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**CHARACTERIZATION OF THE AI-2-DEPENDENT QUORUM SENSING  
PATHWAY IN *AGGREGATIBACTER ACTINOMYCETEMCOMITANS*.**

**Elizabeth A. Novak**

**A dissertation submitted to the faculty of the School of Medicine of the University of  
Louisville in partial fulfillment of the Requirement for the degree of**

**Doctor of Philosophy**

**University of Louisville, School of Medicine  
Department of Microbiology and Immunology**

**December 2011**

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PATHWAY IN *AGGREGATIBACTER ACTINOMYCETEMCOMITANS*.**

By

**Elizabeth A. Novak**

**A Dissertation approved on**

**December 16, 2011**

**by the following Dissertation Committee:**

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**Pascale Alard**

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**George Hajishengallis**

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**James Graham**

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Thank you for everything!!! ☺

-Liz-

## ABSTRACT

### CHARACTERIZATION OF THE AI-2-DEPENDENT QUORUM SENSING PATHWAY IN *AGGREGATIBACTER ACTINOMYCETEMCOMITANS*.

By

**Elizabeth A. Novak**

**December 16, 2011**

Quorum sensing in the oral pathogen *Aggregatibacter actinomycetemcomitans* is dependent upon the soluble signaling molecule AI-2, but it is not known how the initial detection of AI-2 is coupled to the downstream regulation of gene expression that confers complex phenotypes such as biofilm formation and iron acquisition. Here we show that expression of a two-component system encoded by *qseBC* is induced by AI-2 in *A. actinomycetemcomitans* and that induction of *qseBC* requires the AI-2 receptors, LsrB and/or RbsB. Inactivation of the sensor kinase gene *qseC* resulted in a significant reduction of *in vitro* biofilm growth and *in vivo* virulence using a murine model of periodontitis. We also show that *qseBC* regulates the expression of several operons encoding iron acquisition pathways, consistent with previous studies showing that AI-2



regulates iron uptake in *A. actinomycetemcomitans*. However, some AI-2 regulated iron uptake operons were not controlled by QseBC, suggesting that only a subset of iron acquisition systems are co-regulated by AI-2 and QseBC.

Interestingly, the toxin-antitoxin system *mqsRA* is involved in AI-2 and QseBC-dependent regulation of biofilm growth of some strains of *Escherichia coli*. The genome sequence of *A. actinomycetemcomitans* was searched and open reading frames Aa00672 and Aa00673 were shown to exhibit sequence similarity to MqsRA and MazEF, two toxin-antitoxin systems encoding mRNA interferases. Aa00673 was shown by RT-PCR to be co-expressed with Aa00672 and the expression of Aa00672-Aa00673 was decreased in *A. actinomycetemcomitans* strains lacking functional AI-2-receptors or QseC, suggesting that Aa00672-Aa00673 functions downstream from the AI-2 receptors and QseBC in the AI-2 response circuit of *A. actinomycetemcomitans*. We also demonstrate that Aa00673 encodes a toxin, which causes reversible growth inhibition and may function as an RNase; whereas Aa00672 encodes its cognate antitoxin, negating the effects of the toxin. Thus, our results suggest that the QseBC two-component system is part of the AI-2 regulon and in concert with Aa00673, a putative paralog of MqsR, link the detection of AI-2 to the regulation of genes controlling biofilm formation, iron acquisition, and *in vivo* virulence of *A. actinomycetemcomitans*.

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## **CHAPTER ONE: INTRODUCTION**

During the early 1900s, shortly after the prevalent acceptance of both bacteriology and the germ theory, increased awareness of public hygiene resulted in the expectation that most, if not all, diseases would prove to be of infectious origin (125, 186). One popular idea that championed such a belief was the theory of focal infection. This theory proposed that a myriad of systemic diseases were the result of the dissemination of microorganisms or toxic products from a focus of infection (186). The popularity of this theory resulted in the therapeutic extraction of teeth (125). While the theory of focal infection was a product of the intellectual environment of its time, inconsistencies in studies supporting the theory including the inability of healthy tooth extraction to provide relief of illness symptoms, led to its discreditation (125). In recent years, however, the controversial focal infection theory has received a resurgence of support as the potential impact of periodontal diseases is becoming more apparent in systemic health. Current research has focused on the ability of the oral cavity to serve as a reservoir for dissemination of pathogenic and commensal organisms to distant body sites, especially in immunocompromised hosts. The correlation between periodontal disease and systemic diseases such as diabetes, cardiovascular disease (104), respiratory disease (21, 90, 149,

187, 254), and adverse pregnancy outcomes (156, 157) has been and continues to be investigated. A number of epidemiological studies have suggested that oral infection, especially periodontitis, may be a risk factor for many different systemic diseases. On May 25, 2000, the U.S. Surgeon General issued a report on Oral Health, emphasizing that oral health is more than healthy teeth—it is integral to general health and the two should not be considered separate entities (225).

### **Periodontal Disease**

Periodontal disease is a progressive inflammatory disease of the periodontium (tissues surrounding the teeth). Depending on the severity of the disease, these are classified as either gingivitis or periodontitis (Figure 1). Gingivitis, the initial stage of periodontal disease, is an inflammation of the gingival tissue characterized by swelling, redness, and bleeding upon probing of the gingiva (8). Although gingivitis is reversible following proper oral hygiene, if left untreated, it can progress into the more severe, chronic inflammatory condition, periodontitis. Periodontitis is characterized by persistent, uncontrolled inflammation that ultimately causes destruction of the supporting soft tissues surrounding the teeth, loss of gingival fibers and periodontal ligaments, and resorption of the alveolar bone. This can lead to eventual tooth loss and possibly even edentulism (loss of most or all teeth) (10, 169). While treatment and proper oral hygiene can stop the progression of periodontitis, the damage caused cannot be reversed.

Periodontal disease is the most prevalent of bone diseases in humans and has long been implicated as a risk factor in impairment of oral functions and tooth loss, where 10-15% of adults have periodontal disease severe enough to result in tooth loss. While

periodontal disease is prevalent worldwide, it is also serious health problem in the United States (25, 60, 169). Brown *et al* estimate that about 21.8% of adults (22.6 million ) exhibit signs of mild periodontitis, 9.5% (9.9 million) display signs of moderate periodontitis, and about 3.1% (about 3.2 million) exhibit signs of advanced periodontitis (25). The total amount expended for periodontal preventive procedures as well as the treatment of periodontal diseases in the U.S. was \$14.3 billion in 1999 (25). Undoubtedly, innovative methods for the prevention and treatment of periodontal disease would have a major impact on healthcare costs. It is known that periodontal disease can be complicated by certain systemic diseases, and more recently, studies suggest that the reverse may also be true (39, 76, 77, 83, 149, 157, 158, 161, 247, 254). Research proposes that periodontal disease has important systemic implications, and can influence other diseases such as cardiovascular disease (76, 77, 104, 127), pulmonary disease (21, 90, 149, 187, 247, 254), diabetes (65, 83, 161, 222), and adverse pregnancy outcomes (37, 157, 158). Thus, a better understanding of factors that contribute to periodontal disease can be used to develop measures to improve not only oral health, but also overall health of individuals.

The onset and progression of periodontal disease is associated with several risk factors, including gender, age, socio-economic status, nutrition, stress, genetic makeup, and tobacco smoke. Albander *et al* (4) illustrated an increased prevalence of periodontal disease among males (10% greater) as compared with females, confirming estimates produced by the third National Health and Nutrition Examination Survey (NHANES III), which suggested that there was a gender-specific severity of the disease with males having a higher likelihood of developing disease. It was also shown that adults beyond

the age of 30 years had increased levels of periodontitis; whereas after 70 years of age, the progression of severe periodontitis decreased (4). However, individual socio-economic status has an even more significant effect on the progression of periodontal disease. Individuals with higher-income and better insurance providers are more likely to receive necessary treatment compared to uninsured or lower-income patients (4).

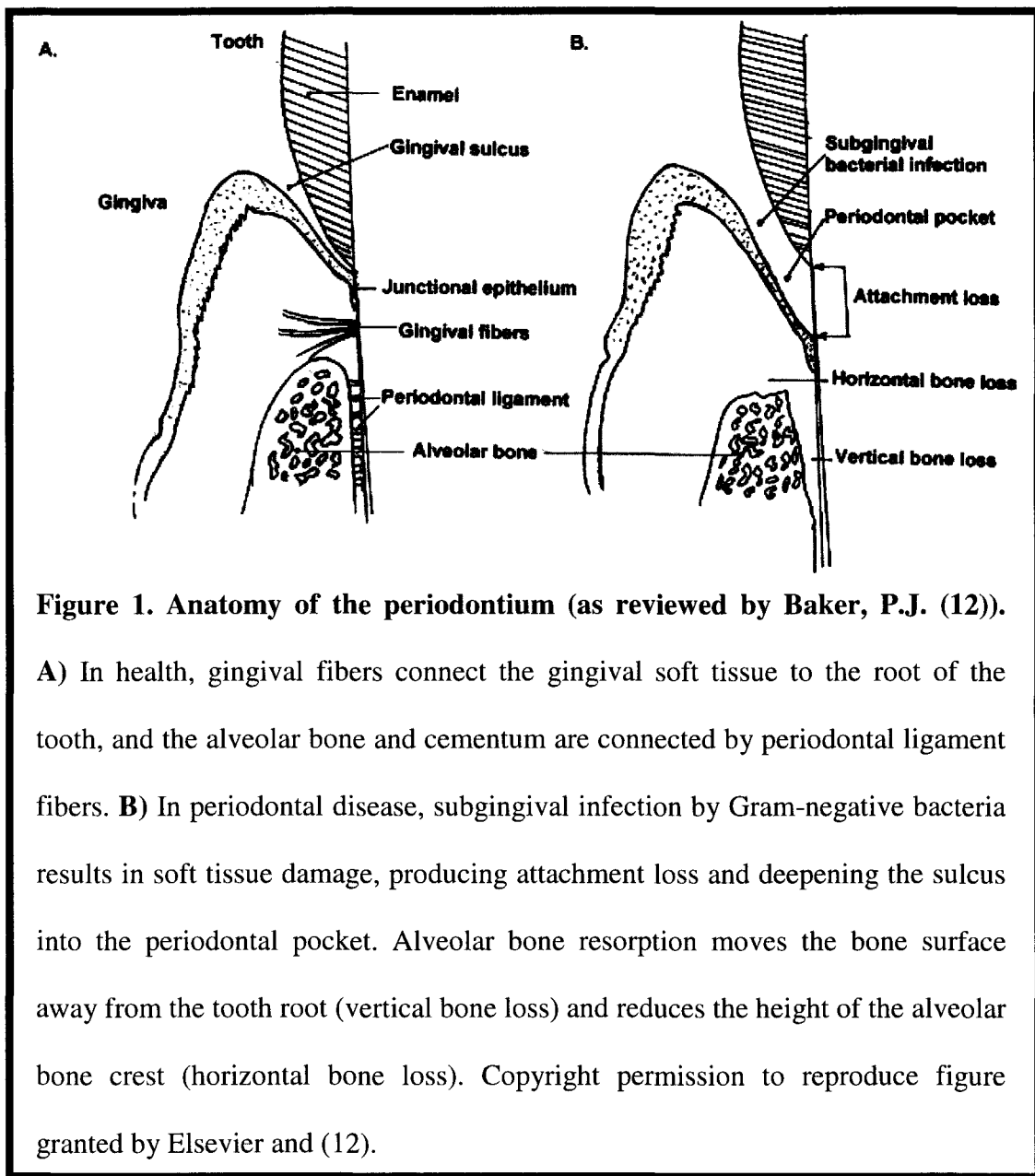
Smoking is considered one of the most important risk factors associated with periodontal disease (182). As such, it has been theorized that increased tobacco usage in populations is directly related to increased numbers of periodontal cases (84, 169). A dose-dependent correlation has been demonstrated between an individual's smoking status and deteriorating periodontal health (18). Smokers are extremely susceptible to periodontitis, and, compared to non-smokers, the disease is often more severe with increased alveolar bone resorption (66), attachment loss, percentage of oral sites with significant attachment loss (67), tooth mobility, and tooth loss (138). Additionally, patients who smoke are more often refractory to treatment than non-smokers (160). Despite conflicting data that tobacco smoke may not influence the sub-gingival microflora (22, 36, 170, 213), recent data strongly indicates that tobacco-induced susceptibility to periodontitis is correlated with populational shifts in the microbial composition of the oral biofilm (28, 49, 61, 69, 95, 198, 226, 229, 251). For example, Umeda *et al* showed that there is an increased risk of *Treponema denticola* inhabiting the oral cavity of smokers (226); while Zambon *et al* reported a higher prevalence of *Aggregatibacter actinomycetemcomitans*, *Tannerella forsythia* and *Porphyromonas gingivalis* in smokers (251). Recent studies also indicate that in addition to changing the composition of the flora, smoking can lead to changes in the activities of residential

bacteria. Bagaitkar *et al* showed that *P. gingivalis* exhibited increased fimbriae expression when grown in medium exposed to tobacco smoke (11).

While all of these factors play a role in the progression and severity of the disease, there is clear evidence that the inflammatory response in the periodontum is initially initiated against the resident microbial biofilm within the subgingival pocket. The oral biofilm consists of several hundred bacterial species, but disease is often associated with a succession in the predominant bacterial species in dental plaque from Gram-positive to Gram-negative. Subgingival colonization by periodontal pathogens is aided by the regulated expression of several immune-evading virulence factors. Bacterial behaviors and the resulting immune response to these pathogens combine to promote inflammatory tissue destruction in periodontitis. The production of various proinflammatory cytokines (i.e. IL-1B, IL-6, TNF $\alpha$ , IL-8, and RANKL) in response to specific Gram-negative bacteria within the oral cavity leads to the activation of osteoclasts (105). Activation of the bone-resorbing osteoclasts (as opposed to bone-forming osteoblasts) eventually results in bone resorption and alveolar bone loss—clinical symptoms of periodontitis (73, 136, 150, 167).

### **Microbial Induction of Periodontal Disease**

Dental plaque is the single most important risk factor associated with the onset and progression of periodontal disease. A hallmark of inflammatory periodontal disease is the increased subgingival colonization by several Gram-negative species, including *A. actinomycetemcomitans*, *Prevotella intermedia*, *Campylobacter rectus*, and a group of three other bacteria designated ‘the red complex’ bacteria (*T. denticola*, *T. forsyntia*, and



*P. gingivalis*) (129). The oral biofilm is one of the most accessible of natural microbial ecosystems—about 50% of the microbes in the oral cavity have been cultivated compared to only about 1% of the population of other ecosystems. It is also complex and potentially

the most pathogenic biofilm present in humans, but its existence is not necessarily detrimental to oral health (194).

A biofilm is a structured community of microorganisms that are adhered to a surface and enclosed in a self-generated matrix that can include proteins, nucleic acids, and carbohydrates. In most cases, biofilms are more resistant to antimicrobial reagents than planktonic cells, and this increased resistance may be mediated by populations of slow-growing cells or persistors, up-regulation of antimicrobial systems (e.g., efflux transporters) in biofilm cells, and semi-permeable intercellular matrices (212). Moreover, the ability of bacteria to adhere tightly to underlying substrata impedes the efficient removal of biofilms by physical or chemical means. In the oral cavity, biofilms form on the surfaces of both hard and soft tissues, and the development of the biofilm depends on interactions between both bacterial cell-surface adhesions and host receptors and bacteria-bacteria interactions. Without the ability to adhere to surfaces in the oral cavity, bacteria are swallowed with the saliva and enter the digestive tract, where they may not survive.

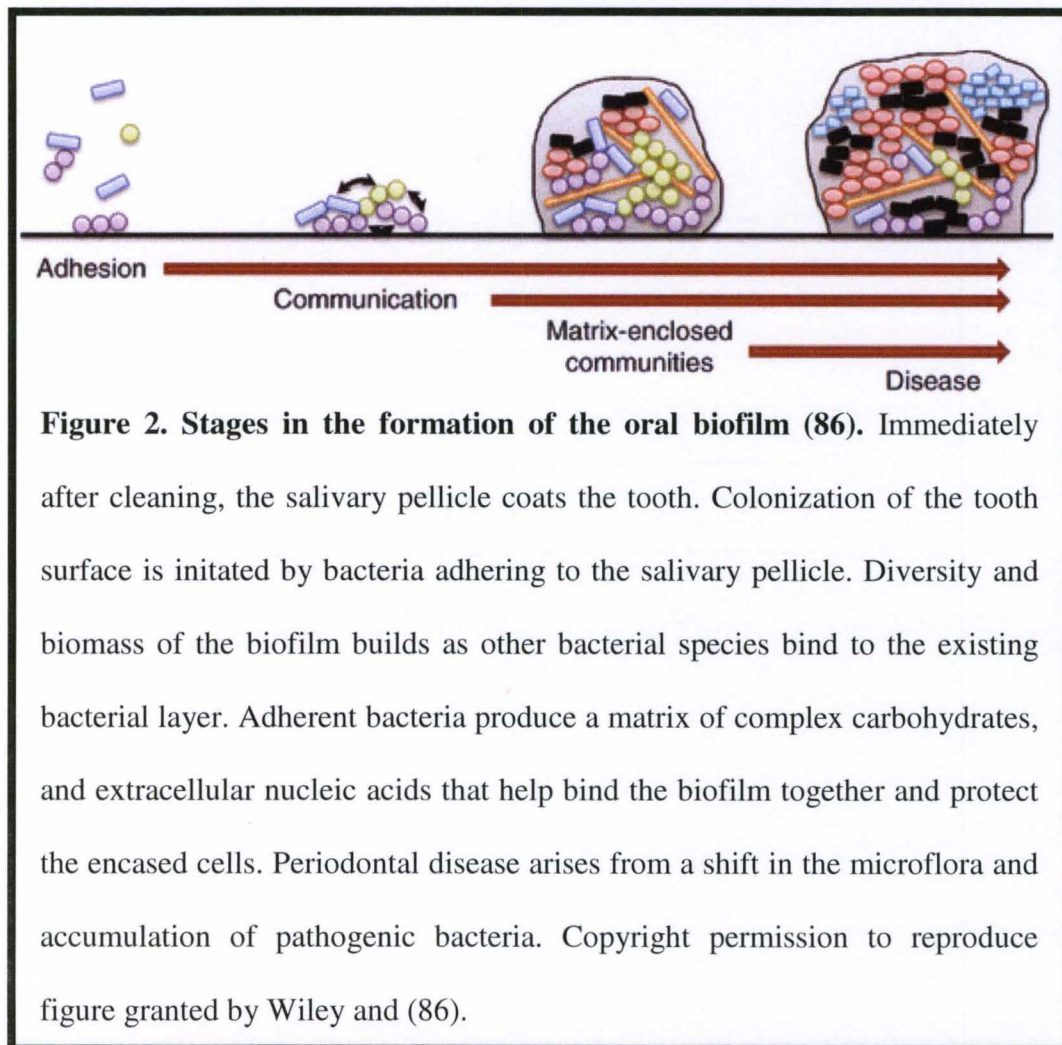
In children, the oral biofilm begins forming on teeth within hours after their eruption from the gingiva while in adults it begins re-forming within minutes after cleaning. Both processes begin with the deposition and formation of the host-derived salivary pellicle. Initial colonizers of the enamel surface—primarily streptococci and actinomyces—recognize and bind to receptors of the salivary pellicle that form on the tooth surface (Figure 2) (40, 42, 86, 155). The initial colonizers also coadhere and coaggregate with each other, successfully forming a bacterial substrate on the enamel of the tooth for other colonizers. Other early colonizers then adhere to and coaggregate with

the already-attached cells, increasing interactions and species diversity of the biofilm. Early colonizers include genera such as *Neisseria*, *Prevotella*, *Eikenella*, *Rothia*, *Porphyromonas*, and *Veillonella* (112). The next stage of colonization comprises the middle colonizers—species not commonly found in initial or early plaque. Middle colonizers must be able to compete with early and initial colonizers, and therefore necessitate different manners of interactions than seen in the early or initial colonizers. For example, *Fusobacterium nucleatum* is capable of binding to many of the initial and early colonizers (6, 109); whereas the early colonizers coaggregate with a specific set of other early colonizers and generally not with any late colonizers (108, 110, 237). Since *F. nucleatum* and other middle colonizers are not often associated with disease (as they colonize the oral cavity before signs of disease are apparent), they are often thought of as the organisms that bridge health and disease (111). The last stage of colonization involves late colonizers, whose presence is often associated with periodontal disease progression. The red complex is microorganisms that are most intimately associated with adult periodontal disease and include *P. gingivalis*, *T. denticola*, and *T. forsythia* (206). *A. actinomycetemcomitans*, another late colonizer, is associated with an aggressive form of periodontitis primarily found in juveniles, but which may also occur in older individuals (72, 203). All four of these species are known to coaggregate with *F. nucleatum*, which is possibly why they appear so late in the oral biofilm.

The oral biofilm consists of several hundred bacterial species living in consortium, establishing a physiologically-compatible microenvironment which allows for metabolic interactions. Metabolic interactions are beneficial interactions that are facilitated by the excretion of a metabolite by one organism that can be utilized as a



nutrient by a different organism; or through the extracellular enzymatic activity of another bacterium which results in the breakdown of a substrate and subsequently creating available nutrients for other organisms. For example, streptococci produce lactic acid as a metabolic waste product, but lactic acid is a major energy source for *A. actinomycetemcomitans*. Brown and Whiteley demonstrated that *A. actinomycetemcomitans* did not grow in medium containing sucrose as the sole carbon source, but it was capable of growing in the presence of *Streptococcus gordonii* because *S. gordonii* converted sucrose to lactate (26). Similarly, interaction of *P. gingivalis* with



*F. nucleatum* facilitates the growth of *P. gingivalis* under aerobic conditions because of the production of carbon dioxide by *F. nucleatum* (41). Grenier *et al* also showed that the production of fatty acids by *P. gingivalis* promotes the growth and biofilm formation of the oral spirochete *T. denticola* (63, 64). Additionally, in the murine subcutaneous lesion model, simultaneous injection of both *P. gingivalis* and *F. nucleatum* resulted in larger lesions and higher morbidity than did injections of either single species alone (48). Thus, these microbial cross-feedings eventually allow for the temporal formation of microbial biofilms in the oral cavity and may also contribute to the increased virulence potential of periodontal pathogens, leading to the development of disease.

#### ***Aggregatibacter actinomycetemcomitans***

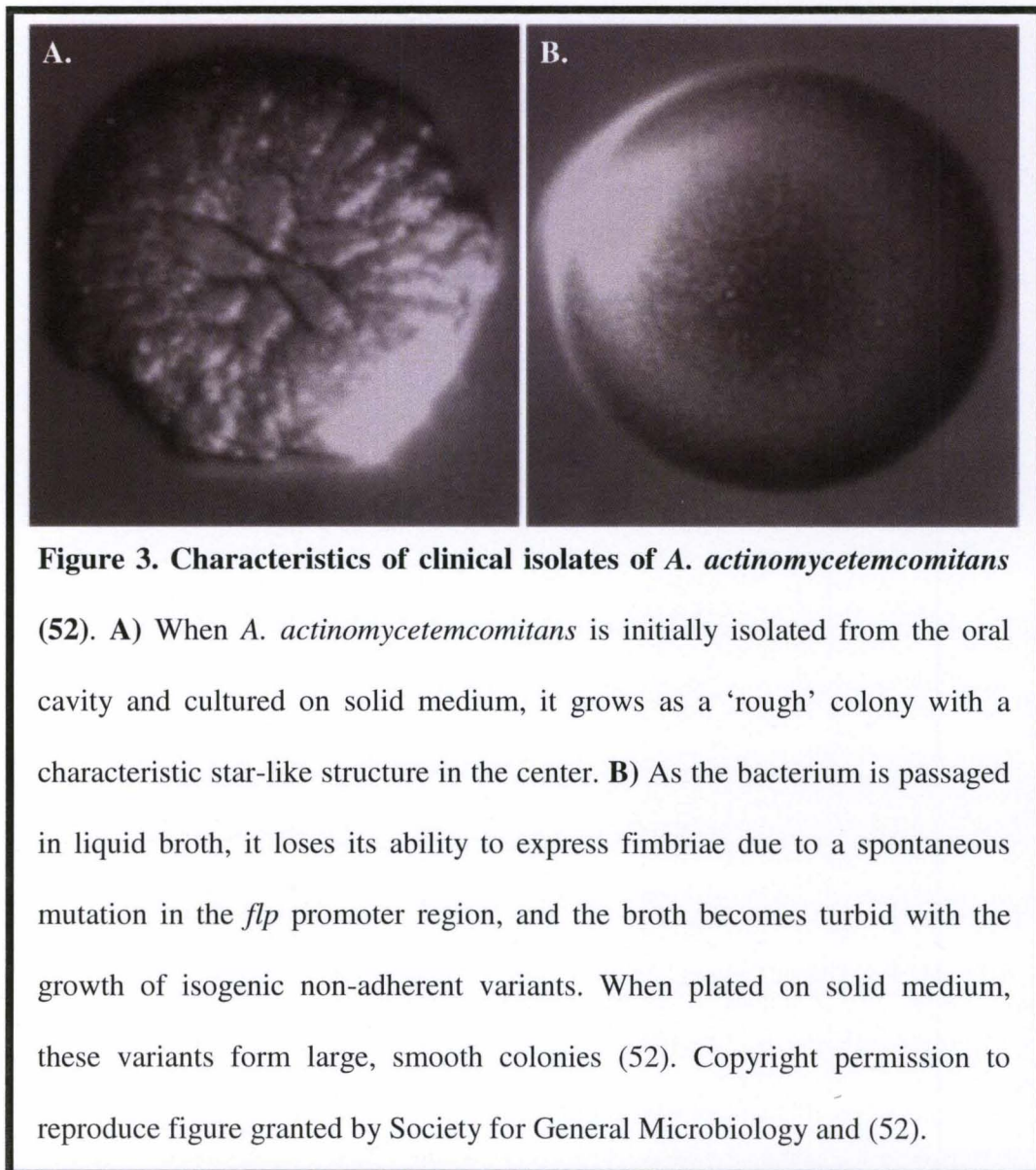
*A. actinomycetemcomitans* (formerly *Actinobacillus actinomycetemcomitans*) is a small, fastidious, CO<sub>2</sub>-requiring coccobacillus (94). It is a facultative anaerobic Gram-negative bacterium that belongs to the family Pasteurellaceae (94). *A. actinomycetemcomitans* was initially isolated from actinomycotic oral lesions along with *Actinomyces israelii*, leading to the derivation of its species name (80). However, although *A. actinomycetemcomitans* was first isolated in 1912 (106), it was never recognized as a member of the oral microbota until 1975 (99). *A. actinomycetemcomitans* has been associated with several systemic infections (e.g. endocarditis (7, 230), brain abscesses (135, 230), bacteremia, infective arthritis (148), and urinary tract infections (223)) and is also strongly associated with localized aggressive periodontitis, an extremely destructive form of periodontal disease that is most common in adolescents (203). As such, most studies of *A. actinomycetemcomitans* have focused on its role in this

disease. If left untreated, localized aggressive periodontitis results in rapid destruction of the supporting structures of the teeth, and eventually leads to loss of alveolar bone and, subsequently, tooth loss—specifically the incisors and first molars (248, 249). Significant geographic differences in the burden of localized aggressive periodontitis have been reported, suggesting that geographical location, socio-economic status, and race are important factors in the prevalence of the disease (183, 204). Nevertheless, *A. actinomycetemcomitans* is a prevalent pathogen in the population and is often found inhabiting the subgingival plaque (204).

*A. actinomycetemcomitans*, like other pathogens, utilizes a myriad of virulence factors to cause disease. There is strong evidence that leukotoxin expression affects disease progression (23, 81, 211). Leukotoxin (LtxA) is a 116-kDa protein that belongs to the repeats-in-toxin (RTX) family of pore-forming toxins exemplified by other Gram-negative bacteria, such as *Escherichia coli* (68, 91, 114, 117). Studies have demonstrated that onset of disease develops at an earlier age in individuals harboring a clonal variant of *A. actinomycetemcomitans* (strain JP2), which expresses high levels of leukotoxin as a result of a deletion in the promoter region upstream from the toxin operon (23, 81, 211). Additionally, disease is more likely to occur in individuals infected with this clone than those infected with other strains of the organism which express only minimal levels of leukotoxin (71). Studies have shown that the target of LtxA is quite specific, which is unusual among RTX toxins, killing only polymorphonuclear leukocytes, monocytes and T-cells in humans, apes, and Old World monkeys (220, 221).

*A. actinomycetemcomitans* also expresses several other potential virulence factors (53), including catalase (45, 147), IgA protease (233), OmpA-like protein (115, 235),

capsular polysaccharide biosynthetic enzymes (246), cytolethal-distending toxin (137, 172, 197), and fimbriae production. Previous studies have demonstrated the ability of *A. actinomycetemcomitans* to bind and invade epithelial (142, 143) and endothelial cells (192), which may be an important mechanism for evading the host immune system and disseminating beyond the initial site of infection (53). Furthermore, fresh clinical isolates



of *A. actinomycetemcomitans* express fimbriae (Figure 3), which allow them to form tenacious biofilms on a variety of solid surfaces, such as hydroxyapatite, glass, and plastic (52, 93, 181, 202). In broth cultures, this bacterium adheres tightly to the culture vessel, forming dense microcolonies (51, 52, 85, 92, 181, 201, 203). The broth remains clear as the biofilm grows; however, older biofilms ultimately have turbid broth from the growth of isogenic non-adherent variants (51, 52, 85, 92, 181, 201, 203). While fimbriae expression may be important in biofilm pathogenesis *in vivo*, it is not the sole determinant in biofilm formation, as isogenic smooth strains are capable of forming biofilms (196). Even so, these fimbriae are thought to enable *A. actinomycetemcomitans* to colonize the tooth surface, persist in the oral cavity, and initiate infection in the presence of salivary flow.

### **Quorum Sensing**

The most common forms of oral diseases, dental caries and periodontal disease, have been linked with the formation and maturation of dental plaque. Furthermore, the populations of pathogens relative to residential microbes are increased at diseased sites, suggesting that major shifts in microbial populations within the oral biofilm may contribute to the disease process (37). Interestingly, the oral cavity experiences constant, continual environmental fluxes, such as pH, temperature, osmolarity, and changes in the nutrient supply, which may contribute, in part, to these microbial population shifts. Oral bacteria are able to detect these fluctuations in the environment and appropriately respond, allowing them to successfully coexist and adapt in dental plaque. However, it is still unknown how host and/or microbial signals are instrumental in orchestrating these

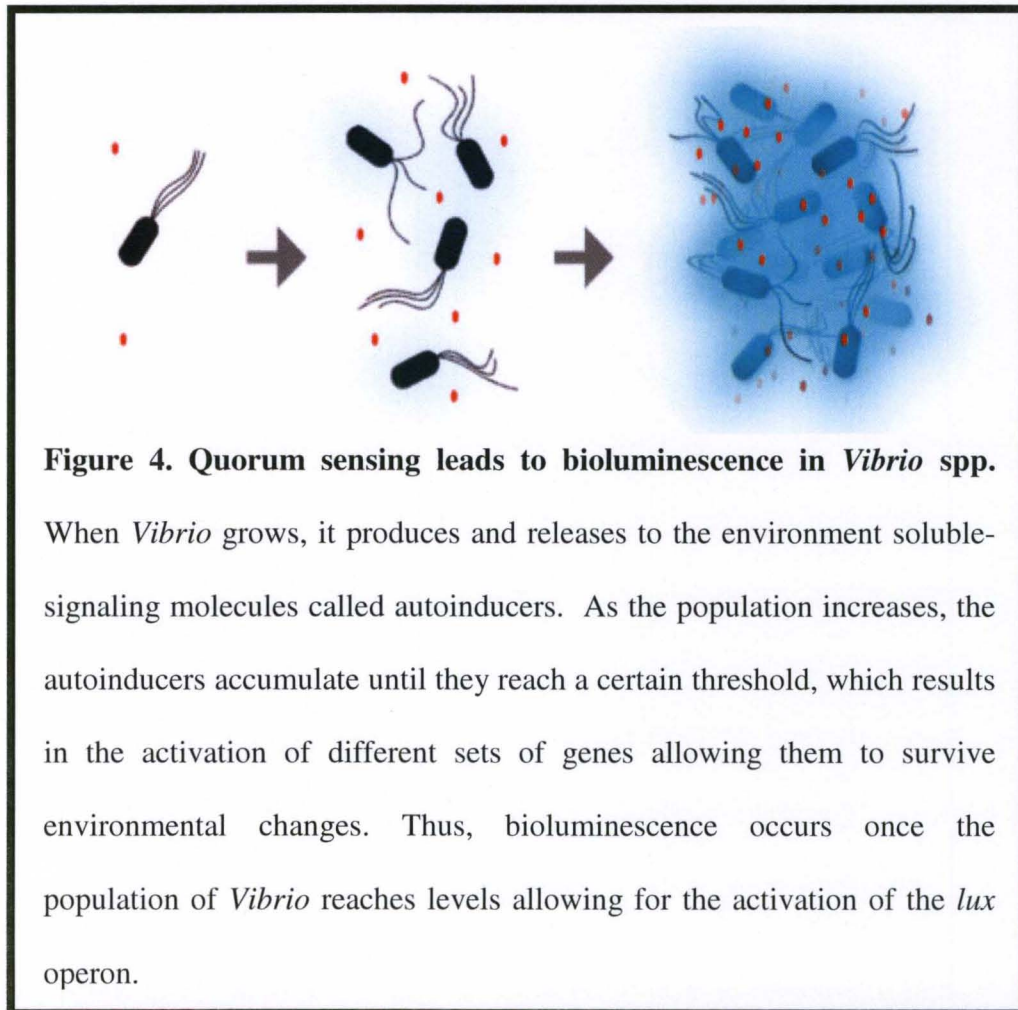
population shifts, or even how bacteria are able to detect changes in their surrounding environment and properly respond.

Both intra- and interspecies communication occurs between bacteria, which enables the bacteria to coordinate their behavior and function, and regulate their gene expression as a community. One mechanism of communication, termed quorum sensing (Figure 4), is a cell-density dependent response (116, 140, 190, 253). In Gram-negative bacteria, quorum sensing is mediated by the production, release, and detection of soluble signal molecules called autoinducers, of which there are a multitude of structures including acylated homoserine lactones (AHL) (74, 162, 166), quinolone derivatives, and furan derivatives (e.g. AI-2) (140, 190, 236, 240). As a population of bacteria expands, the external concentration of autoinducer increases until the rate of secretion is balanced by influx back into the bacterial cell. The population of bacteria is able to detect the autoinducer once it reaches a certain threshold, at which point a signal transduction cascade is initiated, resulting in changes in gene expression, and accordingly behavior, in the community of bacteria. Quorum sensing controls cell-density dependent phenotypes such as virulence-factor expression, biofilm formation, and iron acquisition (14, 74, 236, 238). In this sense, quorum sensing allows single-cell organisms to react coordinately as multi-cellular organisms.

### **Autoinducer-2 Quorum Sensing Pathway**

The contribution of quorum sensing pathways to the development of biofilms was initially characterized in the non-oral organism *Pseudomonas aeruginosa* (38, 163). Indeed, cell-to-cell communication, mediated by the diffusible AHL signals, was shown

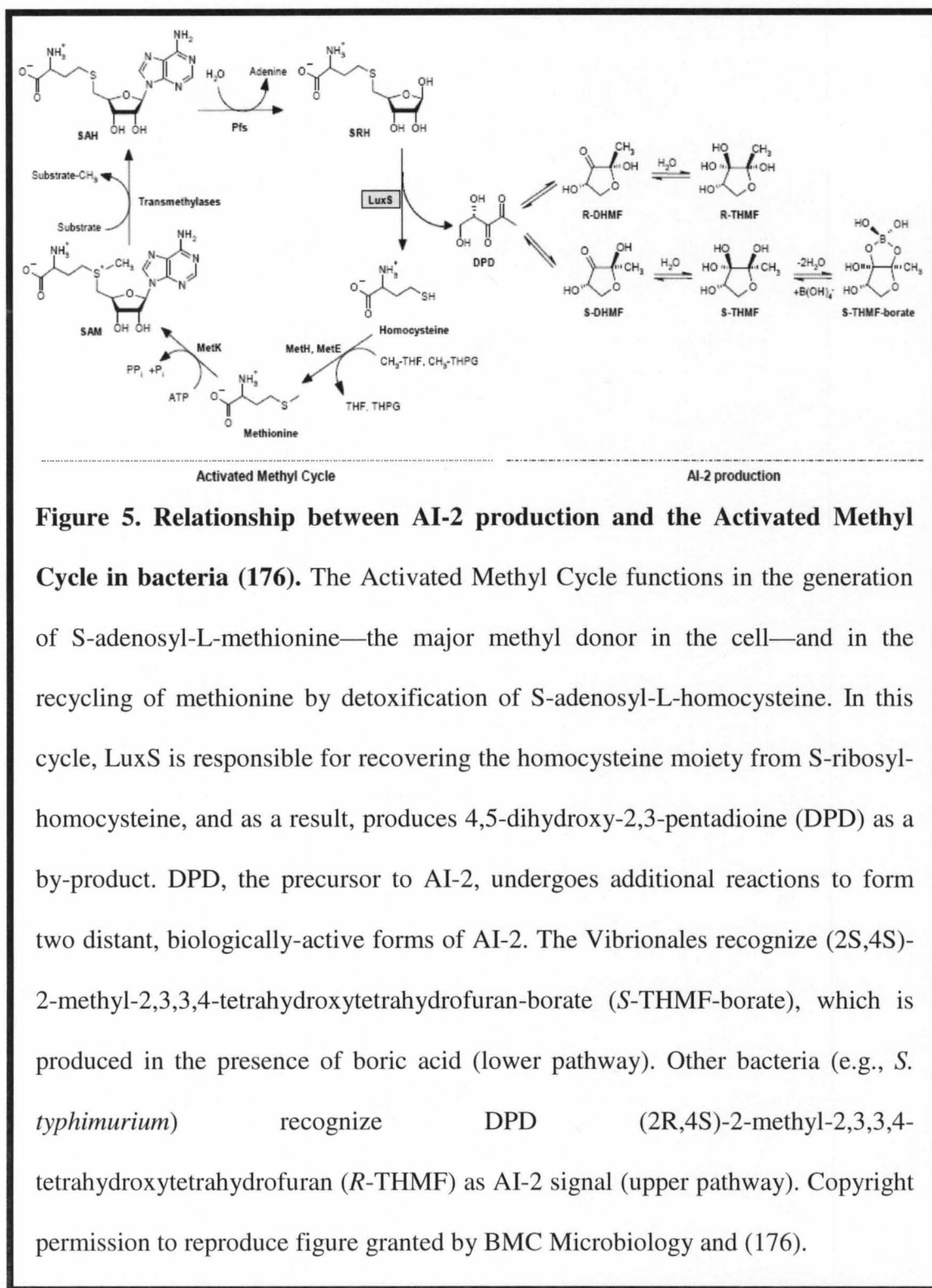
to influence the expression of virulence factors in *P. aeruginosa* (163). Studies that measured biofilm formation on enamel chips implanted in the oral cavity were the first to shed light onto the contribution of soluble autoinducer-like molecules to dental biofilm development (126). However, surveys of oral bacteria utilizing a variety of reporter systems designed to detect AHL autoinducers failed to demonstrate the presence of quorum-sensing signals (58, 237), and queries of the genomes of several oral bacteria failed to identify genes encoding proteins that exhibit similarity to known AHL, suggesting that these AHL-based systems may not be prevalent in oral bacteria.



Studies of *Vibrio harveyi* identified a second type of quorum sensing autoinducer, autoinducer-2 (AI-2) (16), and the *luxS* gene was subsequently shown to be the synthase for this autoinducer (216). Based upon these findings, oral bacteria were then re-examined, using a reporter specific for AI-2, for the presence of AI-2-dependent quorum sensing systems. *P. gingivalis* and *P. intermedia* were shown to produce a soluble molecule that stimulated the AI-2 quorum sensing circuit of the *V. harveyi* reporter strain and induce bioluminescence (58). Subsequently, the genomes of *P. gingivalis* (27, 34), *A. actinomycetemcomitans* (54), and several other species of oral streptococci (19, 139, 141, 234) were shown to have genes encoding polypeptides related to the LuxS protein of *V. harveyi*. The conditioned culture medium from these organisms was also shown to contain an AI-2-like compound that was capable of stimulating the AI-2 quorum sensing circuit of *V. harveyi*. Thus, while the oral bacteria that have been examined to date do not appear to possess AHL-dependent quorum sensing pathways, many do produce an AI-2-like signaling molecule, suggesting that these organisms possess an AI-2 quorum sensing pathway.

Autoinducer-2 requires the *luxS* gene for its production (Figure 5). LuxS is an enzyme that participates in the Activated Methyl Cycle, which functions in the turnover of S-adenosyl-L-homocysteine (SAH) and generation of S-adenosyl-L-methionine (SAM). LuxS cleaves S-ribosyl-L-homocysteine (SRH) to form L-homocysteine and 4,5-dihydroxy-2,3-pentanedione (DPD). Homocysteine is then converted to methionine by the *metH* gene, and ultimately, to S-adenosylmethionine by the *metK* gene. DPD is

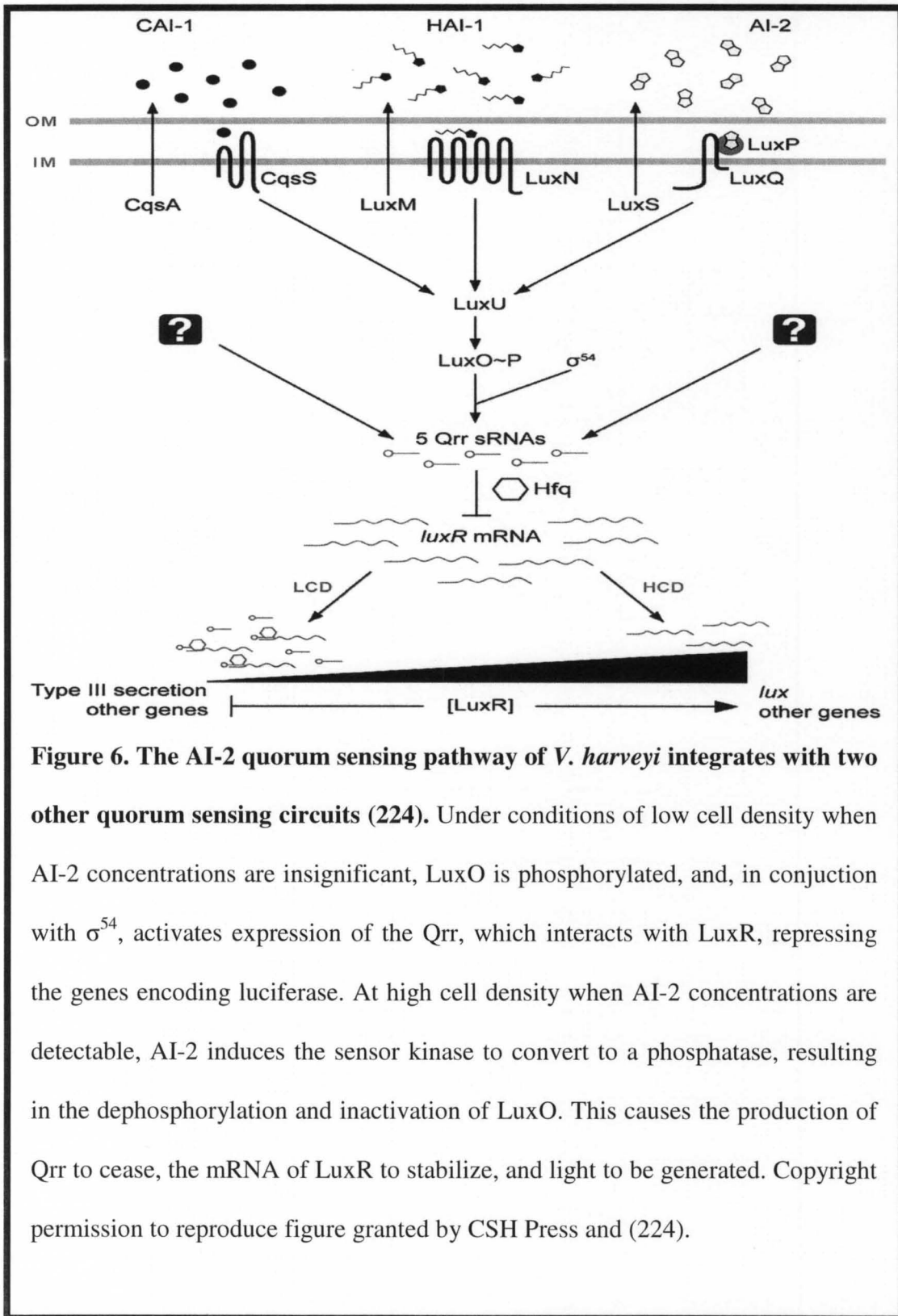




reactive in aqueous solution and undergoes a spontaneous intramolecular cyclization and hydration to yield, in chemical equilibrium, the two known forms of AI-2: (2S,4S)-2-methyl-2,3,3,4-tetrahydroxytetrahydrofuran (*S*-THMF) and (2R,4S)-2-methyl-2,3,3,4-tetrahydroxytetrahydrofuran (*R*-THMF) (144). *S*-THMF is a cis-diol and is able to react with borate ions to form the active quorum-sensing signal, *S*-THMF-borate, which is detected by LuxP, the periplasmic AI-2 receptor of *Vibrio* spp (29). The other stereoisomer, *R*-THMF, is unable to complex borate, but this form is bound by the periplasmic receptor LsrB in *Salmonella enterica* serovar Typhimurium (145).

The *luxS* gene is widely distributed in the Firmicutes and Proteobacteria (122), and as such, many Gram-positive and Gram-negative bacterial species express the protein LuxS and also secrete and respond to a signal that is capable of stimulating bioluminescence in *Vibrio*. The widespread distribution of *luxS* has led to the hypothesis that AI-2 may be a quorum sensing signal capable of facilitating interspecies communication (144, 189). In addition, individual organisms appear capable of adapting LuxS signaling for their own needs, as AI-2 has been shown to mediate the expression of a variety of cellular processes in different organisms. For example, AI-2 regulates type III secretion in enteropathogenic and enterohemorrhagic *E. coli* (207), protease and hemolysin production in *Vibrio vulnificus* and *Streptococcus pyogenes* (100, 130), biofilm formation in *Vibrio cholera*, *Helicobacter pylori*, *Streptococcus mutans*, and *A. actinomycetemcomitans* (19, 35, 70, 139, 141, 196, 245), motility in *E. coli* and *H. pylori* (128, 208), and iron uptake in *P. gingivalis* and *A. actinomycetemcomitans* (54, 55, 87).

The cellular processes regulated by AI-2 in *Vibrio* spp. are induced by a regulatory cascade that is integrated with two other quorum sensing pathways (Figure 6).



**Figure 6. The AI-2 quorum sensing pathway of *V. harveyi* integrates with two other quorum sensing circuits (224).** Under conditions of low cell density when AI-2 concentrations are insignificant, LuxO is phosphorylated, and, in conjunction with  $\sigma^{54}$ , activates expression of the Qrr, which interacts with LuxR, repressing the genes encoding luciferase. At high cell density when AI-2 concentrations are detectable, AI-2 induces the sensor kinase to convert to a phosphatase, resulting in the dephosphorylation and inactivation of LuxO. This causes the production of Qrr to cease, the mRNA of LuxR to stabilize, and light to be generated. Copyright permission to reproduce figure granted by CSH Press and (224).

AI-2 is initially detected and bound by LuxP, a periplasmic AI-2 receptor (29). LuxP then associates with the membrane-bound autophosphorylating histidine sensor kinase protein, LuxQ, which initiates a phosphate-transfer cascade (152). LuxQ transfers a phosphoryl group to LuxU, a histidine-containing phosphotransfer protein, which in turn transfers a phosphate to the  $\sigma^{54}$ -dependent response regulator LuxO. LuxO mediates the expression of several quorum-regulatory RNAs (Qrr) that are bound by Hfq an RNA chaperone. LuxO, in concert with Hfq, coordinate the regulation of the master regulator of the *lux* operon, LuxR (120, 121). Under conditions of low cell density, when AI-2 concentrations are negligible, the LuxQ sensor phosphorylates LuxO. Phosphorylated LuxO, along with  $\sigma^{54}$ , activate expression of Qrr, which destabilizes the mRNA of LuxR, and the genes encoding luciferase are not expressed. In contrast, under conditions of high cell density, when AI-2 concentrations are high, LuxQ functions as a phosphatase, which results in the dephosphorylation of LuxO (57). This inactivates LuxO, which causes the production of Qrr to cease, preventing degradation of LuxR mRNA. LuxR directly activates the *lux* operon (57, 146) and also controls, either directly or indirectly, other known quorum-sensing target genes (199, 217).

### **AI-2-Dependent Quorum Sensing in *A. actinomycetemcomitans***

*A. actinomycetemcomitans* was one of the first oral organisms to be examined for AI-2-dependent quorum sensing systems. Fong *et al* identified the *luxS* gene in *A. actinomycetemcomitans* and demonstrated that an AI-2-like signal in the culture media induced bioluminescence in the *V. harveyi* reporter strain (55). The activity of AI-2 in *A. actinomycetemcomitans* was shown to be maximal during the exponential growth phase

and decreased during late-exponential and stationary phase, implying that AI-2 was either degraded or removed from the culture fluid (54). Inactivation of the *luxS* gene demonstrated that the expression of several virulence factors was influenced by *luxS*. Fong *et al* showed that expression of *ltxCABD* (encoding the RTX leukotoxin) and *afuABC* (encoding a ferric ion transporter) is decreased in a LuxS-deficient organism (55). This suggests that AI-2 may positively regulate leukotoxin expression and iron acquisition (55). While it may be possible that these effects are due to metabolic disruption arising from the alteration of the Activated Methyl Cycle, two factors suggest that the effects were due to the absence of a soluble signaling molecule and not a more general metabolic defect. First, exposing *A. actinomycetemcomitans* to either conditioned medium obtained from a late-exponential culture of *E. coli* AIS (a *luxS*-deficient recombinant strain of *E. coli* transformed with a plasmid-borne copy of *A. actinomycetemcomitans luxS*) or conditioned medium from a late-exponential phase of *A. actinomycetemcomitans* culture led to a two- to threefold increase in *ltxCABD* expression (55). Furthermore, a *luxS*<sup>-</sup> mutation in *P. gingivalis*, another periodontal pathogen, was complemented by conditioned culture medium from *A. actinomycetemcomitans* (54). These results illustrate that the AI-2 signal is capable of complementing the *luxS* mutation alone and is consistent with the hypothesis that AI-2 functions as a multi-species quorum sensing signal.

Additional studies found other *A. actinomycetemcomitans* phenotypes that were dependent on AI-2 (55, 56, 195, 196). Following the initial observation that expression of a ferric-uptake transporter was altered after inactivation of *luxS*, Fong *et al* investigated the expression profile of other iron uptake systems. Expression of several genes encoding

proteins involved in iron acquisition and storage was significantly altered in a *luxS* mutant. For example, genes encoding putative receptors for heme, transferrin, and hemoglobin were downregulated two- to threefold in the *luxS* mutant. Moreover, the expression of *ftnAB*, which encodes an intracellular iron storage protein ferritin, was reduced 50-fold in the mutant organism. The expression of *afuABC*, encoding a periplasmic ferric ion transporter, was reduced approximately eightfold, consistent with earlier observations. Interestingly, inactivation of *luxS* resulted in the increased transcription of a ferric citrate transport operon and another operon encoding a putative periplasmic enterobacterin receptor and ABC transporter (3- and 10-fold, respectively) (55). This type of regulation suggests that AI-2 may function in the adaption of *A. actinomycetemcomitans* from a planktonic lifestyle to a biofilm environment by facilitating a switch from the uptake of iron via chelators (e.g. enterobactin-like siderophores) to the acquisition of iron from host proteins, such as transferrin and hemophores.

Fong *et al* also examined the growth of the parent and *luxS*-deficient strains under iron limitation. The growth rates of both wild-type and *luxS* mutant strains were indistinguishable when cells were grown in iron-replete medium. In addition, wildtype *A. actinomycetemcomitans* also had normal growth in the presence of the ferric iron chelator ethylenediamine-N,N'-bis(2-hydroxyphenylacetic acid) (EDDHA), suggesting that the organism is quite effective in competing with the chelator for iron in the culture medium. On the contrary, the *luxS* mutant failed to divide when grown in medium containing EDDHA. However, complementation of the mutant with a plasmid-borne copy of *luxS* restored growth to wild-type levels under iron limitation. Moreover, the inhibited growth

of the *luxS*<sup>-</sup> mutant under iron limitation was reversed when the bacterial cells were inoculated back into iron-replete medium (55).

Shao *et al* performed subsequent studies which examined the biofilm growth of *A. actinomycetemcomitans* in an open flow chamber and demonstrated that the growth and formation of the *luxS*<sup>-</sup> mutant on saliva-coated coverslips were defective, exhibiting lower total biomass and lower biofilm depth relative to wild-type *A. actinomycetemcomitans*. Restoration of wild-type biofilm growth was achieved by either transforming the mutant strain with a functional copy of the *luxS* gene or by the addition of partially purified AI-2 to the growth medium (196). Furthermore, transformation of the *luxS*<sup>-</sup> mutant with a gene encoding SAH hydrolase, which in the absence of *luxS* restores the activity of the Activated Methyl Cycle, did not fully complement the *luxS*<sup>-</sup> mutation unless AI-2 was also added in trans (196). Collectively, these results imply that iron acquisition and biofilm formation of *A. actinomycetemcomitans* are influenced by AI-2 signaling. Indeed, iron uptake and biofilm formation are intimately associated processes in *A. actinomycetemcomitans* as both are essential for colonization and persistence in the oral cavity.

Analysis of the *A. actinomycetemcomitans* genome also revealed two genes, *lsrB* and *rbsB*, which encode putative periplasmic proteins related to the AI-2 receptor LuxP of *V. harveyi* (88, 195). The *lsrB* gene is within an operon that may be functionally related to the *lsrACDBFGE* operon of *S. enterica* serovar Typhimurium, which encodes an ABC-type transporter that internalizes AI-2. This operon is also located immediately downstream from a divergently transcribed operon, *lsrRK*, in *S. enterica* serovar Typhimurium that encodes a kinase (LsrK) that phosphorylates AI-2 after its

internalization and a master regulator (LsrR) of the *lsrACDBFGE* operon. The *lsrRK* operon is also present upstream of the *lsr* operon in the *A. actinomycetemcomitans* genome (190). In addition, RbsB is similar to a protein expressed by *E. coli*. In *A. actinomycetemcomitans*, it is part of a five gene operon—*rbsDACBK*—which encodes an ABC-type transporter (RbsA and RbsC), a kinase (RbsK), and an enzyme (RbsD) that catalyzes the conversion of ribose from the pyran to the furan form (88). Therefore, there are two distinct mechanisms that *A. actinomycetemcomitans* may be able to utilize to detect and possibly internalize AI-2. *In vitro* competition assays demonstrated that LsrB and RbsB both are capable of binding AI-2 and competitively inhibiting the interaction of AI-2 with cell-associated LuxP. Interestingly, it was shown that RbsB has a higher affinity for *A. actinomycetemcomitans* AI-2 while LsrB interacted at a higher affinity with *V. harveyi* AI-2 (195), suggesting that RbsB and LsrB may differentially interact with AI-2 complexed with borate since the AI-2 recognized by *V. harveyi* is known to contain borate. Additionally, the difference between the kinetics of interaction with AI-2 of each receptor may also allow *A. actinomycetemcomitans* to thrive in areas of low and/or high concentrations of the signaling molecule. Moreover, inactivation of either *lsrB* or *rbsB* resulted in a reduction of the ability of *A. actinomycetemcomitans* to deplete AI-2 from solution relative to the wild-type strain (190). Additionally, both the *lsrB*<sup>-</sup> mutant strain and the *rbsB*<sup>-</sup> mutant strain had a reduction in biofilm formation similar to biofilm formation seen with the *luxS*<sup>-</sup> mutant. Consistent with these results, inactivation of both *lsrB* and *rbsB* had an additive effect not only on the depletion rate of AI-2 by intact cells (190), but also on the ability of mutant strains to form biofilms (196). Taken



together, these results suggest that both RbsB and LsrB interact with AI-2, and that both the Lsr and Rbs transporters are capable of importing the signal into the bacterial cell.

### **Alternative Pathways of AI-2-Dependent Quorum Sensing**

While the early events in AI-2-dependent signaling in *A. actinomycetemcomitans* have been elucidated as well as some of the genes regulated by AI-2, the events that connect detection and internalization to downstream gene expression have yet to be determined. Although proteins that were related to the *Vibrio* AI-2 receptor were readily identified, genes related to the dedicated two-component sensory system (LuxQ sensor, LuxU phosphorelay, and LuxO response regulator proteins) have not been detected in *A. actinomycetemcomitans*. *A. actinomycetemcomitans* does possess several sensor kinases that exhibit similarity to the kinase domain of LuxQ and several response regulators that exhibit similarity to the DNA-binding domain of LuxO, but none of these proteins exhibit significant similarity to the LuxQ sensor domain which interacts with LuxP, or to the LuxO receiver domain which interacts with LuxU. Furthermore, there is no LuxU homolog in the genome of *A. actinomycetemcomitans*. The absence of this dedicated two-component response circuit from *Vibrio* spp. in other organisms has led to the hypothesis that AI-2 does not function as a signaling molecule outside of the *Vibrionales*. Instead, AI-2 may only serve to function in metabolism in these organisms (43, 240). While this may be true for some organisms, the complex phenotypes associated with *luxS* in other bacteria such as *A. actinomycetemcomitans*, and the biochemical complementation of these phenotypes by conditioned medium or synthetic AI-2 is not readily explained by

AI-2 being merely a metabolite; it instead suggests a more direct signaling function for AI-2.

The exact mechanism through which the AI-2 signal information is transduced in organisms that do not express the LuxQ-LuxU-LuxO phosphorelay circuit, like *A. actinomycetemcomitans*, is not known. One potential possibility is that alternative AI-2 circuits exist that depend on or require internalization of AI-2 via the Lsr (or Rbs) transporter. Taga *et al* (218) and Xavier *et al* (242) demonstrated that LsrK functions as a kinase that phosphorylates AI-2 in both *S. enterica* serovar Typhimurium and *E. coli*, respectively, and it is likely that a similar role is performed by LsrK in *A. actinomycetemcomitans*. More recently, it was shown that the product of the LsrK reaction with AI-2 is the phosphorylated form of AI-2 (5-phospho-4-hydroxy-2,3-pentanedione) (242), suggesting that LsrB delivers R-THMF to the transporter for internalization, and once inside the bacterial cell, an equilibrium establishes between the stereoisomeric cyclic forms of AI-2 and the linear form of DPD. Uptake of AI-2 occurs most efficiently during late exponential and stationary growth phases in several organisms, including *A. actinomycetemcomitans*, *E. coli*, and *S. enterica* serovar Typhimurium (54, 218, 241), indicating that a relatively high concentration of AI-2 is needed for LsrK activity and regulation of the *lsr* operon. On the other hand, AI-2 appears to be also internalized by these organisms by *lsr*-independent mechanisms (88, 124, 218), and at times when the expression of the *lsr* operon is low (62, 124). In *A. actinomycetemcomitans*, this internalization may occur via the *rbs* operon; and, in fact, AI-2 interacts with RbsB at a higher affinity than LsrB (195). Therefore, as suggested for *E. coli* by Li *et al*, AI-2 signaling potentially occurs under conditions where LsrK-

mediated phosphorylation rates and subsequent metabolism of AI-2 are low (124). Li *et al* also gave some of the first evidence that LsrR regulates gene expression on a more global basis in *E. coli* (124). To resolve the issue of LsrR regulating the *lsrR* operon as well as other genes outside of the *lsr* operon (genes associated with biofilm formation, e.g., genes for adhesions, polysaccharide export, and fimbriae), Li *et al* proposed that LsrR interacts with both unphosphorylated and phosphorylated forms of AI-2 (124). The primary regulator of the quorum sensing response that influences biofilm-associated genes is the complex of LsrR and unphosphorylated AI-2. The complex of LsrR and phosphorylated AI-2 controls the expression of the *lsr* transporter, essentially functioning as a trigger which terminates quorum sensing by initiating the rapid uptake and metabolic turnover of AI-2 (124). This hypothesis is interesting because it integrates a signaling function and metabolic activity to a single regulatory protein, and it also suggests that, analogous to the LuxR regulator which governs AI-mediated gene expression in *V. harveyi*, LsrR functions as the master regulator of the AI-2 response.

Observations of AI-2-dependent quorum sensing in *A. actinomycetemcomitans* are consistent with the hypothesis proposed by Li *et al* (124) described above. RbsB functions as an AI-2 receptor, and the Rbs transporter may be a potential alternative pathway for Lsr-independent internalization of AI-2 (88, 195). In addition, the levels of AI-2 in cultures of *A. actinomycetemcomitans* are highest during mid- to late-exponential phase, and are quickly reduced when the cells enter stationary phase (54). This is in agreement with the stimulation of the activity of LsrK at high concentrations of AI-2, resulting in rapid phosphorylation of AI-2, the induction of the Lsr transporter and the metabolism of AI-2, and termination of putative LsrR-signaling activity. While *E. coli*

and *A. actinomycetemcomitans* may differ in their regulation of specific genes influenced by AI-2, biofilm formation is affected in both organisms (62, 196). Confirmation of this signaling mechanism in organisms, including *A. actinomycetemcomitans*, requires further identification of genes regulated by AI-2 as well as the interaction of LsrR with unphosphorylated AI-2.

Furthermore, as mentioned earlier, the dedicated two-component signal transduction system that controls the cell density-dependent response of *Vibrio* spp. to AI-2 is conserved in few of these organisms that express LuxS and produce AI-2 (214). Thus, it may be possible that LuxS may primarily serve in the metabolic turnover of SAH and may not play a role in quorum sensing in all organisms; or, alternatively, functions in quorum sensing through different mechanisms.

### **Treatment of Periodontal Disease**

Biofilm growth in the oral cavity is dependent upon AI-2-mediated intra- and interspecies signaling in *A. actinomycetemcomitans* (196). This signaling pathway may allow organisms—specifically pathogens—the ability to rapidly detect and adapt to changes in their local environment, conferring a selective advantage that facilitates expansion of certain microbial populations. If virulence factors are regulated as part of this adaptive response, then the pathogenic potential of the biofilm may also rapidly change, thereby contributing to the onset or progression of disease. Therefore, quorum sensing circuits represent attractive targets for therapeutic intervention to control microbial growth as well as to control and prevent oral diseases associated with microbial biofilms, such as periodontitis. Quorum sensing has already been suggested to be a viable

target in diseases such as cystic fibrosis (79, 151), malaria (47), and other chronic infections (75, 151). Furthermore, naturally-occurring inhibitors of quorum sensing are being studied as drug candidates (75).

The studies characterized in this dissertation further our understanding of AI-2 quorum sensing in the dental pathogen *A. actinomycetemcomitans* both *in vitro* and *in vivo*, and lend a better understanding of the role of AI-2 dependent quorum sensing in periodontal pathogenesis. Overall, these studies define mechanistic differences in the known AI-2 signaling pathways that are present in the Vibrionales and in *A. actinomycetemcomitans*. Elucidating these differences will help develop novel therapies specific for *A. actinomycetemcomitans*-associated diseases, such as aggressive periodontitis. Furthermore, since many other species of bacteria communicate via AI-2 (e.g. *P. gingivalis*), it is important to further define microbial quorum sensing in the oral cavity, where populational shifts in the oral biofilm, which result in overrepresentation of certain microbes, are strongly associated with the onset and progression of disease.

## CHAPTER TWO: MATERIALS AND METHODS

**Bacterial strains and culture conditions.** *A. actinomycetemcomitans* bacterial strains employed in this study are listed in Table 1a. All other bacterial strains are listed in Table 1b. *A. actinomycetemcomitans* JP2 and 652 are afimbriated, smooth colony morphotype strains and were grown at 37°C under microaerophilic conditions in Brain Heart Infusion broth (BHI; Bectin Dickinson and Company (BD), Sparks, Maryland) supplemented with 40 mg of NaHCO<sub>3</sub> (Sigma-Aldrich, St. Louis, MO) per liter. The *luxS*<sup>-</sup> deficient strain of *A. actinomycetemcomitans* was grown as described above, but in medium supplemented with kanamycin (25 µg/ml; Sigma-Aldrich). The *luxS*<sup>-</sup> mutant, complemented with a plasmid borne copy of *luxS*, was grown in BHI supplemented with kanamycin (25 µg/ml) and streptomycin (50 µg/ml; Sigma-Aldrich). The *A. actinomycetemcomitans* *lsrB*<sup>-</sup> and *rbsB*<sup>-</sup> mutant strains were cultured in BHI supplemented with spectinomycin (50 µg/ml; Sigma-Aldrich) and kanamycin (25 µg/ml), respectively. The *A. actinomycetemcomitans* *lsrB*<sup>-</sup>*rbsB*<sup>-</sup> double mutant was cultured in BHI supplemented with kanamycin (25 µg/ml) and spectinomycin (50 µg/ml). The *qseC*<sup>-</sup> mutant was grown in BHI supplemented with spectinomycin (50 µg/ml), and the *qseC*<sup>-</sup> complemented strain was cultured in BHI supplemented with kanamycin (25 µg/ml).

Table 1a. *A. actinomycetemcomitans* strains used in this study

Bacterial Strain	Description	Source
652	Wild-type; minimally leukotoxin strain	(23)
<i>lsrB</i> <sup>-</sup>	<i>lsrB</i> <sup>-</sup> single-receptor mutant:: <i>spec</i>	(195)
<i>rbsB</i> <sup>-</sup>	<i>rbsB</i> <sup>-</sup> single-receptor mutant:: <i>kana</i>	(88)
<i>lsrB</i> <sup>-</sup> <i>rbsB</i> <sup>-</sup>	<i>lsrB</i> <sup>-</sup> <i>rbsB</i> <sup>-</sup> double-receptor mutant:: <i>spec/kana</i>	(195)
<i>qseC</i> <sup>-</sup>	<i>qseC</i> <sup>-</sup> mutant:: <i>spec</i>	This study
<i>qseC</i> <sup>-</sup> comp	<i>qseC</i> <sup>-</sup> mutant complemented with pYGK <i>qseC</i> :: <i>kana</i>	This study
JP2	Wild-type; highly leukotoxic strain	(23)
JP2-12	<i>luxS</i> <sup>-</sup> mutant:: <i>kana</i>	(54)
JP2-12/750	<i>luxS</i> <sup>-</sup> mutant complemented with pJRD215 <i>luxS</i> :: <i>kana/strep</i>	(55)

Table 1b. Other bacterial strains used in this study

Bacterial Strain	Description	Source
<i>E. coli</i> DH5α TqseC	DH5α carrying pGEMTqseC::amp	This study
<i>E. coli</i> DH5α TqseCcomp	DH5α carrying pBSKQseC-spec::spec	This study
<i>E. coli</i> pVT1461	Contains spectinomycin resistance cassette	K. Mintz
<i>E. coli</i> DH5α pGEMT-toxin	DH5α carrying pGEMT-toxin::amp	This study
<i>E. coli</i> DH5α pET30a-toxin	DH5α carrying pET30a-toxin::kana	This study
<i>E. coli</i> BL21 pET30a-toxin	BL21 carrying pET30a-toxin::kana	This study
<i>E. coli</i> DH5α pGEMT-TA	DH5α carrying pGEMT-TA::amp	This study
<i>E. coli</i> DH5α pET30A-TA	DH5α carrying pET30a-TA::kana	This study
<i>E. coli</i> BL21 pET30a-TA	BL21 carrying pET30a-TA::kana	This study
<i>E. coli</i> DH5α pGEMT-toxine/p	DH5α carrying pGEMT-toxine/p::amp	This study
<i>E. coli</i> DH5α pBADgIII/A-toxine/p	DH5α carrying pET30a-toxine/p	This study
<i>E. coli</i> TOP10 pBADgIII/A -toxine/p	BL21 carrying pET30a-toxine/p	This study
<i>P. gingivalis</i> 33277	Wild-type	ATCC
<i>P. gingivalis luxS</i>	33277 <i>luxS</i> mutant::ermF	(34)
<i>V. harveyi</i> BB170	Sensor 1- sensor 2+	(15)



*Porphyromonas gingivalis* strains were grown in reduced Trypticase Soy Broth (TSB; BD) supplemented with yeast extract (1 gram per liter; BD), menadione (1 µg per ml; Sigma-Aldrich), and hemin (5 µg per ml; Sigma-Aldrich). The medium was reduced for 24 hours under anaerobic conditions by equilibration in an atmosphere consisting of 10% CO<sub>2</sub>, 10% H<sub>2</sub>, and 80% N<sub>2</sub>. The *P. gingivalis luxS* mutant (kindly supplied by R. Lamont, University of Florida, Gainesville, FL) was grown as described above, but the medium was supplemented with erythromycin (10 µg/ml; Sigma-Aldrich) immediately before inoculation.

*Vibrio harveyi* BB170 (sensor 1<sup>-</sup> sensor 2<sup>+</sup>) was a gift from B. Bassler (Princeton University) and was grown overnight in AB medium with aeration at 30°C (15). AB medium consists of 0.3 M NaCl, 50 mM MgSO<sub>4</sub>, 0.2% Casamino Acids, 10 mM potassium phosphate (pH 7.0), 1 mM L-arginine, 2% glycerol, 1 µg per ml thiamine, and 10 ng per ml riboflavin. *E. coli* strains were grown in Luria-Bertani (LB) medium (BD) with aeration at 37°C. *E. coli* strains containing plasmid pGEM-T or pYGK were cultured as described above in LB supplemented with 100 µg per ml ampicillin or 25 µg per ml kanamycin, respectively.

**Construction of *qseC* mutant and complemented strain.** The *A. actinomycetemcomitans qseBC* operon was identified from the genomic sequence of strain HK1651 (Los Alamos National Laboratory, <http://www.oralgen.lanl.gov/>) annotated as *ygiX* and *qseC*. However, it appeared that in the HK1651 genome the *qseBC* operon was disrupted by a gene rearrangement. This *qseBC* region was sequenced in strain 652 (the strain used to construct the mutant), and unlike HK1651, the *qseBC*

operon was indeed localized together. Thus, to construct the fragment for inactivation of *qseC*, part of the *ygiX* and *qseC* genes were amplified using genomic DNA of strain 652 as the template with primers P1 and P2 (Table 3a). The following PCR program was used: 94°C for 10 min for 1 cycle, and then 94°C for 30s, 60°C for 1min, and 72°C for 2 min for 30 cycles. The PCR products were then ligated with pGEMT-Easy (Promega, Madison, WI) and transformed into *E. coli* DH5 $\alpha$ . The resulting pGEMTQseC plasmid was purified from *E. coli*, cleaved by digesting with *KpnI/BamHI* and ligated into pBSK. The resulting plasmid, pBSKQseC, was then cleaved with *BamHI* and treated with Alkaline Phosphatase for insertion of a spectinomycin resistance cassette. The spectinomycin resistance cassette was obtained by PCR amplification using plasmid pVT1461 (kindly supplied by K. Mintz, University of Vermont) as template, with primers P3 and P4, and then ligated into pBSKQseC to create pBSKQseC-spec. This plasmid was then transformed into *E. coli* DH5 $\alpha$ , and recombinant clones were confirmed by PCR using primers P1 and P4. Purified pBSKQseC-spec plasmid was introduced into *A. actinomycetemcomitans* 652 by electroporation with ampicillin-resistance and spectinomycin-resistance selection. The lack of QseC transcript in the mutant strain was confirmed by RT-PCR using primers P6 and P7.

To make the *qseC* complemented strain, the entire *qseC* gene was amplified using 652 genomic DNA as template with 5' *qseC*-comp-*HindIII* and 3' *qseC*-comp-*BamHI* primers. The leukotoxin promoter (*ltx-pro*) was also amplified from JP2 genomic DNA using the 5' *ltx-pro-KpnI* and 3' *ltx-pro-HindIII* primers. The PCR program was used: 94°C for 10 min for 1 cycle, and then 94°C for 30s, 60°C for 1min, and 72°C for 2 min for 30 cycles. Both the *qseC* and leukotoxin PCR products were then ligated into

Table 2a. Primers\* used in this study.

Primers	Primer Sequence (5' — 3')	Target Gene	Size (kbp)
P1	<b>GGTACCTCGCCGTGGATTGGTTTACCGAC</b>	<i>ygiX</i> (5' primer)	1.030
P2	<b>GGATCCGGCGTTTATGCGACGGTTTG</b>	<i>qseC</i> (3' primer)	1.030
P3	<b>GGATCCATCGATTTTCGTTTCGTG</b>	SpecR cassette	1.141
P4	<b>GGATCCCATATGCAAGGGTTTAT</b>	SpecR cassette	1.141
P6	TAAGTGGAAATAATTACAGCCTGCG	<i>qseC</i>	0.139
P7	TTGTTGTGCGTCAAACACTTGGTTC	<i>qseC</i>	0.139
5' <i>qseC</i> -comp-HindIII	<b>AAGCTTATGAAACTGAGTAAGTGG</b>	<i>qseC</i>	1.374
3' <i>qseC</i> -comp-BamHI	<b>GGATCCCAACTGAATCTCTGCC</b>	<i>qseC</i>	1.374
5' <i>ltx</i> -pro-KpnI	<b>GGTACCAATGAAAAAAAAACAAAGCG</b>	Leukotoxin promoter	0.308
3' <i>ltx</i> -pro-HindIII	<b>AAGCTTACTCGTTTTTCCTTTTTCATTAG</b>	Leukotoxin promoter	0.308
5' <i>QseBC</i> induction	CGTTTGATTGTCACCTTGTCGCTG	<i>qseC</i>	0.110
3' <i>QseBC</i> induction	TGTTGTGCGTCAAACACTTGGTTC	<i>qseC</i>	0.110
5' <i>qseBC</i> transcript	CCGAAAATGGACGGGCTGGATGTGTTG	<i>ygiX</i> (5' primer)	0.911
3' <i>qseBC</i> transcript	CGCCATTCGTCATCATCTTCCCAG	<i>qseC</i> (3' primer)	0.911
5' <i>qseB</i> sequence	CCGGTTTCGCCGTGGATTGGTTTACC	<i>qseB</i>	1.035
3' <i>qseB</i> sequence	GGCGTTTATGCGACGGTTTGCCACGT	<i>qseB</i>	1.035
5' <i>qseC</i> sequence	CGGGTGGCTAATTAGAACGGGCAG	<i>qseC</i>	1.525
3' <i>qseC</i> sequence	GCACGTCATGCGACCTTTCTATCAAAC	<i>qseC</i>	1.525

\* Bold sequences indicate restriction enzyme sites to facilitate cloning into the appropriate shuttle vectors.

Table 2b. Primers\* used in this study.

Primers	Primer Sequence (5' — 3')	Target Gene	Size (kbp)
5' qseC-aaseq	GCGACGGCGAAAATGGGGAAAACCTTA	qseC aa 130-1230	0.426
3' qseC-aaseq	CGGAGGTAAAACGCCGTTACGCAATAA	qseC aa 130-230	0.426
5' 00696	CGGATTACTCGCCTCACAAGCG	afuA 00696	0.318
3' 0696	CCTTTGGTTTTTGGCCGGATCGC	afuA 00696	0.318
5' 00795	GCGCAGAAAATCGGTGATGTCGTGG	fecB 00795	0.317
3' 00795	GCGATACTTTCCTGACGATAGTGTGC	fecB 00795	0.317
5' 5S rRNA	GCGGGGATCCTGGCGGTGACCTACT	5S-2-rRNA	0.089
3' 5S rRNA	GCGATCTAGACCACCTGAAACCATAACC	5S-2-rRNA	0.089
5' AA00673-TAA	<b>CCATGGAATTGAGACAACAAGCAACGTTAG</b>	AA00673	0.263
3' AA00673-TAA	<b>GGTACCTTAATAGTCCAATTCCTGCGGTAAA</b>	AA00673	0.263
5' AA00673/672	<b>CCATGGAATTGAGACAACAAGCAACGTTAG</b>	AA00673 (5' primer)	0.608
3' AA00673/672	<b>GGTACCTTATCGCAAAGACGTTACCA</b>	AA00672 (3' primer)	0.608
5' AA00673-E/P	<b>CCATGGAATTGAGACAACAAGCAACGTTAG</b>	AA00673	0.263
3' AA00673-E/P	<b>TCTAGATAGTCCAATTCCTGCGGTAAA</b>	AA00673	0.263
5' TA transcript	GGCAATTTACGTACAGTCCCTCAGTTTTCA	AA00673	0.271
3' TA transcript	GGCGATATTTCCCGATTTCCTTTCTTCTT	AA00672	0.271

\* Bold sequences indicate restriction enzyme sites to facilitate cloning into the appropriate shuttle vectors.

pGEMT-Easy (Promega, Madison, WI) and transformed into *E. coli* DH5 $\alpha$ . The resulting pGEMTQseCcomp and pGEMTltx-pro plasmids were purified from *E. coli*. The plasmid pGEMTQseCcomp was cleaved by digesting with HindIII and BamHI and the pGEMTltx-pro plasmid was cleaved by digesting with KpnI and HindIII. Both digested fragments—*qseC* gene and *ltx* promoter—were then ligated into pYGK. The resulting plasmid, pYGKqseC, was transformed into *E. coli*. The plasmid was then purified from an overnight culture of *E. coli*, and then electroporated into the *qseC* mutant for complementation.

**Partial purification of *A. actinomycetemcomitans* AI-2.** An enriched fraction containing AI-2 from *A. actinomycetemcomitans* was produced as described by Sperandio *et al.* (209) for production of AI-2 from *E. coli*. Briefly, an overnight culture of *A. actinomycetemcomitans* cells were diluted 1:20 into fresh medium, cultured to mid-exponential phase (optical density, 0.3) at 37°C, and then harvested by centrifugation. AI-2 was obtained from a 7.2 ml aliquot of the conditioned medium. The culture supernatant was first filtered through a 0.22  $\mu$ m-pore-size filter and then through a Centricon YM-3 3 kDa exclusion filter (Millipore, Bedford, MA). The resulting filtrate was lyophilized, suspended in 1 ml of cold 5 mM sodium phosphate buffer, pH 6.2, and chromatographed on a C18 Sep-Pak reverse-phase column (Waters Company, Milford, MA) according to the manufacturer's instructions. AI-2 activity was assessed by the induction of bioluminescence of *Vibrio harveyi* BB170, and active fractions were lyophilized and stored at 4°C.

**RNA isolation.** Overnight cultures of the appropriate *A. actinomycetemcomitans* strains were diluted 1:20 into fresh BHI medium with or without partially purified AI-2 and incubated at 37°C until the mid-exponential growth phase (O.D. = 0.3) for RNA isolation. Total RNA was isolated from *A. actinomycetemcomitans* cells using the 5 Prime PerfectPure RNA Cell & Tissue kit (5 Prime Inc., Gaithersburg, MD) according to the manufacturer's instructions. To ensure that the samples were free of contaminating genomic DNA, the RNA preparation was digested with RQ RNase-free DNase I (Promega Corporation, Madison, WI). The concentration and purity of each RNA sample were measured via spectrophotometry (ND-1000 Spectrophotometer, NanoDrop Technologies, Inc, Wilmington, DE) and was also assessed by gel electrophoresis. Samples were checked for contamination of genomic DNA by real-time PCR using *A. actinomycetemcomitans* 5s rRNA primers (Table 2b). RNA samples were considered free of significant genomic DNA if no amplification product was detected by real-time PCR after at least 30 cycles of amplification. RNA that was not immediately utilized for a reverse-transcription reaction was aliquoted into 1.5 ml Eppendorf tubes and stored at -80°C until future use.

**cDNA synthesis and qPCR for *qseBC* gene expression.** First-strand cDNA was prepared by using SuperScript III Reverse Transcriptase (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions. Quantitative PCR (qPCR) was performed with the Smart Cycler system (Cepheid, Sunnyvale, CA) in a final reaction volume of 25 µl that contained 100 ng of cDNA, primers for *qseC* (5' QseB Induction and 3' QseB Induction; ~71 µM final concentration), and 1x FastStart SYBER Green Master (Roche,

Indianapolis, IN). The amplification conditions for qPCRs were as follows: denaturation at 95°C for 15 seconds, annealing at 55°C for 30 seconds, and elongation at 72°C for 30 seconds for 35 cycles. The threshold cycle for each qPCR was determined from a second derivative plot of total fluorescence as a function of cycle number by using the software package supplied with the Smart Cycler system. All gene-specific threshold values were normalized against threshold values from primers specific for the *A. actinomycetemcomitans* 5S rRNA gene (~60 µM final concentration). qPCR reactions were carried out in triplicate with consistent results. Each qPCR end-point amplification product was visualized by electrophoresis on 2% agarose gels.

**cDNA synthesis and qPCR analysis of iron-uptake genes.** qPCR was utilized to determine if *qseC* regulated the expression of genes involved in iron-uptake. RNA was isolated from *A. actinomycetemcomitans* strain 652, *qseC*- mutant, *lsrB-rbsB*- double-receptor mutant, JP2, *luxS*- mutant, and *luxS*- complemented strain (see 'RNA isolation' section in Chapter 2) and cDNA was synthesized by using the qScript cDNA supermix (Quanta Biosciences) according to the manufacturer's instructions. The Smart Cycler system (Cepheid) was used for qPCR with the final reaction volume of 40 µl that contained 100ng of cDNA, gene-specific primers (*afuA 00696* and *fecB 00795* ; 300 nM final concentration; Table 3b), and 1x PerfectCTa SYBR Green Fastmix (Quanta Biosciences). The amplification conditions for qPCR reactions were as follows: denaturation at 95°C for 15 seconds, annealing at 55°C for 30 seconds, and elongation at 72°C for 30 seconds for 35 cycles. The threshold cycle for each qPCR was determined from a second derivative plot of total fluorescence as a function of cycle number by using

the software package supplied with the Smart Cycler system. All gene-specific threshold values were normalized against threshold values from primers specific for the *A. actinomycetemcomitans* 5S rRNA gene (300 nM final concentration). qPCRs were carried out in triplicate with consistent results, and each qPCR end-point amplification product was visualized by electrophoresis on 2% agarose gels stained with ethidium bromide for 1 hour 15 minutes at 80 volts.

**Biofilm formation and analysis.** *A. actinomycetemcomitans* biofilms were grown on a saliva-coated coverglass in a polycarbonate flow chamber (model FC81; Biosurface Technologies Corp, Bozeman, MT; chamber dimensions are 50.5 mm by 12.7 mm by 2.54 mm) at a flow rate of 5.8 ml per hour at 25°C, essentially as described by Shao *et al* (196) Briefly, saliva was self-collected, filter-sterilized (pore size 0.22 µm), and incubated with the coverglass (60 mm by 24 mm) for 30 minutes at 37°C. The saliva-coated coverglass was then fixed into the flow chamber and washed with phosphate-buffered saline (PBS; 100 mM NaH<sub>2</sub>PO<sub>4</sub>, 150 mM NaCl) for 10 minutes at a flow rate of 60 ml per hour using a peristaltic pump (Manostat Sarah cassette; Fisher Scientific, Pittsburgh, PA). Overnight cultures of *A. actinomycetemcomitans* were resuspended in PBS at an optical density of 0.5 at 600 nm and inoculated for one hour into the polycarbonate flow chamber. Flow cells were then washed with PBS for 30 minutes at a flow rate of 5.8 ml per hour. Bound cells were fed BHI medium and allowed to grow for 60 hours at a flow rate of 5.8 ml per hour. The resulting biofilm was stained with 0.2 mg/ml fluorescein isothiocyanate (FITC; Sigma-Aldrich) for 1 hour in the dark and then washed with PBS for 2 hours.



Biofilms were visualized using Olympus Fluoview FV500 confocal scanning laser microscope (Olympus, Pittsburgh, PA) under 600x magnification using an argon laser. Confocal images were captured from 9 randomly chosen frames from each flow chamber. Biofilm depth was determined by performing z-plane scans from 0 to 100  $\mu\text{m}$  above the cover glass surface. The total biofilm biomass was determined by integrating fluorescence intensity across the z-stack using the Fluoview FV500 software provided by Olympus. Biofilm depth, total biomass, and biofilm topology were also quantified utilizing COMSTAT image-processing software package (78). Biomass data were analyzed using a pairwise *t* test (Graphpad Software, Inc.) and expressed as the mean  $\pm$  standard deviation calculated from all the frames obtained for a given biofilm. Experiments were carried out in triplicate.

***In vivo* mouse periodontitis model.** To assess *A. actinomycetemcomitans* virulence, a modification of the Baker mouse model of periodontitis was utilized (13). This model measures alveolar bone resorption, the clinical presentation of periodontitis in humans, as an outcome, and has been used previously to assess the virulence of *P. gingivalis*, another periodontal pathogen (168, 232). All animal procedures received Institutional Animal Care and Use Committee approval and were in accordance with Federal guidelines for the care and use of laboratory animals. Specific pathogen-free female Balb/cByJ mice (10 weeks old; Jackson Laboratory, Bar Harbor, ME) were orally infected with wild-type strain 652 or *lsrB*<sup>-</sup>, or *rbsB*<sup>-</sup>, or *lsrB*<sup>-</sup>*rbsB*<sup>-</sup> mutant strains; wild-type strain 652 or *qseC*<sup>-</sup>, or *qseC*<sup>-</sup> complemented strain; wild-type strain JP2, or *luxS*<sup>-</sup>, or *luxS*<sup>-</sup> complemented strains of *A. actinomycetemcomitans*. In some experiments, mice

were infected with wild-type 33277 or *luxS* mutant strains of *P. gingivalis*. Briefly, prior to infection all animals were treated with sulfamethoxazole (final concentration of 800 µg/ml; Sigma-Aldrich) and trimethoprim (final concentration of 400 µg/ml; Sigma-Aldrich) in their water bottles *ad libitum* for 10 days to suppress the normal flora of the mouse oral cavity. After the 10 day antibiotic period, the mice were given regular drinking water without antibiotics for 4 days and then were orally infected a total of five times at 2-day intervals with  $1 \times 10^9$  CFU/ml of bacteria resuspended in 2% carboxymethylcellulose (CMC; MP Biomedicals, Solon, OH) via a blunt-tipped syringe (Fisher Scientific, Pittsburgh, PA). Animals that served as the negative control group (sham-infected) received only 2% CMC without bacteria. Mice were euthanized with CO<sub>2</sub> inhalation and cervical dislocation at 47 days after the last infection (for a total experiment time of 70 days).

**Analysis of alveolar bone resorption.** After euthanization, the mice were decapitated. The mouse heads were boiled for 15 minutes under 15 lb/in<sup>2</sup> of pressure and, subsequently, defleshed. The skulls were then immersed in 3% hydrogen peroxide overnight at room temperature to remove any remaining musculature and washed with deionized water. To ensure the teeth were cleaned of any residual bacteria and/or tissues, skulls were sonicated at 4 volts in 1% bleach for 30 seconds, rinsed with water, and then gently brushed with toothpaste for 30 seconds. They were rinsed clean, swirled in Listerine for 30 seconds, and then once again sonicated at 4 volts in 1% bleach and rinsed clean with deionized water. The cleaned teeth were stained for 30 seconds with 1% Methylene Blue (Ricca Chemical Company, Arlington, TX) and rinsed with deionized

water to remove excess dye. Skulls were allowed to air-dry prior to measurement of the alveolar bone.

To measure loss of alveolar bone, the distance from the Cemento-Enamel Junction (CEJ) to the Alveolar Bone Crest (ABC) was measured at 7 sites on the buccal side of the right and left maxillary molars (for a total of 14 measurements per animal), utilizing a dissecting microscope fitted with a video image marker measurement system (model VIA-170K; Fryer, Huntley, IL) standardized to give measurements in millimeters. Alveolar bone loss was calculated by subtracting the 14-site mean sum total CEJ:ABC distance of each experimental group from the mean sum total of the CEJ:ABC distance of the sham-infected group. Results were expressed as millimeter change in bone, and negative values indicate bone loss compared with sham-infected controls.

#### **Purification of RNA by centrifugation in cesium chloride gradients.**

Overnight cultures of the appropriate *A. actinomycetemcomitans* strains were diluted 1:10 into fresh BHI medium incubated at 37°C until the mid-exponential growth phase ( $OD_{600nm} = 0.3$ ) for RNA isolation. Total RNA was isolated from 200 ml of *A. actinomycetemcomitans* cells using the cesium chloride density gradient centrifugation method similar to Reddy *et al* (174). Initially, cultures were put on ice to stop cellular activities, and 1/20 volume of Buffer A (200 mM Tris-HCl (Sigma-Aldrich) (pH 8.0) , 20 mM EDTA (Sigma-Aldrich), 20 mM sodium azide (Sigma-Aldrich), 20 mM aurintricarboxylic acid (Sigma-Aldrich) was added. The cultures were then centrifuged at 8,000 x g for 15 minutes. The resulting cell pellet was resuspended in 5 ml of STET buffer (50 mM Tris-HCl (pH 7.0), 8% sucrose (Sigma-Aldrich), 5% Triton X-100

(Sigma-Aldrich), 5 mM EDTA). Cell suspensions were extracted with equal volumes of phenol and chloroform, and nucleic acids were precipitated with 1/10 volume of sodium acetate and two volumes of ethanol. The nucleic acid pellet was resuspended in 2 ml of diethyl pyrocarbonate (DEPC)-treated water, and extracted and precipitated twice more as described above. The resulting nucleic acid pellet was resuspended in 2 ml of DEPC-treated water containing 1 g of cesium chloride (RPI, Mount Prospect, IL), layered onto a 2.5 ml cushion of 5.7 M cesium chloride-5 mM EDTA, and centrifuged at 105,000 x g for 18 hours. The RNA pellets were then suspended in 1 ml of DEPC-treated water, precipitated with ethanol, and finally suspended in 1 ml of nuclease-free water (EMD chemicals, Gibbstown, NJ). The concentration and purity of each RNA sample were measured via spectrophotometry (ND-1000 Spectrophotometer) and was also assessed by gel electrophoresis. RNA samples that had contaminating DNA were digested with RQ RNase-free DNase I (Promega). RNA samples were considered free of significant genomic DNA if no amplification product was detected by real-time PCR after at least 30 cycles of amplification using *A. actinomycetemcomitans* 5s rRNA primers. RNA that was not immediately utilized for a reverse-transcription reaction was aliquoted into 1.5 ml eppendorf tubes and stored at -80°C until future use.

**Determination of ORFs Aa00672-Aa00673 operon structure.** In order to determine if AA00672 and AA00673 were co-transcribed in the same operon, cDNA was synthesized by using qScript cDNA supermix (Quanta Biosciences, Gaithersburg, MD) and RNA previously isolated from *A. actinomycetemcomitans* strain 652 (see 'RNA isolation' section in Chapter 2). A reverse-transcription PCR (RT-PCR) reaction was then

set-up as follows: 100ng of cDNA, 1x Platinum PCR supermix (Sigma-Aldrich), primers designed to span both AA00673 and AA00672 (TA-transcript primers—see Table 3b). The amplification conditions for the RT-PCR reaction was as follows: denaturation at 95°C for 30 seconds, annealing at 55°C for 30 seconds, and elongation at 72°C for 30 seconds for 30 cycles. Resulting PCR products were resolved on a 2% agarose gel stained with ethidium bromide for 1 hour 15 minutes at 80 volts.

**Regulation of ORFs Aa00672-Aa00673 by AI-2.** qPCR was utilized to determine if AI-2 regulated the expression of AA00672-AA00673. RNA was isolated from *A. actinomycetemcomitans* strain 652, *qseC* mutant, and *lsrB**rbsB* double-receptor mutant (see ‘RNA isolation’ section in Chapter 2) and cDNA was synthesized by using the qScript cDNA supermix (Quanta Biosciences) according to the manufacturer’s instructions. The cDNA was amplified using the Smart Cycler system (Cepheid) in a final reaction volume of 40µl that contained 100ng of cDNA, gene-specific primers (TA-transcript primers; 300 nM final concentration), and 1x PerfecCTa SYBR Green Fastmix (Quanta Biosciences). The amplification conditions for qPCR reactions were as follows: denaturation at 95°C for 15 seconds, annealing at 55°C for 30 seconds, and elongation at 72°C for 30 seconds for 35 cycles. The threshold cycle for each real-time PCR was determined from a second derivative plot of total fluorescence as a function of cycle number by using the software package supplied with the Smart Cycler system. All gene-specific threshold values were normalized against threshold values from primers specific for the *A. actinomycetemcomitans* 5S rRNA gene (300 nM final concentration). qPCRs were carried out in triplicate with consistent results. Each qPCR end-point amplification

product was visualized by electrophoresis on 2% agarose gels stained with ethidium bromide for 1 hour 15 minutes at 80 volts.

**Construction of Aa00673 and Aa00672-Aa00673 IPTG-inducible vectors.** To construct the fragment that would be cloned into the inducible vector, the entire AA00673 gene was amplified using genomic DNA of strain 652 as the template with primers 5' AA00673-TAA and 3' AA00672-TAA (Table 3). The following PCR program was used: 95°C for 10 min for 1 cycle, and then 95°C for 30s, 55°C for 30 seconds, and 72°C for 30 seconds for 35 cycles. The PCR products were then ligated with pGEMT-Easy (Promega, Madison, WI) overnight at 4°C and then transformed into *E. coli* DH5 $\alpha$ . The resulting pGEMT-AA00673 plasmid was purified from *E. coli*, cleaved by digesting with *KpnI/NcoI* and ligated into the IPTG-inducible pET30a vector overnight at 4°C and then transformed into *E. coli* DH5 $\alpha$ . Potential clones were confirmed by purifying and digesting the resulting plasmid, pET30a-AA00673, with *KpnI/NcoI*. Once the pET30a-AA00673 construct was confirmed to be correct, the plasmid was transformed into *E. coli* BL21. Recombinant clones were confirmed by PCR using primers 5' AA00673-TAA and 3' AA00673-TAA and also by purifying the plasmid from BL21 clones and digesting with *KpnI/NcoI*. Clones which carried the correct construct were stored at -80°C for future use.

The construct for AA00673-AA00672 was made in the same way, except the entire region encoding AA00673 thru AA00672 (see Table 3 for sequences) was amplified from the genomic DNA of *A. actinomycetemcomitans* strain 652 by using the following PCR program: 95°C for 10 min for 1 cycle, and then 95°C for 30s, 55°C for 30

seconds, and 72°C for 1 minute for 35 cycles. The construct was then made following the same procedure as the pET30a-AA00673 construct.

**Growth curve of after induction of Aa00673 or Aa00672-Aa00673.** Overnight cultures of *E. coli* BL21 containing the IPTG-inducible pET30a vector with the toxin (pET40a-AA00673) or the pET30a vector with the TA system (pET30a-AA00673-AA00672) were inoculated (1:10 dilution) into fresh LB medium. Once the OD<sub>600nm</sub> reached 0.4, the cultures were induced by adding 1 mM IPTG. Control cultures were not induced. The optical density was taken every 30 minutes after induction to determine the growth. Growth curves were completed in triplicates. Results are mean ± standard deviations.

**Cell viability after removal of inducer.** In the assay where cell viability was determined, two hours after the cultures were induced, IPTG was removed by washing three times with LB medium in the same volume as the culture. Cultures were then resuspended in LB medium (volume equal to that before washing) and incubated at 37°C. Growth was measured every 30 minutes after removal of IPTG. At certain time points during the growth curve, aliquots were taken and appropriate dilutions were plates on LBkan plates. Plates were incubated at 37°C overnight. Colonies were counted after 24 hours, and the number of Colony Forming Units per ml (CFU/ml) were calculated. Results are mean ± standard deviations.

**Construction of vector with Aa00673 for protein production.** The toxin-expressing plasmid was constructed as follows. The primers (5' AA00673-E/P and 3'

AA00673-E/P) used to amplify the toxin (AA00673) gene were designed with restriction enzymes (NcoI and XbaI, respectively) engineered into the 5' end to allow for easier cloning into the expression vector. The entire AA00673 gene was amplified using genomic DNA from *A. actinomycetemcomitans* strain 652 as the template. The following PCR program was used: 95°C for 10 min for 1 cycle, and then 95°C for 30s, 55°C for 30 seconds, and 72°C for 30 seconds for 35 cycles. The purified PCR product was then ligated into pGEMT-Easy (Promega) overnight at 4°C and then transformed into *E. coli* DH5 $\alpha$ . The resulting pGEMT-AA00673-E/P plasmid and pBAD/gIII A was cleaved by digesting with *NcoI/XbaI*; fragments were purified by gel electrophoresis on a 1% gel and ligated with T4 ligase overnight at 4°C. After transformation into *E. coli* DH5 $\alpha$ , plasmid was purified from potential clones and digested with *NcoI/XbaI* to digest the validity of the construct. The correct plasmid, pBADgIII/A-AA00673-E/P, was then were transformed into chemically-competent *E. coli* TOP10. Recombinant clones were confirmed by PCR using primers 5' AA00673-E/P and 3' AA00673-E/P and also by purifying the plasmid from TOP10 clones and digesting with *NcoI/XbaI*. Clones which carried the correct construct were stored at -80°C for future use.

**Expression and purification of toxin protein.** An overnight culture of *E. coli* TOP10 containing the pBAD/gIII A-AA00673-E/P construct was inoculated (1:20 dilution) into fresh LB medium. The culture were incubated at 37°C in a shaker and was induced with 0.2% arabinose once the OD<sub>600nm</sub> reached 0.8. Growth was continued for another 4 hours, after which time the cells were harvested, centrifuged for 15 minutes at 8,000 x g, and placed at -20°C overnight. The cell pellet was thawed on ice, resuspended



in 10 mM Tris (pH 8.0) containing 10 mg/ml of lysozyme, sonicated three times on ice, and centrifuged at 10,000 x g to remove cellular debris. The supernatant was collected into a clean 50 ml tube. The toxin was purified from the resulting supernatant by affinity chromatography over a copper resin (HisTrap resin; GE Healthcare, Piscataway, NJ). The column was first washed with 25ml of binding buffer (0.02 M sodium phosphate, 0.5 M NaCl, 20 mM imidazole, pH 7.2). The protein extract was then loaded, and the column was subsequently washed with 25 ml of binding buffer and 10ml of 100 mM imidazole elution buffer (0.02 M sodium phosphate, 0.5 M NaCl, 100 mM imidazole, pH 7.2). Bound toxin was eluted with a 10 ml of 500 mM imidazole elution buffer (0.02 M sodium phosphate, 0.5 M NaCl, 500 mM imidazole, pH 7.2). Eluted protein was collected into a clean 15 ml tube. Eluted protein was dialyzed three times against 25 mM phosphate buffer (1.25 mM Na<sub>2</sub>HPO<sub>4</sub>; 1.25 mM NaH<sub>2</sub>PO<sub>4</sub>) with 50 mM NaCl: twice for 4 hours at 4°C and once overnight at 4°C. An aliquot of the dialyzed protein was used to determine protein concentration using the BCA Protein assay (Pierce, Rockford, IL). Purity of the dialyzed protein was assessed by Coomassie blue staining after polyacrylamide gel electrophoresis on a 4-12% Bis-Tris NuPAGE gradient gel (Invitrogen). Confirmation of the correct protein construct with His-Tag was confirmed by a western blot. Murine monoclonal anti-polyHistidine antibody (Sigma-Aldrich), which recognizes the His-Tag, was used as the primary antibody, and Goat anti-mouse IgG conjugated with horseradish Peroxidase (Fisher), which recognizes the mouse primary antibody, was used as the secondary antibody. Diaminobenzidine (Sigma-Aldrich) was used to develop the blot and visualize the antibody-protein complex. Dialyzed protein that was not immediately utilized was lyophilized to dryness.

**Sequencing of *ygiX* and operon structure.** The constructs containing *ygiX* (*qseB*) and *qseC* used for sequencing were constructed as follows. Primers were designed (Table 3) to amplify the region from *ygiX* through *qseC*, the *qseB* gene, the *qseC* gene, and the *qseC* gene amino acids 130-230. Each fragment was amplified from the genome of *A. actinomycetemcomitans* strain 652 using the following PCR program: 95°C for 10 min for 1 cycle, and then 95°C for 30s, 55°C for 30 seconds, and 72°C for 1.5 minutes for 35 cycles. The purified PCR product was then ligated into pGEMT-Easy (Promega) overnight at 4°C and then transformed into *E. coli* DH5 $\alpha$ . The resulting plasmids were purified from *E. coli* and sent to the DNA sequencing core facility at the University of Louisville to be sequenced with T7 and SP6 primers. The operon structure was elucidated by digesting out sequence of the pGEM-T easy vector and assembling the sequence of the *ygiX*-*qseC* region that was returned from the DNA core facility. Sequences were also referenced against the genome of *A. actinomycetemcomitans* strain D11S available from the National Center for Biotechnology Information (NCBI) website (<http://www.ncbi.nlm.nih.gov/gene?Db=genome&Cmd=ShowDetailView&TermToSearch=25327&GeneSearch>).

**RNase activity of AA00673.** In order to determine the RNase activity of AA00673, a Transcript RNA marker (a mixture of 7 defined RNA transcripts, ranging from 0.1 – 1 kb; Sigma-Aldrich) was used. The RNA marker was incubated at 70°C for 5 minutes to denature any secondary structure that may have formed. The RNA marker was then incubated with increasing amounts of affinity purified His-AA00673 (dialyzed

protein but not lyphophilized) in 10 mM Tris-HCl (pH 8.0) containing 1 mM dithiothreitol at 37°C for 15 minutes. The reactions were separated on a 1.2% agarose gel stained with ethidium bromide for 1 hour.

**Statistical Analysis.** Data was evaluated by ANOVA and the Dunn multiple-comparison test using the InStat program (Graphpad). Two-tailed *t* tests were also performed where appropriate (comparison of two groups only). Statistical differences were considered significant at the level of  $p < 0.05$ .

## **CHAPTER THREE: AUTOINDUCER-2 AND QSEC CONTROL BIOFILM AND *IN VIVO* VIRULENCE IN *AGGREGATIBACTER ACTINOMYCETEMCOMITANS***

### **Introduction**

Dental plaque is a complex and dynamic microbial community that forms as a biofilm on the surfaces of teeth and oral tissues (110, 118, 164, 237). It is comprised of over 700 species of bacteria (1, 110, 116, 118, 164, 237) and is the prime etiological agent of three common human oral diseases: dental caries, gingivitis, and periodontal disease (131, 132, 205). Major shifts in microbial populations within the oral biofilm have been associated with the progression of disease, as diseased sites often have increased populations of pathogenic species relative to healthy sites in the oral cavity (131-133). The host and/or microbial signals that contribute to the populational shifts associated with disease are still unknown. However, the oral cavity is subject to continual environmental flux, including changes in pH, temperature, osmolarity and nutrient supply, and it is possible that these stresses contribute in part to microbial population shifts in the biofilm. It is clear that oral bacteria rapidly detect environmental fluctuations and respond appropriately, allowing them to successfully coexist and thrive in the oral cavity (2, 87, 133).

Both intra- and interspecies communication is known to occur between bacteria, and these signaling processes potentially enable the organisms to coordinate their behavior and function by regulating gene expression as a community. One mechanism of communication, termed quorum sensing, is a cell density-dependent response (116, 140, 190, 253), which in Gram-negative bacteria is mediated by the production, release, and detection of soluble signal molecules called autoinducers. A variety of chemical species function as autoinducers, including acylated homoserine lactones (74, 162), quinolone derivatives (166), and furan derivatives (e.g. autoinducer-2 [AI-2]) (140, 190, 236, 240). As a population of bacteria expands, the external concentration of the autoinducer increases until a threshold is attained, at which point a signal transduction cascade is initiated that alters gene expression and behavior of the microbial community. Quorum sensing has been shown to control cell-density dependent behaviors such as the expression of virulence factors, biofilm formation, and iron acquisition in a variety of organisms (14, 74, 237, 238). Thus, it has been suggested that quorum sensing may allow bacteria in a biofilm to react coordinately as a multi-cellular organism to changes in the external environment.

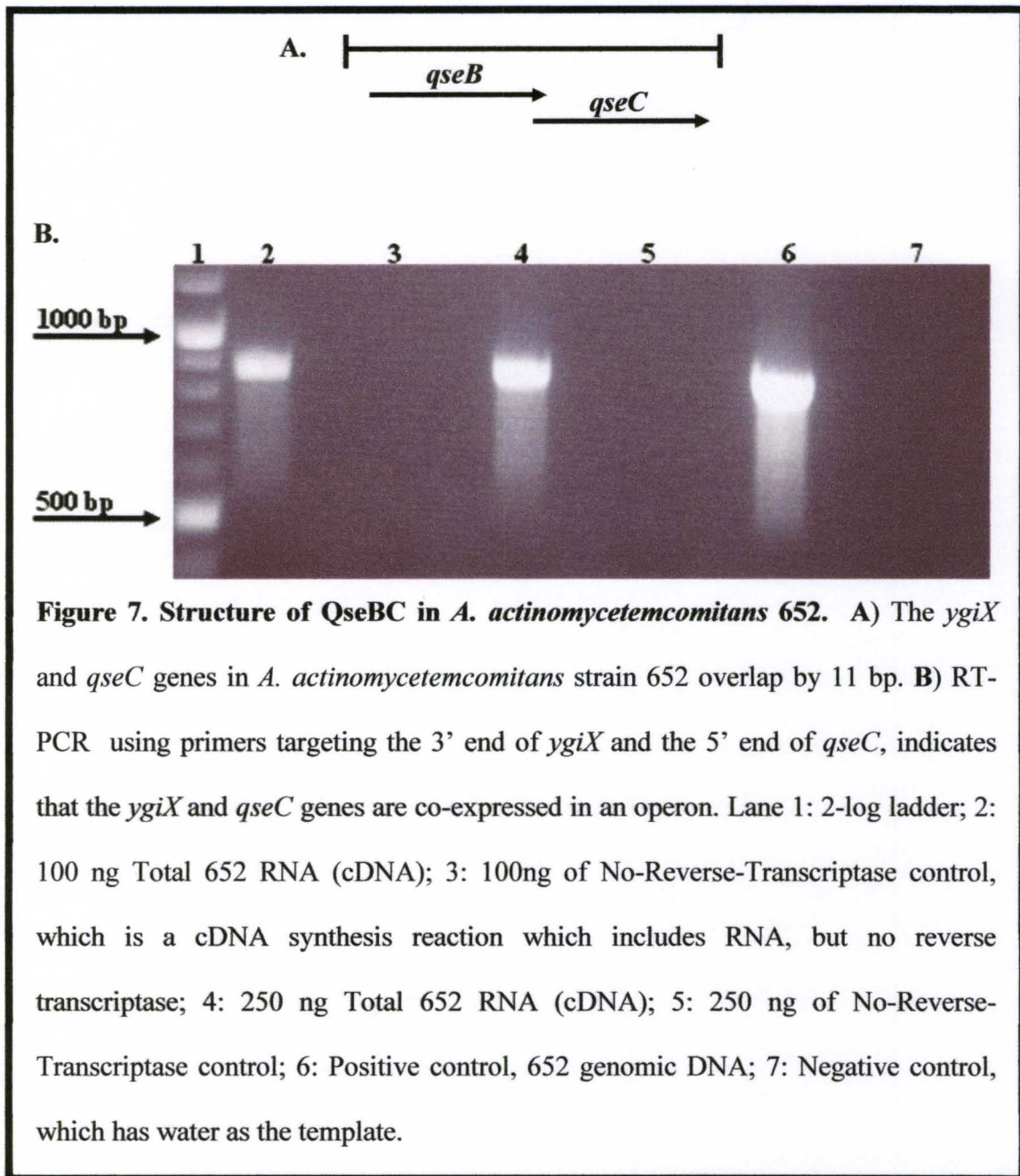
The dental pathogen *Aggregatibacter actinomycetemcomitans*, a Gram-negative organism associated with aggressive forms of periodontitis and other systemic infections (20, 159, 203, 250), possesses an AI-2-dependent quorum sensing system (54). AI-2 produced by *A. actinomycetemcomitans* regulates expression of virulence factors, biofilm formation, and iron uptake and also influences the planktonic growth of the organism under iron limitation (55, 188, 196). *A. actinomycetemcomitans* possesses two periplasmic AI-2 receptors, LsrB and RbsB, both of which are linked to ABC transporters

(88, 195), suggesting that *A. actinomycetemcomitans* may import AI-2. However, exactly how the detection and/or importation of AI-2 is linked to downstream gene regulation remains to be determined. Previous microarray experiments identified the QseBC two-component system as being regulated by LuxS in *A. actinomycetemcomitans* (Demuth, D.R., unpublished). In *E. coli*, the QseBC two-component signal transduction system has been suggested to regulate biofilm formation (62), and *A. actinomycetemcomitans* contains an operon that displays 70-80% sequence similarity to QseBC of *E. coli*. QseBC is also similar to the *feuPQ* two-component system that regulates iron uptake in *Rhizobium leguminosarum*, *Sinorhizobium meliloti*, and *Brucella suis* (46), and iron acquisition is known to be regulated by AI-2 (55) and dramatically influences biofilm formation (177, 178). In this study, we show that *qseBC* is part of the AI-2 regulon, and its induction requires a functional AI-2 receptor. The *qseBC* operon also contributes to biofilm formation and virulence of *A. actinomycetemcomitans*, since inactivation of *qseC* results in reduced biofilm growth and attenuates virulence *in vivo*. These results suggest that QseBC may couple the detection and importation of AI-2 to the downstream regulation of gene expression that controls these processes.

## **Results**

### **AI-2 is required for induction of QseBC in *A. actinomycetemcomitans*:**

In *E. coli*, the QseBC two-component system is induced by AI-2, and furthermore, is part of the AI-2-dependent response circuit that controls biofilm growth (62). The genome of *A. actinomycetemcomitans* encodes a two-component system that is related to QseBC in *E. coli*. These genes are listed as *ygiX* and *qseC* and are annotated as



a sensor histidine kinase (*qseC*) and response regulator (*ygiX*) of a two component signal transduction system ([www.oralgen.lanl.gov](http://www.oralgen.lanl.gov)). In strain 652, the *ygiX* and *qseC* genes overlap by 11bp (Figure 7A) and RT-PCR indicates that they are co-expressed in an

**A.**  
*A. Actinomycescomitans qseB* →  
*E. coli* O157:H7 EDL933 *qseB* →

```

78  LTARDTLDERITGLQRGADDYLCKPFALAEVVARLQALIRRRYGHANPIIQHSRVKFDPN
    LTARD L+ER+ GL+ GADDYLCKPFAL EV ARL+AL+RR G A+ ++H V DP
    LTARDALEERVXGLRLGADDYLCKPFALIEVAARLEALMRRTWGQASNELRHGNVMDPG 137

    QRKVFCDQEIALTTREYKLELFPMLNKEVLRTRDFIEDKLYNWDNEVSSNALEVHIYML
    +R L + + L +E+ LLEL M N RVL R IE+KLY WD EV+SNA+EVH++L
    138 KRIATLAGEPLTLKPKFALLELLMRNAGRVLPRKLEEKLYTWDEEVTSNAVEVHVHML 197

    RQKLGKQFIRTVHGVGYALGK
    R+KLG FIRTVHG+GY LG+
    198 RRKLGSDFIRTVHGIYTLGE 218
  
```

**B.**  
*A. Actinomycescomitans qseC* →  
*E. coli* O157:H7 EDL933 *qseC* →

```

223  LNQPFDRSTSSMLRERRFTSDAAHELRSPLAALRIQTEVAQIAGDDANTREQALDNLTOG
    LNQ F RT +M++PERRFTSDAAHELRSPL AL++QTEVAQ++ DD R++AL L G
    LNQLEARTHAMMVRERRFTSDAAHELRSPLTALKVQTEVAQLSDDDPQARKKALLOLHSG 282

    IDRATQLIEQLLTLRSLDSLQELDNLQEIHWQIVPSLIGDLYPFAEKRDITLPEEIAV
    IDRAT+L+QLLTLRSLDSL L ++ EI E ++ S + D+Y A++ I +
    283 IDRATRLVDQLLTLRSLDSLQELDNLQVAEIPLEDLLQSSVMDIYHTAQQAKIDVRLTLNVQ 342

    PAVKLGQPLLSLMLRNLIDNAIRYCPQGSRIKVRLEKSKIAVEDNGGGVDDADLAKLGO
    + GQPLLSL++RNL+DNA+RY PQGS + V L V DNG GV LA++G+
    343 GIKRTGQPLLSLMLRNLIDNAVRYSPQGSVVDVTLNADNFIVRDNGPGVTPPEARLIGE 402

    RFYRPAQNEKSGGLSIVHRIAELHXYKVRKKN
    RFYRP GQ GSGGLSIV RIA+LH V N
    403 RFYRPPGQTATGSGGLSIVQRIAKLHGMNVEFGN 437
  
```

**Figure 8. Sequence identity of QseB and QseC.** **A)** The amino acid deductions of *A. actinomycescomitans* strain 652 *ygiX* gene display significant sequence identity with *qseB* from *E. coli* O157:H7 as analyzed by blastx. Shown is the 5' end of the *ygiX* sequence in *A. actinomycescomitans*, which exhibits sequence identity (82/141 (58%)) in *E. coli* from amino acid 78 to 218. Sequences from 3' end of *ygiX* show same levels of sequence identity to *E. coli* O157:H7. **B)** The amino acid deductions of *A. actinomycescomitans* strain 652 *qseC* gene display significant sequence identity (119/208 (57%)) with *qseC* from *E. coli* O157:H7 as analyzed by blastx. Shown is the 3' end of the *qseC* sequence in *A. actinomycescomitans*, which exhibits sequence identity in *E. coli* from amino acid 223 to 437. Sequences from 5' end of *qseC* show the same levels of sequence identity to *E. coli* O157:H7.



operon (Figure 7B). The deduced amino acid sequences of *ygiX* and *qseC* from strain 652 exhibit significant sequence identity with QseB and QseC from *E. coli* (Figure 8A and Figure 8B, respectively).

To determine if AI-2 regulates the putative QseBC two-component system in *A. actinomycetemcomitans*, the expression of *qseC* was examined using real-time PCR with RNA isolated from overnight cultures of the *luxS* mutant grown in medium alone or in medium supplemented with partially purified AI-2. As shown in Table 3, the presence of AI-2 in the growth medium resulted in a 14.5-fold induction of *qseC*. As a control, cultures were also grown in medium supplemented with conditioned culture fluid obtained from the *luxS* strain that was subjected to the same purification scheme used to obtain the AI-2 samples. No increase in *qseC* expression was observed under these

<b>Strain</b>	<b>delta (T)<sup>1</sup></b>	<b>delta (delta T)</b>	<b>Fold Induction</b>
<b>JP2-12</b>	-3.1 ± 0.6	0.11 ± 0.04	1.0
<b>JP2-12 + AI-2</b>	-0.66 ± 0.2	1.52 ± 0.3	14.5 ± 4.3
<b>JP2-12 + mock<sup>2</sup></b>	3.6 ± 0.05	0.08 ± 0.01	0.8 ± 0.3
<b>lsrB<sup>-</sup>rbsB<sup>-3</sup></b>	3.2 ± 0.35	0.09 ± 0.13	0.95 ± 0.2

<sup>1</sup> Delta T was calculated by subtracting the cycle threshold determined for the *qseC* reaction from the Ct of the 5S rRNA control.

<sup>2</sup> Mock control represents culture fluid from the *luxS* strain subjected to the purification scheme used to partially purify AI-2.

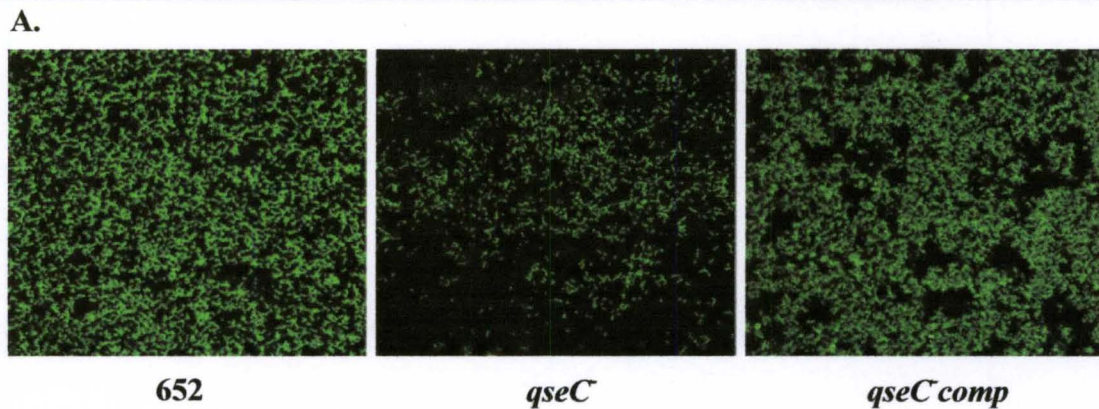
<sup>3</sup> This strain contains a functional copy of *luxS* and produces but cannot respond to AI-2.

conditions. In addition, the induction of *qseC* did not occur in the *lsrB<sup>-</sup>rbsB<sup>-</sup>* strain of *A.*

*actinomycetemcomitans*, which lacks the AI-2 receptors but which can still produce AI-2. This suggests that the QseBC two-component system in *A. actinomycetemcomitans* is regulated by AI-2 and may be linked to the detection and/or importation of AI-2 by the AI-2 receptors.

**Inactivation of *qseC* influences biofilm formation in *A. actinomycetemcomitans*:**

Previous studies demonstrated that biofilm formation by *A. actinomycetemcomitans* was dependent on AI-2-mediated quorum sensing (196), but how the initial detection of the AI-2 signal leads to downstream regulation of gene expression that influences biofilm growth was not determined. To determine if QseBC plays a role in regulating biofilm growth, wild-type, *qseC*<sup>-</sup>, and *qseC*<sup>-</sup> complemented strains of *A. actinomycetemcomitans* were cultured in flow cells as described in Chapter 2. Representative images of the biofilms formed by each of these strains are shown in Figure 9A and illustrate that inactivation of *qseC*<sup>-</sup> resulted in a significant reduction in biofilm growth. Complementation of the mutant with a plasmid-borne copy of *qseC* restored biofilm growth to wild-type levels. Analysis of the biofilms using COMSTAT is shown in Figure 9B. The total biomass ( $p < 0.03$ ), average depth ( $p < 0.02$ ), and maximum depth ( $p < 0.004$ ) of biofilms formed by the *qseC*<sup>-</sup> mutant were significantly reduced relative to wild-type (see Figure 9B). All biofilm growth parameters were restored to wild-type levels in the complemented strain. Indeed, biomass and average biofilm depth for the complemented strain were greater than wild-type, which may reflect gene dosage as *qseC* is expressed from a multi-copy plasmid in the complemented



**B.**

Strain	Biomass ( $\mu\text{m}^3/\mu\text{m}^2$ )	Average biofilm depth ( $\mu\text{m}$ )	Maximum biofilm depth ( $\mu\text{m}$ )
652	$3.86 \pm .83$	$5.77 \pm 0.92$	$37 \pm 1.41$
<i>qseC</i>	$0.32 \pm 0.057$ <sup>1</sup>	$0.4 \pm 0.16$ <sup>3</sup>	$14 \pm 1.41$ <sup>5</sup>
<i>qseC comp</i>	$8.61 \pm 0.23$ <sup>2</sup>	$13.35 \pm 0.72$ <sup>4</sup>	$38.2 \pm 0.71$

Biomass was determined using the COMSTAT package of biofilm analysis software.

<sup>1</sup> p < 0.0265    <sup>2</sup> p < 0.0160    <sup>3</sup> p < 0.0147    <sup>4</sup> p < 0.0116    <sup>5</sup> p < 0.0038

**Figure 9. *qseC* is necessary for biofilm growth in *A. actinomycetemcomitans*.** A) Representative confocal images (in the x/y plane) of 60-hour biofilms formed by wild-type *A. actinomycetemcomitans* (652), *qseC*, and *qseC* complemented strains. The resulting biofilms were quantified using the COMSTAT program. B) Values for microbial biomass, average biofilm depth, and maximum biofilm depth were determined using COMSTAT image-processing software as described in the Materials and Methods. At least nine individual microscopic frames were analyzed for each biofilm experiment, and three independent biofilm experiments were carried out for each strain.

**Table 4a. Expression of the periplasmic iron-compound-binding protein *afuA 00696* in the *qseC* mutant.**

Strain	delta T <sup>1</sup>	delta (delta T)	Fold Change
652	1.34 + 0.44	0.4 + 0.13	1.0
<i>qseC</i>	-0.15 + 0.2	1.11 + 0.15	2.8 + 0.79
<i>qseC</i> comp	2.50 + 0.31	0.18 + 0.04	0.44 + 0.14

<sup>1</sup> delta T was calculated by subtracting the cycle threshold (Ct) determined for the 00696 reaction from the Ct of the 5S rRNA control.

**Table 4b. Expression of the periplasmic iron-compound-binding protein *afuA 00696* in the *luxS* mutant.**

Strain	delta T <sup>1</sup>	delta (delta T)	Fold Change
652	13.14 + 0.08	0.0001 + 6.3E-06	1.0
<i>luxS</i>	9.45 + 0.14	0.001 + 0.00001	12.83 + 1.12
<i>luxS</i> comp	14.5 + 0.17	4.3E-05 + 5.2E-06	0.40 + 0.07

<sup>1</sup> delta T was calculated by subtracting the cycle threshold (Ct) determined for the *afuA 00696* reaction from the Ct of the 5S rRNA control.

**Table 4c. Expression of the putative periplasmic iron-dictrate-binding protein *fecB 00795* in the *qseC* mutant.**

Strain	delta T <sup>1</sup>	delta (delta T)	Fold Change
652	6.64 + 0.08	0.01 + 0.0005	1.0
<i>qseC</i>	4.97 + 0.06	0.03 + 0.0014	3.18 + 0.31
<i>qseC</i> comp	7.42 + 0.05	0.01 + 0.0001	0.58 + 0.01

<sup>1</sup> delta T was calculated by subtracting the cycle threshold (Ct) determined for the *fecB 00795* reaction from the Ct of the 5S rRNA control.

organism. These results suggest that QseC controls biofilm formation by *A. actinomycetemcomitans*.

**Inactivation of *qseC* influences iron acquisition genes in *A. actinomycetemcomitans*:**

Fong *et al* has previously shown that AI-2 regulates the planktonic growth of *A. actinomycetemcomitans* under iron-limitation (54, 55). It was also shown that expression of genes encoding putative *A. actinomycetemcomitans* receptors for heme, transferrin, and hemoglobin was down-regulated while genes for an ABC-type transporter, ferric citrate operon, and another operon encoding a putative enterobactin receptor were up-regulated (55).

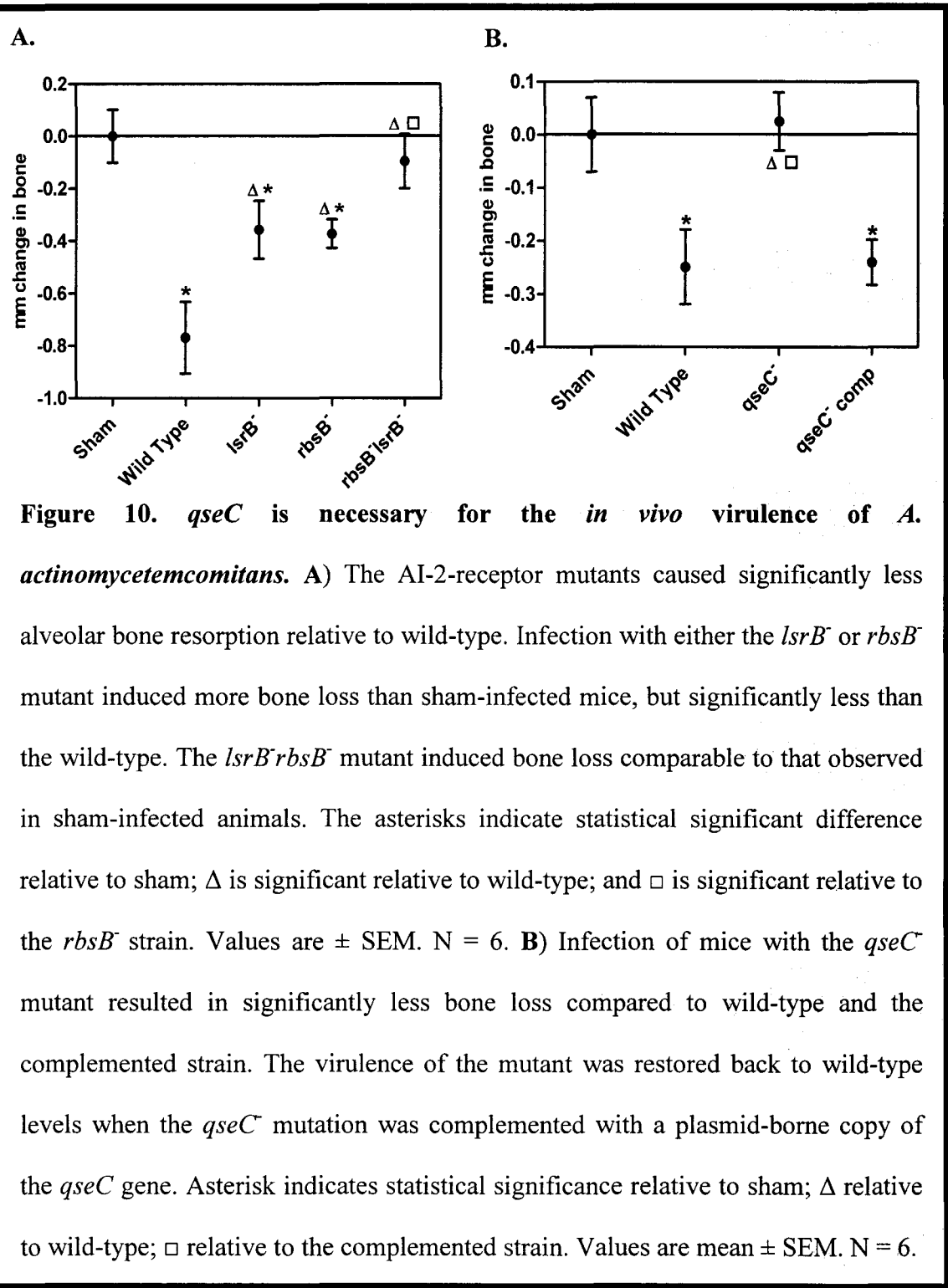
Although AI-2 appears to regulate iron uptake in *A. actinomycetemcomitans*, the mechanism of AI-2-mediated transcriptional regulation of iron acquisition genes has not yet been established. One potential component that may be involved in the regulation of iron-uptake genes in *A. actinomycetemcomitans* is the QseBC two-component system. QseBC shares sequence similarity with FeuPQ two-component system, which is known to regulate iron uptake in other bacteria, such as *Sinorhizobium* and *Brucella suis* (46). To determine if QseBC functions in the transcriptional regulation of iron acquisition genes in *A. actinomycetemcomitans*, the expression of iron-uptake genes in wild-type and QseC-deficient strains were compared by using real-time PCR. Table 4a illustrates that the expression of a putative periplasmic iron-compound-binding protein (*afuA 00696*) is up-regulated 2.8 fold in the *qseC* mutant and is up-regulated 12.8-fold in the *luxS*- mutant (Table 4b). Expression of *fecB 00795*, a putative ferric citrate transport protein, is also

up-regulated in a QseC-deficient background (Table 4c). Furthermore, comparison of the expression of iron-uptake genes in wild-type and the *qseC* mutant indicated that some, but not all *luxS*-regulated iron-uptake genes are regulated by QseBC (Table 5), suggesting that the *luxS* and *qseBC* regulons may overlap. Thus, *qseBC* may regulate a collection of the AI-2-regulated iron-acquisition genes in *A. actinomycetemcomitans*.

<b>Strain</b>	<b><i>fecB</i> 2380</b>	<b><i>afeA</i> 2549</b>	<b><i>afuA</i> 1048</b>	<b><i>hasR</i> 2782</b>	<b><i>pfeA</i> 2151</b>
652	1.0	1.0	1.0	1.0	1.0
<i>qseC</i>	0.50 + 0.63	0.84 + 0.07	0.51 + 0.09	0.40 + 0.04	0.31 + 0.08
<i>qseC</i> comp	0.07 + 0.17	0.01 + 0.003	0.03 + 0.01	0.26 + 0.04	0.29 + 0.003

**The *A. actinomycetemcomitans* *qseC* mutant and AI-2-receptor mutants are less virulent in a murine model of periodontitis.**

The ability of *A. actinomycetemcomitans* to acquire iron and grow in a biofilm is essential for its survival in the oral cavity and is associated with virulence. Our previous studies showed that AI-2-dependent quorum sensing (196) and *qseC* (described above) are essential components that regulate biofilm formation. To determine if AI-2 and *qseC* influence *A. actinomycetemcomitans* virulence, we utilized a murine model of periodontitis (13) in which mice were orally infected with  $1 \times 10^9$  CFU/ml of the wild-type strain, or strains lacking either QseC or the AI-2 receptors. As a control, sham-infected mice were infected only with CMC, the vehicle used to deliver the bacterial suspensions. Alveolar bone loss, one of the clinical outcomes of periodontal disease in humans, was then measured forty-seven days after the last infection. As shown in Figure 10A, mice that were infected with the wild-type ( $p = 0.0002$ ), *lsrB*<sup>-</sup> ( $p = 0.0261$ ), or *rbsB*<sup>-</sup> ( $p =$

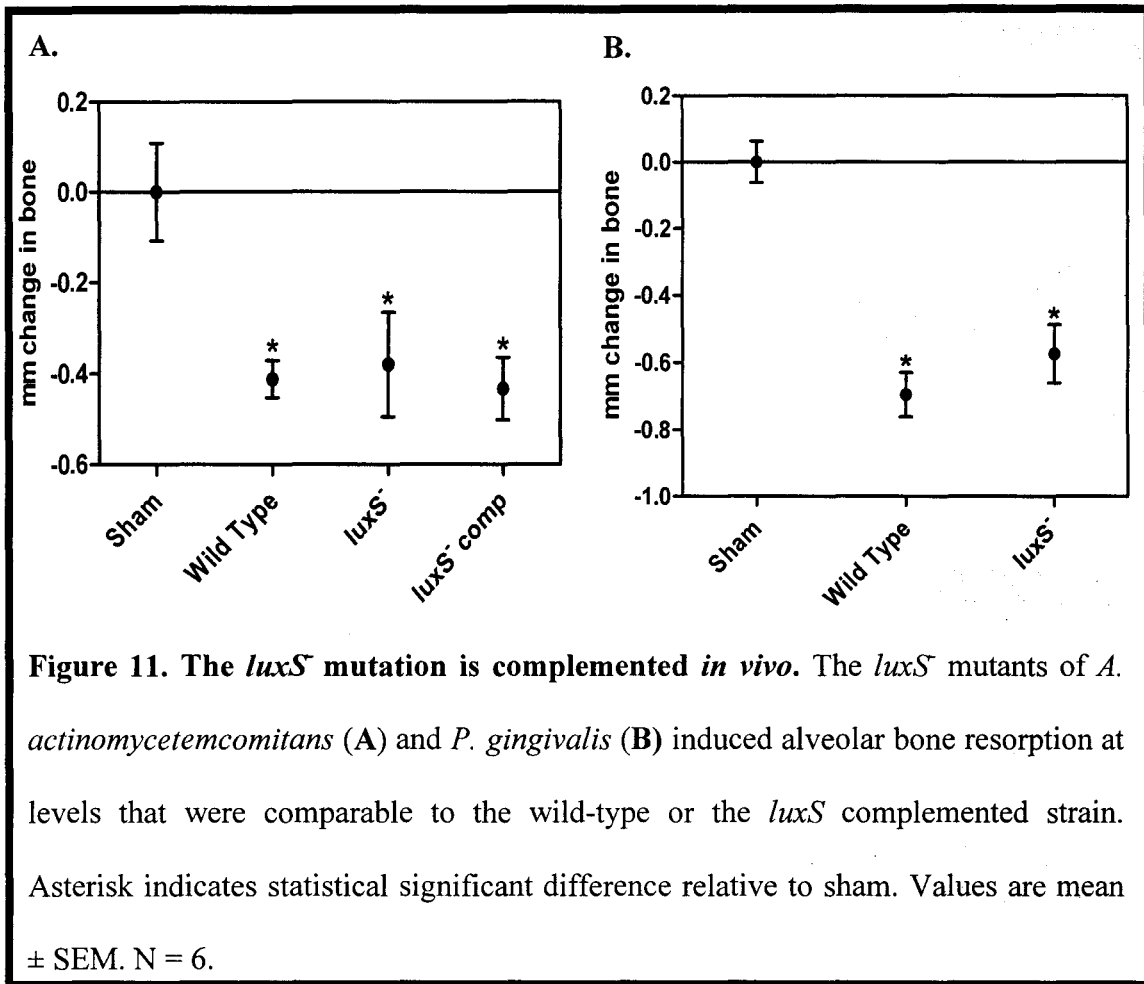


( $p = 0.0036$ ) strains of *A. actinomycetemcomitans* exhibited significantly more alveolar bone resorption relative to sham-infected mice. However, animals infected with either the *lsrB*<sup>-</sup> or *rbsB*<sup>-</sup> mutant strains exhibited a significant reduction in bone resorption relative to wild-type (*lsrB*<sup>-</sup>,  $p = 0.0285$ ; *rbsB*<sup>-</sup>,  $p = 0.0134$ ), suggesting that the detection of AI-2 by either receptor contributes to virulence. Consistent with this, mice that were infected with the *lsrB*<sup>-</sup>*rbsB*<sup>-</sup> double-receptor mutant did not display significant levels of bone resorption compared to the sham-infected controls. As shown in Figure 10B, animals that were infected with the *qseC*<sup>-</sup> mutant exhibited significantly lower bone resorption than wild-type ( $p < 0.02$ ) and were indistinguishable from the sham infected controls. The virulence of the *qseC*<sup>-</sup> mutant was restored to wild-type levels when it was genetically complemented with a plasmid-borne copy of *qseC*. These results suggest that both QseC and the ability to detect AI-2 contribute to the virulence of *A. actinomycetemcomitans*, and that a defective AI-2-dependent quorum sensing pathway negatively impacts the ability of *A. actinomycetemcomitans* to induce alveolar bone resorption.

#### **Inactivation of *luxS* does not influence *A. actinomycetemcomitans* virulence:**

Previous studies showed that a *luxS*<sup>-</sup> mutant of *A. actinomycetemcomitans* formed biofilms that exhibited reduced total biomass and biofilm depth relative to wild-type biofilms (196). Since the *luxS* gene is responsible for the production of AI-2, and AI-2 is essential for the formation of biofilms, we next examined the importance of *luxS* in the *in vivo* virulence of *A. actinomycetemcomitans*. As shown in Figure 11A, the extent of bone resorption induced by *A. actinomycetemcomitans luxS*<sup>-</sup> mutant was indistinguishable from the wild-type or *luxS*<sup>-</sup> complemented strains, but all strains induced alveolar bone





resorption over the sham-infected controls (wild-type  $p = 0.0052$ ; *luxS* mutant  $p = 0.0365$ ; *luxS* complemented  $p = 0.007$ ). Similar results were obtained when animals were infected with wild-type and *luxS* mutant strains of another periodontal pathogen, *P. gingivalis* (see Figure 11B). Thus, inactivation of the AI-2 receptors, but not the AI-2 synthase, affected *A. actinomycetemcomitans* virulence. This suggests that AI-2 produced by indigenous bacteria in the mouse oral cavity may complement the *luxS* mutation in *A. actinomycetemcomitans*.

## Discussion

Dental plaque is a complex oral biofilm comprised of a diverse microbial community, consisting of more than 700 different bacterial species (1, 110, 116, 118, 164, 237) that must co-exist and adapt to the fluctuating environment of the oral cavity. It is likely that both intra- and interspecies communication occurs between these organisms, and that these processes may enable oral organisms to coordinate their behavior and function based on their local environment and the other organisms that occupy the same niche. For *A. actinomycetemcomitans*, this intimate cell-to-cell communication potentially occurs via the quorum sensing circuit dependent upon the soluble signaling molecule AI-2 (54). Indeed, previous studies showed that AI-2 regulates both biofilm formation and various iron acquisition pathways in *A. actinomycetemcomitans* (55, 196). However, it is not known how the initial detection of AI-2 is linked to downstream regulation of gene expression that controls these complex phenotypes. *A. actinomycetemcomitans* does not possess the dedicated two-component system that controls the cell-density-dependent response of *Vibrio* spp. to AI-2 (i.e. the LuxQ sensor kinase, LuxU phosphorelay protein, LuxO response regulator, or the LuxR master regulator of *lux* operon). Instead, the genome of *A. actinomycetemcomitans* encodes two periplasmic proteins, LsrB and RbsB, which function as receptors for AI-2, and each is linked to a putative ABC transporter that may import the signal (88, 195). Thus, the AI-2 response circuit in *A. actinomycetemcomitans* may be similar to that described by Li *et al.* for *E. coli*, in which the LsrR regulator controls the expression of genes involved in biofilm growth as well as regulating the expression of the *lsr* operon when bound to AI-2 that has been phosphorylated by the LsrK kinase (124). AI-2 has also been suggested to

regulate motility and biofilm formation in *E. coli* through the QseBC two-component system (62, 210). The *A. actinomycetemcomitans* genome also encodes the QseBC two-component system, and our results here show that *qseBC* is a part of the AI-2 regulon in *A. actinomycetemcomitans* since *qseBC* is induced by the AI-2 signal itself, and its induction requires a functional AI-2 receptor. Furthermore, AI-2-dependent induction of QseBC in *E. coli* required the MqsR regulator (62). MqsR has been shown to be part of a toxin/anti-toxin system and adopts an  $\alpha/\beta$  fold similar to the RelE family of bacterial RNase toxins (24). Consistent with this, Yamaguchi *et al.* have also shown that MqsR functions as a GCU-specific mRNA interferase (244). MqsR is similar to open reading frame Aa00673 in the *A. actinomycetemcomitans* genome ([www.oralgen.lanl.gov](http://www.oralgen.lanl.gov)), which is co-expressed with Aa00672. Aa00673 and Aa00672 exhibit approximately 65% sequence identity to the HigBA toxin/anti-toxin system of *Vibrio cholerae*, which also functions as an mRNAse (30). This suggests that QseBC may participate in the AI-2 response circuit in *A. actinomycetemcomitans*, and that Aa00673 may contribute to *qseBC* regulation (see Chapter 4).

Consistent with QseBC functioning in the AI-2 quorum sensing pathway, inactivation of the QseC sensor influences the formation of biofilms by *A. actinomycetemcomitans* in that the *qseC* mutant forms biofilms that are reduced in total biomass, average biofilm depth, and maximum biofilm depth relative to biofilms formed by the wild-type strain. The biofilm growth phenotype of the *qseC* strain closely resembled the growth phenotype of the *A. actinomycetemcomitans luxS* mutant previously reported by Shao *et al* (196). Complementation of the *qseC* mutation restored biofilm formation, and indeed, the complemented strain formed biofilms that exhibited

greater average depth and biomass than wild-type. This may be due to the gene dosage of *qseC* in the complemented strain, since *qseC* was expressed from a multi-copy number plasmid in this organism. These data suggest that the QseC sensor, and presumably the QseB response regulator, may be part of the quorum sensing circuit mediated by AI-2 in *A. actinomycetemcomitans*.

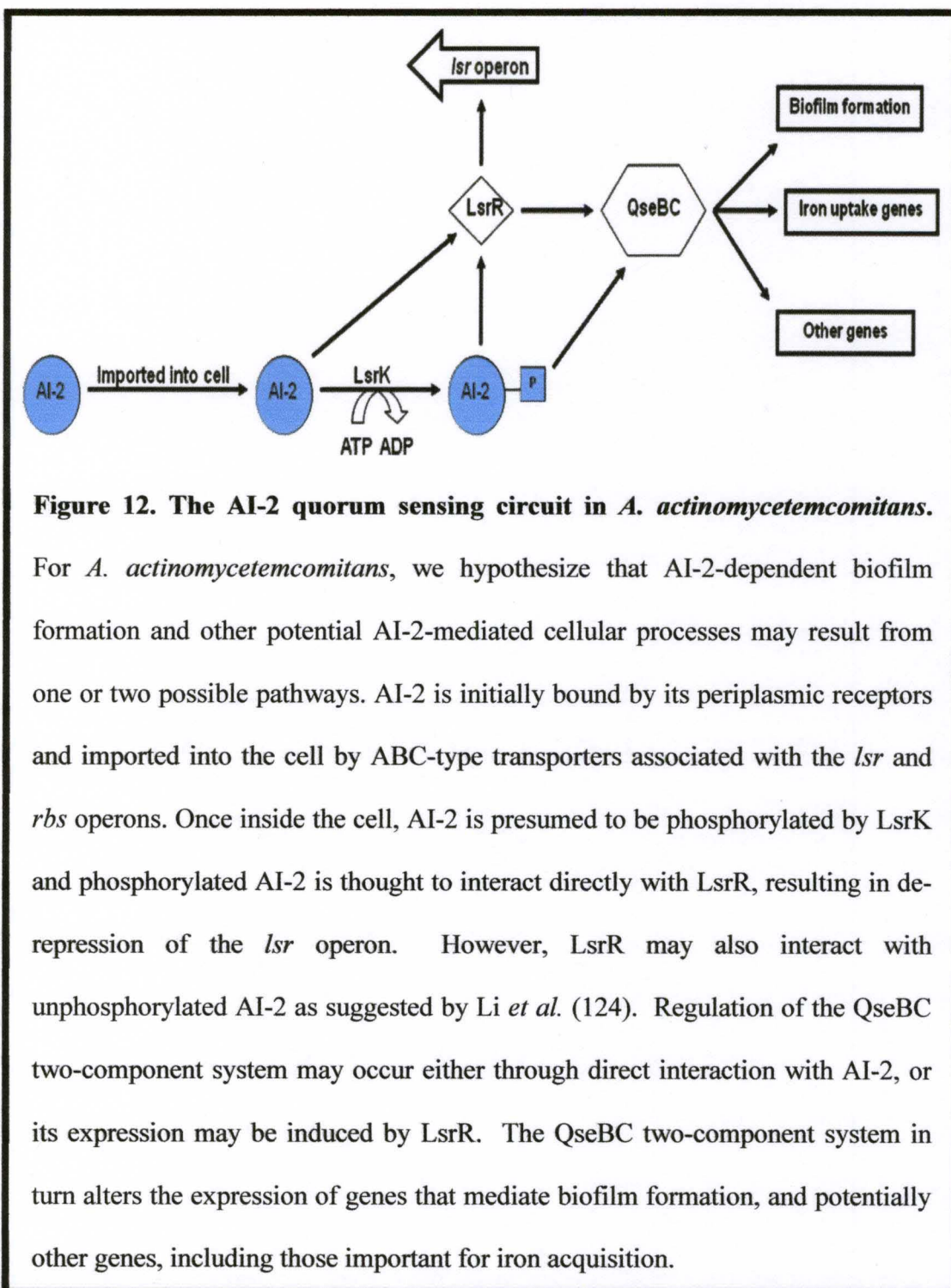
However, although the QseC sensor may be common to the AI-2 signal transduction pathway of both *A. actinomycetemcomitans* and *E. coli*, other aspects of these AI-2 response circuits appear to differ. Sperandio *et al.* have shown that *E. coli* O157:H7 QseC responds to stress hormones (epinephrine/norepinephrine and adrenaline/noradrenaline) as well as the microbial signal autoinducer-3 (AI-3), and that QseC may function in a one-to-many branched signal transduction pathway that activates the QseB, QseF and KdpE response regulators (82, 173). In addition, the QseE sensor kinase was shown to reside downstream of QseC in the adrenergic signal transduction cascade (82). At present, the signal that the *A. actinomycetemcomitans* QseC kinase is sensing is not known, and we have been unsuccessful in identifying AI-3 in *A. actinomycetemcomitans* extracts (D.R. Demuth, unpublished). Furthermore, paralogs of the QseE, QseF and KdpE polypeptides could not be identified in the *A. actinomycetemcomitans* genome at a search stringency that readily detected QseC using the *E. coli* QseC sequence as query. This suggests that the components that reside downstream from QseC in *A. actinomycetemcomitans* may differ from some *E. coli* strains. Interestingly, *E. coli* O157:H7 lacks MqsR and transcription of *qseBC* is independent of AI-2 in this organism.

Biofilm growth in *A. actinomycetemcomitans* is dependent upon AI-2-mediated quorum sensing (196), and formation of biofilms is essential to the *in vivo* survival and virulence of dental pathogens in the oral cavity. Our results show that AI-2-mediated quorum sensing is important for *A. actinomycetemcomitans* virulence *in vivo* since mutants that were deficient in either of the AI-2 receptors (LsrB or RbsB) induced lower levels of alveolar bone resorption than the wild-type strain and a double-receptor mutant was essentially avirulent. These phenotypes are also consistent with the biofilm phenotypes exhibited by these mutant strains (196). The *qseC*<sup>-</sup> mutant was also avirulent and did not induce bone loss over the sham-infected control group. This is consistent with QseC participating in and residing downstream from the AI-2 receptors in the AI-2 response circuit as shown in the model presented in Figure 12. Nonetheless, the exact mechanism(s) through which QseBC contributes to the intracellular response of AI-2 as well as biofilm growth in *A. actinomycetemcomitans* is not known. *A. actinomycetemcomitans* possesses the protein LsrR, which is the master regulator of the *lsr* operon in *Salmonella enteric* (218, 219), and Gonzalez-Barrios *et al.* suggested that AI-2-dependent biofilm growth of *E. coli* requires internalization of AI-2 (62). In *A. actinomycetemcomitans*, the QseBC two-component system may couple the detection/importation of AI-2, either alone or in cooperation with LsrR, to downstream regulation of biofilm formation and other cellular processes.

At present, the sets of genes that are regulated by the QseBC two-component system in *A. actinomycetemcomitans* are not known, but it is of interest that *qseBC* also exhibits sequence similarity to the *feuPQ* two-component system that regulates iron acquisition in several other bacterial species. Several operons that encode iron acquisition

pathways have been previously shown to be regulated by AI-2 (55), and iron availability is also known to dramatically influence biofilm formation by *A. actinomycetemcomitans* (177, 178). Thus, it is possible that the biofilm defect that arises from inactivation of *qseC* occurs in part from the dysregulation of iron acquisition mechanisms. We demonstrated here that the QseC sensor regulates the acquisition of iron by *A. actinomycetemcomitans*. While both *afuA* 00696 and *fecB* 00795 were upregulated in the *qseC* mutant, not all iron-uptake genes showed differential expression in the *qseC* mutant. Our data indicates that the QseBC two-component system regulates the expression of some but not all iron-acquisition genes regulated by AI-2, suggesting that the QseBC regulon may include other components important for biofilm formation. Along these lines, QseBC was shown to regulate biofilm formation and motility in *E. coli* (62). Interestingly, *A. actinomycetemcomitans* is a non-motile organism, which also suggests the *qseBC* regulon mediates the expression of other genes important in biofilm formation.

Since *luxS* was previously shown to influence biofilm growth (196), it was surprising that neither *A. actinomycetemcomitans* nor *P. gingivalis luxS* mutants exhibited attenuated virulence in the murine model. A possible explanation for this result is that the *luxS* mutation was complemented by AI-2 produced by organisms that are indigenous to the murine oral cavity. Consistent with this, McNab *et al.* have shown that AI-2 crosstalk occurs in dual species biofilms of *Streptococcus gordonii* and *P. gingivalis* (139). In this system, dual species biofilms formed efficiently even if one of the strains harbored a *luxS* mutation, but no biofilms formed if both strains were LuxS deficient. Our previous studies also showed that AI-2-mediated crosstalk is possible between *A.*



**Figure 12. The AI-2 quorum sensing circuit in *A. actinomycetemcomitans*.**

For *A. actinomycetemcomitans*, we hypothesize that AI-2-dependent biofilm formation and other potential AI-2-mediated cellular processes may result from one or two possible pathways. AI-2 is initially bound by its periplasmic receptors and imported into the cell by ABC-type transporters associated with the *lsr* and *rbs* operons. Once inside the cell, AI-2 is presumed to be phosphorylated by LsrK and phosphorylated AI-2 is thought to interact directly with LsrR, resulting in de-repression of the *lsr* operon. However, LsrR may also interact with unphosphorylated AI-2 as suggested by Li *et al.* (124). Regulation of the QseBC two-component system may occur either through direct interaction with AI-2, or its expression may be induced by LsrR. The QseBC two-component system in turn alters the expression of genes that mediate biofilm formation, and potentially other genes, including those important for iron acquisition.

*actinomycetemcomitans* and *P. gingivalis*, and that the AI-2 signal of *A. actinomycetemcomitans* is capable of modulating the expression of *luxS*-regulated genes in *P. gingivalis* (54). In addition, Rickard *et al.* showed that the ability of the oral microbes *Actinomyces naeslundii* and *Streptococcus oralis* to form dual-species biofilms in saliva was dependent on AI-2 produced by *S. oralis* (180). The inability to detect or respond to AI-2 prevents the stimulation of the AI-2 response circuit and results in reduced biofilm growth and attenuated virulence, whereas the inability to produce AI-2 was likely overcome by the presence exogenous signal produced by other bacteria in the murine oral cavity.

Since *luxS* was previously shown to control biofilm growth (196), it was surprising that neither *A. actinomycetemcomitans* nor *P. gingivalis luxS* mutant exhibited attenuated virulence in the murine model. A possible explanation for this result is that the *luxS* mutation was complemented by AI-2 produced by organisms that are indigenous to the murine oral cavity. Consistent with this, McNab *et al.* have shown that AI-2 crosstalk occurs in dual species biofilms of *Streptococcus gordonii* and *P. gingivalis* (139). In this system, dual species biofilms formed efficiently even if one of the strains harbored a *luxS* mutation, but no biofilms formed if both strains were LuxS-deficient. Our previous studies also showed that AI-2-mediated crosstalk is possible between *A. actinomycetemcomitans* and *P. gingivalis*, and that the AI-2 signal of *A. actinomycetemcomitans* is capable of modulating the expression of *luxS*-regulated genes in *P. gingivalis* (54). In addition, Rickard *et al.* showed that the ability of the oral microbes *Actinomyces naeslundii* and *Streptococcus oralis* to form dual-species biofilms in saliva was dependent on AI-2 produced by *S. oralis* (180). The inability to detect or



respond to AI-2 prevents the stimulation of the AI-2 response circuit and results in reduced biofilm growth and attenuated virulence, whereas the inability to produce AI-2 was likely overcome by the presence exogenous signal produced by other bacteria in the murine oral cavity.

In summary, we have shown that the QseBC two-component system is induced by AI-2, and that QseC is important for biofilm formation and virulence of *A. actinomycetemcomitans*. Our results suggest that QseC is part of the AI-2 response circuit and resides downstream of the AI-2 receptor proteins LsrB and RbsB. Further definition of the genes regulated by the QseBC two-component system may identify new targets for therapeutic intervention of aggressive periodontitis and other systemic infections associated with *A. actinomycetemcomitans*.

**CHAPTER FOUR: CHARACTERIZATION OF A TOXIN-ANTITOXIN SYSTEM  
INVOLVED IN AI-2-DEPENDENT QUORUM SENSING IN  
*AGGREGATIBACTER ACTINOMYCETEMCOMITANS***

**Introduction**

Periodontal disease is a serious and widespread health ailment worldwide in the adult population. A hallmark of periodontal disease is that it is caused by a complex oral biofilm that is comprised of over 700 species of bacteria. Major shifts in the populations of certain microbes in the oral biofilm have been implemented in the progression of the disease, as diseased sites often have an increase of pathogens relative to residential microbes (131, 132, 134). However, the host and/or microbial shifts that contribute to these populational shifts are still unknown. The oral cavity is not a static environment and is constantly subjected to continual environmental fluxes, such as pH, temperature, nutrient supply, and osmolarity. Amazingly, though, oral bacteria are able to detect and properly respond to these changes, allowing them to successfully coexist and thrive in the oral cavity (2, 87, 134). Intra- and interspecies communication is one mechanism which enables bacteria to respond to their environment by coordinating their behavior and function and regulating gene expression as a community. One mechanism of

communication is quorum sensing, a cell-density dependent response, which is regulated by the production, release, and subsequent detection of signaling molecules called autoinducers in Gram-negative bacteria (116, 140, 190, 253). The external concentration of the autoinducer continues to increase as a population of bacteria expands until a threshold is reached, at which point a signal transduction pathway is initiated, changing gene expression and behavior of the microbial community. Therefore, it has been suggested that quorum sensing may enable bacteria to respond appropriately to changes in the environment, thereby reacting as a multi-cellular organism.

*A. actinomycetemcomitans*, a known periodontal pathogen, is associated with aggressive periodontitis as well as other systemic diseases (20, 159, 203, 250). Previous research has shown that *A. actinomycetemcomitans* possesses an AI-2-dependent quorum sensing circuit (54), and that AI-2 regulates virulence factors (54), biofilm formation (196), iron acquisition (54, 55), and *in vivo* virulence in a mouse model of *A. actinomycetemcomitans* infection (154). While the proteins that were related to the *Vibrio* AI-2 receptor were identified in *A. actinomycetemcomitans*, genes related to the dedicated two-component system (LuxQ sensor, LuxU phosphorelay, LuxO response regulator protein) have not been found in *A. actinomycetemcomitans*. Although the two-component system encoded by QseBC is regulated by AI-2 and controls biofilm growth and virulence in *A. actinomycetemcomitans* (154), as shown in Chapter 3, exactly how the detection and/or internalization of the AI-2 signaling molecule leads to downstream gene regulation of complex phenotypes is not fully known.

The QseBC two-component system is also known to regulate biofilm formation and motility in *E. coli* (62, 210). Previous studies have shown that in some strains of *E.*

*coli* expression of the QseBC two-component system is regulated by MqsR (62). MqsR might potentially be located upstream of QseBC in the AI-2 response circuit in *E. coli*, as deletion of the *mqsR* gene resulted in a 61-fold decrease in QseBC expression and decreased biofilm formation. It has been proposed that the signaling molecule AI-2 stimulates expression of *mqsR*, which then induces expression (either directly or indirectly) of the QseBC two-component system. QseBC then influences gene expression leading to biofilm formation (62). Recently, MqsR was demonstrated to be the toxin of a toxin-antitoxin (TA system) encoded by MqsRA (24), and expression of this TA system is highly expressed in biofilms (62), and has been shown to be a GCU-specific mRNA interferase (244).

The genomes of all free-living bacteria scrutinized thus far encode a number of toxin genes (96, 243), and it is thought that organisms have acquired these systems through horizontal gene transfer (228). Interestingly, the toxins produced by these genes are not intended to kill other bacteria or host cells, but instead are produced intracellularly and are toxic to their host cells, resulting in cell growth arrest and often cell death (50, 227, 228, 243). This process has been termed programmed cell death and has been suggested to be the prokaryotic equivalent of apoptosis (50). Many of these toxins are co-transcribed with their cognate antitoxins in an operon termed Toxin-Antitoxin (TA) systems. The antitoxin and toxin form a stable complex in the cell, suppressing toxicity under normal growth conditions. However, the stability of the antitoxin is significantly lower compared to the toxin; thus any stress causing cellular damage or growth inhibition induces the expression of proteases that alter the equilibrium between toxin and antitoxin, resulting in release of the toxin into the cell (31, 96, 243).

The genome of *A. actinomycetemcomitans* was shown to possess a two-component system that displays 70-80% sequence similarity to the QseBC two-component system of *E. coli*. Furthermore, previous studies demonstrated that AI-2 induces QseBC and a functional AI-2 receptor is needed for induction. In addition, inactivation of *qseC* resulted in a significant decrease in biofilm formation and *in vivo* virulence of *A. actinomycetemcomitans*, suggesting that QseBC is part of the AI-2 regulon (154). Interestingly, *A. actinomycetemcomitans* also possesses open reading frames (ORFs) (Aa0672 and Aa00673) that have sequence similarity to the MqsRA TA system. Both Aa00672 and Aa00673 appear to be located in a pathogenicity island in the *A. actinomycetemcomitans* genome, suggesting that these genes may have been horizontally acquired by *A. actinomycetemcomitans*. In this study, we show that Aa00673 is coexpressed in an operon with Aa00672. Aa00673 encodes a toxin which causes growth inhibition, and this growth inhibition is reversed by its cognate antitoxin, Aa00672. Furthermore, expression of Aa00673 is reduced in both the *lsrB*<sup>-</sup>*rbsB*<sup>-</sup> double AI-2-receptor mutant and the *qseC*<sup>-</sup> mutant, suggesting that stimulation of toxin follows detection and internalization of AI-2 as well as the QseBC two-component system. Additionally, we show that Aa00673 may function as an RNase. Thus, Aa00672 and Aa00673 may regulate the expression of the QseBC two-component system, which in turn, influences downstream expression of genes in AI-2-dependent quorum sensing.

## **Results**

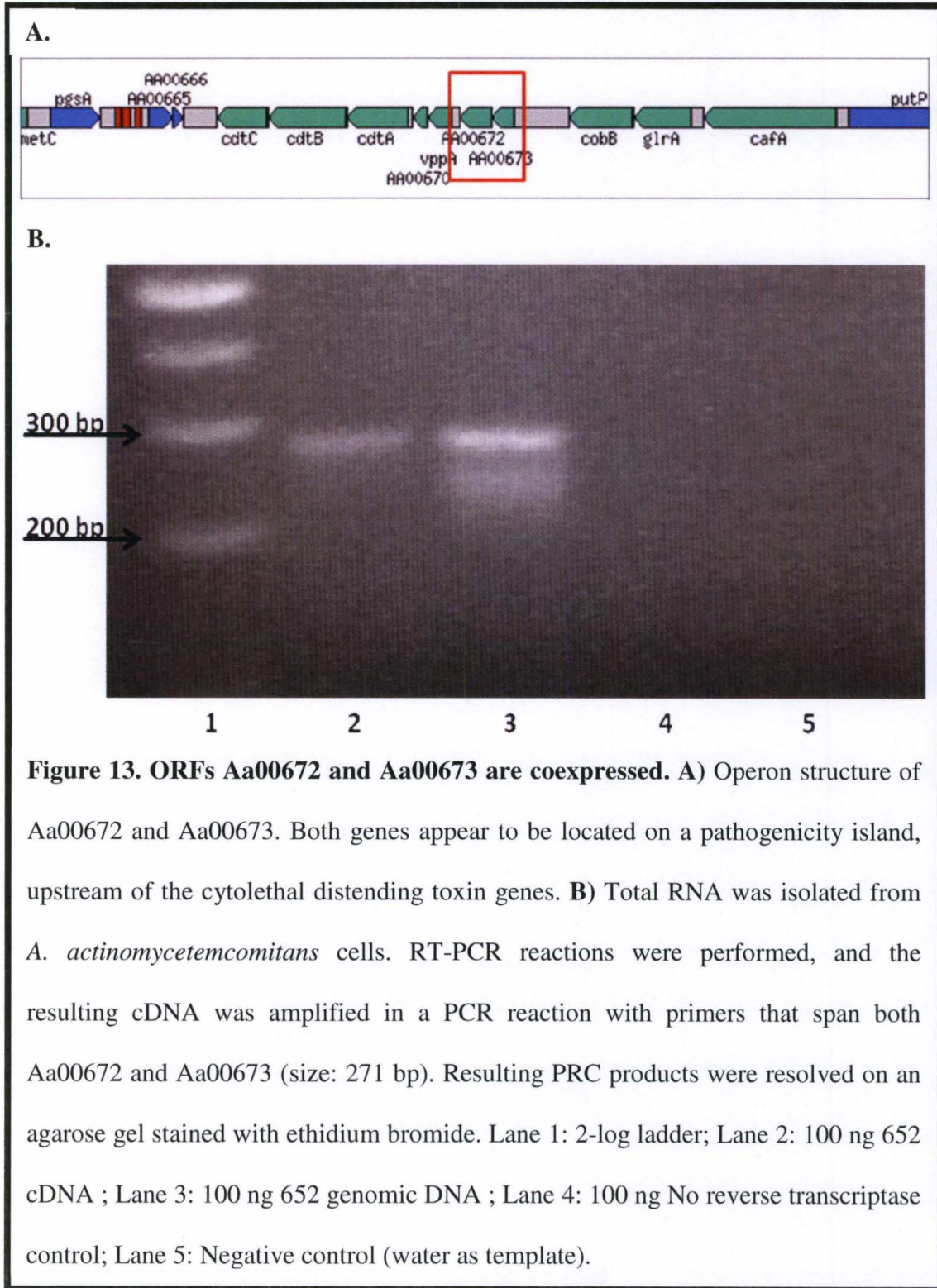
### **ORF Aa00673 and ORF Aa00672 are expressed in an operon:**

The QseBC two-component system has been shown to regulate AI-2-dependent phenotypes in both *A. actinomycetemcomitans* (154) and *E. coli* (62). Gonzalez Barrios et al further demonstrated that *qseBC* are regulated by *mqsR* in *E. coli*, and that *mqsR* might reside upstream of QseBC in the AI-2 signaling circuit (62). Interestingly, the genome of *A. actinomycetemcomitans* encodes several putative TA systems, including ORFs Aa00672 and Aa00673 (Figure 13a), which exhibit similarity to *mqsRA*.

Since most toxins are co-transcribed with their cognate antitoxins in an operon (31, 96, 243), we wanted to determine if ORFs Aa00672 and Aa00673 were co-transcribed together. Aa00672 is a 114-residue protein, and Aa00673 is an 84-residue protein. Reverse-transcription PCR was carried out using cDNA synthesized from total RNA extracted from *A. actinomycetemcomitans* strain 652. As shown in Figure 13b (lane 2), when using 100 ng of cDNA and primers that span the region from Aa00672 to Aa00673 (5' and 3' TA transcript primers; see Table 2b), the expected 271-bp band was detected. This band was not detected in the reaction carried out without the addition of reverse transcriptase (Figure 13b, lane 4). This demonstrate that Aa00673 is co-transcribed with the downstream gene Aa00672.

#### **Aa00673-Aa00672 expression is induced by components in the AI-2 regulon:**

Gonzalez Barrios et al has previously shown that MqsR is a component in the AI-2 response circuit of *E. coli*. Deletion of *mqsR* in *E. coli* resulted in a significant in a 61-fold decrease in the expression of *qseBC*, suggesting that MqsR regulates expression of the QseBC two-componet system in *E. coli* (62). To determine if AI-2 regulates ORF Aa00672 and ORF Aa00673 in *A. actinomycetemcomitans*, the expression of this putative



TA system was examined using real-time PCR with RNA isolated from overnight cultures of the *qseC* mutant and the *lsrB**rbsB* mutant. As shown in Table 5, the expression of the Aa00672-Aa00673 was down-regulated 4.5-fold in the *qseC* mutant. In addition, induction of Aa00672-Aa00673 was not seen in the *lsrB**rbsB* strain of *A. actinomycetemcomitans*, which lacks the AI-2 receptors, but which can still produce AI-2. This suggests that the ORFs Aa00672 and Aa00673 are part of the AI-2 regulon in *A. actinomycetemcomitans*, and is located downstream of detection and internalization of signal as well as the QseBC two-component system.

**Table 6. ORFs Aa00672-Aa00673 are regulated by the AI-2 regulon.**

Strain	delta T <sup>1</sup>	delta (delta T)	Fold Change
652	4.02 ± 0.15	0.062 ± 0.007	1.0
<i>qseC</i>	6.21 ± 0.25	0.014 ± 0.002	0.22 ± 0.019
<i>lsrB</i> <i>rbsB</i>	6.03 ± 0.41	0.016 ± 0.005	0.25 ± 0.047

<sup>1</sup> delta T was calculated by subtracting the cycle threshold (Ct) determined for the 00696 reaction from the Ct of the 5S rRNA control.

**Aa00673 is a toxin and Aa00672 is its cognate antitoxin:**

TA systems are expressed in an operon together, such that under normal growth conditions a stable toxin-antitoxin complex is formed, suppressing the toxic activity of the toxin (31, 96, 243). Since ORFs Aa00672 and ORF Aa00673 in *A. actinomycetemcomitans* were previously shown to be regulated by components of the AI-2 regulon and also co-transcribed together in an operon (see results above), we wanted to determine whether these ORFs actually encode a toxin and an antitoxin. The Aa00673 gene alone or both ORF Aa00672 and ORF Aa00673 together were cloned into the

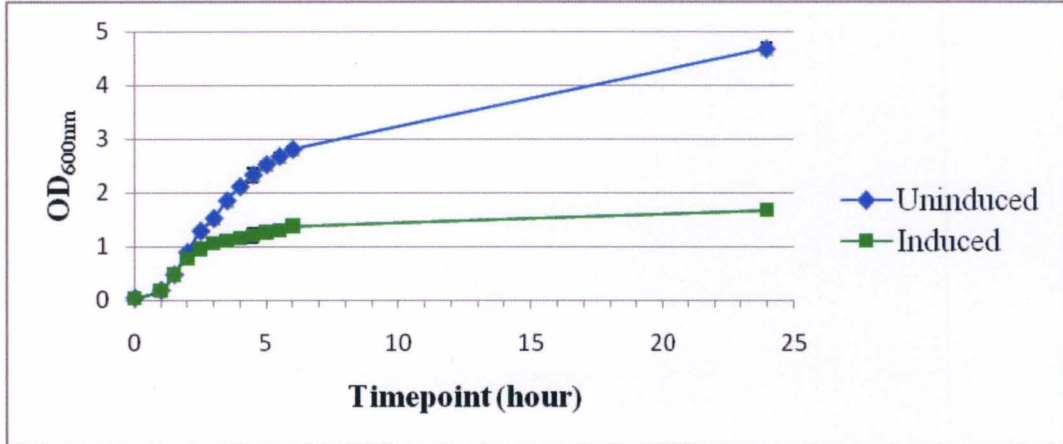


isopropyl  $\beta$ -D-1-thiogalactopyranoside- (IPTG) -inducible pET30a plasmid and transformed into *E. coli*. Uninduced cultures of *E. coli*, which contain the pET30a-Aa00673 construct but were not induced to express it, did not have a defect in cell growth; whereas inhibition of cell growth was visible 30 minutes after induction of Aa00673 in *E. coli* (Figure 14a). However, induction of the construct expressing both Aa00673 and Aa00672 together did not halt cell growth of *E. coli* (Figure 14b). This indicates that Aa00673 encodes a toxin, and Aa00672 encodes its cognate antitoxin, negating the toxic activity of Aa00673.

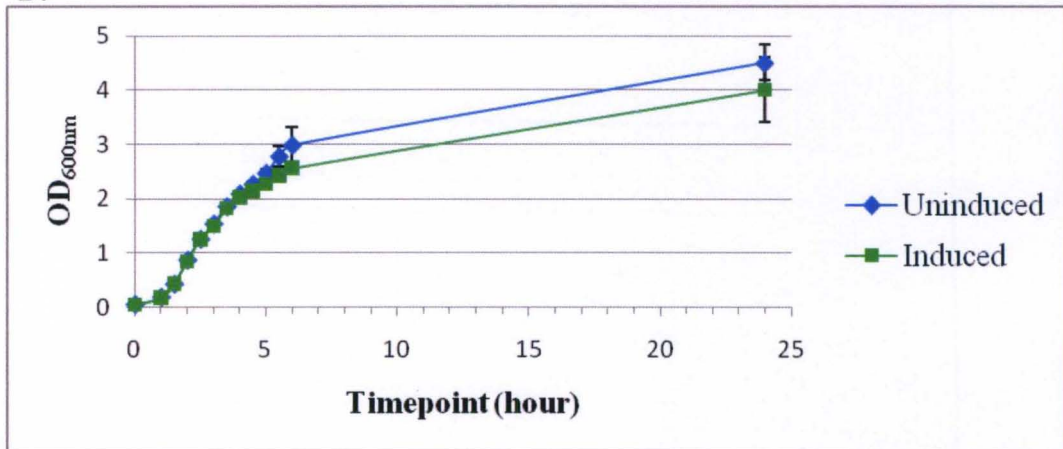
#### **Aa00673 causes reversible growth inhibition:**

Since Aa00673 causes growth inhibition when overexpressed in *E. coli* BL21 cells, we wanted to determine if this inhibition was a bacteriostatic or a bactericidal effect. This was achieved by inducing the cells as previously described, but then two hours after induction, IPTG was removed, the cells were resuspended in fresh LB medium, and the OD<sub>600nm</sub> readings of the *E. coli* cultures resumed every 30 minutes. As shown in Figure 15a, removing IPTG from the induced cultures resulted in a growth rate similar to the uninduced cultures. However, the OD<sub>600nm</sub> of the induced cultures never reached the same level as the uninduced cultures. Furthermore, cell viability plating demonstrated that after removal of the inducer, the induced cells began proliferating again (Figure 15b). Similar to the grow rate, the CFU/ml never reached the same concentration as the uninduced cultures. This phenomenon may be due to the fact that the starting OD<sub>600nm</sub> of the uninduced cultures was higher than the induced cultures, and thus, there would be

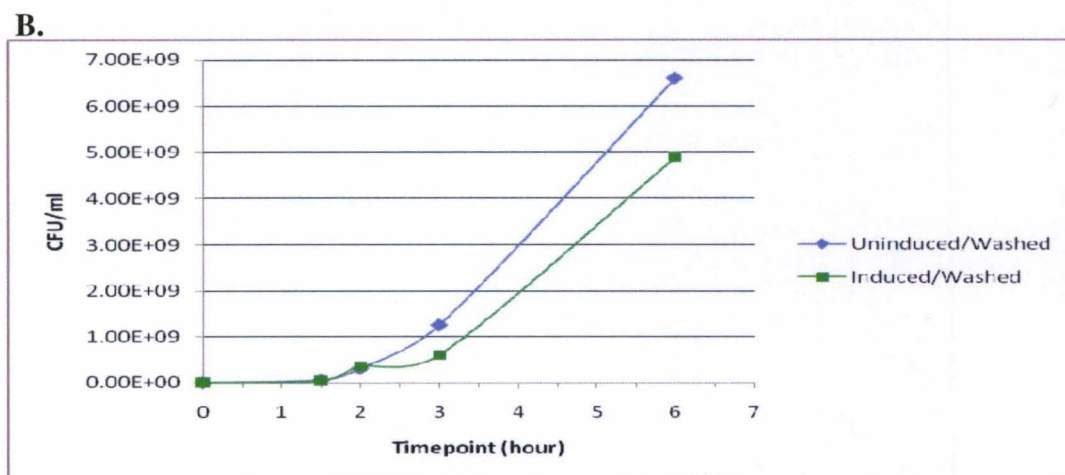
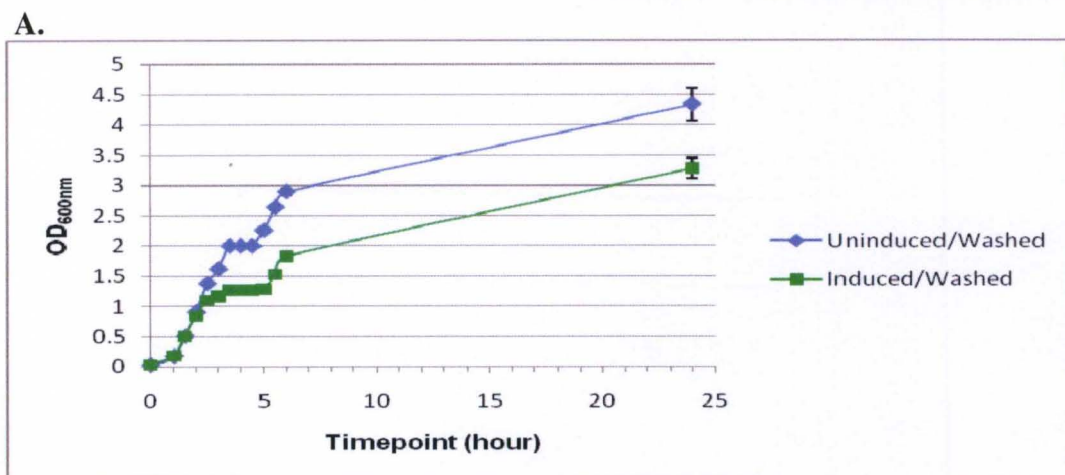
A.



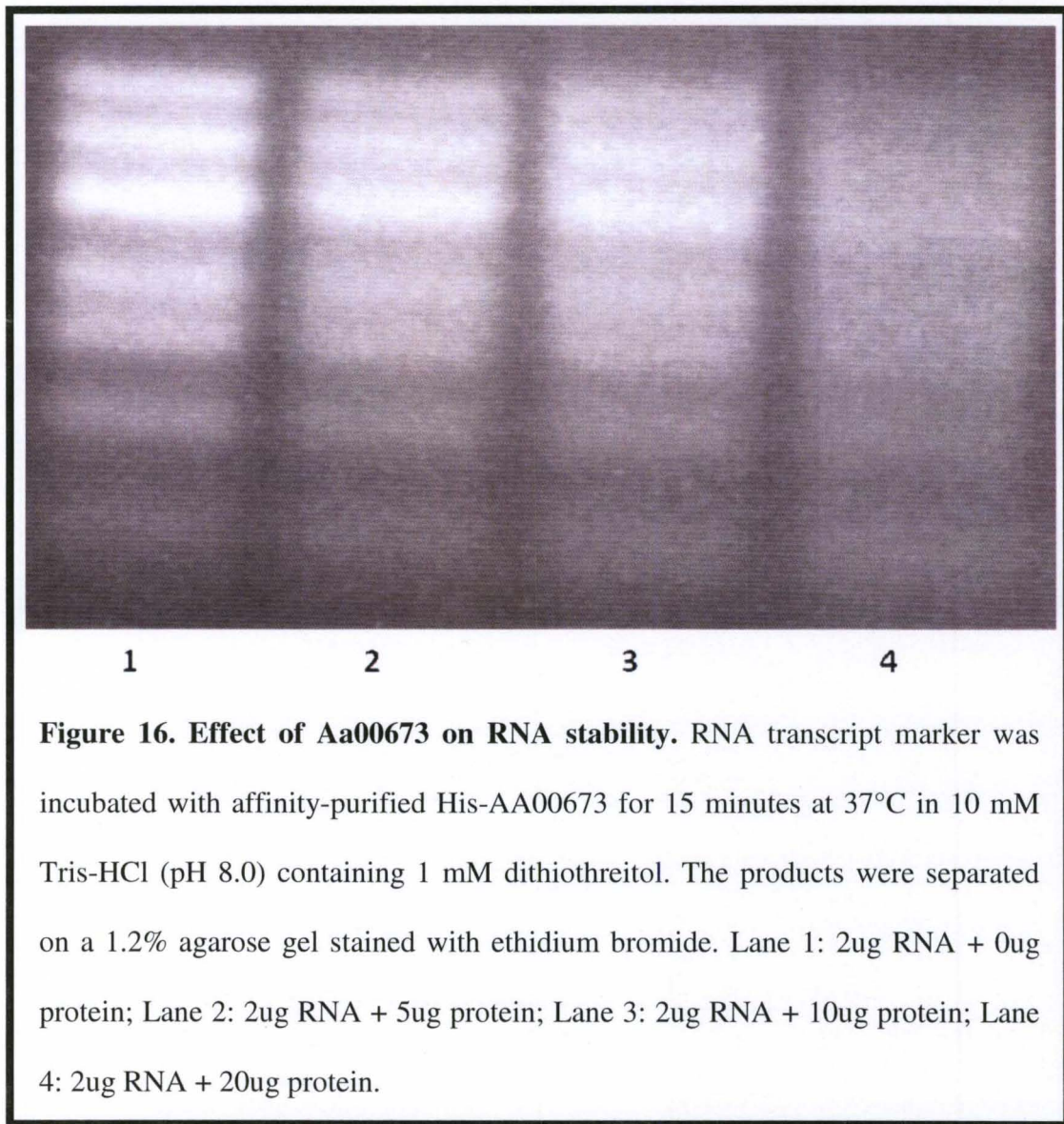
B.



**Figure 14. ORF Aa00673 induces growth arrest that is negated by the expression of ORF Aa00672.** Effect on bacterial growth measured as OD<sub>600nm</sub>. Bacteria were grown without IPTG (blue diamonds), and in the presence of 1 mM IPTG (green squares). Growth was measured every 30 minutes after induction. Results are mean  $\pm$  standard deviation. **A)** *E. coli* BL21 with the pET30a-AA00673 construct was induced at an OD<sub>600nm</sub> of 0.4. Control cultures were not induced. **B)** *E. coli* BL21 with the pET30a-AA00673/AA00672 construct was induced at OD<sub>600nm</sub> of 0.4. Control cultures were not induced.



**Figure 15. ORF Aa00673 induces reversible growth arrest.** Effect on bacterial growth measured as OD<sub>600nm</sub>. Bacteria were grown without IPTG (blue diamonds), and in the presence of 1 mM IPTG (green squares). **A)** *E. coli* BL21 with the pET30a-AA00673 construct was induced at an OD<sub>600nm</sub> of 0.4. Control cultures were not induced. Two hours after induction both induced and uninduced controls were washed 3x, and then resuspended in LB. Results are mean ± standard deviation. **B)** Appropriate dilutions were plated at certain time points throughout the growth curve. Plates were incubated overnight at 37C, colonies counted, and CFU/ml calculated.



more CFUs per ml in the uninduced cultures compared to the induced cultures. These results are consistent with the previous growth curve (Figure 14a), which showed that while induction of Aa00673 resulted in cell growth inhibition, the  $OD_{600nm}$  values increased slightly over time, suggesting that some cells were able to continue to divide

over time. Taken together, these results imply that though Aa00673 inhibits cell growth, it is only a bacteriostatic effect and easily reversible upon removal of the inducer.

### **RNase activity of Aa00673:**

Yamaguchi et al recently demonstrated that MqsR functions as the toxin in the MqsRA TA system in *E. coli* (244), and ORF Aa00673 in *A. actinomycetemcomitans* has sequence similarity to MqsR. In addition, Aa00673 and Aa00672 also show sequence identity (approximately 65%) to the HigBA TA system of *Vibrio cholera*, and the toxin HigB is known to function as an mRNAse (30). Thus, we wanted to see if Aa00673 also functions as an RNase. To determine whether the putative toxin AA00673 functions as an RNase, an RNA transcript marker (7 defined transcripts ranging from 0.1-1 kb) was incubated with increasing amounts of affinity-purified His-AA00673 *in vitro*. Figure 16 shows dose-dependent degradation of the RNA marker as the concentration of purified AA00673 increases, suggesting that Aa00673 may indeed function as an RNase.

### **Discussion**

The environment of the oral cavity is one of constant fluctuations in temperature, pH, osmolarity, and nutrients. In order to survive these conditions, bacteria must be able to appropriately detect, respond, and adapt to their surrounding environment (2, 87, 134). Bacteria express sophisticated mechanisms to sense and quickly react to the challenges presented by the oral cavity, and one of these mechanisms is termed quorum sensing. Quorum sensing is the ability of bacteria to regulate gene expression in response to soluble signaling molecules called autoinducers; thus, quorum sensing allows the

microbial community to coordinately sense the concentration of autoinducer and regulate their gene expression in response (116, 140, 190, 253). One type of quorum sensing circuit, which recognizes the autoinducer AI-2, is common in several Gram-negative and Gram-positive bacteria, and has been hypothesized to be a universal quorum sensing signal (14, 216, 231, 240). However, the AI-2-specific LuxPQUO-response circuit is not present outside of the Vibrionales, and as a result, it has been proposed that AI-2 may not play a role in quorum sensing in all organisms, but instead may function as a by-product of metabolism (43, 240). While this may be true for some bacteria, this is not consistent in those bacteria with phenotypes that are associated with the AI-2 quorum sensing pathway. Consequently, an alternative AI-2 signaling pathway may exist in organisms that lack the LuxPQUO signaling transduction circuit, such as *A. actinomycetemcomitans*.

Our previous studies began elucidating a potential alternative AI-2-dependent quorum sensing circuit in *A. actinomycetemcomitans* involving the QseBC two-component system. We demonstrated that AI-2 induces the QseBC two-component system, and that this two-component system influences cellular processes such as biofilm formation, iron-uptake systems, and *in vivo* virulence in *A. actinomycetemcomitans* (154). Studies by Gonzalez Barrios et al showed that expression of the QseBC two-component system and biofilm formation is induced by the protein MqsR (62). MqsR has been recently characterized as a toxin in a TA system, MqsRA (24). Additionally, MqsR is only present in those strains of *E. coli* where QseBC regulates biofilm formation in an AI-2-dependent manner (62). Interestingly, the genome of *A. actinomycetemcomitans*

encodes ORFs (Aa00672 and Aa00673) which share sequence similarity to MqsR of *E. coli*, suggesting these ORFs may be involved in AI-2-dependent quorum sensing.

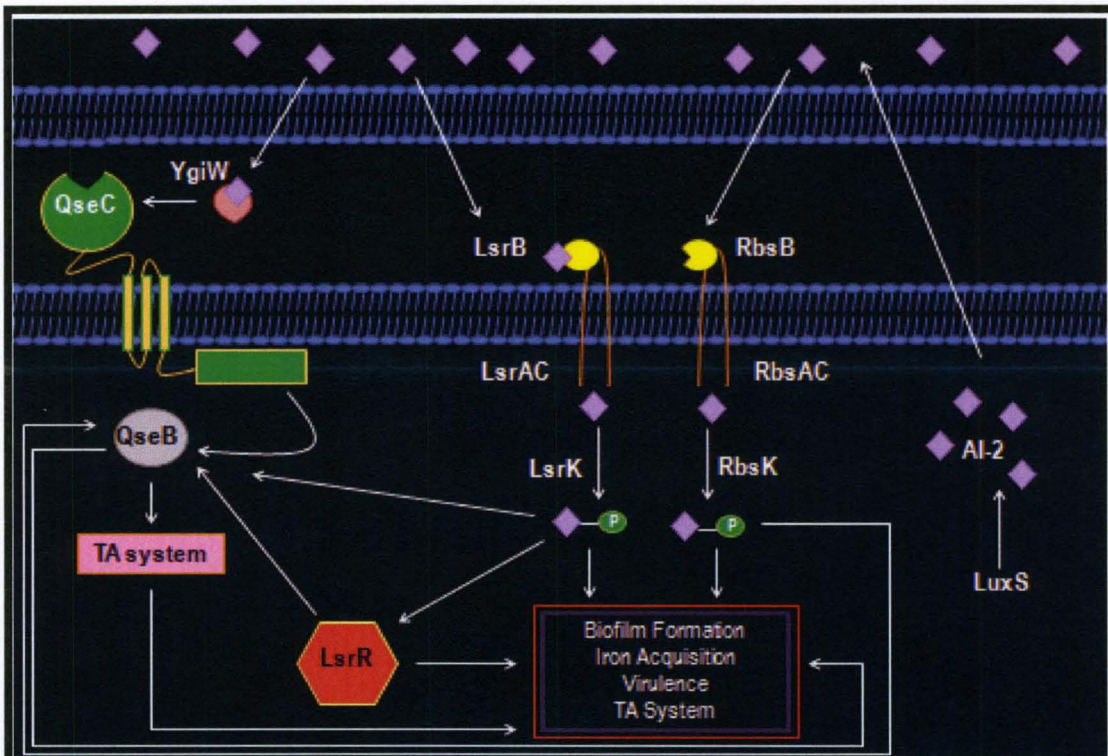
In this work, we demonstrate that the Aa00672 and Aa00673 are co-transcribed in an operon, an important characteristic of TA systems. Additionally, we show that expression of Aa00672 and Aa00673 is not induced in the *qseC* mutant or the *lsrB rbsB* mutant, suggesting that this TA system is a component in the AI-2 signaling transduction cascade. In contrast to *E. coli*, where activation of MqsR influences the expression of *qseBC* (62), it appears that the TA system in *A.actinomycetemcomitans* resides downstream of the QseBC two-component system in the AI-2 regulon (Figure 17), where it is able to post-transcriptionally regulate downstream gene expression in the AI-2-dependent quorum sensing circuit.

We also show that ORF Aa00673 functions as a toxin, inhibiting cell growth, when expressed in *E. coli*. Consistent with this result, expression of the entire TA system (ORF Aa00672-Aa00673) did not result in inhibition of cell growth, indicating that Aa00672 is the cognate antitoxin of Aa00673, and negates its toxic effects on cells. The ability of the toxin to inhibit cell growth, however, is a reversible effect because after removal of IPTG, the growth rate of the induced cultures was similar to the growth rate of the uninduced cultures. It should also be noted that the culture of *E. coli* induced to express the toxin continued to slightly grow over time, which is also consistent with the toxin causing reversible cell growth inhibition. Indeed, the OD<sub>600nm</sub> of the induced cultures never reached the levels of the uninduced cultures, but this may be due to inefficient removal of the inducer.

Toxins that are mRNA interferases degrade mRNA with substrate specificity, and can be viewed as global regulators. Global regulators, such as Hfq and CsrA, regulate gene expression at the post-transcriptional level by differential mRNA decay (153). Yamaguchi et al has recently shown that MqsR functions as a GCU-specific mRNA interferase (244), and the MqsRA was the first TA system to be demonstrated as a global regulator. Deletion of *mqsR* either by replacement with kanamycin resistance cassette or by a transposon insertion resulted in enrichment of 76 transcripts in *E. coli* BW25113 and MG1655 (9, 62, 101). However, these mutations had a polar effect on downstream *mqsA*, so transcriptome studies were also performed where MqsR production in BW25113 wild-type strain was assessed, and it was found that MqsR production triggered a global change in the profile of the transcriptome with 132 transcripts enriched (101). The global change that was observed was due to the mRNA interferase activity of MqsR. Since ORF Aa00673 is a putative paralog of MqsR, we tested whether Aa00673 functions as an RNase. Our results in this study show that ORF Aa00673 may function as an RNase as well. Further analysis is needed to determine if Aa00673 specifically cleaves at GCU sequences as well as to determine additional genes Aa00673 may regulate.

Hundreds of genes, including are known to be differentially regulated during biofilm formation, including stress-associated genes (17, 44, 175, 191). Studies involving mutants of *Streptococcus mutans* which lacked homologues of the *mazF* and *relE* toxin genes did not appear to have an effect on biofilm formation as compared to the parental strain, implying that TA systems did not play a role in biofilm formation (119). However, recent studies have connected the MqsRA TA system to biofilm formation in *E. coli*. A





**Figure 17. Current model of AI-2-dependent quorum sensing in *A. actinomycetemcomitans*.** LuxS produces AI-2 and it is exported outside the cell. Once AI-2 is subsequently detected and internalized into the cell, one outcome is the induction of the QseBC two-component system, which may occur through the regulator LsrR. The QseBC two-component system has been shown to regulate a subset of AI-2-regulated iron-uptake genes, and potentially regulates other genes and operons that may influence quorum-sensing dependent behaviors, such as biofilm formation. The putative TA system (Aa00672-00673) is also downstream of the QseBC system. Thus, this model provides two pathways that can affect gene expression in *A. actinomycetemcomitans*: 1) transcriptionally via QseBC; and 2) post-transcriptionally via the putative TA system.

transcriptome study which identified differentially regulated genes in biofilms showed that the *mqsR* gene was induced in *E. coli* biofilms (175), and follow-up studies corroborated the importance of MqsRA in biofilm formation (62). Additionally, deletion of the RelEB TA system resulted in a 2-fold decrease in biofilm formation at 24 hours in both rich and minimal media (113). Since biofilm formation and quorum sensing are intimately connected in *A. actinomycetemcomitans* (196), and we have shown that ORFs Aa00672-Aa00673 are part of the AI-2 regulon, it is possible that biofilm formation is influenced by this putative TA system in *A. actinomycetemcomitans*. Studies are under way to determine the role ORFs Aa00672-Aa00673 play in biofilm formation in *A. actinomycetemcomitans*.

A phenomenon closely linked to biofilm to formation is that of persister cell formation. Persister cells are a subpopulation of bacteria that are viable after treatment with lethal concentrations of antibiotics (123), but are not drug-resistant mutants because further culturing reverts them back to wild-type (200). Persister cells are typically found in biofilms and in stationary-phase cultures (123), and this phenotype contributes to the antibiotic-tolerance of biofilms (193). It is proposed that TA systems play a major role in persister cell formation, inducing a state of dormancy and allowing the cell to escape the effects of the antibiotics (89, 123). The MqsRA TA system in *E. coli* was the first to be linked to persister cell formation. Deletion of *mqsR* alone and the *mqsRA* locus resulted in a decrease in persister cell formation whereas production of MqsR/MqsA increased persistence (103). Additionally, *mqsR* was shown to be greatly induced in persister cells compared to nonpersister cells (193). Other toxin genes (e.g. *relE*, *higB*, *mazF*, *yafQ*, and *yoeB*) have also been shown to be highly induced in persister cells (98, 107, 193). Thus,

since ORFs Aa00672-Aa00673 in *A. actinomycetemcomitans* have sequence similarity to the toxin MqsR and the HigBA TA system, both of which are known to be involved in persistence, this putative TA system may potentially be involved in persister cell formation in *A. actinomycetemcomitans*. This suggests that ORFs Aa00672-Aa00672 may participate in AI-2-mediated biofilm formation by inducing persistence when the environment of the oral cavity becomes unfavorable (e.g. antibiotic treatment), allowing the cells to survive.

Although we show here that ORFs Aa00672 and Aa00673 appear to function as a TA system in *A. actinomycetemcomitans*, this TA system differs from canonical TA systems in several ways. First, unlike other TA systems, the toxin precedes the antitoxin in the operon in *A. actinomycetemcomitans*. This has only been reported in three other TA systems: *hicAB*, *higBA*, and *mqsRA*. Furthermore, the *higA* gene has its own promoter located with the *higB* gene. Second, in previously characterized TA systems, the toxin is basic while the antitoxin is acidic. The opposite is true in this TA system—the toxin is acidic (pI 5.18) and the antitoxin is basic (pI 8.95). And third, the antitoxin (114 residues) is larger than the toxin (84 residues) in *A. actinomycetemcomitans* whereas the toxin is normally larger. Thus, it appears that Aa00672-Aa00673 in *A. actinomycetemcomitans* differs from the traditional TA system known in other bacteria.

In summary, we have shown that ORFs Aa00672-Aa00673 in *A. actinomycetemcomitans* function as a TA system. Our results suggest that ORFs Aa00672-Aa00673 are part of the AI-regulon, residing downstream of the QseBC two-component system. Further characterization of this TA system in *A. actinomycetemcomitans* may provide a better understanding of the AI-2-dependent

quorum sensing pathway as well as new targets for the treatment of periodontal disease and other systemic infections associated with *A. actinomycetemcomitans*.

## CHAPTER FIVE: SUMMARY

LuxS is widely distributed throughout Gram-negative and Gram-positive bacteria (14, 189, 207, 240), suggesting that AI-2 functions as a universal quorum sensing signal that mediates intra- and interspecies communication (14, 215, 231, 240). Recent studies have proposed that AI-2 may not function as a quorum sensing signal in all bacteria, but instead may just play a metabolic role in the Activated Methyl Cycle (43, 240). Yet, there is also strong evidence that AI-2 behaves as a cell-to-cell communication signal, regulating cellular processes, such as biofilm formation, iron acquisition, and motility through cell-to-cell communication in species of bacteria that lack the conserved two-component response in *Vibrio* spp (62, 196). Thus, AI-2 may function via alternative signaling cascades in organisms such as *A. actinomycetemcomitans* and *E. coli*, which lack the dedicated two-component system in the Vibrionales.

The potential alternative AI-2 quorum sensing pathway that resides downstream from LsrR might be a mechanism through which species of bacteria, such as *A. actinomycetemcomitans* and *E. coli*, communicate without the presence of the dedicated two-component system in the Vibrionales. Gonzalez-Barrios et al demonstrated that Lsr-dependent importation of AI-2 regulates motility and biofilm formation in *E. coli* as well

as QseBC, an AI-2-dependent two-component signal transduction system (62). The genome of *A. actinomycetemcomitans* encodes a two-component signal transduction system that displays 70-80% sequence similarity to QseBC of *E. coli*. While the sequence similarity of QseC extends across the entire gene, the sequence similarity of QseB is only conserved in the receiver domain of the response regulator. The sequence similarity of the DNA binding domain of QseB in *A. actinomycetemcomitans* deviates significantly from its *E. coli* counterpart, which suggests that a different set of genes is regulated by QseB in the dental pathogen (154). This is consistent with the fact that QseBC is known to regulate motility in *E. coli* (60), whereas *A. actinomycetemcomitans* is a non-motile organism. Furthermore, QseBC exhibits significant sequence similarity to the *feuPQ* two-component system that regulates iron uptake in *Rhizobium leguminosarum*, *Sinorhizobium meliloti*, and *Brucella suis* (46). Iron acquisition dramatically influences biofilm formation in *A. actinomycetemcomitans*. We show here that AI-2 induces the QseBC two-component system in *A. actinomycetemcomitans*, and induction follows detection and internalization as a functional AI-2 receptor was needed. QseBC was also shown to regulate biofilm formation, iron acquisition, and *in vivo* virulence of *A. actinomycetemcomitans*. Thus, QseBC may couple the detection and/or importation of AI-2, either alone or in cooperation with LsrR, to downstream regulation of iron acquisition genes and biofilm formation in *A. actinomycetemcomitans* (150).

Additionally, Gonzalez Barrios et al also demonstrated that MqsR regulates expression of QseBC and that *mqsR* might reside upstream of *qseBC* in the AI-2 signaling cascade. Deletion of the *mqsR* gene in *E. coli* led to a 61-fold decrease in *qseBC* expression and to significantly decreased biofilm formation (62). Recently, MqsR was

demonstrated to be the toxin of a toxin/antitoxin system encoded by *mqsRA* (97, 102). This *mqsRA* toxin/antitoxin system is highly expressed in biofilms (60) and has been shown to function as a GCU-specific mRNA interferase (244). The genome of *A. actinomycetemcomitans* encodes several toxin/antitoxin systems, including open reading frames Aa00672 and Aa00673, which exhibit similarity to the toxin/antitoxin systems *mqsRA* and *mazEF*—both of which encode RNA interferases. Aa00672 and Aa00673 appear to reside in a pathogenicity island in the *A. actinomycetemcomitans* genome, suggesting that these genes may have been acquired by *A. actinomycetemcomitans*. Our results here show that Aa00672 and Aa00673 are co-expressed in an operon. We also show that Aa00673 is a toxin, causes reversible growth inhibition, and may function as an RNase; Aa00672 is its cognate antitoxin, negating the effects of the toxin. However unlike in *E. coli*, our studies show that induction of Aa00672-Aa00673 follows QseBC, indicating that QseBC controls Aa00672-Aa00673. Thus, Aa00672 and Aa00673 may influence the expression of QseBC, which, in turn regulates downstream expression of genes in AI-2-dependent quorum sensing.

This dissertation describes the AI-2-dependent quorum sensing circuit in the dental pathogen *A. actinomycetemcomitans*. Recent studies described in this dissertation suggest that an alternative quorum sensing exists in *A. actinomycetemcomitans* and potentially other species of bacteria. This regulatory transduction circuit may connect the detection and internalization of AI-2 by the Lsr and/or Rbs transporters to downstream regulators, such as QseBC and MqsR, which influence cellular processes that contribute to virulence, iron uptake, and biofilm formation. As a result, these alternative AI-2 quorum sensing pathways represent a viable target for novel therapeutic treatments to

control the populations of pathogens and biofilm formation. However, the prevalent distribution of LuxS and AI-2 among bacteria and the likelihood that AI-2 is needed for the establishment of commensal bacteria in the oral biofilm poses a challenge for developing new therapeutics which target AI-2 quorum sensing in bacteria. Previous studies have already reported the effects of differing AI-2 concentrations have on different organisms in the oral biofilm. For example, it has been shown that extremely low concentrations of AI-2 are needed for the association of some commensal organisms whereas Gram-negative oral pathogens produce much higher concentrations of AI-2 (58). As a consequence, developing therapeutic treatments which target the quorum sensing circuit of pathogens without affecting the commensal quorum sensing circuit might be a potential hurdle. Future studies are needed to provide a better understanding of how communication occurs among bacteria which lack the LuxPQUO signaling cascade found in the Vibrionales. This will help facilitate the development of specific quorum sensing inhibitors and may also reveal other cellular components important in the AI-2 quorum sensing circuit which may serve as viable targets for the treatment of biofilm-associated diseases.



## CHAPTER SIX: FUTURE DIRECTIONS

### **The role of the LsrR operon in AI-2-dependent quorum sensing:**

In *S. enterica*, the *lsr* operon is regulated by LsrR which binds to AI-2 that has been phosphorylated by LsrK (239). Furthermore in *E. coli*, LsrR has been shown to regulate other genes as well and may be involved in biofilm growth (62). In addition, LsrR may modulate AI-2 dependent regulation of QseBC. Similar to *E. coli*, the genome of *A. actinomycetemcomitans* possesses both the QseBC regulon as well as the LsrR operon. Previous research has shown that the periplasmic receptors, RbsB and LsrB, not only interact with AI-2 but are also essential for AI-2 dependent biofilm formation by *A. actinomycetemcomitans* (195). Both *lsrB* and *rbsB* are linked to operons—*lsrACDBFG* and *rbsDABCK*, respectively—that encode putative ABC type transporters and other gene products such as putative kinase and aldolase enzymes, which may modify or process AI-2 (88, 195). However, it is unknown whether the response circuit of AI-2 requires these functions or if these functions constitute a pathway to recycle AI-2 after the cellular response has been initiated by LsrB and/or RbsB. In addition, preliminary studies have shown that inactivation of *lsrK* resulted in reduced biofilm growth in *A. actinomycetemcomitans* as compared to wild-type cells and that *qseBC* expression is

reduced in the *lsrK* mutant. Thus, it is important address the hypothesis that the LsrR operon connects AI-2 detection and importation to the expression of QseBC in *A. actinomycetemcomitans*.

**Global regulation of QseBC in *A. actinomycetemcomitans*:**

This dissertation shows that the expression of QseBC is induced by the quorum sensing signal AI-2 in *A. actinomycetemcomitans* and induction of QseBC requires the AI-2 receptors, LsrB and/or RbsB. While the sequence similarity of QseC extends across the entire gene, the sequence similarity of QseB is only conserved in the receiver domain of the response regulator. The sequence similarity of the DNA binding domain of QseB in *A. actinomycetemcomitans* deviates significantly from its *E. coli* counterpart, suggesting that a different set of genes are influenced by QseB in the dental pathogen. This is consistent with the fact that QseBC is known to regulate motility in *E. coli*, whereas *A. actinomycetemcomitans* is a non-motile organism. Thus, in order to better understand the *qseBC* regulon, transcriptome sequencing will be completed comparing the expression profile of wild-type strain 652, *qseC* mutant, and *qseC* complemented strain. Since this approach will most likely generate numerous differentially expressed genes, the results will be initially prioritized by concentrating on the differentially expressed genes that are apt to play a critical role in biofilm formation. The expression of these genes will be confirmed by real-time PCR. The function of the genes that are chosen for further study will be determined by generating isogenic knockout strains and analyzing biofilm formation as previously described.

### **The *ygiW-qseBC* operon in *A. actinomycetemcomitans*:**

Sperandio et al previously demonstrated that the QseC protein in *E. coli* O157:H7 responds to both epinephrine/norepinephrine as well as the microbial signal AI-3, and that QseC may function in a one-to-many-branched signal transduction circuit that activates the response regulators QseB, QseF, and KdpE (82, 173). Additionally, the QseE sensor kinase was shown to be located downstream of QseC in the adrenergic signal transduction cascade (82). Attempts to identify the AI-3 molecule in *A. actinomycetemcomitans* extracts have been unsuccessful. Additionally, paralogs of QseE, QseF, and KdpE polypeptides were not identified in the genome of *A. actinomycetemcomitans*. Presently, the signal that *A. actinomycetemcomitans* QseC kinase senses is not known. We show in this dissertation that *qseBC* are co-transcribed together in an operon. We also have preliminary data that this operon also contains another gene, *ygiW*, which is located upstream of *qseB*, and is also co-transcribed with *qseBC*. *ygiW* is annotated as a periplasmic solute-binding protein in the *A. actinomycetemcomitans* genome, but it is unknown that this point how, or even if, *ygiW* integrates into the QseBC/AI-2 signaling transduction pathway. It is possible that *ygiW* detects a signal (possibly host stress hormones) and relays the signal to QseC, integrating microbe and host signaling. Further studies are needed to understand the role of *ygiW*.

### **The function of ORFs Aa00672-Aa00673 in *A. actinomycetemcomitans*:**

Thus far, the characterization of Aa00673 has been completed by cloning the gene into an expression vector and inducing its expression in *E. coli*. While this approach provided preliminary data about the function of Aa00673, it does not confirm its

functional role in *A. actinomycetemcomitans*. Consequently, it is critical that the function of ORFs Aa00672 and Aa00673 be studied in its native organism. In order to accomplish this, deletions of both of these genes will be constructed, deleting each gene alone and also deleting the entire putative TA system. The resulting mutants will then be analyzed in their ability to form biofilms, regulate iron uptake genes, and influence *in vivo* virulence. The expression of the QseBC two-component system will also be determined in these mutants in order to confirm previous results concerning the location of this putative TA system in the AI-2 regulon. Additionally, since MqsRA was shown to be a global regulator (62), it may be advantageous to also perform transcriptome sequencing on the Aa00672-Aa00673 mutant, comparing its transcripts to wild-type strain 652 and the Aa00672-Aa00673 complemented strain. These results will provide us with a greater understanding of genes that are regulated by this putative TA system and potentially involved in AI-2 regulon, highlighting other components that may serve as targets for therapeutic intervention of biofilm-associated diseases.

**Cellular stresses which induce ORFs Aa00672-Aa00673 in *A. actinomycetemcomitans*:**

Current hypotheses propose that TA systems are induced by cellular stress events, resulting in either programmed cell death (growth inhibition followed by cell death) or reversible cell growth inhibition (3, 32, 33, 165, 184, 185). However, the exact stresses that induce the activation putative TA system Aa00672-Aa00673 in *A. actinomycetemcomitans* are currently obscure. Thus, in order to elucidate the signals which activate this potential TA system, *A. actinomycetemcomitans* cells will be grown

under different stress conditions (e.g. iron-deplete environment, nutrient-limited environment, anaerobic environment, temperature stress, pH stress, antibiotic treatment, etc.), and the expression of Aa00672-Aa00673 will be analyzed by real-time PCR. This will provide preliminary evidence on the signals which activate this TA system. It may also be of interest to analyze the expression of this TA system in both a planktonic culture as well as a biofilm, especially since *A. actinomycetemcomitans* is present in the oral cavity in an adhered microbial community, and it is possible that this putative TA system may be induced in a biofilm setting. Additionally, the genome of *A. actinomycetemcomitans* encodes several other identified putative TA systems. Thus, it may also be advantageous to determine the signals that also activate these systems.

**The function of other TA systems in *A. actinomycetemcomitans*:**

Prokaryotes appear to encode several TA systems in their genome. While not all TA systems act in the same manner, it is possible that each putative TA system allows the cell to respond to specific stresses, allowing for the achievement of one major, redundant function: cell survival. For example, MqsR, MazF, YafQ, and ChpB cleave mRNA at the GCU (244), ACA (252), AAA (171), and ACY (Y = A or G) (59) sites, respectively. However, MazF degrades most mRNAs under antibiotic stress, but the mRNAs which are not degraded are utilized to produce a pool of specific proteins—some are used for toxicity while some are used for cell survival (5). Similarly, MqsR specifically cleaves mRNAs and enriches the mRNA pool for those that encode for DNA replication inhibitor CspD as well as other stress proteins (e. g. RpoS, ClpP, ClpB, and CstA) that help the cell cope with the stress and survive (179). Thus, it seems that under cellular stress, the role

of each TA system may be to not only reduce growth by a specific mechanism, but also to promote the survival of a population of cells.

Although there have been multiple putative TA systems identified in *A. actinomycetemcomitans*, the role of only one—Aa00672-Aa00673—has been investigated and potentially linked to AI-2-dependent quorum sensing. It would be interesting to understand how other TA systems function, whether they are involved in quorum sensing, and if each works towards the same common goal of promoting cell survival. Additionally, it would be interesting to show if any of these putative TA systems signal through each other, forming a network of TA system signaling.

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## CURRICULUM VITAE

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### **Education & Training:**

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Louisville, KY (expected graduation date is December 16, 2011)  
Field of Research: Characterization of the AI-2-dependent quorum sensing  
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M.S., Immunology & Microbiology, University of Louisville 2006-2008  
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### **Educational/Teaching Experience:**

Student Microbiology & Immunology Student (MISO) Mentor 2010-Present  
University of Louisville, Department of Microbiology & Immunology

Clinical Microbiology Laboratory TA, Bellarmine University Fall 2009  
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Volunteer Educator at the Louisville Science Center, Louisville, KY 2008-2009

### **Mentorship:**

- Abhishek Amitkumar Patel, D.M.D. (Oral Biology Masters Student) 2011-Present  
Project: Inhibiting the interaction between *Streptococcus gordonii* sspB and *Porphyromonas gingivalis* Mfa1 using synthetic compounds.
- Naga Srinija-Gummadi, D.M.D (Oral Biology Masters Student) 2011-Present  
Project: Utilizing synthetic compounds to inhibit biofilm formation of *Porphyromonas gingivalis*.
- Whitney Weigel, B.A. (Microbiology & Immunology Ph.D. candidate) 2011-Present  
Project: Functional characterization of TA systems in *Aggregatibacter actinomycetemcomitans*.
- Blair Schneider, B.S. (Microbiology & Immunology Ph.D. candidate) 2011-Present  
Project: Cellular stresses that induce TA Systems in *Aggregatibacter actinomycetemcomitans*.
- Porscha Jefferson, B.A. (DMD/Oral Biology Masters Student) 2010-Present  
Project: Iron Selectivity and Acquisition in *Aggregatibacter actinomycetemcomitans*.
- Emily Revels, B.A. (DMD/Oral Biology Masters Student) 2009-Present  
Project: Molecular Detection of Cytomegalovirus and Epstein-Barr virus in Irreversible Pulpitis and Apical Periodontitis.

### **Service:**

- University of Louisville Graduate Student Council 2006-Present
- University of Louisville, MISO 2006-Present
- MISO President 2010-2011
- MISO Graduate Student Council Representative 2010-2011
- MISO Co-President 2009-2010
- MISO Student Representative on Admissions Committee 2008-2009
- MISO Student Representative on Curriculum Committee 2007-2008
- Berea College Young Alumni Advisory Council 2011-Present

### **Membership in Professional and Scientific Societies:**

- Golden Key National Honor Society 2009-Present

International Association for Dental Research (IADR)	2009-Present
The American Association for the Advancement of Science (AAAS)	2009-Present
American Society of Microbiology (ASM)	2008-Present

### **Awards & Honors:**

2 <sup>nd</sup> place in Doctoral Basic Science Division at Research!Louisville	October 2011
Graduate Student Council Travel Award	March 2011
Arnold Ravin-Muriel Rogers Fellowship, Travel Award Wind River Conference on Prokaryotic Biology	June 2010
Graduate Student Council Travel Award	June 2010
Research!Louisville Travel Award	January 2010
2 <sup>nd</sup> place in Doctoral Basic-Science Division at Research!Louisville	October 2009

### **Invited Talks/Presentations:**

January 2010— Gordon Conference of Sensory Transduction in Microorganisms, Ventura, CA. QseBC and AI-2 control biofilm formation and *in vivo* virulence of *Aggregatibacter actinomycetemcomitans*.

June 2010—Wind River Conference on Prokaryotic Biology, Estes Park, CO. The QseBC two-component system and autoinducer-2 regulate biofilm formation and *in vivo* virulence of *Aggregatibacter actinomycetemcomitans*.

March 2011—International Association for Dental Research Conference, San Diego, CA. Characterization of a toxin-antitoxin system involved in AI-2-dependent quorum sensing in the dental pathogen *Aggregatibacter actinomycetemcomitans*.

### **Grants:**

Funding Source: NIH/NIDCR

Grant Number: DE020240-01 (Ruth L. Kirschstein NRSA-F31)

Title of Research: AI-2-dependent quorum sensing in *Aggregatibacter actinomycetemcomitans*.

Principal Investigator: **Elizabeth A. Novak**

Sponsor: Donald R. Demuth                      Co-Sponsor: George N. Hajishengallis

Support per year (maximum of 3 years): \$29,259.00

### **Publications:**

Daep, C.A., **Novak, E.A.**, Lamont, R.J. and Demuth, D.R. 2011. Structural dissection and *in vivo* effectiveness of a peptide inhibitor of *Porphyromonas gingivalis* adherence to *Streptococcus gordonii*. *Infect. Immun.* 79:67-74.

Daep, C.A., **Novak, E.A.**, Lamont, R.J. and Demuth, D.R. 2010. Selective substitution of amino acids limits proteolytic cleavage and improves the bioactivity of an anti-biofilm peptide that targets the periodontal pathogen, *Porphyromonas gingivalis*. *Peptides.* 31:2173-2178.

**Novak, E.A.**, Shao, H., Daep, C.A. and Demuth, D.R. 2010. Autoinducer-2 and QseC control biofilm formation and *in vivo* virulence of *A. actinomycetemcomitans*. *Infect. Immun.* 78:2919-2926.

### **Book Chapters:**

D.R. Demuth, **E.A. Novak**, and H.J. Shao. 2011. Alternative autoinducer-2 quorum sensing response circuits: impact on microbial community development. In *Oral Microbial Communities: Genomic Inquiry and Interspecies Communication*. Paul E. Kolenbrander, Ed. ASM Press, Washington, D.C., USA.

### **Abstracts:**

HanJuan Shao, **Elizabeth A. Novak**, Deanna James and Donald R. Demuth. 2006. Heterologous autoinducer-2 signals influence biofilm growth of *A. actinomycetemcomitans*. Midwest Microbiology Meeting, Cincinnati, OH.

HanJuan Shao, **Elizabeth A. Novak**, Deanna James and Donald R. Demuth. 2007. Autoinducer-2 is required for biofilm growth of *A. actinomycetemcomitans* on a saliva coated surface. IADR conference, New Orleans, LA.

**Elizabeth A. Novak**, HanJuan Shao and Donald R. Demuth. Oral infection with *Aggregatibacter (Actinobacillus) actinomycetemcomitans* results in alveolar bone loss in mice. Research!Louisville, Louisville, KY.

**Elizabeth A. Novak**, HanJuan Shao and Donald R. Demuth. 2009. AI-2 dependent quorum sensing is required for iron acquisition and virulence in the dental pathogen *Aggregatibacter actinomycetemcomitans*. IADR conference, Miami, FL.

**Elizabeth A. Novak**, HanJuan Shao, and Donald R. Demuth. 2009. QseBC and autoinducer-2 control biofilm formation and *in vivo* virulence in the dental pathogen *Aggregatibacter actinomycetemcomitans*. Research!Louisville, Louisville, KY.

**Elizabeth A. Novak**, HanJuan Shao, and Donald R. Demuth. 2009. Biofilm formation and *in vivo* virulence are controlled by the QseBC two-component system and autoinducer-2 *in vivo* virulence in the dental pathogen *Aggregatibacter actinomycetemcomitans*. KY/TN American Society for Microbiology Branch Meeting, Knoxville, TN.

**Elizabeth A. Novak**, HanJuan Shao, and Donald R. Demuth. 2010. QseBC and autoinducer-2 control biofilm formation and *in vivo* virulence of *Aggregatibacter actinomycetemcomitans*. Gordon Conference of Sensory Transduction in Microorganisms, Ventura, CA.

**Elizabeth A. Novak**, HanJuan Shao, and Donald R. Demuth. 2010. QseBC and autoinducer-2 control biofilm formation and *in vivo* virulence of *Aggregatibacter actinomycetemcomitans*. National Institute for Dental and Craniofacial Research (NIDCR) Trainee Poster Session, Washington D.C.

**Elizabeth A. Novak**, HanJuan Shao, and Donald R. Demuth. 2010. QseBC and autoinducer-2 control biofilm formation and *in vivo* virulence of *Aggregatibacter actinomycetemcomitans*. American Association for Dental Research (AADR), Washington D.C.

Porscha Jefferson, **Elizabeth A. Novak**, and Donald R. Demuth. 2010. The Role of Iron in the Growth of the Dental Pathogen *Aggregatibacter actinomycetemcomitans*. Research!Louisville, Louisville, KY.

**Elizabeth A. Novak** and Donald R. Demuth. 2010. Characterization of a Toxin/Antitoxin System Involved in AI-2-Dependent Quorum Sensing in the Dental Pathogen *Aggregatibacter (Actinobacillus) actinomycetemcomitans*. Research!Louisville, Louisville, KY.

**Elizabeth A. Novak** and Donald R. Demuth. 2011. Characterization of a Toxin-Antitoxin System Involved in AI-2-Dependent Quorum Sensing in the Dental Pathogen *Aggregatibacter (Actinobacillus) actinomycetemcomitans*. National Institute for Dental and Craniofacial Research (NIDCR) Trainee Poster Session, San Diego, CA.

**Elizabeth A. Novak** and Donald R. Demuth. 2011. Characterization of a Toxin-Antitoxin System Involved in AI-2-Dependent Quorum Sensing in the Dental Pathogen *Aggregatibacter actinomycetemcomitans*. Graduate Research Symposium, Louisville, KY.

Porscha Jefferson, **Elizabeth A. Novak**, and Donald R. Demuth. 2011. The Role of Iron in the Growth of the Dental Pathogen *Aggregatibacter actinomycetemcomitans*. Research!Louisville, Louisville, KY.

Naga Srinija-Gummadi, **Elizabeth A. Novak**, and Donald R. Demuth. 2011. Conditions for *S. gordonii* - *P. gingivalis* biofilm formation. Research!Louisville, Louisville, KY.

**Elizabeth A. Novak** and Donald R. Demuth. 2011. Identification of the AI-2-Dependent Quorum Sensing in the Dental Pathogen *Aggregatibacter actinomycetemcomitans*. National Institute for Dental and Research!Louisville, Louisville, KY.

Porscha Jefferson, **Elizabeth A. Novak**, and Donald R. Demuth. 2011. The Role of Iron in the Growth of the Dental Pathogen *Aggregatibacter actinomycetemcomitans*. Hinman Student Research Symposium, Memphis, TN.