilis transposon Tn4351. P. gingivalis MSM-3 grew poorly when supplies with hemin as a sole source of iron; however growth was observed with hemoglobin or inorganic iron. P. gingivalis MSM-3 grown under hemin-replete or hemin-deplete conditions bound and transported less \(^{14}C\)hemin and \(^{59}Fe\)hemin than did the parental strain A7436. At 4 hr, P. gingivalis MSM-3 grown under hemin-replete conditions transported only 10,000 pmol of hemin per mg total cellular protein, or 14% of the amount transported by P. gingivalis A7436 grown under similar
conditions. Unlike *P. gingivalis* A7436, hemin binding and transport by *P. gingivalis* MSM-3 were not tightly regulated by hemin or iron. Examination of *P. gingivalis* MSM-3 cultures by electron microscopy revealed an overproduction of membrane vesicles, and determination of the dry weight of purified vesicles indicated that *P. gingivalis* MSM-3 produced twice the amount of membrane vesicles as did strain A7436. Extracellular vesicles isolated from *P. gingivalis* MSM-3 were found to express higher hemagglutination titers, as well as enhanced hemolytic and arginine-X-specific protease activities. *In vivo* studies revealed that *P. gingivalis* MSM-3 was more infectious and invasive than the parent strain, as indicated by secondary lesion formation and death in experimental mice.

Genetic analysis has revealed that while Tn4351 has inserted into a non-coding region (60 bp downstream from a known *P. gingivalis* insertion sequence element, IS1126), its insertion into the host chromosome resulted in the mobility of IS1126. We have determined that *P. gingivalis* MSM-3 contains two additional copies of IS1126 as compared to the parent strain. The insertion site of one of the additional IS1126 elements has been sequenced. Genetic analysis reveals that it contains numerous open reading frames and does not provide evidence of an open reading frame which may encode for a gene involved in iron acquisition. However, approximately 1 kb downstream of the insertion site, a 1.014 kb open reading
frame (designated hemB) has been located. Analysis has revealed that this open reading frame shares homology with several genes that encode for hemin/iron receptors or iron-regulated outer membrane proteins.

Taken together, the results presented in this study provide evidence for the coordinate regulation of *P. gingivalis* virulence factors by hemin. Additionally, the capability of mobilization of an insertion sequence element, a phenomenon not previously reported in *P. gingivalis*, has been demonstrated. Further characterization of hemB and the second additional IS1126 element's insertion site may provide evidence for the role of specific proteins involved in hemin/iron acquisition by *P. gingivalis*. 
CHARACTERIZATION OF A Tn4351-GENERATED HEMIN UPTAKE MUTANT OF *Porphyromonas gingivalis*: EVIDENCE FOR THE COORDINATE REGULATION OF VIRULENCE FACTORS BY HEMIN

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CHAPTER ONE: INTRODUCTION

In 1921, Oliver and Wherry (53) isolated a small, anaerobic, Gram negative rod from a variety of sites, including the oral cavity, urine, human feces and respiratory tract, as well as from postsurgical infections. This rod, when grown on blood agar plates, produced colonies with black pigmentation. The pigment was considered to be melanin, and the culture was named Bacterium melaninogenicum. This bacterium was first described in the third edition of Bergey's Manual of Determinative Bacteriology (1) as Haemophilus melaninogenicus because better growth was obtained on solid medium containing X and V growth factors, characteristic of the members of the genus Haemophilus.

Prevot in 1938 (57) regrouped several species into new genera and species. Prevot proposed that the name Haemophilus melaninogenicus be changed to Ristella melaninogenica. However, the name was not generally accepted, and the fifth edition of Bergey's Manual retained Bacteroides melaninogenicus. In 1947, Schwabacher et. al. (59) proposed a new name for B. melaninogenicus based principally on pigmentation. Because the organism had previously been classified in the group Fusiformis, Schwabacher et. al. suggested Fusiformis nigrescens. This name did not take
precedence, and *Bacteroides melaninogenicus* was retained as the proper description of the organism.

Although Courant and co-workers (10) showed biochemical and immunological heterogeneity among strains of *B. melaninogenicus*, only one species of black pigmented *Bacteroides* (BPB) was recognized until 1970: *B. melaninogenicus*. Along with an increased understanding of microbial physiology and the usefulness of end product analysis as a tool in taxonomy, it soon became clear that *B. melaninogenicus* could be taxonomically divided into several "subspecies" as a result of their fermentative activities. Therefore, the saccharolytic strains of *B. melaninogenicus* subsp. *melaninogenicus*, which was strongly fermentative, and *B. melaninogenicus* subsp. *intermedius*, which was weakly fermentative. Asaccharolytic strains, that is, those which did not lower the pH of a glucose-based growth medium, were grouped in *B. melaninogenicus* subsp. *asaccharolyticus*. Studies showed that the biochemical and genetic characteristics of the saccharolytic and asaccharolytic strains were sufficiently different so as to justify the elevation of the asaccharolytic subspecies to the species level. Additionally, oral and nonoral *B. melaninogenicus* species were separated based on their genetic heterogeneity, particularly among the asaccharolytic strains. This led Coykendall et al (11) to propose the new species *B. gingivalis* for the asaccharolytic BPB strains isolated from oral sites.
B. asaccharolyticus was retained for the nonfermentative Bacteroides sp. isolated from nonoral sites. In 1988, Shah and Collins (61) proposed the reclassification of Bacteroides asaccharolyticus and Bacteroides into a new group, Porphyromonas. This reclassification has been widely accepted and Bacteroides gingivalis is currently referred to as Porphyromonas gingivalis, or P. gingivalis.

\[
\begin{align*}
\text{Bacteroides melaninogenicus} & \\
\text{Haemophilus melaninogenicus} & \\
\text{Ristella melaninogenica} & \\
\text{Fusiformis nigrescens} & \\
\text{Bacteroides melaninogenicus} & \\
\text{Bacteroides melaninogenicus subsp. asaccharolyticus} & \\
\text{Bacteroides asaccharolyticus} & \\
\text{Bacteroides gingivalis} & \\
\text{Porphyromonas gingivalis} & 
\end{align*}
\]

Figure 1. Chronology for establishment of the asaccharolytic BPB species.

Periodontal disease, a term that encompasses a range of inflammatory and destructive conditions of the tissues surrounding the teeth, is initiated by the overgrowth of bacteria in the gingival crevice (66,67) causing tissue damage by a combination of direct action of toxic bacterial products that include endotoxins(27) and hydrolytic enzymes and
stimulation of destructive host immunological responses. Several distinct clinical categories of periodontitis can be distinguished, but the most common form is adult periodontitis, with Porphyromonas gingivalis being generally regarded as the major pathogen involved in the induction and/or progression of this disease (32). Compared to quiescent sites, sites in the periodontium which show progressive disease contain elevated numbers of cultivable P. gingivalis and the serum IgG antibody response to this organism is elevated in cases with a history of destructive disease as compared to controls (8). Eradication of P. gingivalis from the subgingival microbial population correlates with resolution of the disease. However, the precise role of the organism in the initiation and progression of the disease is unclear.

Iron is an essential nutrient for the growth and metabolism of essentially all prokaryotic organisms, as well as for the initiation and progression of many infectious diseases. The 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 and to use it in their own metabolic processes. P. gingivalis may face the problem of an extremely low availability of free iron in the initial stage of the infection process (50). The host defends itself against bacterial iron appropriation by the synthesis of a large
number of iron-binding proteins (IBPs) including hemosiderin, ferritin, hemoglobin, haptoglobin, and hemopexin. In addition, the high-affinity IBPs such as serum albumin, transferrin, found in serum, and lactoferrin, found in mucosal secretions, are also involved in the sequestering and transport of host iron. 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 (5). A number of pathogens have evolved mechanisms for interacting with iron and iron-containing compounds.

In the facultative intracellular parasite Legionella pneumophila, the agent of Legionnaires’ disease, a gene that promotes hemin binding has been identified. Designated hbp for hemin binding protein, it is predicted to encode for a secreted 15.5-kDa protein. An hbp mutant displayed a 42% reduction in hemin binding, confirming that hbp potentiates hemin acquisition in L. pneumophila (51). In Plesiomonas shigelloides, an organism associated with outbreaks of diarrheal disease and food poisoning in humans, DNA sequences encoding hemolysin production and DNA sequences encoding for the ability to use hemin or hemoglobin as a sole iron source have been identified, cloned and expressed in Escherichia coli. The abilities to use hemin and hemoglobin
as iron sources were closely linked and the cloned sequences encoded the ability to transport the porphyrin, as well as iron into the cells (15).

*Neisseria gonorrhoea*, the gram-negative causative agent of the sexually transmitted disease gonorrhoea, expresses both transferrin and lactoferrin receptors to acquire iron from these host binding proteins. The transferrin receptor consists of a complex of 2 transferrin binding proteins (Tbps): a relatively conserved Tbp A and an antigenically and size variable Tbp B. Tbp B may function to make the process of iron utilization more efficient and/or more specific for iron-loaded transferrin. Iron acquisition from lactoferrin, like that described for transferrin, may also require 2 outer membrane proteins, Lbp A and Lbp B. Although the requirement for Lbp A in lactoferrin binding and utilization has been confirmed, the role of Lbp B has not yet been defined (2). Once bound to their receptors, iron is removed from transferrin and lactoferrin in an energy-dependent manner; however neither transferrin nor lactoferrin is internalized. Following removal of iron from transferrin, it is bound by the ferric binding protein, Fbp A, which functions within the periplasm as a shuttle from transferrin to the cytoplasmic membrane.

The transport of iron from heme and heme-containing compounds in the pathogenic *Neisseria* appears to involve several outer membrane receptors. Putative heme-binding
proteins have been isolated from *N. meningitides* and *N. gonorrhoea*, (35,36) however, the precise function of these proteins has not been defined. A 76-kDal iron regulated outer membrane receptor, Frp B, has been suggested to play a role in heme utilization, although a function in heme uptake has not been documented. The existence of a receptor for hemoglobin as well as hemoglobin bound to haptoglobin, Hpu, has been demonstrated in *N. meningitides* (38).

Iron plays an important role in periodontal infection with *P. gingivalis*, as this bacterium requires iron (i.e. hemin) as an essential growth factor. Accumulated hemin (also known variously as protoheme and heme depending upon the oxidation state of the iron atom) is the cause of the characteristic black-brown coloration of colonies of the BFBs when grown on blood agar. Heme consists of the tetrapyrrole molecule protoporphyrin IX, into the center of which iron is ligated (69):

![Figure 2. Model of hemin molecule. (Abbreviations: M=methyl; P=propionate; V=vinyl)](image)

*P. gingivalis* is unable to synthesize protoporphyrin IX, which it requires as the prosthetic group of cytochrome b.
The latter serves as an electron sink during amino acid fermentation. Studies have shown that hemin is capable of satisfying *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 (5). Bramanti and Holt (5) have demonstrated that, in vitro, *P. gingivalis* is capable of utilizing a broad range of hemin-containing compounds such as hemoglobin, methemoglobin, myoglobin, hemin-saturated serum albumin, lactoperoxidase, cytochrome c and catalase.

*P. gingivalis* is also capable of growth with nonhemin iron sources, including ferric and ferrous inorganic iron, and human transferrin (5). These findings are in agreement with results obtained in our laboratory. Additionally, our hemin binding and internalization studies have demonstrated that *P. gingivalis* transports the entire hemin moiety into the cell and that the binding and accumulation of hemin are induced by growth of cultures in the presence of hemin (20). The diversity of iron substrates utilized by *P. gingivalis* may explain why it is such a formidable pathogen in the periodontal disease process (5).

In addition to its role as an essential nutrient, iron also serves as a regulatory signal in many organisms, influencing the expression of a wide variety of virulence factors (39). The most extensively studied iron uptake system that is used by many aerobic organisms depends on the
production of siderophores. Siderophores are low-molecular mass iron chelators that can remove Fe$^{3+}$ complexed to transferrin and lactoferrin (62). In organisms such as E. coli, genes involved in the production of siderophores are transcriptionally regulated by the availability of iron through the ferric uptake regulator protein, Fur. Fur acts as a classic negative repressor, using Fe$^{2+}$ as a corepressor, and binds to the promoter region of Fur-regulated genes. More specifically, Fur binds to a 19 bp DNA sequence called the "fur box". Under iron limited cell conditions, the Fur-Fe$^{2+}$ complex does not bind to the promoter(s) of the gene(s) it regulates, and the genes are thus transcribed. Regulation of iron-regulated genes by a Fur homolog is widespread and has been described in such genetically diverse organisms such a Y. pestis, V. cholera, V. vulnificus, P. aeruginosa, H. influenza, L. pneumophila, B. pertussis, S. typhimurium, N. meningitides.

The influence of iron or hemin on the expression of several putative virulence factors produced by P. gingivalis has recently been well documented (7,44,48). However, the role of hemin in the regulation of specific virulence genes has not been precisely defined. Studies have demonstrated that hemin modulates the virulence of P. gingivalis. Growth of P. gingivalis under conditions of hemin limitation has been reported to affect the numbers and/or levels of several putative virulence factors, specifically, the number of
extracellular vesicles (ECV), and the levels of hemolytic, proteolytic and hemagglutinating activities (65). Of the members of the black-pigmented Bacteroides spp., P. gingivalis appears to be most virulent in experimental animals, as it repeatedly causes spreading, inflammatory infections when it is monoinfected. Many virulence determinants of P. gingivalis are extracellular products or cell surface components which have been intensively investigated.

Vesicles result from the budding (extrusion) of the outer cell membrane of Gram-negative bacteria. These structures, ranging in size from 20 to 500 nm, can either be attached to or released from the bacterial cell surface. They have alternatively been called vesicles, microvesicles, blebs, outer membrane fragments or membrane vesicles. These vesicles have been found in different genera, such as Porphyromonas, E. coli (70), Haemophilus (16) and Neisseria (17). McKee et al. have shown that when viewed by electron microscopy, P. gingivalis W50 cells grown under hemin limitation appeared to be either coccobacillary or short rods possessing large numbers of extracellular vesicles which could be seen both surrounding the cell surface and free in the environment (47).

Extracellular vesicles have been shown to exhibit proteolytic and collagenolytic activities, as well as to be capable of hemagglutinating erythrocytes. The vesicles also promote bacterial adherence between homologous P. gingivalis strains in addition to mediating attachment between non-
coaggregating bacterial species (24). A growing body of literature suggests that ECV are a virulence factor of *P. gingivalis*. Their small size could easily permit them to cross epithelial barriers that are otherwise impermeable to whole cells. The vesicles could serve as a vehicle for toxins and various proteolytic enzymes, as well as indirectly extending the bacterial cell's capacity to obtain nutrients. These membranous vesicles could also compete for antibodies and thus impede the specific antibacterial immune defense.

Proteases produced by black-pigmented *Bacteroides* species may play an important role in the development of periodontal disease, as they have the potential to destroy periodontal tissues (29). Collagen is the major constituent of the gingival connective tissues, and while this triple-helix protein is resistant to a wide variety of proteolytic enzymes, it is degraded by bacterial and tissue collagenases, both of which occur in the mouth (45).

Some types of periodontal disease are characterized by the loss of gingival connective tissues. The ability of *P. gingivalis* to degrade collagen was first shown by Gibbons and MacDonald in 1961 (23). They demonstrated that the collagenase enzyme was not affected by high salt concentrations and functioned optimally under neutral conditions. The enzyme was found to be synthesized intracellularly, with all of the enzymatic activity being cell associated. As opposed to eukaryotic collagenases which
cleave undenatured collagen at a single site, the collagenase from *P. gingivalis* hydrolyses collagen into small peptides. Although 3 types of caseinolytic proteases (Pase-A, Pase-B and Pase-C) have been isolated and purified from culture supernatants of *P. gingivalis* 381 (29), much attention is focused upon the thiol activated trypsin-like protease activity of this organism. This activity is designated as such because it has the same specificity for synthetic substrate as trypsin. Trypsin-like protease activity is also often used in taxonomy to distinguish *P. gingivalis* from other black pigmented species in the subgingival microbial plaque and has been shown to regulated by hemin levels (7). This enzymatic activity is found by using appropriate chromogenic substrates such as nitroanilide derivatives of C-terminal arginyl peptides. *P. gingivalis* proteinases have been a target for purification and characterization since 1984, but interest in this system has recently increased.

In early 1995, at least 39 apparently distinct proteolytic enzymes had been claimed as having been purified from *P. gingivalis*. However, they differed in molecular mass, susceptibility to synthetic inhibitors, association with hemagglutinin activity and ability to degrade a variety of proteins. More recently, names were given prematurely to a variety of *P. gingivalis* proteinases that were impure, poorly characterized or equivalent to already well-established enzymes. Most were classified as thiol-dependent enzymes.
cleaving synthetic substrates with arginine and/or lysine in the P1 position. A mechanism for resolving the confusion surrounding the apparent heterogeneity of the P. gingivalis proteinase system was the cloning, sequencing and expression of the genes encoding proteolytic enzymes produced by this bacterium. So far, 9 genes have been described, including 6: *prtH*, *agp*, *rgpl*, *cpgR* and *prpR1*, that apparently encode one or more arginine-X-specific proteinases. Except for *prtH* and *cpgR*, the translated part of each gene encodes a prepropeptide, a catalytic domain and a hemagglutinin domain (56). Remarkably, the structures of the prepropeptides and of the catalytic domains are virtually identical for a variety of bacterial strains, with the differences between the sequences being confined to the region encoding the hemagglutinin domain. The *prtR* and *prtH* genes have a high degree of nucleotide sequence identity with *rgpl*. The *rgpl* and *agp* genes are identical except that *agp* appears to be missing 2139 internal bases encoding part of the hemagglutinin domain. However, regardless of the differences in gene structure within the hemagglutinin domain, it can be concluded that the primary structure of the catalytic domain of the arginine-arginine-X-specific proteinases is nearly identical in all strains of *P. gingivalis* tested to date, and that the proteinase is synthesized as a larger precursor protein containing the prepropeptide, catalytic and hemagglutinin domains (56).
In addition to the rpgl-like gene(s), the gene encoding a lysine-X-specific cysteine proteinase (kgp) has also been cloned and sequenced. Significantly, this gene encodes a polyprotein consisting of a prepropeptide, a catalytic domain and a hemagglutinin domain(s), which is identical to that encoded by rpgl. Studies have shown that the arginine-X-specific proteinases occur as 110, 95, 70-90 and 50 kDa proteins, regardless of the P. gingivalis strain or method of cultivation. The first 2 forms are found as noncovalent complexes of the 50 kDa catalytic domain with hemagglutinin, while the others are single chain enzymes (56). the predominant form the lysine-X-specific proteinase is a noncovalent complex of the 60 kDa catalytic domain with hemagglutinin.

As the first purified enzyme shared several properties with the enzyme clostripain, the acronym gingipain (P. gingivalis + clostripain = gingipain) was initially proposed (56). Later, this name was used with the prefix Arg (Arg-gingipain, RGP) or Lys (Lys-gingipain, KGP) to distinguish between the Arg-X and Lys-X-specific proteinases (56). These data explain why several forms of cysteine proteinases with various molecular masses and both with and without hemagglutinin activity have been purified: each is the product of proteolytic processing of a large precursor polyprotein, translated from the rpgl gene or its homologs, or from the kpg gene (56). Both whole cells and culture
supernatant of *P. gingivalis* can hydrolyze and inactivate human plasma proteins involved in the host defense of periodontal tissues, including immunoglobulins, iron binding proteins and protease inhibitors (12). The proteolytic destruction of immunoglobulins can result in an increase in bacterial adherence, a decrease in bacterial lysis due to complement, and a reduction of phagocytosis, as well as a decrease in antibody-neutralizing ability against toxins and enzymes. Degradation of immunoglobulins can thus paralyze locally the host defense mechanisms and may permit bacterial invasion of tissues.

Colonization, the initial event in the establishment of disease, requires the adherence of bacteria to host tissues. Therefore, bacterial surface components which mediate bacterial adherence are considered to be important virulence factors. In the oral cavity, bacterial can attach to host tissues as well as to bacteria in pre-formed plaque. The nature of the binding sites on teeth and oral tissues to which periodontopathic bacteria, including *P. gingivalis*, attach has not been well established. *In vitro*, *P. gingivalis* can attach to and agglutinate erythrocytes, adhere in high numbers to human buccal epithelial cells (52), KB epithelial cells (21), and to surfaces of Gram-positive bacteria present in plaque (60). In addition, this species will adhere to untreated and saliva-treated hydroxyapatite (SHA), but in comparatively low numbers (64).
Boyd and McBride(4) have reported the isolation of an outer membrane complex containing hemagglutinating activity. Their results have shown that hemagglutinating activity was contained in the membrane fraction consisting of low-molecular weight lipopolysaccharide, protein and loosely bound lipid. They also demonstrated that removing fimbria from *P. gingivalis* had no effect on the hemagglutinating activity of whole cells. Kay et al.(34) have demonstrated that in *P. gingivalis* W50, hemagglutinating activity of extracellular vesicle was associated only with the extracellular membrane.

In 1989, Progulske-Fox et al.(58) reported the expression and function of a *P. gingivalis* hemagglutinin gene (3.2 kb) in *E. coli*. Immunoelectron-microscopic studies using the antiserum to clone ST2 indicate that the product of the cloned gene (hemagglutinin) is located on the cell surface of *P. gingivalis*. The hemagglutinating activity of *P. gingivalis* is most likely attributed to the hemagglutinin domain of the *rpgl* gene (56), which itself is approximately 3 kb. It is interesting that the inhibition of attachment of *P. gingivalis* to erythrocytes is mediated by a low concentration of L-arginine. It is possible that arginine functions as a contact residue between the bacterial cell receptor and its counterpart on the erythrocyte during agglutination.

Hemolysins are cytolytic toxins found in a broad diversity of organisms. They are named for their capacity to
lyse erythrocytes, but many are toxic to other cell types as well. Microbial hemolysins are generally considered to be virulence factors, although the relative contribution of hemolysins to disease is variable among microbes and different host species. Many microbial hemolysins also lyse leukocytes and other host cells.

Hemolysin genes have been cloned from many microorganisms. The RTX family of toxins, named for a series of tandem repeats in their structural proteins, are found in *E. coli*, *A. suis*, *V. vulgarius* and *M. morganii*. The RTX toxins exhibit a wide range of target cell specificities. Pore formation is postulated to be the mechanism of the cytolytic activity for these toxins (49). Recently, Palmer and Munson (54) have reported on the cloning and characterization of the genes encoding the hemolysin of *Haemophilus ducreyi*. Two genes, designated *hhdB* and *hhdA*, have been identified. The gene *hhdB* is 1596 bp in length, while the *hhdA* gene, located 88 bp downstream of the *hhdB* gene, is 3525 bp in length.

Chu et al. (8) have investigated *P. gingivalis* W50, W83, A7A1-28, and ATCC 33277 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 the late-exponential-early-stationary growth phase. The enzyme was determined to be 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. 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 excess heme (8).

Recently, Holt and Karunakaran (33) have reported on the cloning of 2 distinct hemolysin genes from *P. gingivalis* in *E. coli*. These 2 genes, isolated by screening genomic DNA libraries of *P. gingivalis* on sheep blood agar, each confer a hemolytic phenotype in *E. coli*. Results obtained from physical maps and Southern blot analysis indicate a considerable degree of divergence between the nucleotide sequences of the 2 genes. Analysis of the recombinant plasmids in *E. coli* suggested that one gene encoded for a polypeptide of MW 48 kDa, while a second gene encoded for a polypeptide of 18 kDa. In agreement with results obtained by Chu et al., the levels of hemolysin activity increased when *E. coli* harboring these hemolysin genes was subjected to iron starvation. Hemolytic activity may enhance the survival of the microbe when confronted with a host immune response. Additionally, erythrolysis has been proposed to be a mechanism for iron acquisition in iron deficient microenvironments, such as the periodontal pocket.
Genetic manipulation of *P. gingivalis* has been limiting due, in part, to lack of efficient genetic systems. The transposable element Tn4351 was initially described on the conjugative plasmid pBF4, originally isolated from *Bacteroides fragilis*. Tn4351 carries two antibiotic resistance genes (Fig 8). One gene, which codes resistance to erythromycin (Em<sup>r</sup>) and clindamycin (Cc<sup>r</sup>), is expressed in *Bacteroides* spp. but not in *E. coli* (63). The other gene, which codes for resistance to tetracycline (Tc<sup>r</sup>), is expressed in aerobically grown *E. coli*, but not in anaerobically grown *E. coli* or in *Bacteroides* spp. To distinguish this Tc<sup>r</sup> from other Tc<sup>r</sup> genes that are found in *Bacteroides* spp. and in *E. coli*, it has been designated *Tc<sup>r</sup>*. Both the Em<sup>r</sup>-Cc<sup>r</sup> gene and the *Tc<sup>r</sup>* genes are contained within a 3.8 kb EcoRI fragment of Tn4351 (63). In order to develop a method for introduction of Tn4351 into various *Bacteroides* species, Tn4351 was cloned into the broad-host-range plasmid R751 (R751::Tn4351). Shoemaker et al. (63) have shown that Tn4351 can transpose from several different R751::Tn4351 plasmids into the *Bacteroides* chromosome and that R751 is not maintained in *Bacteroides* spp. Additionally, Hwa et al. (30) have demonstrated that direct repeats flanking Tn4351 are insertion sequences elements.

Although previous studies support the concept of regulation of *P. gingivalis* virulence factors in response to hemin, evidence at the molecular level has been lacking. Due to the genetic similarity between *P. gingivalis* and...
Bacteroides, our laboratory has developed the Tn4351 mutagenesis system for *P. gingivalis*. This involves the introduction of Tn4351 into *P. gingivalis* A7436 by conjugation using a broad adaptation of the procedure of Shoemaker et al. (63), which was specifically modified for *P. gingivalis*. Tn4351, carried on pR751, is capable of transferring into a wide variety of organisms; however, pR751 is not capable of replication in *P. gingivalis* and once the plasmid is transferred, Tn4351 excises and integrates into the host chromosome. The development of this system for transpositional mutagenesis for *P. gingivalis* has allowed our laboratory to create genetically defined mutant strains of *P. gingivalis* wildtype strain A7436. In this study, one such mutant, designated MSM-3, has been characterized. The characterization of this mutant has allowed us to begin to address basic questions regarding the role of hemin in the regulation of expression of putative *P. gingivalis* virulence factors. The overall goal of this work was the characterization of this mutant in terms of its putative virulence factors; specifically, arginine-X-specific protease, hemagglutinating, and hemolytic activities as well as the production of extracellular vesicles. The assessment of the aforementioned putative virulence factors of *P. gingivalis* MSM-3 may provide evidence that virulence factors of *P. gingivalis* are coordinately regulated by hemin. In addition, the mobilization of an insertion sequence element, IS1126, has
been demonstrated. The insertion site of one of the two additional copies of IS1126 has been sequenced and characterized. Genetic analysis of this region (and that adjacent to it) may give an initial description of a putative heme regulated gene in P. gingivalis.
CHAPTER TWO: MATERIALS AND METHODS

Bacterial strains and growth conditions. P. gingivalis A7436 and E. coli HB101 (R751::Tn4351) were used in these studies. P. gingivalis A7436 was typically maintained on anaerobic blood agar (ABA; Remel, Lenexa, Kans.) at 37°C in an anaerobic chamber (Coy Laboratory Products, Inc.) with 85% N₂, 5% H₂ and 10% CO₂. Following incubation at 37°C for 3 days, cultures were inoculated into basal medium (BM; 10 g of trypticase peptone, 0.2 g of tryptophan, 2.5 g of NaCl, 0.1 g of sodium sulfite, and 0.4 g of cysteine per liter) and incubated at 37°C (under anaerobic conditions) for 24 h. This culture served as the inoculum into BM, BM supplemented with 1.5 µM hemin (BMH), 1.3 µM hemoglobin, 20 µM ferric chloride or 20 µM ferrous chloride, Schaedler broth (SB; Difco Laboratories, Detroit, Mich.), SB supplemented with 20 µg of dipyridyl per mL, or anaerobe broth MIC (Difco). Growth was monitored at A₆₆₀ with a Beckman DU8 spectrophotometer.

Transpositional mutagenesis and isolation of P. gingivalis MSM-3. Tn4351 was introduced into P. gingivalis A7436 by a broad adaptation of the procedure of Shoemaker et al. (63) as modified for P. gingivalis (22). P. gingivalis was subcultured from an ABA plate into 10 mL of SB and grown anaerobically to an A₆₆₀ of between 0.3 and 0.5 (early
logarithmic growth). *E. coli* R751::Tn4352 (63) was subcultured into LB and grown aerobically at 37°C to an A₆₆₀ of 0.2. Samples (1.0 mL of *P. gingivalis* and 0.1 mL of *E. coli*) were removed, centrifuged at 12,000 xg, and resuspended in 0.05 mL of SB and LB, respectively. Conjugations were performed under anaerobic conditions, using 125-mL flasks containing 25 mL of prereduced blood agar on which a 0.45-µm-pore-size HAWP filter (Millipore) was aseptically placed. *P. gingivalis* and *E. coli* cultures were spotted on filters, the flasks were tightly stoppered and removed from the chamber, and 1.0 mL of air was added to each flask with a hypodermic needle and syringe. Stoppered flasks were incubated overnight (18 to 24 h) at 37°C aerobically. Cultures containing *P. gingivalis* and *E. coli* only were also spotted onto filters as described above and served as controls. Following incubation, filters were removed from flasks, cells were washed off by vortexing in 2.0 mL of SB, and cultures were incubated at room temperature for an additional 45 minutes. Cultures were serially diluted and plated on selective media (ABA plates containing geneticin [50.0 µg/mL] and erythromycin [1.0 µg/mL]). CFU per milliliter of input cultures was determined by plating *E. coli* on LB plates containing tetracycline and *P. gingivalis* on ABA plates containing geneticin. Plates were incubated anaerobically for 7 to 10 days, and potential transconjugants were restreaked on selective media and incubated anaerobically at 37°C; transconjugants were
typically maintained on selective media and grown anaerobically. *P. gingivalis* MSM-3 was initially selected for further characterization on the basis on its nonpigmented colony phenotype on ABA and was typically maintained on selective media and grown anaerobically.

To confirm the insertion of Tn4351 into the *P. gingivalis* chromosome, chromosomal DNA was prepared from *P. gingivalis* MSM-3, digested with EcoRI, HindIII or PstI as instructed by the manufacturer (Stratagene, Inc.), and electrophoresed on a 0.8% agarose gel in 1x Tris-borate-EDTA buffer (20V) for 16 h. Gels were probed by Southern blot analysis using pVOH1, a plasmid that contains the entire transposon Tn4351, or R751 (63). Briefly, nitrocellulose filters were incubated for 16 to 24 h at 42°C in hybridization buffer (25 mM KPO₄ [pH 7.4], 5x SSC (1x SSC is 0.15 M salmon sperm DNA per mL, 1.0% sodium dodecyl sulfate [SDS]). Following hybridization, membranes were washed twice with 2x SSC-0.1% SDS for 15 min at room temperature, once for 15 min in 0.5x SSC-0.1% SDS, and, if necessary, once with 0.1x SSC-1.0% SDS for 30 min at 42°C.

**Binding and transport of radiolabeled hemin.** [¹⁴C]hemin and [⁵⁹Fe]hemin (University of Leeds Innovations Ltd., Leeds, England, and Dupont Co., Wilmington, Del.) were used in studies to examine the transport of hemin and the Fe from hemin by *P. gingivalis* (20). All glassware was washed with nitric acid (10%) and thoroughly rinsed in deionized water before use. *P. gingivalis* cultures were grown in BM or BMH to
the exponential phase of growth, and each culture was divided into two separate flasks. Cultures were washed in phosphate-buffered saline (PBS) and resuspended in BM to an OD$_{660}$ of 0.10. To one flask of cells grown in BM and one flask of cells grown in BMH was added 20 μM potassium cyanate (KCN) and cells were then incubated at 37°C under anaerobic conditions for 1 h. $[^{14}\text{C}]$hemin (1.5 μM; specific activity, 112 Ci/mol) or $[^{59}\text{Fe}]$hemin (4.0 μM; specific activity, 83 Ci/mol) was added to all cultures, and cultures were then incubated for an additional 24 h at 37°C under anaerobic conditions. $[^{14}\text{C}]$hemin or $[^{59}\text{Fe}]$hemin was added to flasks containing BM or BMH only, which served as negative controls. Duplicate samples of cells were removed from each flask at 0, 0.5, 1, 4 and 24 h, diluted into 10 mL of 0.1 M sodium citrate buffer (pH 7.4) containing 1.0 μM MgCl$_2$ and 0.25 mM CaCl$_2$, and filtered twice through 0.45-μm-pore-size cellulose acetate filters. Filters were washed twice with 3 mL of 0.02 N NaOH in 50% ethanol and air dried overnight, and the amount of cell-associated $[^{14}\text{C}]$hemin or $[^{59}\text{Fe}]$hemin was determined by liquid scintillation spectrometry. Cell-associated $^{14}\text{C}$ or $^{59}\text{Fe}$ is expressed as picomoles/per milligram of total cellular protein. Hemin uptake or Fe uptake from hemin is calculated as the difference between the $[^{14}\text{C}]$hemin or $[^{59}\text{Fe}]$hemin associated with untreated cultures and potassium-cyanate treated cultures and is expressed as picomoles/per milligram of total cellular protein for each time point.
Isolation of extracellular vesicles. Extracellular membrane vesicles were isolated from *P. gingivalis* A7436 and MSM-3 as described by Bourgeau and Mayrand (3). *P. gingivalis* cultures were centrifuged for 20 min at 10,000 xg (4°C), and the supernatant was concentrated 50-fold by passage through an ultrafiltration system (membrane molecular weight cutoff, 10,000; Millipore). Concentrated supernatants were subjected to light ultrasonic treatment (5 s) to remove extracellular vesicles attached to cell surfaces. The suspension was centrifuged twice at 10,000 xg (4°C) for 30 min, and the resulting supernatant was dialyzed overnight against 50 mM Tris-HCl buffer (pH 9.5). Vesicles were collected by ultracentrifugation at 100,000 xg for 2 h, and the pellet was resuspended in 0.5 ml of distilled water and lyophilized.

Hemolytic activity. Bacterial suspensions (whole cell or supernatant fractions) were washed once in NCN (3 M sodium citrate, 0.9% NaCl [pH 6.8]) buffer and used in the hemolysis assay. Sheep erythrocytes (Remel) were washed with NCN buffer until the supernatant was visually free of hemoglobin pigment. Hemolytic activity of whole cells or supernatant fractions was determined by mixing 0.8 mL of a 2.5% sheep erythrocyte suspension (approximately 5 x 10^8 cells in NCN buffer) with 0.2 mL of each bacterial fraction. Mixtures were incubated for 2 h with slow shaking at 37°C. Intact erythrocytes were removed by centrifugation (8,000 xg, 1 min), and the amount of lysis was measured by the quantification of hemoglobin release.
as determined at $A_{405}$. Control erythrocytes were incubated as described above but without the addition of bacterial cells. The hemolytic activity of vesicle preparations was determined as described above, using vesicles at a concentration of 0.1 mg/mL. Complete erythrocyte lysis was obtained by mixing 0.2 mL of a 10% sheep erythrocyte solution in 0.8 mL of distilled water. The percent lysis of the experimental sample was calculated relative to the value for this complete lysis control.

**BAPNA assay.** Arginine-X-specific protease activity was assessed by using N-α-benzoyl-DL-arginine-p-nitroanilide (BAPNA) by a modification of the procedure described by Mayrand and Grenier (46). *P. gingivalis* cultures (0.1 mL) were centrifuged at 10,000 xg for 10 min (4°C); the supernatant was removed and transferred to a fresh Eppendorf tube. The remaining pellet was resuspended in 1.0 mL of 1.0 M PBS (pH 7.3). Supernatants, bacterial cells or vesicle preparations (0.05 mL prepared as described above) were then added to 0.2 mL of 0.05 M Tris maleate (pH 7.5), 1.25 mL of 0.001 M BAPNA (in 0.05 M Tris maleate [pH 7.5]) and 0.075 mL of 1.0 M cysteine. Mixtures were incubated with shaking at 37°C for a period of 2 h at 250 rpm. Following incubation, 0.250 mL of 5 N acetic acid was added and the samples were read spectrophotometrically at $A_{410}$.

**Hemagglutination assay.** The hemagglutination assay was performed in V-bottom microtiter plates (Dynatech Laboratories
Inc., Alexandria, Va.) (58). Sheep erythrocytes were washed three times with 0.14 M PBS (pH 7.2) and resuspended to a final concentration of 0.2% (vol/vol). *P. gingivalis* cultures were washed twice in PBS resuspended to an OD$_{660}$ of 0.5, and diluted in a twofold series with PBS; 0.05 mL was added to the wells. After addition of an equal volume of erythrocytes, plates were stored for 16 h at 4°C and then examined for evidence of hemagglutination. Sheep erythrocytes incubated with PBS only served as negative controls. Positive hemagglutination was observed as a pinkish carpet coating the bottom of the well. Negative hemagglutination was observed as a bright red disk consisting of settled erythrocytes. The titer was expressed as the reciprocal of the highest dilution showing evidence of agglutination.

**Electron microscopy.** Late-logarithmic phase cultures of *P. gingivalis* A7436 or MSM-3 were centrifuged (10,000 xg for 5 min) and washed twice in PBS. Concentrated cultures were fixed first at room temperature for 2 h with 3.6% (vol/vol) glutaraldehyde in 0.1 M cacodylate buffer (pH 7.2) and then at room temperature in osmium tetroxide in 0.1 M phosphate buffer (pH 7.2) for 1 h (9). Chamber fluid samples obtained from *P. gingivalis*-infected mice (day 2) were diluted 1:10 in PBS and fixed in glutaraldehyde as described above. Grids were viewed in a JEM-1200 EX electron microscope.

**Experimental animals.** Eight-week-old female BALB/c mice (Charles River Laboratories, Wilmington, Mass.) were
surgically implanted with subcutaneous chambers. Chambers were inoculated with \textit{P. gingivalis} A7436 or MSM-3 grown in anaerobe broth MIC (10^7 to 10^8 CFU), and mice were examined daily for general appearance, primary and/or secondary abscess formation, and health status. Chamber fluid was removed daily with a hypodermic needle and syringe for bacteriological culture and microscopic examination as described previously (19). All surviving animals were sacrificed 30 days post-inoculation, and sera were separated from blood obtained by cardiac puncture.

**DNA sequencing and computer analysis.** DNA sequencing was performed using the PRISM™ Ready Reaction DyeDeoxy™ Terminator Cycle Sequencing Kit (Perkin-Elmer, Foster City, CA) and 373A DNA Sequencer (Applied Biosystems). Briefly, primer and template (1 ug) were brought to a final volume of 12 uL and 8 uL of terminator ready reaction mix was added. This reaction mixture was overlaid with 40 uL of light mineral oil. Tubes were placed in the Thermal Cycler, and cycling was begun as follows: Rapid thermal ramp to 96°C, 96°C for 30 sec, rapid thermal ramp to 50°C, 50°C for 15 sec, rapid thermal ramp to 60°C, 60°C for 4 min. (repeat cycling steps for 25 cycles), rapid thermal ramp to 4°C. Extension products were purified and samples were prepared for loading onto the gel. Computer analysis was performed using the IntelliGenetics Suite program.

**Protein concentration.** Total protein content was determined
for all samples by use of a bicinchoninic acid protein assay with bovine serum albumin as the standard (Pierce, Rockford Ill.).
CHAPTER THREE: RESULTS

Isolation of *P. gingivalis* MSM-3. Previous studies have reported that the storage of protohemin by *P. gingivalis* results in a dark pigmentation phenotype when cultures are grown on blood agar plates and provides its black-pigmented *Bacteroides* character (7). The absence of pigmentation in *P. gingivalis* may thus correlate with a decrease in the ability of the organism to bind or transport hemin. Therefore, the initial screening for Tn4351-generated insertional mutants of *P. gingivalis* defective in hemin utilization was based upon the loss of pigmentation as observed on ABA plates. *P. gingivalis* MSM-3 was randomly chosen from several nonpigmented transconjugants and characterized in terms of its ultrastructure, putative virulence factors and ability to bind and transport hemin. Insertion of Tn4351 into the *P. gingivalis* MSM-3 chromosome was confirmed by Southern blot analysis (Figure 3). In *P. gingivalis* MSM-3 chromosomal DNA digested with EcoRI, a simple insertion of Tn4351 is indicated by the strongly hybridizing 3.8 kb fragment, plus the two additional hybridizing fragments which are representative of junction fragments (Figure 3, Lane 2). Southern blot analysis of *P. gingivalis* MSM-3 DNA with \(^{32}\)P-labeled pR751 failed to show any hybridization of the conjugal plasmid (data not
shown). Transfer of *P. gingivalis* MSM-3 to non-selective medium (ABA) for 10 sequential passages did not result in the loss of erythromycin resistance.
Figure 3. Hybridization analysis of *P. gingivalis* MSM-3. *P. gingivalis* MSM-3 was digested with EcoRI, separated by agarose gel electrophoresis, and transferred to nitrocellulose. The resulting Southern blot was probed with $^{32}$P-labeled pVOH1. Lanes: MSM-3 (undigested MSM-3 chromosomal DNA); MSM-3, EcoR1 (EcoR1 digested MSM-3 chromosomal DNA). Size markers are indicated on the right. Hybridization was not observed with *P. gingivalis* A7436 digested with EcoRI when pR751 was used as the probe (data not shown).
Growth of *P. gingivalis* MSM-3 *in vitro*. On the basis on its nonpigmented colony phenotype, *P. gingivalis* MSM-3 was postulated to be defective in hemin storage or utilization. Therefore, the growth of *P. gingivalis* MSM-3 was examined in a minimal medium (BM) supplemented with several different iron sources. *P. gingivalis* MSM-3 grew poorly in BMH as compared to the parent strain A7436. Although *P. gingivalis* MSM-3 grew poorly with hemin, it was capable of growth with hemoglobin as the sole iron source (Table 1). Inorganic iron in either the ferrous or ferric form (FeCl₂ or FeCl₃) was also found to support the growth of *P. gingivalis* MSM-3 (Table 1). However, *P. gingivalis* MSM-3 did not grow as well as strain A7436 in BM supplemented with hemoglobin or ferric chloride (Table 1). Previous experiments performed in our laboratory (20) have shown that SB and ABA contain sufficient inorganic iron to sustain bacterial growth, and therefore *P. gingivalis* MSM-3 is most likely utilizing inorganic iron sources present in the complex medium for growth. Additionally, growth of *P. gingivalis* MSM-3 was inhibited to the same extent as that of the parent strain in complex medium (SB) made iron limiting by the addition of the iron chelator dipyridyl (Table 1).
Table 1. Growth of *P. gingivalis* A7436 and MSM-3 with different iron sources

<table>
<thead>
<tr>
<th>Growth Medium</th>
<th>OD&lt;sub&gt;660&lt;/sub&gt;&lt;sup&gt;2&lt;/sup&gt;</th>
<th>A7436</th>
<th>MSM-3</th>
</tr>
</thead>
<tbody>
<tr>
<td>BM</td>
<td>0.450</td>
<td>0.131</td>
<td></td>
</tr>
<tr>
<td>BMH</td>
<td>1.10</td>
<td>0.131</td>
<td></td>
</tr>
<tr>
<td>BM + 1.3 uM hemoglobin</td>
<td>0.85</td>
<td>0.644</td>
<td></td>
</tr>
<tr>
<td>BM + 20 uM ferric chloride</td>
<td>0.964</td>
<td>0.504</td>
<td></td>
</tr>
<tr>
<td>BM + 20 uM ferrous chloride</td>
<td>0.594</td>
<td>0.630</td>
<td></td>
</tr>
<tr>
<td>SB</td>
<td>1.2</td>
<td>0.84</td>
<td></td>
</tr>
<tr>
<td>SB + 200 uM dipyridyl</td>
<td>0.82</td>
<td>0.84</td>
<td></td>
</tr>
<tr>
<td>Anaerobic broth MIC</td>
<td>1.52</td>
<td>1.53</td>
<td></td>
</tr>
</tbody>
</table>

<sup>1</sup>*P. gingivalis* cultures were inoculated into BM and at 37°C under anaerobic condition for 24 h. This culture served as the inoculum into the indicated media.

<sup>2</sup>Growth of *P. gingivalis* A7436 or MSM-3 was monitored at A<sub>660</sub> with a Beckman DU8 spectrophotometer, and the OD<sub>660</sub> was recorded when cells reached stationary phase. Results are representative of two to four separate experiments.
Binding and transport of $^{[14}C$]hemin and $^{[59}Fe$]hemin. To examine the ability of P. gingivalis MSM-3 to bind and transport hemin, $^{[14}C$]hemin or $^{[59}Fe$]hemin was used as the sole iron source during growth in BM. For these studies, cultures of P. gingivalis A7436 and MSM-3 were grown under either hemin-deplete (BM) or hemin-replete (BMH) conditions for 24 h and reinoculated into fresh BM. Binding and transport were determined under growing conditions by supplementation of cultures with $^{[14}C$]hemin (1.5 uM) or $^{[59}Fe$]hemin (4.0 uM). The time course of $^{[14}C$]hemin binding and uptake during logarithmic growth of strains A7436 and MSM-3 is shown in Figure 4.

Hemin was bound quickly to P. gingivalis A7436 and the amount of hemin bound increased over time, with the maximal binding by 24 h (Figure 4A). In agreement with previous studies (20), binding of hemin by P. gingivalis A7436 was enhanced by growth in hemin-replete conditions (BMH) (65% more hemin bound than in cultures grown in hemin-deplete conditions as detected at 24 h). P. gingivalis MSM-3 grown in either hemin-replete or hemin-deplete conditions bound less hemin than did A7436. P. gingivalis MSM-3 grown in hemin-replete conditions bound 60,000 pmol of hemin per mg total protein as detected at 24 h, or 92% less than the parent strain. In addition, a constant amount of hemin was bound by hemin-depleted cultures of P. gingivalis MSM-3 throughout the entire growth period. Although cultures of P. gingivalis MSM-3 grown in hemin-replete conditions bound more hemin than did hemin-deplete
cultures, this value represented an increase of only 27% as
detected at 24 h (Figure 4A). Cutler et al. (14) have
examined the ability of P. gingivalis A7436 and MSM-3
lipopolysaccharide (LPS) to bind hemin. Results show that
growing P. gingivalis A7436 under hemin-replete conditions
increased the hemin-binding capacity of the LPS. The binding
of hemin to the LPS of P. gingivalis MSM-3 was shown to not be
induced by hemin, as LPS of P. gingivalis MSM-3 grown under
hemin-replete and hemin-deplete conditions bound comparable
amount of hemin.

Cultures of P. gingivalis A7436 initially grown in hemin
conditions and sampled at 1 h transported 43,000 pmol of hemin
per mg total protein (Figure 4B) (21). In marked contrast, P.
gingivalis MSM-3 grown under similar conditions transported
7,000 pmol of hemin per mg of protein, or only 16% of the
amount transported by P. gingivalis A7436. At all time points
examined, hemin transport by P. gingivalis A7436 was induced
by hemin-replete conditions. In contrast, hemin transport by
P. gingivalis MSM-3 was not enhanced by growth in hemin-
replete conditions; this was consistent throughout the entire
growth cycle (Figure 4B). Thus, hemin transport in P.
gingivalis MSM-3 does not appear to be regulated by hemin as
has been previously observed in P. gingivalis A7436. Studies
performed in our laboratory have also examined the ability of
P. gingivalis MSM-3 to transport the iron from hemin into the
cell. In agreement with results obtained when [14C]hemin was
used, *P. gingivalis* MSM-3 transported less \[^{59}\text{Fe}]\text{hemin}\) than did *P. gingivalis* A7436 (data not shown). Thus, *P. gingivalis* MSM-3 accumulates less hemin as well as less Fe from hemin compared with *P. gingivalis* A7436.
Figure 4. Hemin binding and accumulation by P. gingivalis A7436 and MSM-3. Cultures of P. gingivalis A7436 were grown under either hemin-deplete or hemin-replete conditions, cells were suspended in fresh BM, and hemin binding (A) and hemin uptake (B) were determined under growing conditions as a function of time. The amount of hemin bound and hemin internalization were determined as stated in Materials and Methods. □, A7436 grown in BM; ■, A7436 grown in BMH; ▲, MSM-3 grown in BM; ▼, MSM-3 grown in BMH.
Ultrastructure and quantification of extracellular vesicles of *P. gingivalis* A7436 and MSM-3. Results obtained from both growth experiments and hemin transport studies indicated that *P. gingivalis* MSM-3 could not efficiently utilize hemin because of a defect in hemin binding and transport. It was hypothesized that the inability of *P. gingivalis* MSM-3 to efficiently transport hemin into the cell could result in low levels of intracellular hemin, thus rendering cultures in a hemin limited state. Previous studies have shown (47) that under conditions of nutrient starvation, *P. gingivalis* is capable of altering its morphology. *P. gingivalis* MSM-3 was therefore examined by electron microscopy to determine if the defect in hemin utilization resulted in ultrastructural changes. Analysis of *P. gingivalis* MSM-3 by electron microscopy revealed extensive blebbing of outer membranes, with large numbers of vesicles visible extracellularly (Figure 5B) (21). Although extracellular vesicles were also observed in electron micrographs of *P. gingivalis* A7436, they were fewer in number when compared with *P. gingivalis* MSM-3 (Figure 5A).
Figure 5A. Electron micrograph of *P. gingivalis* A7436 (culture grown in SB). Magnification: x120,000.
Figure 5B. Electron micrograph of *P. gingivalis* MSM-3 (culture grown in SB). Magnification: x120,000.
Extracellular vesicles from *P. gingivalis* A7436 and MSM-3 were then purified. Cultures of *P. gingivalis* MSM-3 grown in either ABA or ABA (hemin-replete media) produced more vesicles than did *P. gingivalis* A7436 (Table 2). *P. gingivalis* MSM-3 grown in ABA produced approximately twice the quantity of vesicles as did *P. gingivalis* A7436 grown similarly (Table 2). These results were consistent in over four separate extractions and are statistically significant at P < 0.05. Additionally, for both *P. gingivalis* A7436 and MSM-3, cells grown in ABA produced approximately twice the quantity of vesicles compared with cultures grown in SB (Table 2).

Table 2. Yield of extracellular vesicles from *P. gingivalis* A7436 and MSM-3

<table>
<thead>
<tr>
<th>Strain</th>
<th>Growth medium</th>
<th>Dry Weight (in mg)</th>
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<tbody>
<tr>
<td></td>
<td></td>
<td>5.9 ± 0.2</td>
</tr>
<tr>
<td></td>
<td></td>
<td>11.6 ± 1.9</td>
</tr>
<tr>
<td>A7436</td>
<td>SB</td>
<td></td>
</tr>
<tr>
<td></td>
<td>AB</td>
<td></td>
</tr>
<tr>
<td>MSM-3</td>
<td>SB</td>
<td>8.9 ± 0.4</td>
</tr>
<tr>
<td></td>
<td>AB</td>
<td>20.9 ± 1.0</td>
</tr>
</tbody>
</table>

1. *P. gingivalis* A7436 and MSM-3 were grown in either Schaedler (SB) or Anaerobe (AB) broth (500 ml culture), cells were harvested at late exponential growth, and vesicles were prepared as described in Materials and Methods.

2. Following extraction, vesicles were lyophilized and dry weight was determined. Results are means of two separate experiments ± standard deviations.
Hemolytic activity. Previous studies (8) have indicated that *P. gingivalis* produces an active hemolytic activity that is cell bound and associated with the outer membrane. Growth of *P. gingivalis* under hemin limitation has also been reported to result in an increase in hemolytic activity in both outer membrane and outer membrane vesicle fractions (65). In agreement with these studies, whole cell, supernatant and vesicle preparations from *P. gingivalis* MSM-3 exhibited increased hemolytic activity compared with similar preparations from *P. gingivalis* A7436 (21) (Table 3). Using equivalent quantities of protein (0.1 mg/ml), vesicle preparations from *P. gingivalis* MSM-3 has a statistically significant increase in hemolytic activity compared with vesicles from *P. gingivalis* A7436 (Table 3). When one accounts for the increased production of vesicles by *P. gingivalis* MSM-3, the total hemolytic activity associated with *P. gingivalis* MSM-3 is approximately fourfold greater than that of *P. gingivalis* A7436 cultures.
Table 3. Hemolytic Activity of \textit{P. gingivalis} A7436 and MSM-3

<table>
<thead>
<tr>
<th>Strain</th>
<th>Whole cell fraction</th>
<th>Supernatant fraction</th>
<th>Vesicle Fraction</th>
</tr>
</thead>
<tbody>
<tr>
<td>A7436</td>
<td>29 ± 1.0</td>
<td>8.6 ± 0.5</td>
<td>16 ± 3.0</td>
</tr>
<tr>
<td>MSM-3</td>
<td>34 ± 1.0</td>
<td>9.4 ± 0.3</td>
<td>44² ± 3.0</td>
</tr>
</tbody>
</table>

\(^1\)Expressed as the percentage of sheep erythrocytes lysed after 2 hours. Results are the averages of three experiments ± standard deviation.

\(^2\)P < 0.05.
Arginine-X-specific protease activity. Until recently, this activity had been designated as "trypsin-like" protease activity, as the protease(s) involved have the same specificity for synthetic substrate as trypsin. Studies by Travis et al. (56) have shown that, in \textit{P. gingivalis}, the arginine-X-specific protease activity is encoded by the gene \textit{rgpl}, or one of its homologs. Previous studies (26) of this activity by \textit{P. gingivalis} have demonstrated that the production of this type of protease is induced by growth under conditions of hemin limitation. To determine if the hemin transport defect in \textit{P. gingivalis} MSM-3 could alter the expression of arginine-X-specific protease activity, the ability of \textit{P. gingivalis} MSM-3 to cleave the synthetic substrate BApNA was examined. As shown in Table 4, whole cell and supernatant fractions of \textit{P. gingivalis} A7436 and MSM-3 were found to exhibit comparable levels of arginine-X-specific protease activity. In contrast, the arginine-X-specific protease activity associated with the \textit{P. gingivalis} MSM-3 extracellular vesicle fraction was 3.3-fold greater than the activity associated with the vesicles isolated from \textit{P. gingivalis} A7436. When one accounts for the greater quantity of vesicles produced by cultures of \textit{P. gingivalis} MSM-3 (Table 2), the total arginine-X-specific protease activity associated with \textit{P. gingivalis} MSM-3 vesicles is approximately sixfold greater than that associated with \textit{P. gingivalis} A7436 vesicle fractions (21).
Table 4. Arginine-X-specific protease activity of P. gingivalis A7436 and MSM-3

<table>
<thead>
<tr>
<th>Strain</th>
<th>Whole cell fraction</th>
<th>Supernatant fraction</th>
<th>Vesicle fraction</th>
</tr>
</thead>
<tbody>
<tr>
<td>A7436</td>
<td>1,224 ± 91</td>
<td>3,808 ± 637</td>
<td>1,817 ± 965</td>
</tr>
<tr>
<td>MSM-3</td>
<td>1,346 ± 278</td>
<td>4,663 ± 37</td>
<td>7,863² ± 875</td>
</tr>
</tbody>
</table>

³Determined by using the synthetic substrate BApNA as described in Materials and Methods. Results are averages of two to three experiments ± standard deviation.
²P < 0.05.
Hemagglutination. Recent reports (56) have shown that the
rgpl gene possesses a domain which encodes for a
hemagglutinin. Thus, the hemagglutinating ability of P.
gingivalis is postulated to be associated with the rgpl gene.
Extracellular vesicles isolated from hemin-limited cultures
have been previously shown (48) to hemagglutinate erythrocytes
to higher titers than vesicles isolated from cultures grown in
hemin-excess. The ability of P. gingivalis MSM-3 and A7436 to
agglutinate erythrocytes was therefore examined. Using the
hemagglutination assay of Progulske-Fox et al. (58), whole
cells of P. gingivalis A7436 and MSM-3 were found to exhibit
hemagglutination titers of 1:16 and 1:32, respectively (Figure
6). In agreement with previous studies (48), hemagglutinating
activity was concentrated in the extracellular vesicle
fractions. Vesicle preparations from P. gingivalis A7436
exhibited a hemagglutination titer of 1:2,048; vesicles from
P. gingivalis MSM-3 exhibited a hemagglutination titer of
1:4,096 (21). These results indicate that P. gingivalis MSM-3
has a greater affinity for erythrocytes, which may relate to
the increased hemolytic activity observed for this strain.
Figure 6. Hemagglutination of whole cells (WC) and extracellular vesicles (ECV) prepared from P. gingivalis A7436 and MSM-3. Results are representative of 3 separate experiments. A7436 and MSM-3 WC: rows 1 to 8, serial two-fold dilutions of WC preparations; top row A7436 and MSM-3 ECV: top row two-fold dilutions of ECV preparations; bottom row A7436 and MSM-3. Hemagglutination titers were as follows: A7436 WC, 1:16; MSM-3 WC, 1:32; A7436 ECV, 1:2,048; MSM-3 ECV, 1:4,096.
Growth of *P. gingivalis* A7436 and MSM-3 in vivo. Previous studies performed in our laboratory, in agreement with others (6), have shown that the growth of *P. gingivalis* under hemin limitation results in enhanced virulence. To determine if the hemin utilization defect in *P. gingivalis* MSM-3 could influence its pathogenic potential, growth of *P. gingivalis* A7436 and MSM-3 was examined using the mouse subcutaneous chamber model. *P. gingivalis* is an invasive strain when inoculated in mice, producing ulcerated lesions distant from the injection site, septicemia, and death (19). Strain A7436 appears to invade mouse polymorphonuclear leukocytes in vivo and has also been observed to be highly resistant to phagocytosis by human polymorphonuclear leukocytes in vitro (13). It has been advantageous to use this virulent strain of *P. gingivalis* so that mutants can be examined both in vitro and in vivo for the expression of specific virulence factors.

When injected into subcutaneous chambers implanted in mice, *P. gingivalis* A7436 invaded the subcutaneous tissue, causing secondary necrotic lesions and death of animals (Table 5). Injection of *P. gingivalis* MSM-3 into subcutaneous chambers also resulted in secondary necrotic abdominal lesions and death; however, at an equivalent inoculum (10^8 CFU), *P. gingivalis* MSM-3 was more virulent than *P. gingivalis* A7436. Two days post-inoculation, 100% of mice infected with *P. gingivalis* MSM-3, but only 50% of mice infected with *P. gingivalis* A7436, exhibited abdominal lesions. Three days
post-inoculation, 100% of mice infected with *P. gingivalis* MSM-3 died; in contrast, only 25% of mice infected with *P. gingivalis* A7436 died (Table 5). At an inoculum of $10^7$ CFU, 40% of mice infected with *P. gingivalis* MSM-3, but none of the mice infected with an equivalent inoculum of *P. gingivalis* A7436 displayed abdominal lesions (Table 5) (21).

Chamber fluid was cultured from infected animals throughout the course of the experiment to correlate bacterial growth within chambers with the pathology observed in mice. Inoculation of *P. gingivalis* A7436 resulted in the growth of these organisms for up to 21 days postinoculation. *P. gingivalis* MSM-3 was also cultured from infected mice throughout the course of infection and up to the day each mouse died (data not shown). All bacterial samples of chamber fluid obtained from *P. gingivalis* MSM-3-infected mice grew on erythromycin-containing media. In agreement with our in vivo results, analysis of chamber fluid from *P. gingivalis* MSM-3 showed extensive blebbing of the outer membrane of isolated bacterial cells and large numbers of extracellular vesicles (Figure 7).
Table 5. Pathogenicity in mice following infection with *P. gingivalis*

<table>
<thead>
<tr>
<th>CFU</th>
<th>% with abdominal lesions (day[s] lesions first appeared)</th>
<th>Cachexia (ruffled fur)</th>
<th>% Death (day first death observed)</th>
</tr>
</thead>
<tbody>
<tr>
<td>A7436</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>$10^8$</td>
<td>50 (3-6)</td>
<td>Severe</td>
<td>25 (3-6)</td>
</tr>
<tr>
<td>$10^7$</td>
<td>0</td>
<td>Mild</td>
<td>0</td>
</tr>
<tr>
<td>MSM-3</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>$10^8$</td>
<td>100 (2)</td>
<td>Severe</td>
<td>100 (3)</td>
</tr>
<tr>
<td>$10^7$</td>
<td>40 (30)</td>
<td>Moderate</td>
<td></td>
</tr>
</tbody>
</table>

Mice (10 per group) were infected by inoculation of $10^8$ or $10^7$ CFU of each strain. Results presented are from one experiment and are representative of four separate experiments.
Figure 7. Chamber fluid obtained from P. gingivalis MSM-3 infected mice 2 day post-inoculation (1:10 dilution). Magnification: x120,000.
Genetic characterization of *P. gingivalis* MSM-3. *P. gingivalis* MSM-3 was next examined to determine the insertion site of Tn4351 (Tn4351 is depicted in Figure 8).

![Transposon Tn4351. Restriction sites and insertion sequences are indicated.](image)

Southern blot analysis of HindIII-digested *P. gingivalis* MSM-3 genomic DNA with pVOH1 as a probe revealed a 5 kb fragment (data not shown) containing the partial *ermF* gene, the entire tetracycline-resistance gene, and IS4351 attached to the chromosomal junction fragment. Using the tetracycline gene as a selective marker, this fragment was cloned from *P. gingivalis* MSM-3 into plasmid pGEM3Zf(-).

Following identification of the Tn4351-specific sequence in the recombinant plasmids, an AvaI-AvaI fragment of Tn4351, which contains a portion of IS4351 sequence attached to the chromosomal junction fragment and the multiple cloning site, was purified and used as a probe to screen the *P. gingivalis* A7436 cosmid library for the wildtype clones containing the insertion site. Cosmid DNA from positive clones was digested with HindIII and analyzed by Southern blot hybridization using
the previous AvaI-AvaI restriction fragment as a probe. A 5.3 kb DNA fragment was identified and sequenced. Computer analysis of the nucleotide sequence of the 5.3 kb fragment using Intelligenetics Suite revealed that, in the MSM-3 genome, Tn4351 had inserted 60 bp upstream (a non-coding region) from an IS1126 element (Figure 9).

IS1126 is the first IS element identified in P. gingivalis (43). The nucleotide sequence of the element in P. gingivalis MSM-3 is 98% identical to the IS1126 purified from P. gingivalis W83 as previously reported by Maley and Roberts (43). This IS1126 located downstream of the Tn4351 insertion (designated IS1126_{\text{Tn}}) is 1334 bp in length with 12 bp imperfect inverted repeats at either end (Figure 9). When compared to the previously reported sequence of IS1126 (43), a 4 bp deletion in the major open reading frame (ORF) presumably representing the IS1126 transposase was noted, which resulted in the interruption of this ORF. Identified also in this 5.3 kb region are two long ORFs. Open reading frame 1 (ORF1) encodes for a putative 449 amino acid protein. The protein encoded by ORF2 showed strong homology to the polynucleotide phosphorylase in E. coli.

![Figure 9. Localization of Tn4351 in P. gingivalis MSM-3. Tn4351 has inserted 60 bp upstream of IS1126 (1334 bp).](image-url)
Examination of IS1126 in P. gingivalis MSM-3. To determine the number of copies of IS1126 in P. gingivalis MSM-3, Southern blot analysis was performed with P. gingivalis A7436 and MSM-3 genomic DNA digested with BamHI (there is no BamHI site within the IS1126 sequence) and probed with a fragment isolated from IS1126. Two extra bands of approximately 4 kb and 5 kb were present in P. gingivalis MSM-3, indicating the existence of two additional of IS1126 in this strain as compared to the wildtype strain A7436 (data not shown), which may be responsible for the mutation in P. gingivalis MSM-3.

This is the first report of mobilization of an IS element in P. gingivalis. To examine one of the additional IS1126 elements in P. gingivalis MSM-3, a 4 kb BamHI restriction fragment was subsequently cloned from MSM-3 into pGEM3Zf(-) and the nucleotide sequence of this new IS1126 element (designated IS1126\textsubscript{MSM}) and its junction fragments was analyzed. Nucleotide sequence of downstream chromosomal sequences revealed that 387 bp of terminal sequence displayed 96% homology with the sequence of the prtT cysteine protease gene of P. gingivalis ATCC 53977 (42); the duplicated IS1126 was identified as being located 322 bp downstream from the prtT gene (Fig 10).

![Fig. 10. Location of IS1126 on the P. gingivalis genome.](image-url)
A radiolabeled oligonucleotide contained within the junction fragment was next used to screen the *P. gingivalis* A7436 SuperCos 1 cosmid library. A *HindIII-HindIII* fragment of approximately 7 kb from a clone was subcloned, and nucleotide sequence analysis confirmed that IS1126 was not present in the corresponding region of the wild type genome (data not shown). The complete sequence of this region and the sequence of an open reading frame adjacent to it (1.014 kb; designated *hemB*) on the *P. gingivalis* A7436 genome is shown in Figure 11. The point of insertion of IS1126 into the *P. gingivalis* genome and the open reading frame of *hemB* are shown.
3'ATAGCTACGAAGCTTTATGCTTTGCACCGTCATAAAGAGAAAAAGGTTTCCAC
5'TATCGATGCTTCCAATAATGCAAAAAACGTTGCGATTTTTTCTTTTTTCAAAAGGTG 954
3'TTCAGCATTTAAGGGCTGATATTCTCTGCTAAACTTGACCCTGTACCCGAGA
5'AGTCGTAATGTTTACGCATTAAGAGCTATTGTTGGCGCAAGAATGTAAGGGAAGGTG 1007
3'GTTGCGATACTGCAAGCAGAGTTAACCGTACATCCCTCAATCTCCACCAACC
5'CACCGCTATGACGTTCGTCTCAATTGTGGCGAAAGTGGTAAGGGAAAAGGTGG 1060
3'TTCAGCATTTAAGGGCTGATATTCTCTGCTAAACTTGACCCTGTACCCGAGA
5'AGTCGTAATGTTTACGCATTAAGAGCTATTGTTGGCGCAAGAATGTAAGGGAAGGTG 1113
3'TGCTTTATGACATCTCAAGAGCCGGCCATAAATGCTGATTTGCATCTCTTCCTCTC
5'ACGGGATATGATCAGATTTTCTCGGCCGTATTACAGTCAAGTCAGAAGGAGAC 1166
3'GACTACCAGTAGTCATCTACATTATAATGCAAGGCAATGGGCTGCTTCTTCCTCCT
5'CTCATGGGCTCAGATAATTATCAGAAGAGAGGCTTGGCCTAAGGATATTACC 1219
3'CCGGTCTTCTTCACTGCTTCCGTATAACGAGTTCACCAAACCAGCTAAAGAGAT
5'GGCCAAAGAAGACCCCGACCGGATTTGGCCTTCCGAGATTTCTCTCA 1272
3'GCCAGGACGCCAGGGTGAAATGGTGACGACTGCTTTCTCTCTGAGAAGAAGCAAC
5'CGGTCTCTGGGCGACTCTCCATCAAGCTGAGCTAAGAGAGTTCTTCCTCGTG 1325
3'CACGACTCTACCCATTATAACGGTTTCACGTCAAGTGACTGATAGTCCTACGTCTT
5'GTGCTGATGTTAATATGCTGACTGATACAGATGTTAAGGCAGAAGGAAAGGAGGAC 1378
3'GAGCAGGCCAGCTTTCTCTCAGATGGAAAGGCAAGATATTGATGTTGGGCAATAT
5'CTCTAAAAAGGAGTCACTCATACTTTCTCTATACAGTTAACAAAGGTTAA 1431
3'CTCCTTTTTATCTATTTCTACTCTACTTCTGATGCTGGAGGATGCTGAGAAGGAG
5'GAGGGAAATATGAAAAGTGTAGTAACAAAGCAGCCCTCATCGGCCTGCTTTT 1483
3'GAAATCATATTATATATGAGGGCATGCAGTTATCGTGCTATCTGACATTCTCCG
5'CTTCTCAGATAGTATATTGTACATCGTATTACCTCTAACCTCCGATCAGGCTGGG 1536
3'TGTGGTACGATAGGCTTTAGATCGAATATCCACTTATATCCACATTACAGGAGC
5'ACACATCGTATCCGGCAGATGCTGAGGATGCTGATGCTGAGGAGGAGGTTGAGC 1589
3'GCATGTCGACGCAAGCAATTTTCTACATGGACAGGGGGTTCACCAAAAGTCCCG
5'CGTACGTGCTTCAGCTAAGAGATGTCGACATTTTTTCACCAACAAAAAGGTGTTGAC 1642
3'GTTTTCTATGGTTCGTATGGAGGTGAGGAAAGTAAAACTGCGTAGCAGGTATAT
5'CAAAGATAATCAGATAGTATACCCCATCTCGGCACACCTCGGCACACTCCG 1695
3'AAGAAGGACCTTCAAGATGGTATAGGTCCACAGGCTCTAGTTCTGAGTACGAG
5'TTCTCTGGGCGACTCTCCATCAAGCTGAGCTAAGAGAGTTCTTCCTCGTG 1748
3'GTCCTAAACTGCTTTTCAAGATAGAAGAAAAGAAAAAGCATCAGCTCCTCGT
5'CAGGGATTTTACGAAAGTGCTATCTCTCTTCTTCTCGTGATGGGAAATGTTTTC 1801
Figure 11. Nucleotide sequence of the insertion site of XS1126 into the P. gingivalis genome. The end of the prtT gene’s sequence appears in italics. The 3’—>5’ sequence appears in bold print, while the 5’—>3’ sequence appears in normal print. An asterisk (*) denotes the insertion site of XS1126. The 1.014 kb open reading frame of hemB is denoted by open to closed arrows (i.e. < --- >). The start and stop codons of hemB are double underlined.

Genetic analysis using IntelliGenetics Suite revealed numerous open reading frames in the sequence of the IS1126 insertion site. Additionally, no homology of this region to genes involved in hemin binding and/or transport was found. Interestingly, analysis of the open reading frame of hemB
revealed homology not only to a gene that encodes for a hemin receptor in *Yersinia enterocolitica* but also several genes whose products have been shown to be involved in the acquisition of iron (receptors for siderophores such as enterobactin) and iron regulated outer membrane protein receptors (i.e. colicins and irg A) (Table 6).

**Table 6. Identity of hemB to genes involved in iron acquisition**

<table>
<thead>
<tr>
<th>Gene</th>
<th>General Description</th>
<th>% Identity to hemB</th>
</tr>
</thead>
<tbody>
<tr>
<td>irgA</td>
<td><em>Vibrio cholerae</em> iron-regulated OMP</td>
<td>28</td>
</tr>
<tr>
<td>cirA</td>
<td><em>Escherichia coli</em> colicin receptor</td>
<td>27</td>
</tr>
<tr>
<td>fepA</td>
<td><em>Escherichia coli</em> ferric enterobactin receptor</td>
<td>26</td>
</tr>
<tr>
<td>hemR</td>
<td><em>Yersinia enterocolitica</em> hemin receptor</td>
<td>25</td>
</tr>
<tr>
<td>fhuE</td>
<td><em>Escherichia coli</em> coprogen receptor</td>
<td>25</td>
</tr>
<tr>
<td>pFeA</td>
<td><em>Pseudomonas aeruginosa</em> enterobactin receptor</td>
<td>24</td>
</tr>
<tr>
<td>fhuA</td>
<td><em>Escherichia coli</em> ferrichrome receptor</td>
<td>24</td>
</tr>
</tbody>
</table>

Experiments are currently in progress to mutate the open reading frame of hemB in an effort to identify its function. Also, the second additional IS1126 insertion in *P. gingivalis* MSM-3 must be isolated and characterized. These data may result in the identification of additional iron (hemin) regulated genes in *P. gingivalis.*
CHAPTER FOUR: DISCUSSION

A number of gram-negative bacterial pathogens are capable of utilizing hemin as a source of iron and, of those, a few can also utilize hemin as a porphyrin source. Studies have shown that *E. coli*, *Y. pestis*, and *S. typhimurium* are normally unable to utilize hemin as a porphyrin source since their outer membranes are impermeable to hemin (20). By contrast, *Y. enterocolitica*, *S. flexneri* and *H. influenzae* can utilize hemin as both an iron source and porphyrin source since hemin can permeate their outer membrane (25). Previous studies in our laboratory have demonstrated that both $^{[14}C]$hemin and $^{[59}Fe]$hemin are accumulated by *P. gingivalis* A7436. This indicates that the iron and the porphyrin ring (i.e. the entire hemin moiety) is taken into the cell. These studies also demonstrated that hemin accumulation was inhibited by the addition of potassium cyanide (KCN) to *P. gingivalis* cultures. Cyanide is an inhibitor of the electron transport chain; it blocks the reduction of oxygen catalyzed by cytochrome aa$_3$ (37). This result indicates that hemin is transported into *P. gingivalis* by an energy-requiring process.

In *E. coli*, *Y. enterocolitica*, *S. typhimurium*, *H. influenzae* and *H. ducreyi*, energy for transport of ligands across the outer membrane is provided by the TonB protein
TonB spans the periplasmic space and physically interacts with outer membrane receptors at a highly conserved region called the TonB box. This interaction is thought to lead to a conformational change in the receptor protein, permitting the transport of the ligands across the outer membrane into the periplasmic space. The TonB protein is required for a variety of energy-dependent outer membrane processes, including uptake of iron-siderophore complexes, vitamin B12, the action of many colicins and the irreversible step in binding of bacteriophages such as T1 and 80. The TonB protein has recently been shown to be required for the transport of hemin in Y. enterocolitica, V. cholerae and H. influenzae (68,78,31). The energy-dependence of hemin transport in P. gingivalis suggests that a TonB analog anchored in the cytoplasmic membrane may also be present in P. gingivalis and function in the transport of hemin.

It is well documented that competitive growth and survival of bacterial pathogens require an adaptive response on the part of the microorganism to the specific growth environment of the host. A number of environmental factors, including pH, osmolarity, temperature, and iron concentration have been shown to coordinately regulate the expression of bacterial genes. In the mammalian host, free iron is rapidly sequestered by iron binding proteins, making its acquisition by invading pathogens extremely difficult. The acquisition of iron is thus one of the most important adaptive bacterial
responses for bacterial pathogens. Like other pathogenic microorganisms, *P. gingivalis* appears to sense and respond to hemin levels within the environment by altering the expression of several putative virulence factors; however, the molecular mechanisms by which hemin regulates the expression of specific *P. gingivalis* genes has not been defined.

The inability of the mutant strain *P. gingivalis* MSM-3 to utilize hemin for growth was associated with a decrease in both hemin binding and transport. The defect in hemin utilization may occur during binding of hemin to the cell surface and may result from the inability of *P. gingivalis* MSM-3 to store or remove hemin from the outer membrane. The inability of *P. gingivalis* MSM-3 to efficiently transport hemin into the cell would thus result in low intracellular hemin levels. This inference is consistent with the observations of enhanced expression of several putative virulence factors by *P. gingivalis* MSM-3. The increased production of extracellular vesicles and the associated increase of proteolytic, hemolytic and hemagglutination activities of *P. gingivalis* MSM-3 may be responsible for the enhanced pathogenicity of this organism. Of the large number of potential virulence factors *P. gingivalis* produces, potent proteolytic activity is thought to be particularly relevant in the disease process. The increased proteolytic activity of *P. gingivalis* MSM-3 may contribute to the enhanced invasiveness exhibited in the mouse subcutaneous chamber.
model. Proteases may also play a biological role in the growth of *P. gingivalis* by releasing small peptides, amino acids, and hemin-related compounds from proteins present in natural environments. In early 1995, protease activity was believed to have been attributed to as many as 39 distinct proteinases, some poorly characterized. Studies have demonstrated (56) that the arginine-X-specific protease activity of *P. gingivalis* is due to the *rgpl* gene. This gene is synthesized as a polyprotein containing a prepropeptide, a catalytic domain and a hemagglutinin domain.

The agglutination of erythrocytes by *P. gingivalis* may correlate with this organism's ability to lyse erythrocytes. Upon lysis, hemoglobin (Hb) released from erythrocytes is immediately bound by haptoglobin (Hp) in the host environment. Lustbader et al. (41) have demonstrated that bacterial proteases are capable of degrading the Hb-Hp complex. Proteases were shown not to attack the Hb of the complex, but rather cut the Hp in various places (41). This preferential attack of the complex Hp probably weakens the stabilization of the Hb, allowing for the removal of heme (18). Thus, the proteolytic capability of *P. gingivalis* may contribute to the degradation of this host hemoglobin-sequestering protein and liberate hemoglobin for binding and uptake into the cell (62). Results presented here have shown that *P. gingivalis* MSM-3 is capable of efficiently utilizing hemoglobin for growth. This may indicate multiple uptake systems for heme and heme-
containing compounds or that heme and hemoglobin are transported by different mechanisms. This quality is most likely attributed to the enhanced proteolytic and hemagglutinating activities, concentrated in the extracellular vesicle fraction, which are encoded by the rgpl gene. The regulation of expression of the *P. gingivalis* proteolytic and hemolytic activities may be a logical stress-induced response to nutrient deprivation, although it is currently not known if they are transcriptionally regulated.

The observed increase in expression of several *P. gingivalis* virulence factors in MSM-3 suggests that the mutation in *P. gingivalis* MSM-3 may result from an insertion in a hemin-responsive regulatory gene which controls the expression of hemolytic and arginine-X-specific protease/hemagglutinating activities, as well as the production of extracellular vesicles. Disruption of the hemin-responsive regulator would result in the enhanced expression of these factors. While this hypothesis is consistent with the regulation of hemin-responsive genes by a negative regulator such as Fur, it does not account for the inability of *P. gingivalis* MSM-3 to bind and transport hemin. In *E. coli*, Fur controls the expression of genes required for iron utilization (i.e. siderophore transport systems as well as ferric dicitrate transport). These systems are maximally expressed under conditions of iron limitation as a result of regulation by Fur at the transcriptional level. However, our
previous studies (20) indicate that in *P. gingivalis*, hemin binding and transport are induced by hemin, and thus regulation of genes required for hemin transport in *P. gingivalis* appears to be positively regulated.

Alternatively, the mutation may be due to the inactivation of a gene required for hemin transport. The resulting inability to transport hemin would render the cell in a hemin-limited state. Assuming a Fur-like system is operative, this would result in the derepression of genes regulated by the *P. gingivalis* Fur-like protein, resulting in enhanced expression of virulence genes. Preliminary studies in our laboratory indicate that *P. gingivalis* A7436 produces a Fur-like protein as assessed by Western blot (immunoblot) analysis using antisera raised to *P. aeruginosa* Fur. Using FURTA (the Fur Titration Assay) as described by Hantke et al. (25), we are currently working to identify genes in *P. gingivalis* which may be regulated by Fur. Involvement of a Fur-like protein in the regulation of hemin-responsive genes in *P. gingivalis* does not necessarily exclude the involvement of other regulatory factors. Recent studies with *V. cholerae*, *P. aeruginosa* and *S. typhimurium* indicate that the expression of a number of virulence determinants by iron may require more than one regulatory factor (39,40).

The successful establishment of a pathogen within a specific niche requires the ability of the pathogen to sense the specific environmental conditions of the host and to
regulate the expression of virulence genes accordingly. It is interesting to speculate that in response to hemin limitation, *P. gingivalis* is capable of turning on the expression of several factors which appear to be involved in the virulence potential of this organism. *P. gingivalis* is found in inflamed periodontal tissue, which is typically rich in erythrocytes (50). Because of the obligate requirement for iron or hemin by *P. gingivalis*, the expression of a hemin uptake system appears to be an important survival mechanism within this niche. The results of our studies support the concept of coordinate regulation of several putative *P. gingivalis* virulence genes in response to hemin.

To date, our laboratory has further characterized the genetic lesion of *P. gingivalis* MSM-3. Genetic analysis has revealed that Tn4351 has inserted into a non-coding region of the *P. gingivalis* MSM-3 chromosome. Additional studies have demonstrated that *P. gingivalis* MSM-3 has two additional copies of one of several previously identified insertion sequences, IS1126. This finding suggests that the integration of Tn4351 into the *P. gingivalis* MSM-3 chromosome resulted in the mobility of IS1126. The region of *P. gingivalis* A7436 DNA, homologous to the region containing the additional insertion sequence in *P. gingivalis* MSM-3, has been cloned and the area disrupted by IS1126 has been sequenced. Our studies show no homology to previously identified genes which have a role in iron binding and/or transport. The *hemB* open reading
frame, located approximately 1 kb downstream of the IS1126 insertion site, has been found by our laboratory and, independently by the lab of Dr. H. Kuramitsu (personal communication), to be homologous to genes involved in the iron acquisition process. This may suggest that the insertion of IS1126_{MSM} upstream of hemB may have produced a polar effect. Experiments are currently in progress to determine if hemB is transcriptionally regulated by hemin. In addition, our laboratory is currently attempting to mutate hemB in an effort to identify its function. We are also characterizing the second insertion of IS1126 in P. gingivalis MSM-3. This will allow us to begin to define potential regulatory determinants associated with the pathogenicity of P. gingivalis as well as identify specific proteins and genes involved in hemin binding and transport.
REFERENCES


