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AI-2 signaling in the *Streptococcus anginosus* group

By

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Preface

The present thesis and appending papers (I-III) are submitted in partial fulfillment of the requirements for the Degree of Philosophiae Doctor at the Faculty of Dentistry, University of Oslo, Oslo, Norway.
The papers will be referred to by their Roman numerals throughout the thesis text in the following order

Paper I: Fernanda C. Petersen, **Nibras A.M. Ahmed,** Alioddin Naemi and Anne Aamdal Scheie. 2006. LuxS-mediated signalling in *Streptococcus anginosus* and its role in biofilm formation. Antonie van Leeuwenhoek. 90: 109-121.

Paper II: **Nibras A.M. Ahmed,** Fernanda C. Petersen and Anne Aamdal Scheie. 2008. Biofilm formation and AI-2 signaling in *Streptococcus intermedius*: role of thermal and pH factors. Oral Microbiology and Immunology. *In press*.

Paper III: **Nibras A.M. Ahmed,** Fernanda C. Petersen and Anne Aamdal Scheie**.** 2007**.** AI-2 quorum sensing affects antibiotic susceptibility in *Streptococcus anginosus.* Journal of Antimicrobial Chemotherapy. 60: 49–53.

Introduction

1. Anginosus streptococci in health and disease

Streptococci are members of the vast indigenous flora that inhabit the human oral cavity, upper respiratory-, gastrointestinal- and urogenital-tract. Streptococci are the first predominant colonizers to establish in the oral cavity of neonates, subsequently mediating attachment of other bacterial species to host tissue [41].

Streptococcus anginosus, *Streptococcus intermedius* and *Streptococcus constellatus*; collectively termed the *Streptococcus anginosus group*, are phylogenetically related streptococci [99]. The *S. anginosus* group, occasionally termed "*Streptococcus milleri* group" display hemolytic and serologic diversity, yet share core physiological traits [76]. In the oral cavity, the *S. anginosus* group strains are commensally present in saliva, supra- and sub-gingival plaque, with *S. anginosus* being the most prevalent [12, 99, 101]. The *S. anginosus* group may also be isolated from various other body sites, including the throat and vagina [99].

Isolates of *S. anginosus* are commonly detected in root canals of primary and secondary endodontic infections [8, 25], whereas *S. intermedius* may also colonize oral surgical sutures and periodontal lesions [67, 83]. Laine *et al.* displayed that members of the *S. anginosus* group were also prevalent in cases of failed dental implants [43]. In contrast to other viridans streptococci, numerous studies and case reports also display frequent isolation of the *S. anginosus* group from oral [20, 46] head and neck- [30], central nervous system-, hepatic-, and pulmonary-abscesses [2, 68, 74]. Due to limitations of traditional identification methods, studies frequently report the presence of *S. anginosus* group collectively. However, genetic studies using 16s rRNA indicate that *S. anginosus* and *S. intermedius* are not equally associated with abscess formation. *S. intermedius* is frequently detected as a solitary isolate in abscesses and are associated with deep-seated, highly invasive, purulent infections, especially that of the central nervous system [12, 13, 100]. *S. anginosus,* on the other hand, is more commonly associated with superficial abscess formation in a poly-microbial environment [12]. *S. anginosus* is often isolated from septicemia, gastrointestinal- and genitourinary- tracts abscesses [12, 13, 100].

The presence of the *S. anginosus* group, particularly *S. anginosus*, may pose a risk for developing infective endocarditis [40, 71, 104]. Woo *et al.* found higher mortality rates in *S. anginosus* endocarditis than in cases caused by other streptococci [104]. Furthermore, the

pyogenic tendency of the *S. anginosus* group may induce suppurative cardiac complications in endocarditis patients [31, 45]. In addition to heart lesions, *S. anginosus* DNA is also more frequently detected in cancerous and pre-cancerous tissues of oral squamous carcinoma, esophageal and gastric cancer than from normal control tissue samples [60, 78, 80]. Various studies suggest that *S. anginosus* infections may invoke the dysplasia and the carcinogenic process by inducing inflammation and damaging human DNA [79, 80 61, 78].

S. anginosus group strains may enter the blood stream, lymphatic system or submucosal connective tissue following surgical dental procedures, chronic oral infections or even simple daily hygiene procedures [71, 73, 77, 92]. Experimental abscess induction in mice from subcutaneously injected human plaque samples revealed that the *S. anginosus* group was the most prevalent bacteria despite their initial low presence in the injected dental plaque [66]. *S. intermedius* from septic oral lesions may also predispose to hepatic, intracranial or pulmonary abscesses [10, 97]. *S. anginosus* DNA isolated from oral squamous cell carcinoma corresponds to *S. anginosus* DNA found in the patient's dental plaque, suggesting that *S. anginosus* infection in cancer tissue may be derived from the individual's dental plaque [80]. Renvert *et al.* observed that periodontitis patients with high *S. intermedius* serum load might also be more subjected to contract acute coronary disease [71]. Thus, despite being commensal organisms, members of the *S. anginosus* group display wide pathogenic potential that necessitates further investigations.

2. Quorum Sensing

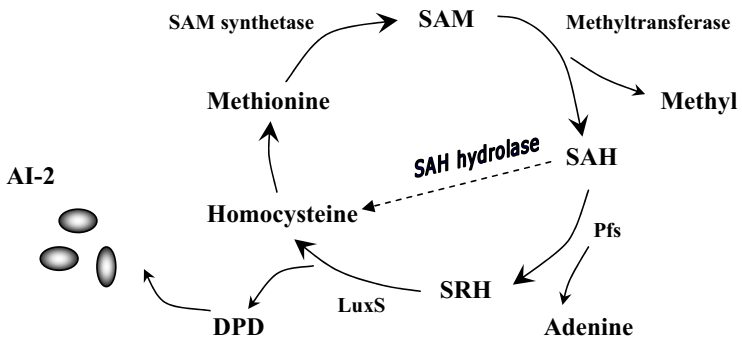
Quorum sensing is the process of signal dependent bacterial gene expression in response to their population and external stimuli. Thus, signal dependent gene regulation gives bacteria the flexibility of altering gene expression, in unison, as a consequence of changes in their community or surrounding environmental conditions.

Tomasz (1965) demonstrated that extracellular substances synthesized by bacteria controlled pneumococcal competence in a synchronized fashion, providing the first evidence of a signal regulated population behavior in bacteria [93]. In 1970, Nealson *et al.* detected a growth-dependent pattern of light emission in marine bacterium *Vibrio fischeri* [63]. Accumulation of diffusible, auto-synthesized extracellular signals, called autoinducers (AI), in the bacterial culture medium triggered transcription of the luminescence gene (*lux*). The luminescence increases 1000 folds beyond certain culture concentrations, suggesting that

bacteria regulate genes in a density dependent manner [37]. Addition of mature culture fluid stimulated luminescence in diluted cultures of *V. fischeri*, indicating a positive association between light intensity and autoinducer concentration [62]. Later, Greenberg *et al.* reported that supernatants from other non-bioluminescent marine bacteria also stimulated light production in *Vibrio harveyi* [27]. Bassler *et al.* subsequently described a signal system with a second set of autoinducers in *V. harveyi*, designated autoinducer-2 (AI-2) [4]. Cell-free suspensions of several other Gram-positive [6, 52, 58] and Gram-negative [1, 5, 11, 21, 35] bacterial species induced luminescence in *V. harveyi*, suggesting AI-2 to be involved in a dual intra- and inter-species communication system [3]. Luminescence induction in *V. harveyi* paved the way for surveying AI-2 production and investigating potential communication within and across bacteria.

The AI-2 signals are byproducts of the bacterial activated methyl cycle (Figure 1). The activated methyl cycle recycles S-adenosylmethionine (SAM), the main methyl donor in eubacterial, archaebacterial and eukaryotic cells [15, 94]. SAM is converted to methyl and S-adenosylhomocysteine (SAH), a highly toxic metabolite that inhibits SAM-dependent methyltransferase. SAH may be detoxified through a two-step enzymatic process whereby S-adenosylhomocysteine nucleosidase (Pfs enzyme) converts SAH to adenine and S-ribosylhomocysteine (SRH). S-ribosylhomocysteinase (LuxS enzyme) then catalyzes SRH to homocysteine and 4,5-dihydroxy-2,3-pentanedione (DPD) [81, 94]. Alternatively, in several organisms including *Eukarya*, *Archaea*, *α-Proteobacteria*, *Actinobacteria* and *Cyanobacteria*, SAH is recycled in one-step by SAH hydrolase (SahH) to homocysteine, thereby bypassing DPD production [15, 86, 94].

Figure 1: The Activated Methyl Cycle



DPD is a highly reactive product that spontaneously rearranges to form a range of structurally similar cyclic derivatives, recognized by bacterial species and collectively termed AI-2 signals [81]. Therefore DPD is regarded as an AI-2 precursor common to all AI-2 signaling bacteria. LuxS is coded for by *luxS*, a well conserved gene in several species [89]. Genome analyses indicate that the metabolic pathway and LuxS enzyme necessary for AI-2 synthesis is widely conserved, however, bacteria may utilize different AI-2 sensing mechanisms [86]. In order to exist in either a synergistic or antagonistic relationship with other organisms, bacteria may also display alterations in AI-2 uptake or degradation patterns [95, 105].

Various pathogens including *Streptococcus pyogenes, Streptococcus pneumoniae, Vibrio cholerae* and *Escherichia coli* display AI-2 signaling associated with virulence factors including biofilm formation, toxin production and bacterial-host interactions [26, 36, 52, 54, 110].

Data from microarray studies on *E. coli* suggest LuxS to be a global bacterial regulator that may influence protein synthesis, stress response and cell division [84]. AI-2 associated behavior may also correspond to certain environmental challenges. In the oral cavity, AI-2-like activity has been detected in the periodontal pathogens *Porphyromonas gingivalis, Actinobacillus actinomycetemcomitans, Fusobacterium nucleatum* and *Prevotella intermedia* [11, 21, 22]. *Streptococcus mutans* and *Streptococcus gordonii* also display AI-2 activity relevant to inter and intra-species biofilm formation and carbohydrate metabolism [57, 109]. However, since streptococci are primary colonizers of host tissue, more comprehensive investigations on the role of AI-2 signaling in streptococci are needed.

3. Biofilms and host environments

Until late 20th century, bacteria were thought to exist in a solitary state, incapable of performing complex tasks as those in higher hierarchy organisms. It is now apparent that, whether in our bodies or in nature, bacteria frequently exist in a cooperative society of sessile, matrix-enclosed aggregates known as biofilms. Biofilm formation is a gene-controlled process for the purpose of bacterial survival that is initiated by attachment, cell division and aggregation of primary colonizing species from the planktonic population [34]. Gradually, a climax community develops as early colonizers provide attachment substrates for the subsequent colonizers.

The oral cavity and other intrinsic body surfaces may harbor a miscellaneous combination of microorganisms in an ecological equilibrium with the host. When the equilibrium is

shifted to the advantage of specific organisms, disease and infection may ensue [55]. Thus, medical-device contamination, infective endocarditis, cystic fibrosis pneumonia, dental caries, endodontic and periodontal infections occur in sequelae to establishment of harmful microbial complexes [25, 28, 55, 83]. Restricted penetration, persister formation, antibiotic inactivation and genetic stress response render biofilms resilient to innate host defenses and antimicrobial agents [39, 53]. For example, *Pseudomonas aeruginosa* biofilms are 10-1000 times more resistant to antibiotic treatment than planktonic cells [64]. *S. anginosus* biofilm cells from dental root canals are 28 times more likely to survive after alkaline treatment than their planktonic counterparts [9]. Thus, biofilms represent a critical obstacle in the treatment of chronic infections.

A wide range of microenvironments may exist within the biofilm structure. Host environments may also vary in oxygen tension, temperature, pH and nutrient availability. Low oxygen levels in deep-seated infections may attract anaerobic bacteria. In the oral cavity, temperatures shift from alveolar bone tissue to the oral mucosa and from posterior to anterior regions [96]. Infection and inflammation of bacterial origin is frequently accompanied by a febrile response or hypothermia. For instance, fever may accompany periodontitis and purulent infections as a result of endogenous pyrogen interleukin-1 accumulation [42, 56]. Variations in pH may influence the ecological selection of bacterial species under health and diseased conditions [55]. The alkaline shifts following periodontal inflammation, for example, may stimulate growth of Gram negative bacteria while acidic milieus may favor colonization of cariogenic bacteria [55]. Abscesses of bacterial etiology may also predispose to acidic environments [82].

In addition to being causative agents in disease, streptococci represent the majority of primary colonizers in oral biofilms [65]. Therefore, potential interference in streptococcal biofilm formation may hinder attachment of successive organisms. Studies of *S. mutans* and *S. gordonii luxS* mutants display alteration in biofilm abundance and microscopic architecture [6, 57, 58, 109]. However, no studies have investigated the role of AI-2 signaling in biofilm formation by the *S. anginosus* group.

4. Antibiotics

Antibiotics are chemotherapeutic agents that possess antimicrobial activity at low concentrations, inflicting minimum toxicity to the host. Antibiotics target basic bacterial

cellular structures causing either a lethal (bactericidal) or suppressive (bacteriostatic) effect. Generally, antibiotics are classified into three categories (1) antibiotics that target cell-wall synthesis: consisting of β-lactams and vancomycin, (2) antibiotics that target RNA and protein synthesis: comprising macrolides, tetracycline and aminoglycosides, and (3) antibiotics that target bacterial DNA replication and repair: known as fluoroquinolones [98].

Bacteria may resist the antibiotic effect through efflux pumps, antibiotic inactivation or modification of susceptible targets [98]. Often a subpopulation of bacteria may survive antibiotic doses by adopting a temporary, dormant, low-metabolic phenotype known as persisters [85]. Altered antibiotic susceptibility may also be attributed to induction of bacterial stress genes [50]. Interestingly, Fux *et al.* observed that antibiotic tolerance increased when bacterial pellets were resuspended in concentrated versus diluted supernatants, proposing a possible role for accumulation of metabolic waste or intercellular signals in antibiotic stress [23].

Bacterial resistance to various antibiotic treatments is spreading globally [98]. *S. anginosus* group infections remain moderately susceptible to ampicillin and erythromycin, although resistant patterns are emerging [38, 49]. Ampicillin is a β -lactam that acts on penicillin-binding proteins preventing normal cross-linking of peptide chains in the peptidoglycan layer and rendering it susceptible to lyses by changing osmotic pressure [98]. Erythromycin is a bacteriostatic antibiotic macrolide that suppresses protein synthesis by targeting the 23S rRNA and relevant proteins in the peptidyl transferase center of the ribosome, thereby preventing elongation of protein chains [98].

Pseudomonas aeruginosa is innately resistant to a wide spectrum of antibiotics due to the presence of multi-drug efflux pumps. Evans *et al*. showed that these efflux systems also regulate intracellular AI-1 levels, AI-1 production and subsequent expression of AI-1 dependent virulence genes [19]. However, no studies have yet investigated whether antibiotic susceptibility is associated with AI-2 signaling.

Major aim:

To investigate AI-2 signaling in *S. anginosus* and *S. intermedius*.

Specific aims

1. To investigate AI-2 levels in culture supernatants of oral streptococci during growth, with special focus on *S. anginosus* and *S. intermedius*.
2. To study the possible role of AI-2 signaling in *S. anginosus* and *S. intermedius* biofilm formation under different environmental conditions.
3. To examine the involvement of AI-2 signaling in *S. anginosus* antibiotic susceptibility.

Summary of results

1. AI-2 production and *luxS* identification (Paper I and II)

The *S. anginosus* group was investigated for its ability to produce detectable AI-2 signals. Supernatants were collected from the *S. anginosus* group and other oral streptococci at various growth phases. The supernatants were exposed to the AI-2 biosensor *V. harveyi* BB170 and bioluminescence was measured. All oral streptococci, including the *S. anginosus* group, exhibited detectable levels of AI-2 in culture supernatants (Paper I). When comparing AI-2 bioluminescence induction among oral streptococci, the *S. anginosus* group showed marked AI-2 activity reaching optimum levels at early exponential phase (Paper I and II).

The *luxS* gene was identified in *S. anginosus* and *S. intermedius* by PCR amplification and sequencing with primers designed to anneal with conserved *luxS* regions in other streptococci. Real-time PCR displayed that maximum *S. anginosus luxS* expression preceded peak AI-2 bioluminescent induction in *V. harveyi*.

The *luxS* gene which encodes LuxS was inactivated in *S. anginosus* and *S. intermedius*. The resultant mutants were employed in functional analysis of the *S. anginosus* group AI-2 /LuxS signaling, biofilm formation and antibiotic susceptibility.

2. Role of AI-2 in growth and biofilm formation (Paper I and II)

The role of AI-2/LuxS in *S. anginosus* and *S. intermedius* biofilm formation was investigated. The *luxS* mutants SA001 and SI006 formed significantly less biofilm compared to their respective *S. anginosus* and *S. intermedius* wild type phenotypes (50 % and 30 %, respectively). Inactivation of *luxS* did not influence planktonic growth in *S. anginosus* or *S. intermedius*, highlighting the role of AI-2 communication in biofilm formation and not in growth. When observing biofilm architecture under scanning electron microscopy, *S. anginosus* displayed higher aggregation patterns compared to the *luxS* mutants, indicating a role for AI-2 signaling in biofilm structural complexity (Paper I). To confirm the role of AI-2 signaling in biofilm formation, the AI-2 precursor (DPD) was supplemented to the *S. intermedius luxS* mutant. Biofilm formation increased in *S. intermedius luxS* mutant to wild

type levels, indicating that biofilm defects were actually due to defective AI-2 signaling in the *luxS* mutants (Paper II).

3. Influence of environmental factors on AI-2 signaling, growth and biofilm formation (Paper I and II)

The role of AI-2 in *S. anginosus* and *S. intermedius* biofilm formation was investigated under various atmospheric, thermal, acidic and pH levels. *S. anginosus* wild type and its *luxS* mutant biofilm formation were favored under an aerobic atmosphere. *S. anginosus luxS* mutant displayed, however, significant defective biofilm formation compared to its wild type counterpart under both aerobic and anaerobic atmospheres (Paper I). Growth and biofilm formation were enhanced in *S. intermedius* wild type and its *luxS* mutant under warmer and more acidic environments (Paper II). At various pH levels, the *luxS* mutant formed less biofilm compared to *S. intermedius* wild type, however, the role of AI-2/LuxS in biofilm formation was not detected at temperatures other than 37 °C. Bioluminescence induction in *V. harveyi* confirmed that at 37 °C, *S. intermedius* displayed higher AI-2 levels than at other temperatures.

4. The role of AI-2 in antibiotic susceptibility (Paper III)

We wanted to test whether AI-2 influenced antibiotic susceptibility in *S. anginosus*. *S. anginosus* wild type and its *luxS* mutant were exposed to sub-inhibitory concentrations of ampicillin and erythromycin. We observed, for the first time that AI-2 signaling affects antibiotic susceptibility to ampicillin and erythromycin. *S. anginosus luxS* mutant displayed higher susceptibility to sub-inhibitory antibiotic concentrations, showing reduced growth and viability compared to the wild type. Supplementation with *S. anginosus* wild type supernatants significantly increased growth of *S. anginosus luxS* mutant in the presence of ampicillin or erythromycin. AI-2 precursor complementation significantly increased viability of *S. anginosus luxS* mutant when exposed to either antibiotic.

General Discussion

1. AI-2 in the *S. anginosus group* and other oral streptococci

In this study we addressed the presence of AI-2 in the major early colonizers of the oral cavity, namely, oral streptococci. Since most studies had focused on AI-2 communication in *S. mutans*, and *S. gordonii*, information on AI-2 production by other oral streptococci remained scarce at the commencement of this study. We confirmed that AI-2 production is widespread among oral streptococci indicating that AI-2 intercellular communication may be broadly employed among these species (Paper I). Generally, AI-2 levels in oral streptococci were pronounced during early-mid exponential phase. Similarly, a trend of early AI-2 expression has been encountered in other bacterial species [1, 7, 32, 48]. Compared to the other streptococci, the *S. anginosus* group displayed prominent AI-2 induction especially at early growth phase. Early accumulation of AI-2 signals may play an essential role in commencing community regulated behavior in competitive oral environments. Since the *S. anginosus* group is found in normal oral flora and in infectious environments, we also inquired whether growth at physiological temperatures between 35 ° C and 39 ° C may influence the pattern of AI-2 production in *S. intermedius*. We observed that growth at average body temperatures (37 ° C) yielded 4-5 fold greater extracellular AI-2 levels at early exponential phase than growth below or above this temperature (Paper II). Thus, temperature change (hyperpyrexia or hypopyrexia) and other cardinal symptoms of infection, may affect bacterial signaling patterns.

V. harveyi bioluminescence assay is dependent on the presence of extracellular AI-2 or AI-2- like molecules in the medium. When AI-2 binds to the periplasmic protein receptor (LuxP), AI-2 activates the *lux* operon through a signal transduction cascade [94]. The level of extracellular AI-2 may vary throughout growth depending, for instance, on production, binding/uptake and degradation rates. Therefore, higher AI-2 levels may be a result of accelerated AI-2 biosynthesis, reduced binding/uptake or repressed degradation. The decline in AI-2 sensing at stationary phase may be due to AI-2 degradation, altered expression of the AI-2 synthase (LuxS) or altered environmental conditions [88, 107]. In addition to actual changes in AI-2 levels, the bioluminescent induction in *V. harveyi* is influenced by several factors. For example, various studies have shown that light induction may vary with pH and glucose availability in response to catabolic repression [16]. Media components may also define AI-2

levels and bioluminescence as a consequence of nutrient stress or metabolic waste accumulation [1, 35, 87, 88]. Furthermore, the variation in *V. harveyi* BB170 bioluminescence may be a consequence of structural difference in AI-2 forms produced by different bacterial species. Lowery *et al*. observed that various structural variants of DPD, the AI-2 precursor, may all interact with the LuxP sensor protein in *V. harveyi*. However, molecules with distant deviation from the DPD core structure resulted in lower bioluminescent induction [51]. DPD is a highly reactive compound that rearranges into several distinct forms of AI-2, structurally defined by the surrounding environment [59]. Therefore, bacteria that produce AI-2 signaling activity, as assayed by the *V. harveyi* bioluminescence assay, do not necessarily secrete AI-2 in the same chemical form. When growing in a multispecies environment, bacterial species may recognize particular forms of AI-2. For example, the boron containing AI-2 produced by *V. harveyi* is not recognizable by *Salmonella typhimurium* AI-2 sensor [59]. In a multispecies environment, a wide spectrum of AI-2 molecules would allow bacteria the advantage of selective communication with other species [59, 81].

2. *S. anginosus* and *S. intermedius luxS*

Since the discovery of AI-2 production in *V. harveyi*, several bacterial species have shown similar AI-2 communication triggered by *luxS* homologues [106]. In this study, conserved *luxS* regions in other streptococci were used to identify the presence of *S. anginosus* and *S. intermedius luxS* (Paper I and II). The predicted LuxS protein of *S. intermedius* (Genebank, accession number DQ836241) displayed 90 % identity and 96 % similarity to *S. anginosus* LuxS (Genebank, accession number DQ067600, Paper I). In both species, LuxS displayed more than 80 % homology to LuxS orthologues of other sequenced streptococci, including *S. pneumoniae*, *S. pyogenes, S. mutans*, *S. gordonii* and *Streptococcus oralis*. All conserved amino acids involved in *Bacillus subtilis* LuxS enzymatic activity [29] were identified in *S. anginosus* and *S. intermedius* LuxS.

Genetic inactivation of *S. anginosus* and *S. intermedius luxS* offer the advantage of testing phenotypic changes in AI-2 deficient mutants compared to their wild type counterparts. Therefore, gene interruption using plasmid chromosomal integration was used to obstruct *luxS* expression in *S. anginosus* and *S. intermedius* (Paper I and II). The plasmid carried a kanamycin resistance cassette to isolate *luxS* mutants from wild type bacteria [91].

S. anginosus luxS expression reached highest values at early exponential phase, preceding maximum extracellular supernatant AI-2 activity, as measured by the bioluminescence assay (Paper I). Since LuxS is the AI-2 synthase in the activated methyl cycle, AI-2 production may be dependent on *luxS* expression. Variation in *luxS* expression during growth has also been observed in *P. gingivalis* [32], *Klebsiella pneumoniae* [1] and *Clostridium difficile* [7]. However, the *luxS* expression profile does not always follow extracellular AI-2 levels in all bacteria. In *S. typhimurium,* for example, *luxS* expression remains constitutive throughout growth phase, whereas *pfs* transcription follows AI-2 production [5]. These results indicate that AI-2 production is regulated at the level of LuxS substrate (SRH) availability (Figure 1) [5]. Although our results suggest that changes in *luxS* transcription levels play a significant role in *S. anginosus* AI-2 production, other regulatory mechanisms can not be excluded.

Several bacteria may employ AI-2/LuxS in metabolic and signaling functions [47, 94]. LuxS has a crucial function in detoxification of SAH and in the metabolism of methionine and cysteine [103]. Changes in gene expression due to inactivation of *luxS* could thus result from defective methionine/cysteine metabolism or toxic SAH accumulation rather than lack of AI-2 /LuxS communication *per se*. However, in addition to LuxS, SAH may be detoxified through SAH hydrolase, Pfs with LuxS or a combination of these mechanisms (Figure 1) [94]. The *luxS* inactivation and subsequent DPD supplementation did not disturb growth of *S. anginosus* or *S. intermedius* (Paper II and III) indicating that *S. anginosus* and *S. intermedius luxS* mutants may possess alternative pathways to perform SAH detoxification.

3. The role of AI-2 in *S. anginosus* and *S. intermedius* biofilm formation

In vivo biofilms comprise a consortium of bacterial species in juxtaposition with the host tissue. Exchange of signaling molecules among and within bacterial species may influence mature biofilm structures. We therefore investigated whether the *S. anginosus* group employed AI-2 signaling in biofilm formation. We found that *S. anginosus* and *S. intermedius luxS* mutants were respectively 50 % and 30 % defective in biofilm formation and displayed altered biofilm architecture at 37 °C (Paper I and II). In several bacteria, including most oral streptococci, *luxS* inactivation is frequently associated with defective biofilm formation either in biomass or architecture [1, 6, 26, 57, 58, 69, 72]. Occasionally, *luxS* inactivation may result in a mutant with increased biofilm formation, as in *Staphylococcus epidermidis* [108]. The specific mechanism instigating biofilm distortions in *luxS* mutants are not well defined. However,

several studies indicate that *luxS* may affect biofilm formation through diverse pathways. For example, the role of *luxS* expression in biofilm formation is linked to altered polysaccharide formation in *S. epidermidis* and *Salmonella* species [69, 108]. In *E. coli*, *luxS* inactivation affects the surface charge, a critical factor in initial bacterial attachment [18]. In *S. mutans, luxS* inactivation influences the expression of several glucosyltransferase genes [109]. Since *luxS* functions as a global regulator in several bacteria [48, 50, 84], biofilm formation may be defined through various genetic pathways. In *E. coli*, for instance, AI-2 regulator proteins (LsrR and LsrK) affect biofilm architecture through the coordinated regulation of several biofilm-related genes, including those involved in capsular-polysaccharide formation and auto-aggregation genes [47]. Further studies are required to investigate the mechanisms behind defective biofilm formation in *S. anginosus* and *S. intermedius luxS* mutants.

Since extracellular AI-2 production reached maximum levels at 37 °C, we proceeded to investigate whether AI-2 production at different temperatures may consequently influence biofilm formation in *S. intermedius* (Paper II). Incubation within physiological temperatures ranging between 35 °C - 41 °C implied that the role of *luxS* in biofilm formation was confined to 37 °C in *S. intermedius* (Paper II). No significant changes in biofilm formation were observed between *S. intermedius* wild type and its *luxS* mutant at temperatures below or above 37 °C. These results confirmed a positive association between extracellular AI-2 levels in *S. intermedius* and its role in biofilm formation. Previous studies have shown that AI-2 has high heat stability up to 100 °C, indicating that the incubation temperatures used in our study had no effect on AI-2 [33].

Bacteria are commonly subjected to a wide variety of challenges within host environments, which may influence AI-2 production [17, 88]. Therefore, in addition to temperature, the role of *luxS* in *S. anginosus* group biofilm formation was examined under various oxygen tensions, growth media and pH levels (Paper I and II). Unlike temperature, the role of *luxS* in *S. anginosus* and *S. intermedius* biofilm formation was not influenced by atmospheric conditions, medium type or pH levels (Paper I and II). These results indicate that, excluding temperature changes, the role of AI-2 in biofilm formation may be present under a variety of environmental conditions in the *S. anginosus* group.

4. The role of environmental conditions on growth and biofilm formation

In addition to AI-2, in our study other environmental factors also influenced growth and biofilm formation in the *S. anginosus* group. Despite similar planktonic growth rate, *S. anginosus* biofilm formation was favored under an aerobic as opposed to an anaerobic atmosphere (Paper I). *S. intermedius* displayed accelerated growth rate and increased biofilm formation at higher temperatures and under acidic environments (Paper II). Growth and biofilm formation may be influenced by several environmental conditions as a result of altered stress responses. In *E. coli,* for example, shifting incubation temperatures from 23°C to 37 °C may alter the expression of a broad range of genes, including those involved in general stress responses and biofilm formation [102]. In *S. pyogenes,* microarray studies indicate that high temperatures reaching 40 °C activated heat shock proteins enabling optimum growth at high temperatures [14]. Our results may reflect oral conditions that favor growth and biofilm formation in the *S. anginosus* group. For instance, *S. intermedius* biofilms may thrive in infected periodontal pockets and purulent niches where temperatures rise and acidic conditions may prevail [42, 82]. Whether in the oral cavity or other parts of the human body, in health or disease, bacteria constantly encounter challenging environments that necessitate flexible genetic systems to facilitate colonization and survival in preferable ecological niches.

5. The role of AI-2 in *S. anginosus* antibiotic susceptibility

The association between AI-2 signaling and antibiotic susceptibility was demonstrated for the first time in our study (Paper III). *S. anginosus luxS* mutant displayed diminished ability to survive several subinhibitory concentrations of ampicillin and erythromycin compared to wild type strain. *S. anginosus luxS* exposed to various concentrations of ampicillin and erythromycin displayed reduced growth compared to the wild type. Upon antibiotic exposure, the effect of *luxS* inactivation on bacterial cell viability was evident at mid-exponential and stationary phase. Ampicillin and erythromycin have two distinct mechanisms of action on the bacterial cell structures; ampicillin functions on the penicillin binding proteins disturbing cell wall synthesis while erythromycin inhibits protein synthesis by binding to the ribosomes [98]. Since the role of *luxS* on antibiotic susceptibility was independent of the antibacterial targets, these results may refer to generalized reduction in stress responses of the *S. anginosus luxS*

mutant. In *S. mutans*, several genes related to stress and protein synthesis exhibited AI-2 regulated changes [90].

The effect of *S. anginosus luxS* inactivation on antibiotic susceptibility was concentration dependent and exhibited maximum differences between *S. anginosus* wild type and its *luxS* mutant at a point when subinhibitory concentrations reduced growth of the wild type by approximately 25-50 %. In *S. pneumoniae*, real time gene expression profiling revealed that *luxS* was significantly upregulated when exposed to 50 % minimum inhibitory concentration of penicillin (MIC_{50}) [75]. In Gram negative bacteria, antibiotics at low concentrations may alter gene transcription through mechanisms other than the antibacterial inhibitory effect [24]. For example, in *S. typhimurium*, low concentrations of antibiotics with different modes of action affects the activities of diverse promoters, including those involved in quorum-sensing [24]. Up regulation of quorum-sensing system regulons at low antibiotic concentrations would allow bacteria to adopt a protective strategy under ominous sub-lethal doses. In *S. mutans*, for example, the TetR protein regulator is AI-2 dependent [90]. The TetR family of repressors, named after one of its most genetically characterized member, the TetR protein, is found in numerous bacterial species, including several streptococci and *Vibrio* species [70]. TetR controls the expression of the *tet* genes, which encode tetracycline efflux pumps. The TetR family of proteins participates in several regulatory networks including metabolism, complex quorum-sensing circuits, multidrug resistance, virulence and antibiotic biosynthesis [70]. Further studies are necessary to explore the role of AI-2 in the expression of multidrug resistant regulators in *S. anginosus*.

6. AI-2 complementation assays

The diverse roles of AI-2 in fundamental cellular processes and its broad influence on gene expression suggest LuxS to be a global bacterial regulator [48, 50, 84]. Since LuxS occupies an integral metabolic position in the activated methyl cycle, altered *luxS* mutant phenotypes may result from pleiotropic effects rather than genuine signaling functions. In *S. mutans* and *Lactobacillus rhamnosus*, for example, DPD supplementation did not restore certain phenotypic changes in *luxS* mutants [44, 90]. To investigate the signaling nature of AI-2 in the *S. anginosus* group, it was necessary to complement the defective biofilm formation and reduced antibiotic susceptibility in the *luxS* mutant strains. AI-2 complementation may be demonstrated using wild type supernatant or by addition of the AI-2 precursor DPD. Conditioning media

prepared from wild type species provide feasible complementation but may prove not to be particularly well defined to AI-2 molecules due to the diversity of supernatant composition. Since DPD is the core molecule parent to all AI-2 derivatives, addition of DPD allows specific AI-2 complementation. Various DPD concentrations were tested for their ability to restore *luxS* mutant defects in biofilm formation and antibiotic susceptibility to their wild type levels in the *S. anginosus* group. We observed that DPD restored antibiotic susceptibility and biofilm formation in *S. anginosus* and *S. intermedius luxS* mutants to their respective wild type traits at a specific concentration range (Paper II and III). Optimum complementation of antibiotic susceptibility in *S. anginosus luxS* mutant was attained between 1.5-1.8 nM DPD and in *S. intermedius luxS* biofilm formation was restored at a range of 0.8-8 nM DPD. The DPD complementation range in the *S. anginosus* group was consistent with complementation assays of dual-biofilm formation in other oral bacteria [72].

Concluding remarks

The current thesis addresses autoinducer 2 communication in the *S. anginosus* group and discusses its potential role in biofilm formation and antibiotic susceptibility.

The sessile, durable nature of biofilms makes them major culprits in persistent infections and fortifies them against antibiotics. Current antibiotic effectiveness is impeded by resistant organisms, predicting potential emergence of incurable infections. Traditional mechanisms of disease treatment target sensitive bacterial structures that are apt to genetic modulation and resistance. Therefore, the panel of microbial treatment should be extended to include novel bacterial targets. The AI-2 communication system is unique in its wide presence across Gram positive and Gram negative bacterial species. Future prophylactic procedures that target AI-2 signals may hamper biofilm formation and increase antibiotic susceptibility concomitantly. Understanding how bacteria employ their population behavior during health and disease may contribute to novel infection control strategies.

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