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The Role of Porphyromonas gingivalis Lysine Specific Protease, KGP in Hemin Transport and Pigment Accumulation

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THE ROLE OF THE *Porphyromonas gingivalis* LYSINE SPECIFIC PROTEASE, *KGP* IN HEMIN TRANSPORT AND PIGMENT ACCUMULATION

A thesis submitted in partial fulfillment of the requirements for the degree of Masters of Science in Physiology at Virginia Commonwealth University.

by

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Abstract

THE ROLE OF THE *Porphyromonas gingivalis* LYSINE SPECIFIC PROTEASE, *KGP* IN HEMIN TRANSPORT AND PIGMENT ACCUMULATION

By Anuj Sareen Phull, B.S

A thesis submitted in partial fulfillment of the requirements for the degree of Masters of Science in Physiology at Virginia Commonwealth University.

Virginia Commonwealth University, 2009

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Porphyromonas gingivalis, a gram-negative anaerobic bacterium, is implicated as a major etiological agent in the initiation and progression of severe forms of periodontal disease. It has been reported that gingivitis and periodontal disease affect roughly 50.3% and 35% of the adult population, respectively. Therefore, approximately over 85% of the adult population may suffer from some form of gingival disease. *Porphyromonas gingivalis*, an established periodontopathogen, requires hemin for growth. Although multiple hemin uptake systems appear to be present in this organism, their specific role in hemin uptake and virulence remains unknown. Pigmentation is thought to result from the accumulation of iron protoporphyrin IX (FePPIX) derived from erythrocyte hemoglobin. It has been observed that mutations abolishing activity of the Lys-X specific cysteine

protease, *Kgp*, resulted in loss of black pigmentation of *P. gingivalis* W83; they were less virulent than their wild-type counterparts. Thus, we have observed that *Kgp* degradation of fibrinogen deregulates the clotting cascade, thereby minimizing the availability of free erythrocytes. Additionally, *Kgp* binds erythrocytes and degrades them, releasing hemoglobin. The interference with mechanisms involved in the accumulation of black pigmentation may be significant in controlling the pathogenic potential of *P. gingivalis.*

These results suggest that Lys-gingipain protease is a principal protein involved in acquisition of hemin from hemoglobin as well as a major factor in transport, by affecting the accumulation of FePPIX on the bacterial cell surface. Microarray analysis indicates a change in the expression of key enzymes and proteins required for hemin uptake, iron storage, electron transport and oxidative stress. Therefore, interference with mechanisms involved in accumulation of black pigmentation may be significant in controlling the pathogenic potential of *P. gingivalis.*

List of Abbreviations

Abbreviation	Definition
α	alpha
β	beta
μg	microgram
μL	microlitre
°C	degree Celsius
BHI	Brain Heart Infusion
cDNA	Complementary DNA
DNA	Deoxyribonucleic acid
E. coli	*Escherichia coli*
FBS	Fetal Bovine Serum
IL-1	Interleukin-1
LB	Luria Bertani broth
LPS	Lipopolysaccharide
LRR	Leucine rich repeats
MCV	Medical College of Virginia
mL	milliliter
OD	Optical density

PCR	Polymerase Chain Reaction
Pg	*Porphyromonas gingivalis*
PI3K	Phosphoinositide-3OH kinase
p-value	Probability value
PPIX	Protoporphyrin-IX
PTLBW	Pre Term Low Birth Weight
RNA	Ribonucleic acid
RT	Reverse transcriptase
TLR	Toll-like receptors
TNF-α	Tumor-necrosis factor alpha
VCU	Virginia Commonwealth University
WT	Wild type

Introduction

A. Periodontal Disease

Porphyromonas gingivalis, a gram-negative anaerobic bacterium, is implicated as a major etiological agent in the initiation and progression of severe forms of periodontal disease [1, 2]. The disease is due to an imbalance of oral homeostasis, caused by a balance disruption between the oral flora and the host response. When this balance is compromised, aggressive pathogens are able to invade the epithelial barrier and accumulate. The indigenous microbiota of the mouth exist in a state of homeostasis with the host, except when the microbiota is disturbed, such as when the mucosal surface is damaged, or when the immune system is compromised. As soon as bacterial invasion of the pulp has taken place, both nonspecific inflammation and the specific immunologic response of the host have a profound effect on the progression of the disease [3,4].

The mouth harbors an abundant and diverse complex microbial community, representing over $1x10^{10}$ microorganisms. This complex oral microbiota contains more than 700 different bacterial species [9]. An overwhelming majority of these organisms are non-pathogenic and actually serve as an integral component in maintaining a healthy immune system. Periodontal lesions are estimated to be among the most common bacterial infections in the United States. It has been reported that gingivitis and periodontal disease affect roughly 50.3% and 35% of the adult population, respectively [5, 6]. Therefore, approximately over 85% of the adult population may suffer from some form of gingival disease.

Periodontal disease has been classified into multiple diagnostic categories, which are dependent upon the age of onset, rate of progression, and response level to clinical therapy [7]. Additionally, periodontitis may be classified by patient type, by rate of progression, and by the extent of affected region, such as localized or generalized. It may also be classified according to the size of lesions; initial, moderate, or advanced breakdown, as well as by effectiveness of treatment. Although no such classification is currently available, it has been proposed that a classification system based on specific infectious agent origin would facilitate treatment decisions [8].

A study performed by Dr. Ashimoto, in order to detect the presence of certain bacteria in patients with mild to severe forms of periodontal disease, found that the following bacterial strains were present: *Actinobacillus actinomycetemcomitans*, *Tannerella forsythia*, *Campylobacter rectus*, *Eikenella corrodens*, *Porphyromonas gingivalis*, *Prevotella intermedia*, *Prevotella nigrescens* and *Treponema denticola* [9]. When examining patients with advanced periodontitis, the four bacteria present in the highest concentration were *T. forsythia*, *E. corrodens*, *C. rectus* and *P. gingivalis* respectively [9]. Among these bacterial species, *P. gingivalis* is believed to be a primary contributor to periodontal disease [10]. This is because *P. gingivalis* possesses a remarkable array of virulence factors [4, 11], immune response suppressors, as well as proteolytic enzymes that degrade host tissues and adherence factors that promote bacterial survival [12].

Figure 1. Initiation and Progression of Periodontal Disease

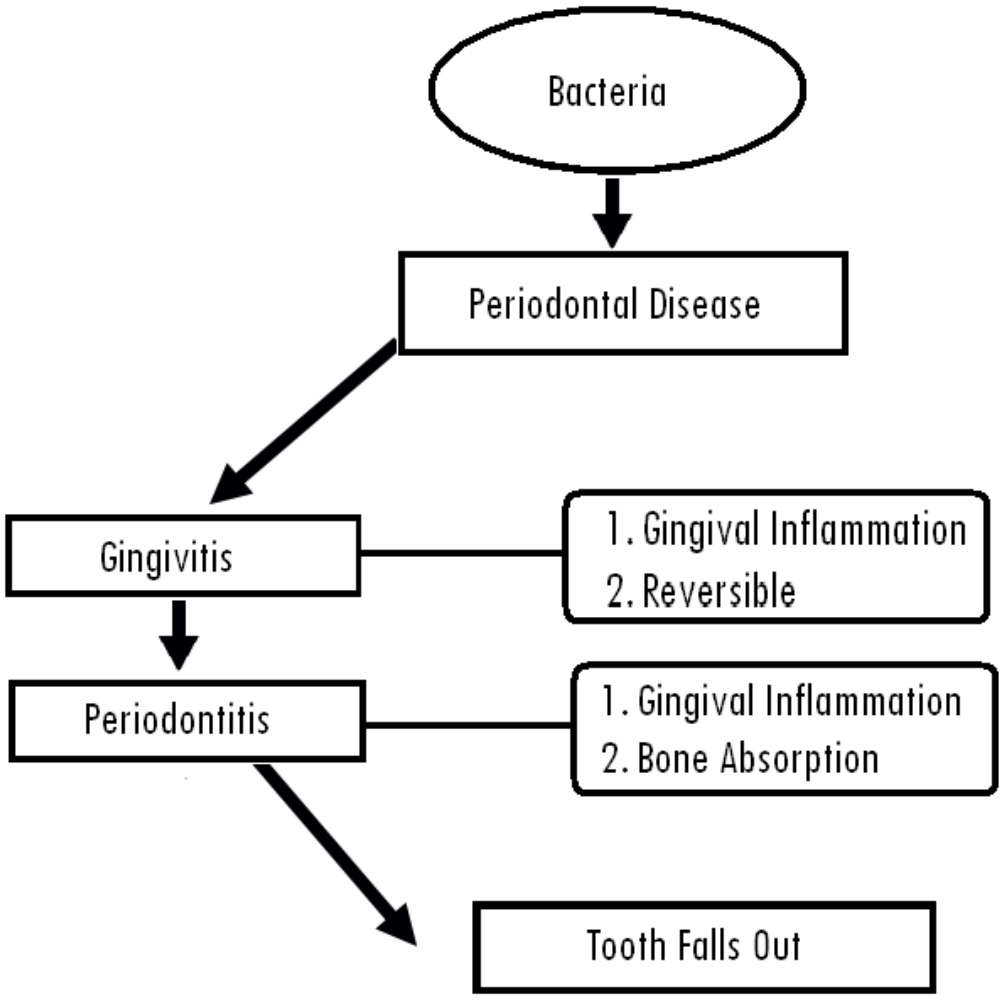
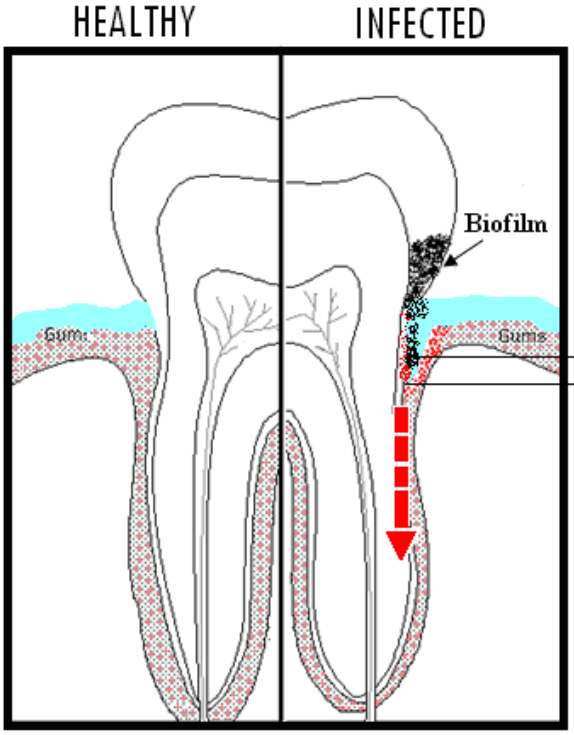

Bacteria
Periodontal Disease
Gingivitis
1. Gingival Inflammation
2. Reversible
Periodontitis
1. Gingival Inflammation
2. Bone Absorption
Tooth Falls Out

Figure 2. Pathogens Involved In The Progression of Periodontitis

Pathogens shed from biofilm aggregated on tooth enamel and proceed to invade host cells.

Adapted from (Lamont et al. 1998) and

(http://www.enchantedlearning.com/subjects/anatomy/teeth/tooth_bw.GIF)


HEALTHY
INFECTED
Biofilm
Gums
Gums
Tissue that anchors teeth to bone becomes inflamed
Pockets form as gums separate from teeth

B. Pathogenesis of Periodontitis

Microbes utilize the environment provided by the host in order to gain nutrients, which are essential for growth. This may result in disruptions of the balance between host and microbes, resulting in pathogenesis. The perturbation may be caused by alterations in the host defense or by changes in the microbiota [13]. However, these opportunistic pathogens are capable of accessing normally sterile area of the oral cavity such as dental pulp or periradicular tissue. The steps in the development of an endodontic infection include microbial invasion, multiplication and pathogenic activity. Therefore the effective cooperation between the host's innate and adaptive immune response keeps the growth of the microbiotica under constant observation. Together, commencial bacteria and the host defense system prevent pathogenic bacterial intrusion into local tissues through a variety of mechanisms [14].

Bacterial communities that are embedded in the matrix of polymers are called biofilms and are comprised of bacterial and salivary origin. It develops naturally on teeth, and forms part of the defense system of the host by aiding in colonization resistance against microorganisms [15]. Plaque is typically found at protected and stagnant surfaces, which are therefore at the greatest risk of disease. However, if plaque is not removed regularly, it can lead to calculus, dental carries and ultimately gingivitis. It is also recognized that oral microorganisms damage tissue directly by their toxins and histolytic enzymes. Tissue can also be damaged indirectly by excessive inflammatory reaction of the tissue itself in response to toxins [16]. Electron, fluorescent and confocal scanning laser microscopy techniques have detected virulent bacteria within epithelial and dendritic cells

in tissues with advanced periodontitis [17]. This finding confirms intracellular invasion of the host cell, as the primary means of infection by oral pathogens [18].

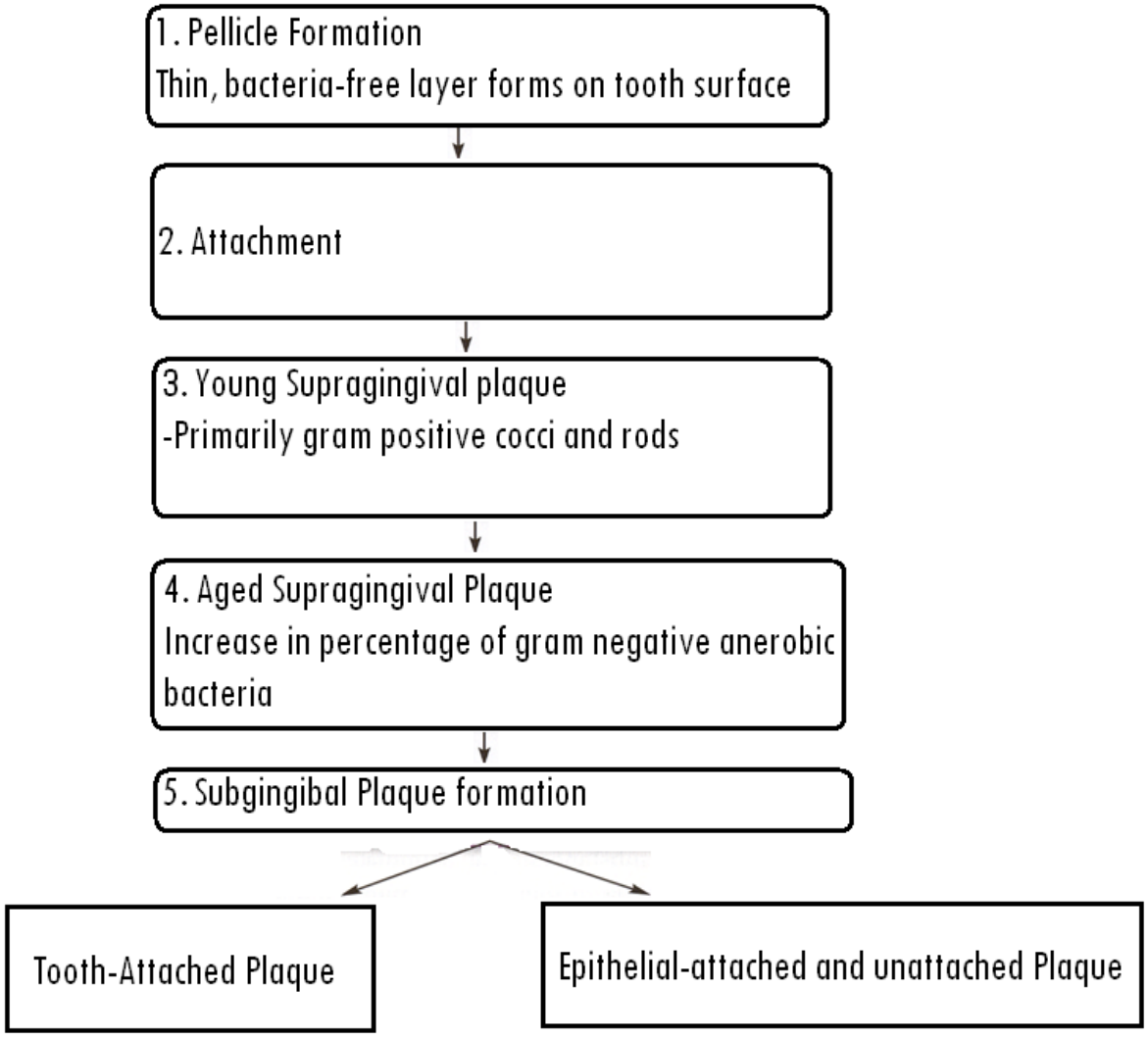
I. Colonization of Oral Cavity by *P. gingivalis*

The initial attachment of bacteria begins with pellicle formation, which is a thin coat of salivary proteins that adhere to the tooth surface. Specific saliva components, such as proline-rich proteins and statherin, serve as receptors for P. *gingivalis*, thereby allowing it to bind to solid surfaces. This formation facilitates the bacterial attachment to the fimbriae, which are hair-like structures on the pellicle [19]. Additionally, bacterial motility is also facilitated by saliva, which acts to transport it to different locations within the oral cavity and areas of the host body. However, the adhesion or interaction of bacteria with salivary histatins, cystatins, mucin glycoprotein MG2, and fibronectin, will inhibit bacterial growth as well as result in apoptosis of the infected cells [19].

Colonization occurs once the tooth has been covered with the attached bacteria, primarily by gram-positive faculatative cocci, the major streptococci species present [20]. Next, coaggregation results in the formation of a complex array of different bacteria linked together. After the plaque forms, the gingival margin becomes inflamed and swollen [21]. The biofilm then proceeds to extend into the sub-gingival region and thrives in the protected environment, which permits long-term survival. These conditions allow for the formation of a mature sub-gingival plaque bioflim. The accumulation of biofilm as well as increased susceptibility of the host are key, for the progression of periodontitis [22].

Figure 3. Phases of Plaque Formation

This figure depicts the various stages in pellicle formation and plaque adherence. Abstracted from Nield-Gehrig JS and Willmann DE. Foundations of Periodontics for the Dental Hygienist. Philadelphia: Lippincott Williams & Wilkins 2003:67-73.Phase of Plaque Formation.

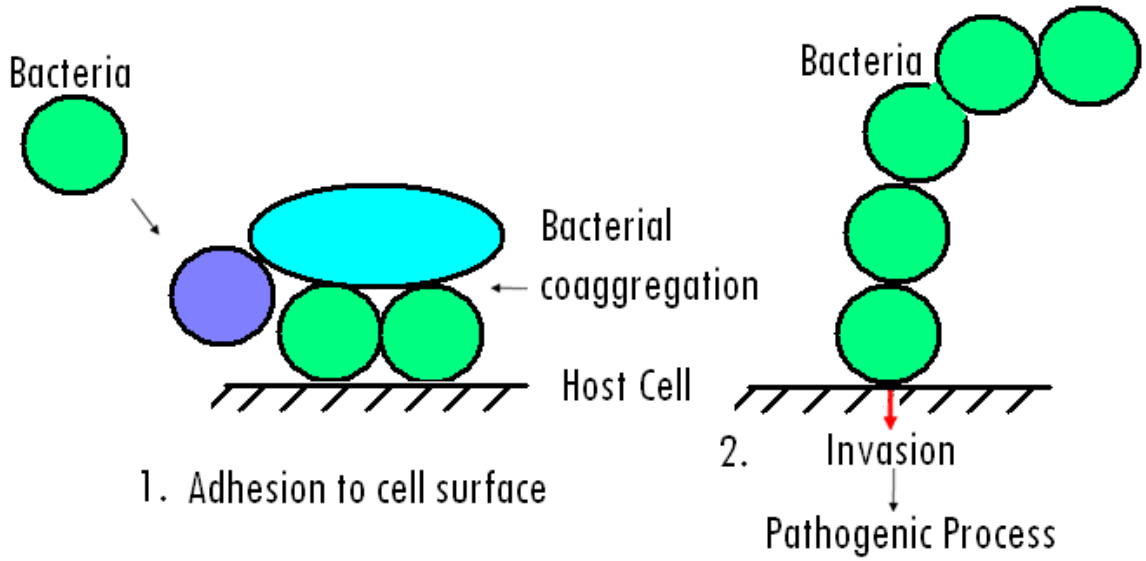

1. Pellicle Formation
Thin, bacteria-free layer forms on tooth surface
2. Attachment
3. Young Supragingival plaque
-Primarily gram positive cocci and rods
4. Aged Supragingival Plaque
Increase in percentage of gram negative anerobic bacteria
5. Subgingibal Plaque formation
Tooth-Attached Plaque
Epithelial-attached and unattached Plaque

II. Predicted Mechanism of Invasion

P. gingivalis has the ability to invade gingival epithelial cells within twenty minutes of interaction [17]. It has been proposed that, FimA, which codes for bacterial fimbriae, plays a significant role in adhesion to host epithelial cells [22]. The sensitivity of *FimA* to environmental cues is indicative of its role in invasion and progression of disease. Prior to infection, *P. ginigvalis* expression of fimbriae is documented at high levels, thereby optimizing bacterial adhesion to tooth surfaces [4]. Additionally, hemin concentrations affect FimA expression, since low nutrient levels are associated with decreased fimbrial expression. Therefore, subgingival locations with inadequate levels of nutrients are avoided, since they prevent bacterial adhesion and mobility.

Once the bacteria have replicated, invaded and degraded host tissue, FimA expression is reduced, potentially to avoid any host immune response [4]. One proposed mechanism of *P. gingivalis* invasion is fimbrial interaction with gingival epithelial cell β1 integrin receptors [17]. This particular interaction leads to the engulfment of bacterial cells. Although, the mechanism of bacterial uptake is still unclear, there is evidence of strong cytoskeletal rearrangement. It is clear that focal adhesion kinase (FAK) and paxillin are phosphorylated upon invasion, thereby influencing actin and microtubule dynamics [17]. According to a study performed by Yilmaz et al, *P. gingivalis* invasion was significantly decreased, yet surprisingly not completely inhibited, in FimA knockout models [17], which serves as an indication there are alternative modes of invasion.

Figure 4. Initial Association between Pathogenic Bacteria and Host Cell
This figure was adapted from (www.nihon-u.ac.jp/arish/prof/prof_l_hamada_e.html). This depicts the initial association and subsequent invasion of pathogenic bacteria to the host cell surface.


Bacteria
Bacterial
coaggregation
Host Cell
1. Adhesion to cell surface
Bacteria
2. Invasion
Pathogenic Process

C. Factors Influencing Microbial Growth

I. Environment

Environmental conditions on teeth are not uniform, which creates a gradient of biological and chemical factors that influence the growth and resistance of the present microflora. These differences are reflected in variations among the composition of the community at particular sites such as the gingival crevis, aproximal regions, smooth surfaces, and pits and fissures [19]. The fissure plaque is more affected by saliva, while the gingival crevicular fluid (GCF) has a greater impact on plaque in the gingival crevice. This latter site also has a lower redox potential (Eh) and is colonized by higher numbers of anaerobes, especially proteolytic species which obtain key growth factors from the catabolism of host proteins and glycoproteins in GCF [20].

II. Temperature, pH and Oxygen

Additional factors affecting bacterial growth are temperature, pH, and oxygen availability. Temperature primarily affects the enzymes of the microorganisms, allowing a faster growth rate by increasing enzyme activity, until key enzymes are denatured. Most bacteria have an optimum pH for growth in the range 6.5-7.5 [21]. Acidophilic bacteria can grow at a low pH, as many of them produced acid as end products of metabolism. Organisms found in periodontal disease, such as *Porphyromonas gingivalis,* tend to rely on protein and peptide breakdown, for growth, since it produces slightly alkaline end products [22,23].

Oxygen requirements vary, ranging from obligate aerobes to facultative anaerobes and microaerophiles to obligate anaerobes [24]. An obligatory anaerobic organism, whose

energy generating pathways do not require molecular oxygen, demonstrates a high degree of sensitivity to oxygen. Therefore bacteria in the center of a micro colony may live in a strict anaerobic environment, while other bacteria at the edges of the fluid channels can live in an aerobic environment [23]. Therefore, the biofilm structure provides a wide range of customized living environments with differing pH, nutrient availability, oxygen, allowing bacteria with different physiological needs to survive.

III. Nutrients

The growth of a pathogen is dependent upon its ability to scavenge essential nutrients; iron plays a crucial role in the establishment and progression of an infection.

D. Periodontal Disease Implications

The most common form of periodontitis affects about 15% of the population, making it one of the most common chronic infections disease in humans. In the United States alone, more than 100 million people have shown measurable periodontal bone loss [24]. The disease is a chronic inflammatory condition, acting just below the gum line in the sulcus. Soft tissue swelling traps the plaque in the pocket. As the tissues are damaged, the sulcus develops into a pocket. While in the pocket, it causes the attachment of the tooth and its supporting tissue to break down. The severity of the disease is generally associated with the depth of the pocket. Infection is likely and a tooth abscess may also develop, thereby increasing the rate of bone destruction [25].

Although the destructive cycle starts with the accumulation of plaque, there are a number of other factors that can contribute to and aggravate the condition including heredity, tobacco, diabetes, pregnancy, Downs syndrome leukemia, immune deficiencies, vitamin C deficiency and pregnancy [26]. Additionally, smoking is a significant risk factor for periodontal disease since it creates a favorable environment for harmful bacteria and interferes with the normal mechanisms for limiting bacterial growth in the mouth. A diet deficient in calcium, vitamins C and B, can also contribute to periodontal disease, since calcium is important in maintaining the integrity of bones, including those that support our teeth [27].

Table 1. List of Factors Contributing to Periodontal Disease

- Tobacco smoking or chewing
- Systemic diseases such as diabetes, cancer, or AIDS
- Prescription medication:
    I. Steroids
    II. Anti-epilepsy drugs
    III. Cancer therapy drugs
    IV. Calcium channel blockers
    V. Oral contraceptives
- Crooked teeth
- Crowded teeth
- Fillings that have become defective
- Increased hormone level during pregnancy
- Poorly fitting crowns, bridges, and fillings
- Poor diet
- Genetics
- Clenching and grinding teeth
- Emotional or psychological stress

E. Periodontitis and diabetes

Periodontitis has been referred to as the sixth complication of diabetes [28]. The results of the study performed by et al. Loe, indicates that diabetic patients were twice as likely as non-diabetic subjects to have attachment loss associated with periodontal disease. People with type 1 and type 2 diabetes appear equally susceptible to periodontal disease and tooth loss. The severity of the periodontal disease is usually related to the duration of the diabetes [28, 29] as measured by the glycosylated hemoglobin. The concentration of glycosylated hemoglobin in serum is a direct function of the time that hemoglobin is exposed to elevated glucose levels [29].

Additionally, gingivitis and periodontitis impair the body's ability to utilize insulin, which in turn makes diabetes more difficult to control [30]. Severe periodontal disease can increase blood sugar, contributing to increased periods of time when the body functions with a high blood sugar. This in turn places diabetics at increased risk for diabetic complications. Children with IDDM (insulin-dependent diabetes mellitus) are also at risk for gum problems. For these individuals the best protection against periodontal disease is good diabetic control, since studies show that controlling blood sugar levels is proven to lower the risk of some of the complications of diabetes, including heart disease, never damage, and periodontal disease.

F. Periodontitis and Pregnancy

Preterm low birth weight (PTLBW) infant treatment in hospitals for respiratory distress, jaundice, malnutrition, and anemia is approximated to cost over $5 billion annually [31]. A recently proposed mechanism of labor suggests that intra-amnionic levels of mediators, including interleukins (IL-1, IL-6), tumor necrosis factor alpha (TNF-a), and prostaglandins, specifically PGE_2, will continue to increase steadily throughout pregnancy until a threshold is reached, at which labor is induced. This raises the possibility that in the presence of infection, the elevated levels of mediators may trigger preterm birth, thereby resulting in low birth weight [32]. It has been hypothesized that microbial products, such as lipopolysaccharide (LPS), reach the uterus of the bloodstream and induce cytokine release in the membrane. The increase in cytokine release will result in increased prostaglandin production, thus increased uterine muscle contraction. Therefore inflammatory mediators such as cytokines and prostaglandins, when produced in the periodontal tissues in response to LPS stimulation, may also pose a real threat to the fetoplacental unit, thereby increasing the risk of PTLBW [33].

G. Porphyromonas gingivalis

Porphyromonas gingivalis is a gram-negative anaerobic bacterium, whose virulence is dependent upon hemin acquisition. Hemin is required for synthesis of the cytochrome b subunit of *P. gingivalis* fumarate reductase. Additionally, hemin is a critical growth factor for the bacterium, since the reduction of fumarate to succinate is the central pathway for generation of metabolic energy in *P. gingivalis*. Due to the lack of enzymes,

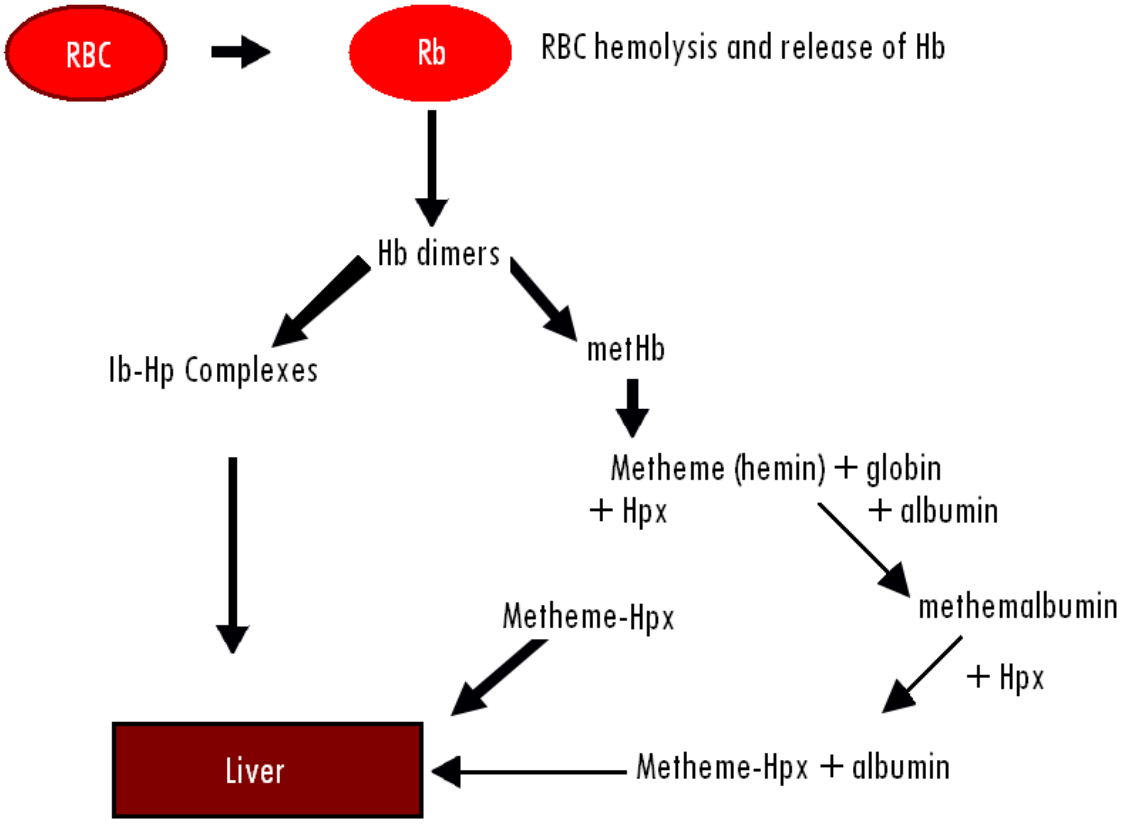
5-aminolevulinic acid synthase and porphobilinogen deaminiase, *P. gingivalis* is unable to synthesize protoporphyrin IX, which is the precursor of hemin. Therefore, an exogenous supply of hemin must be provided, to facilitate growth.

H. Hemin

Hemin serves as a valuable source of iron for most host-associated microorganisms [35]. As with other pathogens, a requirement for the *in vivo* growth of *P. gingivalis* is that the organism must be capable of obtaining iron from the host. Hemin, an iron protoporphyrin IX, is an indispensable cofactor for proteins, allowing for proper metabolism and oxidative stress protection. Erythrocytes are the major carrier of hemin in the host, while hemolysis results in the release of hemoprotein and hemoglobin. The host proteins bind hemin with a high affinity of $10^{-12\ M}$ for hemin-hemopexin [36] and 10^{-8}M for heme-albuin [37]. The major source of hemin is hemoglobin, however it is also rapidly and strongly bound by haptoglobin, with an affinity less than 10^{-15} M for the hemoglobin-haptoglobin complex [38]. Therefore, successful bacterial pathogens must be able to acquire the hemin from the host scavenging proteins either by proteolytic degradation or by expression of high affinity binding receptors, capable of sequestering the hemin from the host hemin-binding proteins.

Figure 5. Availability of Hemin under Physiological Conditions
Upon vascular trauma, red blood cells are released. Following RBC lysis, hemoglobin (Hb) is released. The released hemoglobin is rapidly bound by haptoglobin (Hp) and the complex is transported to liver. Any remaining free hemin is bound by a lower affinity protein, albumin. Abstracted from [39].

Figure 5. Availability of Hemin in physiological conditions

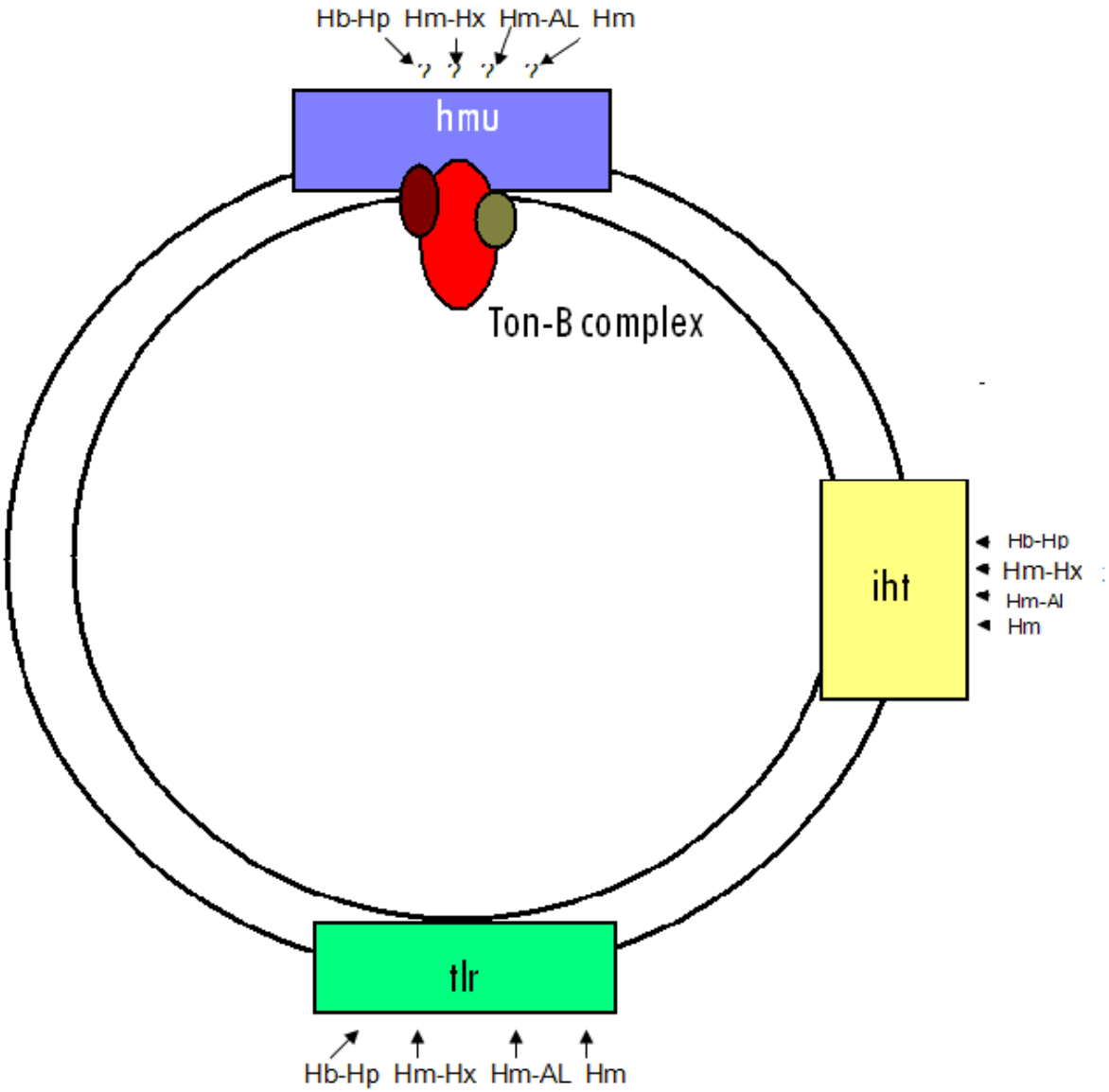


I. Hemoglobinase Activity

The lab of Dr. Lewis has determined that *P. gingivalis* produces a potent hemoglobinase, capable of binding and degrading hemoglobin, thereby releasing hemin [39]. The released hemin is then acquired and stored by *P. gingivalis* on its cell surface. Therefore, the characteristic pigmentation produced by *P. gingivais* colonies appears to be due to the accumulation of hemin and is thought to serve as a mechanism for hemin storage [40]. The ability of *P. gingivalis* to store hemin appears to provide a nutritional advantage for the survival of this pathogen in the iron-limited environment of the healthy periodontal pocket.

Multiple hemin binding and transport proteins have been identified in *P. gingivalis*, experimentally. Three multigenic clusters, encoding proteins thought to be involved with the hemin acquisition pathway have been detected in the genome of *P. gingivalis* W83 [41]. The first Locus, ihtABCDE, is comprised of five open reading frames (ORF's) coding for TonB-dependent receptor (IhtA), a lipoprotein (ihtB), a periplasmic binding protein (ihtC), permease (ihtD), and cytoplasmic ATP binding protein (ihtE). The growth of hemin-limited *P gingivalis* is inhibited by preincubation with IhtB-specific antisera, thereby indicating the role of this locus in hemin uptake [42]. The second locus has a similar composition in that a TonB-dependent receptor, encoding gene, is followed by a putative ATP –binding cassette hemin transport system, htrABCD [43].

Figure 6. Schematic Representation of the Hemin Uptake Mechanism Present in *P. gingivalis* W83. Hb-Hp (hemoglobin-haptoglobin complexes), Hm-Hx (hemin-hemopexin complexes), Hm-Al(hemin-albumin complexes), Hm (hemin)


Hb-Hp
Hm-Hx
Hm-AL
Hm
hmu
Ton-B complex
iht
Hb-Hp
Hm-Hx
Hm-Al
Hm
tlr
Hb-Hp
Hm-Hx
Hm-AL
Hm

J. Protease Activity and Kgp

Proteases play a major role in *P. gingivalis* virulence by directly degrading host tissue, activating host pro-enzymes, or neutralizing host immune systems [44]. Additionally, proteases have the ability to degrade immunoglobulins, inactivate cytokines and their receptors, degrade host tissue, activate host proenzymes, increase vascular permeability, and promote bleeding. There is a strong correlation between gingival protease activity and periodontal inflammation [45]. Extracellular Arg-x- and Lys-x-specific cysteine proteinases are considered important virulence factors and pathogenic markers for *Porphyromonas gingivalis*, a major factor in chronic periodontitis. The genes: *rgpA* and *rgpB* encode an Arg-x-specific proteinase and adhesins (RgpA), an Arg-x-specific proteinase (RgpB). On the other hand, k*gp* encodes a Lys-x-specific proteinase and adhesins (Kgp). The lysine specific proteinase gene appears to be identical to the *prtP* gene characterized as a lysine and arginine-specific protease [46]. Some specific functions have now been assigned to two of the enzymes, RgpA and Kgp [47], however the roles of the PrtT and Tpr enzymes in *P. gingivalis* remain uncertain.

It has been proposed that the adherence of *P. gingivalis* to erythrocytes [48], fibronectin, and the collagenous substrata [49], is mediated in part by such proteases at the cell surface. Several investigators have indicated interesting results for the correlation of the protease and hemagglutinin activities in *P. gingivalis* [48,49]: arginine-specific cysteine protease (arginine-gingipain)-deficient mutants exhibited decreased hemagglutinating activity, which suggests notable properties of this protease [50]. Recent

enzymatic and molecular cloning analysis directly revealed that these proteases are composed of two distinct domains: one for proteolytic activity and the other for hemagglutinating activity [51, 52, 53].

Figure 7. Schematic Representation of the Processing of the RgpA , RgpB, and Kgp

The white areas indicate the catalytic domains of the proteinase, the shaded areas indicate the adhesins. ↑ marks the proposed outer membrane attachment to LPS. Image is abstracted from (Role of RgpA, RgpB, and Kgp Proteinases in Virulence of *Porphyromonas gingivalis* W50 in a Murine Lesion Model)

Neil M. O'Brien-Simpson, Rita A. Paolini, Brigitte Hoffmann, Nada Slakeski, Stuart G. Dashper, and Eric C. Reynolds

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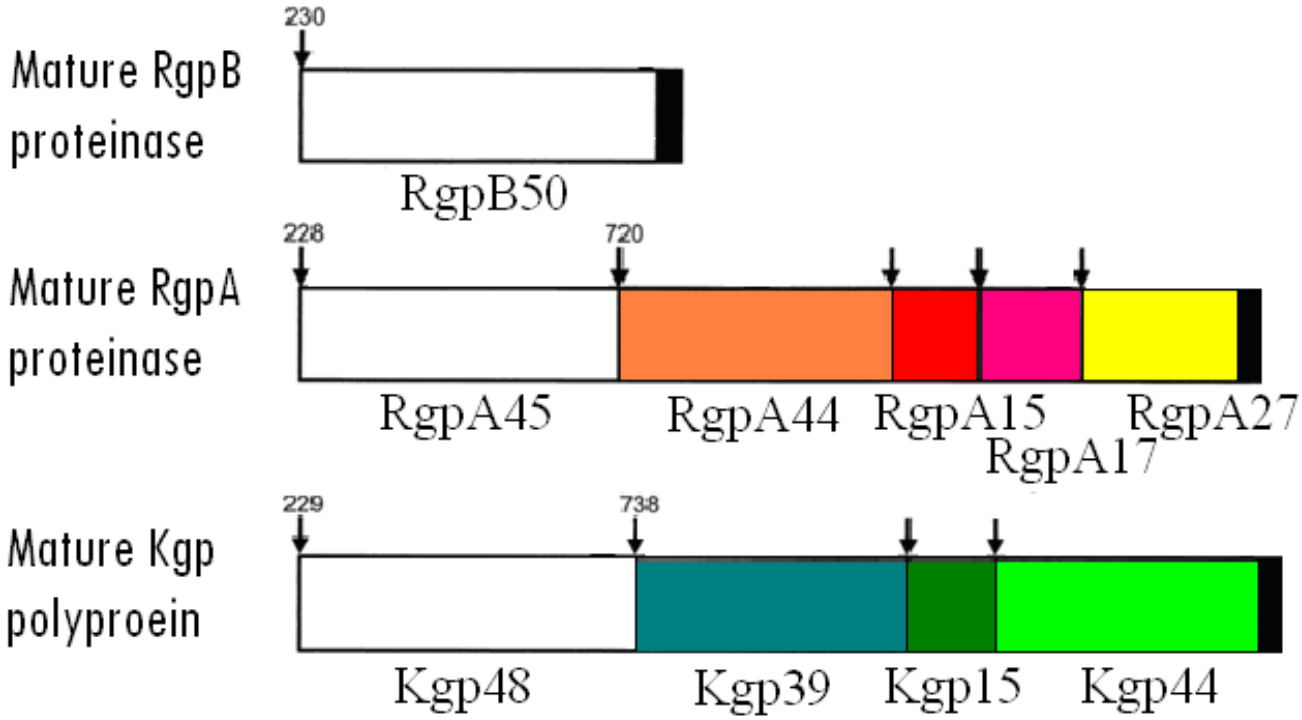

Mature RgpB proteinase
230
RgpB50
Mature RgpA proteinase
228
720
RgpA45
RgpA44
RgpA15
RgpA27
RgpA17
Mature Kgp polyproein
229
738
Kgp48
Kgp39
Kgp15
Kgp44

It has been suggested that expression of *P. gingivalis* protease is regulated by the growth environment. Since the hemagglutinating and hemolytic potentials of mutant strains were reduced but not eliminated, it was hypothesized that this protease played a role in acquisition of FePPIX from hemoglobin. [54,55,56] In contrast to Arg-gingipain, Lys-gingipain was not inhibited by hemin, suggesting that this protease played a role near the cell surface where high concentrations of hemin grant the black pigmentation. Human hemoglobin contains 11 Lys residues in the α chain and 10 Lys residues in the β chain. In contrast, there are only three Arg residues in each of the α and β chains. These observations are consistent with human hemoglobin being a preferred substrate for Lys-gingipain but not Arg-gingipain. The ability of the Lys-gingipain to cleave human hemoglobin at Lys residues was confirmed by electrospray ionization and Fourier transform mass spectrometry of hemoglobin fragments, resulting from digestion with the purified protease.

Our lab was able to detect several of the predicted hemoglobin fragments rendered by digestion with purified Lys-gingipain. Thus, it was postulated that Lys-gingipain of *P. gingivalis* is a hemoglobinase which plays a role in heme and iron uptake by affecting the accumulation of FePPIX on the bacterial cell surface. Additionally, the laboratory was also the first to identify a lysine protease as a major virulence factor, by constructing an allelic exchange mutant that was defective in the Lys-X specific protease (Lys-gingipain), in order to demonstrate its role as a virulence factor [39].

Figure 8. Schematic Representation of Human Blood Hemoglobin, Containing Iron at the Center

Heme
(Fe-protoporphyrin IX)

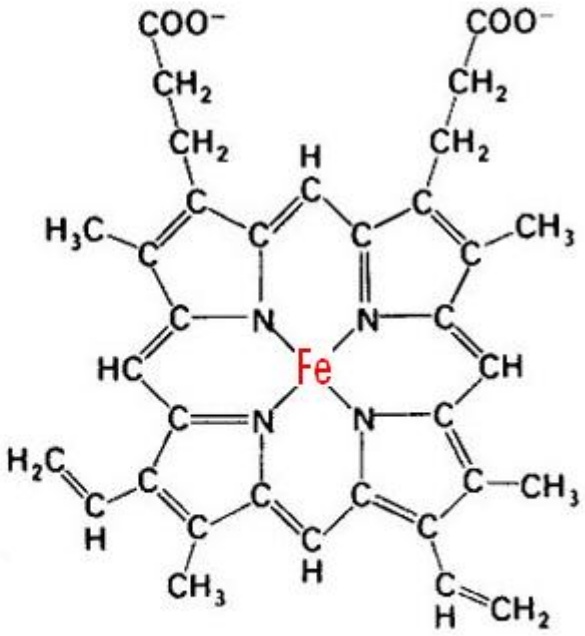
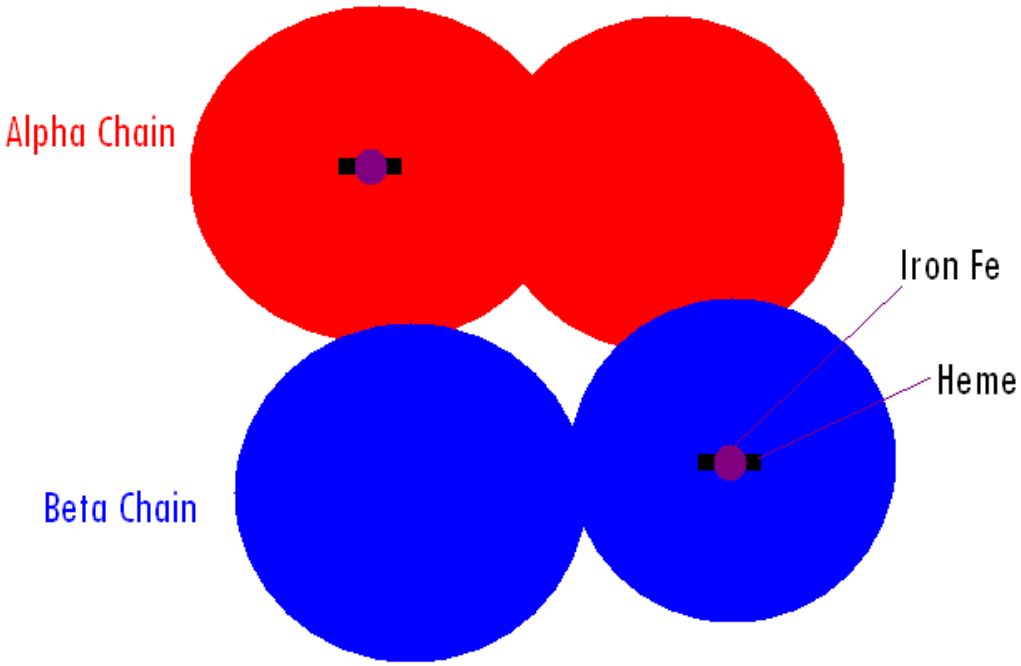


Figure 9. Schematic of human hemoglobin polypeptide chain, with identified alpha and beta chains. Abstracted from University of Miami (http://www.iit.edu/~smile/guests/hemoglobin.html)

Table 2. Hemoglobin chart identifying alpha and beta chain detail



HEMOGLOBIN

Alpha chain	Beta chain	Delta chain	Epilson chain
141	146 15,867	146 15,924	146 16,071

K. Research Objectives

The importance of hemin/ iron for growth and virulence of *P. gingivalis* is well documented, however there are gaps in knowledge of the mechanisms involved in hemin/iron uptake and the role that hemin/iron may play in gene regulation. In particular the study will focus on Kgp and its role in hemin acquisition and gene regulation.

Two Aims were addressed in this study:

Aim 1. Test the hypothesis that Kgp is involved in synthesis and maturation of other hemin-binding components present in outer membrane ultimately leading to altered pigment accumulation on bacterial cell surface and hemin transport.

Aim2. Examine the differences in gene regulation between wild type *P. gingivalis* strain and the Kgp deficient strain.

MATERIALS AND METHODS

A. Bacterial Strains and Plasmids

Table 3. Outline of the bacterial strains and plasmids used during experimentation

	Strain	**Plasmid**	**Description**	**Reference**
P. gingivalis	W83	Parental Strain		Lewis and Macrina 1999
	V2577	Kgp deficient strain		Lewis and Macrina 1999
E. coli	One Shot® Top 10 cells	Chemically competent cells		Invitrogen (Catalog Number: C404003)
	V2198	pVA2198	2-kb *ermF-ermAM* cassette between *Kpn*I and *Bam*HI	Fletcher and Macrina 1995

B. Media Growth Conditions

I. *Porphyromonas gingivalis* W83

Porphyromonas gingivalis was grown in brain heart infusion broth (BHI) (Difco Laboratories, Detroit, MI) along with: hemin (5µg/ml; Sigma, St. Louis, MO), vitamin K_3 (0.5 µg/ml; Sigma,St Louis, MO), yeast extract (5mg/ml) and cysteine (1%). In order to prepare a solid medium, Agar (2%) was added. The cultures were prepared under anaerobic conditions composed of 10%H_2, 10%CO_2, 80%N_2 at 37 ˚C. Blood agar plates (TSA II, 5% sheep blood; BBL, Cockeysville, MD) were required to observe hemin uptake from *P. gingivalis* as well as our *Kgp* mutant. Clindamycin (0.5µg/ml), erythromycin (300µg/ml), and carbenicillin (50µg/ml) were also required for selection and maintenance of the strains.

During the growth analysis, *P. gingivalis* strains were prepared in Mycoplasma broth (1 liter contained: beef heart infusion [2.0g], pancreatic digest of casein [7.0g], brain extract [3.0g], yeast extract [3.0g], sodium chloride [5.0g], L-cysteine [1.0g], vitamin K_3 [0.5mg]).

II. *Escherichia coli*

E. coli was grown at 37˚C in Luria-Bertani (LB) medium (Invitrogen; Catalog Number: 12780-029) or on LB agar. Erythromycin (300 µg/ml) and kanamycin (25 µg/ml) were used as required for selection and maintenance of strains.

C. *Porphyromonas gingivalis* Cell Culture Preparation

P. gingivalis W83 strain (Table 3) was spread on TSA II blood agar plates and grown anaerobically at 37°C. V2577, Kgp deficient strain, was spread on TSA II blood agar plates treated with clindomycin (0.5 μg/ml) and allowed to grow anaerobically at 37°C. After approximately 3-7 days after preparation colonies were observed on the plates. The appropriate colonies were then selected and inoculated into BHI medium, where they grew anaerobically at 37°C. They were inoculated for approximately 12-48 hours in order to obtain an optical density of 0.5 and 1.6, respectively, when measured at wavelength 660 nm. This method was used in preparation for RNA isolation.

Table 4. *kgp* Gene Information

Gene Information
Gene Size: 5193 kb
Gene ID: PG1605

D. Generation of *P. gingivalis* Mutant Strains

I. Isolation of *erm*F-*erm*AM

The erythromycin gene, 2.1 kb, was isolated from the pVA2198, and used as a selection factor for the mutant strain. While isolating the pVA2198, *Eco*RI *and Bam*HI were used as restriction sites, since they are present on either side of the *erm* gene. A restriction digest was prepared using both *Eco*RI *and Bam*HI, (New England Biolabs) at 37°C for 2 hours. After the digestion was complete a 1% agarose gel was prepared in order to run the DNA through a gel electrophoresis (BioRad). The band size of interest measured 2.1kb, and once located, was sliced from the gel using a sterile scalpel. The sliced gel was then eluted, using Qiagen® MinElute Gel Extraction Kit (Catalog Number: 28606). The eluted gel now contained the isolated *erm*F gene, whose restriction sites are *Bam*HI and *Eco*RI.

II. Ligation

The e*rm* gene was isolated and then inserted into a pUC19® vector supplied by NEB (pUC19 Vector® Catalog Number: N3041L). pUC19® is a commonly used plasmid cloning vector in *E.coli*, since it has a high copy number. It has a 54 base-pair multiple cloning site polylinker that contains unique sites for 13 different restriction endonucleases. The ligation procedure was performed according to the recommended Invitrogen protocol. The ermF-ermAM cassette was ligated into the *KpnI* and *BamHI* cleaved pUC19 vector

carrying a 450 base *Hind*III fragment encompassing the prepropeptide region of the prtP gene. Thus we created plasmid with the pUC19 vector containing *kgp* and *erm*.

III. Transformation

The pUC19-*kgp-erm* plasmid was transformed into One Shot® Top 10 cells supplied by Invitrogen (Table 3). Approximately 1.0µL ligation mixture was introduced to 50µL of chemically competent cells through a 30 second heat shock at 42°C. The transformation procedure was performed according to the recommended Invitrogen protocol. After the heat shock, 500µL of SOC medium was added to the sample, and placed in the shaker apparatus for 1 hour at 37°C, while rotating at 225RPM. The prepared transformation mixture was then plated on LB, agar and kanamycin (30µg/ml) solid media and allowed to grow at 37°C overnight. The pUC19-*kgp-erm* vector includes the genes for erythromycin, allowing for selective growth. The colonies were then screened through restriction digestion and gel electrophoresis analysis. The digestion was done with *Eco*RI (New England Biolabs), since this was a proven restriction site. This construct was used to electroporate *P. gingivalis* W83. The subsequent clindamycin-resistant colonies were analyzed by Southern blot analysis to confirm the disruption of the *kgp* gene.

E. Growth Study

P. gingivalis W83 strain and *Kgp* deficient strain were prepared from inoculation of cells derived from colonies grown on TSA II blood agar plates. The colonies were inoculated into 5 mL of BHI media with hemin. The wild type required 4-5 passages, while the mutant strain only required 3. During the first passage, 1ml of BHI inoculated

culture was added to a Mycoplasma solution, in a 1: 5 dilution. (1mL BHI grown sample with 4mL Mycoplasma) and allowed to incubate for 24 hours at 37°C. The second passage consisted of a 1: 10 dilution, which included 1mL of the previous sample and 9mL of fresh mycoplasma. The third dilution was also a 1:10, while the fourth dilution was a 1:20 dilution. After obtaining 5 passages for the wild type and 3 passages for the Kgp deficient strain, the sample was added to 4 different growth media in order to determine growth under each of these hemin-controlled conditions.

These hemin depleted conditions served to inoculate basal medium and basal media with a range of concentrations of a number of supplements including: hemin (750nM - 0.9nM), PPIX (750 nM – 0.9 nM), bovine hemoglobin + BSA (1nM – 1.5 x 10^{-2} nM hemoglobin equivalent), bovine hemoglobin-haptoglobin complex (1nM – 1.5 x 10^{-2} nM hemoglobin equivalent).. Hemoglobin haptoglobin complex was constructed from mixing hemoglobin with haptoglobin in a 1:2 ratio (wt/wt) for a period of 30 minutes, at room temperature. The concentrations of hemoglobin-haptoglobin and hemin-albumin with each a minimum of 50% saturated. 1mM of TLCK was included in the growth studies in order to prevent the degradation of hemoproteins by *P. gingivalis* protease inhibitor N-alpha-p-tosl-L-lysine chloromethl ketone.

F. Microarray

I. Bacterial Culture

The strains for both *P. gingivalis* W83 and the *Kgp* deficient mutant were cultured under the same anaerobic conditions; however they were harvested at two different growth points: (1) 0.05 OD and (2) 1.6 OD. The optical density was measured with a spectrophotometer DU® 640, at the 660 wavelength. The 5mL culture was aliquoted into 15 ml tubes and centrifuged at 8,000 rpm for 15 min at 4°C, in order to form the pellet. Next, the supernatant was removed, by using a sterile Pasteur pipette, and the pellet was saved at -80°C.

II. RNA Isolation

Prior to RNA isolation, the bench and pipettes were sprayed with DNAse solution (Ambion Inc., Foster City, CA). RNA was prepared using the Qiagen® RNeasy Mini Columns Kit (Valencia, CA Catalog Number: 74104). The isolation was done according to the Qiagen® protocol with some modifications. Bacterial cells from 5ml of culture were suspended in 500µl of RLT buffer. This mixture was then transferred to a tube containing cell lysing beads, FastPrep®-24 tubes (MP Biomedicals Lot. 6911-500-119847). At this point, 500 µl of Acid phenol: chloroform was added to the tubes, while remaining under the ventilation hood, while keeping the samples on ice. Next, the cells were lysed with a FastPrep™ FP120 BIO 101 ThermoSavant, at speed 6 for 45 seconds. The tubes were then incubated on ice for five minutes. In order to separate the different components of the cell, the tube was centrifuged at 13000 RPM for 10 minutes at 4C. The supernatant containing

the RNA was harvested and mixed with iced 500µl of 100% ethanol. The mixture was pipetted into RNeasy columns and the remaining purification steps were completed according to the Qiagen® protocol. While on the column, a 30 minute DNase treatment was included to degrade any residual DNA. The RNA was eluted with 50µl of nuclease free-water and concentration of RNA was measured using NanoDrop spectrophotometer ND-1000.

III. cDNA Generation

While generating cDNA, 10 µg of total RNA was resuspended in 12.0 µl of dH_2O RNase Free Water (Qiagen®), which gave a concentration of approximately 0.80 µg/µl. Additionally, 3 µl of Random Primers (Stratagene® FairPlay® III Microarray Labeling Kit) were added and incubated at 70°C for 10 minutes. A cocktail of 2.4 µl of 10x Affinity Script RT Buffer, 1 µl of 20x dNTP mix, 1.5 µl of 0.1M DTT, .5 µl of RNase Block (40 U/µl), 2 µl of Affinity Script HC/RT and 2 µl of Superscript III Reverse Transcriptase was prepared (Stratagene® FairPlay® III Microarray Labeling Kit) and added to the RNA mixture. The reaction was incubated overnight at 42°C, however not to exceed 12 hours. The reaction was stopped with 10 µl of 1N NaOH and incubated at 70°C for 10 minutes. Next, the samples were neutralized with 10 µl of 1M HCl. The cDNA was purified according to the recommended Qiagen® PCR Purification Kit protocol, however $NaHCO_3$ was used during the elution step and a phosphate wash buffer was used during the second wash, rather than EB Buffer.

IV. Labeling

Samples were labeled with 5 µl of Cy-3 or Cy-5 dye (GE Healthcare Lot: 366152) and allowed to stand for 1.5 hours at room temperature, while remaining in the dark. The Cy-5 was used for the mutant strain, while the Cy-3 was used for the wild-type. In order to stop the reaction, 5 µl of 4M hydroxylamine was added and left at room temperature for 15 minutes, while still remaining in the dark. In order to terminate the reaction, 5µl of of 4M hydroxylamine was added to each sample.

After the reactions were terminated, the samples were purified. At this time, purification was also performed in the dark, to protect the light-sensitive dyes. The Qiagen MinElute PCR Purification Kit protocol was followed. The only adjustment made from the protocol was for the elution step which was done twice using 10µl of the Buffer EB, resulting in a final volume of 20µl.

V. Pre-Hybridization

The *P. gingivalis* microarray slides (The Institute for Genomic Research; version 1) were incubated with a pre-hybridization buffer for 2 hours at 42°C. 50 ml of pre-hybridization buffer was filtered and prepared for every 5 slides: 12.5 ml of 20x SSC (Ultrapure™ Invitrogen® Catalog Number: 15557-036), 500 µl of 10% SDS (GIBCO Invitrogen® Lot. 561591), .5 g of BSA powder (Fisher Biotech; Fair Lawn, NJ) and 37 ml of dH_2O.

During the pre-hybridization step 50mL of pre-hybridization buffer was made by mixing 12.5mL of 20x SCC, 500µl of 10% SDS, 0.5gm of BSA powder, and 37mL of MilliQ water. The buffer was then filtered with a 0.22µm Mini-Miser Filter. Afterwards, the filtered buffer was transferred to a clean Coplin jar and preheated to 42°C for approximately 30 minutes. In order to pre-hybridize the array slides, the slides were cleaned with kim wipes and were placed in the preheated Coplin jar for 1 hour at 42, with their labels facing up.

The pre-hybridized slides were then washed in three different pre-hybridization wash solutions, as illustrated in Table 4. The first buffer contained 100ml of SSC, 20 mL of SDS, and 880mL of dH2. The second buffer was composed of 100mL of SSC and 900mL of dH20. The last buffer had 10mL of SSC, 25µL of DTT, and 990mL of dH20. The pre-hybridization slides were removed from the Coplin jar and were washed in each buffer twice. The slides were placed in each of the buffers for varying amounts of time: Buffer 1 for 15 minutes for each wash, while 10 minutes for buffer 2 and 5 minutes for buffer 3. Each buffer was heated in the hot bath to 55°C prior to use. After washing, the slides were centrifuged at 1000 RPM for approximately 30 seconds at room temperature to dry. After drying, the slides were examined against the light to check for any streaks or spots, if present the slides were rewashed.

Table 4. Wash buffer preparation and protocol for microarray slides..

Pre-Hybridization Wash			**Post-Hybridization Wash**	
Wash I	100 ml SSC 20 ml SDS 880 ml dH_2O	* Wash slides with 500 ml Wash I @ (55°C) * Shake for 20 minutes	**Wash I**	100 ml SSC 20 ml SDS 25 μl 1M DTT 880 ml dH_2O
Wash II	100 ml SSC 900 ml dH_2O	* Wash slides with 500 ml Wash II @ (55°C) * Shake for 10 minutes	**Wash II**	100 ml SSC 25μ 1M DTT 900 ml dH_2O
Wash III	10 ml SSC 25 μl 1M DTT 990 ml dH_2O	* Wash slides with 500 ml Wash III buffer @ (55°C) * Shake for 5 minutes	**Wash III**	10 ml SSC 990 ml dH_2O

V. Hybridization

Immediately after the pre-hybridization step, the hybridization step followed, to achieve optimal results. To prepare for hybridization, the samples were combined with dyes Cy3, and Cy5, in the dark and were purified once more, while following the Qiagen MinElute PCR Purification kit. After the DNA was eluted twice with Buffer EB, the samples were purified using the Qiagen® PCR Purification Kit protocol and spun in a SpeedVac centrifuge (SpeedVac Concentrator Savant DNA120) for 15-25 minutes until a pellet was formed.

The hybridized cDNA pellet was resuspended in 80 μl of hybridization buffer: 400μl formamide, 250 μl 20x SSC, 10 μl 10% SDS, 1 μl .1M DTT, 339 dH_2O and 60 μl of Salmon Sperm DNA (Ambion® Catalog Number: AM9680); and heated at 95°C for 5 minutes. The cDNA was vortexed and heated a second time, meanwhile 24mm x 60mm microscope glass cover slips were gently placed over the array printed area of the slide. It was important to ensure that no air bubbles were between the cover slip and he slide and that no excessive pressure was placed on the cover slip. Next, 75 μl of the sample was loaded onto washed microarray slides. Slides were incubated overnight at 42°C. After incubation, the slides were washed again and coated with DyeSaver^2 (Genisphere Inc.; Hatfield, PA) to protect from light.

After approximately 12 hours, the slides were taken out of the microarray chamber and were washed with post-hybridization buffers. Three post-hybridization buffers were prepared, as illustrated in Table 4**.** The first buffer consisted of 100mL of SSC, 20mL of

SDS, 25 μl 1M DTT, and 880 ml dH_2O. The DTT serves to reduce background 'noise', when examining the micro arrays. The second buffer contained 100mL of SCC, 25μl of 1M DTT, and 900 ml dH_2O. Additionally, the third buffer only consisted of two components; 10 ml SSC and 990 ml dH_2O. The first buffer was preheated to 55C prior to washing; the slides were washed vigorously in this buffer to ensure that the cover slips fell off and then allowed to stay for 15 minutes. Next, the slides were washed in buffer 2 twice, for 10 minutes. Following the buffer 2 wash, the slides were washed for 5 minutes in buffer 3. In order to dry the slides for scanning, the slides were centrifuged for 30 seconds at room temperature.

VI. Microarray Data Analysis

The Axon 4200 Scanner Program GenePix pro v6 was used to scan and acquire label signals from the microarray slides. The slides were placed in the scanner with the bar code facing down. First the slides were fast-previewed, and appropriate changes were made in the both the red and green exposure in order to maximize clarity. After the appropriate modifications, determined by examining the histogram, the detailed scan was performed. The signal intensities associated with each gene was detected with Axon4200 scanner, was analyzed for statistical significance using ArrayAssist software (Stratagene Co. La Jolla, CA). Two arrays were used for each analysis, each containing four spots of the gene, thus a total of eight measurements were taken for each gene. The data was first normalized, using Lowess normalization. Next the Significance Analysis for Oral Pathogen Microarrays tools were used SAOPMD) comparing the expression of the mutant

to the parental strain. The software uses Linear Models for Microarray Data (LIMMA) for statistical interface. The ratio represented by florescent dye Cy-5/Cy-3 represents the change in mRNA expression. If the value is greater than one, this indicates increased mRNA expression of the strain labeled with Cy-5, whereas a value less than 1 indicates a decrease in expression of the Cy-5 labeled strain. Genes with a Cy-5/Cy-3 ratio <0.5 and >1.5 when comparing the strains, were considered to have significantly altered mRNA expression.

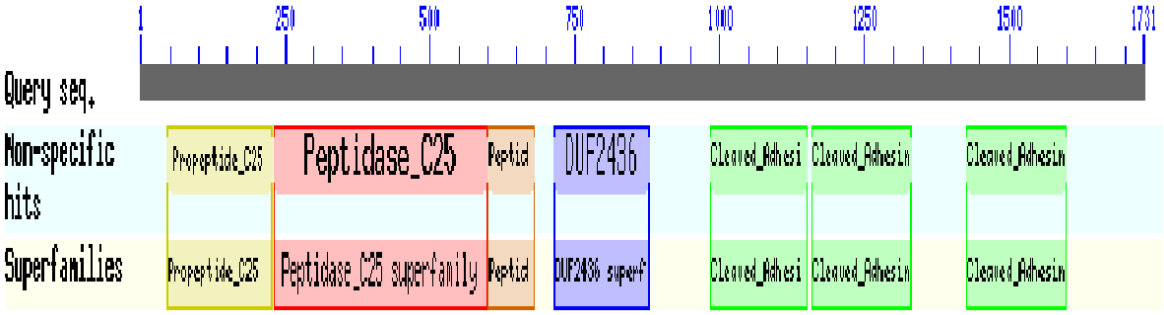
RESULTS

A. Bioinformatical Analysis of *Kgp*

Gingipains have demonstrated an integral role in *P. gingivalis* hemoprotein utilization. *Kgp* is a multidomain polypeptide composed of a signal peptide, pre-propeptide, protease domain, and hemagglutinin/adhesion domain. NCBI protein blast search reveals the pro-peptide domain is found at the N terminal end of the members of the C25 peptidase family. The cleaved adhesion domain is found in a group of hemagglutinins and peptidases, that form in *Porphyromonas gingivalis* from components of the major extracellular virulence complex RgpA-Kgp - a mixture of proteinases and adhesins. These domains are cleaved from the original polyprotein and form part of the adhesins.

Figure 10. NCBI Protein Blast Results

Kgp is a multidomain polypeptide composed of a signal peptide, pre-propeptide, protease domain, and hemagglutinin/adhesion domain.


1
250
500
750
1000
1250
1500
1731
Query seq.
Non-specific hits
Superfamilies
Propeptide_C25
Peptidase_C25
Peptid
DUF2436
Cleaved_Adhesi
Cleaved_Adhesin
Propeptide_C25
Peptidase_C25 superfamily
Peptid
DUF2436 superf
Cleaved_Adhesi
Cleaved_Adhesin

B. Mutant Construction

The *kgp* gene fragment was excised with *Hind*III and inserted into pUC19. The *erm*F-*erm*AM gene was isolated from pVA2198 vector. The recombinant pUC19-*kgp* and *erm*F-*erm*AM, was transformed into Shot $_{R}$ Top 10 cells (Invitrogen). The transformation was carried out in accordance with the Invitrogen protocol. The transformation mix as then plated on LB agar plates, supplemented with erythromycin (300 µg/ml), which served to act as an antibiotic screen. Once the colonies were verified, DNA was isolated and electroporated (BioRad®) into *P. gingivalis* electrocompetent cells. The *kgp* gene was interrupted with the plasmid giving a band size of approximately 5kb (See Fig. 12).

Figure 11. pUC19 Vector Schematic

pUC19 is a commonly used *E.* coli plasmid cloning vector. It is double stranded and contains a multiple cloning site polylinker, which contains 13 different restriction endonucleases. Abstracted from (Yanisch-Perron, C., Vieira, J. and Messing, J. (1985) Gene 33, 103-119).

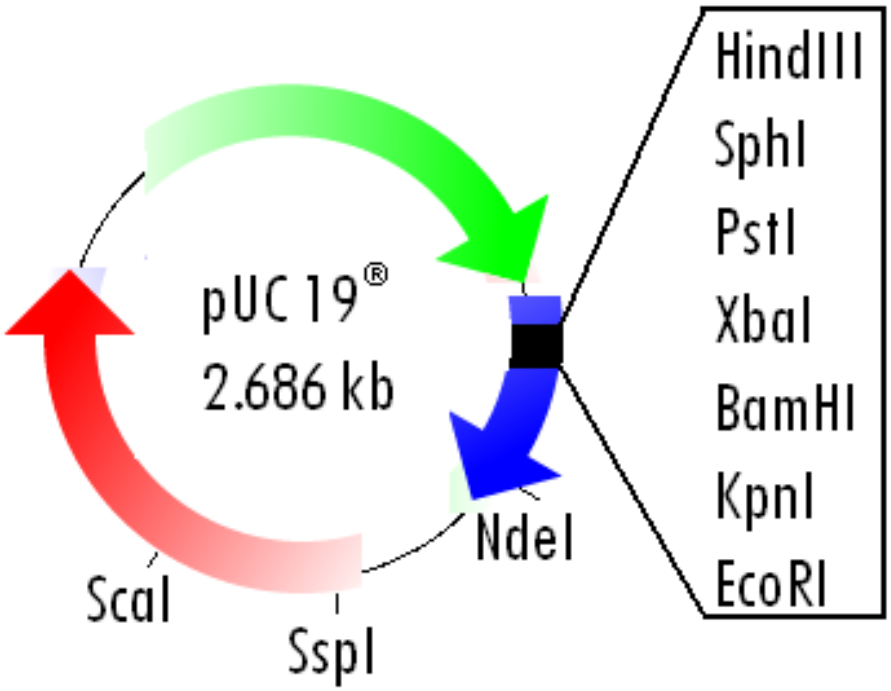

pUC 19®
2.686 kb
HindIII
SphI
PstI
XbaI
BamHI
KpnI
EcoRI
NdeI
ScaI
SspI

Figure 12. Allelic Exchange Mutant Construction

The allelic-exchange mutation of the k*gp* gene was made by ligating the *erm*F-*erm*AM cassette into the *kpn*I-*Bam*H1-cleaved pUC19 vector. This construct was used to electroporate *P. gingivalis* W83. As shown the entire recombinant plasmid is inserted into the *kgp* gene by single homologous recombination involving the 0.45Kb *kgp* sequence.

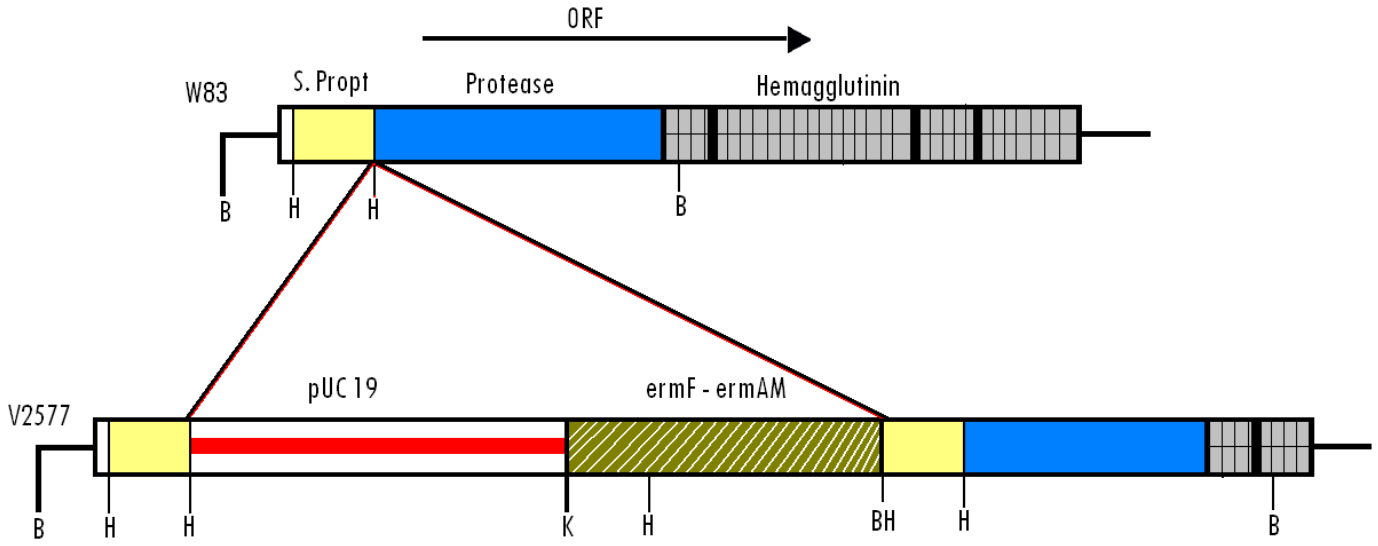

ORF
W83
S. Propt
Protease
Hemagglutinin
B
H
H
B
V2577
pUC 19
ermF - ermAM
B
H
H
K
H
BH
H
B

Figure 13: Hemoglobinase Activity

Illustration of amino acid sequence in human alpha and beta hemoglobin chains. The Lysine residues are indicated in boldface. Amino acid sequences of peptides demonstrated by ESI-FTICR spectroscopy are shaded. Image abstracted from Dr. Janina P. Lewis [38].

Hemoglobin Alpha Chain

VLSPADKTNVKAAWGKVGAHAGEYGAEALERMFLSFPTTKTYFPHFDLSHGSAQVKGHGKKVADALTNA
VAHDDMPNALSALSDLHAHKLRVDPVNFKLLSCHLLVTLAAHPAEFTPVHASDKFLASVTVLTSKYR

Hemoglobin Beta Chain

VHLTPEEKSAVTALWGKVNVDEVGGEALGRLLVVYPWTQRFFESFGDLSTPDAVMGNPKVKAHGKKVLGAFSD
GLAHDNLKGTFATSELHCDKLHVDEPENFRLLGNVLVCVLAHHFGKEFTPPVQAAYQKVVAGVANALAHKYH

C. Pigmentation

The formation of black pigmentation of *P. gingivalis* grown on blood agar plates is the result of an accumulation of iron protoporphyrin IX (FePPIX), which was derived from erythrocytes present in the growth medium (Figure 10). Abolishing the activity of the Lysine-X-specific protease, Kgp, resulted in the lack of pigmentation of *P. gingivalis.* Therefore, the Kgp deficient mutants appear white in color, while the wild type *P. gingivalis* retains its black pigmentation. Proteases are essential for generating free amino acids and peptides, which in turn form the main carbons and nitrogen source for eventual growth of the organism. Additionally, Okamoto et al. (1996) reported that Lys-X protease activity was reduced approximately 20-30% of levels in the wild-type parent strain. This finding was further confirmed by an insertional mutant of Kgp (IS195) in strain W83, which is associated with reduced Arg-X activity (Lewis & Macrina, 1999).

Our results confirm that KGP is involved in the acquisition and perhaps storage of hemin. This is further supported by Nakayama et al, stating that a hemin-binding peptide is intragenically encoded by *kgp* and this might function as part of the hemin storage mechanism at the cell surface. Additionally, this is also supported by Dr. Lewis et at, who demonstrated that the enzyme is an efficient hemoglobinase and hence may serve to release protein-bound hemin into the extracellular environment.

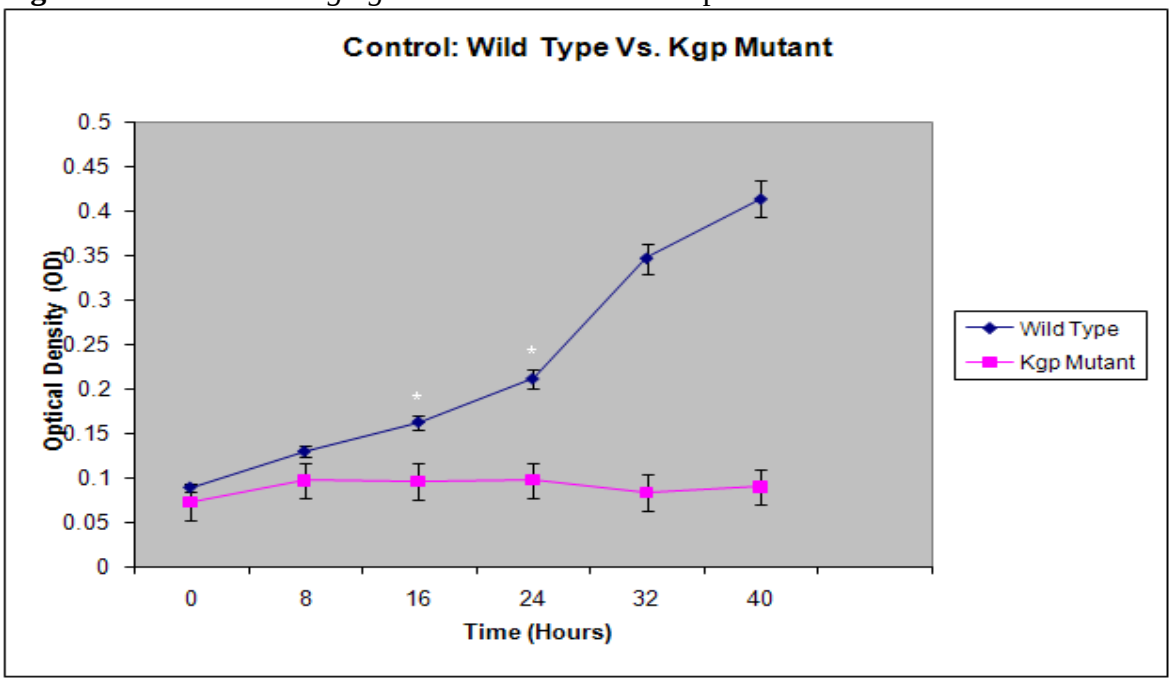
Figure 13. *Kgp* Vs. Wild Type Pigmentation

This illustrates the accumulation of black pigment by *P. gingivalis* strains grown on blood agar. *P. gingivalis* strains were inoculated in BHI medium and then streaked onto blood agar medium. They were incubated for 5 days anaerobically at 37°C followed by one week anaerobically at room temperature. Two phenotypes are shown; the wild type and the kgp mutant. The black-pigmented growth was formed by the wild-type strain, W83 (on left side). The mutation abolishing the Lys-gingipain activity (V2577) resulted in loss of pigmentation, represented by the white, non-pigmented growth (on right side).

D. Growth Study Analysis

The graphs in figures 15-19 below, illustrate the growth of wild-type W83 vs the *kgp* mutant strains at 8 hour time intervals, measured at 660nm (OD_{660}). The study was performed under various hemin supplemented conditions. Initially each of the strains was deprived of hemin, by inoculation in mycoplasma medium. The wild type required 4-5 passages, while the mutant strain only required 3 passages. These hemin depleted conditions served to inoculate basal media with a range of concentrations of a number of supplements including: hemin (750nM - 0.9nM), PPIX (750 nM – 0.9 nM), bovine hemoglobin + BSA (1nM – 1.5 x 10^{-2} nM hemoglobin equivalent), bovine hemoglobin-haptoglobin complex (1nM – 1.5 x 10^{-2} nM hemoglobin equivalent). Hemoglobin haptoglobin complex was constructed from mixing hemoglobin with haptoglobin in a 1:2 ratio (wt/wt) for a period of 30 minutes, at room temperature. The concentrations of hemoglobin-haptoglobin and hemin-albumin were each, a minimum of 50% saturated. 1mM of TLCK was included in the growth studies in order to prevent the degradation of hemoproteins by P. gingivalis protease inhibitor N-alpha-p-tosl-L-lysine chloromethl ketone.

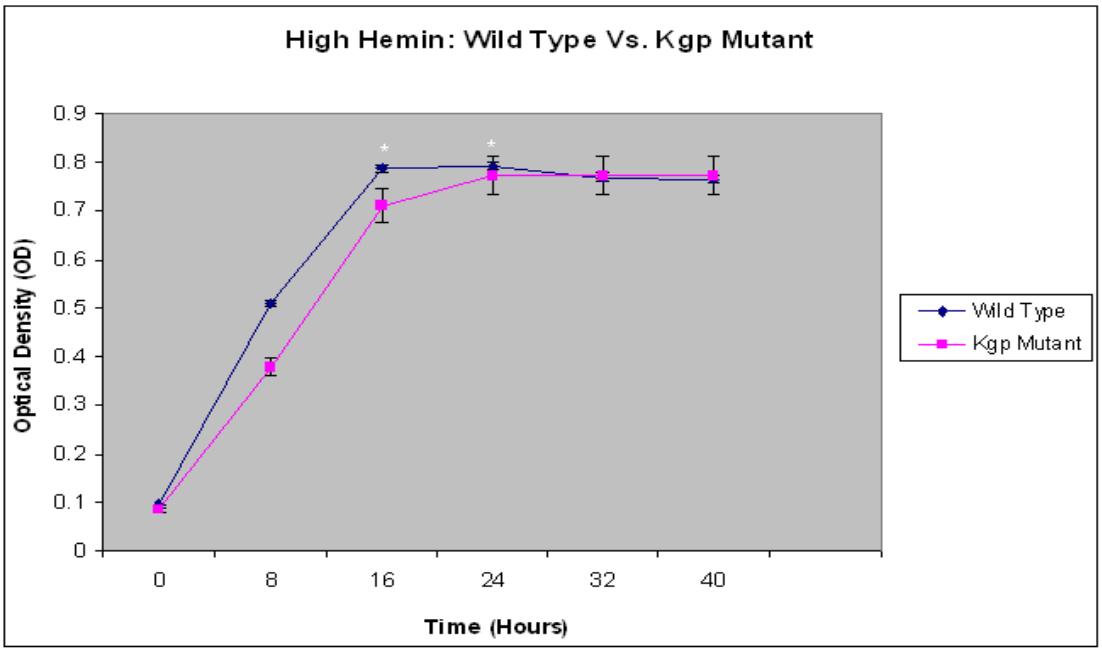
Figure 15: Growth of *P. gingivalis* strains in media depleted of hemin



White asterisk at 24 hours: P= 0.00056 White asterisk at 32 hours: P=0.00012

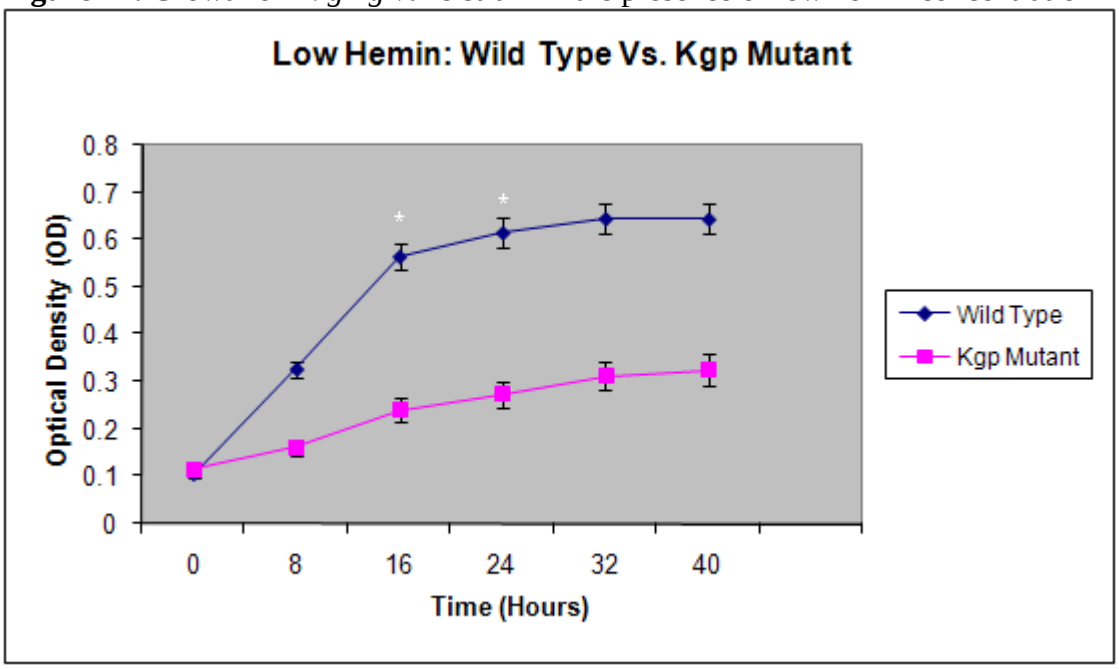
The control experiment, which lacked a hemin source, produced desirable results in the mutant as well as the wild type, with the exception of a slight increase after a 16 hour period. As expected, both the wild type and mutant show significantly reduced growth in media devoid of hemin sources. The mutant begins to show reduced growth after the 16 incubation period, due to inadequate nutrient availability. These results indicate that bacterial survival for both the mutant and wild type is dependent upon nutrient availability; strains produced limited growth under nutrient void conditions.

Figure 16: Growth of *P. gingivalis* strain in the presence of high hemin concentration



White asterisk at 16 hours: P=0.00292 White asterisk at 24 hours: P=0.0016

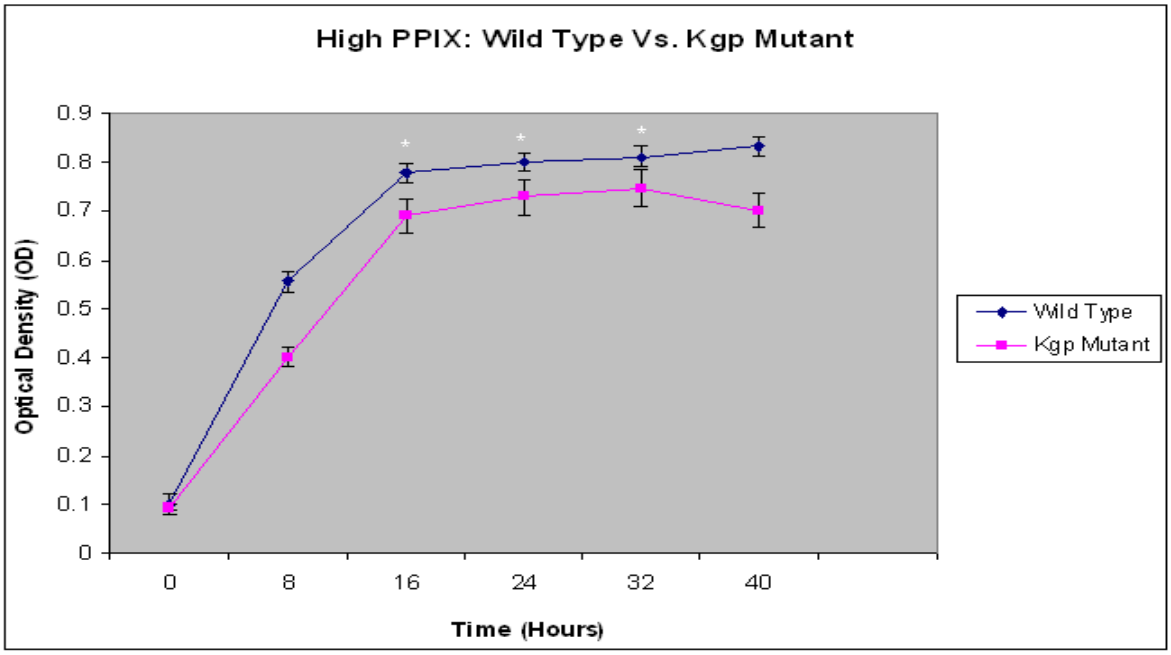
Figure 17: Growth of *P. gingivalis* strain in the presence of low hemin concentration



White asterisk at 16 hours: P= 0.000031 White asterisk at 24 hours: P=0.000124

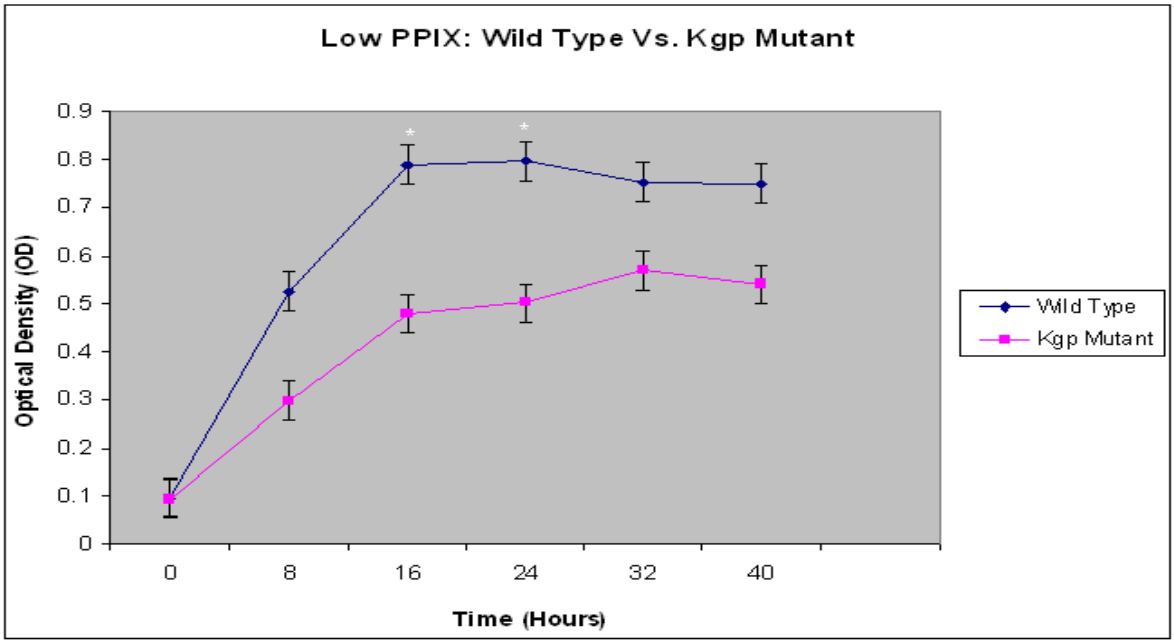
When using hemin as a growth source, both wild type and *Kgp* mutant strains were able to grow under conditions of high hemin, obtaining an OD_{660} of approximately 0.77, an increase of roughly six fold compared to hemin depleted conditions. Significant growth was expected, since hemin, the iron containing protoporphyrin is an essential nutrient for growth. However, an unexpected occurrence was the growth of the *Kgp* mutant under conditions of low hemin. After the 40 inoculation the wild type achieved an OD_{660} of approximately 0.65, while the *Kgp* mutant has an OD_{660} of about 0.35. This indicates that the *Kgp* mutant does contain the necessary machinery to utilize hemin.

Figure 18: Growth of *P. gingivalis* strain in the presence of high PPIX concentrations



White asterisk at 16 hours: P= 0.00218, 24 hours: P= 0.00088, 32 hours: P= 0.00099

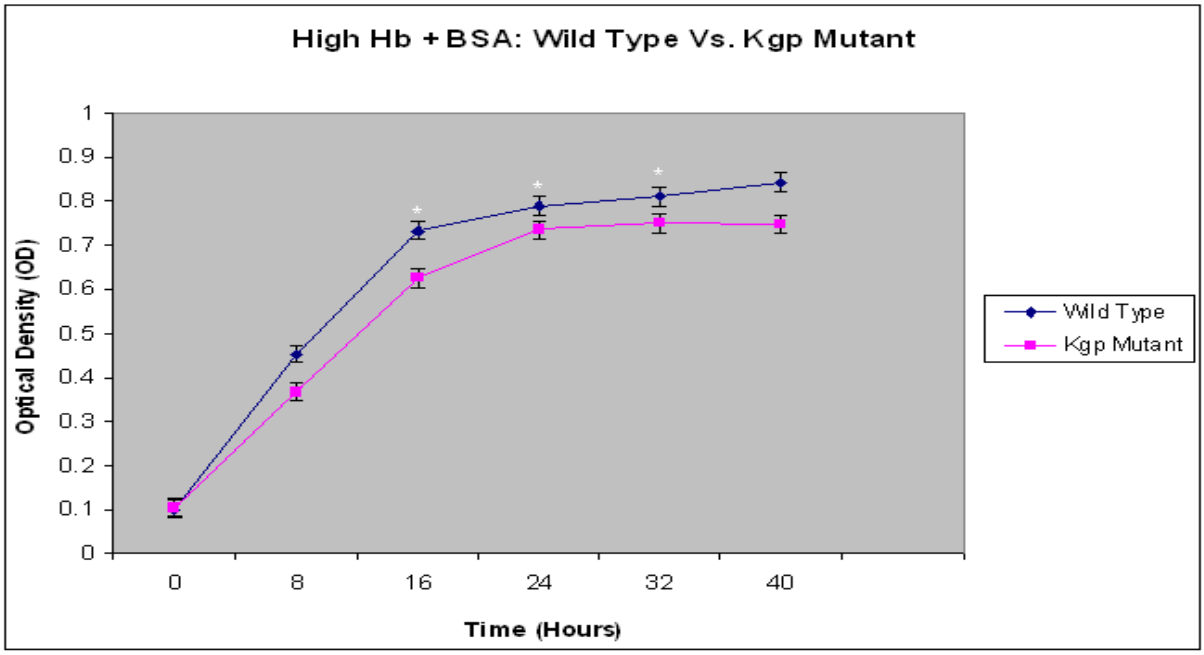
Figure 19: Growth of *P. gingivalis* strain in the presence of low PPIX concentrations



White asterisk at 16 hours: P= 0.0000514 White asterisk at 24 hours: P=0.000108

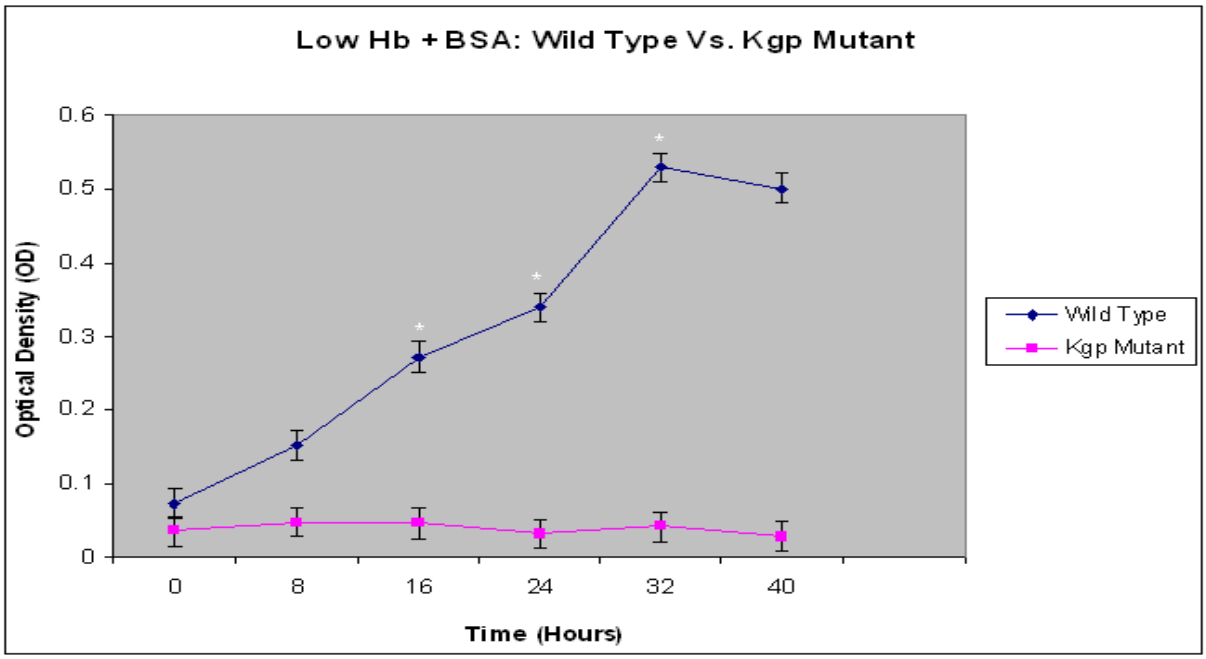
Protoporphyrin IX, is a direct precursor to heme, that combines with ferrous iron to form the heme of hemoglobin and with ferric iron to form the prosthetic groups of substances such as myoglobin. PPIX proved to be the best growth source for both the wild type and kgp mutant strain, under both high and low conditions (Figure 16). Under conditions of high PPIX there is a drastic spike in growth until the 16^{th} hour of observation, reaching an OD_{660} of approximately 0.80 in the wild type and 0.70 in the kgp mutant. After the 16^{th} hour these values begin to taper off, resulting in a nearly similar final OD_{660} of wild type 0.78, and mutant 0.70. Under low concentrations of PPIX both the wild type and kgp mutant have comparable growth initially, however after the first 8 hours the wild type has a more drastic increase in growth, (OD_{660} 0.78) compared to kgp mutant (OD_{660} 0.48). This difference may be due to trace amounts of hemin present in the wild type, despite the multiple passages. After 40 hours of incubation the wild type strain attained an OD_{660} of 0.75, which is a six fold difference from the control. On the other hand, after 40 hour incubation, the kgp mutant achieved an OD_{660} of 0.55, which is six times greater than the control and nearly two times greater growth than conditions of low hemin.

Figure 20: Growth of *P. gingivalis* in the presence of high Hb + BSA concentrations



White asterisk at 16 hours: P= 0.00266, 24 hours: P= 0.000586, 32 hours: P= 0.00106

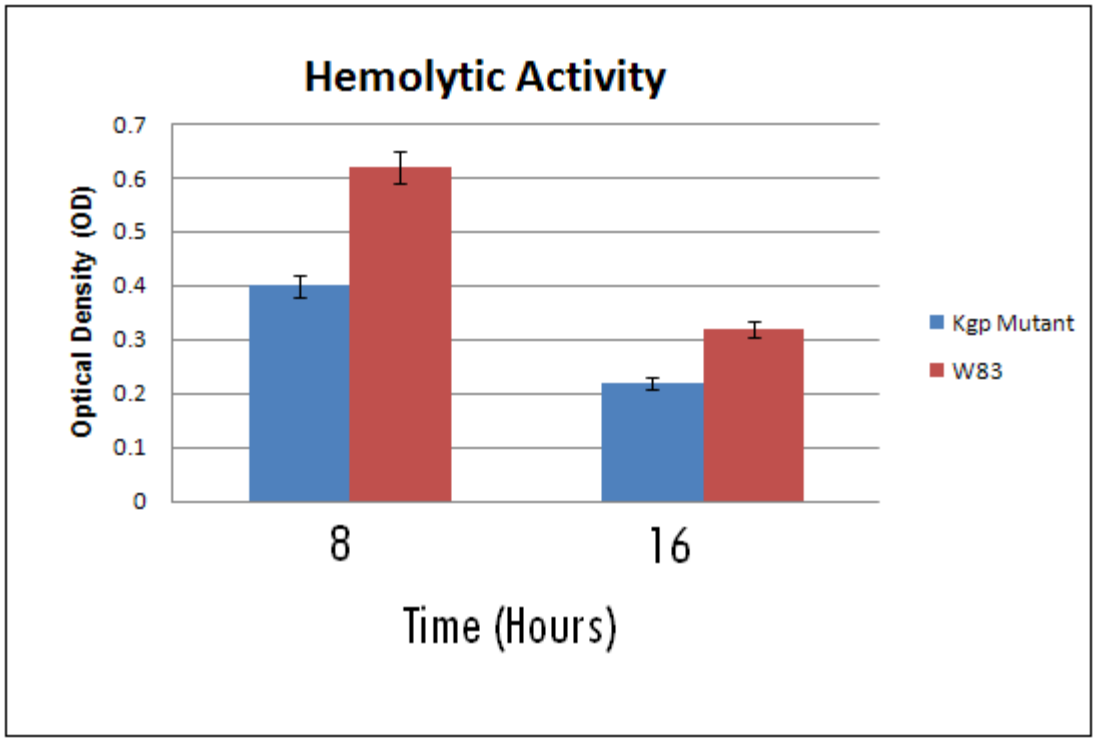
Figure 21: Growth of *P. gingivalis* in the presence of low Hb + BSA concentrations



White asterisk at 16 hours: P= 0.00028, 24 hours: P= 0.000311, 32 hours: P= 0.00065

Growth under conditions of Hb + BSA showed the greatest difference between the wild type and mutant strain under low concentration (Figure 17). Hb or hemoglobin is the iron-containing oxygen transport metalloprotein in red blood cells. BSA or bovine serum albumin is used as a nutrient in cell culture as well as for its stabilization properties. Strains of both wild type and kgp mutant grew well under high concentrations of Hb + BSA, achieving OD_{660} values of 0.843 and 0.74 respectively. The growth contrast is present under conditions of low concentrations of Hb +BSA, in which the kgp mutant has practically no growth. Under low concentrations of hemoglobin and BSA the wild type attains an OD_{660} of 0.501 and the kgp mutant 0.028. These results clearly indicate that the kgp mutant lacks hemoglobinase machinery, which would be required to breakdown the hemoglobin in order to acquire hemin. This finding is also supported by Shi et al., indicating that since *Kgp* mutants grow very poorly in medium containing albumin as the sole source of carbon and nitrogen, there is reasonable evidence that *Kgp* has an important nutritional role.

Figure 22. *Kgp* vs Wild Type W83 hemoglobinase activity
Hemolysis of erythrocytes was monitored by the change of absorbance for red pigment resulting from release of hemoglobin from the lysed cells at 520 nm in assays using 36 mg of extracellular vesicles or 50 ml of cells. The Hemolytic activity of the *Kgp*-deficient mutant was significantly lower than the haemolytic activity of the wild-type strain. Abstracted from J.P Lewis, et al. [39]


Hemolytic Activity
Optical Density (OD)
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
8
16
Time (Hours)
Kgp Mutant
W83

A. Microarray Analysis

Preliminary results indicate a change in the expression of key enzymes and proteins required for hemin uptake, iron storage, electron transport and oxidative stress. Table 6 depicts the up-regulated genes present in the *kgp* mutant, compared to the wild type W83; Cy-5 is the labelled mutant, while Cy-3 is the labelled wild type. There was a significant up regulation in both thioredoxin and superoxide dismutase, which is important since they are both involved in oxidative stress mechanisms.

Thioredoxin was significantly unregulated in the *Kgp* mutant, with a Cy-5/Cy-3 ratio of 3.6. Thioredoxin are small disulfide-containing redox proteins, which serve as a general protein disulfide oxidoreductase. It interacts with a broad range of proteins by a redox mechanism based on reversible oxidation of two cysteine thiol groups to a disulfide, accompanied by the transfer of two electrons and two protons. In the NADPH-dependent protein disulphide reduction, thioredoxin reductase catalyses the reduction of oxidised thioredoxin (trx) by NADPH using FAD and its redox-active disulphide. Superoxide dismutase is an enzyme that removes the superoxide radical, by disproportionating it to O_2 and hydrogen peroxide (H_2O_2). As such, they are an important antioxidant defense in nearly all cells exposed to oxygen.

Genes involved in metabolism such as L-aspartate oxidase and glutamate dehydrogenase were upregulated in the *kgp* mutant strain. L-aspartate oxidase contained a mutant: wild type ratio of 1.02, while glutamate dehydrogenase contained a mutant: wild type ratio of 0.89. L-Aspartate oxidase is a monomeric flavoprotein, involved in

catalyzing the first step in the de novo biosynthetic pathway for pyridine nucleotide formation.

The mutant strain exhibited an up regulation in the expression of genes involved in iron uptake and transport such as hemin-binding protein FetB, rubrerythrin, and ferritin. Hemin-binding protein FetB contained a mutant: wild type ratio of 0.76. This is in conjunction with Olczak et al, stating that FetB is involved in iron hemin transport and may function to assimilate hemin. Rubrerythrin contained a mutant: wild type ratio of 0.604. It is a fusion protein containing an N-terminal di-iron binding domain and a C - terminal domain homologous to rubredoxin. Other studies have also shown that it has been implicated in oxidative stress protection in anaerobic bacteria [55], by a proposed mechanism of catalytic reduction of intracellular hydrogen peroxide. Additionally, there was a significant up-regulation in ferritin, mutant: wild type ratio 0.794, one of the intracellular iron-storage proteins also thought to contribute to the protection of organisms against oxidative stress generated by intracellular free iron.

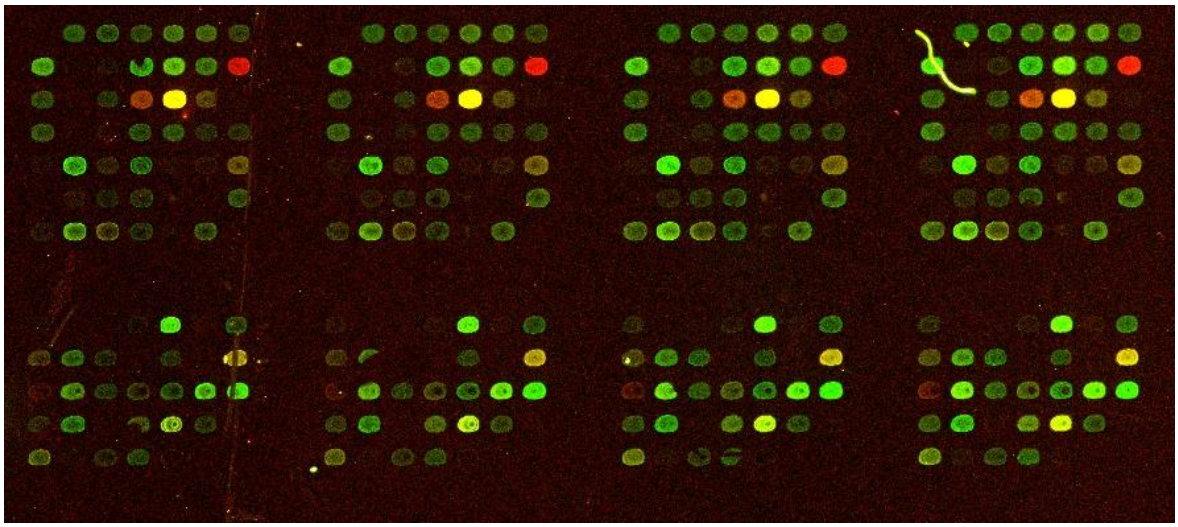
Lipoprotein RagB contained a 1.76 fold increase in the *kgp* mutant. A distinguishing characteristics and putative virulence property of *Porphyromonas gingivalis* is the ability to agglutinate erythrocytes. In the *kgp* mutant strain, we have up-regulation of hemagglutinin genes; Hag A, Hag C, and Hag E. This is important since hemagglutinins function as adhesions, bind RBC's, therefore playin a role in acquisition of hemin and are required for the virulence of *P. gingivalis*. According to a study performed

by Dr. Song et al. HagB is involved in the adherence of *P. gingivalis* to human primary endothelial cells.

Approximately 25% of the genes up-regulated encoded for hypothetical proteins, whose functions are not yet determined (data now shown). These results indicate that *kgp* does not exclusively regulate genes based on internal conditions, but must also regulate genes dependent on other signalling cues.

Figure 23. Microarray Slide

Change in gene expression of *kgp* mutant as compared to wild type W83. The wile type strain, containing the *kgp* gene was labeled with Cy-5, while the mutant strain, lacking lysine-gingipain activity (*kgp*) was labeled with Cy-3. Green and red signals indicate a change in gene expression, up regulation and down regulation, respectively. Yellow signals indicate a lack of change in gene expression.



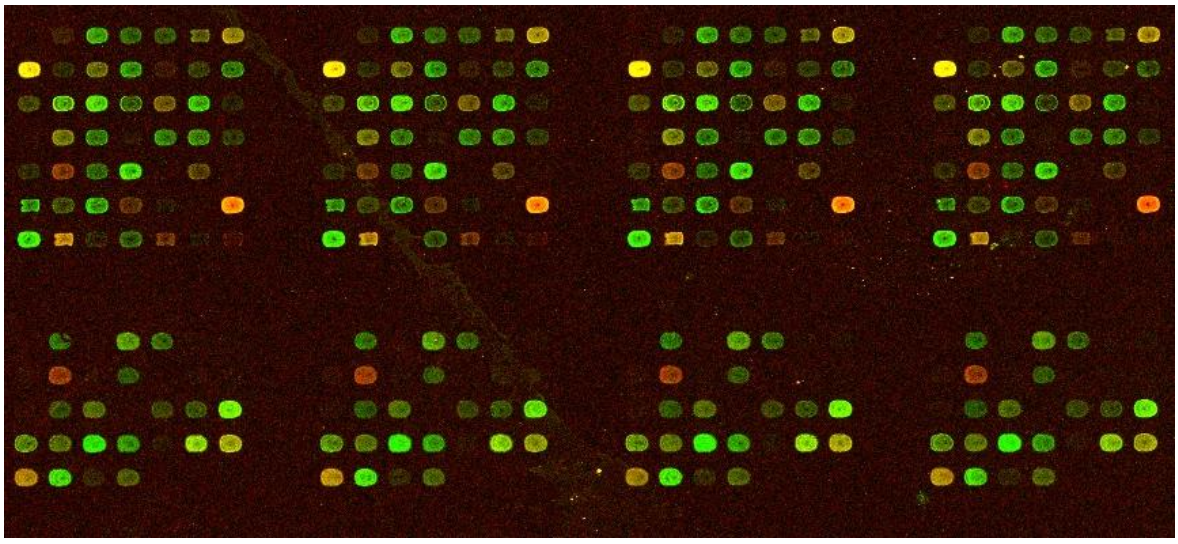


Table 6: Up-regulated Genes in Kgp Mutant

Gene ID [1]	Common Name [2]	M	Ratio[3] Cy5/Cy3	P	Repeat [4]
PG0275	thioredoxin family protein	1.614766	3.062620	0.000002	11
PG1545	superoxide dismutase, Fe-Mn	0.989946	1.986111	0.092923	12
PG1576	L-aspartate oxidase	1.015047	2.020969	0.062765	14
PG1044	iron dependent repressor, putative	0.853005	1.806259	0.007800	14
PG1975	hemagglutinin protein HagC	1.026027	2.036408	0.006756	16
PG0186	lipoprotein RagB	0.816891	1.761606	0.014340	16
PG0669	heme-binding protein FetB	0.746001	1.677138	0.000892	16
PG1837	hemagglutinin protein HagA	1.056220	2.079477	0.527206	20
PG1232	glutamate dehydrogenase, NAD-specific	0.888167	1.850823	0.000025	20
PG2024	hemagglutinin protein HagE	0.795668	1.735880	0.002137	20
PG1286	ferritin	0.793505	1.733280	0.071609	20
PG0195	rubrerythrin	0.604030	1.519956	0.115180	20

[1] Gene ID according to JCVI (formerly TIGR)
[2] Common Name
[3] Ratio of Cy-5/Cy-3 fluorescently labeled samples
[4] Number of spots on the PG slide used for analysis

Table 7: Down-regulated Genes in Kgp Mutant

Gene ID [1]	Common Name [2]	M	Ratio [3] Cy-5/Cy-3	P [4]	Repeat [5]
PG1897	thiamine pyrophosphokinase	-1.129068	0.457211	0.023672	12
PG0433	tetrapyrrole methylase family protein	-0.979430	0.507180	0.012752	12
PG1820	cytochrome c nitrite reductase, catalytic subunit NrfA	-0.648357	0.638006	0.511460	12
PG1551	hmuY protein	0.972158	0.509743	0.016648	13
PG1844	hemagglutinin protein HagD	-2.193055	0.218688	0.000010	14
PG1225	ABC transporter, ATP-binding protein	-0.744573	0.596845	0.066400	14
PG0801	polyA polymerase family protein	-0.511944	0.701277	0.211212	16
PG1072	MutS family protein	-0.467543	0.723195	0.416819	16
PG2222	acyltransferase, HtrB-MsbB family	-0.561304	0.677689	0.172990	17
PG2035	tRNA (guanine-N1)-methyltransferase	-0.533358	0.690945	0.218264	18
PG1996	deoxyribose-phosphate aldolase	-0.487456	0.713282	0.011260	18
PG1368	glucose-6-phosphate isomerase	-0.498831	0.707680	0.001877	19
PG0398	recF protein	-0.492564	0.710761	0.064281	19
PG1858	flavodoxin	-0.457599	0.728197	0.006664	19
PG1213	ribonuclease H	-0.666258	0.630139	0.146274	20
PG1156	S4 domain protein	-0.618953	0.651144	0.062204	20
PG0635	ribosomal protein L11 methyltransferase	-0.597727	0.660794	0.104999	20
PG0292	chromate transport protein, putative, authentic point mutation	-0.565199	0.675862	0.368481	20
PG0777	electron transfer flavoprotein, beta subunit	-0.529416	0.692835	0.015037	20
PG0910	FHA domain protein	-0.499708	0.707250	0.175153	20
PG1078	electron transfer flavoprotein, alpha subunit	-0.466636	0.723650	0.579111	20
PG0802	alpha keto acid dehydrogenase complex, E3 component, lipoamide dehydrogenase	-0.437579	0.738373	0.223340	20
PG1346	glycosyl transferase, group 1 family protein	-0.421828	0.746478	0.227681	20
PG0646	iron compound ABC transporter, ATP-binding protein	-0.405362	0.755047	0.008104	20
PG1346	glycosyl transferase, group 1 family protein	-0.421828	0.746478	0.227681	20

[1] Gene ID according to JCVI (formerly TIGR)
[2] Common Name
[3] Ratio of Cy-5/Cy-3 fluorescently labeled samples
[4] Number of spots on the PG slide used for analysis

Thiamine pyrophosphokinase showed a significant reduction in expression, with a mutant: wild type ratio of about 0.46. It functions to transfer a pyrophosphate group from a nucleoside triphosphate, such as ATP, to the hydroxyl group of thiamin to produce thiamin pyrophosphate.

There is a significant decrease in expression of the following genes: Hmu Y, glycosyltransferase, and immunoreactive proteins. HmuY contains a Cy-5/Cy-3 ratio of approximately 0.5097. It is a putative heme-binding lipoprotein that is located in the outer membrane. It is part of an operon together with a gene encoding an outer-membrane hemin utilization receptor (HmuR). Under normal conditions the HmuY protein is present as a homodimer, however in the presence of hemin in may form tetramers. According to a study by Wojciechowski et al. the function of HmuY occurs in conjuction with HmuR, an outer-membrane heme transporter. This is further supported by Dr.Lewis et. Al, stating that HmuY and HmuR are likely to function together as a two-component hemin receptor, however haemin binding to HmuY is specific.

According to an article by Lan et al, reverse polymerase chain reaction (PCR) analysis indicates that hagD expression is regulated by hemin concentration. Our experimental results indicate a mutant: wild type ratio of approximately 0.2187. We see reduced expression of hagD, due to the reduced hemin concentration, which is in turn a result of P.*gingivalis's* inability to acquire and utilize hemin.

DISCUSSION

In order to examine if inactivation of the gingipain function would affect the ability of *P. gingivalis* to utilize heme for growth, a Lysine-gingipain deficient model, or Kgp mutant was created. The existence of nonpigmented variants of *P. gingivalis* has been found, but the mechanism of pigment accumulation from erythrocytes has not been previously explained. Other work has suggested that pigmentation may be related to Kgp activity, we have demonstrated that the black pigmentation of *P. gingivalis* colonies observed on blood agar plates is dependent on Kgp activity. The results indicate that growth was adversely affected during early phases as well as late exponential and stationary growth phases. This suggests that Lysine gingipain is required for growth initially and also during mid-late exponential growth phases.

Porphyromonas gingivalis is a major etiological agent in the initiation as well as progression of periodontal disease. Its virulence is made possible by intricate gene regulation, particularly those involved in hemin acquisition and storage. The observations from our experimentation lead us to conclude that *Kgp* production is necessary for the provision of large amounts of hemin from hemoglobin. The hemin accumulates on the cell surface, ultimately leading to pigment formation [44]. However, other factors, including surface binding components, may be involved in the pigmentation process. Our results indicate that the Lys-gingipain is in fact a hemoglobinase. This observation suggests that this enzyme plays a role in heme and iron acquisition by efficient extraction of iron

protoporphyrin from hemoglobin. This is in turn accumulated on the bacterial cell surface; black pigmentation.

The main source of hemin in gingival crevicular fluid is hemoglobin derived from lysed erythrocytes. The concentrations of hemoglobin may vary depending on the degree of bleeding associated with the periodontal tissue destruction. Hemoglobin, however, is not available to P. *gingivalis* as it is rapidly and irreversibly bound by the serum protein, haptoglobin. In order to overcome the scavenging effect of haptoglobin P. *gingivalis* must have effective mechanisms for removal of hemin from the hemoglobin-haptoglobin complex.

In order to examine the importance of individual hemin uptake loci in the utilization of hemin and various hemoproteins, we have monitored the ability of the P. *gingivalis* parental and mutant strains devoid of various hemin uptake systems, to use hemin and hemoproteins: hemoglobin, hemoglobin-haptoglobin, heme-hemopexin, and hemin-albumin. Our results indicate that hemin is required for growth of both the wild type and *kgp* mutant strains. We attribute the slight discrepancy in growth between the two strains under conditions void of hemin, to trace amounts of hemin being present in the wild type, despite multiple passages. After the 16^{th} hour of inoculation, we notice slight growth in the wild type strain, while the mutant strain demonstrates a decrease in growth. Trace amounts of hemin may have allowed the wild type to grow slightly, while the mutant strain showed a decrease in growth due to unavailable nutrient source.

It is important to note that we do see growth in our mutant under conditions of low hemin, which further proves that although the *Kgp* mutant lacks hemoglobinase activity, it still contains contain the necessary machinery to utilize hemin. This is also supported by reduced growth under conditions of low Hb + BSA, under which hemoglobin does not appear to be broken down and utilized by the *Kgp* mutant strain. *Kgp* is capable of hydrolyzing protein substrates such as casein, hemoglobin, human IgG, and IgA. The ability of *Kgp* to hydrolyze hemoglobin is due to the alpha chain containing 11-Lys residues and the beta chain containing 10, which in turn affects the accumulation of iron protoporphyrin IX on the bacterial surface. Additionally, *kgp* is also a potent fibrinogen-digesting enzyme, as supported by Scott et al. This de-regulates the clotting cascade by causing a rapid decrease in clottable fibrinogen, resulting in the inhibition of fibrin polymerization by cleavage products.

Microarray results indicate a significant up-regulation of oxidative stress factors in the mutant strain, which is a key oxygen defense enzyme, responsible for breaking down molecular oxygen into hydrogen peroxide. The major outer-membrane proteins RagA and RagB of *Porphyromonas gingivalis* are considered to form a receptor complex that is functionally linked to TonB. RagA and RagB were originally identified as immunodominant surface antigens recognized by sera from periodontitis patients [44]. It has been reported that *ragB*, which is located adjacent to and downstream of *ragA*, is co-transcribed with *ragA* as a polycistronic message. According to Bonass *et al*. RagA has homology to TonB-linked outer-membrane receptors, which are involved in the recognition

and active transport of specific external ligands by a wide range of Gram-negative species [44].

Additionally the down regulation of the glycosyltransferase gene in our mutant strain leads us to believe that it may play a role in pigmentation, due to its role in the transfer of monosacharaide units. It has been associated with *P. gingivalis* auto aggregation as well as attachment to epithelial cells, suggesting that it might have an important role in the pathogenicity of *P. gingivalis*, possibly by adhesion regulation. It is speculated that pigmentation may be a form of sequestering and detoxifying of hemin, since it has been shown to have antibacterial effects at high concentrations. This is interesting, as the deteriorating effect of hemin on bacteria can be reduced in the presence of thiol reagents. We see an upregulation of these reagents in our *Kgp* mutant, which act to bind free oxygen and thereby reduce the hemin-mediated oxygen radical cell damage. Therefore, interference with mechanisms involved in accumulation of black pigmentation may be significant in controlling the pathogenic potential of *P. gingivalis.*

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