ROLE OF PG1237 A NOVEL TRANSCRIPTIONAL REGULATOR IN BIOFILM FORMATION IN *PORPHYROMONAS GINGIVALIS*

By

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To All The People Who Supported Me During My Graduate Program

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Chronic periodontitis is a widely prevalent disease in the United States and one of the major organisms implicated in its etiology is *Porphyromonas gingivalis.* Biofilm formation with primary colonizers of the tooth such as *Streptococcus gordonii* is one of the main mechanisms by which *P. gingivalis* colonizes the tooth and the periodontium. Understanding the biology and mechanism of *P. gingivalis* biofilm formation is crucial for the development of more effective treatments against chronic periodontitis.

The luxR family of transcriptional regulators are widely found in prokaryotes and have been found to regulate biofilm formation and other virulence properties in many bacteria. In this study the role of PG1237, a LuxR family transcriptional regulator in homotypic and heterotypic biofilm formation of *P. gingivalis* was investigated.

We characterized the role of PG1237 through the use of mutants that lack PG1237 and showed that PG1237 activity constrains both monospecies biofilm development and community development with the primary oral biofilm constituent *Streptococcus gordonii*. We found that PG1237 regulates transcriptional activity of *luxS* and thus impacts AI-2-dependent signalling in biofilm communities. Also PG1237 regulates production of Mfa, the minor fimbriae protein that is necessary for community

formation with *S. gordonii*. We also found that PG1237 directly regulates *luxS* and *mfa1* by binding to their promoter regions.

In the absence of PG1237 transcription of *luxS* and *mfa1* was increased in the mutant resulting in both greater homotypic biofilm formation as well as greater heterotypic community formation with *S. gordonii*. We also showed that *Ltp1*, a low molecular weight tyrosine phosphatase which has a regulatory role in biofilm formation might be regulating the expression of PG1237. Complementation of the *pg1237* mutation with wild-type *pg1237* restored homotypic biofilm formation as well as *luxS* and *mfa1* expression to wild type levels.

Collectively, these results show that PG1237, a LuxR family transcriptional regulator controls and regulates several pathways that are important for the biofilm formation of *P. gingivalis* .

CHAPTER 1
INTRODUCTION TO PERIODONTAL DISEASE AND *PORPHYROMONAS GINGIVALIS*

Periodontal Disease- A Significant Health Concern

Periodontal diseases are a group of inflammatory diseases that affect the supporting tissues of the dentition. The most prevalent periodontal diseases result from the interaction of specific bacterial species with components of the host immune response in disease susceptible individuals. They are currently classified as plaque-induced gingival diseases, early onset, chronic adult and aggressive periodontitis (Armitage 1999). Plaque-induced gingival diseases are limited to the gingivae (gingivitis) and are characterized by erythema, edema, hemorrhage and enlargement of the gingival tissues. Plaque- induced gingivitis is nearly pandemic in children and young adults and is reversible with plaque removal. In contrast, early onset, chronic and aggressive periodontitis are irreversible forms of periodontal disease that culminate in tooth loss if left untreated. Estimates of the prevalence of periodontitis vary with the clinical criteria used to define disease status; however, the Third National Health and Nutrition Survey (NHANES III) reported a 14% prevalence of moderate to severe periodontitis in the United States population >20 years of age (Oliver et al. 1998).

The inflammatory lesion in periodontitis extends from the gingiva to include deeper connective tissues resulting in the loss of periodontal ligament and alveolar bone . Gingival epithelium migrates into the area of periodontal ligament and alveolar bone destruction, creating a periodontal pocket around the affected tooth. The formation of a periodontal pocket is a characteristic feature of periodontitis in humans. Recruited into the connective tissue adjacent to the periodontal pocket is an intense cellular infiltrate

consisting of polymorphonuclear leucocytes, monocytes/macrophages, B and T cell lymphocytes (Ebersole et al. 2000)

The periodontal pocket is colonized by bacteria that exist in a stratified, highly ordered ecosystem, termed a dental biofilm or plaque, consisting of bacteria, bacterial products such as endotoxin/LPS and an extracellular matrix of polysaccharides, proteins and inorganic compounds. The organization of the dental biofilm optimizes bacterial cell proliferation, while providing protection from host defence mechanisms as well as externally applied anti-microbials. However, discreet complexes of bacterial species have been described in association with periodontal disease status and progression (Socransky and Haffajee 2005). Plaque samples from periodontally healthy subjects consist largely of Gram-positive aerobic species. A shift towards increasing numbers of Gram-negative species, including the appearance of *Fusobacterium nucleatum* and various *Treponema* species, occurs in samples from subjects with plaque-induced gingivitis. The shift towards Gram-negative bacteria increases in plaque samples from subjects with chronic periodontitis with nearly 85% of the bacterial species reported as Gram-negative anaerobic or facultative anaerobic species including *Aggregatibacter actinomycetemcomitans* serotypes a and b, *Campylobacter rectus, Porphyromonas gingivalis, Prevotella intermedia, Tannerella forsythia* and *Treponema denticola*. In particular, *P. gingivalis, T. forsythia* and *T. denticola* have been associated with the progressing plaque front in chronic periodontitis (Dzink et al. 1988).

However, colonization with a specific periodontal pathogen appears necessary but not sufficient for periodontal disease progression, since the majority of sites colonized remain quiescent for extended periods of time. During progression of the periodontal

lesion, the host may be exposed to increased antigenic challenge in sites with active extracellular matrix destruction since serum IgG antibody to *P. gingivalis* in particular has been reported to be elevated in subjects with periodontitis and further elevated with disease progression (Craig et al. 2002).

Risk Factors

Evidence supports the existence of several risk factors which increase an individual's likelihood of developing periodontal disease. Some of the major risk factors are poor oral hygiene, pregnancy, use of oral contraceptives and menopause also increase the risk of periodontitis (Boggess, 2008). Other major risk factors are cigarette smoking and poorly controlled diabetes mellitus. Obesity, osteoporosis, socioeconomic status and HIV infection were also found to be risk factors (Borrell, 2005). There is a genetic predisposition of certain individuals to periodontal disease (Loos et al. 2005). Herpes virus infection (Wu et al. 2006) and autoimmune diseases such as Crohn's disease, multiple sclerosis, rheumatoid arthritis are also associated with a higher incidence of periodontal disease. Also deficiency of vitamin C could contribute to periodontal disease (Amaliya et al. 2007). Therefore, a multitude of factors can influence the development and progression of periodontal disease.

Dental Plaque-Relationship Of Specific Organisms To Periodontal Disease

Socransky (Socransky and Haffajee 1992) proposed the following criteria for an organism to be a periodontal pathogen:

1. ASSOCIATION: A pathogen should be found more frequently and in higher numbers in disease states than in healthy states
2. Elimination: Elimination of the pathogen should be accompanied by elimination or remission of the disease.

3. Host Response**:** There should be evidence of a host response to a specific pathogen which is causing tissue damage.

4. Virulence Factors**:** Properties of a putative pathogen that may function to damage the host tissues should be demonstrated.

5. Animal Studies**:** The ability of a putative pathogen to function in producing disease should be demonstrated in an animal model system.

The two periodontal pathogens that have most thoroughly fulfilled Socransky's criteria are *Aggregatibacter actinomycetemcomitans* in the form of periodontal disease known as localized aggressive periodontitis (LAP), and *Porphyromonas gingivalis* in the form of periodontal disease known as adult periodontitis or chronic periodontitis

Porphyromonas Gingivalis

General characteristics

Porphyromonas gingivalis is a black pigmented, Gram negative, anaerobic, asaccharolytic rod that is the major cause of chronic periodontitis. This bacteria is also implicated as a possible risk factor for the development of certain types of cardiovascular disease (Renvert et al. 2006) and pregnancy complications (Contreras et al. 2006).

In vitro growth of *P. gingivalis* is routinely performed on blood agar plates supplemented with hemin and vitamin K_1. Early colonies of *P. gingivalis* appear tan in color but after several days of growth, the colonies become strongly black-pigmented. This characteristic results from the storage of acquired heme on the bacterial cell surface (Lamont and Jenkinson, 1998). *P. gingivalis* obligately requires iron for growth and hemin satisfies this requirement (Lamont and Jenkinson, 1998). *P. gingivalis* has to rely on small peptide molecules for its nutrition because of its inability to effectively utilize sugars as an energy source (Lamont and Jenkinson, 1998). This nutrient

requirement requires *P. gingivalis* to express several proteases which along with hydrolyzing proteins into small peptides and amino acids, also simultaneously can damage host tissues.

P. gingivalis has been characterized into six serogroups designated K1-K6 based on the antigenicity of its capsular carbohydrates (Laine et al. 2006). Also, the LPS of *P. gingivalis* has been characterized into three serogroups, O1 to O3. Bacteria isolated from disease sites display a wide variety of K and O antigen combinations and chronic periodontitis does not appear to rise from any single specific combination. *Porphyromonas gingivalis fimA* which encodes fimbrillin, a subunit of fimbriae, has been classified into 6 genotypes (types I to V and Ib) (Kuboniwa et al. 2009), based on their nucleotide sequences. Among the *P. gingivalis*-positive healthy adults, the most prevalent *fimA* type is type I followed by type V. In contrast, a majority of the periodontitis patients carry type II *fimA* organisms followed by type IV. There are both disease-associated and non-disease-associated strains of *P. gingivalis,* and their infectious traits influencing periodontal health status can be differentiated based on the clonal variation of *fimA* genes. (Amano et al. 2000).

P. gingivalis has the ability to invade gingival epithelial cells (Lamont et al.1995; Tribble et al. 2006). The mechanism of invasion involves cytoskeletal arrangement (Lamont and Jenkinson 1998).The recently described haloacid dehalogenase family phosphatase SerB, has been demonstrated to play an important role in the invasion process (Tribble et al. 2006). This bacteria also has the ability to override host cell apoptotic programs which probably maintains a favorable intracellular environment for bacterial survival.

Virulence factors

Any factor that increases the ability of a pathogen to damage its host tissue is considered a virulence factor. The presence of a capsule in *P. gingivalis* has been considered an important anti-phagocytic virulence factor by many investigators. A strong relationship was found to exist between the extent of *P. gingivalis* encapsulation and several important biological functions that could have a significant effect on its ability to function as an oral pathogen. The highly encapsulated *P. gingivalis* strains exhibit decreased auto agglutination, lower buoyant densities and are more hydrophilic than the less encapsulated strains. Increased encapsulation was also correlated with increased resistance to phagocytosis, serum resistance,and decreased induction of polymorphonuclearleukocyte chemiluminescence (Holt, 2000).The decreased tendency for the highly encapsulated strains to be phagocytosed has been proposed due to the increased hydrophilicity of the strains and their decreased ability to activate the alternate complement pathway.

P. gingivalis produces major *fimbriae* which are composed of FimA subunits and are associated with the ability to adhere to different surfaces. Different regions of *P. gingivalis* fimbriae have been found to interact with various substrates such as lactoferrin, fibronectin and erythrocytes demonstrating the dynamic adhering properties of this bacterial structure (Lamont and Jenkinson 1998). FimA mutants were shown to have impaired adherence as well as a deficiency in invading host cells (Weinberg et al. 1997).

The FimA mutant phenotype allowed for the discovery of minor *fimbriae* (Hamada et al. 1996). The minor *fimbriae* are composed of Mfa subunits encoded by the *mfa1* gene. *mfa1* mutant strains were completely incapable of forming biofilms (Lin et al.

2006). This provides evidence that minor *fimbriae* are important for cell-cell interactions in *P. gingivalis*. Also, minor *fimbriae* have demonstrated interaction with the SspA/B antigen of *Streptococcus gordonii*. It is evident that minor *fimbriae* complement the adhesive characteristics of major fimbriae and both are important *P. gingivalis* virulence factors.

The most potent and important proteinases produced are Arg-X and Lys-X proteases or gingipains. It is estimated that around 85% of proteolytic activity of *P. gingivalis* is attributable to gingipains (Potempa et al. 2007). Some functions of gingipains are that they give *P. gingivalis* the ability to affect gingival crevicular fluid production by inducing vascular permeability, increasing inflammation through activation of blood coagulation pathway and preventing blood clotting through degradation of fibrinogen (Imamura, 2003). Also gingipains promote *in vivo* colonization of *P. gingivalis* and by their proteolytic activity cause formation of periodontal pocket and eventual tooth loss (Potempa et al. 2001). They also prevent activation of leukocytes and they bind and lyse erythrocytes for heme acquisition (Imamura, 2003).

P. gingivalis lipopolysaccharide (LPS) is also an important virulence factor. It is less immunostimulatory than LPS from enteric bacteria such as *E. coli*. It functions by inducing chemokine induction, natural killer cell activation, induction of tumor necrosis factor alpha and nitrous oxide. LPS stimulates the production of interleukin IL-1, IL-6 and IL-8 in human gingival fibroblasts, which in turn activates osteoclasts *in vitro* (Wang et al. 1999).

P. gingivalis also possesses several hemagglutinins and hemolysins. These molecules also help in iron acquisition through binding of erythrocytes and their

eventual lysis. Most notable of the hemagglutinins are the proteins HagA-E (Progulske-Fox, 1995). Iron acquisition is associated with quorum sensing systems in *P. gingivalis. P. gingivalis* shows a strong preference for iron in the form of hemin and expresses a number of heme/hemin uptake systems. These hemin uptake mechanisms include the TonB-linked outer membrane hemin binding proteins HmuR and Tlr and the hemin binding lipoprotein FetB (IhtB). Hemin uptake mechanisms are regulated by an external AI-2 signal produced by the action of LuxS. Furthermore, it is apparent that the AI-2 signal allows *P. gingivalis* to switch between different mechanisms of hemin and iron uptake as dictated by environmental conditions. (James et al. 2006).

CHAPTER 2
ORAL BIOFILM FORMATION AND ITS REGULATION

Biofilms-Their General Properties

A biofilm is an aggregate of microorganisms in which cells are stuck to each other and/or to a surface. These adherent cells are frequently embedded within a self-produced matrix of extracellular polymeric substance (EPS). Biofilm EPS, which is also referred to as "slime," is a polymeric mass of DNA, proteins and polysaccharides. Biofilms may form on living or non-living surfaces, and represent a prevalent mode of microbial life in natural, industrial and hospital settings (Stoodley et al. 2004). The cells of a microorganism growing in a biofilm are physiologically distinct from planktonic cells of the same organism, which by contrast, are single-cells that may float or swim in a liquid medium. Microbes form a biofilm in response to many factors, which may include cellular recognition of specific or non-specific attachment sites on a surface, nutritional cues, or in some cases, by exposure of planktonic cells to sub-inhibitory concentrations of antibiotics (Karatan et al. 2009). When a cell switches to the biofilm mode of growth, it undergoes a phenotypic shift in behavior in which large suites of genes are differentially regulated. Biofilms are ubiquitous. Nearly every species of microorganism, not only bacteria and archaea, have mechanisms by which they can adhere to surfaces and to each other.

Some common properties of bacterial biofilms are microorganisms are arranged in microcolonies, the microcolonies are surrounded by a protective matrix, microorganisms have different communication systems such as Quorum sensing and microorganisms in the biofilm are more resistant to antibiotics, antimicrobials, and host response as compared to planktonic cells.

The stages of biofilm development are initial attachment, irreversible attachment, maturation and dispersal.

Biofilms have been found to be involved in a wide variety of microbial infections in the body. Infectious processes in which biofilms have been implicated include common problems such as urinary tract infections, catheter infections, middle-ear infections, formation of dental plaque, gingivitis, coating contact lenses , and less common but more lethal processes such as endocarditis, infections in cystic fibrosis, and infections of permanent indwelling devices such as joint prostheses and heart valves (Lewis, 2001). More recently it has been noted that bacterial biofilms may impair cutaneous wound healing and reduce topical antibacterial efficiency in healing or treating infected skin wounds (Suman et al. 2009).

Dental Plaque-An Oral Biofilm

Dental plaque is a complex and dynamic biofilm that accumulates through the sequential and ordered colonization of over 500 different species of bacteria (Marsh, 1995). In addition to the bacterial cells, plaque contains a small number of epithelial cells, leukocytes, and macrophages. The cells are contained within an extracellular matrix, which is formed from bacterial products and saliva. The extracellular matrix contains protein, polysaccharide and lipids. Inorganic components are also found in dental plaque; largely calcium and phosphorus which are primarily derived from saliva. The inorganic content of plaque is greatly increased with the development of calculus. The process of calculus formation involves the calcification of dental plaque.

Dental plaque can be classified in several different ways. Plaque can be classified as supragingival or subgingival based on its relationship to the gingival margin. Supragingival plaque is evident on the tooth above the gingival margin. Plaque can also

be classified by its relationship to the tooth surface, as either attached or unattached plaque. The unattached subgingival plaque is more closely associated with the wall of the subgingival tissues than is the attached plaque. Lastly, plaque has been classified by association with disease state as "health-associated" or "disease-associated". The latter classification is related to differences in the microbial composition of dental plaque in health versus disease (Schwartz et al.1995).

The pellicle-coated tooth surface is colonized by Gram-positive bacteria such as *Streptococcus sanguis* and *Actinomyces naeslundii*. These organisms are examples of the "primary colonizers" of dental plaque. Bacterial surface molecules interact with components of the dental pellicle to enable the bacteria to attach or adhere to the pellicle-coated tooth surface. For example, specific protein molecules found as part of the bacterial fimbriae on both *Streptococcus sanguis* and *Actinomyces naeslundii* interact with specific proteins of the pellicle (the proline-rich proteins) with a "lock and key" mechanism that results in the bacteria firmly sticking to the pellicle-coating on the tooth surface (Mergenhagen et al.1987). Within a short time after cleaning a tooth, these Gram-positive species may be found on the tooth surface.

After the initial colonization of the tooth surface, plaque increases by two distinct mechanisms: 1) the multiplication of bacteria already attached to the tooth surface, and 2) the subsequent attachment and multiplication of new bacterial species to cells of bacteria already present in the plaque mass. The secondary colonizers include Gram-negative species such as *Fusobacterium nucleatum, Prevotella intermedia*, and *Capnocytophaga* species. A key property of these microorganisms appears to be the ability to adhere to Gram-positive species already present in the existing plaque mass.

These organisms would typically be found in plaque after 1 to 3 days of accumulation. When the structure of dental plaque from this time period is observed, the presence of a complex array of bacterial cocci, rods and filaments is apparent (Sbordone et al. 2003).

After one week of plaque accumulation, other Gram-negative species may also be present in plaque. These species represent what is considered to be the "tertiary colonizers", and include *Porphyromonas gingivalis,* the oral spirochetes (*Treponema* species) and other Gram negative anaerobes. The structural characteristics of dental plaque in this time period reveal complex patterns of bacterial cells of cocci, rods, fusiform, filaments, and spirochetes. In particular, specific associations of different bacterial forms have been observed. For example, the adherence of cocci to filaments results in a typical form referred to as "test-tube brushes" or "corn-cob" structures. The structural interactions of the bacteria probably are partially a reflection of the complex metabolic interactions that are known to occur between different plaque microorganisms. One example of this is the production of succinic acid from *Campylobacter* species that is known to be used as a growth factor by *Porphyromonas gingivalis* (Takahashi 2005).

The overall pattern observed in dental plaque development is a very characteristic shift from the early predominance of Gram-positive facultative microorganisms to the later predominance of Gram-negative anaerobic microorganisms, as the plaque mass accumulates and matures. This developmental progression is also reflected in the shifts in predominant microorganisms that are observed in the transition from health to disease. Studies of plaque taken from sites of health or disease and examined either

microscopically or by culturing have demonstrated distinct differences in health versus disease-associated microbial populations (Socransky et al. 2000).

Quorum Sensing In Biofilm Communities

Population density is used by bacteria as a cue for the regulation of diverse cellular functions. Quorum (density-dependent) sensing involves the synthesis of small signalling molecules that accumulate in the local environment. As the bacterial population density increases, the concentration of signalling molecules also increases until a threshold, or quorum, is reached, triggering the expression of target genes. In Gram-negative bacteria, the best-described and most widespread signalling molecule is acyl homoserine lactone (AHL) (Fuqua et al.., 2001). In the archetypal quorum-sensing system of the marine symbiotic bacterium *Vibrio fischeri*, AHL is synthesized by LuxI protein. At a threshold concentration, the membrane-diffusible AHL interacts with and activates the intracellular LuxR transcriptional activator resulting, in the case of *V. fischeri*, in expression of genes required for bioluminescence. LuxI/R homologues have now been described in a large group of diverse bacteria including numerous plant and animal pathogens and, in many instances, two or more LuxI/R systems may function in a single species. AHL-based quorum sensing is known to regulate a range of cellular functions including bioluminescence, motility, production of secondary metabolites, expression of virulence factors and plasmid conjugal transfer (Fuqua et al.., 2001). Signalling through AHLs has also been shown to be important for biofilm formation in some organisms (Davies et al.., 1998).

It became clear from molecular studies of *V. harveyi* that there existed a second signalling system, based on an unrelated autoinducer (AI-2), that was species non-specific (Bassler et al.., 1994). The *luxS* gene encoding AI-2 synthase revealed no

similarity to other autoinducer synthase genes (Surette et al.1999). Moreover, by using a mutant strain of *V. harveyi* able to detect AI-2 but not AI-1, it was soon demonstrated that culture supernatants of strains of *Escherichia coli* and *Salmonella enterica* serovar Typhimurium (*S*. typhimurium) were able to induce bioluminescence, indicating that AI-2 production was not restricted to *V. harveyi* and that interspecies communication was possible (Surette et al.., 1999). Following its discovery in *V. harveyi*, various homologues of *luxS* in a wide range of organisms, including a number of important human pathogens were found. A role for LuxS signaling as both a global regulator and as important for expression of virulence traits is thus emerging. In *Porphyromonas gingivalis*, AI-2 can control expression of genes involved in hemin uptake and in haemagglutination (Chang et al. 2002). In *A. actinomycetemcomitans* , production of leukotoxin, an important virulence factor, and iron-uptake proteins is regulated in response to LuxS (Fong et al. 2001). The *luxS* gene is also present in a range of pathogenic Gram-positive bacteria including *Streptococcus mutans*, *Streptococcus pyogenes* and *Clostridium perfringens* (Lyon et al. 2001; Ohtani et al. 2002; Wen & Burne2 004).

Biofilm Formation And Its Regulation In Porphyromonas Gingivalis

Biofilm development in general proceeds through a series of ordered developmental steps ie. surface attachment, microcolony formation and biofilm maturation(Stanley and Lazazzera 2004). In the case of *P. gingivalis-S. gordonii* consortia, the first step is a multivalent coadhesive interaction mediated by two distinct adhesin receptor pairs. The *P. gingivalis* long fimbriae (FimA) bind to glyceraldehyde-3-phosphate dehydrogenase (GAPDH) present on the streptococcal surface (Maeda et al.., 2004). In addition, the *P. gingivalis* short fimbriae (Mfa) engage the streptococcal

SspA/B (antigen I/II) adhesins (Park et al. 2005) through an approximately 27 aa binding epitope of SspA/B termed BAR (Daep et al. 2006). Following co-adhesion, LuxS dependent signaling is required for further development of the heterotypic biofilm communities (McNab et al. 2003). With regard to monospecies *P. gingivalis* biofilms, initial attachment depends on an internalin family protein, InlJ (Capestany et al. 2006), and development requires expression of the short *fimbriae* and the universal stress protein UspA (Lin et al. 2006). Limitation of biofilm development appears to be important for *P. gingivalis,* and such mechanisms in general are thought to arise in order to optimize exposure to oxygen (either maximal or minimal), or to facilitate influx of nutrients and efflux of waste (Rainey and Rainey 2003).

Key players in regulating *p. Gingivalis* biofilm formation

LuxS

LuxS is an enzyme that converts S-ribosylhomocysteine (RH) to 4,5-dihydroxy-2,3-pentanedione (DPD) and homocysteine (Schauder et al. 2001). DPD gets spontaneously converted to AI-2 which is required for formation of mixed species biofilm with *Streptococcus gordonii* (McNab 2003)

Ltp1

Ltp1 is a low molecular weight tyrosine phosphatase. It has a role in constraining both monospecies biofilm formation as well as mixed species community formation with *S. gordonii.* It constrains biofilm formation by regulating exopolysaccharide synthesis as well as regulation of *luxS* at the transcriptional level. Thus, *Ltp1* limits cell-to-cell communication as well as exopolysaccharide synthesis. Deletion mutants of *ltp1*showed both increased monospecies and mixed species community formation proving that *Ltp1* negatively regulates biofilm formation (Maeda et al. 2008).

Mfa

Mfa is the minor fimbriael protein that is the main building block of minor fimbriae. Mfa is required for development of both monospecies as well as mixed species biofilm formation with *S. gordonii* through its interaction with SspA/B. *mfa1* deletion mutants showed no monospecies or mixed species biofilm formation indicating that Mfa is critical for *P. gingivalis* biofilm formation.

Pg1237-A Luxr Family Novel Transcriptional Regulator

DNA sequence analysis shows that PG1237 belongs to the LuxR family. Sequence analysis showed that it contained the helix-turn –helix domain at its C terminal which is a characteristic of proteins belonging to the LuxR family. Also, the PG1237 helix-turn-helix domain showed a high degree of similarity to HTH domain of GerE, a LuxR family transcriptional regulator which regulates spore formation in *B.subtilis*.

Similar to the expression regulated by other members of LuxR family, expression of the *pg1237* gene is regulated in a cell density-dependent manner. Generally, LuxR proteins sense the autoinducers, acyl-homoserine lactones, which are synthesized by members of the LuxI protein family (Fuqua, 1996). However, LuxI and acyl-homoserine lactones have not been found in *P. gingivalis*.

Previous work has shown that PG1237 is required for the expression of *hmuY* and *hmuR*, but not other iron acquisition-related genes, such as *fetB* and *tlr*, which also encode hemin binding proteins (Wu et al. 2009).It was shown that PG1237 controls the expression of the *hmu* operon in a cell density dependent manner (Wu et al. 2009). Interestingly, another cell density-dependent sensory system, LuxS-AI-2, appears to be also involved in the regulation of hemin and iron acquisition pathways in *P. gingivalis*.

LuxS proteins function as a key synthase that generates an autoinducer, AI-2. Previous studies have shown that expression levels of Ton-linked hemin binding protein (Tlr) and the lysine-specific protease Kgp are reduced in a *luxS* mutant, whereas expression levels of some other iron acquisition-related genes, including *hmuR* , are upregulated in the mutant (James et al. 2006).These results suggest there could be a potential interplay between regulatory pathways of PG1237 and LuxS and that PG1237 could be regulating other virulence properties of *P. gingivalis* such as biofilm formation by regulating LuxS pathway. Hence, we decided to investigate the role of PG1237 in both monospecies and mixed species biofilm formation with *S. gordonii*.

CHAPTER 3
MATERIALS AND METHODS

Bacteria and Culture Conditions

P. gingivalis strains ATCC 33277, Δ*ltp1* (Simionato et al. , 2006), Δ*1237* (Wu et al. 2009), CΔ1237and Δ*luxS* (McNab et al. 2003) were cultured in Trypticase Soy Broth (TSB), supplemented with hemin (5 μg ml^{-1}) and menadione (1 μg ml^{-1}), anaerobically at 37°C (85% N_2, 10% H_2, 5% CO_2) . When necessary, erythromycin at 10 μg ml^{-1}, tetracycline at 1 μg ml^{-1}, or gentamicin at 100 μg ml^{-1} were incorporated into the medium. *S. gordonii* DL1 and *S.* cristatus CC5A were cultured anaerobically at 37°C in Brain Heart Infusion (BHI). *Escherichia coli* strains were grown aerobically at 37°C in LB media containing when necessary kanamycin (50 μg ml^{-1}) and chloramphenicol (30 μg ml^{-1}).

Table 3-1 List of bacterial strains and plasmids used

Strain or Plasmid	Source
P. gingivalis ATCC 33277	American Type Culture Collection
P. gingivalis 33277 Δ*1237*	Laboratory stock (Wu)
P. gingivalis 33277 Δ*ltp1*	Laboratory stock (Maeda)
P. gingivalis 33277 Δ*luxS*	Laboratory stock (McNab)
P. gingivalis 33277 CΔ1237	This study
TUNER DE3 pET30b-1237	Laboratory stock
S. gordonii DL1	Laboratory stock
S.cristatus CC5A	Laboratory stock
pT COW	Laboratory stock
E.coli J53	Laboratory stock
E.coli R751	Laboratory stock
TOP10 Competent Cells	Invitrogen
BL21 (DE3) Competent Cells	Novagen

Construction of Mutant and Complemented Strains :

Insertional pg1237 mutant was a kind gift from Dr. Xie at University of Tennessee. An insertional pg1237 mutant was generated by using ligation-independent cloning of

PCR-mediated mutagenesis. A 2.1-kb *ermF-ermAM* cassette was introduced into the pg1237 gene by three steps of PCR to yield a pg1237-*erm*-pg1237 DNA fragment.The fragment was then introduced into *P. gingivalis* 33277 by electroporation. The pg1237-deficient mutant was generated via a double crossover event that replaces the pg1237 gene with the pg1237-*erm*-pg1237 DNA fragment in the 33277 chromosome. The mutants were selected on TSB plates containing erythromycin (5 µg/ml). The insertional mutation was confirmed by PCR analysis, and the mutants were designated *P. gingivalis* 1237E (Wu et al. 2009).

Reverse transcription PCR showed that PG1236 and PG1237 form an operon. A DNA sequence including the PG1236 and PG1237 ORF's along with 300 bp upstream of the PG1236 initiation codon and 100 bp downstream of the PG1237 termination codon was amplified from *P. gingivalis* chromosomal DNA, The primers were engineered to contain the following restriction sites : NheI-EagI. The shuttle vector plasmid pT-COW was digested with the appropriate restriction enzymes to allow cloning of the PCR product into the *tetC* region. The resulting plasmids were transformed into *E. coli* TOP10 and selected on ampicillin(100µg/ml) plates. Colonies were screened by colony PCR and restriction digestion. The plasmids with the correct restriction profile were designated pT-*1237* and these plasmids were introduced into the Δ*1237* strain by conjugation. The conjugation reaction mixture also contained helper *E. coli* J53 containing *R751,* an IncP plasmid used to mobilize vectors from *E. coli* to a *Bacteroides* recipient (Shoemaker 1987). The conjugation mixture had a donor-to-recipient ratio of 0.2 .The mating was performed in a candle jar on pre-reduced blood agar plates for 16 h, and transconjugants were selected with gentamicin and tetracycline. The presence of

wild type *pg1237* gene was confirmed by PCR. The resulting strains were designated CΔ*1237.*

Production of PG1237 Recombinant Protein:

The DNA fragment encoding ORF of PG1237 was amplified by PCR with primers r1237F-EcoRV and r1237R-HindIII, which produced a 600-bp PCR product (Wu et al. 2009).The PCR products were then cloned into pCRII-TOPO (Invitrogen, Carlsbad, CA). The recombinant PG1237 (rPG1237) was expressed in *E. coli* by using a pET protein expression system (Novagen, Madison, WI). The DNA fragment of pg1237 was subcloned into the pET-30b downstream of a histidine tag. The rPG1237 was expressed in *E. coli* BL21(DE3) cells carrying the pET-30b/pg1237 plasmid in the presence of IPTG (isopropyl-β-$_D$-thiogalactopyranoside) and kanamycin(50µg/ml). His-tagged rPG1237 was purified with Ni^{2+}-charged His-bind resin (Novagen, Madison, WI) The protein was washed on the column with 60 mM imidazole and then eluted with 1M imidazole. Purity was greater than 97% as determined by SDS-PAGE Coomassie staining. Protein was then dialysed against TBS.

Homotypic *P. gingivalis* Biofilm Formation :

Homotypic biofilm formation by *P. gingivalis* was quantified by a microtiter plate assay (O'Toole and Kolter 1998), as adapted for *P. gingivalis* (Capestany et al. 2008). Parental and mutant strains in early log phase (2×10^8 cells) were incubated in microtiter plate wells at 37°C anaerobically for 24 h. The resulting biofilms were washed thrice with PBS, stained with 1% crystal violet, and destained with 95% ethanol. Absorbance at 595 nm was determined with a Benchmark microplate reader. Biofilm assays were repeated independently three times with each strain in triplicate and analyzed with a Student's unpaired two-tailed t test.

Heterotypic P. gingivalis-S. gordonii Biofilms:

Heterotypic *P. gingivalis*-*S. gordonii* communities were generated and analyzed as described previously (Kuboniwa et al. 2006). *S. gordonii* cells were labeled with hexidium iodide (15 μg ml^{-1}) (Kuboniwa et al. 2006), then cultured for 16 h anaerobically with rocking in a CultureWell coverglass system (Grace Biolabs). Fluorescein-labeled *P. gingivalis* cells (2×10^6 in pre-reduced PBS)(Kuboniwa et al. 2006) were reacted with the *S. gordonii* biofilm for 24 h anaerobically at 37°C with rocking. The resultant communities were examined on a Yokogawa spinning disc confocal scanning laser microscope system with a 60x 1.4 N.A. objective. Images were digitally reconstructed (2D image; *x-z* section, y-z section and *x-y* section, 3D image; x-y-z section), and quantitation of *P. gingivalis*-specific fluorescence was determined with Imaris software (Bitplane). Quantitation of *S. gordonii*-specific fluorescence ensured equivalent levels of the streptococcal substratum were present in each experiment.

Biofilm assays were repeated independently three times with each strain in triplicate and analyzed with a Student's unpaired two-tailed t test. Area, volume and biofilm height were calculated using Image J software.

Quantitative Real Time RT-PCR:

Primers (Table 2) for real time RT-PCR were designed by using Primer 3 software. 16S rRNA was included as a control. Predicted product sizes were in the 100 to 200 bp range. Bacterial cells were grown upto desired O.D and RNA was extracted using the RNeasy mini kit (QIAGEN). The iScript cDNA synthesis kit (Bio-Rad) was used to generate cDNA from RNA (1 μg) templates. Specific DNA standards for the genes under investigation were synthesized from chromosomal DNA using standard PCR. Real time RT-PCR was performed on a Bio-Rad iCycler using SYBR Green Supermix

(Bio-Rad). Results were analyzed with the iCycler iQ Optical System software version 3.0a. The melt curve profiles were examined to verify a single peak for each sample, and transcript copy number was calculated as described (Yin et al. 2001). RNA extracts were prepared in duplicate from independent experiments and cDNA samples were loaded in triplicate.

Table 3-2. Primers Used in this Study

Gene	Primer Sequence
16S	F AGGAACTCCGATTGCGAAGG
	R TCGTTTACTGCGTGGACTACC
luxS	F GAATGAAAGAGCCCAATCG
	R GTAATGGCCTCGCATCAG
Mfa1	F TGCGGCGAAGTCGTAATG
	R ATCTTCAGCACTCTCCACAAG
Ltp1	F TTCAGCAGTAGCGGTATTCACG
	R TGCGGATAGGGAGGAGTTGTC
1237	F CCACGCCACAGTAGAGGAAT
	R GCTCCTCGCCAATCTCTTTG
Gppx	F AGTTTCTCCTTGCAGCCAAA
	R ATGGTGGAGCAACCTACGAC

Electrophoretic Mobility Shift Assay :

Electrophoretic mobility shift assays (EMSA) were performed using the LightShift chemiluminescent EMSA kit (Pierce, Rockford, IL) as described previously (Wu et al. 2007). Biotin-labeled DNA fragments were generated by using 5' biotin-incorporated primers.The binding of rPG1237 to DNA was carried out in a 20-µl reaction mixture containing 20 fmol biotin-labeled DNA,10 mM Tris (pH 7.5), 50 mM KCl,1 mM dithiothreitol, 10 ng/µl poly(dI-dC), 2% glycerol, 0.05% NP-40, and 2 mM MgCl with various amounts of purified rPG1237 protein (0, 0.5 , 1 and 2 µg) at room temperature for 30 min. Samples were then loaded and run into a 5% nondenaturing polyacrylamide gel in 0.5 x Tris-borate-EDTA buffer. The DNA and protein complexes were then transferred to a positively charged nylon membrane (380 mA, 30 min). The biotin end-

labeled DNA was detected using the streptavidin-horseradish peroxidase conjugate and the chemiluminescent substrate. Each EMSA was repeated three times.

Consortia of Oral Bacteria:

Bacteria were cultivated to mid-exponential phase, harvested, washed and resuspended in phosphate-buffered saline (PBS) to an OD_{600} of 1.0. *P. gingivalis* parent or mutant strains (10^8) were mixed with an equal number of *S. gordonii* or *S. cristatus* cells in 1 ml of PBS. The cell mixtures were pelleted by centrifugation (10,000 g for 1 min) and incubated at 37°C anaerobically for 24 hours.

The total RNA was extracted using RNeasy mini kit (QIAGEN). The iScript cDNA synthesis kit (Bio-Rad) was used to generate cDNA from RNA (1 µg) templates. Specific DNA standards for the genes under investigation were synthesized from chromosomal DNA using standard PCR. Real time RT-PCR was performed as described previously.

Detection of Mfa and FimA :

For Western blotting, *P. gingivalis* strains in early-log phase ($O.D_{600}$ 0.5), mid-log phase ($O.D_{600}$ 0.7) and late-log phase ($O.D_{600}$1.0) were lysed with bacterial cell lysis buffer (Sigma) and the proteins were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (10% gel) and transferred to a nitrocellulose membrane by electroblotting. Membranes were blocked using 5% milk in TBS-T (TBS buffer containing 0.01% Tween-20) and reacted with rabbit polyclonal antibodies against recombinant Mfa (rMfa) (1:20,000) or rFimA, (1:10,000) overnight at 4°C. The next day the membranes were reacted with horseradish peroxidase-conjugated goat anti-rabbit secondary antibody (1:5,000). Bound antibody was detected with an enhanced chemiluminescence system (Amersham).

General Molecular Techniques

Recombinant-DNA techniques were performed as described previously (Sambrook et al. 2001). Restriction enzymes and DNA-modifying enzymes were purchased from New England BioLabs. Chromosomal DNA was isolated using a Wizard genomic DNA purification kit (Promega). RNA was extracted using RNeasy mini kit from QIAGEN. Plasmid DNA was isolated from *E. coli* by using a Plasmid mini prep kit from PROMEGA. Standard PCR conditions were 95°C for 6 min and 30 cycles of 94°C for 30 s, 58°C for 30 s, and 72°C for 3 min, followed by a final extension at 72°C for 7 min. E.coli electrocompetent cells were prepared by suspending early-log-phase cells in chilled distilled water (Dower et al. 1988). For electroporation, cells were incubated with 2 µg of a plasmid in water and pulsed with a Bio-Rad gene pulser at 2.5 kV.

Statistical Analysis

The Student's unpaired two tailed t-test was used to determine relationship of distribution of *P. gingivalis* wild type and mutant stain in monospecies biofilm formation and of *P. gingivalis* wild type and mutant stain with *S. gordonii* in heterotypic community formation. Statistical significance was set at p value < 0.05.

The role of PG1237 in expression of *mfa1, luxS, ltp1, 16S, hmuR* in *P. gingivalis, S. gordonii* and *S. cristatus* strains were analysed using Student's unpaired two tailed t-test. Statistical significance was set at p value < 0.001.

CHAPTER 4
RESULTS

Homotypic Biofilm Formation by P. gingivalis is Enhanced in the Absence of PG1237

PG1237 is a LuxR family transcriptional regulator which is thought to have a regulatory role in the LuxS pathway. Hence we wanted to investigate if PG1237 has any role in *P. gingivalis* biofilm formation.The ability of *P. gingivalis* to form biofilms on oral surfaces contributes to the persistence of the organism and increases the potential for periodontal tissue destruction. Hence we investigated whether PG1237-mediated control functions to regulate the accumulation of homotypic *P. gingivalis* biofilms. We examined the relative biofilm forming capabilities of the *pg1237* mutant strain of *P. gingivalis* compared to the parent strain. Monospecies biofilm formation was first tested in the microtiter plate assay with crystal violet staining (O'Toole and Kolter 1998). As shown in Fig.4-1 the *pg1237* mutant showed greater biofilm accumulation than the parental strain, and biofilm formation by the complemented *pg1237* strain was restored to wild-type levels. Hence, PG1237 is required to constrain *P. gingivalis* homotypic biofilm development, and in the absence of PG1237, more luxuriant biofilms are formed by *P. gingivalis*.

For construction of the complemented mutant a DNA sequence including the PG1236 and PG1237ORF's along with 300 bp upstream of the PG1236 initiation codon and 100 bp downstream of the PG1237 termination codon was amplified from *P. gingivalis* chromosomal DNA, The primers were engineered to contain the following restriction sites : NheI-EagI. The shuttle vector plasmid pT-COW was digested with the appropriate restriction enzymes to allow cloning of the PCR product into the *tetC* region. The resulting plasmids were transformed into *E. coli* TOP10 and selected on ampicillin

(100µg/ml) plates. Colonies were screened by colony PCR and restriction digestion. The plasmids with the correct restriction profile were designated pT-*1237* and these plasmids were introduced into the Δ*1237* strain by conjugation. The conjugation reaction mixture also contained helper *E. coli* J53 containing *R751,* an IncP plasmid used to mobilize vectors from *E. coli* to a *Bacteroides* recipient (Shoemaker 1987). The presence of wild type *pg1237* gene was confirmed by PCR. The resulting strains were designated CΔ*1237.*

PG1237 Deficiency Enhances Accumulation of Heterotypic *P. gingivalis-S. gordonii* Communities

The biofilms that develop in vivo on oral surfaces are complex multispecies communities (Rosan and Lamont, 2000). Successful bacterial colonizers of oral biofilms, therefore, are frequently capable of interacting synergistically with other biofilm inhabitants. Of particular relevance to this study, the presence of a substratum of the antecedent colonizer *S. gordonii* stimulates attachment and biofilm formation by *P. gingivalis* (Kuboniwa et al. 2006). Accumulation of *P. gingivalis* into heterotypic communities with *S. gordonii* proceeds through a series of interactive events (Kuboniwa et al. 2006). *P. gingivalis* cells are first recruited from the fluid to the solid phase where accretion into rudimentary biofilm microcolonies occurs. To investigate the role of PG1237 in the development of heterotypic *P. gingivalis-S. gordonii* communities, the accumulation of *P. gingivalis* parental and mutant strains on substrata of *S. gordonii* was examined.

A biofilm of hexidium iodide-labeled *S. gordonii* cells was first generated on glass coverslips. Fluorescein-labeled *P. gingivalis* cells were reacted anaerobically with the *S. gordonii* biofilm, and accumulations of heterotypic biofilms were observed by confocal

microscopy as described above. As the *P. gingivalis* cells were suspended in buffer, any increase in number is unlikely to be due to cell division. Therefore, this assay models one of the early stages of biofilm development: the recruitment, coadhesion, and accumulation of *P. gingivalis* cells from the planktonic phase into a sessile biofilm. This process is contingent on interbacterial signaling events and occurs prior to a further increase in number through growth and division (Kuboniwa et al. 2006). *x-y* and *x-z* images of the heterotypic *P. gingivalis-S. gordonii* biofilms are shown in Fig.. 4-2 A and B. *S. gordonii* cells developed biofilms that extensively covered the glass surface, and cells of parental *P. gingivalis*, formed a few discrete microcolony accumulations clearly separated from each other, whereas the *pg1237*mutant showed both increased number and size of microcolonies (Fig. 4-2 B and D).

The total fluorescence analysis performed using Imaris software showed statistically significant (ie. p value < 0.05) increased accumulation of the mutant compared to the wild type level (Fig.4-3). In addition to total fluorescence, biofilm parameters such as height, area and volume analysed using Image J software were significantly elevated (ie. p value < 0.05) in the mutant as compared to the wild type (Fig. 4-4 to Fig. 4-6). These results suggest that PG1237 has a role in constraining development of heterotypic *P. gingivalis* communities.

luxS and *mfa1* Production are Controlled by PG1237

Maturation of dual and single species *P. gingivalis* biofilms also requires the activity of the AI-2 family of signaling molecules (McNab, 2003). We reasoned, therefore, that PG1237 may also control expression of the LuxS enzyme that is responsible for AI-2 formation. *P. gingivalis* is one of only a few organisms in which LuxS production and AI-2 activity is regulated at the level of *luxS* transcription (James et

al. 2006). Expression of *luxS* in planktonic cells of wild type and mutant *P. gingivalis* was monitored by real time RT-PCR. Loss of PG1237 resulted in an approximately 3 fold increase in the levels of *luxS* mRNA (Fig. 4-7). Thus, enhanced biofilm formation by the PG1237 mutant may arise from an increase in AI-2 mediated signaling .

Minor fimbriae are also important for the maturation of dual and single species *P. gingivalis* biofilms. Hence, we wanted to investigate whether PG1237 controls transcription of *mfa1*. Expression of *mfa1* in planktonic cells of wild type and mutant *P. gingivalis* was monitored by real time RT-PCR. Loss of PG1237 resulted in an approximately 10 fold increase in the levels of *mfa1* mRNA (Fig. 4-7). Thus, increased expression of the minor fimbriae in addition to increased *luxS* expression may be responsible for the enhanced biofilm formation by the *pg1237* mutant.

luxS Production is Controlled by PG1237 in a Cell Density Dependent Manner

It has been shown that PG1237 is differentially expressed during different growth phases (Wu et al. 2008). Hence, we wanted to investigate whether PG1237 regulates luxS production in a growth phase or cell density dependent manner.

Wild type and *pg1237* mutant strains were grown to O.D600 of 0.5 (early log phase), 0.7 (mid log phase) and 1.0 (late log phase). RNAs were extracted from the bacterial cells collected from the different time points, and expression levels of *luxS* were determined using real time RT-PCR

Loss of PG1237 resulted in an approximately 9 fold increase in the levels of luxS mRNA at O.D600 of 0.5 (Fig.4-8). At O.D600 of 0.7 and 1.0 the fold increase in levels of luxS mRNA were much less pronounced almost returning to wild type levels at O.D600 of 1.0.

These results show that PG1237 regulates production of LuxS in a growth phase phase or cell density dependent manner.

AI- 2 Represses PG1237 and Ltp1 Production

We know that AI-2 is required for the for the formation of both mono biofilms and mixed species communities of *P. gingivalis*.(McNab et al. 2003) Hence, we wanted to investigate the effect of AI-2 on PG1237 and Ltp1 production.

We used Δ*luxS* ie.*luxS* deletion mutant as this strain would have no production of AI-2 as LuxS is the enzyme required for AI-2 production. RNA was extracted from the wild type andΔ *luxS* strains and pg1237 expression levels were measured using real time RT-PCR.

Loss of AI-2 resulted in an approximately 3 fold increase in the levels of *pg1237*and *ltp1* (fig.4-9) in the mutant as compared to the wild type strain.

These results show that AI-2 represses PG1237and Ltp1 production.

***Ltp1* Causes Increased Production of PG1237 in a *P. gingivalis* biofilm**

Previous studies have shown that *Ltp1* regulates both dual and single species biofilm formation in *P. gingivalis* by regulating *luxS* production and exopolysaccharide production (Maeda et al. 2008). We wanted to investigate whether *Ltp1* could be regulating biofilm formation by regulating PG1237 production.

RNA was extracted from wild typeΔ *ltp1* and CΔ*ltp1* biofilms grown anaerobically for 24 hours and *pg1237* mRNA levels were determined using real time RT-PCR. Loss of *ltp1* resulted in approximately a 2.5 fold decrease in *pg1237* levels in the mutant as compared to the wild type and the expression of *pg1237* returned to wild type levels in the complemented strain (Fig.4-10) showing that the change in *pg1237* mRNA levels in the mutant was indeed due to the loss of *ltp1*.

These results suggest that *Ltp1* regulates *P. gingivalis* biofilm formation by increasing the production of PG1237.

Interaction of *S. gordonii* with *P. gingivalis* Causes Increased PG1237 and Decreased Mfa1 Production

Previous studies have shown that contact with *S. gordonii* caused decreased *mfa1* promoter activity in *P. gingivalis* (Park et al. 2006). We wanted to test the involvement of PG1237 in this down regulation of *mfa1.*

We extracted RNA from a *S. gordonii-P. gingivalis* community grown for 24 hours anaerobically and measured expression levels of *mfa1* and *pg1237*. We saw an approximately 2.5 fold decrease in the levels of *mfa1* and a 2.6 fold increase in the levels of *pg1237* in the mixed biofilm as compared to *P. gingivalis* only biofilm (Fig.4-11). To ensure that these effects were not the result of a non-specific effect on global mRNA levels, the same experiments were done on *S. cristatus-P. gingivalis* community and we saw no change in the mRNA levels of *mfa1* and *pg1237* as compared to *P. gingivalis* only biofilm.

These results suggest that contact with *S. gordonii* regulates biofilm formation by down regulating *mfa1* production via. up-regulating *pg1237* expression.

Interaction of *S. gordonii* with Δ*1237* Causes Increased Production of LuxS and Mfa1

In this study we have seen that the Δ *1237* mutant shows both increased monospecies biofilm as well as mixed biofilm formation with *S. gordonii* as compared to the wild type strain. We also saw increased expression of *luxS* and *mfa1* in the mutant as compared to the wild type using planktonic cells.We wanted to investigate whether the increased biofilm formation of the mutant with *S. gordonii* could be due to increased production of LuxS and Mfa1.

We extracted RNA from a *S. gordonii*- wild type *P. gingivalis* and *S. gordonii*-Δ*1237* communities grown for 24 hours anaerobically and measured expression levels of *mfa1* and *luxS*. We saw an approximately 2.5 fold decrease in the levels of *mfa1* and a 3 fold decrease in the levels of *luxS* in the wild type mixed community as compared to *P. gingivalis* only biofilm (Fig.4-12). In contrast in theΔ *1237* mixed community there was a 2.2 fold increase in *luxS* levels and a 3 fold increase of *mfa1* levels as compared to Δ*1237* only biofilm (Fig.4-12).To ensure that these effects were not the result of a non-specific effect on global mRNA levels the same experiments were done on *S. cristatus*-wild type *P. gingivalis* and *S. cristatus*-Δ*1237* communities and we saw no change in the mRNA levels of *mfa1* and *luxS* as compared to *P. gingivalis* only biofilm.

These results suggest that PG1237 regulates *P. gingivalis* biofilm formation with *S. gordonii* by regulating expression of *luxS* and *mfa1* and that the increased *S. gordonii*-Δ*1237* community formation is due to increased LuxS and Mfa1 production.

Interaction of *S. gordonii* with *P. gingivalis* Causes Increased *Ltp1* Production

Previous studies have shown that loss of *Ltp1* resulted in increased *S. gordonii-P. gingivalis* biofilm formation as compared to the parental strain (Simionato et al. 2004).suggesting that *Ltp1* had a role in regulating biofilm formation. Hence, we wanted to investigate whether contact between *S. gordonii* and *P .gingivalis* caused a change in the expression level of *ltp1.*

We extracted RNA from a *S. gordonii*- wild type *P. gingivalis* and *S. gordonii*-Δ*1237* community grown for 24 hours anaerobically and measured expression levels of *ltp1*. We saw an approximately 2.5 fold increase in the levels of *ltp1* in the wild type mixed community as compared to *P. gingivalis* only biofilm (Fig.4-13). In the Δ*1237* mixed community there was a 1.7 fold increase in *ltp1* levels as compared toΔ *1237*

only biofilm (Fig.4-13).To ensure that these effects were not the result of a non-specific effect on global mRNA levels the same experiments were done on *S. cristatus*-wild type *P. gingivalis* and *S. cristatus*-∆*1237* communities and we saw no change in the mRNA levels of *ltp1* as compared to *P. gingivalis* only biofilm.

These results strongly suggest that *Ltp1* has a regulatory role in *S. gordonii-P. gingivalis* community formation.

Binding of PG1237 Protein to the Promoter Region of *luxS* and *mfa1*

In this study we have shown that PG1237 regulates expression of luxS in both planktonic and biofilm *P. gingivalis* cells by using real time RT-PCR analysis. Hence, we wanted to investigate whether PG1237 directly regulates *luxS* expression.

To determine if the transcriptional regulator PG1237 directly interacts with the promoter region of *luxS* and *mfa1*, we performed an EMSA. *pfs* is the gene upstream to *luxS* and *pfs* and *luxS* form an operon. DNA fragments 200bp upstream of *pfs* initiation codon and 200bp upstream of *mfa1* initiation codon were generated by PCR with the 5 biotin-labeled primers (Table 2). The promoter region of *fimA*, a gene-encoding component of the long fimbriae of *P. gingivalis*, was used as a control. rPG1237 was expressed in a pET expression system and purified from E. coli. As shown in Fig.4-14 and Fig. 4-15. the DNA fragments upstream of *pfs* and *mfa1* were shifted in the presence of the rPG1237. As the concentration of rPG1237 was increased, the retarded protein-DNA complex became more evident, with a parallel loss of uncomplexed DNA. We also saw that DNA binding activity of rPG1237 was blocked in the presence of excess unbiotinylated DNA, and that no DNA shift was detected when rPG1237 was incubated with the promoter region of *fimA*. This suggests that the shift we saw was specific.

These results suggest that PG1237 directly regulates expression of *luxS* and *mfa1* by binding to their promoter region.

PG1237 Regulates Expression of Mfa protein

In this study we have shown that PG1237 regulates expression of mfa1 in both planktonic and biofilm *P. gingivalis* cells by using real time RT-PCR analysis. Hence, we wanted to investigate whether the increased *mfa1* expression at the transcriptional level in the PG1237 mutant translated into increased Mfa protein production in the mutant.

Wild type and Δ1237 strains were grown to early, mid and late log phase .Total bacterial proteins were extracted at the different growth phases using cell lytic buffer and probed with antibodies to FimA, Mfa by Western blotting. The blot analysis showed that expression of Mfa was increased in the 1237 mutant at early log phase ie.O.D600 of 0.5 (Fig.4-17). No change in expression of Mfa was seen during mid and late log phases. No change in expression of FimA was detected in the mutant (Fig.4-18) confirming that Mfa is indeed induced in the mutant.

These results suggest that the increased *mfa1* expression at the transcriptional level in the PG1237 mutant translated into increased Mfa protein production in the mutant and that PG1237 has a role in regulating expression of Mfa protein.

Complementation of Δ*1237* with Wild-type *1237* Restored Production of LuxS and Mfa1 to Wild-type Levels

To confirm expression changes of *luxS* and *mfa1* we saw in the Δ*1237* mutant was due to the loss of *1237*, we complemented the mutant strain with a wild-type *1237* allele.

Expression of *luxS* and *mfa1*in planktonic cells of wild type, mutant and complemented mutant *P. gingivalis* was monitored by real time RT-PCR.

Complementation of mutant restored expression levels of *luxS* and *mfa1* to almost wild-type levels (Fig. 4-19).

These results suggest that the expression changes we found in the Δ1237 mutant were due to the loss of *1237*.

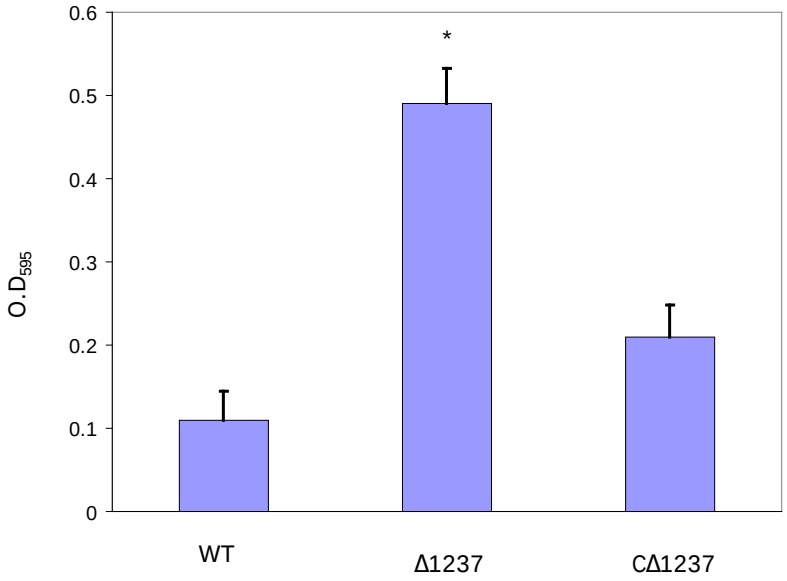


Figure 4-1. PG1237 controls homotypic *P. gingivalis* biofilm growth. Microtiter plate biofilms after 24 h were stained with crystal violet and washed, and then the crystal violet was released with 95% ethanol. Biofilm accumulation was measured by absorbance at 595 nm for parental strain 33277 and *pg1237* mutant Δ*1237* and complemented strain CΔ1237. * denotes *P* values of <0.05 (*t* test) for comparison to 33277.

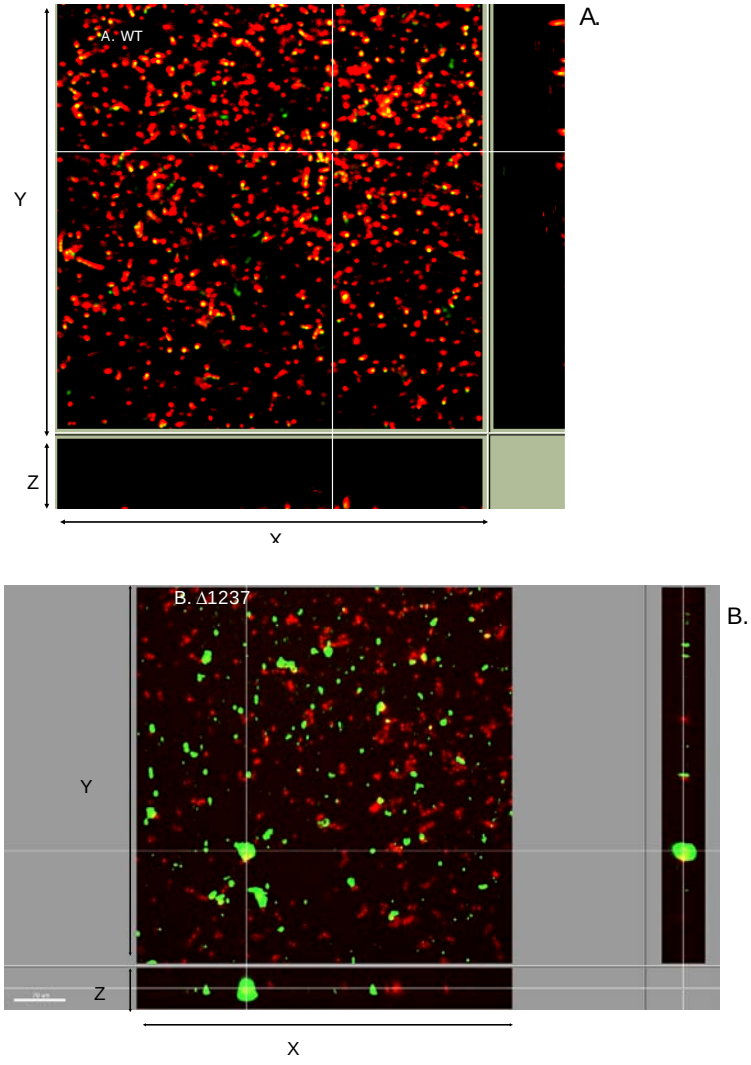
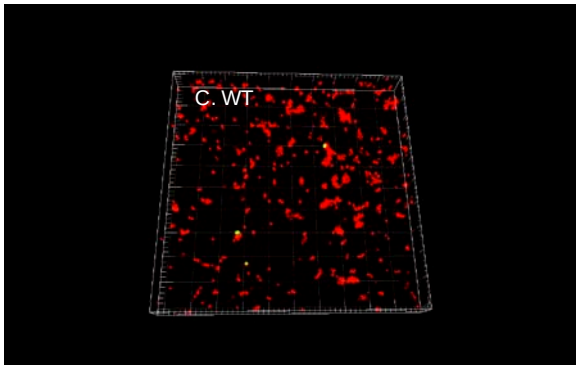
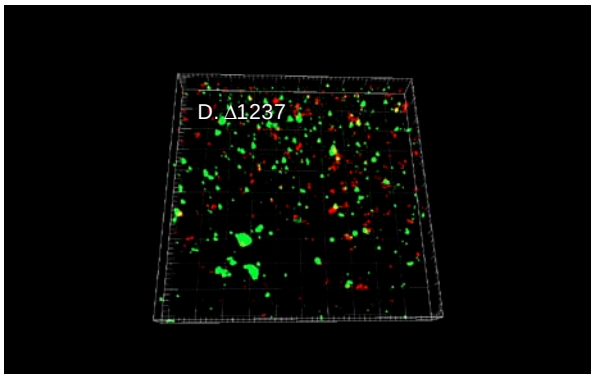


Figure 4-2. PG1237 regulates heterotypic *P. gingivalis*-*S. gordonii* biofilms. *S. gordonii* stained with hexidium iodide (red) was cultured on glass plates. *P. gingivalis* strains were stained with fluorescein (green) and reacted with the *S. gordonii* biofilms for 24 h. Colocalized bacteria appear yellow. Mixed-species biofilm accumulations were viewed by confocal microscopy. A)Confocal laser scanning microscopy images of *x-y* and *x-z* projections of parental strain 33277 B)Confocal laser scanning microscopy images of *x-y* and *x-z* projections of mutant strain Δ1237 C)3-D view of parental strain 33277 D)3-D view of mutant strain Δ1237



C.



D.

Figure 4-2. PG1237 regulates heterotypic *P. gingivalis*-*S. gordonii* biofilms. *S. gordonii* stained with hexidium iodide (red) was cultured on glass plates. *P. gingivalis* strains were stained with fluorescein (green) and reacted with the *S. gordonii* biofilms for 24 h. Colocalized bacteria appear yellow. Mixed-species biofilm accumulations were viewed by confocal microscopy. A)Confocal laser scanning microscopy images of *x-y* and *x-z* projections of parental strain 33277 B)Confocal laser scanning microscopy images of *x-y* and *x-z* projections of mutant strain Δ1237 C)3-D view of parental strain 33277 D)3-D view of mutant strainΔ1237

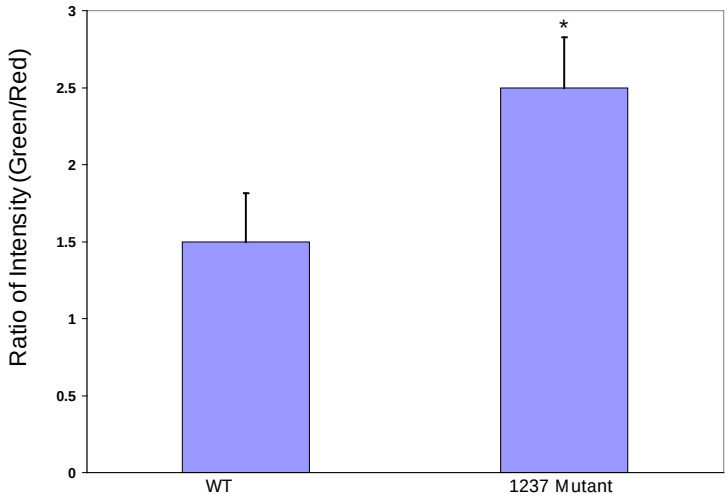


Figure 4-3. Ratio of *P .gingivalis/S. gordonii* in 33277 vs. Δ1237 strain in *P. gingivalis-S. gordonii* community. * denotes p-value<0.05 (*t* test) in comparison to 33277.

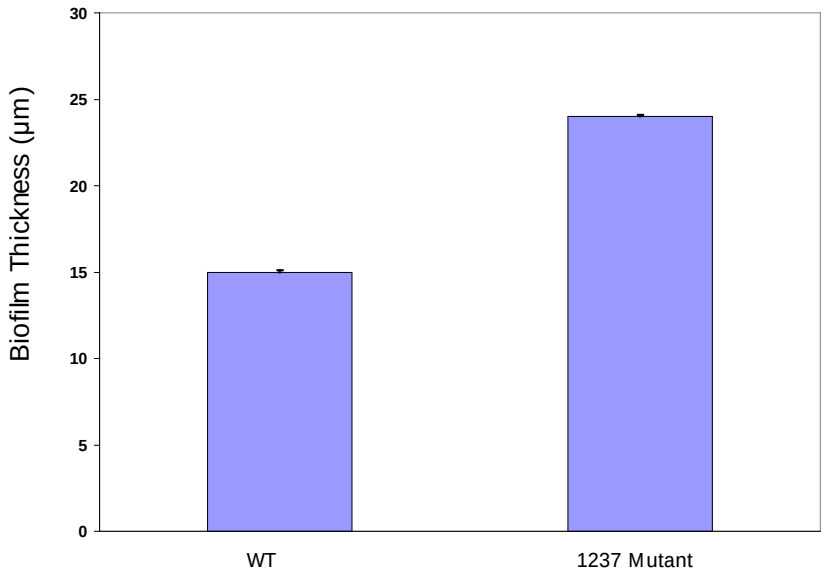


Figure 4-4. Biofilm thickness of 33277 vs.Δ1237 strain in *P. gingivalis-S. gordonii* community. * denotes p-value<0.05 (*t* test) in comparison to 33277.

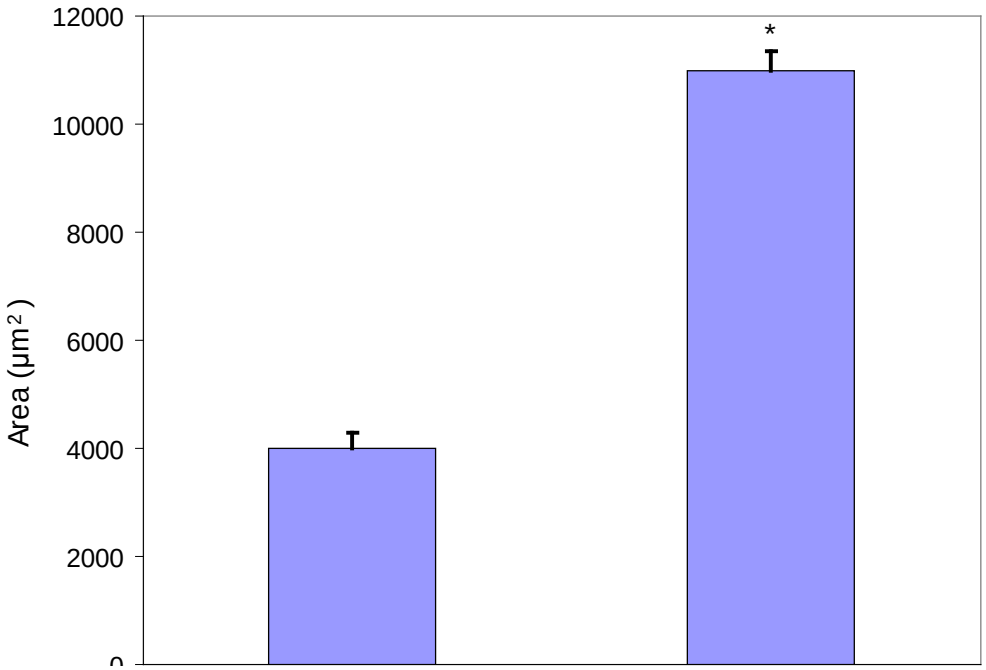


Figure 4-5. Total *P. gingivalis* accumulation in 33277 vs. ∆1237 strain in *P. gingivalis-S. gordonii* community measured by area analysis. * denotes p-value<0.05 (*t* test) in comparison to 33277

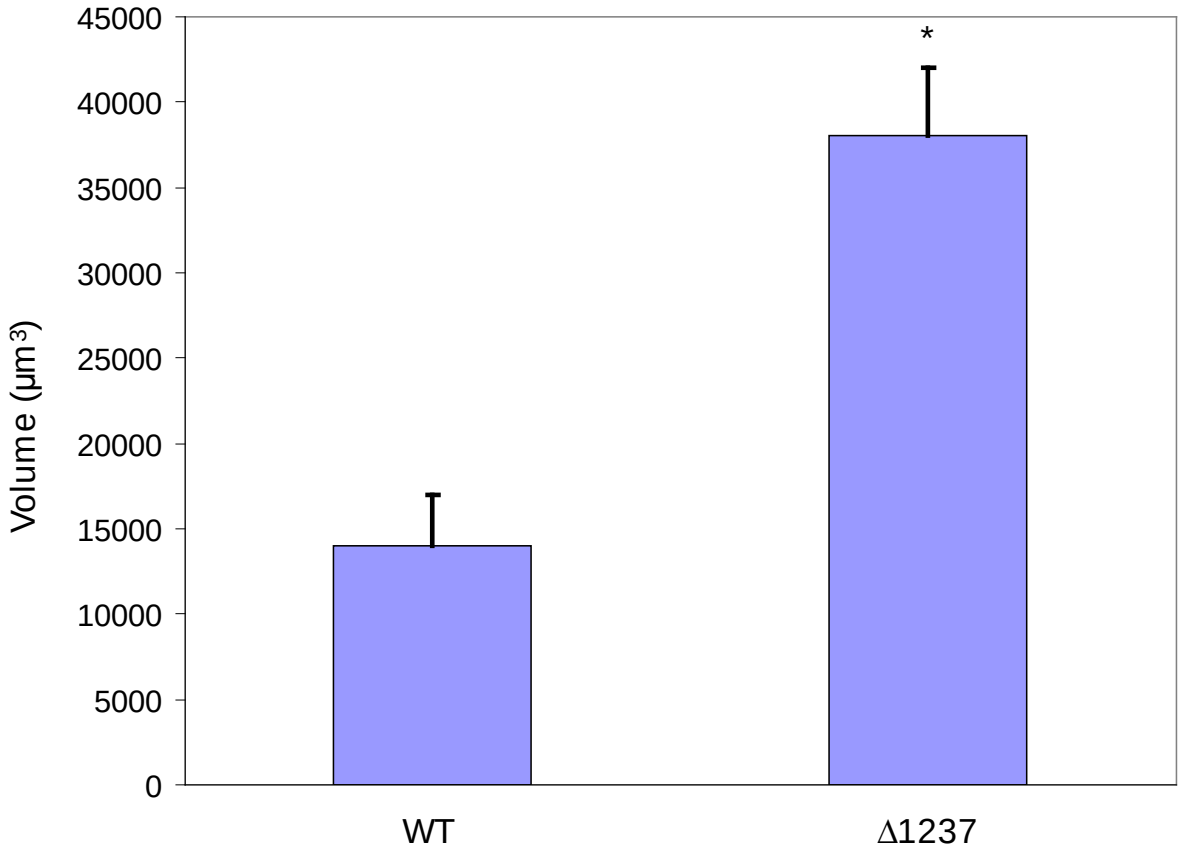


Figure 4-6. Total *P. gingivalis* accumulation in 33277 vs. Δ1237 strain in *P. gingivalis-S. gordonii* community measured by volume analysis. * denotes p-value<0.05 (*t* test) in comparison to 33277.

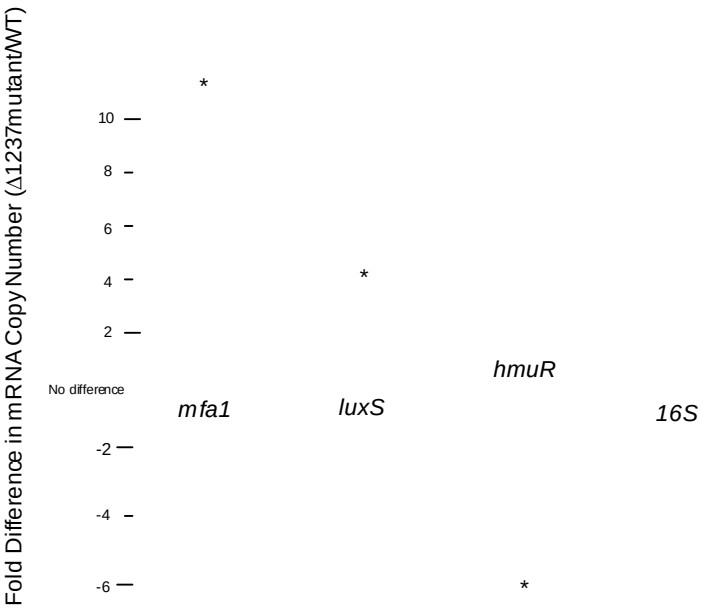


Figure 4-7. *mfa1* and *luxS* genes are differentially expressed in the Δ1237 mutant. Gene expression was measured by quantitative RT-PCR on wild-type (WT) or Δ1237 cultures grown to early (OD_{600} 0.5) exponential phase. Fold change was calculated by dividing the copy number of the gene transcript (per microgram of RNA) in the Δ1237 mutant by the copy number in the WT. The 16S rRNA gene was used as a control. Significant differences ($P < 0.001$ by *t* test) are labeled with asterisks. *hmuR* was used as a positive control as other groups have previously shown that *hmuR* is down-regulated in Δ1237 mutant .

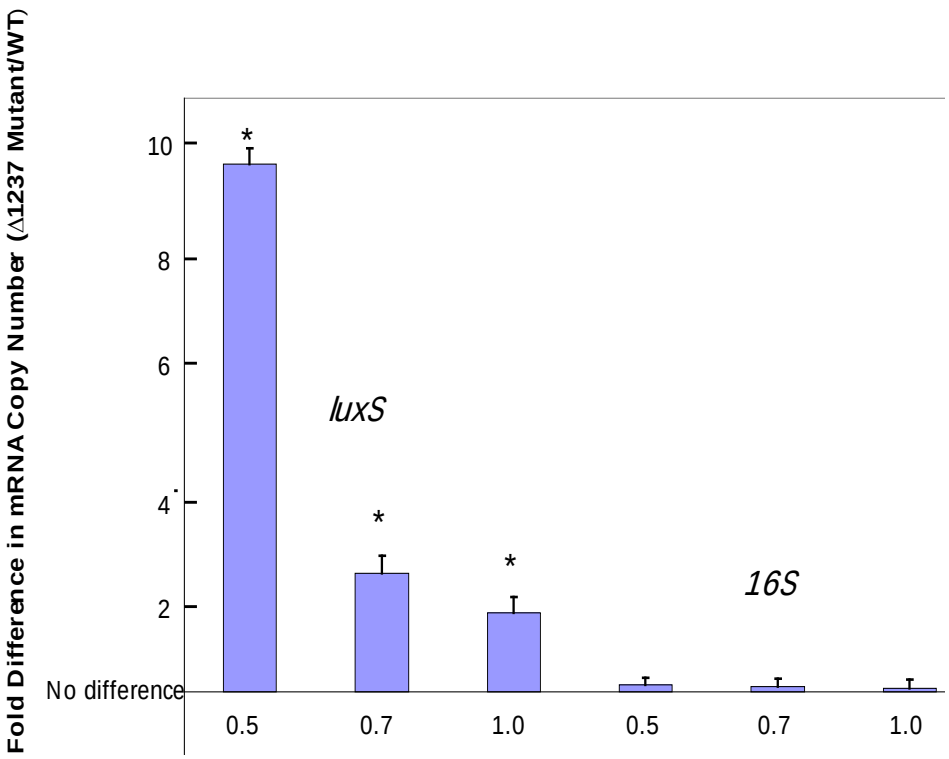


Figure 4-8. *luxS* gene is differentially expressed in the Δ1237 mutant in a growth phase dependent manner. Gene expression was measured by quantitative RT-PCR on wild-type (WT) or Δ1237 cultures grown to early (OD_{600}, 0.5), middle (OD_{600}, 0.7) or late (OD_{600}, 1.0) exponential phase. Fold change was calculated by dividing the copy number of the gene transcript (per microgram of RNA) in the Δ1237 mutant by the copy number in the WT. The 16S rRNA gene was used as a control. Significant differences ($P < 0.001$ by *t* test) are labeled with asterisks

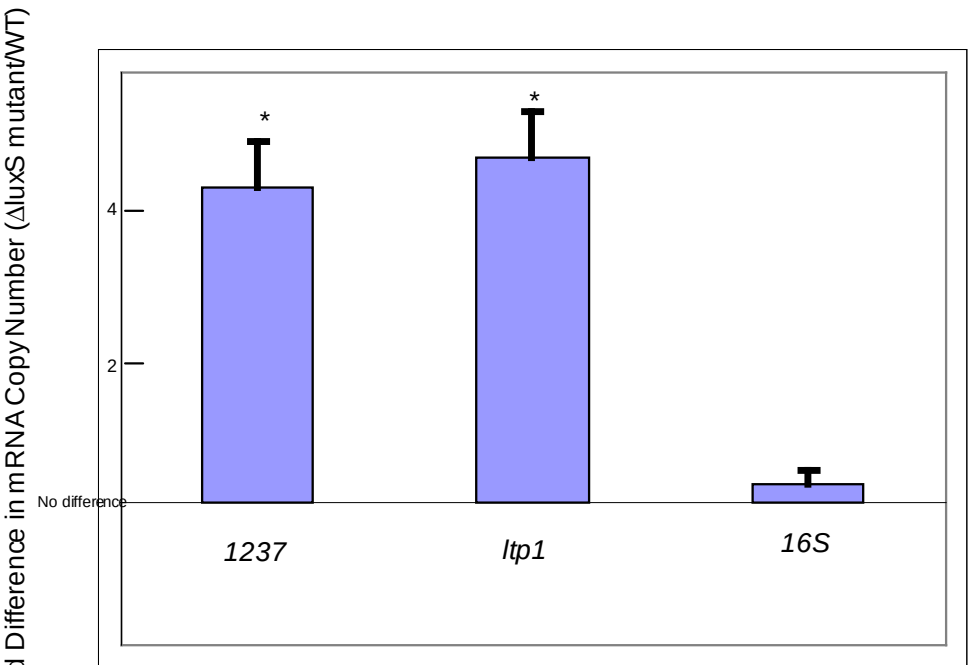


Figure 4-9. *1237* and *ltp1* genes are differentially expressed in the ΔluxS mutant. Gene expression was measured by quantitative RT-PCR on wild-type (WT) or Δ luxS cultures grown to mid (OD_{600}, 0.7) exponential phase. Fold change was calculated by dividing the copy number of the gene transcript (per microgram of RNA) in the ΔluxS mutant by the copy number in the WT. The 16S rRNA gene was used as a control. Significant differences ($P < 0.001$ by *t* test) are labeled with asterisks.

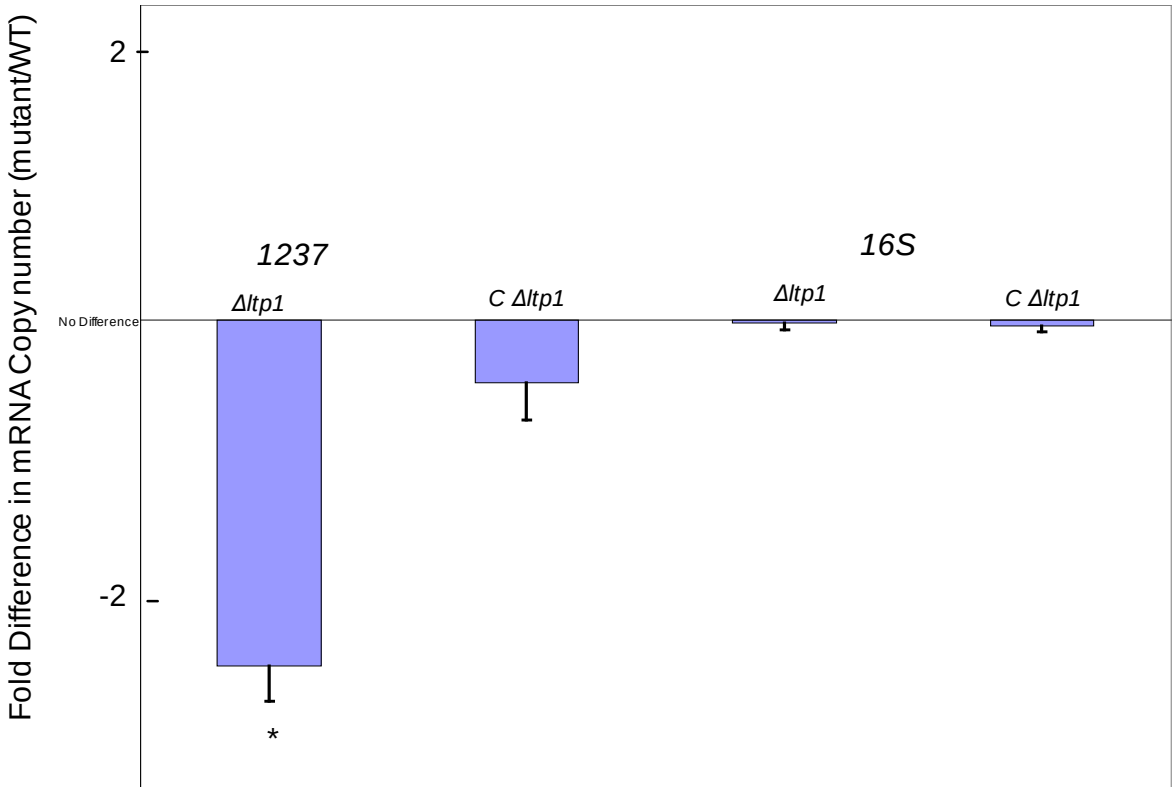


Figure 4-10. The *P. gingivalis1237* gene is differentially expressed in the Δ*ltp1* mutant biofilm. Gene expression was measured by quantitative RT-PCR on wild-type (WT) or Δ*ltp1* or CΔ*ltp1* biofilm grown for 24 hours anaerobically. Fold change was calculated by dividing the copy number of the gene transcript (per microgram of RNA) in the Δ*ltp1* mutant by the copy number in the WT. The 16S rRNA gene was used as a control. Significant differences ($P < 0.001$ by *t* test) are labeled with asterisks.

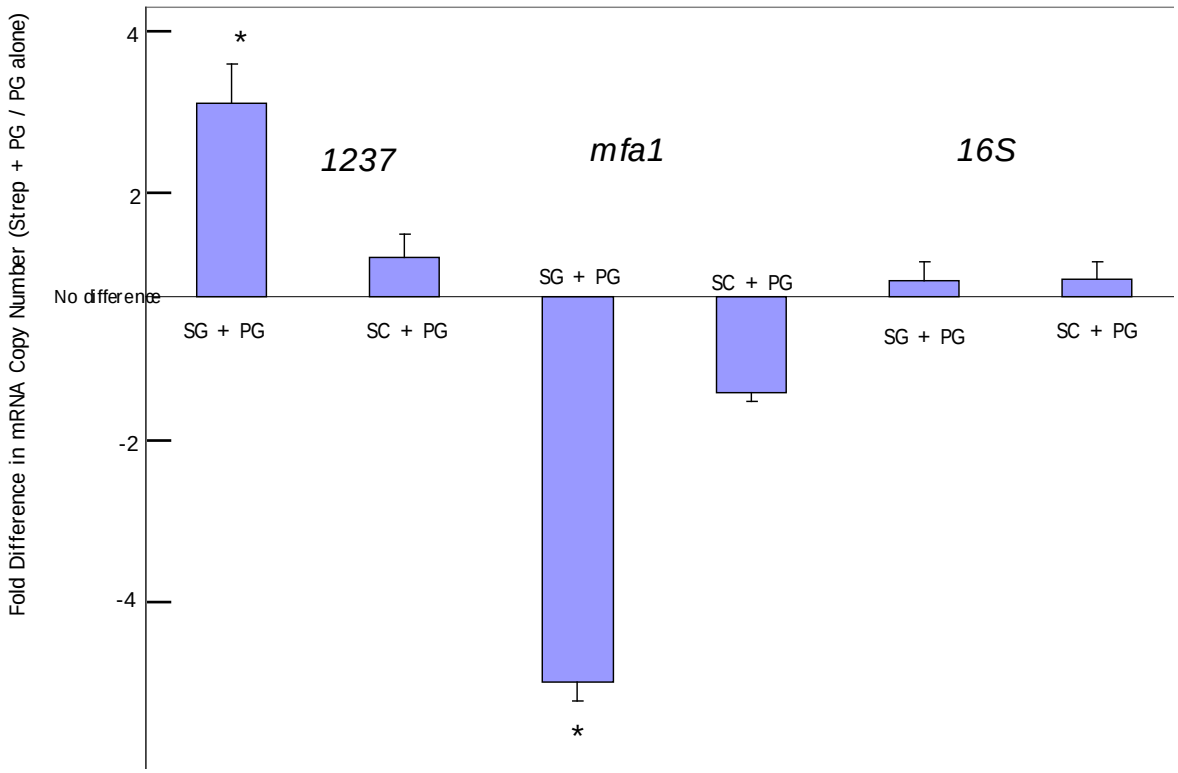


Figure 4-11. *1237* and *mfa1*genes are differentially expressed in the *P. gingivalis-S. gordonii* community as compared to *P. gingivalis* mono biofilm. Gene expression was measured by quantitative RT-PCR on *P. gingivalis-S. gordonii, P. gingivalis-S. cristatus* or *P. gingivalis* mono biofilm grown for 24 hours anaerobically. Fold change was calculated by dividing the copy number of the gene transcript (per microgram of RNA) in the *S. gordonii* or *S. cristatus* community with *P. gingivalis* by the copy number in the *P. gingivalis* mono biofilm. The 16S rRNA gene was used as a control. Significant differences ($P < 0.001$ by *t* test) are labeled with asterisks. SG=*Streptococcus gordonii,* SC=*Streptococcus cristatus*

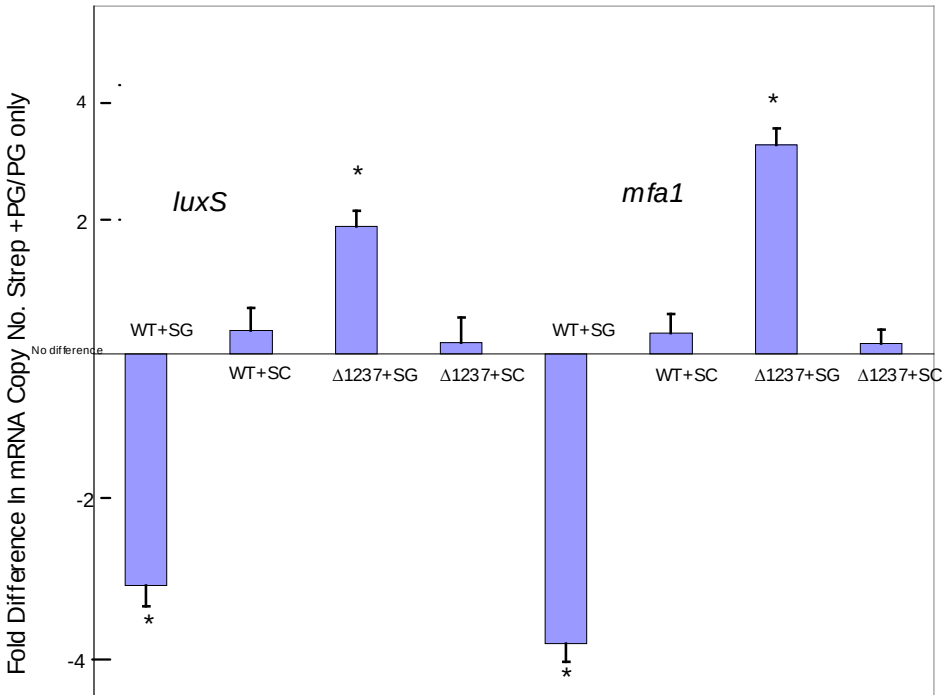


Figure 4-12. *luxS* and *mfa1*genes are differentially expressed in the *P. gingivalis-S. gordonii* community as compared to *P. gingivalis* mono biofilm. Gene expression was measured by quantitative RT-PCR on *P. gingivalis-S. gordonii, P. gingivalis-S. cristatus* or *P. gingivalis* mono biofilm grown for 24 hours anaerobically. Fold change was calculated by dividing the copy number of the gene transcript (per microgram of RNA) in the *S. gordonii* or *S. cristatus* community with *P. gingivalis* by the copy number in the *P. gingivalis* mono biofilm. The 16S rRNA gene was used as a control. Significant differences ($P < 0.001$ by *t* test) are labeled with asterisks. SG=*Streptococcus gordonii,* SC=*Streptococcus cristatus*

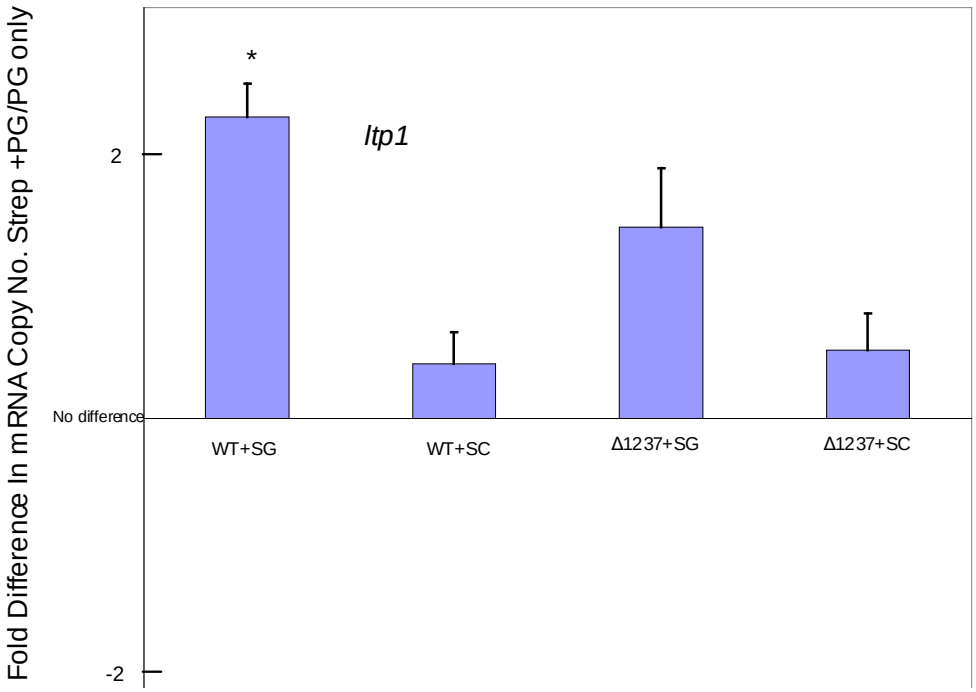


Figure 4-13. *ltp1* gene is differentially expressed in the *P. gingivalis-S. gordonii* community as compared to *P. gingivalis* mono biofilm. Gene expression was measured by quantitative RT-PCR on *P. gingivalis-S. gordonii, P. gingivalis-S. cristatus* or *P. gingivalis* mono biofilm grown for 24 hours anaerobically. Fold change was calculated by dividing the copy number of the gene transcript (per microgram of RNA) in the *S. gordonii* or *S. cristatus* community with *P. gingivalis* by the copy number in the *P. gingivalis* mono biofilm. The 16S rRNA gene was used as a control. Significant differences ($P < 0.001$ by *t* test) are labeled with asterisks. SG=*Streptococcus gordonii,* SC=*Streptococcus cristatus*

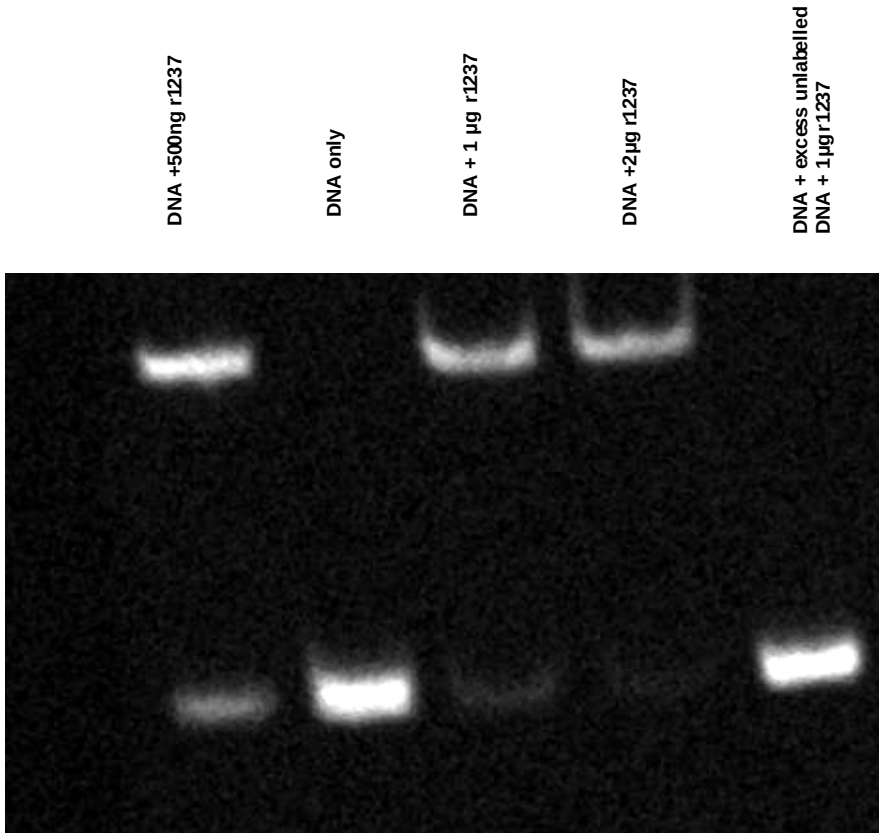


Figure 4-14. Interaction of rPG1237 with the promoter region of luxS. EMSA were performed in the presence or absence of rPG1237 and 20 fmol biotin-labeled DNA . Increasing amounts of rPG1237(500 ng to 2 µg) were used in the assays. For testing specificity of binding 200-fold excess amounts of competitor oligonucleotide was added to the reaction mixture with the labeled probe.

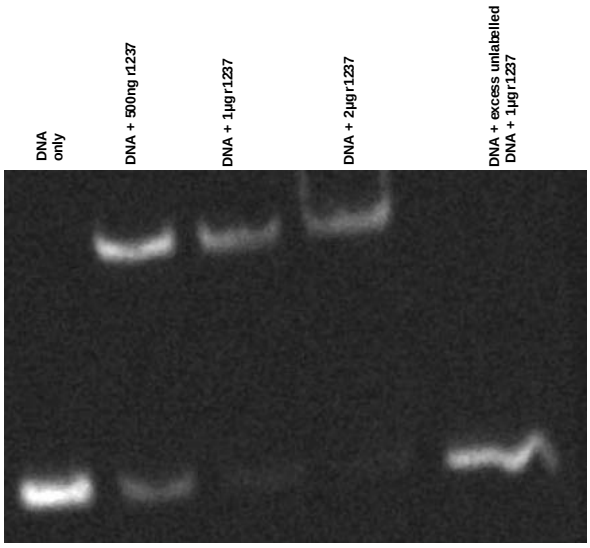


Figure 4-15. Interaction of rPG1237 with the promoter region of *mfa1.* EMSA were performed in the presence or absence of rPG1237 and 20 fmol biotin-labeled DNA . Increasing amounts of rPG1237(500ng to 2 µg) were used in the assays. For testing specificity of binding 200-fold excess amounts of competitor oligonucleotide was added to the reaction mixture with the labeled probe.

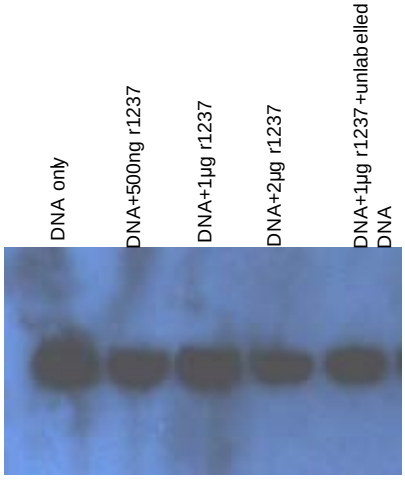


Figure 4-16. Interaction of rPG1237 with the promoter region of *fimA*. EMSA were performed in the presence or absence of rPG1237 and 20 fmol biotin-labeled DNA . Increasing amounts of rPG1237(500 ng to 2 µg) were used in the assays.

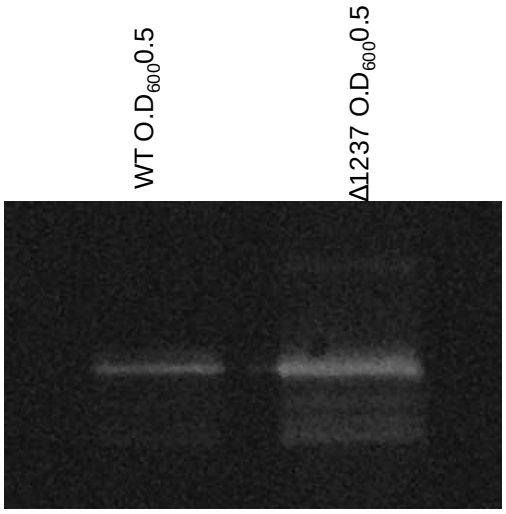


Figure 4-17. Expression of Mfa is increased in the absence of PG1237. Proteins were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (10% gel) and transferred to a nitrocellulose membrane by electroblotting. Membranes were blocked using 5% milk in TBS-T (TBS buffer containing 0.01% Tween-20) and reacted with rabbit polyclonal antibodies against recombinant Mfa (rMfa) (1:20,000) overnight at 4°C. The next day the membranes were reacted with horseradish peroxidase-conjugated goat anti-rabbit secondary antibody (1:5,000). Bound antibody was detected with an enhanced chemiluminescence system (Amersham).

Figure 4-18. Expression of FimA is not increased in the absence of PG1237. Proteins were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (10% gel) and transferred to a nitrocellulose membrane by electroblotting. Membranes were blocked using 5% milk in TBS-T (TBS buffer containing 0.01% Tween-20) and reacted with rabbit polyclonal antibodies against recombinant FimA (rFimA) (1:10,000) overnight at 4°C.The next day the membranes were reacted with horseradish peroxidase-conjugated goat anti-rabbit secondary antibody (1:5,000). Bound antibody was detected with an enhanced chemiluminescence system (Amersham).

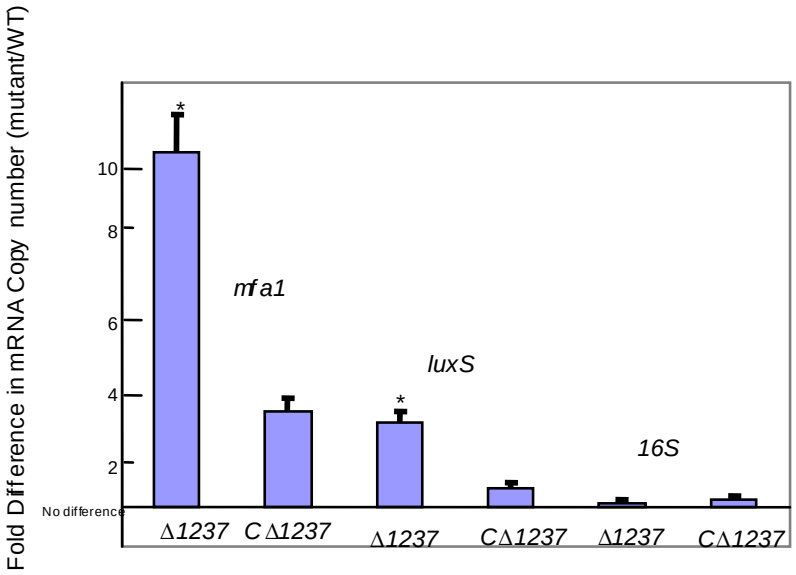


Figure 4-19. Complementation of the Δ1237 mutant with the wild-type (WT) *1237* gene restores expression of *luxS* and *mfa1* genes to close to WT levels.The transcript copy number (per microgram of RNA) was determined by real-time PCR for the WT, CΔ1237, or Δ1237 strain grown to early exponential phase. The 16S rRNA gene was used as a control. Significant differences ($P < 0.001$ by *t* test) are labeled with asterisks.

CHAPTER 5
DISCUSSION

An association between LuxR family transcriptional regulators and biofilm formation has been observed with other bacteria. It has been reported that VanT, a LuxR family transcriptional regulator regulates biofilm formation in *Vibrio anguillarum* and that a *vanT* mutant of *V.anguillarum* could not form biofilms properly (Croxatto et al. 2002). Similarly, in *Pseudomonas aeruginosa*, mutation of *lasR* causes reduced biofilm formation hence showing that LasR, a LuxR family transcriptional regulator regulates biofilm formation in *P.aeruginosa* (Pesci et al. 1997). It was also reported that LeuO, a LuxR family transcriptional regulator is important for biofilm formation in *Vibrio cholerae* (Stratmann et al. 2008). Thus a number of organisms utilize LuxR transcriptional regulators to regulate their biofilm formation.

LuxR-family proteins such as PG1237 for which there is no obvious cognate LuxI synthase are known as LuxR Solos or Orphans. Unlike synthase-associated LuxR proteins, orphan LuxR homologs do not directly control the synthesis of autoinducers, but can interact with them to expand the existing regulatory network of the bacterium. AHLs appear to be the most prevalent activating signal for orphan LuxR homologs, though other mechanisms of regulatory action such as heterodimer formation or activation by plant signals also exist (Ledgham et al. 2001, Ferluga et al. 2002).These proteins are found in bacteria which possess a complete AHL QS system as well as in bacteria that do not. It is emerging that these proteins could allow bacteria to respond to endogenous and exogenous signals produced by their neighbours and have diverse roles in bacterial interspecies communication.

Several factors can justify the increased prevalence of orphan LuxR regulators in quorum-sensing systems of bacteria. These regulators can utilize the existing quorum-sensing signal in the bacteria and alleviate the cost associated with making additional signal molecules. Gain of response regulators also leads to expansion of the existing regulatory networks. For instance, QscR of *P. aeruginosa* utilize the existing AHL signal molecules to extend their regulatory control beyond that of the cognate LuxR/I pair (Lequette et al. 2006). Also, orphan LuxR regulators could be recruited for perceiving exogenous signals for intercellular communication. Several instances of intercellular communication have been reported for orphan LuxR regulators for eg. in *Serratia* sp. ATCC39006, carbapenem synthesis by CarR is modulated by the interspecies communication system of LuxS/AI-2 (Coulthurst et al. 2005).

Genetic and structural studies indicated that nine residues are identical in at least 95% of LuxR-type proteins (Zhang et al. 2002). Six of those residues in the N-terminal domain (W57, Y61, D70, P71, W85, and G113) were involved in binding to the cognate autoinducer while three in the C-terminal domain (E178, L182, and G188) were associated with DNA binding (Nasser and Reverchon 2007). Structure-function analysis has indicated that the DNA-binding domain is largely conserved, while the autoinducer-binding domain tends to vary in several LuxR-type proteins, perhaps to accommodate the variety of activating signals (Bottomley et al. 2007). PG1237 followed this pattern as it contained none of the six conserved N-terminal residues at its N-terminal but contained two of the three conserved C-terminal residues at its C-terminus. This indicates that some signal other than auto inducer binding might be regulating PG1237. Sequence analysis indicated that PG1237 contains the well conserved DNA-binding,

helix-turn-helix (HTH) domain of about 65 amino acids, present in transcription regulators of the LuxR/FixJ family of response regulators. HTH domain of PG1237 showed 43% homology with HTH domain of LuxR from *V. fischeri* and 68% homology with HTH domain of GerE from *B. subtilis.* Sequence analysis showed that PG1237 belongs to GerE family.

Biofilm development in general proceeds through a series of ordered developmental steps (Stanley et al. 2004). In the case of *P. gingivalis-S. gordonii* consortia, the first step is a multivalent coadhesive interaction mediated by two distinct adhesin receptor pairs. The *P. gingivalis* long fimbriae (FimA) bind to glyceraldehyde-3-phosphate dehydrogenase (GAPDH) present on the streptococcal surface (Maeda et al.., 2004). In addition, the *P. gingivalis* short *fimbriae* (Mfa) engage the streptococcal SspA/B (antigen I/II) adhesins (Park et al.., 2005) through an approximately 27 amino acid binding epitope of SspA/B termed BAR (Daep et al. 2006, Demuth et al. 2001).. Following co-adhesion, LuxS dependent signaling is required for further development of the heterotypic biofilm communities (McNab et al. 2003). With regard to monospecies *P. gingivalis* biofilms, initial attachment depends on an internalin family protein, InlJ (Capestany et al. 2006), and development requires expression of the short *fimbriae* (Mfa). Limitation of biofilm development appears to be important for *P. gingivalis,* and such mechanisms in general are thought to arise in order to optimize exposure to oxygen (either maximal or minimal), or to facilitate influx of nutrients and efflux of waste (Rainey and Rainey 2003).

P. gingivalis has the potential to be an aggressive pathogen in periodontal disease. The organism colonizes the biofilms that accumulate on oral non-shedding

surfaces and can damage periodontal tissues through the production of arginine and lysine specific proteases and other toxic factors. Paradoxically, *P. gingivalis* is also present in the oral cavity in the absence of tissue destruction, implying that mechanisms to restrain pathogenic potential are operational in the organism. The results of this study indicate that the LuxR family transcriptional regulator, PG 1237 occupies a central position in regulating several aspects of *P. gingivalis* virulence such as biofilm formation and hemin uptake.

In the absence of PG1237 , *mfa1* and *luxS* were upregulated determined by real time RT PCR in planktonic cells as well as *P. gingivalis-S. gordonii* communities. Phenotypically the *pg1237* mutant showed increased monospecies biofilm formation as well as increased community formation with *S. gordonii.* Using electrophoretic mobility shift assay (EMSA) we showed that PG1237 binds to the promoter regions of *mfa1* and *luxS*. Complementation of the mutant with wild-type pg1237 led to restoration of monospecies biofilm formation and expression of mfa1 and luxS to wild- type levels.

Control of *P. gingivalis* biofilm development by PG1237 was associated with a decrease in both *luxS* and *mfa1* expression. Mfa (minor fimbriael protein) is necessary for development of both monospecies *P. gingivalis* biofilms and heterotypic *P. gingivalis-S. gordonii* communities. Mutants of *P. gingivalis* that lack Mfa show impaired formation of both monospecies biofilms (Lin et al. 2006) and heterotypic biofilms with *S. gordonii* (Park et al. 2005). PG1237 constrains biofilm formation by decreasing Mfa production both at the transcriptional level and at the protein processing level. AI-2 dependent signaling is required for recruitment of *P. gingivalis* cells from the fluid phase and incorporation into the sessile communities. Consistent with this, PG1237 activity in

P. gingivalis biofilm cells negatively regulates expression of LuxS, an enzyme responsible for AI-2 production. Thus, PG1237 exerts negative regulation of biofilm development through limitation of cell-cell communication, in addition to minor fimbriael protein production.

Ltp1 is a low molecular weight tyrosine phosphatase that was found to be differentially regulated in *P. gingivalis* biofilms. *Ltp1* functions in constraining both monospecies biofilm formation as well as community formation with *S. gordonii* by regulating exopolysaccharide synthesis as well as *luxS* transcription. The expression of *pg1237* was reduced in the *ltp1* mutant biofilm determined using real time RT-PCR suggesting that *Ltp1* in a biofilm increases the production of PG1237 which would then target luxS and mfa1 to limit biofilm formation. Hence, *Ltp1* and PG1237 link together for the purpose of controlling biofilm and mixed species communities formation.

The results of the current study would thus allow us to propose the following model (Fig. 5-1) During early-exponential growth LuxS produces AI-2 which suppresses the production of *Ltp1* and PG1237 which in turn leads to increased Mfa production leading to biofilm formation. Subsequently, as the *P. gingivalis* community becomes established , the LuxS and AI-2 levels are reduced which cause increased production of *Ltp1* and PG1237 which in turn repress the production of LuxS and Mfa causing constrained biofilm formation. This is a good example of a positive feedback system. However this positive feedback is growth phase or optical density dependent. Using real time RT-PCR we saw that there was not much regulation of luxS expression by PG1237 during middle and late log phase ie. O.D 0.7 and 1.0. This suggests that the positive feedback between AI-2, PG1237 and *Ltp1* is mainly active during early log or early exponential

phase. This model predicts that a PG1237-deficient mutant would show an increased biofilm phenotype which was indeed exhibited by our *pg1237* mutant increasing our confidence in the model.

The results of this study also allow us to propose the following model in terms of heterotypic *P. gingivalis-S. gordonii* communities.(Fig. 5-2) Contact of *S. gordonii* and *P. gingivalis* causes increased production of *Ltp1* which in turn causes increased production of PG1237 which represses LuxS and Mfa production leading to constrained heterotypic *P. gingivalis-S. gordonii* community formation. This model predicts that a PG1237-deficient mutant would show an increased heterotypic *P. gingivalis-S. gordonii* biofilm phenotype which was indeed exhibited by our pg1237 mutant increasing our confidence in the model.

The results of this study suggest that both PG1237 and *Ltp1* could be potential targets for drugs and other therapeutics being developed in the treatment of chronic periodontitis.

Further research involving DNAse I footprinting to map the binding region of PG1237 to *mfa1* and *luxS* , electrophoretic mobility shift assay to see whether PG1237 is auto-induced , heterotypic *P. gingivalis-S. gordonii* biofilm assays with complemented PG1237 strains and potential interaction between *Ltp1* and PG1237 still needs to be carried out.

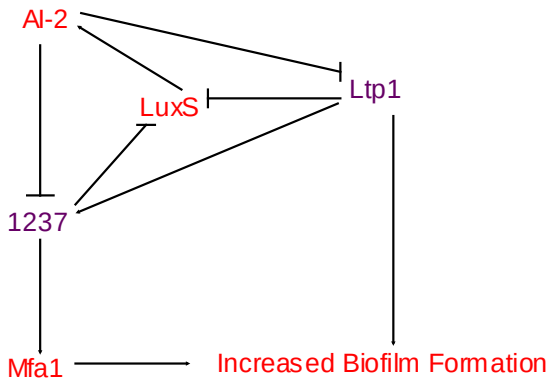


Figure 5-1. Schematic representation of the AI-2-PG1237-*Ltp1* positive feedback model
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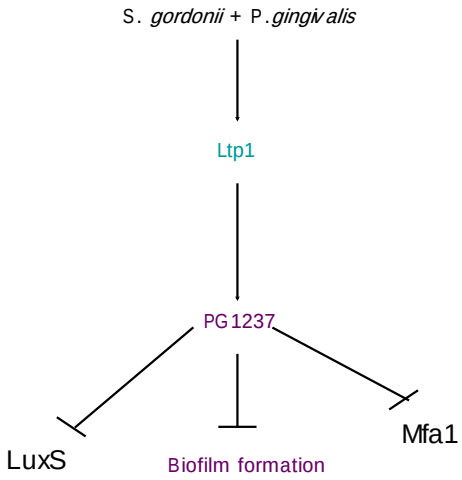

Figure 5-2. Schematic representation of functionality of *Ltp1* and PG1237 in constraining *P. gingivalis-S. gordonii* community formation.

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BIOGRAPHICAL SKETCH

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