Characterization of a putative TonB deficient Porphyromonas gingivalis mutant

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Characterization of a putative TonB deficient Porphyromonas gingivalis mutant

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

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<td>µL</td>
<td>Micro liter</td>
</tr>
<tr>
<td>°C</td>
<td>Degree Celsius</td>
</tr>
<tr>
<td>bp</td>
<td>Base pairs</td>
</tr>
<tr>
<td>cDNA</td>
<td>Complementary DNA</td>
</tr>
<tr>
<td>DNA</td>
<td>Deoxyribonucleic acid</td>
</tr>
<tr>
<td>DNase</td>
<td>Deoxyribonuclease</td>
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<tr>
<td><em>E. coli</em></td>
<td><em>Escherichia coli</em></td>
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<tr>
<td>kb</td>
<td>Kilobase pairs</td>
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<tr>
<td>LPS</td>
<td>Lipopolysaccharide</td>
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<tr>
<td>OD</td>
<td>Optical Density</td>
</tr>
<tr>
<td>BHI</td>
<td>Brain-heart infusion</td>
</tr>
<tr>
<td>nm</td>
<td>Nanometer</td>
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<tr>
<td>W83</td>
<td>Wild type <em>P. gingivalis</em> W83 strain</td>
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<tr>
<td>V3128</td>
<td>Mutant <em>P. gingivalis</em> with disrupted PG0785 gene</td>
</tr>
<tr>
<td>ml</td>
<td>Milliliter</td>
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<tr>
<td>AA</td>
<td>Amino Acid</td>
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CHARACTERIZATION OF A PUTATIVE TONB DEFICIENT PORPHYROMONAS GINGIVALIS MUTANT

By: Soheil Rostami, B.S.

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

Virginia Commonwealth University, 2014

Major Director: Janina P. Lewis, Ph.D., Philips Institute of Oral Health Research

Porphyromonas gingivalis is one of the major bacterial pathogens responsible for the initiation and progression of periodontal disease. The bacterium requires hemin uptake for its growth and has developed sophisticated mechanisms to extract hemin from hemin containing proteins in the oral cavity. Hemin first binds to receptors on the surface of P. gingivalis and is then taken up in an energy dependent manner. TonB is an inner membrane bound protein that spans the periplasm and is believed to be involved in the passage of hemin through the double membrane of P. gingivalis. However, the TonB protein in P. gingivalis is yet to be identified. We identified PG0785 as a possible P. gingivalis TonB based on its bioinformatics data showing similarity to other known TonB proteins. We generated a P. gingivalis mutant lacking a functional PG0785 and then characterized the mutant to determine the role of PG0785. We performed metal content and protease assays, virulence studies and transcriptional and translational analysis of our mutant and wild type P. gingivalis strains.

Phenotypic studies showed that the mutant cannot accumulate hemin on its surface. The mutant has significantly lower levels of iron compared to wild type based
on metal content assays. The mutant also has significantly lower protease activity compared to the wild type. Virulence studies showed that the mutant interacted and invaded eukaryotic cells at much lower levels than the wild type. These results allowed us to speculate that PG0785 is very important in binding of hemin to surface of *P. gingivalis*. PG0785 also plays an important role in iron uptake, protease activity and virulence of *P. gingivalis*. Transcriptional and translational analyses have shown that numerous TonB related genes, metal uptake genes, hemin uptake genes and genes related to virulence have been differentially regulated in the mutant lacking a functional PG0785 gene compared to the wild type strain. In conclusion we believe that based on our results PG0785 is a putative *P. gingivalis* TonB protein that plays a significant role in the biology of *P. gingivalis*. 
CHAPTER 1: BACKGROUND AND SIGNIFICANCE

1.1 Introduction

*Porphyromonas gingivalis* is a Gram-negative, rod-shaped, anaerobic bacteria that belongs to the phylum Bacteriodetes. Biological studies have shown that *P. gingivalis* is one of the main etiological agents involved in initiation and progression of periodontal disease. Periodontal disease is one of the most common inflammatory diseases worldwide and is believed to affect as much as 35% of the US population. *P. gingivalis* contains many virulence factors that allow it to escape the host immune response and colonize the sub-gingival area and periodontal pockets. These virulence factors are also associated with the release of many pro-inflammatory cytokines which lead to the extensive inflammatory response seen during periodontal disease.

*P. gingivalis* requires iron for its growth and it mainly acquires iron in the form of hemin derived from hemoproteins present in the oral cavity. *P. gingivalis* contains proteases that degrade hemin containing proteins such as hemoglobin which leads to release of hemin. Hemin then binds to the surface of *P. gingivalis* and is then taken up in an energy dependent manner. The TonB/ExbB/ExbD complex is believed to play a major role in the passage of hemin through the double-membrane of *P. gingivalis*. The inner membrane of the bacterium generates a proton motive force (pmf) that is transferred via ExbB/ExbD to TonB. TonB spans the periplasm and interacts with outer-membrane receptors that have bound hemin. This interaction leads to the plug domain of these receptors to be removed from inside of the receptors which then allows for hemin to be taken up through the receptor.
1.2 Periodontal Disease

Periodontal disease is a chronic inflammatory disease that is characterized by an increased inflammatory response affecting the periodontium. The increased levels of cytokines and polymorphonuclear leukocyte (PMN) in gingival tissue lead to alveolar bone loss and destruction of connective tissue and periodontal ligament. Periodontal disease develops from pre-existing gingivitis, although not all gingivitis progresses to periodontal disease (Brown et al., 1989). Recently it’s been estimated that as many as 75% of Americans are affected by gingivitis and 35% have some form of periodontal disease (Amar et al., 2003; Albandar et al., 1999). Clinically a periodontal pocket of 4 mm or more is associated with periodontal disease and a pocket of 6 mm or more is associated with advanced or severe form of the disease (Elter et al., 2004; Hujoel et al., 2000).

The trigger for the initiation of disease is the accumulation of microorganisms in a biofilm known as plaque (Smalley et al., 2002). Initially it was believed that only gram-negative microorganisms contribute to periodontal disease. However, recent reports have show that gram-positive bacteria can also be cultured from diseased sites suggesting that both gram-negative and gram-positive bacteria contribute to the disease (Kumar et al., 2005). Over 700 bacterial species are present in the periodontal pockets but only around 10-20 bacterial species play a major role in pathogenesis of periodontal disease (Amar et al., 2003; Socransky and Haffajee, 1994). Some of these bacterial species include Porphyromonas gingivalis, Tannerella forsythia, Aggregatibacter actinomycetemcomitans, Prevotella intermedia, Prevotella melaninogenica and Fusobacterium nucleatum (Teles et al., 2013). The ‘red complex’ consists of
Porphyromonas gingivalis, Treponema denticola, and Tannerella forsythia which are thought to be three major periodontal pathogens (Holt et al., 2005). P. gingivalis has been detected in as much as 85% of disease sites and is frequently associated with severe periodontal disease (Yang et al., 2004). The major pathogenic bacteria associated with the disease have developed mechanisms to evade the host immune response which allows them to colonize and invade the gingival epithelial cells. They also contain mediators that lead to induction of an inflammatory response which can lead to connective tissue damage and alveolar bone loss (Mahanonda et al., 1991; Darveau et al., 1997).

Periodontal disease is associated with multiple risk factors that are thought to increase the probability of the disease. Some of these risk factors include the microbial plaque found in the periodontal pocket, increased age, smoking and systemic diseases such as diabetes mellitus (Horning et al., 1992; Stoltenberg et al., 1993, Oliver and Tervonen, 1993). It is also believed that conditions that affect the antibacterial defense mechanisms such as human immunodeficiency virus (HIV) and neutrophil disorders can also increase the risk of periodontal disease (Loesche et al., 2001). Periodontal disease has also been implicated as a risk factor for several systemic diseases. The increased inflammatory responses due to increased inflammatory cytokines and C-reactive species can increase the risk for coronary heart disease, preterm low birth weight, stroke and artheroclerosis (Bahekar et al., 2007; Lopez et al., 2002; Pussinen et al., 2004)
1.3 Porphyromonas gingivalis

*Porphyromonas gingivalis* is a Gram-negative, rod-shaped, anaerobic bacteria that belongs to the phylum Bacteroidetes (Shah and Collins, 1988). When grown on blood plates, the bacterial colonies initially display a white color, however, eventually the colonies become dark pigmented due to the accumulation of heme on their surface (Smalley et al. 1998). *P. gingivalis* is asaccharolytic due to its inability to break down sugars and carbohydrates. As a result the bacterium utilizes energy produced from proteolytic breakdown of various proteins. *P. gingivalis* is believed to be one of the main causative pathogens in initiation and progression of periodontal disease (Socransky and Haffajee, 1994). It belongs to a group of three species known as the “red complex”. This complex includes *Porphyromonas gingivalis, Treponema denticola* and *Tannerella forsythia*, all of which have been strongly associated with diseased sites during periodontal disease (Holt et al., 2005). These three pathogenic species can cause a change in the microbiota of the oral cavity and induce an inflammatory response that can lead to periodontal disease. As a result, *P. gingivalis* is believed to be a “keystone pathogen”. A “keystone pathogen” is pathogen that can remodel a normal microbiota into a disease-provoking microbiota which can lead to initiation of inflammatory diseases (Hajishengallis et al., 2012).

*P. gingivalis* is mainly found in the oral cavity where it can colonize the periodontal pocket and invade the gingival epithelial cells (Sandros et al., 1995; Matto et al., 1998). It has also been cultured from other body parts such as the umbilical cord, coronary artery endothelial cells, lung abscesses and atherosclerotic plaques (Beck et al., 1998; Dorn et al., 2001; Okuda et al., 2005; Kozarov et al., 2005). It is a late
colonizer in the oral cavity and requires other established organisms to create the necessary environmental conditions for it to be able to proliferate. These conditions include providing attachment sites, growth substrates and reduction of oxygen to low enough levels that would allow for survival of anaerobic bacteria (Nonaka et al., 2001; Kamaguchi et al., 2003).

During colonization and invasion of the oral tissue, reactive oxidative species such as $\text{O}_2^\cdot$, $\text{HO}^\cdot$, and $\text{H}_2\text{O}_2$ are found in the oral environment (Imlay, 2002; Park et al., 1992). Exposure of *P. gingivalis* to this oxidative stress condition poses a challenge to the survival and proliferation of the bacteria. However, the bacterium has established effective protective mechanisms against this oxidative stress. *P. gingivalis* contains superoxide dismutase which provides protection against atmospheric oxygen (Lynch & Kuramitsu, 1998). It also contains OxyR, a redox-sensitive protein, and rubrerythrin which provide resistance to $\text{H}_2\text{O}_2$ exposure and aerotolerance (Harley et al., 1981; Sztukowska et al., 2002). The bacteria can also withstand nitrosative stress conditions by expressing HcpR, a regulator of the hybrid cluster protein (Hcp). The hydroxylamine produced from nitrite or nitric oxide is reduced into water and ammonia by Hcp (Wolfe et al., 2002). The HcpR of *P. gingivalis* can bind to the *hcp* promoter causing elevated levels of Hcp which protects the bacteria against nitrosative stress (Lewis et al., 2012).

Evasion of the host immune response is necessary for the survival of *P. gingivalis* during colonization and invasion. Interleukin-8 (IL-8) is essential for local defense against organisms by recruiting neutrophils to the site of infection (Baggiolini et al., 1992). *P. gingivalis* can inhibit gingival epithelial cells from producing IL-8 which prevents recruitment of neutrophils to the target site (Darveau et al., 1998). It’s been
reported that the capsule of *P. gingivalis* can also prevent activation of the complement pathway (Brunner et al., 2010). Gingipain-dependent degradation of C3 also allows the bacteria to suppress complement activation (Popadiak et al., 2007). After evading the immune response and establishing colonization, the bacterial mediators will cause an increase in production of inflammatory cytokines such as IL-1β, tumor necrosis factor-α (TNF-α), IL-6, and IL-8 (Darveau, 2010; Baker et al., 1999). This leads to stimulation of an inflammatory response which stimulates bone and tissue destruction in the periodontal area (Lindemann et al., 1988).

Iron is an essential component for the growth of nearly all organisms. *P. gingivalis* obtains iron in the form of hemin (iron and protoporphyrin IX) which becomes available after proteolytical break down of hemin containing proteins such as hemoglobin (Schifferle et al., 1996; Shizukuishi et al., 1995). Accumulation of hemin on the cell surface of *P. gingivalis* gives it its black pigmentation and protects the bacteria from oxidative stress (Smalley et al., 1998).

### 1.4 Virulence Factors of *P. gingivalis*

#### 1.4.1. Capsule

The capsule is an extracellular, hydrophilic polysaccharide coating the surface of the bacteria (Bayer and Bayer, 1994). It has been implicated in evasion of the immune response by down-regulating phagocytosis, opsonization and the complement system (Domenico et al., 1994; Noel et al., 1992). Studies have shown that *P. gingivalis* strains that express capsule have an increase in resistance to phagocytosis compared to non-encapsulated strains (Sundqvist et al., 1991; Van Winkelhoff et al. 1993). This increase
in resistance could be due to both increased hydrophilicity of the different strains and a
decrease in activation of the complement pathway (Schifferle et al., 1993).

Encapsulated *P. gingivalis* strains have also been demonstrated to reduce
production of cytokines such as IL-1, IL-6 and IL-8 which further supports the role of
capsule in evasion of the immune response (Brunner et al., 2010). The adaptive
response has also been shown to be down-regulated and less effective in response to
*P. gingivalis* strains containing a capsule (Wilensky, 2009). In a recent study by Lewis et
al., mice that were infected with a non-encapsulated mutant of *P. gingivalis* W50 had a
much higher survival rate than those treated with an encapsulated *P. gingivalis* W50
(Singh et al., 2011). This result demonstrates that encapsulated strains of *P. gingivalis*
indeed have an increase in virulence compared to non-encapsulated strains.

1.4.2. Lipopolysaccharide

Lipopolysaccharides (LPS) are found on the outer membrane of gram-negative
bacteria such as *P. gingivalis*. LPS plays a crucial role in mediating inflammation and
stimulation of pro-inflammatory cytokines which produce an inflammatory response that
leads to bone resorption. The Lipid-A moiety of LPS is believed to mediate binding of
LPS to host receptors and its composition is dependent of varying hemin concentrations
(Cutler et al., 1996). The tetra-acylated form of the Lipid-A moiety is a Toll-Like
Receptor 4 (TLR4) antagonist and the penta-acylated form is a TLR4 agonists (Nemoto
et al., 2006; Darveau et al., 2004). When hemin concentration is low, the penta-acylated
form is abundant which allows LPS to bind and activate TLR4 (Al-Qutub et al., 2006).
Activation of TLR4 triggers activation of many inflammatory signaling pathways including nuclear factor-kB (NF-kB), c-Jun N-terminal kinase (JNK) and p38 mitogen-activated protein kinase (p38MAPK) (Wang et al., 2002; Ip and Davis, 1998; Lu et al., 2001). Activation of these pathways lead to stimulation of many inflammatory cytokines such as interleukin-1β (IL-1β), tumor necrosis factor-α (TNF-α) and IL-6 which lead to initiation of an inflammatory response (Chiang et al., 1999; Nishida et al., 2001).

1.4.3. Fimbriae

*P. gingivalis* expresses two distinct fimbriae on its cell surface. Type I or major fimbriae are long, filamentous components on the cell surface and are encoded by the *fimA* gene and range from 41 to 49 kDa in size depending on the strain (Lee et al., 1991). They have many roles including cell adhesion and invasion, mediating inflammatory response, biofilm formation and immune evasion. Recent studies have shown that *P. gingivalis* mutants deficient in FimA had both reduced adhesion and a significant reduction in invasion of human oral epithelial cells (Njoroge et al., 1997; Weinberg et al., 1997). Type I fimbria play a role in bone resorption by stimulating release of pro-inflammatory cytokines. These fimbriae can stimulate TLR2 and CD14 which promotes release of pro-inflammatory cytokines such as TNF-a, IL-1B, IL-8 and IL-6 from epithelial and endothelial cells (Asai et al., 2001; Davey et al., 2008; Ogawa et al., 2002). A previous study demonstrated that rats infected with a *fimA* deficient *P. gingivalis* mutant showed reduced bone loss compared to mice infected with the wildtype strain further supporting the role of fimbriae in bone degradation during periodontal disease (Malek et al., 1994).
Type I fimbriae have also been shown to play a role in evasion of cell-mediated immunity. It's been demonstrated that these fimbria can interact with complement receptor 3 (CR3) causing inhibition of IL-12. This leads to reduction in levels of interferon-γ (IFN-γ), an activator of cell-mediated immunity, thus promoting the survival of *P. gingivalis* (Hajishengallis et al., 2007; Hajishengallis, 2011). Type I fimbriae mediate co-adhesion of *P. gingivalis* with many other bacteria such as *Treponema denticola* and *Streptococcus oralis* and promote biofilm formation (Hashimoto et al., 2003; Maeda et al., 2004; Kuboniwa et al., 2009).

Type II or minor fimbriae are short, secondary structures that are encoded by the *mfa1* gene and have a molecular mass of 67 kDa (Hamada et al., 1996). Their role is less known than type I fimbriae but they have been shown to play a role in cell adhesion and invasion, biofilm formation and inflammatory response. Certain strains of *P. gingivalis* such as W50 and W83 do not possess type I fimbriae and are only covered with type II fimbriae. These strains are still invasive indicating that type II fimbriae play a role in invasion of epithelial cells. A recent study demonstrated that an *mfa1*-deficient *P. gingivalis* mutant had reduced invasion of epithelial cells which supports the role of type II fimbriae in adhesion and invasion (Umemoto and Hamada, 2003). Type II fimbriae have also been shown to be necessary for interaction of *P. gingivalis* with *Streptococcus gordonii* during biofilm formation (Park et al., 2005). This indicates that both type I and type II fimbriae play a role in biofilm formation. Type II fimbriae can mediate bone resorption by inducing an inflammatory response via release of cytokines such as IL-1α, IL-1β, IL-6 and TNF-α (Hamada et al., 2002).
1.4.4. Gingipains

*P. gingivalis* proteases are referred to as gingipains. Gingipains can be found as soluble, extracellular entities but they are mostly located on the outer membrane of the bacteria. They are a group of trypsin-like cysteine proteinases that are believed to be responsible for at least 85% percent of the proteolytic activity of *P. gingivalis* (Potempa et al. 2003). There are at least three genes that encode these cysteine proteinases. These include *rgpA* which encodes arginine-specific gingipain A (RgpA), *rgpB* which codes for arginine-specific gingipain B (RgpB) and the *kgp* gene which codes for a lysine-specific gingipain (Kgp) (Lewis et al., 1998, Potempa et al. 1995).

The RgpA contains a signal sequence, an arginine-specific catalytic domain and a C-terminal hemagglutinin/adhesion domain (Pavloff et al., 1995). The rgpB domain however, only codes for a signal sequence and a catalytic domain (Mikolajczyk-Pawlinska et al., 1998). Just like RgpA, Kgp contains a polyprotein which consists of a profragment containing a signal sequence, a lysine-specific catalytic domain and a C-terminal hemagglutinin/adhesion domain (Pavloff et al., 1997). The C-terminal adhesion domain of the gingipains is responsible for binding of the bacteria to a variety of substrates such as hemoglobin, fibrinogen, fibronectin, transferrin and red blood cells (Pike et al., 1996; Nakayama et al., 1998; Brochu et al., 2001). The adhesion domain has also been demonstrated to play a role in co-colonization of *P. gingivalis* with other bacteria in the periodontal pockets during plaque formation (Kamaguchi et al., 2003). The catalytic domain of the gingipains is responsible for degradation of many host proteins such as plasma proteins, extracellular matrix proteins, cytokines and host cell surface proteins (Sroka et al., 2001; Potempa et al., 2003).
Gingipains have many different biological roles and contribute greatly to the virulence of \textit{P. gingivalis}. They can play a role in establishment of chronic inflammation by stimulating expression of receptors in neutrophils, epithelial cells and fibroblasts which lead to induction of pro-inflammatory cytokine response (Lourbakos et al., 1997; Lourbakos et al., 2001; Belibasakis et al., 2010). Gingipains can deteriorate the cell-mediated immune response by cleaving multiple T-cell receptors, such as CD2, CD4 and CD8 (Kitamura et al., 2002). Arginine-gingipains also have a significant role in allowing the bacteria to evade the complement system by cleaving the C5 and C3 molecules. Cleaving the C5 molecule prevents recruitment of PMNs and degradation of the C3 molecule leads to a decrease in bacterial opsonization (Wingrove et al., 1992; Imamura et al., 2001; Sroka et al., 2001).

One of the major roles of gingipains is their ability to degrade heme containing proteins which are essential for \textit{P. gingivalis} growth. Recent studies have demonstrated that lysine-gingipains play a major role in binding and degrading hemoglobin and other heme containing proteins (Lewis et al., 1999). It has also been shown that arginine-gingipains play a major role in degrading hemoglobin (Fujimura et al., 1998).

\textbf{1.4.5. Hemagglutinin}

\textit{P. gingivalis} contains five hemagglutinin proteins encoded by \textit{hagA}, \textit{hagB}, \textit{hagC}, \textit{hagD} and \textit{hagE} genes. Mutations in the \textit{hagA}, \textit{hagB} and \textit{hagC} genes caused a reduction in the hemagglutinating activity of the bacteria which suggests that these genes are important in hemagglutination of the \textit{P. gingivalis} (Lepine et al., 1996). Hemagglutitins are thought to promote colonization of \textit{P. gingivalis} by allowing for
adhering of the bacteria with the host epithelial cells (Duncan et al., 1996). These proteins also play a role in hemin uptake by \textit{P. gingivalis} by mediating the binding of the bacteria to erythrocytes in the periodontal pocket (Lepine and Progulske-Fox, 1996).

1.5 Iron

1.5.1. Iron Uptake

Iron is an essential nutrient for the growth of almost all organisms. Biologically, iron participates in many processes such as photosynthesis, methanogenesis, \textit{H}2 production and consumption, respiration, tricholoracetic acid cycle, oxygen transport and DNA biosynthesis. Iron exits in two forms: the reduced form as a ferrous iron (Fe\textsuperscript{2+}) or the oxidized form as ferric iron (Fe\textsuperscript{3+}). Ferrous iron is much more soluble in water and it is highly reactive (Boukhalfa & Crumbliss, 2002). Because of its ability to be reduced or oxidized, iron is an essential component of life. However, free iron is also capable of catalyzing the conversion of hydrogen peroxides into free radicals which can lead to high toxicity. Due to their high reactivity, these radicals can react with nucleic acids, proteins and lipids and produce significant damage that could kill the cells. As a result the free iron is sequestered into protein-bound forms to prevent the increase in free radicals and toxicity. Many high affinity binding proteins such as transferrin, lactoferrin and ferritin bind free iron (Otto et al., 1992). However, majority of iron is often found as heme in proteins that are associated with transporting oxygen such as hemoglobin or myoglobin. It is believed that as much as 95\% of heme is bound to proteins (Bridges & Seligman, 1995). This leads to low free iron concentrations inside the host which does not support bacterial life. As a result organisms have developed specific mechanisms to extract iron from proteins in order to successfully survive. Some of these pathogens
contain small iron-chelating molecules called siderophores (Cornelissen & Sparling, 1994). These are high-affinity Fe$^{3+}$ scavengers which can outcompete the host’s scavenging ability and sequester iron from iron-containing proteins. Other bacteria such as *P. gingivalis* do not contain siderophores and rely on the proteolytic degradation of hemin-binding proteins and/or expression of binding receptors that are capable of wrestling the iron from proteins. Lysis of erythrocytes releases hemoglobin which is then rapidly bound to haptoglobin, hemopexin and albumin (Hwang & Greer 1980; Hrkal et al., 1974; Beaven et al., 1974).

### 1.5.2. *P. gingivalis* and Iron

Iron plays a crucial role in the growth and virulence of *P. gingivalis*. Reduction of fumarate to succinate by fumarate reductase is a crucial pathway in production of energy in *P. gingivalis*. Hemin is essential for the synthesis of cytochrome b subunit of the fumarate reductase in *P. gingivalis* (Macy et al., 1975). The bacterium does not have the ability to produce protoporphyrin IX, a component of hemin, and thus relies on exogenous uptake of hemin for growth (Shah et al., 1979). Hemin also has the ability to bind to surface of *P. gingivalis* which protects the bacteria against oxidative stress (Smalley et al., 1998). Hemin also plays a role in protection of *P. gingivalis* against nitrosative conditions. Hemin is required for the binding of HcpR to the hcp promoter leading to elevated levels of Hcp which protects the bacteria from nitrosative stress (Lewis et al., 2012). Recent studies have demonstrated that this bacteria uses hemin from hemoglobin more effectively than other iron binding proteins, although transferrin and lactoferrin have both been shown to support the growth of the organism (Shizukuishi et al., 1995; Lamont & Jenkinson, 1998). *P. gingivalis* has developed
mechanisms for both lysing of red blood cells which releases hemoglobin and lysing of hemoglobin which releases hemin. As discussed earlier, this organism encodes a family of proteases termed gingipains. These gingipains contain an N-terminal proteolytic domain and a C-terminal adhesion domain which has the ability to bind substrates such as red blood cells (Curtis et al., 2001; Imamura, 2003). *P. gingivalis* also contains hemagglutinins such as HagB and HagC which are also capable of binding erythrocytes (Progulske-Fox et al. 1995). After binding the erythrocytes, the various hemolysins of *P. gingivalis* cause lysing of the erythrocytes which leads to the release of hemoglobin (Chu et al., 1991; Nelson et al., 2003). The Kgp and Rgp gingipains of the bacterium will then bind hemoglobin and then degrade the hemoglobin which leads to the release of hemin (Lewis et al., 1999; Sroka et al., 2001). Once hemin is released from hemoglobin, *P. gingivalis* must bind it via receptors on its surface and transport it across its double membrane. Recently it’s been demonstrated that three multigenic clusters exist in the bacteria that are believed to encode proteins involved in hemin uptake pathways (Nelson et al., 2003). The first locus, *ihtABCDE*, is composed of five open reading frames (ORFs). The ORFs encode for a TonB-dependent outer-membrane receptor (*ihtA*), lipoprotein (*ihtB*), periplasmic binding protein (*ihtC*), permease (*ihtD*) and cytoplasmic ATP-binding protein (*ihtE*) (Dashper et al., 2000). The second locus is composed of a TonB-dependent receptor (Tla) followed by an ATP-binding cassette hemin-transport system known as *htrABCD* (Aduse-Opoku et al., 1997; Slakeski et al., 2000). The third locus, *hmu*, also has a role in hemin uptake (Lewis et al., 2001). The locus consists of *hmuY* and *hmuR* which are co-transcribed and repressed in presence of iron (Simpson et al., 2000). Recent studies have demonstrated that the HmuY protein is
capable of binding hemin and HmuR protein can bind both hemin and hemoglobin (Liu et al., 2006; Olcazak et al., 2006). The hmu locus also consists of the hmuSTUV locus which could be involved in encoding proteins that process and transport the hemin molecule from periplasmic space through the inner membrane.

1.5.3. TonB

Transport of heme across the double-membrane of P. gingivalis is a critical step in the growth of the bacteria. Due to its large size, heme cannot diffuse across the membrane and relies on an energy dependent active transport. The active transport of this molecule involves an outer membrane receptor, a periplasmic binding protein and an inner membrane ATP-binding cassette transporter (Nikaido, 2003). The inner-membrane generates a proton motor force (pmf) that promotes active transport through the outer-membrane (Bradbeer, 1993). The periplasm space is estimated to span a distance anywhere from 15-71 nm (DePamphlis and Adler, 1971). As a result, a mechanism is required to transport the pmf of the inner-membrane to the outer-membrane.

The TonB, ExbB, and ExbD complex have been proposed to provide the linkage between the inner-membrane and the outer-membrane. ExbB is a 26 kDa cytoplasmic membrane protein consisting of three transmembrane domains and a cytoplasmic domain (Kampfenkel and Braun, 1993). ExbD is a 17 kDa cytoplasmic membrane protein which consists of an N-terminal transmembrane domain and C-terminal domain found in the periplasm (Kampfenkel and Braun, 1992). These two proteins are believed to be involved in the coupling of TonB to the pmf generated by the inner-membrane (Larsen et al., 1999). Previous studies have shown that mutations in the ExbB and
ExbD genes in *E. coli* caused a 90% decrease in the activity of TonB, suggesting that
the ExbB/ExbD complex is required for TonB activity (Ahmer et al., 1995). TonB is
believed to be constantly reenergized by the pmf due to its conversion from its high-
affinity outer membrane associated state to its high-affinity cytoplasmic membrane
associated state by the ExbB/ExbD complex (Letain and Postle, 1997). TonB is a 26
kDa protein that has a single helical N-terminal transmembrane domain, a proline-rich
domain and a C-terminal domain located in the periplasm. The hydrophobic sequence
of the N-terminal domain promotes translocation of TonB into the cytoplasmic
membrane. The proline-rich spacer spans the periplasm and confers flexibility to TonB
(Kohler et al, 2010). The C-terminal domain allows for interaction of TonB with the outer
membrane transporters. Recent studies suggest that TonB forms homodimers which is
in contrast to previous studies that suggested TonB exists as a monomer (Sauter et al.,
2003; Moeck et al., 2001). The structure of TonB allows for linkage of the inner and
outer-membrane of bacteria which promotes transduction of the pmf. Outer-membrane
receptors are composed of a 22 strand Beta-barrel structure with an N-terminal plug
located inside the beta-barrel. The N-terminal plug contains a seven amino acid
sequence called the TonB-box which allows for interaction with the C-terminal domain
of TonB (Larsen et al., 1997). During their interaction, TonB box adopts a beta-strand
conformation that allows for pairing with the three-stranded beta-sheet of TonB
(Pawelek et al., 2006; Shultis et al., 2006). Rotation of the TonB in the periplasm causes
removal of the plug domain from inside of the receptor. This rotation is energized by the
electrochemical gradient that is produced from the pmf which is physically connected to
the TonB by the ExbB/ExbD complex (Jordan et al., 2013). Interaction of TonB with the outer-membrane TonB dependent receptors can be seen in Figure 1.

Recently four models have been proposed for the mechanism of TonB action. The first model proposed, the propeller model, suggests that TonB exists as a dimer (Cascales et al., 2001). In this model TonB interacts with the TonB-box and the ExbB/ExbD complex use the pmf gradient of the cytoplasmic membrane to generate a rotary motion of TonB. This causes a conformational change or release of the plug from the beta-barrel structure which then allows for transport across the membrane receptor.

The second model, the shuttle model, suggests that TonB is associated with both the outer membrane and the cytoplasmic membrane (Letain and Postle, 1997). In this model, the pmf of the cytoplasmic membrane is used by the ExbB/ExbD complex to convert TonB from an unenergized state to an energized state. The TonB then detaches from the cytoplasmic membrane and spans the periplasm to interact with the TonB-box. The energy released from TonB causes a conformational change in the plug domain which then allows for transport across the receptor.

The pulling model suggests that both the plug domain and the TonB-TonB-box complex are 4-stranded beta-sheets (Brockwell et al., 2003; Gumbart et al., 2007). In this model, the TonB spans across the periplasm, interacts with the TonB-box and applies a force on the plug domain. The force applied on the plug domain causes unfolding of the 4-stranded Beta-sheets which allows for transport of substrates across the membrane.

Finally, the PBP-assisted model is based on the interaction of TonB with FhuD which is the periplasmic siderophore binding protein (Carter et al., 2006). In this model,
TonB first presents FhuD to FhuA which is an outer membrane transporter. TonB then interacts with the TonB-box which leads to a conformational change in the plug domain that leads to transport of the siderophore. Finally TonB moves FhuD to FhuBC, the inner membrane transporter, which allows movement of the siderophore into the cytoplasm. The PBP-assisted model is not associated with the uptake of heme in \textit{P. gingivalis} as this bacteria does not use siderophores.

Currently no \textit{P. gingivalis} TonB-coding genes have been identified and thus nothing is known about the exact role, mechanism or structure of \textit{P. gingivalis} TonB. Since TonB has been proposed to play a role in hemin uptake and \textit{P. gingivalis} requires hemin for growth, then further investigation of a possible \textit{P. gingivalis} TonB protein could provide useful information about the exact mechanism of hemin uptake in \textit{P. gingivalis}. To further investigate the role of \textit{P. gingivalis} TonB, we wanted to generate a \textit{P. gingivalis} mutant that was deficient in a putative TonB and characterize the mutant to see the roles that this putative TonB plays in the biology of \textit{P. gingivalis}. 
Figure 1: Cartoon representation of the TonB/ExbD/ExbB complex. The proton motive force generated in the inner membrane is transferred via ExbD/ExbB to TonB. TonB spans the periplasm and interacts via its C-terminal domain with the TonB box of the plug domain of TonB dependent receptors. This allows for removal of the plug domain from inside of the receptor which allows for hemin to pass through.
CHAPTER 2: HYPOTHESIS AND AIMS

2.1 Hypothesis

We hypothesize that *P. gingivalis* candidate protein PG0785 could be a possible TonB protein.

2.2 Research Objective and Aims

The main objective of this research is to investigate the role of candidate protein PG0785 in *P. gingivalis*. The objective and aims of the project are depicted in Figure 2.

**Aim 1:** Generate a *P. gingivalis* mutant with a non-functional PG0785 gene using:

I. Electroporation to transform the disrupted PG0785 construct into *P. gingivalis* W83 genome. This allows for incorporation of a non-functional PG0785 into the genome of W83.

**Aim 2:** Determine metal content of V3128 and W83 *P. gingivalis* strains using:

I. ICP-MS metal content analysis which ionizes the samples with inductively coupled plasma and then separates and quantifies the ions by a mass spectrometer. This allows for quantification of the metal levels in all our samples.

II. Colorimetric Ferrozine Assay which allows for quantitation of iron in our cultured samples. Both ferric and ferrous iron levels can be quantified using this method.

**Aim 3:** Determine arginine-specific and lysine-specific cysteine protease activity of mutant and wild type *P. gingivalis* using:
I. Protease assays which is a colorimetric assay that allows for measurement of protease activity. Interaction of arginine-specific proteases with BAPNA and interaction of lysine-specific proteases with Z-Lys-pNA causes the pNA portion to be cleaved off resulting in a yellow color in our samples. More intense colors correlate to higher protease activity.

**Aim 4:** Determine virulence ability of V3182 and W83 *P. gingivalis* using:

I. Total interaction and invasion assays of V3128 and W83 with HUVEC cells. Both V3128 and W83 will be allowed to interact with HUVEC cells and the cultures are then plated on blood plates. Colonies are counted to determine how V3128 and W83 interact and invade eukaryotic cells.

II. Flow Cytometry is an alternative method that allows for measurement of interaction and invasion of V3128 and W83 with eukaryotic cells.

**Aim 5:** Transcriptional and translational analysis of V3128 and W83 *P. gingivalis* using:

I. Proteomics which allows us to identify proteins that have been differentially regulated in V3128 compared to W83.

II. RNA isolation and library generation which allows us to identify genes that have been differentially regulated in V3128 compared to W83.
Figure 2: Flowchart of the project aims and methods. Project hypothesis and aims are listed in the above figure.
CHAPTER 3: MATERIALS AND METHODS

3.1 Mutant Generation

3.1.2. Growth Media Conditions

Porphyromonas gingivalis strain W83 was grown anaerobically at an atmosphere of 10% H₂, 10% CO₂ and 80% N₂ at 37° C. Bacteria liquid cultures were prepared in Brain-Heart infusion broth (BHI; Difco Laboratories, Detroit, MI) with Hemin (5 µg/ml; Sigma, St. Louis, MO), yeast extract (5 mg/ml), cysteine (1 mg/ml; Sigma, St. Louis, MO) and Vitamin K1 or K2 (1 µg/mL; Sigma, St. Louis, MO).

3.1.3. P. gingivalis Cell Preparation

Both P. gingivalis V2802 and V2984 W83 strains were grown anaerobically on blood agar plates (TSA II, 5% sheep blood; BBL, Cockeysville, MD). From blood plates, strains were then grown in 3 ml BHI broth overnight and then again in 1:10 BHI broth dilutions overnight. Bacteria from the 1:10 dilutions were then grown in 150 mL BHI broth overnight. Cultures were grown until at least an optical density (OD) of 0.300 was attained. Cultures were then washed twice with electroporation butter (10% Glycerol; Fisher BioReagents) and stored in at -80° C.

3.1.4. Transformation

Transformation was done with both W83 strains. PG0785, PG1912 and PG1913 were cloned into the pCR 2.1 vector using NrlI/Smal, BsmBI/Smal and NrlI/ClaI restriction sites respectively. pCR2.1PG0785, pCR2.1PG1912 and pCR2.1PG1913 plasmids were transformed into P. gingivalis W83 electrocompetent cells using the
electroporation method. 10 µL of each plasmid was mixed with 100µL of electroporation cells in Electroporation Cuvettes (BIO-RAD, Hercules, CA) and electroporated at 2,500 mV using a Gene-Pulser (BIO-RAD, Hercules, CA). 500 µL BHI was added to each cuvette and cells were grown overnight. From cuvettes containing the 500 µL culture, 300 µL was then spread on blood agar plates containing 20 µL Clindamycin (0.5 mg/ml). The plates were then incubated anaerobically for 7 days at 37° C for colonies to appear.

The plates were inspected for the presence of bacterial colonies. PCR was done on selected colonies using the GoTaq® Long PCR Master Mix (Promega). This was done to screen for transformation of constructs into the \textit{P. gingivalis} genome. The PCR consisted of 35 cycles consisting of denaturing at 94° C for 30 seconds, annealing at 52° C for 30 seconds and extension at 65° for 5 minutes. Gel electrophoresis was used to confirm the size of the fragment. The electroporation mechanism is shown in Figure 4.
Figure 3: Electroporation method. Plasmids and *P. gingivalis* W83 cells are mixed together in electroporation cuvettes and electroporated at 2500 mV. Bacterial cells are then grown in 500 µL of BHI overnight at 37° C. 300 µL of the overnight culture is spread on blood plates containing 20 µL of clindamycin. The plates are incubated anaerobically for 7 days at 37° C. Colonies are selected from blood plates and screened by PCR.
3.2 Identification of a putative TonB gene

Bioinformatics was done for PG0785. The protein and nucleotide sequence of PG0785 are indicated in Table 1. The Basic Local Alignment Search Tool (BLAST®) was used to search the nucleotide and protein sequence of the gene against *P. gingivalis* W83 database. This was used to look at how identical the PG0785 gene is compared to genes found in other species. The C-terminal sequence of PG0785 was used to attain the structure of C-terminal region of PG0785 using one to one threading by Phyre (Kelley and Sternberg, 2009). Chimera was used to generate an overlay of the structure of *P. gingivalis* PG0785 and the structure of the C-terminal TonB protein of *E. coli*.

**Table 1: Nucleotide and protein sequence of PG0785**

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<th>Type of Sequence</th>
<th>Sequence of PG0785</th>
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<td>Nucleotide</td>
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</table>
3.3 Eukaryotic Cell Invasion and Total Interaction

3.3.1. Bacterial Cell Preparation

Wild type *P. gingivalis* (W83) and mutant *P. gingivalis* (V3128) from blood plates were inoculated in 4 mls of BHI broth to an OD of 0.5 at 660 nm. Bacteria were centrifuged at 7500 rpm for 10 minutes to obtain pellets. Pellets were then washed twice with 2 mls Phosphate Buffered Saline (PBS, 0.137 M NaCl, 0.0027 M KCl, 0.010 M Na$_2$HPO$_4$, 0.0018 M KH$_2$PO$_4$) and suspended in 2 mls of HUVEC media (Lifeline Cell Technology, Table 2).

3.3.2. Total Interaction Assay

Human umbilical vein endothelial cells (HUVEC) were grown in 6-well plates until at least 80% confluence. Cells in each well were then washed 3 times with PBS and suspended in 2 mls of HUVEC media (Table 2). Bacteria were then added to each well with a multiplicity of infection (MOI) of 100. W83 were added to 3 wells and V3128 were added to the remaining 3 wells. Bacteria and HUVEC cells were incubated for 30 minutes before each well was washed twice with PBS. Immediately 2 mls of BHI broth
with 1% saponin (Riedel-de Haen, Germany) was added to each well for 15 minutes to determine the number of adhered and internalized bacteria. Liquid from each well was then collected and serially diluted (1:10 and 1:100). 200 µL from the original tube and dilutions were spread on blood plates and incubated anaerobically for 7 days at 37°C until colonies appeared.

3.3.3. Invasion Assay

HUVEC cells were grown in 6-well plates until at least 80% confluence. Cells in each well were then washed 3X with 2 mls of PBS and suspended in 2 mls of HUVEC media. Bacteria were then added to each well with an MOI of 100. W83 was added to 3 wells and V3128 were added to the remaining 3 wells. Bacteria and cells were incubated for 30 minutes and then 160 µL of Metronidazole (50 mg/ml; SIGMA®) and 6 µL of Gentamycin (50 mg/ml; SIGMA®) were added to each well for 1 hour to kill externally adhered bacteria. 2 mls of BHI broth with 1 % saponin was added to each well for 15 minutes to determine the number of adhered and internalized bacteria. Liquid from each well was collected and serially diluted (1:10 and 10:100). 200 µL from the undiluted tube and dilutions were spread on blood plates and incubated anaerobically at 37°C until colonies appeared (Typically around 7-10 days).
Table 2: HUVEC Media

<table>
<thead>
<tr>
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<th>Part Number</th>
<th>Volume to add</th>
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<tbody>
<tr>
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<td>475 mL</td>
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<tr>
<td>VascuLife VEGF LifeFactors Kit</td>
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<td>LS-1012</td>
<td>10 mL</td>
<td>2%</td>
</tr>
<tr>
<td>L-Glutamine LifeFactor</td>
<td>LS-1013</td>
<td>25 mL</td>
<td>10 mM</td>
</tr>
<tr>
<td>rh IGF-1 LifeFactor</td>
<td>LS-1014</td>
<td>0.5 mL</td>
<td>15 ng/mL</td>
</tr>
<tr>
<td>rh EGF LifeFactor</td>
<td>LS-1015</td>
<td>0.5 mL</td>
<td>5 ng/mL</td>
</tr>
<tr>
<td>rh VEGF LifeFactor</td>
<td>LS-1016</td>
<td>0.5 mL</td>
<td>5 ng/mL</td>
</tr>
<tr>
<td>Heparin Sulfate LifeFactor</td>
<td>LS-1017</td>
<td>0.5 mL</td>
<td>0.75 units/mL</td>
</tr>
<tr>
<td>Optional Supplements</td>
<td>Part Number</td>
<td>Volume to add</td>
<td>Concentrations of Supplement</td>
</tr>
<tr>
<td>Phenol Red Supplement</td>
<td>LS-1009</td>
<td>1.0 mL</td>
<td>33 mM</td>
</tr>
<tr>
<td>Antimicrobial Supplement: Penicillin, Streptomycin and Amphotericin B</td>
<td>LS-1011</td>
<td>1.0 mL</td>
<td>Penicillin 10,000 Units/mL, Streptomycin 10,000 µg/mL, Amphotericin B 25 µg/mL</td>
</tr>
</tbody>
</table>
3.3.4. Flow Cytometry

Total interaction and invasion assays were also done using flow cytometry. On the day of the reading, W83 and V3128 from blood plates were inoculated in 3 mls of BHI media to obtain an OD between 0.5 and 0.7 at 660 nm. Cultures were then centrifuged at 7000 rpm for 5 minutes. Pellets were then washed twice with PBS and re-suspended in 1 ml of PBS. 1 µL of fluorescein-5-EX, succinimidyl ester (FITC) (Life Technologies™) was added to each sample and incubated for 1 hour at 4°C. Samples were then washed again with PBS and re-suspended in HUVEC media. For total interaction, bacterial cells were added to HUVEC cells at an MOI of 100 and incubated for 30 minutes. For invasion, bacteria were added to HUVEC cells and the mixture was diluted with 0.4% Trypan Blue which quenches extracellular fluorescence and thus only allows for counting of bacteria that have invaded cells. Samples were then taken to the Flow Core Facility at VCU (4th floor, MMRB) for fluorescence measurement using the BD FACS Canto II flow cytometer.

3.4 Proteomics

W83 and V3128 from blood plates were inoculated in 4 mls of BHI broth to an OD between 1 - 1.4 at 660 nm. Cultures were then centrifuged at 7500 rpm for 10 minutes to obtain pellets. Pellets were washed twice with sterile distilled water and re-suspended in 3 mls of 100 mM Tris-HCL. Cells were then lysed using the sonication method and 1 µL of Benzonase (Sigma) was added to each sample for 30 minutes to degrade DNA and RNA. 600 µL of 10% Sodium Dodecyl Sulfate (SDS) Solution (Invitrogen) was then added to each sample for 30 minutes to unfold the proteins.
Samples were then centrifuged at 7500 rpm for 10 minutes and supernatant was collected. Both W83 and V3128 proteomic samples were then ran on NuPage 10% Bis-Tris Gel (Life Technologies™) and lanes of interested were excised from the gel. Both proteomic solutions and gel bands were submitted for mass spectrometry analysis to the VCU Chemical and Proteomic Mass Spectrometry Core Facility.

3.5 RNA Library generation

3.5.1. RNA Isolation

RNA was isolated from W83 and V3128 using the Qiagen RNeasy® kit. The isolated RNA samples were then treated with the DNA-free™ DNase Treatment and Removal Reagents kit (Life Technologies™) to remove all DNA. The DNAse treated RNA samples were then treated with the Epicentre Ribo-Zero™ Magnetic Kit (Gram-Negative Bacteria) to deplete rRNA. The rRNA depleted RNA samples were then ready for sequencing.

3.5.2. RNA Library Generation

The rRNA depleted RNA samples were treated with the SMARTer® Stranded RNA-Seq Kit (Clontech® Laboratories). This kit was used to synthesize cDNA, purify cDNA, amplify RNA-sequence library using PCR and purify the RNA-sequence library (Figure 4). The generated library was then sent to the VCU Nucleic Acid Research Facilities to be validated and sequenced.
Figure 4: Schematic representation of SMARTer® Stranded RNA-Seq Library generation adapted from Clontech Laboratories protocol. The Ovation® Complete Prokaryotic RNA-Seq DR Multiplex Systems 1-8 and 9-16 (NuGEN) kit was also used to generated RNA library. This kit did not require a separate rRNA removal treatment because it allowed for non-rRNA library generation by selective priming during first and second strand cDNA synthesis. This kit allowed for synthesis of double stranded cDNA, fragmentation and purification of cDNA, end repair to generate blunt ends, adaptor ligation, strand selection to leave only one cDNA strand and PCR amplification to produce the library. The generated library was then sent to the VCU Nucleic Acid Research Facilities to be validated and sequenced.
3.6 Metal Content Assay

3.6.1. ICP-MS

W83 and V3128 from blood and BHI plates were inoculated in 4 mls of BHI to obtain an OD between 1 and 1.4 at 660 nm. Cultures were centrifuged at 7500 rpm for 10 minutes to harvest bacterial cells. Bacterial pellets were washed twice with 4 mls of Chelex Buffer (0.05 M Hepes, 0.05 M NaCl), suspended in 3 mls Chelex Buffer with 8 M Urea and incubated for 1 hour at room temperature. Cells were then lysed using the sonication method and centrifuged at 14,800 rpm for 30 minutes. Supernatant was then collected and used for ICP-MS analysis to determine metal content.

3.6.2. Colorimetric Ferrozine Assay

W83 and V3128 strains from blood and BHI plates were prepared the same way as section 3.6.1. Ammonium ferrous sulfate was used as our standard. Standards of 0.5, 1.0, 2 and 2.5 ppm were created from the ammonium ferrous sulfate stock solution. 500 µL of each standard and sample was added to different spectrophotometric cuvettes followed by 50 µL of 0.01M Ferrozine (SIGMA) and 25 µL of 5M ammonium acetate. The OD of each sample was measured after 24 hours using mass spectrophotometer set at 562 nm.

3.7 Protease Assay

3.7.1. Bacterial Preparation

W83 and V3128 strains were grown on both blood plates and BHI plates. They were inoculated in 0.5 mls of BHI to an OD between 1-1.4 at 660 nm. Samples were
centrifuged at 8000 rpm for 10 minutes, supernatant was removed and pellets were suspended in 0.5 mls of PBS.

### 3.7.2. Arginine-Specific Protease Assay

Tris-HCl, L-Cysteine (SIGMA®), CaCl₂ (SIGMA®), TCEP Nα-Benzoyl-DL-arginine 4-nitroanilide hydrochloride (BAPNA) (SIGMA®), W83 and V3128 samples and distilled water were mixed in VIS cuvettes as designated in Table 3. Reactions were done at 37°C and OD was measured at 405 nm after 30 minutes, 60 minutes, 90 minutes and 180 minutes of incubation.

**Table 3: Arginine-specific protease activity**

<table>
<thead>
<tr>
<th>Arginine-specific activity</th>
<th>Reagent</th>
<th>Concentration</th>
<th>Volume</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Tris-HCl, pH 7.5</td>
<td>50 mM</td>
<td>400 µL</td>
</tr>
<tr>
<td></td>
<td>L-Cysteine</td>
<td>500 mM</td>
<td>5 µL</td>
</tr>
<tr>
<td></td>
<td>CaCl₂</td>
<td>500 mM</td>
<td>5 µL</td>
</tr>
<tr>
<td></td>
<td>TCEP</td>
<td>1 mM</td>
<td>5 µL</td>
</tr>
<tr>
<td></td>
<td>BAPNA</td>
<td>0.0043 g/ml of dimethyl sulfoxide (DMSO) (SIGMA®)</td>
<td>50 µL</td>
</tr>
<tr>
<td></td>
<td>Sample</td>
<td>Approximately 25 µg</td>
<td>15-30 µL</td>
</tr>
<tr>
<td></td>
<td>Water</td>
<td></td>
<td>30 µL</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Total Volume = 500 µL</td>
</tr>
</tbody>
</table>
3.7.3. Lysine-Specific Protease Assay

Tris-HCl, L-Cysteine (SIGMA®), TCEP, Z-Lys-pNA (SIGMA®), W83 and V3128 samples and distilled water were mixed in VIS cuvettes as designated by the Table 4. Reactions were done at 37°C and OD was measured at 405 nm after 30 minutes, 60 minutes, 90 minutes and 180 minutes of incubation.

**Table 4: Lysine-specific protease activity**

<table>
<thead>
<tr>
<th>Lysine-specific activity</th>
<th>Reagent</th>
<th>Concentration</th>
<th>Volume</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Tris-HCl, pH 7.5</td>
<td>50 mM</td>
<td>400 µL</td>
</tr>
<tr>
<td></td>
<td>L-Cysteine</td>
<td>500 mM</td>
<td>5 µL</td>
</tr>
<tr>
<td></td>
<td>TCEP</td>
<td>1 mM</td>
<td>5 µL</td>
</tr>
<tr>
<td></td>
<td>Z-Lys-pNA</td>
<td>0.0049 g/ml of dimethyl sulfoxide (DMSO) (SIGMA®)</td>
<td>25 µL</td>
</tr>
<tr>
<td></td>
<td>Sample</td>
<td>Approximately 25 µg</td>
<td>15-30 µL</td>
</tr>
<tr>
<td></td>
<td>Water</td>
<td></td>
<td>60 µL</td>
</tr>
</tbody>
</table>

**Total Volume = 500 µL**
4.1 Identification of a putative TonB gene

PG0707 and PG0785 are designated as the same gene according to the Los Alamos and TIGR databases. BLAST was used to search the nucleotide and protein sequence of PG0785/0707 against the *P. gingivalis* W83 database. Results of the nucleotide sequence are seen in Table 5. There were four genes found that had TonB related features. The nucleotide sequence of PG0785 had a 79% similarity to the sequence of *Bacteriodes vulgatus* ATCC 8482, 78% similarity to *Haliscomenobacter hydrossis* DSM 1100, 77% similarity to *Tannerella forsythia* ATCC 43037 and 76% to *Bacteriodes xylanisolvens* XB1A. The results of the protein sequence similarities are seen on Table 6. The protein sequence of PG0785 had 33% similarity with Chain B of Btub:tonB complex, 32% similarity with Chain A of *E. coli* TonB, 32% similarity with Chain C of TonB in complex with FhuA outer membrane receptor and 31% similarity with Chain A of TonB2 of *Vibrio anguillarum*.

**Table 5: Nucleotide sequence similarities of PG0785 with other sequences**

<table>
<thead>
<tr>
<th>Nucleotide Sequence</th>
<th>Similarity</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Bacteriodes vulgates</em> ATCC 8482 Feature – Outer membrane protein TonB</td>
<td>79%</td>
</tr>
<tr>
<td><em>Haliscomenobacter hydrossis</em> DSM 1100 Feature – TonB family protein</td>
<td>78%</td>
</tr>
<tr>
<td><em>Tannerella forsythia</em> ATCC 43037 Feature – TonB dependent receptor</td>
<td>77%</td>
</tr>
<tr>
<td><em>Bacteriodes xylanisolvens</em> XB1A Feature – Outer membrane transport energization protein TonB</td>
<td>76%</td>
</tr>
</tbody>
</table>
Table 6: Protein sequence similarities of PG0785 with other sequences

<table>
<thead>
<tr>
<th>Protein Sequence</th>
<th>Similarity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Chain B, Structure Of The Btub:Tonb Complex</td>
<td>33%</td>
</tr>
<tr>
<td>Chain A, Solution Structure Of Escherichia coli TonB</td>
<td>32%</td>
</tr>
<tr>
<td>Chain C, Crystal Structure Of TonB In Complex With FhuA, E. coli Outer Membrane Receptor For Ferrichrome</td>
<td>32%</td>
</tr>
<tr>
<td>Chain A, Molecular Characterization Of The TonB2 Protein From Vibrio anguillarum</td>
<td>31%</td>
</tr>
</tbody>
</table>

The homology model of the C-terminal structure of PG0785 was attained using one to one threading by Phyre (Kelley and Sternburg, 2009). The protein sequence of PG0785 is 230 amino acids long. Amino acids 155-230 (75 AA’s) correlate to the C-terminal region and were used to generate this structure. This structure can be seen on Figure 5. The C-terminal domain structure of E. coli TonB (1IHR) was attained from Protein Data Bank (PDB). C-terminal residues 164-239 (76 AA’s) were used to generate this structure. Chimera was used to generate an overlay of the homology model of C-terminal domain of PG0785 and C-terminal domain of E. coli TonB. This structure can be seen in Figure 6.
**Figure 5: Homology Model of the C-terminal Domain of PG0785.** Amino acids 155-230 (75 AA’s) correlating to the C-terminal sequence of PG0785 were used to create the homology model using Phyre. The blue portion of this model represents the N-terminal region and the red portion represents the C-terminal region.

**Figure 6: Overlay of the C-terminal Domains of E. coli TonB (1IHR) and PG0785.** The C-terminal domain of TonB from *E. coli* is represented by the red model. The blue model represents the homology model of the C-terminal domain of PG0785. The overlay was made using the Chimera software.
4.2 Mutant Generation

In order to be able to determine the role of TonB in *P. gingivalis* our first aim was to generate a putative TonB deficient mutant. PG0785 is believed to be a possible TonB protein. A previous student in Dr. Lewis’ lab cloned PG0785, PG1912 and PG1913 into a pCR2.1 TA vector. Using restriction enzymes, an ErmF-AM cassette was inserted in the middle of the PG0785, PG1912 and PG1913 gene to disrupt their function. We will refer to these plasmids as pCR2.1PG0785, pCR2.1PG1912 and pCR2.1PG1913. PCR was done on the pCR2.10785 plasmid using primers for PG0785 and then analyzed by 1% agarose gel to determine the size of the fragment (Figure 7). pCR2.1PG1913, pCR2.1PG1912 and pCR2.1PG0785 plasmids were electroporated into *P. gingivalis* W83 (W83) electroporation cells and spread on blood plates with clindamycin. They were then grown at 37°C in an anaerobic chamber until colonies appeared. Colonies were then picked, screened using PCR and analyzed by 1% agarose gel electrophoresis. Figures 6, 7 and 8 show the results of our PCR products. Blood plates containing colonies for pCR2.1PG1913 + W83 (Figure 8) and pCR2.1PG1912 + W83 (Figure 9) did not have a fragment at 3 kb meaning that the PG1913 + ErmF-AM cassette construct and PG1912 + ErmF-AM cassette construct were not inserted in the genome of W83. PCR was done on a colony picked from a blood plate containing pCR2.1PG0785 + W83 (Figure 10). Figure 10 shows the electrophoresis gel done on the PCR product. A 3 kb fragment can be seen in lane 3 meaning that the PG0785 + ErmF-AM cassette was successfully inserted the genome of W83. In lane 3 there are two specific bands at 3 kb and around 1 kb. This means that this was most likely a double crossover event meaning that part of the intact gene has remained. If there was
only one band at 3 kb in lane 3 then it would suggest a single crossover event had taken place.

Figure 7: Restriction enzyme profile of pCR2.1PG0785 plasmid PCR products. The construct consisting of the ErmF-AM cassette and surrounding PG0785 was amplified by PCR. Lane 1: Ladder; Lane 2: pCR2.1PG0785 plasmid 1:10 dilution; Lane 3: pCR2.1PG0785 plasmid 1:100 dilution; Lane 3: Genomic DNA. Both pCR2.1PG0785 dilutions had a fragment at 3 kb compared to the genomic DNA which did not have a fragment at 3 kb. This suggests that any mutant that has the construct successfully inserted in its genome should have the same 3 kb fragment.
Figure 8: Restriction enzyme profile of the PCR product of selected pCR2.1PG1913 + W83 colonies. Selected colonies were used as template and amplified by PCR. PCR products were analyzed by gel electrophoresis. Lane 1: Ladder; Lane 2: pCR2.1PG1913 + V2802 W83; Lane 3: pCR2.1PG1913 + V2984 W83; Lane 4: pCR2.1PG1913 + V2984 W83; Lane 5: pCR2.1PG1913 + V2802 W83; Lane 6: pCR2.1PG1913 + V2984 W83; Lane 7: pCR2.1PG1913 + V2984 W83; Lane 8: Genomic DNA. Lanes 2-7 represent the PCR product of selected pCR2.1PG1913 + W83 colonies from blood plates. None of the colonies selected had a fragment at 3 kb (white box) meaning that the construct was not inserted into the genome of W83.
Figure 9: Restriction enzyme profile of the PCR product of selected pCR2.1PG1912 + W83 colonies. Selected colonies were used as template and amplified by PCR. PCR products were analyzed by gel electrophoresis. Lane 1: Ladder; Lane 2: pCR2.1PG1912 + 2984 W83; Lane 3: pCR2.1PG1912 + 2984 W83; Lane 4: pCR2.1PG1912 + 2984 W83; Lane 5: pCR2.1PG1912 + 2984 W83; Lane 6: Genomic DNA. Lanes 2-5 represent the PCR product of selected pCR2.1PG1912 plasmid + W83 colonies from blood plates. None of the colonies selected had a fragment at 3 kb (white box) meaning the construct was not inserted into the genome of W83.
Figure 10: Restriction enzyme profile of the PCR product of selected pCR2.1PG0785 + W83 colony. Selected colonies were used as template and amplified by PCR. PCR products were analyzed by gel electrophoresis. Lane 1: Ladder; Lane 2: pCR2.1PG0785 + 2984 W83 on BP; Lane 3: pCR2.1PG0785 + 2984 W83 on BP + Clindamycin; Lane 4: Genomic DNA. Lane 3 represents the PCR product of pCR2.1PG0785 plasmid + W83 colony selected from blood plate. There is a fragment at 3 kb (white box) suggesting that the construct consisting of PG0785 + the ErmF-AM cassette was inserted into the genome of W83.

4.3 Mutant Growth

W83 grew well in BHI broth compared to the putative TonB deficient mutant (V3128) which did not grow in BHI broth. As a result both strains were repeatedly grown
on blood and BHI plates. The V3128 strain was always grown on blood and BHI plates containing clindamycin. Both W83 and V3128 strain grew well on blood plates. W83 would usually become dark pigmented after a week on blood plates compared to V3128 which never became dark pigmented on blood plates. W83 also grew well on BHI plates but never became dark pigmented. V3128 would usually not grow on BHI plates after 3 times of re-plating. As a result, once V3128 on BHI plates stopped growing, V3128 from blood plates had to be used to again grow V3128 on BHI plates. Since V3128 did not grow in BHI broth we could not produce accurate growth curves. Based on their growth on blood plates, usually after 6-7 hours both strains would start to grow on both blood and BHI plates. After 24 hours both strains would grow enough to be ready to use for experiments. Figure 11 shows W83 and V3128 on blood and BHI plates.

**W83 and V3128 on blood plates**

![A. W83 on blood plate](image1)

![B. V3128 on blood plate](image2)

**Figure 11: Blood plates containing W83 and V3128.** W83 and V3128 were grown anaerobically on blood plates at 37°C for 7 days. (A) Blood plate containing W83. (B) Blood plate containing V3128.
4.4 Eukaryotic Cell Invasion and Total Interaction

4.4.1. Total Interaction and Invasion Assay

We wanted to find out how virulence of V3128 would be affected compared to W83. To do so we performed total interaction and invasion assays to see how V3128 interacts and invades eukaryotic cells. W83, V3128 and HUVEC cells were prepared as indicated in the “Materials and Methods” section. For the total interaction assay, bacteria were incubated with HUVEC cells and cultures were then spread on blood plates from the undiluted tubes, 1:10 dilution tubes and 1:100 dilution tubes. For the invasion assay, bacteria were incubated with HUVEC cells and antibiotics were then added to kill any extracellular bacteria so that only bacteria that had invaded the HUVEC cells would be able to grow. Cultures were then spread on blood plates from the undiluted tubes, 1:10 dilution tubes and 1:100 dilution tubes. Colonies were counted after 1-2 weeks.

Figure 12 shows plates that contained colonies for the total interaction assay and Figure 13 shows plates that contained colonies for the invasion assay. Four biological repeats were done and the number of colonies from each experiment was averaged together. Results of the total interaction assay are shown on Figure 14. For the total interaction assay, W83 from undiluted tubes had an average of 1556 colonies compared to V3128 from undiluted tubes which had an average of 23 colonies. W83 from 1:10 dilution tubes had an average of 552 colonies compared to V3128 from 1:10 dilution which had an average of 181 colonies. W83 from 1:100 dilutions had an average of 88 colonies compared to V3128 from 1:100 dilutions which had an average of 73 colonies.
These results show that W83 interacts with HUVEC cells at significantly higher levels than V3128.

Results of the invasion assay are shown in Figure 15. For invasion assay, W83 from undiluted tubes had an average of 1366 colonies compared to V3128 from undiluted tubes which had an average of 142 colonies. W83 from 1:10 dilutions had an average of 511 colonies compared to V3128 from 1:10 dilutions which had an average of 29 colonies. W83 from 1:100 dilutions had an average of 68 colonies compared to V3128 from 1:100 dilutions which had an average of 5 colonies. These results show that W83 has significantly higher ability to invade eukaryotic cells than V3128.
Figure 12: Blood plates containing colonies for the total interaction assay.

Cultures from undiluted tubes, 1:10 dilution tubes and 1:100 dilution tubes were spread on blood plates and colonies were counted after 1 week. W83 from undiluted tubes (A), W83 from 1:10 dilution tubes (B), W83 from 1:100 dilution tubes (C), V3128 from undiluted tubes (D), V3128 from 1:10 dilution tubes (E) and V3128 from 1:100 dilution tubes (F).
Figure 13: Blood plates containing colonies for the invasion assay. Cultures from undiluted tubes, 1:10 dilution tubes and 1:100 dilution tubes were spread on blood plates and colonies were counted after 1 week. W83 from undiluted tubes (A), W83 from 1:10 dilution tubes (B), W83 from 1:100 dilution tubes (C), V3128 from undiluted tubes (D), V3128 from 1:10 dilution tubes (E) and V3128 from 1:100 dilution tubes (F).
Figure 14: Total Interaction Assay for V3128 and W83 strains. W83 and V3128 were inoculated with HUVEC cells in 6 well plates. Cultures were spread on blood plates from undiluted tube, 1:10 dilution and 1:100 dilution and colonies were counted after one week. Yellow bars represent W83 from blood plates and black bars represent V3128 from blood plates. The average number of colonies from each plate is represented by the Y-axis.
**Figure 15: Invasion Assay of V3128 and W83 strains.** W83 and V3128 were inoculated with HUVEC cells in 6 well plates. Antibiotics were added to kill extracellular bacteria so that only bacteria that had invaded HUVEC cells could survive. Cultures were spread on blood plates from undiluted tube, 1:10 dilution and 1:100 dilution and colonies were counted after one week. Yellow bars represent W83 from blood plates and black bars represent V3128 from blood plates. The number of colonies is represented by the Y-axis.

### 4.4.2. Flow Cytometry

Flow cytometry was done on both V3128 and W83 for total interaction and invasion. Four biological repeats were done and the median fluorescence (MF) of each experiment was averaged together. Results can be seen in Figure 16. V3128 had an
MF of 202 for total interaction and an MF of 195.75 for invasion. W83 had an MF of 180.5 for total interaction and an MF of 170.5 for invasion.

Figure 16: Flow cytometry results for total interaction and invasion. V3128 and W83 were labeled with FITC. They were then inoculated with HUVEC cells. Yellow bars represent the total interaction by V3128 and W83. Blue bars represent invasion by V3128 and W83. The Y-axis represents the median fluorescence measured.

4.5 Proteomics

Proteomics was done on both W83 and V3128 strains to identify major proteins up-regulated and down-regulated. Bands were excised out of gel and send for analysis. Figure 17 shows the bands that were excised out of the gel. The samples were analyzed using the Sequest search algorithm against *P. gingivalis* database. Figure 18 shows the peptides and proteins that were identified from the gel slices. The major proteins identified were lipoprotein RagB (ragB) represented by band 6 in Figure 17, RagA protein (ragA) represented by band 1, 4-hydroxybutyryl-CoA dehydratase (abfD)
represented by band 5, hemmagglutinin proteins HagA and HagE represented by bands 9 and 10, outer membrane lipoprotein Omp28 (omp28), immunoreactive 43 kDa antigen PG32 represented by band 14, ABC transporter ATP-binding protein represented by band 12, glutamate dehydrogenase, NAD-specific (GDH), arginine-specific cysteine proteinase (PrtRII) and Dps family protein.

Figure 17: V3128 and W83 proteomic products ran on protein gel. Lane 1: Ladder; Lane 2: V3128 from blood plate; Lane 3: W83 from blood plate. Bands 1, 5, 6, 9, 12 and 14 from V3128 and bands 1, 5, 6, 9, 19 and 14 from W83 were excised out of gel and sent for analysis to identify proteins from the gel.
Figure 18: Peptides and proteins identified from gel slices using Sequest search algorithm. Gel slices were analyzed using the sequest search algorithm to identify proteins. The major proteins identified were lipoprotein RagB (RagB), RagA protein (RagA), 4-hydroxybutyryl-CoA dehydratase (AbfD), hemagglutinin protein HagA and HagE, outer membrane lipoprotein Omp28 (Omp28), immunoreactive 43 kDa antigen PG32, ABC transporter ATP-binding protein, NAD-specific glutamate dehydrogenase (GDH), arginine-specific cysteine proteinase (PrtRII) and a Dps family protein.
4.6 RNA Library Generation

W83 and V3128 RNA were isolated and used to generate RNA libraries as described in the “Materials and Methods” section. Three sets of libraries were generated on different dates. Samples were then sent to the VCU Sanger Hall sequencing center to be validated and sequenced.

4.6.1 Validation of the RNA-Seq Library using the BioAnalyzer

The bioanalyzer results for our samples are shown in Figures 19, 20, 21, 22 and 23. The results indicated that the RNA libraries were ready for processing and sequencing since all of the samples had peaks less than 1000 base pairs.

Figure 19: Electropherogram results from bioanalyzer of V3128 and W83 RNA from 3/06/2014.
Figure 20: Electropherogram results from bioanalyzer of W83 RNA from 04/10/2014

Figure 21: Electropherogram results from bioanalyzer of V3128 RNA from 04/10/2014
Figure 22: Electropherogram results from bioanalyzer of W83 RNA from 04/14/2014

Figure 23: Electropherogram results from bioanalyzer of V3128 RNA from 04/14/2014
4.6.2. High throughput sequencing of RNA libraries

The samples were sequenced using Rapid flow cell (VCU Sanger Hall). From the high throughput sequencing results, we were able to align the DNA fragments along the *P. gingivalis* genome. Across the whole genome were areas of high copy numbers which can be seen in Figure 24.

![High throughput sequencing reads aligned with P. gingivalis W83 genome. The y-axis represents the number of reads and the x-axis represents the region of the genome.](image)

**Figure 24:** High throughput sequencing reads aligned with *P. gingivalis* W83 genome. The y-axis represents the number of reads and the x-axis represents the region of the genome.

4.6.3. Statistical Analysis

We performed statistical analysis of our transcriptomic results by looking at the reads and fold changes of the genes. Figure 25 shows that 16s rRNA had the highest reads recorded. By looking at the fold changes we were able to determine which genes had been up-regulated and down-regulated in V3128 compared to W83. Table 7 lists the number of genes regulated from RNA samples prepared on 3/06/14. Table 8 lists the number of genes which were up-regulated and down-regulated in RNA samples from 4/10/2014. Table 9 lists the number of genes which were up-regulated and down-regulated in RNA samples from 4/14/2014. Figure 26 shows the most down-regulated
and up-regulated genes from RNA samples prepared on 3/06/2014. These include PG1511, PG0337, PG1710, PG0711, PG0835, PG1167, PG2104, PG2224, PG0043, PG0042, PG0787 and PG1234. Figure 27 shows the most down-regulated and up-regulated genes from RNA samples prepared on 4/10/2014. Some of the most down-regulated genes include PG1310, PG0717, PG1312, PG1109, PG1858, PG0566, PG1522, PG1690, PG1498, PG1311, \( atpK \), PG0785. Some of the up-regulated genes include \( rpsR \), PG0326, PG0065, PG0302, PG0071, \( lpxA \), PG0069, PG0223, \( lpxB \), PG0086, \( abfT-1 \), PG0475. Figure 28 shows the most down-regulated and up-regulated genes from RNA samples prepared on 4/14/2014. Some of the most down-regulated genes include PG0785, PG1779, PG0439, \( murC \), PG0059, PG0843, \( radC \), PG2006, PG1407, \( uraA \), PG1042, PG1667. Some of the most up-regulate genes include \( rpsR \), PG1528, PG0217, PG0327, \( ngrC \), \( fabF \), PG1156, PG0430, \( lpxB \), \( uvrB \), PG1954, PG0133. The variability in expression of the genes seen in figure 26, 27 and 28 could be due to the fact that each time V3128 grows somewhat differently each time it is grown on a new plate. We were interested to see how TonB-related genes, metal regulation genes, hemin regulation genes and genes involved in virulence of \( P.\ gingivalis \) were regulated in V3128 compared to W83.

Table 10 lists genes of interest that were down-regulated and up-regulated. PG0785, which is our putative TonB gene, was down-regulated in all three samples. PG0707, PG0668, PG1899 and PG2008 are all putative TonB dependent receptors which are down-regulated in two of the three sequencing samples. \( hmuR \), \( hmuY \) and \( tlr \) are genes involved in hemin binding on surface of \( P.\ gingivalis \) and they were down-regulated in all three samples with the exception of \( hmuR \) which was up-regulated in
RNA sequencing sample from 4/14/2014. Pg0646, PG0647, PG0648, PG0671, PG0672 are all genes involved in the iron ABC transporter which is crucial for uptake of iron in *P. gingivalis*. These genes have been down-regulated in two of the three sequences and up-regulated in one of the sequences. RagA is also involved in iron uptake and it has been down-regulated in two of the three sequences. PG1312 is believed to be a *capA* gene involved in capsulation of *P. gingivalis*. It was slightly down-regulated in RNA sample from 3/06/14, significantly down-regulated in RNA sequence from 4/10/14 (3rd most down-regulated gene) and slightly up-regulated in RNA sequence from 4/14/2014. PG1975, PG1837 and PG1972 are all hemagglutinin genes which are involved in adherence of the bacteria with epithelial cells which is important for the virulence of *P. gingivalis*. PG1975 is down-regulated in all three sequences, PG1837 is down-regulated in two of the three sequences and PG1972 is also down-regulated in two of the three sequences. The arginine-specific protease (*prtRII*) gene was slightly up-regulated in two of the sequences and significantly down-regulated in the other sequence.

**Figure 25: All sequence reads aligned to *P. gingivalis* W83 genome.** The y-axis represents the intensity of individual reads and x-axis represents regions of the genome. The selected region in this figure shows that the 16s rRNA intensities are the four largest peaks.
Table 7: Number of genes up-regulated and down-regulated from samples prepared on 3/06/2014

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Table 8: Number of genes up-regulated and down-regulated from samples prepared on 4/10/2014

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Table 9: Number of genes up-regulated and down-regulated from samples prepared on 4/14/2014

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<td>11</td>
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Figure 26: Genes up-regulated and down-regulated from RNA samples (3/06/2014). * “A” depicts the most down-regulated genes in V3128 compared to W83 while “B” depicts the most up-regulated genes in V3128 compared to W83. (1) Gene identification in *P. gingivalis*. (2) The fold change is comparing the RPKM values of V3128 to W83. (3) RPKM: the reads by kilobase transcript per million reads is reflective of the number of reads for each respective gene in W83. (4) RPKM: the reads by kilobase transcript per million reads is reflective of the number of reads of each respective gene in V3128.
Figure 27: Genes up-regulated and down-regulated from RNA samples (4/10/2014). * “A” depicts the most down-regulated genes in V3128 compared to W83 while “B” depicts the most up-regulated genes in V3128 compared to W83. (1) Gene identification in *P. gingivalis*. (2) The fold change is comparing the RPKM values of V3128 to W83. (3) RPKM: the reads by kilobase transcript per million reads is reflective of the number of reads for each respective gene in W83. (4) RPKM: the reads by kilobase transcript per million reads is reflective of the number of reads of each respective gene in V3128.
**Figure 28: Genes up-regulated and down-regulated from RNA samples**

(4/14/2014). *“A”* depicts the most down-regulated genes in V3128 compared to W83 while “B” depicts the most up-regulated genes in V3128 compared to W83. (1) Gene identification in *P. gingivalis*. (2) The fold change is comparing the RPKM values of V3128 to W83. (3) RPKM: the reads by kilobase transcript per million reads is reflective of the number of reads for each respective gene in W83. (4) RPKM: the reads by kilobase transcript per million reads is reflective of the number of reads of each respective gene in V3128.

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* This table contains our genes of interest. (1) Gene identification obtained from NCBI. (2) Description of the respective gene. (3) Fold changes of all three RNA sequencing samples. It compares the RPKM values of V3128 to W83. The fold change values tell us if our genes of interest have been up-regulated or down-regulated in V3128 compared to W83.
4.7 Metal Content Assay

To determine if PG0785 played a role in metal regulation, we performed metal content assays on both W83 and V3128. W83 and V3128 strains from blood and BHI plates were prepared as indicated in the “Materials and Methods” section.

4.7.1 Metal Content using ICP-MS Analysis

Samples were analyzed by using an ICP-MS to determine the amount of iron (Fe), magnesium (Mg), manganese (Mn), zinc (Zn), nickel (Ni), copper (Cu) and cobalt (Co) in each sample. Metal content results for Ni, Cu, Zn and Co are shown on Figure 29 and results for Fe, Mg and Mn are shown on Figure 30. For Ni, W83 from blood plate had a ppt of 27,706 compared to V3128 from blood plate which had a ppt of 33,219. W83 from BHI plate had a ppt of 30,146 compared to V3128 from BHI plate which had a ppt of 33,549. For Cu, W83 from blood plate had a ppt of 37,483 compared to V3128 from blood plate which had a ppt of 22,106. W83 from BHI plate had a ppt of 69,501 compared to V3128 from BHI plate which had a ppt of 46,020. For Zn, W83 from blood plate had a ppt of 68,643 compared to V3128 from blood plate which had a ppt of 140,527. W83 from BHI plate had a ppt of 72,208 compared to V3128 from BHI plate which had a ppt of 82,752. For Co, W83 from blood plate had a ppt of 6,434 compared to V3128 from blood plate which had a ppt of 7,401. W83 from BHI plate had a ppt of 15,582 compared to V3128 from BHI plate which had a ppt of 53,706. These results indicated that there was not a significant difference between V3128 and W83 in uptake of Ni, Cu, Zn and Co. In fact, V3128 had higher values for these metals than W83.
For Mg, W83 from blood plates had a PPT of 3,079,268 compared to V3128 from blood plates which had 743,942. W83 from BHI plates had a ppt of 1,210,178 compared to V3128 which had a ppt of 425,705. For Mn, W83 from blood plate had a ppt of 372,233 compared to V3128 from blood plate which had a ppt of 86,091. W83 from BHI plate had a ppt of 150,636 compared to V3128 from BHI plate which had a ppt of 161,591. For Fe, W83 from blood plate had a ppt of 3,395,185 compared to V3128 from blood plate which had a ppt of 164,824. W83 from BHI plate had a ppt of 137,910 compared to V3128 from BHI plate which had a ppt of 203,222. These results indicate that W83 had significantly higher values of Fe, Mn and Mg than V3128 suggesting that PG0785 could play a major role in uptake of these metals.

**Figure 29: Ni, Cu, Zn and Co content of V3128 and W83 metals measured.** Samples were analyzed using ICP-MS. Blue bars represent V3128 from BHI, green bars represent W83 from BHI, Red bars represent V3128 from BP and purple bars represent W83 from BP. Metal concentrations are in parts per trillion (PPT)
Figure 30: Fe, Mn and Mg content of V3128 and W83. Samples were analyzed using ICP-MS. Blue bars represent V3128 from BHI, green bars represent W83 from BHI, Red bars represent V3128 from BP and purple bars represent W83 from BP. Metal concentrations are in parts per trillion (PPT).

### 4.7.2 Metal Content using Ferrozine Assay

The ICP-MS we were using had major maintenance issues and as a result we decided to perform ferrozine assay as an alternative method for quantifying the Fe concentration in our samples. Samples were prepared using the same protocol as samples for ICP-MS analysis. Samples were then treated with Ferrozine which interacts with ferrous iron and forms a purple colored solution. The absorbance was measured at 562 nm using a mass spectrophotometer. Five sets of experiments were done with freshly prepared samples. The absorbance of the four standards (0.5 ppm = 0.87, 1 ppm = 0.198, 2 ppm = 0.398 and 2.5 ppm = 0.490) were measured and used to make a
scatter plot (Figure 31). The plot gave us an $R^2 = 0.997$ and an equation set at $y = 0.2101x$. This equation was used to determine the Fe concentration of our samples. Table 11 shows the concentration of Fe from each sample in ppm. The results show that there was no significant difference in Fe concentration between V3128 and W83.

**Figure 31: Plot of standards.** Ferrozine was added to the standards and the absorbance of each standard was measured at 562 nm. The y-axis represents the absorbance of the standards. The plot gave us an $R^2$ value of 0.9972 and an equation set at $y = 0.2102x$. This equation was used to determine the Fe concentration of our samples.

**Table 11: Concentration of Fe from V3128 and W83 using the ferrozine assay**

<table>
<thead>
<tr>
<th>Bacterial Samples(1)</th>
<th>Absorbance (562 nm)(2)</th>
<th>Concentration (PPM) of Fe(3)</th>
</tr>
</thead>
<tbody>
<tr>
<td>V3128 from blood plate</td>
<td>0.1430</td>
<td>0.6803</td>
</tr>
<tr>
<td>W83 from blood plate</td>
<td>0.1462</td>
<td>0.6960</td>
</tr>
<tr>
<td>V3128 from BHI plate</td>
<td>0.1431</td>
<td>0.6808</td>
</tr>
<tr>
<td>---------------------</td>
<td>--------</td>
<td>--------</td>
</tr>
<tr>
<td>W83 from BHI plate</td>
<td>0.0847</td>
<td>0.4029</td>
</tr>
</tbody>
</table>

* (1) Indicates the bacterial samples used. (2) The absorbance measurements of our bacterial samples. (3) The Fe concentrations of the bacterial samples.

### 4.8 Protease Assay

Protease assays were done to see if PG0785 could play a role in protease activity. Three biological repeats of the protease assay were done for W83 and V3128 strains grown on blood and BHI plates. The absorbance at 405 nm for each experiment was measured at 30, 60, 90 and 180 minutes. The results for all three experiments were averaged together. The absorbance for the arginine protease activity of W83 from blood plates had an average of 0.88 at 30 minutes, 1.27 at 60 minutes, 1.99 at 90 minutes and 2.7 at 180 minutes. The absorbance of V3128 from blood plates was 0.252 at 30 minutes, 0.321 at 60 minutes, 0.371 at 90 minutes and 0.475 at 180 minutes. Figure 32 shows a plot of the arginine protease activity for W83 and V3128 strains from blood plates. The absorbance of W83 from BHI plates was 1.47 at 30 minutes, 2.46 at 60 minutes, 2.99 at 90 minutes and 3.40 at 180 minutes. The absorbance of V3128 from BHI plates was 0.239 at 30 minutes, 0.293 at 60 minutes, 0.356 at 90 minutes and 0.437 at 180 minutes. Figure 33 shows a plot of the arginine protease activity for W83 and V3128 strains from BHI plates. These results showed that W83 from both blood and BHI plates had significantly higher arginine-specific protease activity than V3128.
The absorbance for the lysine protease activity of W83 from blood plates was 0.421 at 30 minutes, 0.733 at 60 minutes, 0.923 at 90 minutes and 1.540 at 180 minutes. The absorbance for the V3128 from blood plates was 0.180 at 30 minutes, 0.236 at 60 minutes, 0.300 at 90 minutes and 0.470 at 180 minutes. Figure 34 shows the plot of the lysine protease activity for W83 and V3128 strains from blood plates. The absorbance for the W83 from BHI plates was 0.561 at 30 minutes, 0.993 at 60 minutes, 1.272 at 90 minutes and 2.005 at 180 minutes. The absorbance for V3128 was 0.180 at 30 minutes, 0.231 at 60 minutes, 0.274 at 90 minutes and 0.415 at 180 minutes. Figure 35 shows the plot of the lysine protease activity for W83 and V3128 from BHI plates. These results indicate that W83 from both blood and BHI plates has significantly higher lysine-specific protease activity than V3128.
Figure 32: Arginine protease activity of W83 and V3128 grown on blood plates.

V3128 and W83 from blood plates were prepared as indicated in the “Materials and Methods” section. BAPNA was added to each sample and absorbance was measured at 405 nm at 30, 60, 90 and 180 minutes. The red squares represent W83 from blood plates and blue diamonds represent V3128 from blood plates. The x-axis represent the time in minutes of the absorbance recordings. The y-axis represents the activity of proteases by monitoring absorbance at 405 nm.
Figure 33: Arginine protease activity of W83 and V3128 grown on BHI plates.

V3128 and W83 from blood plates were prepared as indicated in the “Materials and Methods” section. BAPNA was added to each sample and absorbance was measured at 405 nm at 30, 60, 90 and 180 minutes. The red squares represent W83 from blood plates and blue diamonds represent V3128 from blood plates. The x-axis represent the time in minutes of the absorbance recordings. The y-axis represents the activity of proteases by monitoring absorbance at 405 nm.
Figure 34: Lysine protease activity of W83 and V3128 grown on blood plates.

V3128 and W83 from blood plates were prepared as indicated in the “Materials and Methods” section. Z-Lys-pNA was added to each sample and absorbance was measured at 405 nm at 30, 60, 90 and 180 minutes. The red squares represent W83 from blood plates and blue diamonds represent V3128 from blood plates. The x-axis represent the time in minutes of the absorbance recordings. The y-axis represents the activity of proteases by monitoring absorbance at 405 nm.
Figure 35: Lysine protease activity of W83 and V3128 grown on BHI plates. V3128 and W83 from blood plates were prepared as indicated in the “Materials and Methods” section. Z-Lys-pNA was added to each sample and absorbance was measured at 405 nm at 30, 60, 90 and 180 minutes. The red squares represent W83 from blood plates and blue diamonds represent V3128 from blood plates. The x-axis represent the time in minutes of the absorbance recordings. The y-axis represents the activity of proteases by monitoring absorbance at 405 nm.
CHAPTER 5: DISCUSSION

Iron uptake is vital for bacterial survival inside the host. *P. gingivalis* is one of the main etiological agents involved in initiation and progression of periodontal disease. *P. gingivalis* survival inside the periodontal pocket relies on its ability to take up iron in the form of hemin which it does by proteolytic breakdown of hemin containing proteins. The TonB/ExbB/ExbD is believed to play a major role in uptake of hemin. However, currently nothing is known the role and mechanism of TonB in *P. gingivalis*. As a result we generated a putative TonB deficient *P. gingivalis* mutant to further investigate the role of TonB in *P. gingivalis*.

Using bioinformatics, our lab identified two genes, PG0785/0707 and PG1912 that were further investigated as putative TonB genes. BLAST results for PG0785 showed that the nucleotide sequence of this gene had sequence similarities to four other genes that had TonB related features (Table 5). BLAST analysis also showed that the protein sequences of PG0785 had 33% similarity with Chain B of Btub:tonB complex, 32% similarity with Chain A of *E. coli* TonB, 32% similarity with Chain C of TonB in complex with FhuA outer membrane receptor and 31% similarity with Chain A of TonB2 of *Vibrio anguillarum* (Table 6). We also generated a homology model for PG0785 (Figure 5) using one to one threading by Phyre. We then used chimera to overlay this homology model of PG0785 with Chain A of C-terminal domain of *E. coli* TonB (Figure 6). There were some minor differences between the structures but overall they showed very strong similarities with each other. All of the bioinformatics information led us to believe that PG0785 would be a good candidate to further investigate as a possible *P. gingivalis* TonB protein.
In order to test our hypothesis, we first aimed to generate a *P. gingivalis* mutant that had a disrupted PG0785 gene. We were provided pCR2.1PG0785 plasmid which had a non-functional PG0785 gene + ErmF-AM cassette construct. PCR was first done on pCR2.1PG0785 plasmid to determine the size of the construct. Lanes 2 and 3 in Figure 7 show that the construct had a fragment at 3 kb when ran on 1% agarose gel. We expected that successful transformation would yield mutants that would have the same size 3 kb fragment. We used electroporation to try and transform pCR2.1PG0785 into wild type *P. gingivalis* W83 (W83) cells. Selected colonies were screened using PCR and ran on 1% agarose gel. Lane 3 in Figure 10 shows that a pCR2.1PG0785 + W83 colony selected from a blood plate had a 3 kb fragment suggesting that the PG0785 + ErmF-AM cassette construct had been inserted into the W83 genome and we had successfully generated a *P. gingivalis* mutant that had a non-functioning PG0785 gene. We refer to this mutant as V3128. We were never able to generate a *P. gingivalis* mutant that had PG1912 knocked out.

There were significant differences between the phenotype of W83 and V3128 when grown on blood and BHI plates. One of the key characteristics of *P. gingivalis* is its ability to become dark pigmented on blood plates due to accumulation of hemin on its surface. When grown on blood plates, our W83 became dark pigmented after one week on blood plates (Figure 11). However, V3128 never became dark pigmented on blood plates meaning that hemin could not accumulate on its surface (Figure 9). This suggested that PG0785 could potentially play a pivotal role in binding of hemin to the surface of *P. gingivalis*.
Our second aim was to determine the metal content of V3128 to determine whether PG0785 plays role in metal uptake. Multiple studies have suggested that TonB is involved in uptake of hemin which is critical for growth of bacteria. We performed comparative metal content assays between W83 and V3128. We grew both strains on blood and BHI plates and used an ICP-MS to quantify the amount of metal in each sample. We expect our wild type strain to contain significantly higher levels of iron than the mutant strain. Figure 29 shows the content for Ni, Cu, Zn and Co. We can see that in W83 there was not a significant increase in any of these metals compared to the mutant. In fact V3128 had elevated levels of Ni, Zn and Co. Figure 30 shows the results for Mg, Mn and Fe. W83 had significantly higher concentrations of Mg and Mn compared to V3128. But more importantly, Fe levels were significantly higher in W83 from blood plates than V3128 from blood plates. In fact W83 had an iron content of 3,395,185 which was the highest recorded metal.

*P. gingivalis* acquires iron in the form of hemin. Our results showed that V3128 contains significantly less iron but we cannot make the assumption that V3128 also has significantly less hemin levels until we perform hemin uptake analysis. Based on these results we can see that PG0785 potentially plays an important role in iron uptake since V3128 has significantly less iron concentration than W83. Unfortunately the ICP-MS we were using became damaged and as a result we were only able to perform one metal content assay using ICP-MS.

We decided to perform colorimetric ferrozine assays to further analyze the Fe content of our samples. We expected W83 from blood plates to have the highest absorbance and concentration. Table 11 shows the results of our ferrozine assays. We
can see that W83 from blood plate had very similar Fe concentration to V3128. One reason could be the method that we used to prepare our samples. The protocol we were using for the ferrozine assay did not indicate how to prepare samples. As a result we prepared our samples for the ferrozine assay just like we did for ICP-MS. This might not have been a suitable way of preparing the samples, especially since it took about 24 hours for the color change compared to the 10 minutes that was indicated in the protocol. We also realized that our results from the ferrozine assay were not useful because this assay measured the ferric iron levels. However, we were interested in the ferrous iron levels because that is the form of iron that *P. gingivalis* requires for its growth. In order to get useful results, this ferrozine assay needs to be done in an anaerobic chamber.

Our third aim was to determine the protease activity of V3128 and W83. Protease assays were done to compare the arginine-specific and lysine-specific cysteine protease activity of both V3128 and W83. The results of the three experiments were averaged together and are shown in Figures 32, 33, 34 and 35. Figures 32 and 33 show that W83 from both blood and BHI plates has significantly higher arginine-specific proteases activity than V3128 from blood and BHI plates. Figures 34 and 35 also show that W83 has significantly higher lysine-specific protease activity than V3128 from blood and BHI plates. Since V3128 was previously shown to contain significantly less iron than W83, we can assume that the low iron levels could potentially play a significant role in production of proteases in *P. gingivalis*. A study by Carman et al. showed that *P. gingivalis* W50 strains grown in absence of hemin had significantly less trypsin-like protease activity than strains grown in presence of hemin (Carman et al., 1990). Hemin
is made from iron and protoporphyn IX which means that low hemin levels could mean low iron levels. These results suggest that PG0785 could be crucial to the protease activity of \textit{P. gingivalis}.

Our fourth aim was to determine the virulence ability of V3128 and W83 by performing total interaction and invasion assays. Our results showed that PG0785 can indirectly play a crucial role in \textit{P. gingivalis} virulence. W83 and V3128 from blood plates were used for both total interaction and invasion assays. The number of colonies from all five replicates was averaged together and the results can be seen in Figures 14 and 15. We can see based on these results, that W83 had a significantly elevated level of interaction with HUVEC cells than V3128. W83 also invaded HUVEC cells at a significantly higher level than V3128. These results suggest that the virulence of V3128 has significantly decreased compared to W83. This decrease in virulence could be due to the lack of hemin attachment to the surface of V3128 and decrease in iron uptake by V3128. Hemin on the surface of \textit{P. gingivalis} can form $\mu$-oxo dimers which are two Iron (III) Meso-Tetraphenylporphines connected together by an oxygen. Generation of these $\mu$-oxo dimers helps protect the bacterium in presence of reactive oxygen compounds (Smalley et al., 1998). We have demonstrated that V3128 cannot accumulate hemin on its surface and thus could be damaged in presence of reactive oxygen species. Studies have also demonstrated that \textit{P. gingivalis} grown in hemin-limited conditions have decreased virulence in mouse models than those grown in hemin-excess conditions (McKee et al., 1986). Another group found that a \textit{P. gingivalis} mutant defective in \textit{prtH} protease had significantly less virulence than wild type (Fletcher et al., 1995). These two studies show that low hemin/iron concentrations and decreased protease activity can
lead to a decreased virulence. We have already demonstrated that V3128 has significantly lower iron levels and protease activity compared to W83 which could be one reason why V3128 virulence is decreased compared to W83.

Flow cytometry was also done to compare the interaction and invasion ability of V3128 with W83. Both V3128 and W83 were labeled with FITC which allows us to measure fluorescence using a flow cytometer. For total interaction, bacteria were incubated with HUVEC cells and fluorescence was measured. For invasion, bacteria were allowed to incubate with HUVEC cells and then Trypan blue was added to kill extracellular cells so that only the fluorescence of invading bacteria could be measured. We expected that W83 would have higher fluorescence for both interaction and invasion. However, what we saw with our results was the V3128 and W83 had higher median fluorescence then invasion but overall V3128 had higher numbers then W83 (Figure16). Although we had shown that interaction has higher fluorescence than invasion, we could not figure out why V3128 had higher numbers. We believe that the protocol possibly needs to be changed in order for flow cytometry to give us better numbers.

Proteomics was done on both W83 and V3128 to identify proteins that had been up-regulated and down-regulated. Both solutions and gel bands (Figure 16) were prepared and sent for analysis. Unfortunately we did not get usable results for our solutions. This could be due to protocol that we used to prepare our samples. The proteomics center was also down for two months thus we could not submit any more samples. Our gel bands, however, did yield some proteins that we were able to analyze using our transcriptomics results. The major proteins identified from gel bands were
lipoprotein RagB, RagA, 4-hydroxybutyryl-CoA dehydratase (abfD), arginine-specific cysteine protease, hemmagglutinin protein HagA and HagE, outer membrane lipoprotein Omp28 (omp28), immunoreactive 43 kDa antigen PG32, ABC transporter ATP-binding protein, NAD-specific glutamate dehydrogenase (GDH), peptidylarginine deiminase and a Dps family protein. Based on our RNA sequencing results which we will discuss later we were able to determine that ABC Transporter ATP-binding protein, HagA, HagE, arginine-specific cysteine protease, RagA and RagB were all down regulated and Omp28 was up-regulated. RagA and RagB are both TonB linked receptors on the surface of *P. gingivalis* and the fact that both were down-regulated in V3128 which has a non-functional PG0785 gene tells us that PG0785 could be a putative TonB protein. Arginine-protease activity was also down-regulated which correlates with our protease assay results which show that protease activity of V3128 had significantly decreased compared to W83.

The sequencing results allowed us to align the reads of the cDNA fragments with the *P. gingivalis* genome and to compare reads of W83 and V3128. Figure 25 shows that the four largest reads from the libraries were 16s rRNA. The kit we used was supposed to deplete rRNA but we have shown that it has not completely depleted the rRNA. We would like our libraries to have very low rRNA levels and thus we plan on generating another library from samples that have first been treated to have rRNA removed. Tables 7, 8 and 9 list the number of genes up-regulated and down-regulated in V3128 based on their fold change. Figures 26, 27 and 28 show the most up-regulated and down-regulated genes from the three different RNA sets. Our genes of interest
included TonB-related genes, metal uptake genes, hemin uptake genes and genes involved in virulence.

We expected that our RNA sequencing results would show that PG0785 would be one of the most down-regulated genes in V3128 since this is the gene that we knocked out. Our first sequencing result from 03/06/2014 showed that PG0785 was down-regulated but it was not one of the most down-regulated genes as we expected. However, our RNA sequencing result from 4/10/2014 showed that PG0785 was the most down-regulated gene. Our third RNA sequencing result from 4/14/2014 showed that PG0785 was the 12th most down-regulated gene. This showed that we have significantly down-regulated the gene for our candidate TonB protein. Further analysis showed that many other TonB linked genes had also been down-regulated.

PG0668, PG0707, PG1899 and PG2008 are all identified as coding for a putative TonB linked outer-membrane receptors which could play a role in hemin binding. All four have been down-regulated in our RNA sequencing sample from 4/10. This is very encouraging because we would expect that down-regulation of TonB would lead to down-regulation of TonB-linked outer-membrane receptors. Three other interesting genes that have been down-regulated are hmuR, hmuY and tlr. HmuR is a TonB-linked outer membrane receptor that is believed to bind hemin and transport it across the outer membrane (Simpson et al., 2000). Our results showed that this gene was down-regulated in two of the three sequencing results. Down-regulation of this gene could explain why V3128 does not accumulate hemin on its surface compared to W83. HmuY is also a hemin binding protein and Tlr is TonB-linked hemin binding receptor on the surface of P. gingivalis (Nelson et al., 2003). The genes coding for these
proteins have been down-regulated in all three of our RNA sequencing results which provides further evidence for the lack of hemin accumulation on surface of *P. gingivalis* and could also affect hemin transport across the cell.

PG1875 is believed to be a hemolysin involved in breakdown of hemin containing proteins which allows for release of hemin and uptake by *P. gingivalis*. This gene has been down-regulated in all three RNA sequencing results. Down-regulation of this gene hinders the ability of V3128 to breakdown hemin containing proteins leading to a decrease in available hemin and iron. This could also attribute to the lack of hemin accumulation on surface of V3128 and the significantly lower iron levels seen in our metal content assay result. Since *P. gingivalis* obtains the majority of its iron in the form of hemin, then down-regulation of these genes could also attribute to the decrease of iron in V3128. Down-regulation of PG0668, PG0707, PG1899, PG2008, *hmuR*, *hmuY*, *tlr* and PG1875 in V3128 tells us that PG0785 could be very important in binding and uptake of hemin in *P. gingivalis*, a process that TonB is believed to play an important role in.

We were also interested in genes coding for proteins involved in virulence of *P. gingivalis* to determine if PG0785 plays a role in virulence of the bacterium. Hemagglutinins are important in the adherence of the bacteria with eukaryotic cells and play a major role in virulence of *P. gingivalis*. Our RNA sequencing results showed that hemagglutinin genes *hagA*, *hagB* and *hagC* were mostly down-regulated. This could attribute to the significant decrease seen in the virulence of V3128. The *capA* gene is involved in encapsulation of *P. gingivalis*. Capsules help protect the bacteria against the immune system and also play an important role in virulence of *P. gingivalis*. The *capA*
gene was significantly down-regulated in one of our RNA sequences. Down-regulation of this gene could also attribute to the significant decrease seen in virulence of V3128. Proteases are involved in breaking down hemin-containing proteins and also play a major role in virulence of P. gingivalis. Arginine-specific cysteine protease (PrtRII) was slightly up-regulated in two of the RNA sequences and significantly down-regulated in one of the sequences. Down-regulation of this gene could provide evidence for both decrease of virulence and protease activity seen in V3128. Hemin on surface of P. gingivalis also plays a role in protecting the bacteria from reactive oxygen species. Down-regulation of gene involved in hemin-binding could also provide evidence for the decrease in virulence of V3128. Overall, knocking out PG0785 resulted in mostly down-regulation of genes involved in virulence which indicates that PG0785 could play a significant role in virulence of P. gingivalis.

We have already shown that iron levels had been significantly reduced in V3128. As a result we expected that genes involved in iron uptake would be down-regulated in V3128. PG0646, PG0647, PG0648, PG0671 and PG0672 are all genes coding for proteins that are part of the iron compound ABC transporter which is involved in uptake of iron in P. gingivalis (Nelson et al., 2003). Two of our sequencing results showed that all five genes had been down-regulated in V3128. Down regulation of these genes could explain why there is significantly lower level of iron in V3128 compared to W83. RagA is a TonB linked outer-membrane receptor involved in iron uptake (Nelson et al., 2003). The ragA gene was also down-regulated in two of our sequencing results. Down-regulation of this gene could also provide evidence for the significant decrease of iron in
V3128. Overall down-regulation of these genes due to mutation of PG0785 shows that PG0785 could play an important role in iron uptake.

In conclusion, we were able to demonstrate that PG0785 is important in binding of hemin to surface of *P. gingivalis*. We were also able to show that PG0785 plays an important role in uptake of iron, protease activity and virulence of *P. gingivalis*. V3128 contains significantly less iron than W83. This lack of iron due to disruption of PG0785 could be the reason why protease activity and virulence of *P. gingivalis* were significantly decreased. Translational and transcriptional analysis of V3128 showed us that numerous TonB-related genes, metal uptake genes, hemin uptake genes and genes involved in virulence had been down-regulated in V3128. Down-regulation of these genes suggests that PG0785 is a very important protein in the biology of *P. gingivalis*. It is important to note that the TonB protein in other bacteria has also been shown to play important roles in the biology of those bacteria. Although we have shown that PG0785 plays an important role in *P. gingivalis*, we cannot make the assumption that PG0785 codes for the TonB protein. TonB is involved in energy transduction in other bacterial species thus further investigation of mechanisms of energy transduction in *P. gingivalis* needs to be done. If PG0785 is shown to play a crucial role in energy transduction in *P. gingivalis* then that provides more definitive evidence that PG0785 is the TonB protein of *P. gingivalis*. 
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