CHARACTERIZATION OF MUTANTS OF THE *Porphyromonas gingivalis* STRAIN W83

By

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To my parents, Gloria and Joseph Rainho
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TABLE OF CONTENTS

ACKNOWLEDGMENTS ...............................................................................................................4

LIST OF TABLES ...........................................................................................................................7

LIST OF FIGURES .........................................................................................................................8

ABSTRACT ...................................................................................................................................10

CHAPTER

1 INTRODUCTION ..................................................................................................................11

Porphyromonas gingivalis ......................................................................................................13
  Biofilms ...........................................................................................................................14
  Fimbriae ...........................................................................................................................16
  Hemagglutinins/ Hemolysis ............................................................................................17
  Gingipains ........................................................................................................................18
  Adhesion, Invasion and Persistence ................................................................................20

2 INVASION AND PERSISTENCE STUDIES .......................................................................24

Introduction .............................................................................................................................24
Material and Methods .............................................................................................................25
  Bacterial Strains and Growth Conditions ........................................................................25
  Mutant Construction ........................................................................................................26
  Growth Curves .................................................................................................................26
  Cell Culture .....................................................................................................................27
  Adhesion Assays .............................................................................................................27
  Invasion Assay................................................................................................................28
Results .....................................................................................................................................28
  Growth Curve ................................................................................................................28
  Adherence ........................................................................................................................28
  Invasion ...........................................................................................................................29
  Persistence ........................................................................................................................29
Discussion ...............................................................................................................................30

3 BIOFILM ...................................................................................................................... ........39

Introduction .............................................................................................................................39
Material and Methods .............................................................................................................40
  Bacterial Strains and Growth Conditions ........................................................................40
  Mutant Construction ........................................................................................................40
  Homotypic Biofilm ..........................................................................................................40
  Fluorescently labeling Porphyromonas gingivalis for Homotypic Biofilm ....................41
<table>
<thead>
<tr>
<th>Table</th>
<th>Description</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>2-1</td>
<td>Primers used for mutant construction in <em>P. gingivalis</em> W83. Genes were mutated by double crossover allelic exchange.</td>
<td>33</td>
</tr>
<tr>
<td>3-1</td>
<td>Chi square analysis values of biofilm peaks &gt;120 in intensity for <em>P. gingivalis</em> wild-type W83 strain and mutant W83Δ0092 at 24 and 48 hours.</td>
<td>50</td>
</tr>
<tr>
<td>3-2</td>
<td>Chi square analysis values of biofilm peaks &gt;120 in intensity for <em>P. gingivalis</em> wild-type W83 strain and mutant W83Δ1683 at 24 and 48 hours.</td>
<td>50</td>
</tr>
<tr>
<td>4-1</td>
<td>Phenotype summary. Summary of results of mutants and wild type.</td>
<td>70</td>
</tr>
</tbody>
</table>
LIST OF FIGURES

<table>
<thead>
<tr>
<th>Figure</th>
<th>Description</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>2-1</td>
<td>Bioinformatic analysis of the putative operon for <em>P. gingivalis</em> genes PG0091, PG0092, PG0093, and PG0094.</td>
<td>34</td>
</tr>
<tr>
<td>2-2</td>
<td>Bioinformatic analysis of the putative operon for <em>P. gingivalis</em> genes PG1681, PG1682, PG1683, and PG1684.</td>
<td>34</td>
</tr>
<tr>
<td>2-3</td>
<td>Growth curve of <em>P. gingivalis</em> strains W83, W83Δ0092 and W83Δ1683 in sTSB media</td>
<td>34</td>
</tr>
<tr>
<td>2-4</td>
<td>Adherence of <em>P. gingivalis</em> to HCAEC of strain W83 and W83Δ0092 in the presence of Cytochalasin D.</td>
<td>35</td>
</tr>
<tr>
<td>2-5</td>
<td>Adherence of <em>P. gingivalis</em> to HCAEC of strain W83 and W83Δ1683 in the presence of Cytochalasin D.</td>
<td>36</td>
</tr>
<tr>
<td>2-6</td>
<td>Invasion and Persistence of HCAEC for <em>P. gingivalis</em> W83 and W83Δ0092 in sTSB media</td>
<td>37</td>
</tr>
<tr>
<td>2-7</td>
<td>Invasion and Persistence of HCAEC by <em>P. gingivalis</em> W83 and W83Δ1683 in sTSB media</td>
<td>38</td>
</tr>
<tr>
<td>3-1</td>
<td>Microtiter plate monospecies biofilm production by <em>P. gingivalis</em> W83 and W83Δ0092 in sTSB media</td>
<td>47</td>
</tr>
<tr>
<td>3-2</td>
<td>Microtiter plate monospecies biofilm production by <em>P. gingivalis</em> W83 and W83Δ1683 in sTSB media</td>
<td>48</td>
</tr>
<tr>
<td>3-3</td>
<td>Confocal micrographs of monospecies biofilm production of <em>P. gingivalis</em> W83 and W83Δ0092 at 24 and 48 h in sTSB</td>
<td>49</td>
</tr>
<tr>
<td>3-4</td>
<td>Confocal micrographs of monospecies biofilm production of <em>P. gingivalis</em> W83 and W83Δ1683 at 24 and 48 h in sTSB media</td>
<td>51</td>
</tr>
<tr>
<td>3-5</td>
<td>Scanning electron micrographs of monospecies biofilm production of <em>P. gingivalis</em> mutants and wild type at 24 and 48 h in sTSB media</td>
<td>52</td>
</tr>
<tr>
<td>3-6</td>
<td>Mixed species biofilms of <em>P. gingivalis</em> W83, W83Δ0092 and <em>F. nucleatum</em> 22586 in sTSB media</td>
<td>53</td>
</tr>
<tr>
<td>3-7</td>
<td>Mixed species biofilms of <em>P. gingivalis</em> W83, W83Δ1683 and <em>F. nucleatum</em> 22586 in sTSB media</td>
<td>54</td>
</tr>
<tr>
<td>4-1</td>
<td>Electron micrograph of negative stains of <em>P. gingivalis</em> strains W83 and W83Δ0092 in sTSB and sBHI media</td>
<td>63</td>
</tr>
</tbody>
</table>
4-2. Electron micrograph of negative stains of *P. gingivalis* strains W83 and W83Δ1683 in sTSB and sBHI media. I. .................................................................64

4-3. Hemagglutination of *P. gingivalis* wild-type W83, W83Δ0092 and W83Δ1683 when grown in sTSB media.................................................................65

4-4. Hemagglutination of *P. gingivalis* wild-type W83, W83Δ0092 and W83Δ1683 when grown in sBHI media.................................................................65

4-5. Adherence to HCAEC for *P. gingivalis* W83 and W83Δ0092 in sBHI media. ..............66

4-6. Adherence to HCAEC by *P. gingivalis* W83 and W83Δ1683 in sBHI media. ..............67

4-7. Adherence of *P. gingivalis* W83 to HCAEC, previously grown in sTSB and sBHI media.................................................................68

4-8. Microtiter plate homotypic biofilm production by *P. gingivalis* W83 and W83Δ1683 in sBHI media.................................................................68

4-9. Microtiter plate homotypic biofilm production by *P. gingivalis* W83 and W83Δ0092 in sBHI media.................................................................69

4-10. Microtiter plate monospecies biofilm production by *P. gingivalis* W83 in sTSB and sBHI media. .................................................................69
CHARACTERIZATION OF MUTANTS OF THE *PORPHYROMONAS GINGIVALIS* STRAIN W83

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Periodontal disease is the second most common infectious disease, affecting 50 to 90% of adults world wide. *Porphyromonas gingivalis* is an important etiologic agent in the development of periodontal disease and may be involved in cardiovascular disease as well. The objective of this study was to characterize two genes of *P. gingivalis*: encoding a putative ABC transporter PG0092 and encoding PG1683 a putative glycosyl hydrolase/alpha amylase, both of which in previous studies were found to be up-regulated during host cell invasion. Previously, double crossover mutants of these genes were constructed in strain W83. For this study, mutant strains were tested in assays of adherence, invasion and persistence using human coronary artery endothelial cells, for biofilm development, hemagglutination and the presence of fimbriae in order to begin to characterize the roles of these genes during *P. gingivalis* infection. The effect of growth conditions on phenotype and gene expression was found to be critical to this analysis. These studies have provided additional information regarding the role of these genes in *P. gingivalis* pathogenesis.
Periodontal disease is the second most common infectious disease of humans, affecting 50 to 90% of adults worldwide and causing pathological changes to the supporting tissues of the teeth (1). Periodontal disease is a group of diseases that varies in severity, from mild and reversible inflammation of the gum (gingiva) to chronic damage of the periodontal tissues which include the gingival soft tissues, periodontal ligament, and alveolar bone. The most severe cases result in eventual exfoliation of the affected teeth (2). The initiation and progression of the disease is associated with the presence of a variety of organisms, making its bacterial etiology complex. In fact many of the pathogenic bacteria are present in individuals that are periodontally healthy and can exist with the host in collective harmony (2). As the ecological balance between bacterial and host factors shift, the result is a change in the quantity of specific organisms, alteration in gene expression and pathogenicity, and alteration of particular host factors (2). There is a shift from predominantly gram positive bacteria in a state of health to predominantly gram negatives in diseased conditions. Aggregatibacter actinomycetemcomitans, Porphyromonas gingivalis, and Tannerella forsythia are among the Gram negative microorganisms that are considered significant etiologic agents of periodontitis (3). P. gingivalis, Treponema denticola, and T. forsythia are a group of organisms frequently found together in the subgingival plaque and are members of the “red complex”, and have been strongly linked to advanced periodontal lesions (4). The members of the red complex, which are all Gram negative anaerobic bacteria, promote an immunodestructive host response leading to disease (5). These bacteria express a number of virulence factors, allowing the bacteria to colonize sites in the subgingiva, leading to disruption of the host defense system and invasion and destruction of the
periodontal tissues (5). It is this host immune response to the bacterial challenge that is the primary etiologic factor of periodontitis (5).

Numerous groups have reported associations between poor dental health and coronary heart disease, particularly atherosclerosis. For example, studies by Mattila have shown an association between cardiovascular disease (CVD) and periodontal disease, demonstrated a significant association between dental infections and severe coronary atheroma and reported that subjects with periodontitis were found to have a 25% increased risk of coronary heart disease (CHD) compared to those with minimal periodontal disease (6). There is also experimental evidence proving an epidemiological link between periodontal disease and atherosclerosis, including an association between \textit{P. gingivalis} and atherosclerosis in humans (7-10). For example, genomic DNA of periodontal pathogens including \textit{P. gingivalis} DNA, has been identified in atheromatous plaques (11). Animal studies have also proven that a relationship exists since oral infection with \textit{P. gingivalis} accelerates early atherosclerosis in apolipoprotein E-deficient (\textit{apo}-E) mice (12-14). Furthermore, a more extensive accumulation of lipids in the aortas of rabbits in which periodontitis had been induced was found than in periodontally healthy rabbits (15). Additionally, intravenous injection of \textit{P. gingivalis} in pigs lead to coronary disease and atherogenesis (16). Others in the Progulske-Fox laboratory have also been able to show that human atherosclerotic plaque contains live \textit{P. gingivalis} and \textit{A. actinomycetemcomitans} which, can in fact, adhere to and invade endothelial cells (17, 18). Recently, a study by Amar et al., with \textit{apo}-E mice, has concluded that the presence and invasion of \textit{Porphyromonas gingivalis} is critical to the progression of atherosclerosis (19).

Atheroma development and thromboembolic phenomena in cardiovascular diseases are thought to progress with systemic exposure to inflammatory mediators of periodontal origin (20,
21). These localized cytokines that are produced in response to periodontal infections can cause a systemic effect (20). The cytokines that are involved in artherosclerosis in vascular cells include IL-6, VCAM-1, MCP-1, IL-1, TNF-alpha, and IL-8 (22). Most often bacteria entry into host cells elicits cytokine production including IL-1B, IL-6 and IL-8, which in turn will attract phagocytes to this area (23). Since periodontal disease is also an inflammatory condition, periodontal infections may contribute to the progression of atherosclerosis simply by increasing inflammation (19). Invasion of coronary artery cells by periodontal bacterial species may initiate and/or aggravate an inflammatory response associated with atherosclerosis. In the gingival crevice, oral microorganisms cross the inflamed gingival barrier and enter the circulatory system (24). Therefore, interactions between *P. gingivalis* and the endothelial layer of the arteries may have a significant effect on the progression of atherosclerosis. Thus *P. gingivalis* can gain access to the vasculature and interact directly with the endothelial layer, by adhering to and invading the endothelial cells. This adherence/invasion then likely triggers signal transduction pathways, leading to an amplified inflammatory response and atheroma formation.

**Porphyromonas gingivalis**

*Porphyromonas gingivalis* (formerly *Bacteroides gingivalis*) is an obligately anaerobic, nonmotile, non-spore-forming, gram-negative bacterium that requires hemin as an iron source. It is a rod shaped organism that is asaccharolytic and acquires its source of energy and carbon from small peptides. It is believed that *Porphyromonas gingivalis* initially colonizes the mouth and is transmitted via infected individuals. It colonizes by attaching to available surfaces including components found in saliva that form a pellicle on oral surfaces or to other plaque bacteria. For example, it co-aggregates with some oral streptococci, *Actinomyces naeslundii*, and other late colonizers such as *Fusobacterium nucleatum*, *Treponema denticola* and *Tannerella forsythia*.
This co-aggregation likely not only promotes colonization but also nutritional interrelationships and intercellular signaling mechanisms (31).

_P. gingivalis_ produces several virulence factors as protection from host defenses allowing the bacteria to survive and thrive in the host. These virulence factors include a capsule, lipopolysaccharide, fimbriae, outer membrane proteins, such as hemagglutinins, outer membrane vesicles and a variety of enzymes including the Rgp and Kgp gingipains (23). Fimbriae are important for adherence to bacterial cells and receptors (see Fimbriae section). Hemagglutinins are proteins that serve as adhesins but also may allow for the attachment to erythrocytes that can be lysed to provide iron for nutrition and growth (see Hemagglutinins/Hemolysis section). Gingipain proteases have been shown to be important for adherence to the host cell and other bacteria in the host environment and cleave polypeptides at arginine and lysine residues. The gingipains also help support the growth of _P. gingivalis_ in vivo, inhibit host defense mechanisms and are involved in direct tissue destruction (23) (see Gingipains section). The functional and genetic determinants of these virulence factors are inevitably linked and allow for the invasive potential and subsequent overgrowth of _P. gingivalis_ resulting in disease activity (2).

**Biofilms**

Dental plaque is a biofilm of a complex organization of bacteria that develops in the oral cavity. Saliva coats the surfaces of target tissues and the primary colonizers, which are predominantly gram positive species, express biochemical components that allow for their attachment to many host derived molecules on this salivary pellicle (26, 32, 33). When the biofilm is not consistently removed, the biofilm thickens and gram negative bacteria, often containing components that allow them to adhere to early colonizers, begin to colonize the gingival area. This colonization of many bacteria leads to inflammation and the destruction of
host tissues supporting the teeth and can lead to the pathogenesis and the chronic disease known as periodontal disease (34).

Biofilm formation occurs in a series of steps. First, the primary colonizers must adhere to a surface, which brings about the alteration of their gene expression, allowing them to adjust to the environment of the surface to which they have attached (35). Assuming the environment is beneficial for growth, the bacteria will continue to grow on this surface, recruiting additional bacteria, eventually developing into an established biofilm encased in a matrix (35). Ultimately, the cycle is completed with the detachment of bacteria, which then disseminate and colonize new surfaces (35). The biofilm formation process is complex and requires the expression and coordinated regulation of many genes (35). Specific bacterial species will clump together when forming a biofilm, allowing for acquisition of nutrients, protection from mediators of host immunity and possibly exchange of genetic information (36).

*P. gingivalis* can adhere to the salivary pellicle but colonization in the oral plaque is delayed until the oxygen tension is reduced by predecessor organisms (37). *P. gingivalis* has been shown to autoaggregate and form homotypic biofilms. It has also been shown to colonize with oral streptococci, for example *S. gordonii*, and *A. naeslundii*, and form *in vitro* biofilms (37). The highly proteolytic properties of *gingivalis* allow this bacterium to colonize the subgingival plaque area and allow for other bacteria, which lack this capability, to co-localize with *P. gingivalis* and to benefit from the significant extracellular hydrolytic activity (33, 38). It frequently coexists with other bacteria associated with periodontal disease, such as *F. nucleatum*, *Prevotella intermedia*, *Treponema denticola*, and *Tannerella forsythia* (33, 39-42). *P. gingivalis* is vulnerable to pH levels lower than 6.5 and is sensitive to oxygen. Interestingly, it has been shown that *P. gingivalis* can grow in the presence of increased oxygen levels, as high as 20% O₂,
when co-cultured with *Fusobacterium nucleatum* (43). Studies have also shown that *F. nucleatum* can maintain an optimum pH and satisfy the CO₂ requirement for *P. gingivalis* growth and when co-infected can lead to increased expression of virulence factors and subsequent infection (43). Thus beyond supporting adherence and aiding in bacterial progression, co-aggregation may present additional benefits to species that interact.

**Fimbriae**

The initial step in bacteria colonization is adherence. For most bacteria, fimbriae, proteinaceous hair-like projections from the surface of bacteria, mediate specific adhesion to surfaces (44-47). The major fimbriae of *P. gingivalis* interact with salivary receptors for *P. gingivalis* including proline-rich proteins, glycoproteins, and statherin (2, 37). *P. gingivalis* has been shown to express more than one type of fimbriae; the major fimbriae (FimA), the minor fimbriae (mFa1) which are 67kDa in size and Pg-II which are 72kDa (48, 49). Depending on the strain, FimA fimbriae vary in size, between 41 and 49kDa proteins, are 3 to 5nm wide and 0.3 to 3.0μm long (2, 50). However, not all strains of *P. gingivalis* express FimA (51). FimA facilitates the adhesion and invasion of oral epithelial cells and are expressed in both noninvasive and invasive strains (51, 52). The minor fimbriae are involved in the development of micro-colonies and *P. gingivalis* biofilm maturation (53). *P. gingivalis* fimbriae may represent unique classes of gram-negative fimbriae, since there are no homologies to fimbrial proteins of other bacteria, when comparing protein sequences (16). Fimbriae of *P. gingivalis*, can facilitate adherence to a range of oral substrates and molecules.

FimA is the major structural component of *P. gingivalis* major fimbriae, but it is now known that additional proteins are part of the fimbrial structure and are encoded by genes directly downstream of FimA (54-56). The accessory proteins, including FimC, FimD, and FimE, with molecular masses of 50, 80 and 60 kDa, respectively, contribute considerably to *P.
*P. gingivalis* virulence but yet only comprise of $\leq 1\%$ of the fimbrial protein (54, 55). Studies in the mouse periodontitis model have shown a dramatical loss in virulence in *P. gingivalis* mutants that express *fim*A but are devoid of these accessory proteins (54).

Fimbriae and gingipains have been shown to act together in regulating *P. gingivalis* biofilm development (57). Specifically, a mutant deficient in FimA was able to initiate biofilm formation but was unable to form a mature biofilm (57). In this same study, the role of Mfa was found to be suppressive in the regulation of biofilm development. (57). The relationship of biofilm development and fimbriae remain unclear. For example, long fimbriae have been shown to suppress autoaggregation where small fimbriae have been shown to enhance it (57). In a second study, mutation of long fimbriae showed negligible autoaggregation and a mutant devoid of short fimbriae showed enhanced autoaggregation (58). Therefore, additional studies need to be done to fully understand the role of fimbriae in biofilm development.

**Hemagglutinins/ Hemolysis**

Hemagglutinin proteins are expressed by a number of bacterial species and are known virulence factors. *P. gingivalis* is reported to express at least five hemagglutinins that aid microbial binding to erythrocytes and host cells (2, 20). Hemagglutination genes in *P.gingivalis* include *hag* A, *hag* B, *hag* C, *hag* D, and *hag* E, which have all been cloned and sequenced (59-63). There are significant homologies among some of the hemagglutinins including *Hag* A and *Hag* D, 73.8% homology; *Hag* A and *Hag* E, 93% homology; and *Hag* B and *Hag* C, 98% homology (20, 61). *Hag* B induces proinflammatory cytokine responses in several rodent models, and is involved in *P. gingivalis* adherence to HCAEC, but is not sufficient for invasion into host cells (20, 64). *P. gingivalis* hemagglutinin activities may be complexed with lipopolysaccharide (LPS)(65), lipids on the cell surface (65) or released as 40-kDa activity designated as exhemagglutinin (2, 66). Some of the hemagglutinins, specifically *Hag* A, *Hag* D
and Hag E, have significant sequence homology to the gingipains, and associations have been found between hemagglutinating and proteolytic activities (20, 67-71). The *P. gingivalis* gingipains, as well as several other adhesion proteins, are complexed together to form a “hemagglutination complex”, all of which are transcribed from the same gene. Both the 50-kDa Arg-gingipain molecule and 60-kDa Lys-gingipain molecule are complexed with a 44-kDa hemagglutinin (72). It has also been suggested that fimbriae must be complexed to HA-Ag2 for full hemagglutination expression (73). The *P. gingivalis* hemagglutinins, along with the hemolysins and other enzymatic activities may promote colonization by aiding in the acquisition of hemin or iron (74, 75).

The ability of pathogens to grow in a particular niche requires the ability to acquire nutrients in that niche. Iron has a crucial role in the establishment and progression of an infection (76). An abundance of iron can be found intra-cellularly in the form of hemoglobin, ferritin or heme proteins. Thus many pathogens that occupy intracellular niches can utilize heme directly (23). *P. gingivalis* similarly has developed mechanisms that allow for the capture of iron and hemin, which are required for its growth (23). *P. gingivalis* is able to utilize a broad range of hemin containing compounds and is capable of storing hemin-containing compounds on its cell surface, giving *P. gingivalis* its black pigment (23). This likely allows the survival of *P. gingivalis* in a healthy periodontal pocket, which has limited iron concentrations.

**Gingipains**

*P. gingivalis* contains many hydrolytic, proteolytic and lipolytic enzymes that play significant roles in its virulence (23). The proteinases, especially the cysteine proteinases that cleave polypeptides at arginine and lysine residues have been given the most attention (23). These Arg- and Lys-proteinases are referred to as gingipains. Proteinases are important for adherence to the host cell and to other bacteria in the host environment, they help support the
growth of \textit{P. gingivalis} \textit{in vivo}, inhibit host defense mechanisms and are likely involved in direct tissue destruction (23). Proteases can be post-translationally processed for secretion into the extracellular milieu or localized to the cell surface (57). \textit{In vivo} experiments indicate that \textit{P. gingivalis} proteinases are functionally important since the species is asachrolytic and expresses an elaborate proteolytic system that serves to provide nutrients in the form of small peptides and amino acids, leading to tissue destruction (77).

In \textit{P. gingivalis}, there are at least three different genes that encode two cysteine arginine gingipains, \textit{rgp}A and \textit{rgp}B, and a lysine gingipain, \textit{kgp} (23). The polyproteins that encompass proteinases, Rgp and Kgp, are proteolytically processed and contain C-terminal adhesion domain (78). Specifically, \textit{rgp}A and \textit{kgp} contain separate adhesion/hemagglutinin domains that are catalytic and are non covalent complexes. The proteolytic activity of Kgp is vital in hydrolyzing the hemoglobin protein rapidly and therefore the RgpA-Kgp complexes may play a role in the disruption of vascular cells and binding and quick degradation of hemoglobin for \textit{P. gingivalis} heme assimiliation (78, 79). RgpB is also a proteinase on the cell surface that is very similar to that of RgpA, however it lacks the C-terminal adhesion binding motif that is found in the RgpA and Kgp catalytic domains (80).

Gingipains play a role in a variety of important functions, such as maturation of fimbriae, host protein amino acid uptake, bacterial housekeeping functions, development and infection (81). For example, studies have shown that the RgpA-Kgp proteinase-adhesion complexes are involved in colonization of \textit{P. gingivalis} by binding to crevicular epithelial cells and binding to other bacteria in the subgingival plaque, through the adhesion domains A1 and A3 (78, 82). Sustained colonization of \textit{P. gingivalis} is facilitated by the degradation of macrophage CD14 by gingipains, which inhibits activation of the leukocytes through the lipopolysaccharide (LPS)
receptor (81). Additionally, studies conclude that *P. gingivalis*, specifically HRgpA, a product of *rgpA* and RgpB, have the ability to activate the kallikrein/kinin pathway which induces vascular permeability and in turn activates the blood coagulation system (83-86). Theoretically this can be linked with the production of gingival crevicular fluid, which provide nutrients, and inflammation progression in the periodontitis site, leading to alveolar bone loss (78). Gingipains are also involved in the bleeding tendency at sites of periodontitis, even though the 3 gingipains degrade fibrinogen/fibrin. Kgp is the most potent enzyme in this regard (87). A recent study has also shown that gingipains are important in biofilm formation, Kgp in suppression and regulation of biofilm development, whereas Rgp affects morphology and biofilm volume (57). Furthermore, indications that polyphenolic inhibitors of gingipains can prevent both homotypic (*P. gingivalis*) and heterotypic ( *P. gingivalis* and *F. nucleatum*) biofilm formation, have been reported (88). In addition, a *P. gingivalis* mutant lacking Rgp lost the ability to form synergistic biofilms with *Treponema denticola* (89). However, more studies must be completed in order to better understand the roles of gingipains in biofilm development.

**Adhesion, Invasion and Persistence**

*P. gingivalis* has a variety of virulence factors which contribute to its pathogenesis and aid in its colonization, modulation of the host immune system and nutrient acquisition in the periodontal site. Colonization is an important step in pathogenecity and can be facilitated by adhesions, invasions and cell signaling effecting molecules. Adhesion molecules, such as fimbriae and hemagglutinins, allow bacteria to interact with host cells as well as other bacteria. Adherence can also be promoted by gingipains and its hemagglutinins domains, aid in maturation of fimbriae and exposure of cryptic epitopes (23, 90, 91). Proteases can destroy tissues during the progression of disease and allow for spreading of *P. gingivalis* into deeper tissues (23). Factors expressed by *P. gingivalis* shield the bacteria from clearance by the immune
system. Once in the host, \textit{P. gingivalis} can acquire nutrients with the help of proteases, hemagglutinins and hemolysins (23, 83, 92, 93). Given these complex molecular mechanisms \textit{P. gingivalis} is an interesting microorganism to study. An important tool to study its virulence is the analysis of site-directed mutants.

In order to find an appropriate niche for colonization invading bacteria must breach the outer barrier of the host, avoid continuous host cell fluid movement and cilia action. Adherence is the key step that allows for the initial colonization and subsequent ability of the microorganisms to be internalized by nonphagocytic cells. Bacterial pathogens adhere to eukaryotic surfaces through adhesins and interact with receptors on the cells’ surfaces. Bacterial components that function as adhesins include fimbriae, flagella, lipopolysaccharides, polysaccharides, capsules, micro-vesicles and outer membrane proteins such as hemagglutinins (23, 94). Bacteria can avoid the immune system by invading host cells and persisting in these cells.

Several bacterial pathogens including \textit{Brucella abortus}, enteropathogenic \textit{E. coli} (EPEC), \textit{Listeria monocytogenes}, \textit{Salmonella} spp., \textit{Shigella flexneri} and \textit{Yersinia} spp invade nonphagocytic cells (95-97). These bacteria are internalized into host cells via a ligand-receptor interaction which activates host cell signals to direct the entry of the bacteria. The signals induce cytoskeletal rearrangements which then facilitates bacterial internalization. It has been shown that internalization can be prevented for many species of pathogenic bacteria by an actin polymerization inhibitor, Cytochalasin D (96-100). Once the bacteria have invaded they can be free in the cytoplasm of enclosed in phagocytic vacuoles. Some pathogens, for example, \textit{S. flexneri} and \textit{A. actinomycetemcomitans} have been shown to evade these vacuoles, where as \textit{Salmonella} spp. and \textit{Yersinia} spp, remain in the vacuoles (101-103).
*P. gingivalis* has been reported to invade multiple cell types including macrophages (9), bovine aortic endothelial cells (BAEC), fetal bovine heart endothelial cells (FBHEC), human umbilical vein endothelial cells (HUVEC) (104), dendritic cells (105), and KB cells (51). Our lab has reported the invasion of HCAEC and coronary artery smooth muscle cells by *P. gingivalis* (18). The results show that invasion is strain specific with some strains being highly invasive, while others are not invasive at all (106).

Even though *Porphyromonas gingivalis* gains access to the circulatory system and has been proven to invade HCAEC, the mechanism by which *P. gingivalis* invasion affects the expression of molecules and cytokines involved in atherosclerosis is unknown (23). Previous researchers in the Progulske-Fox laboratory have established that *P. gingivalis* adheres to the cell surface of HCAEC within the first 15 minutes of co-culture. *P. gingivalis* is then internalized via lipid rafts and incorporated into an early phagosome. The early phagosome then fuses with a double membrane-membrane bound early autophagosome derived from the rough endoplasmic reticulum. However, when this autophagosome is suppressed by wortmannin, *P. gingivalis* instead transmits to a late phagosome and phagolysosome where the bacteria are degraded. Thus, the survival of *P. gingivalis* in HCAEC depends upon infection and the activation of autophagy in HCAEC. The bacteria is sorted to a vacuole that is similar to an autophagosome. However, *P. gingivalis* prevents maturation of the autophagosome into an autolysosome. Therefore, *P. gingivalis* may remain in a “replicating vacuole” which has characteristics similar to late autophagosomes, but in which the bacteria replicate and persist for many hours. Thus *P. gingivalis* enters an endothelial cell, it traffics through the autophagic vacuole and is able to prevent the final degradative steps, thereby establishing itself in a niche where it can survive and
replicate. In the first 24 hours of co-culture *P. gingivalis* promotes the survival of its endothelial host cell and does not induce apoptosis.
CHAPTER 2
INVASION AND PERSISTENCE STUDIES

Introduction

A microorganism’s ability to invade and persist within host tissues can provide an intracellular niche that allows for access to nutrients and evasion of the host’s immune mechanisms. *P. gingivalis* can invade HCAEC as well as many other cells types, as described earlier (18). *P. gingivalis* expresses multiple adhesins, both fimbrial and non fimbrial, to attach to host cells. Examples of these are specifically FimA (fimbrial) and HagB (nonfimbrial) (51, 52, 64, 107-109). However, the initial steps of internalization for *P. gingivalis*, especially with regard to HCAEC, are not well understood. Some evidence has shown that *P. gingivalis* interacts with lipid rafts which can provide a portal for entry into host eukaryotic cells, but our knowledge is limited of the role of lipid rafts in bacterial invasion (109). Once *P. gingivalis* is inside the endothelial cell, it traffics to the autophagic pathway. *P. gingivalis* induces and suppresses death in these cells, allowing for a microenvironment favorable for its replication (109). Once *P. gingivalis* leaves the oral cavity and enters the circulatory system, it likely invades HCAEC, thereby evading systemic immune defenses also inducing an inflammatory response (20). Thus *P. gingivalis* may allow for the acceleration of atheroma formation by triggering signal transduction pathways, leading to an amplified inflammatory response and foam cell formation (10).

The purpose of this study was to investigate how *P. gingivalis* mutants W83Δ0092 and W83Δ1683 interact with HCAEC as a model system for the endothelial layer of the vasculature. This is the layer that lines the lumen of the circulatory vessels and is the site of the initiation of artherosclerosis. Prior to this study, microarray analysis of *P. gingivalis* W83 genes expressed during invasion of HCAEC were completed (110). A group of genes that were determined to be
up-regulated at different time points during invasion were mutated by allelic replacement (9). Two of these mutants were chosen for this study. The first mutant is Δ0092, a putative ABC transporter with 77% homology to a Bacteroides fragilis (putative) ABC transporter gene (Figure 2-1). Dr. Liu in the Department of Bioinformatics at the University of Florida predicted that it was in an operon with PG0091, PG0093 and PG0094. PG0091 and PG0092, are putative ABC transporters (transport and binding proteins, substrate unknown), PG0093 is a HIYD family secretion protein (transport and binding protein), and PG0094 is a putative outer membrane efflux protein (transport and binding proteins, unknown substrate). The second mutant chosen for this study is Δ1683, a putative glycosyl hydrolase / alpha amylase with 78% homology to a Parabacteroides distasonis gene. Dr. Liu predicted that this gene was in an operon with PG1681, PG1682, PG1684 (Figure 2-3). PG1681 is predicted to be a glycogen debranching enzyme and to play a role in energy metabolism (biosynthesis and degradation of polysaccharides), PG1682 is predicted to be a glycosyl transferase that plays a role in the cell envelope (biosynthesis and degradation of surface polysaccharides and lipopolysaccharides), and PG1684 is a hypothetical protein.

The following experiments were performed to investigate the role of P. gingivalis genes PG1683 and PG0092 in adherence to and invasion of HCAEC. HCAEC were chosen because of their significance in atherosclerosis.

**Material and Methods**

**Bacterial Strains and Growth Conditions**

Strain W83 (the type strain) was isolated during the 1950’s from an undocumented oral human infection by H. Werner (111). This strain and mutants (see below) were stored as at -80°C. For broth culture, the bacteria were grown in tryptic soy broth (sTSB), supplemented with vitamin K₁, hemin, yeast extract and L-cysteine hydrochloride. P. gingivalis strains were also
maintained on blood agar plates (sBAP), supplemented with vitamin K₁, hemin, yeast extract and L-cysteine hydrochloride, as previously described (112). In all cases *P. gingivalis* strains were cultured in a Coy anaerobic chamber (Ann Arbor, MI) with an atmosphere of 10% H₂, 5% CO₂, and 85% N₂.

**Mutant Construction**

The mutant strains used in this study were constructed by Dr. Paulo Rodrigues. For reference purposes the following is a brief description of their construction. *P. gingivalis* W83Δ1683 and W83Δ0092 were constructed as double crossover mutants by allelic replacement using an erythromycin cassette as described previously (112). Briefly, the upstream and downstream regions of the PG1683 gene were amplified by PCR with gene-specific primers (Insert A, FW 5’ CTGGCTGCCCCGACACAAGATAG 3’ and RV 5’ GCGCAGCCTACCGGTGTTTACAC 3’; Insert B, FW 5’ CTCCGAATCCATGGCTGAG 3’ and RV 5’ GTTTCGATCGGGCTGAAGTTGC 3’). The upstream and downstream regions of the PG0092 gene were amplified by PCR with gene-specific primers (Insert A, FW PG0092 5’CATGGTGACCGGAAGAAGAAGA 3’ and RV 5’ GCCAACGCGTCGAAAAAG 3; Insert B, FW 5’ TCTCTGCGCCGTATTGAGATGTT 3’ and RV 5’ ACGGCGGTTACCAGTATGTCCA 3’). The PCR amplicons were cloned into the suicide vector pPR-UF1. This vector was developed in this laboratory by Dr. Paulo Rodrigues (110).

**Growth Curves**

The growth rate of *P. gingivalis* wild type W83 and mutants W83Δ1683 and W83Δ0092 were determined. Overnight cultures in sTSB were used to inoculate 100 ml of sTSB at an OD₅₅₀ of 0.1, in triplicate. Every two hours 1.0 ml of culture was taken for optical density measurements at 550nm. Experiments were completed in triplicate.
Cell Culture

Human coronary artery endothelial cells (HCAEC) (Lonza, Walkerville, MD) were maintained in minimum essential medium (Lonza) supplemented with fetal bovine serum with EGM-2-MV singlequots’ (Lonza), according to the supplier’s instructions with the following: FBA, hydrocortisone, hFGF, VEGF, R3-IGF-1, ascorbic acid, hEGF and heparin. Cells were cultured in 75-cm² flasks (Starstest, Newton, NC) at 37°C in a humidified atmosphere of 5% CO₂. Confluent monolayers were split by treatment with Hank’s Balanced Salt Solution (HBSS) (Mediatech, Manassas, VA) and trypsin-versene (BioWhittaker, Walkersville, MD). The cells were obtained from the company at passage 3 and they were not passaged more than 3 other times in our laboratory.

Adhesion Assays

HCAEC were seeded at 1x10^5/well into 24-well cell culture plates (Costar, Corning, NY) and grown overnight to confluence. The cells were then incubated with 5 μg/ml Cytochalasin D (Sigma-Aldrich, St. Louis, MO) for 30 minutes, followed by the addition of anaerobically grown cultures of P. gingivalis at a concentration of 1x10^7 CFU/ml and 5 μg/ml Cytochalasin D in EBM-2 antibiotic free cell culture medium. The bacterial culture was also plated for determination of the colony forming unit (CFU) for standardization. The 24-well plate (Costar) was then incubated without agitation at 37°C/5%CO₂ for 1 hr. The cells were washed three times with warm EBM-2 media containing 5% fetal bovine serum (EGM-FBS), and then lysed (pipeting up and down) with warm sterile water for 20 minutes in a 37°C incubator. Cell lysates were serially diluted and adherent bacteria were plated on sBAP for enumeration. All individual cell culture experiments were performed in quintuplicate wells, and each experiment was completed twice.
Invasion Assay

Approximately $10^5$ HCAEC were seeded in 24 well tissue culture plates (Costar) and washed three times with antibiotic free EBM-2 media. An overnight culture $1 \times 10^7$ CFU/ml of \textit{P. gingivalis} was re-suspended in fresh warmed antibiotic-free EBM-2 media and 1.0 ml was added to HCAEC for a 1.5 h infection period at 37°C in the 5% CO$_2$ incubator. The bacteria suspension was then removed and the cells were subsequently washed with warmed EBM-2 media. Extracellular bacteria were killed by adding 1.0 ml of warmed EBM-2 containing 300ug/ml Gentamycin (Sigma-Aldrich) and 200ug/ml Metronidazole (Sigma-Aldrich) with incubation for 1 hour at 37°C in the 5% CO$_2$ incubator. The medium was removed and the cells washed three times with EBM-2. Cells were lysed after a 20 minute incubation at 37°C/5% CO$_2$ with distilled sterile water and the lysate was plated on sBAP for enumeration of the intracellular bacteria (2.5 h invasion time point). For additional time points, cells were incubated with fresh antibiotic free EBM-2 and at 6, 24, and 48 h of infection. All plates were incubated at 37°C in an anaerobic chamber and colonies were grown for up to 10 days before enumeration. Individual invasion assays were performed in quintuplicate wells and completed twice.

Results

Growth Curve

The growth rate of \textit{P. gingivalis} wild-type strain W83, W83Δ0092 and W83Δ1683 were determined every two hours, using a growth curve assay (Figure 2-3). The growth curves did not reveal a difference in \textit{in vitro} growth as a result of the mutations.

Adherence

The adherence of \textit{P. gingivalis} strain W83 and the two mutant strains to HCAEC were measured after 30 minutes of co-culture using an adherence assay and are recorded as percent inoculum. Cytocholsin D inhibits the polymerization, or remodeling, of the actin cytoskeleton of
the host cell which normally forms a vesicle around the target particle/substance, allowing for endocytosis, in the case of *P. gingivalis* (18). Thus this toxin prevents *P. gingivalis* from entering the HCAEC by inhibiting the endocytosis normally induced by the *P. gingivalis* when it binds to Beta-1 integrin (18). This inhibition then allows the quantification of the number of *P. gingivalis* adhering to the cell surface in the absence invasion (Figure 2-4 and 2-5). Interestingly, mutant PG0092 demonstrated a 150% increase in adherence to HCAEC when compared to the wild-type strain, as determined using the Student’s *t*-test and One Way ANOVA (2.2 fold, *p*<0.05). Similarly, the PG1683 mutant demonstrated an 80% increase in adherence to the HCAEC when compared to the wild type (1.4 fold, *p*<0.05).

**Invasion**

The ability of *P. gingivalis* W83 and the two mutant strains to invade HCAEC was determined using an antibiotic protection assay at 2.5 hours of co-culture, and is reported as percent inoculum (Figure 2-6 and 2-7). Mutations in genes PG0092 and PG1683 showed no effect (Two-way ANOVA) on invasion when compared to the wild-type strain W83.

**Persistence**

The ability of *P. gingivalis* strain W83 and the two mutants to persist longer than 2.5 hours of co-culture within HCAEC was examined using a modified antibiotic protection assay. In order to measure persistence, multiple plates were setup at various time points. Following antibiotic treatment to eradicate extra-cellular bacteria, antibiotic free medium was added and the cells were incubated for an additional 3.5, 21.5, and 45.5 hours. At post–inoculation time: 6, 24, and 48 hours, the number of bacteria present in the medium was determined by plating serial dilutions on sBAP. To determine the number of bacteria still present inside the cells, the cells were washed, lysed, diluted and colonies were grown for up to 10 days before enumeration. The
individual persistence experiments were performed using quintuplicate wells and each experiment was performed two times.

The number of bacteria that persisted compared to the wild-type strain, is shown in Figures 2-6 and 2-7. The results are expressed as the percentage of that strain recovered at post-inoculation of 2.5 hours. Throughout, post-inoculation times of 6, 24 and 48 hours, there was no significant differences found when comparing to wild-type and mutant strains as determined by two-way ANOVA. As post inoculation times increased, fewer bacteria were recovered from the cells in both mutants and wild-type.

**Discussion**

The ability of an intracellular pathogen to invade and persist within a cell is important for its ability to cause disease. The understanding of the relationship between *P. gingivalis* and atherosclerosis depends on elucidating how this bacterium is able to enter endothelial cells. The testing of site-directed mutants in the HCAEC invasion model can be used as a tool to identify genes important for the survival of *P. gingivalis* within these host cells. To this end, the ability of *P. gingivalis* W83 and two mutant strains were evaluated for their ability to attach to, invade and persist in HCAEC.

PG0092 is classified as a putative ABC transporter in the TIGR database. Surprisingly, this mutant demonstrated a significant increase in adherence, but no difference in invasion or persistence, were observed when compared to the parent strain W83. This may indicate that entry/invasion is actually impaired in this mutant, assuming that attached bacteria all have an equal ability or likelihood of entry. Thus, if this mutant adheres to the HCAEC at numbers 2.2 times greater than the wild-type strain it would be expected that the number of mutant bacteria that invaded would also be 2.2 times greater. However, this was not the case, indicating that the Δ0092 mutant is less able to enter/invas HCAEC once it has attached to the cell surface. One
explanation for this could be that whatever change in surface properties of the mutant that allows for increased interaction with the host cell surface decreases its ability to signal conditions in the cell that allows/facilitates entry. Another possibility is that the mutant is more likely to autoaggregate and that larger aggregates of \textit{P. gingivalis} are less able to enter the cell. Presently, there is no evidence to support this, and this mutant did not demonstrate an increased ability to form monotypic biofilms but did produce morphologically different biofilms higher than the wild type strain (see chapter 3). Concerning persistence, the mutation in PG0092 resulted in no change compared to the parent, W83. A mutation in a putative ABC transporter, as in PG0092, may prevent transport of some, as yet, unidentified molecule that is involved in adherence and or aggregation but not in internalization. However, this is purely speculation at this time because the substrate for this putative ABC transporter, or even if PG0092 is a transporter is unknown.

PG1683 is identified as a putative glycosyl hydrolase/alpha amylase [on the \textit{P. gingivalis} W83 genome TIGR database]. The loss of this gene product also demonstrated increased adherence to HCAEC and no change in the invasion of HCAEC. Therefore, the mutation may functionally be similar to the PG0092 mutation but mechanistically different. The mutation in the putative glycosyl hydrolase may allow for increased adherence of the bacteria to one another but prevent internalization. Alternatively, glycosyl hydrolases can break down carbohydrates on the surface of the host cell which may otherwise reveal a cryptic epitope or receptor, facilitating adherence. The mutation may also modify \textit{P. gingivalis} surface carbohydrates allowing for increased \textit{P. gingivalis} adherence but no increase in internalization.

For both mutants, no difference was detected in persistence in HCAEC. \textit{P. gingivalis} tends to be difficult to culture from invasion assays after an extended period of time. Studies have shown that after 48 hours of co-culture, viable \textit{P. gingivalis} can no longer be recovered.
vitro (113). However, when co-incubated with fresh HCAEC, viable *P. gingivalis* can be recovered. Therefore uncultivable *P. gingivalis* can exit the initially infected cells and subsequently enter and multiply in “fresh” HCAEC (113). Future studies which include culturing in fresh HCAEC, need to be completed to obtain conclusive about persistence of these mutants.

An important factor in bacterial intracellular pathogenesis is the ability of bacteria to adhere to enter and traffic within host cells. The mechanisms of adherence and invasion are complex and require multiple molecules and coordination and regulation of gene expression. Additional studies are needed to more completely understand how *P. gingivalis* is able to invade and persist. However, this study indicates that among those genes involved in at least the initial events of these interactions are PG0092 and PG1683.
Table 2-1. Primers used for mutant construction in *P. gingivalis* W83. Genes were mutated by double crossover allelic exchange.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Primer name</th>
<th>Sequence (5’→3’)</th>
</tr>
</thead>
<tbody>
<tr>
<td>PG0092</td>
<td>0092 5’F</td>
<td>CATGGTCGACGGGAAGAAGAGA</td>
</tr>
<tr>
<td></td>
<td>0092 5’ R</td>
<td>GCCAACGCGTCGCAAAAAAAG</td>
</tr>
<tr>
<td></td>
<td>0092 3’ F</td>
<td>TTCCTGCCCGTATTTGAGATGTT</td>
</tr>
<tr>
<td></td>
<td>0092 3’ R</td>
<td>ACGGCCGGTACCAGTATGTCCA</td>
</tr>
<tr>
<td>PG1683</td>
<td>1683 5’F</td>
<td>CTGGCTGCCCGACACAAGATAG</td>
</tr>
<tr>
<td></td>
<td>1683 5’R</td>
<td>GCGCAGCCTACCCGGTTTTACAC</td>
</tr>
<tr>
<td></td>
<td>1683 3’F</td>
<td>CTCCGCAATCCATGGCTGAG</td>
</tr>
<tr>
<td></td>
<td>1683 3’ R</td>
<td>GTTTCGATCGGGCTGAAGTTGC</td>
</tr>
</tbody>
</table>
Figure 2-1. Bioinformatic analysis of the putative operon for \textit{P. gingivalis} genes PG0091, PG0092, PG0093, and PG0094.

Figure 2-2. Bioinformatic analysis of the putative operon for \textit{P. gingivalis} genes PG1681, PG1682, PG1683, and PG1684.

Figure 2-3. Growth curve of \textit{P. gingivalis} strains W83, W83\Delta 0092 and W83\Delta 1683 in sTSB media. Growth curve of mutants compared to the wild type strain W83 over time. Student’s t-test was used for statistical comparisons.
Figure 2-4. Adherence of *P. gingivalis* to HCAEC of strain W83 and W83Δ0092 in the presence of Cytochalasin D. Cells were pre-exposed to Cytochalasin D (to prevent internalization) for 1 hour in sTSB media. *P. gingivalis* W83 and W83Δ0092 were allowed to adhere to cells for 30 minutes at an MOI of 100 and nonadherent bacteria were washed away. Cells were lysed with 1 ml of water for 20 minutes. The adherent bacteria were serially diluted with PBS and plated for enumeration on sBAP to measure adherent bacteria. Mutant W83Δ0092 demonstrated an increase in adherent ability to HCAEC at 30 minutes when compared to the wild-type (P < 0.05). Two-way ANOVA was used for statistical comparisons. Asterisks indicate a significant difference.
Adherence of *P. gingivalis* to HCAEC of strain W83 and W83Δ1683 in the presence of Cytochalasin D. Cells were pre-exposed to Cytochalasin D (to prevent internalization) for 1 hour in sTSB media. *P. gingivalis* W83 and W83Δ1683 were allowed to adhere to cells for 30 minutes at an MOI of 100 and nonadherent bacteria were washed away. Cells were lysed with 1 ml of water for 20 minutes. The adherent bacteria were serially diluted with PBS and plated for enumeration on sBAP to measure adherent bacteria. Mutant W83Δ1683 demonstrated an increase in adherent ability to HCAEC at 30 minutes when compared to the wild type. (P < 0.05). Two-way ANOVA was used for statistical comparisons. Asterisks indicate a significant difference.
Figure 2-6. Invasion and Persistence of HCAEC for *P. gingivalis* W83 and W83Δ0092 in sTSB media. *P. gingivalis* W83 and W83Δ0092 were allowed to invade the HCAEC at a MOI of 100 for 1.5 hours. The wells were washed with EBM-2 media and 300 µg/ml gentamycin and 400 µg/ml metronidazole were added to kill extracellular bacteria. After incubation for 1 additional hour, the cells were washed with EBM-2 media and lysed with 1ml of water for 20 minutes. The intracellular bacteria were diluted in EBM-2 and plated for enumeration on blood agar plates to quantify invasion and persistence over 48 h. Mutant PG0092 demonstrated no difference when compared to the wild type in invasion and persistence. ANOVA was used to make statistical comparisons.
Figure 2-7. Invasion and Persistence of HCAEC by *P. gingivalis* W83 and W83Δ1683 in sTSB media. *P. gingivalis* W83 and W83Δ1683 were allowed to invade the HCAEC at a MOI of 100 for 1.5 hours. The wells were washed with EBM-2 media and 300 µg/ml gentamycin and 400 µg/ml metronidazole were added to kill extracellular bacteria. After incubation for 1 additional hour, the cells were washed with EBM-2 media and lysed with 1ml of water for 20 minutes. The intracellular bacteria were diluted in EBM-2 and plated for enumeration on blood agar plates to quantify invasion and persistence over 48 h. W83Δ1683 demonstrated no difference when compared to the wild type in invasion and persistence. ANOVA was used to make statistical comparisons.
CHAPTER 3
BIOFILM

Introduction

Biofilm is a complex organization of aggregated bacteria. Biofilm in the gingival area leads to inflammation and destruction of host tissues leading to pathogenesis and the chronic disease known as periodontal disease (34). *P. gingivalis* is a secondary colonizer that is able to bind to salivary receptors, aggregate with itself and co-aggregate with many other bacteria such as *Fusobacterium nucleatum*. As previously mentioned, the complexity of biofilm formation is likely to require the coordinated expression of multiple genes (35). Biofilm formation allows for acquisition of nutrients, protection from mediators of host immunity and possibly exchange of genetic information (36).

The goal of this study was to determine the role, if any, of two *P. gingivalis* proteins, PG0092, a putative ABC transporter and PG1683, a putative glycosyl hydrolase/alpha amylase, in biofilm development. A previous study reported the importance of a putative ABC transporter in the negative regulation of biofilm formation in *Listeria monocytogenes* (114). Another study characterized a glycosyl hydrolase (NghA) of *Y. pseudotuberculosis* and determined its role in reducing biofilm formation of *Y. pestis* and *S. epidermis* biofilms in vitro (115). Yet another study confirmed that glycosyl hydrolases (PGA) of *A. actinomycetemcomitans* and *A. pleuropneumoniae* biofilms (PGA) are involved in biofilm matrix polysaccharide synthesis and may play a role in intercellular adhesion and cellular detachment and dispersal (116). Based on these studies in other pathogens, the purpose of our study was to compare biofilm formation between the wild-type *P. gingivalis* W83 strain and mutants W83Δ0092 and W83Δ1683. Homotypic biofilm height, biofilm volume and biofilm structure were determined using a crystal violet assay, spinning confocal microscopy and scanning electron microscopy, respectively.
Further studies were completed to test differences in heterotypic biofilm formation with *P. gingivalis* and *Fusobacterium nucleatum 22586*.

**Material and Methods**

**Bacterial Strains and Growth Conditions**

Bacterial strains and growth conditions were maintained as previously described in Chapter 2.

**Mutant Construction**

Mutations were constructed as previously described in Chapter 2.

**Homotypic Biofilm**

To determine homotypic biofilm formation by *P. gingivalis*, strain W83 and mutants W83Δ0092 and W83Δ1683 were grown anaerobically overnight in sTSB at 37°C and then subcultured to an OD 1.0. Five replicates of 500 µl of the *P. gingivalis* cultures were inoculated into wells of a 48-well microtiter plate (Nunclon, Denmark). After 12, 24, 48, 72 and 96 hrs in an anaerobic chamber, the resulting biofilms were washed three times with pre-reduced PBS and stained with 100µl of filtered 1% crystal violet in water for 15 minutes. After staining, the plates were washed three times with sterile distilled water by immersing the 24 well plate (Costar) in a bowl of water, shaking the excess water into the sink and blotting the plate on paper towels. The biofilms were then visualized as circles at the bottom of each well and biofilm production was quantitatively analyzed by adding 200µl of 95% ethanol for 15 minutes to destain the wells. Fifty µl of the ethanol from each well were then pipeted in triplicate into a 96 flat bottom well plate (Costar). A Benchmark microplate reader (Bio-Rad Laboratories) was used to measure absorbance at 595nm. The average OD of the control wells was calculated and subtracted from the OD of sample wells, the averages were determined and graphed with standard deviations.
ANOVA was used for statistical analysis. Independent biofilm assays were repeated twice with each strain in quintuplicate.

**Flourescently labeling Porphyromonas gingivalis for Homotypic Biofilm**

For homotypic biofilm height and volume measurements determined by confocal microscopy, 1 ml of *P. gingivalis* wild type W83, mutant W83Δ0092 or W83Δ1683, grown anaerobically overnight in sTSB at 37°C, was centrifuged at 5000 g/4 min and washed twice in 1 ml pre-reduced PBS (Mediatech). The bacterial cells were then re-suspended in pre-reduced PBS and 1µl of 5-(6)-carboxyfluorescein-succinimidyl ester (fluorescein isothiocyanate [FITC], 4µg ml⁻¹; Molecular probes C1311, green fluorescence), from a stock solution of 10 mg/ml in DMSO. The bacterial cells were then covered with aluminum foil and incubated on a rolling platform at 4°C for 30 minutes. The labeled cells were then centrifuged for 4 min at 5000 g, washed three times and resuspended in 1x PBS (Mediatech). The OD₅₅₀ of the bacterial suspension was adjusted to a concentration of 2x10⁸ CFU/ml and 200µl of each labeled bacterial preparation (5x10⁷ CFU/well) was pipeted into a 16 well chamber coverglass system (Nalge Nunc International, Rochester, NY). The slides were covered with aluminum foil and incubated anaerobically with rocking at 37°C for 24 and 48h. At each time point, the supernatant was exchanged with fresh PBS and biofilm development on the coverglass bottom was observed by spinning disk confocal consisting of a CSU10 Yokagowa confocal scan head, a Roper Cascade II EMCCD 512b camera, ASI X, Y, and peizo Z computer controlled stage, three lasers for 3 channel fluorescent imaging attached to a Leica DMIRB inverted microscope. This system was controlled by the open source software package Micro-manager (http://www.micro-manager.org/). Quantification of *P. gingivalis* specific fluorescence biofilm height and biofilm volume was described using the following Image J plugins: Micro Manager, Volume Viewer, Plot Profile and Volume Renderer. Biofilm assays were repeated twice and in duplicate.
Heterotypic Biofilm (P. gingivalis and F. nucleatum 22586)

Fusobacterium nucleatum 22586, which was kindly provided by Dr. Clay Walker, University of Florida, was used with P. gingivalis for studies on heterotypic biofilms which were completed using the microtiter plate assay described in the previous section (Homotypic Biofilm). Bacterial suspensions of P. gingivalis were prepared from anaerobically grown overnight cultures in sTSB whereas bacterial suspensions of F. nucleatum 22586 were grown anaerobically at 37°C in sTSB for three days. Different ratios of parental and mutant P. gingivalis strains were mixed with F. nucleatum 22586 in sTSB, aliquoted into 24 well plates (Costar, Corning, NY), and incubated anaerobically at 37°C for 24 and 48 h. In addition, F. nucleatum 2256 was added to a preformed 24h P. gingivalis homotypic biofilm. For this, the medium was removed and F. nucleatum 22586 was added on top for an additional 24 h. The reverse was also done. After the incubation period, the liquid was removed from the wells for all experiments and the wells were washed three times with pre-reduced PBS (Mediatech), allowing for removal of loosely associated bacteria. The remaining cells (biofilm) were stained with 100µl of filtered 1% crystal violet in water for 15 minutes and plates were washed three times with sterile distilled water by the immersion technique described above. Biofilm production was quantitively analyzed by adding 50µl of 95% ethanol to destain the wells and 50µl from each well was pipeted in triplicate into wells of a 96 flat bottom well plate. The intensity at OD$_{595}$ of the crystal violet present in the destaining solution was measured using a Benchmark microplate reader (Bio-Rad Laboratories). The average OD from the control wells was subtracted from the OD of all sample wells and the averages and standard deviations of the samples were calculated. Two-way ANOVA was used for statistical analysis. Independent biofilm assays were repeated twice with each strain in quintuplicate.
Results

Homotypic Biofilm

Overall mass of homotypic biofilm using P. gingivalis W83, W83Δ0092 or W83Δ1683 was measured using a crystal violet staining assay at absorbance 595 nm over 24, 48, 72 and 96 hours. As shown in Figures 3-1 and 3-2, no difference was found in biofilm production for the two mutants when compared to wild type strain W83, under the conditions described. The data also indicate that biofilm production using this assay and media reached its maximum volume at 48 hours.

For biofilm structure visualization and quantification, biofilms were also generated on 16-well chamber coverglass systems, stained with FITC (as described earlier) and examined by confocal laser microscopy. The images were observed at 490 wavelength and analyzed with Image J to determine biofilm volume and biofilm height (Figure 3-3). The biofilm formation of the PG0092 mutants showed no statistical difference in biofilm volume or average biofilm height. A more detailed analysis of the proportional height, compared to W83, indicated that mutant ΔPG0092 formed sporadic taller biofilm colonies than the wild-type. Chi square analysis was used to statistically analyze proportions of wild-type versus mutant peak heights greater than 120 in intensity and demonstrated a difference among the strains. A peak intensity of 120 was chosen since it is roughly 50% of the largest peak intensity of the highest intensity observed. When comparing W83 to W83Δ0092 at 24 h, W83 had 0.33% of peaks greater than the 120 peak intensity with a 95% confidence of 0.16 to 0.60%, while W83Δ0092 had 20.3% peaks greater than the 120 peak intensity with a 95% confidence of 18.9 to 21.7%. At 48h, strain W83 had 6.9% peaks greater than the 120 peak intensity (95% CI 6.0 to 7.9%) and W83Δ0092 had 30.4% peaks greater than 120 peak intensity (95% CI 28.7 to 32.0%). Thus the biofilm formed by W83Δ0092 contained a greater proportion of high biofilm peaks than did the W83 biofilm (Table
The scanning electron microscopy biofilm pictures of this mutant appeared sparser than that of the wild-type W83 strain (Figure 3-5).

When comparing biofilms of W83 to W83Δ1683 at 24h using chi square analysis (Figure 3.4 A), W83Δ1683 had no peaks greater than the 120 peak intensity (95% CI 0 to 0.0012%), but at 48 h, 11.9% of its peaks had an intensity of greater than 120 (95% CI 10.8 to 13.1%). W83Δ1683 had greater biofilm height at 48h (P value <0.05) when compared to wild-type W83 but there was no statistical difference at 24 h. Visually, mutant 1683 had patchy and chain-like formation of biofilm colonies compared to the formation of wild-type biofilm with evenly spread dispersed micro-colonies (Figure 3-4, 3-5).

Heterotypic Biofilm

Heterotypic biofilm was measured using a crystal violet staining assay over 24 and 48 hours with mixtures of P. gingivalis strain W83 and Fusobacterium nucleatum strain 22586. No differences were determined with the P. gingivalis mutant W83Δ1683 and Fusobacterium nucleatum 22586 compared to P. gingivalis wild type strain W83. As shown in Figure 3-6, the only significant difference found was with the addition of mutant W83Δ0092 grown initially for 24 hours on the microtiter plate, followed by the addition of Fusobacterium nucleatum 22586 on top, when compared to the same conditions of W83, (P value < 0.05). It was also determined that heterotypic biofilm with the initial bacteria (mutants and wild-type) grown on the plate for 24 hours and later adding a second type of bacteria, F. nucleatum 22586, resulted in greater biofilm production than the homotypic biofilms or when F. nucleatum 22586 was grown first.

Discussion

Oral biofilm development is vital to the progression of periodontal disease. It allows for the colonization of multiple bacterial species in the oral cavity, leading ultimately to inflammation, destruction of the supporting tissues of the teeth and bone resorption. When
comparing our mutants to *P. gingivalis* wild type strain W83, no difference was determined in homotypic biofilm production, biofilm volume, or average biofilm height formation. However more detailed analysis of proportions of biofilm height formation revealed differences in biofilms of the mutants compared to that of W83. These differences indicate that mutations of either of these genes, PG1683 and PG0092, affect *P. gingivalis* biofilm morphology. How the difference in morphology translates into biofilm “pathogenicity” is not known at this time.

As stated above, a study by Zhu et al., has shown that another *Listeria monocytogenes* putative ABC transporter functions as a negative regulator of biofilm formation (114). This appears not to be the case with this *P. gingivalis* putative ABC transporter with respect to monospecies biofilms. However, it could be that other ABC transporters compensate for the loss of the PG0092 putative ABC transporter or that PG0092 is in fact, not an ABC transporter. Regardless of its function, this protein plays a role in biofilm formation, since a mutation in PG0092 resulted in a difference in biofilm peak height. Thus loss of function of this gene resulted in higher biofilm peaks and more profound aggregation within mono-species and mixed species biofilms. This putative ABC transporter may, however, be a negative regulator of heterotypic biofilm formed by *P. gingivalis* and *F. nucleatum*, since in the case of the Δ0092 mutant, the loss of this gene resulted in greater formation at 48 h. Further studies must be completed to further characterize this gene’s substrate (function) and phenotype. Heterotypic biofilms of *P. gingivalis* and *F. nucleatum* have been shown to aggregate well with one another and promote biofilm production (117). Studies reported here, also indicate that all strains of *P. gingivalis* tested make more biofilm in the presence of *F. nucleatum* 22586 than homotypic biofilm. This further substantiates that, *P. gingivalis* and *F. nucleatum* assist each other with co-aggregation, as previously discussed.
Recent studies have concluded that glycosyl hydrolases are important for the amount of biofilm formed, may function as a biofilm matrix polysaccharide, and may be important in intercellular adhesion, cellular detachment and dispersal (115, 116). The mutation in PG1683, a putative glycosyl hydrolase/alpha amylase, resulted in a change in biofilm height. Thus the loss of this gene function affects biofilm structure in that higher chain-like peaks form compared to wild type biofilm which forms dispersed micro-colonies. It may be that this putative glycosyl hydrolase plays a role in enhancing mono-species aggregation. When this mutant was grown as a mixed-species biofilm with *F. nucleatum* 22586, there was more abundant accumulation of biofilm compared to the monospecies biofilm, but no differences were observed compared to the wild type strain dual species biofilm.

It is reasonable to speculate that the differences detected in biofilm structure may be related to differences in adhesion to HCAEC as reported in Chapter 2. The change in architecture of the *P. gingivalis* surface structure(s) likely mediate changes in both adherence and biofilm formation.
Figure 3-1. Microtiter plate monospecies biofilm production by \textit{P. gingivalis} W83 and W83\textDelta0092 in sTSB media. \textit{P. gingivalis} biofilms were stained with 1\% crystal violet. A) Biofilms of \textit{P. gingivalis} W83 and W83\textDelta0092 at 24, 48, 72 and 96 h. Two-way ANOVA was used for statistical analysis. No differences were determined for mutant compared to wild-type.
Figure 3-2. Microtiter plate monospecies biofilm production by *P. gingivalis* W83 and W83Δ1683 in sTSB media. *P. gingivalis* biofilms were stained with 1% crystal violet. A) Biofilms of *P. gingivalis* W83 and W83Δ1683 at 24, 48, 72 and 96 h. Two-way ANOVA was used for statistical analysis. No differences were determined for mutant compared to wild-type.
Figure 3-3. Confocal micrographs of monospecies biofilm production of *P. gingivalis* W83 and W83Δ0092 at 24 and 48 h in sTSB. Confocal laser scanning microscopy projections of monospecies biofilm formation by *P. gingivalis* strains A) W83 at 24 h B) W83 at 48 h hand C) W83Δ0092 at 24 h D) W83Δ0092 at 48 h. *P. gingivalis* was pre-stained with FITC (green). Magnification 60X. E) Total biofilm volume analysis of a 268.6 by 268.6 µm x-y section of *P. gingivalis* strains W83 and W83Δ0092. F) Total biofilm height analysis of a 268.6 by 268.6 µm x-y section of *P. gingivalis* W83 and W83Δ0092. The horizontal pictures placed under each confocal picture represents cross sections of each biofilm used to determine peak height. Two-way ANOVA was used for statistical analysis.
Table 3-1. Chi square analysis values of biofilm peaks >120 in intensity for *P. gingivalis* wild-type W83 strain and mutant W83Δ0092 at 24 and 48 hours.

<table>
<thead>
<tr>
<th>Strain</th>
<th>24 h</th>
<th>48 h</th>
</tr>
</thead>
<tbody>
<tr>
<td>W83</td>
<td>0.33% (95% CI 0.16 - 0.60%)</td>
<td>6.9% (95% CI 6.0 – 7.9%)</td>
</tr>
<tr>
<td>W83Δ0092</td>
<td>20.3% (95% CI 18.9 – 21.7%)</td>
<td>30.4% (95% CI 28.7 – 32.0%)</td>
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</tbody>
</table>

Table 3-2. Chi square analysis values of biofilm peaks >120 in intensity for *P. gingivalis* wild-type W83 strain and mutant W83Δ1683 at 24 and 48 hours.

<table>
<thead>
<tr>
<th>Strain</th>
<th>24 h</th>
<th>48 h</th>
</tr>
</thead>
<tbody>
<tr>
<td>W83</td>
<td>0.33% (95% CI 0.16 - 0.60%)</td>
<td>6.9% (95% CI 6.0 – 7.9%)</td>
</tr>
<tr>
<td>W83Δ1683</td>
<td>0% (95% CI 0-0.0012%)</td>
<td>11.9% (10.8 – 131%)</td>
</tr>
</tbody>
</table>
Figure 3-4. Confocal micrographs of monospecies biofilm production of *P. gingivalis* W83 and W83Δ1683 at 24 and 48 h in sTSB media. Confocal laser scanning microscopy projections of monospecies biofilm formation by *P. gingivalis* strains. A) W83 at 24 h B) W83 at 48 h. C) W83Δ1683 at 24 h D) W83Δ1683 at 48 h. *P. gingivalis* was pre-stained with FITC (green). Magnification 60X. E) Total biofilm volume analysis of a 268.6 by 268.6 µm x-y section of *P. gingivalis* strains W83 and W83Δ1683. F) Total biofilm height analysis of a 268.6 by 268.6 µm x-y section of *P. gingivalis* W83 and W83Δ1683. The horizontal pictures placed under each confocal picture represents cross sections of each biofilm used to determine peak height. Two-way ANOVA was used for statistical analysis.
Figure 3-5. Scanning electron micrographs of monospecies biofilm production of \textit{P. gingivalis} mutants and wild type at 24 and 48 h in sTSB media. A) W83 at 24 h B) W83Δ0092 at 24 h. C) W83Δ1683 at 24 h. D) W83 at 48 h. E) W83Δ0092 at 48 h. F) W83Δ1683 at 48 h.
Figure 3-6. Mixed species biofilms of *P. gingivalis* W83, W83Δ0092 and *F. nucleatum* 22586 in sTSB media. A) Biofilms at 24 and 48 h grouped by time and separated by strain. B) Biofilms at 24 and 48 h grouped as strains and separated by time. Asterisks indicate a significant difference. Two-way ANOVA was used to make statistical comparisons.
Figure 3-7. Mixed species biofilms of *P. gingivalis* W83, W83Δ1683 and *F. nucleatum* 22586 in sTSB media. A) Biofilms at 24 and 48 h grouped by time and separated by strain. B) Biofilms at 24 and 48 h grouped as strains and separated by time. Two-way ANOVA was used for statistical analysis.
CHAPTER 4
VIRULENCE FACTORS AND ENVIRONMENTAL SIGNALS

Introduction

A microorganism’s ability to survive within different environmental niches is dependent upon its ability to adapt and respond to environmental cues by regulating the expression of its genes (118). For a pathogen, adapting to a new environment quickly can determine the microorganism’s rate of colonization, proliferation and successful infection (118). The coordinated expression of bacterial virulence genes during an infectious process is vital for adherence, penetration, replication and colonization of host tissues and cells (118). Therefore, the bacterium must adapt to its varying niche by turning on and off different virulence factors as a response to different environmental signals in various stages of infection (118). As discussed earlier, *P. gingivalis* has mechanisms to acquire nutrients, such as hemin, which is limited in the subgingival crevice and is required for its growth. For example, *P. gingivalis* grown in a heme limiting environment, showed a decrease in transcription of a specific set of genes, believed to be the result of severe stress (119, 120). Another study showed that *P. gingivalis* grown under various conditions of growth resulted in different expression patterns of virulence factors (121).

The expression of various factors on the surface of bacteria that are associated with adhesion and invasion include fimbriae, flagella, LPS, polysaccharide, micro vesicles and outer membrane vesicles (118).

The fimbriae of *P. gingivalis* are among its most significant virulence factors and are important for host cell interactions such as adherence and invasion. A report from the Hanley laboratory measured the length of fimbriae present on the surface of several *P. gingivalis* strains, including W83 (122). However, our laboratory was unsuccessful in detecting fimbriae on the surface of W83. Of significance, was the fact that our growth conditions for W83 were different
from those used by the Hanley laboratory. The purpose of this study was to compare the expression of fimbriae by W83 under the two different conditions of growth as well as determine the phenotypes of two W83 mutants in the varying conditions. To this end, *P. gingivalis* wild-type W83 and mutants W83Δ0092 and W83Δ1683 were evaluated for biofilm formation, the production of fimbriae, adhesion, invasion, and hemagglutination activity in response to conditions of growth.

**Material and Methods**

**Bacterial Strains and Growth Conditions**

Bacterial strains and growth conditions were maintained as previously described in Chapter 2, as well as maintained and transferred biweekly on Reinforced Clostridial Medium (Difco, Sparks, MD) plates (sRCP) supplemented with menadione, KNO₃ and NaHCO₃ as previously described (122). For liquid growth, *P. gingivalis* strains were also grown in Brain Heart infusion Broth (sBHI), yeast extract, glucose, NaHCO₃, KNO₃ and menadione as previously described (122). All *P. gingivalis* cultures were incubated in a Coy anaerobic chamber (Ann Arbor, MI) at 37°C with an atmosphere of 10% H₂, 5% CO₂, and 85% N₂.

**Mutant Construction**

Mutations were constructed as previously described in Chapter 2.

**Fimbriae Negative Staining for Electron Microscopy**

*Porphyromonas gingivalis* strain W83 and mutants W83Δ0092 and W83Δ1683 were grown anaerobically overnight at 37°C in either sTSB or sBHI for 24 hours. Negative staining and examination by electron microscopy was performed as described previously (123). Briefly, the bacteria were washed three times in water, resuspended in PBS with 1% BSA and one drop of this suspension was then applied to copper grids (300 mesh, EMS, Hatfield, PA). Ten microliters of 1 per cent w/v methylamine tungstate (Ted Pella, Redding, CA) was added to one
drop of the *P. gingivalis* suspension on a grid to negatively stain the bacteria for 45 seconds. Any excess fluid was then wicked away with filter paper. The negatively stained grids were then examined by Debra Akin at the University of Florida, Department of Anatomy and Cell Biology, using a JEOL 100CX electron microscope.

**Hemagglutination Assay**

*P. gingivalis* wild type W83 and mutants W83Δ0092 and W83Δ1683, grown overnight in sTSB or sBHI, were centrifuged at 5000 g and washed 3 times with PBS. The bacterial cells were then resuspended in PBS to an OD$_{660}$ of 2.0. Sheep erythrocytes (QUAD-FIVE) were centrifuged at 400 g/5min/4°C, washed twice with PBS and re-suspended in PBS to a final concentration of 2%. Aliquots of 100ul from each bacterial suspension were serially diluted twofold with PBS into 96 well v-shaped microtiter plates (Costar). An equal volume of 2% sheep erythrocytes was then added to the bacterial suspensions and mixed with each dilution. After incubation for 2h at 4°C, the hemagglutination titer was assessed as the last dilution that showed complete agglutination (pellet could not be observed). The plate was further incubated for 16 h at 4°C and observed again. Hemagglutination assays were performed twice for each strain.

**Adherence Assay**

Adherence assays were performed as previously described in the Material and Methods section of Chapter 3.

**Results**

**Fimbriae**

The presence of fimbriae was determined using electron microscopy, as previously described. No fimbriae were detected on any of the strains, wild-type or mutants, when they were cultured in sTSB (Figure 4-1 and 4-2). However, when grown in BHI, long, peritrichous fimbriae
on strain W83 were evident (Figure 4-1 and 4-2). In contrast, no fimbriae could be observed on strains W83Δ0092 and W83Δ1683 when grown in sBHI.

In experiments done by Debra Akin, Western blot analysis using specific antisera indicated that the structural gene of major fimbriae, fimA, is being expressed in all three strains W83, Δ0092 and Δ1683 (data not shown), but neither wild-type nor mutants express the minor fimbriae, MfaI, when grown in either media. (data not shown)

**Hemagglutination**

Hemagglutination activity of wild type strain W83 and mutants W83Δ0092 and W83Δ1683 was measured using an hemagglutination assay. Serial dilutions of each strain were incubated with sheep erythrocytes at 4°C overnight. The titer was defined as the last dilution that showed full agglutination, which was determined by the lack of a pellet. As is shown in Figure 4-3, the hemagglutinin titers of strains W83 Δ0092, and Δ1683 were all 1:128, when grown in sTSB. Thus no difference in hemagglutination was detected when both mutants W83Δ0092 and W83Δ1683 were compared to the wild-type strain. However, as is evident in Figure 4-4, when grown in sBHI, the hemagglutinin titer of W83 and W83Δ0092 were 1:8, but W83Δ1683 did not demonstrate any hemagglutination activity at all.

**Adherence Assay**

The adherence of *P. gingivalis* strain W83 and the two mutants which had been cultured in the media described by Handley (sBHI) to HCAEC was measured at 30 minutes of co-culture using the previously described adherence assay and was recorded as percent inoculum (Figure 4-5 and 4-6). As previously discussed, cytocholsin D inhibits the polymerization of the actin cytoskeleton of the host cell, thereby preventing endocytosis, and thus entry of *P. gingivalis* (18). Consequently, the number of *P. gingivalis* that adhere to the cell surface without complication of invasion can be quantified. As can be seen in Figure 4-5 a mutation in PG0092 resulted in a 51%
decrease in adherence to HCAEC, as compared to the parental W83 strain, similarly the mutation in PG1683 resulted in 60% (as analyzed using the Student’s t-test and One Way ANOVA (2.1 fold, P<0.05). Given this difference in adherence of the mutants as compared to W83, for reference, a comparison of the adherence of W83 grown in the two media was also done. Interestingly, W83 grown in sBHI was slightly (1.2 times) less adherent than when it was grown in sTSB and this difference was statistically different (P<0.05) (Figure 4-7).

Biofilm

Homotypic biofilm production by P. gingivalis wild type W83 and mutants W83Δ0092 and W83Δ1683 when grown overnight in sBHI was determined using the crystal violet assay over 96 hours. Drastic differences were observed for biofilm formation by W83Δ1683 when compared to W83 (Figure 4-8). In fact, a mutation in this putative glycosyl hydrolase/alpha amylase gene resulted in the total loss of biofilm formation. In strain W83Δ0092 (Figure 4-9), a putative mutant in an ABC transporter, demonstrated a 0.9x reduced ability to form biofilm (0.9 fold) was observed when compared to wild type W83 and this difference was not statistically significant. For all strains, biofilm formation remained essentially constant over 96 hrs. Similar to adherence experiments, the amount of biofilm formed by the parent strain grown in the two media was also compared. As can be seen in Figure 4-10, there was a significant difference in biofilm formation comparing W83 grown in sTSB versus sBHI at all time points. However the culture grown in sTSB formed more biofilm early, through 48 h, but by 72 h, the BHI grown culture formed significantly more biofilm, which continued through 96 h. This difference at the later time points was due to the sBHI cultured cells maintaining a greater amount of the 24 – 48 h biofilm as compared to the sTSB cultured cells.
Discussion

Characterizing mutants involved in adhesion, hemagglutination, protease activity, biofilm development, and fimbriae formation allows us to better understand to what extent these individual gene products influence the course of infection and disease. As discussed earlier, *P. gingivalis* is able to colonize, evade host responses and adapt to its environment in order to acquire nutrients and allow for the progression of this disease. The data reported here clearly indicate that when characterizing genes in bacteria, cultural conditions should be taken into consideration since differences in conditions of growth will likely result in a change in gene expression and, consequently, possibly virulence. In our studies, changing growth media in some cases completely changed the phenotype of mutated genes compared to the wild type strain.

Gene PG1683, as determined by the TIGR institute, encodes a putative glycosyl hydrolase/alpha amylase. In earlier chapters, it was reported that W83Δ1683, grown in sTSB exhibited no difference in invasion, fimbriae formation and most homotypic and heterotypic biofilm measurements, as compared to W83. The most significant difference found was an increase in adherence. However, the experiments reported in this chapter demonstrate that W83Δ1683 has a drastic phenotypic difference when cultured in sBHI. In this study, it was determined that a mutation in PG1683 resulted in the loss of hemagglutination activity, decreased adherence to host cells and the inability to form biofilms when the mutant was cultured in sBHI. A glycosyl hydrolase can be important for the degradation/synthesis of carbohydrates and may logically change carbohydrate surface structures. Given these phenotypes, it is logical to conclude that one or more surface molecules of W83 are absent or significantly changed in W83Δ1683.

As reported in earlier chapters, the phenotype found for PG0092 was an increase in adherence and biofilm structural formation, when cultured in sTSB. However, when this strain was cultured in sBHI, it did not express structural fimbriae and exhibited a significant decrease
in adherence as compared to W83. No differences were observed in hemagglutination or invasion compared to W83. The TIGR institute lists PG0092 as a putative ABC transporter. Although, it is difficult to make conclusions on the function of this gene without first characterizing its substrate, it is possible that it is involved in fimbriae formation. As discussed in the introduction, ABC transporters are trans-membrane proteins that utilize the energy of ATP to carry out various biological processes and in other bacteria have been reported to be important in the regulation of biofilm formation. However this does not seem to be the case for this *P. gingivalis* putative ABC transporter in the sBHI environment. Additional studies need to be completed in order to more fully characterize the function of this gene product.

The negative stains/electron microscopy analysis revealed long fimbriae present on the surface of W83 but not present on the surface of W83Δ1683, when grown in sBHI. However, no fimbriae were visible on strain W83Δ0092 either, when grown in sBHI, and this strain exhibited no reduction in hemagglutination activity and only a slight reduction in biofilm formation. Thus the presence/absence of fimbriae alone cannot account for the loss of phenotype for the W83Δ1683 mutant. However the loss of the expression of intact fimbriae by the mutants is significant. Interestingly, Western blots indicated that the proteins for both the major fimbriae (FimA) are expressed in these strains, when grown in both sTSB and sBHI media but the minor fimbriae were not expressed for either condition (data not shown, Debra Akin personal communication). However, when this mutant was maintained in sTSB no fimbriae could be detected by electron microscopy. These data thus indicate that FimA is expressed but intact fimbriae are not formed. Therefore, the loss of these gene product results in the inability to produce functional fimbriae, and this phenotype is environment specific.
Analysis of the parental strain when grown in different media resulted in some interesting observations. First, the adherence of strain W83 to HCAECs was compared when grown in both media and the data indicated a significant difference in phenotype. When W83 was cultured in sBHI, a reduction in adherence was found and determined to be statistically different from the sTSB grown culture. These results are puzzling since fimbriae are expressed when W83 is grown in sBHI but not in the sTSB media. This may indicate that fimbriae are not involved in the specific adherence mechanism responsible for this difference and it is known that many other \textit{P. gingivalis} surface structures mediate adherence as well. In fact, W83 hemagglutination is reduced when it is grown in sBHI versus sTSB as discussed in the introduction hemagglutination is important for adherence. Additionally, homotypic biofilm formation was statistically different between cultures from the two media. When grown in sTSB, the W83 biofilm increased at 48 h but then decreased over the 72 and 96 h. However, in sBHI media, the W83 biofilm remained constant over the 96 h. The resulting biofilm phenotype may be due to the expression of fimbriae on the surface of W83 when grown in sBHI, which may mediate increased adherence of \textit{P. gingivalis} cells and partially inhibits the detachment, and dissemination stages of biofilm formation.

Experiments reported in this chapter indicate that when testing mutants in different media, the phenotype can be different. The data presented here demonstrate that the sBHI medium promotes the expression of some genes that are not expressed in sTSB. The reverse is also undoubtedly true. Table 4-1, summarizes the phenotypes of the W83 parental genes and mutants when grown in both media. It would be interesting to complete microarray analysis of the expression of W83 genes when W83 is cultured in these two media.
Figure 4-1. Electron micrograph of negative stains of *P. gingivalis* strains W83 and W83Δ0092 in sTSB and sBHI media. A) W83 grown in sTSB. (B) Strain W83 grown in sBHI C) Strain W83Δ0092 grown in sTSB D) Strain W83Δ0092 grown in sBHI.
Figure 4-2. Electron micrograph of negative stains of *P. gingivalis* strains W83 and W83Δ1683 in sTSB and sBHI media. A) W83 grown in sTSB. (B) W83 grown in sBHI. C) W83Δ1683 grown in sTSB (D) W83Δ1683 grown in sBHI.
Figure 4-3. Hemagglutination of *P. gingivalis* wild-type W83, W83Δ0092 and W83Δ1683 when grown in sTSB media. The titer was defined as the last dilution that showed full agglutination.

Figure 4-4. Hemagglutination of *P. gingivalis* wild-type W83, W83Δ0092 and W83Δ1683 when grown in sBHI media. The titer was defined as the last dilution that showed full agglutination.
Figure 4-5. Adherence to HCAEC for *P. gingivalis* W83 and W83Δ0092 in sBHI media. Cells were pre-exposed to Cytochalasin D (to prevents internalization) for 1 hour. *P. gingivalis* W83 and W83Δ0092 (with Cytochalasin D) were allowed to adhere to cells for 30 minutes at an MOI of 100 and non-adherent bacteria were washed away. Cells were lysed with 1 ml of water for 20 minutes. The adherent bacteria were diluted with PBS and plated for enumeration on blood agar plates to measure adherent bacteria. Mutant W83Δ0092 demonstrated an increase in adherent ability of HCAEC at 30 minutes when compared to the wild-type. (P < 0.05). Two-way ANOVA was used for statistical comparisons. Asterisks indicate a significant difference.
Figure 4-6. Adherence to HCAEC by *P. gingivalis* W83 and W83Δ1683 in sBHI media. Cells were pre-exposed to Cytochalasin D (to prevent internalization) for 1 hour. *P. gingivalis* W83 and W83Δ1683 (with Cytochalasin D) were allowed to adhere to cells for 30 minutes at an MOI of 100 and nonadherent bacteria were washed away. Cells were lysed with 1 ml of water for 20 minutes. The adherent bacteria were diluted with PBS and plated for enumeration on blood agar plates to measure adherent bacteria. Mutant W83Δ1683 demonstrated an increase in adherent ability of HCAEC at 30 minutes when compared to the wild type. (P < 0.05). ANOVA was used for statistical comparisons. * Asterisks indicate significant difference. Asterisks indicate a significant difference.
Figure 4-7. Adherence of *P. gingivalis* W83 to HCAEC, previously grown in sTSB and sBHI media. Cells were pre-exposed to Cytochalasin D (to prevent internalization) for 1 hour. *P. gingivalis* W83 (with Cytochalasin D) from sTSB and sBHI were allowed to adhere to cells for 30 minutes at an MOI of 100 and non-adherent bacteria were washed away. Cells were lysed with 1 ml of water for 20 minutes. The adherent bacteria were diluted with PBS and plated for enumeration on blood agar plates. Student’s *t*-test was used for statistical comparisons. Asterisks indicate significant difference.

Figure 4-8. Microtiter plate homotypic biofilm production by *P. gingivalis* W83 and W83Δ1683 in sBHI media. *P. gingivalis* biofilms were stained with 1% crystal violet. Biofilms of *P. gingivalis* W83 and W83Δ1683 at 24, 48, 72 and 96 h. Two-way ANOVA was used for statistical comparisons. Asterisks indicate significant difference (P value < 0.05).
Figure 4-9. Microtiter plate homotypic biofilm production by *P. gingivalis* W83 and W83Δ0092 in sBHI media. *P. gingivalis* biofilms were stained with 1% crystal violet. Biofilms of *P. gingivalis* W83 and W83Δ0092 at 24, 48, 72 and 96 h. Two-way ANOVA was used for statistical comparisons.

Figure 4-10. Microtiter plate monospecies biofilm production by *P. gingivalis* W83 in sTSB and sBHI media. *P. gingivalis* biofilms were stained with 1% crystal violet at 24, 48, 72 and 96 h. Student’s *t*-test was used to make statistical comparisons. Asterisks indicate significant difference.
Table 4-1. Phenotype summary. Summary of results of mutants and wild type.

| Strain | Adhesion | Invasion | Biofilm | Fimbriae | HA | sTSB | | |
|--------|----------|----------|---------|----------|----|------| | |
| W83    | +        | +        | +       | -        | +  | +    | + | + |
| 0092   | ↑        | +        | +       | -        | +  | -    | - | - |
| 1683   | ↑        | +        | +       | -        | +  | -    | - | - |

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<tr>
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</tr>
<tr>
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<td>↓</td>
</tr>
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<td>Biofilm</td>
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CHAPTER 5
DISCUSSION

According to the Center for Disease Control, periodontal disease is the second most common infectious disease in the world. It has more recently been associated with cardiovascular and other systemic diseases. It is believed that Porphyromonas gingivalis, an etiologic agent of periodontal disease, is able to enter the bloodstream via daily routines, such as toothbrushing and flossing, and can invade the human coronary artery endothelial cells that line the blood vessel walls of the human artery, (51).

The studies reported here focused on characterizing two mutants of Porphyromonas gingivalis wild type strain W83 and their abilities to invade and adhere to HCAEC, as well as to determination of the importance of these genes in other virulence mechanisms. Such analysis allows us to advance our knowledge of P. gingivalis as a pathogen and more completely understand the importance of this pathogen in infections and in cardiovascular disease. The two genes that were the focus of this investigation, PG0092 and PG1683, were chosen because they were previously found to be up-regulated during invasion of HCAEC at several time points. The up-regulation of genes under specific conditions suggests a function or role for the gene products in these conditions. In the model of invasion of HCAEC, up-regulation of genes at earlier time points may suggest their involvement in adherence and/or entry whereas expression at later time points may suggest a function in persistence. PG1683 was found to be up-regulated at 5 min, 1 and 2.5 h, therefore indicating a likely purpose in earlier events such as adherence, entry and trafficking, whereas PG0092 was found to be up-regulated at 2.5 h, indicating possible importance in trafficking and/or persistence. Prior to the work reported here, mutants in PG0092 and PG1683 had been constructed by allelic replacement to allow for mutational analysis to be completed.
To this end and as reported here, mutational analysis was used to examine the role of these genes in multiple assays related to virulence/pathogenesis. The two mutants were initially tested in adherence, invasion, and persistence assays. Both mutants, when grown in sTSB, demonstrated an increase in adherence to HCAEC but no difference in invasive capabilities at 2.5 and 6 h, when compared to the parental wild type strain, W83. In extended invasion assays, the mutants and wild type all showed reduced persistence at 24 and 48 hrs. This was not surprising since *P. gingivalis* is difficult to culture at later time points (17). In addition, the mutants were tested in other virulence assays. Neither mutants, when grown in sTSB, exhibited a difference in hemolysis, hemmaglutination, or gingipain activity. Both mutant and wild type strains were devoid of fimbriae when grown overnight in sTSB.

The mutants were further characterized as to biofilm phenotype. When grown in sTSB, no differences were detected for both mutants compared to the wild type in homotypic biofilm formation. Scanning electron microscopy analysis of homotypic biofilm illustrated a difference in biofilm aggregation in both mutants when compared to wild-type W83 in that the mutants sporadically aggregated in long chains, and wild type strain W83 formed a flat, evenly distributed biofilm. Confocal microscopy also indicated that the mutants formed taller peaks of biofilm when compared to W83. Differences were also determined using a heterotypic biofilm model, containing *Porphyromonas gingivalis* and *Fusobacterium nucleatum* 22586, which showed an increase in total biofilm production for W83Δ0092 at 48 hrs. All three strains made significantly more heterotypic than homotypic biofilm.

Contrastingly, different phenotypes were observed when the *P. gingivalis* strains were cultured in a different medium (122). The most significant changes were changes in adherence, hemagglutination, biofilm formation and the presence/absence of fimbriae. When grown in
sBHI, mutant W83Δ1683, was no longer able to agglutinate erythrocytes, did not form intact fimbriae and was totally impaired in biofilm formation, all in contrast to wild type W83. When mutant W83Δ0092 was cultured in this medium, it did not form fimbriae and was partially impaired in its ability to form biofilms, as compared to W83. Interestingly, all three strains had a significantly higher hemagglutination titers when cultured in sTSB as compared to sBHI.

**Gene PG0092 (Putative ABC Transporter)**

PG0092 is classified as a putative ABC transporter in the TIGR database and is predicted to span the entire inner and outer membrane. The gene is homologous to an ABC transporter in *Bacteroides fragilis* and the predicted operon contains a HIYD family secretion protein. The expression profile of this gene was evaluated during invasion of HCAEC and was up-regulated 3.5 fold at 2.5 hours. In sTSB, a mutation in PG0092 did show an affect on adherence (an increase) but not invasion or persistence. However, when cultured in sBHI an opposite phenotype was observed, with a decrease in adherence and biofilm formation. This putative ABC transporter may facilitate the transport of some molecule that affects adherence. If adherence is changed, perhaps the dynamics of bacterial entry is affected. It is likely that the difference in adherence is the result of a change in surface structure.

A change in surface structure may also explain the affect on biofilm formation. When mutant W83Δ0092 was cultured in sTSB an increase in biofilm height was also observed, suggesting that this mutation increases aggregation as well as adherence. However, when grown in sBHI, this mutant showed a reduction in the formation of homotypic biofilm and adherence, showing an opposite phenotype. Interestingly, a study has shown that an ABC transporter of *Listeria monocytogenes* up-regulates biofilm formation (114). The change in biofilm formation may be related to the lack of fimbriae in the new media when compared to the wildtype. Regardless of mechanism, the data clearly indicate that PG0092 is important in adherence even if
opposite affects are observed when cultured in two conditions. This mutation may be preventing the transport of a molecule that is responsible for surface structure formation or the transport of a virulence factor that contributes to adherence. It will this be important to further characterize this mutant regarding the function of PG0092 transport.

**Gene PG1683 (Putative Glycosyl Hydrolase/Alpha Amylase)**

PG1683 is listed as a putative glycosyl hydrolase/alpha amylase in the TIGR database. This gene is homologous to a gene in *Parabacteroides distasonis* and its predicted operon contains a glycogen debranching enzyme and a glycosyl transferase. Since this gene was found to be up-regulated at 0.5 min, 1 and 2.5 hours, this indicates that the gene most likely plays an important role during the earlier stages of invasion of HCAECs. However, when grown in sTSB, this mutant showed no difference in invasion, persistence, hemagglutination, gingipain activity, or fimbriae expression. The only differences determined were in adherence (an increase) and biofilm height formation. Most significantly, however, when grown in sBHI, this mutant exhibited a drastic reduction in adherence, homotypic biofilm formation, fimbriae expression and hemagglutination.

Although the basis of this phenotype is unknown, it can be speculated that PG1683 may be involved in the assembly of surface structures including functional fimbriae, since when this mutant was cultured in sBHI no fimbriae were detected by electron microscopy in contrast to W83. Since the data it is indicated that W83Δ1683 expresses the major structural fimbrial protein, FimA, it is likely that it is not able to assemble functional fimbriae. Therefore, the loss of this gene product results in the inability to produce fimbriae in this environment. The reduction in adherence and biofilm formation observed with this mutant may be due to the lack of fimbriae. This gene (including PG1683) may also be able to degrade carbohydrates on the surface of the host or other cells, exposing an epitope (cryptitope) or adhesion receptor that allow
for increased adhesion and/or changes in carbohydrate surface structures. Through this same mechanism, the absence of this process may lead to decrease hemagglutination, adherence, and fimbriae expression.

**Further Directions**

The experiments completed on these two mutants have raised several questions. Since the mutants had different phenotypes when grown in different media, environmental factors must be responsible for a change in gene expression. Consequently, the identification of the component(s) of the media responsible for the difference in the phenotypes of the mutants should be determined. Thus a biochemical analysis of the media components and the identification of the environmental signal in the sBHI medium will be significant for future characterization of these mutants. In addition the examination of biofilms grown in the BHI broth and using confocal laser and scanning electron microscopy imaging techniques may prove inciteful. It will also be important to characterize the substrate for the putative ABC transporter (PG0092), determine or confirm the function of both genes and to characterize both of the operons. The construction of complemented strains would be helpful in establishing that these mutations did not result in polar effects.

*P. gingivalis* is internalized into HCAEC, turns on autophagy and then traffics within autophagic vacuoles. However, since *P. gingivalis* is an obligate anaerobe, the question of how it survives within eukaryotic cells is significant. One reasonable hypothesis is that it “creates” a microenvironment within the endothelial cell vesicle that is anaerobic. However this remains an important question and studies need to be completed to analyze the process by which *P. gingivalis* can survive in aerobic cells. It is also important to study how the mutant genes, PG0092 and PG1683, might interact and affect vascular endothelial cells including the role of these genes in establishing a microenvironment for *P. gingivalis* intracellularly. These mutants
may interact different from the wild-type with vascular endothelial cells and possibly provide us with a better understanding of the intracellular trafficking of *P. gingivalis* in HCAEC.

The characterization of the phenotype of mutants in these genes has provided additional information regarding the role of these genes in *P. gingivalis* pathogenesis. However, the elucidation of the significance and roles of these genes in pathogenesis, both oral and nonoral, associated with *P. gingivalis* infections will require additional studies. The important question is to find the important factor in the environment that is leading to changes in phenotype and gene expression.
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BIOGRAPHICAL SKETCH

Jennifer Nina Rainho was born in Newark, NJ, to Portuguese immigrants, Gloria and Joseph Rainho. She attended Queen of Peace High School in North Arlington, NJ and graduated in May 1999. In 2002, Jennifer earned an Associate of Art’s degree in Business at Union County College in Cranford, NJ. Jennifer went on to attend Kean University in Union, NJ, where she served as a Secretary for the Student Organization Inc. and the American Chemical Society. She was a member of Beta Beta Beta Biological Honors Society and Theta Phi Alpha Fraternity Inc. During 2004, Jennifer began research in the laboratory of Dr. My Abdelmajid Kassem of the Botany Department in which she assisted in the mapping of soybean traits and was co-author of two published peer reviewed papers. In December 2005, she graduated from Kean University with a Bachelors of Science in Biology and worked as a temporary employee at Colgate-Palmolive Corporation. In the fall of 2007, Jennifer entered the College of Medicine’s M.S. program at the University of Florida and began conducting research in the laboratory of Dr. Progulske-Fox. During her time at the University of Florida, Jennifer placed first at the Seventh Annual University of Florida College of Dentistry Research Day in the Master’s/Resident division. In the fall of 2009, Jennifer will be enrolling in the Ph.D. Program in Biomedical Sciences at the University of Miami.