AI-2 quorum sensing affects antibiotic susceptibility in *Streptococcus anginosus*

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**Objectives:** The concern over rising antibiotic resistance necessitates exploration of alternative approaches in antimicrobial therapy. Bacterial communities use the auto-inducer 2 (AI-2) quorum sensing signal at a specific threshold level for intra- and interspecies communication in order to regulate virulence behaviour. AI-2 signal production occurs in bacteria that possess a *luxS* homologue. In this study, we investigate for the first time the association between AI-2 signalling and susceptibility to antibiotics.

**Methods:** *Streptococcus anginosus* wild-type and its isogenic *luxS* mutant SA001 were exposed to erythromycin and ampicillin. Susceptibility to erythromycin and ampicillin was determined by measuring the cell density and viability. Complementation assays were conducted by exposing the mutant to wild-type supernatant or to the AI-2 precursor molecule dihydroxy-2,3-pentanediione (DPD).

**Results:** Disruption of *luxS* in *S. anginosus* resulted in a mutant with increased susceptibility to erythromycin and ampicillin. Supernatant from *S. anginosus* wild-type partially restored growth of SA001 in the presence of the two antibiotics. DPD restored growth of the *luxS* mutant in the presence of erythromycin and ampicillin to values similar to that of *S. anginosus* wild-type.

**Conclusions:** Our results indicate that *luxS*-based AI-2 communication is associated with antibiotic susceptibility. Targeting the AI-2 signal communication may present a novel approach in antimicrobial therapy.

Keywords: *luxS*, DPD, ampicillin, erythromycin, signalling

**Introduction**

High-density bacterial populations display decreased susceptibility to antibiotic treatment. This has been explained by persister cells, activation of stress response genes or nutrient limitation resulting in low metabolic activity and decreased cell permeability. More recently, Fux *et al.* also suggested the involvement of metabolic waste or extracellular signalling molecules in the bacterial response to antimicrobial stress. The identification of such extracellular signals remains, however, elusive.

The recognition that bacteria use a chemical language of signalling molecules in a process called quorum sensing (QS) has led to a new understanding of bacterial behaviour in response to their environment. QS implies that bacteria sense each other by detecting a threshold accumulation of secreted signals called auto-inducers (AIs), resulting in the regulation of several genes. One of the QS signals is AI-2, suggested to mediate communication among and between species. Studies have shown that AI-2 signals are an interconvertible set of molecules that form as a result of spontaneous rearrangement of dihydroxy-2,3-pentanediione (DPD). The synthesis of DPD is catalysed by the LuxS enzyme; a product of the highly conserved and widespread bacterial *luxS* gene. Many studies have highlighted the significance of *luxS* in the biological processes of various Gram-positive and Gram-negative bacterial species including biofilm formation, carbohydrate metabolism and virulence expression.

The *Streptococcus anginosus* group is well recognized for its prevalence in brain and liver abscesses, gastrointestinal and genitourinary tract infections as well as in infective endocarditis. *S. anginosus* has, in addition, been associated with dysplasia of oesophagus, oesophageal cancer, gastric cancer and oral squamous cell carcinoma.

The β-lactam group of antibiotics such as ampicillin has routinely been the drug of choice for prophylactic strategies and treatment of mixed bacterial infections in oropharyngeal regions. The emergence of resistance to β-lactams has led to an
alternative prescription of erythromycin and other macrolides, in addition to their use in penicillin-allergic patients. The *S. anginosus* group shows intermediate susceptibility to β-lactams and macrolide antibiotics, although resistance patterns are increasing.¹⁷,¹⁸

We recently showed that *S. anginosus* possesses a *luxS* homologue and that inactivation of *S. anginosus* *luxS* resulted in a mutant with significantly reduced biofilm formation.¹⁹ We wanted to investigate whether AI-2 signalling influenced other functions in *S. anginosus*. The aim of the current study was to test the hypothesis that AI-2 QS communication may affect the antibiotic susceptibility of *S. anginosus*. Here, we show that *luxS* inactivation results in a mutant with increased susceptibility to erythromycin and ampicillin.

### Materials and methods

**Bacterial strains and culture conditions**

The *S. anginosus* strains used were the wild-type strain NCTC 10713 (WT) and its isogenic AI-2 communication defective *luxS* mutant SA001 (*luxS::psf151::luxS; Kan*'), constructed by insertional inactivation of the *luxS* gene, as described previously.¹⁹

Bacterial cells were stored at −20°C. Before each experiment, bacterial cells were grown on Todd–Hewitt agar plates (Difco Laboratories, Detroit, MI, USA) for 24 h at 37°C in a 5% CO₂ aerobic atmosphere. Colonies were then cultivated overnight in tryptone soy broth (TSB; Oxoid, UK). In the first overnight cultures, the *luxS* mutant SA001 was supplied with kanamycin 500 mg/L (Sigma-Aldrich, St Louis, MO, USA). *S. anginosus* WT and SA001 from first overnight growth were inoculated in TSB for second overnight growth.

**Measurement of bacterial growth**

TSB without or with erythromycin or ampicillin, added in the ranges of 0.01–0.2 and 0.02–0.1 mg/L, respectively, was dispensed into 96-microwell plates (Nunclon Surface; NUNC A/S, Denmark). *S. anginosus* WT or SA001 was then inoculated to a final concentration of ~7 × 10⁶ cfu/mL into microtitre wells. The plates were incubated at 37°C in a 5% CO₂ aerobic atmosphere. Growth of *S. anginosus* WT and SA001 was monitored from early exponential until stationary phase by optical density absorbance measurements at 595 nm (OD₅₉₅) in a Wallac 1420 Victor multilabel plate reader (Wallac Oy, Turku, Finland). This allowed monitoring of antibiotic susceptibility during AI-2 production in *S. anginosus*.

Bacterial cells were stored at 8°C in air and viable counts were determined after exposure to antibiotics at 6 and 24 h. At mid-exponential phase (6 h), viable counts were determined after overnight incubation at 37°C in a 5% CO₂ aerobic atmosphere. Experiments were repeated four times with two parallels.

As optimal AI-2 induction in *S. anginosus* occurs at mid-exponential phase (6 h),¹⁹ *S. anginosus* WT and *luxS* mutant SA001 with or without erythromycin (0.02 mg/L) and ampicillin (0.05 mg/L) supplementation were plated to assess viability after 6 h of growth. Overnight cultures with or without erythromycin (0.02 and 0.06 mg/L) and ampicillin (0.05 and 0.08 mg/L) were likewise assessed. Erythromycin and ampicillin were added at time 0. Appropriate dilutions of bacterial cultures were plated on TSB agar plates and viable counts were determined after overnight incubation at 37°C in a 5% CO₂ aerobic atmosphere. Experiments were repeated four times with two parallels.

### AI-2 complementation

To determine whether AI-2 produced by WT would compensate for the lack of AI-2 production in SA001, supernatants from growing *S. anginosus* WT were allowed to seep through 0.2 μm pore filter inserts (Nunc tissue culture inserts, eight-well strip; Nalge Nunc International, Naperville, IL, USA) to cultures of SA001 for 24 h. SA001 was inoculated in the lower compartment (160 μL) and either SA001 or *S. anginosus* WT was added in the upper filters (60 μL). This system allowed the combination of the supernatants and extracellular signal molecules while keeping the bacterial cells separated, as described previously.²⁰ Erythromycin and ampicillin were added to final concentrations of 0.06 and 0.05 mg/L, respectively.

OD₅₉₅ of SA001 was measured after 24 h.

To verify the association between AI-2 communication and altered susceptibility to antibiotics, the pre-AI-2 molecule DPD (Omn Scientific Inc., TX, USA) was used to complement the *luxS* mutant SA001. To determine the appropriate concentration, DPD was added in a range of 0.08–102 nM. *S. anginosus* WT and *luxS* mutant SA001 inoculated to a final concentration of ~7 × 10⁶ cfu/mL into TSB containing DPD and erythromycin (0.02–2 mg/L) or ampicillin (0.02–0.1 mg/L) were dispensed into 96-well microtitre plates. Appropriate dilutions from mid-exponential growth (6 h) and overnight bacterial cultures (24 h) were plated on TSB agar plates. The plates were incubated at 37°C in 5% CO₂ in air and viable counts were determined after 48 h. Experiments were repeated four times with two parallels.

### Statistical analysis

The Wilcoxon signed-rank test and the paired t-test (SigmaStat 3.1, USA) were used for statistical comparisons. The level of statistical significance was set at *P* ≤ 0.05.

### Results

**Inactivation of luxS in *S. anginosus* resulted in increased antibiotic susceptibility**

Figure 1 displays the growth levels reached by both *S. anginosus* WT and SA001 at mid-exponential (6 h) and at stationary phase (24 h) in the presence or absence of various erythromycin and ampicillin concentrations. In the absence of antibiotics, *S. anginosus* NCTC 10713 WT and its AI-2 communication defective *luxS* mutant SA001 reached similar growth values after 6 and 24 h, respectively. SA001 displayed, however, increased susceptibility to erythromycin or ampicillin when compared with *S. anginosus* WT. The difference in the growth curves between WT and SA001 in the presence of antibiotics was already evident at early exponential growth phase (data not shown) and continued throughout stationary phase. The concentrations of erythromycin and ampicillin needed to reduce growth by 50% were approximately 3 and 1.5 times higher, respectively, for WT than for SA001.

To assess the viability of bacterial cells that were exposed to antibiotics, viable counts were determined after exposure to antibiotics at 6 and 24 h. At mid-exponential phase (6 h), viable counts were 3 times lower in SA001 in the presence of 0.02 mg/L erythromycin and 1.7 times lower in the presence 0.05 mg/L ampicillin when compared with *S. anginosus* WT (Table 1). Overnight growth of SA001 resulted in 2.4–19.9 times lower viable counts in the presence of 0.02–0.06 mg/L...
AI-2 and antibiotic susceptibility

To examine whether the higher antibiotic susceptibility of SA001 is reversed by exposure to WT supernatant, SA001 was co-cultured with S. anginosus WT. The strains were separated by filter compartments, allowing free diffusion of extracellular substances as described previously. When SA001 was supplied with supernatant from S. anginosus WT, it exhibited higher growth in the presence of erythromycin and ampicillin than when it was supplied with its own supernatant (Figure 2). These results indicate that a substance or extracellular signal present in WT supernatant, most probably AI-2, was involved in decreasing antibiotic susceptibility of SA001.

AI-2 signal complementation reversed antibiotic susceptibility of SA001

Table 1. cfu of S. anginosus with or without erythromycin (ERY) or ampicillin (AMP)

<table>
<thead>
<tr>
<th>Growth condition</th>
<th>S. anginosus WT (n = 8)</th>
<th>SA001 (n = 8)</th>
</tr>
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<tbody>
<tr>
<td>6 h control (without antibiotics)</td>
<td>339 (20.2)</td>
<td>355 (24.5)</td>
</tr>
<tr>
<td>ERY (0.02 mg/L)</td>
<td>164 (15.7)</td>
<td>55 (5.1) a</td>
</tr>
<tr>
<td>AMP (0.05 mg/L)</td>
<td>197 (14.0)</td>
<td>113 (8.2) a</td>
</tr>
<tr>
<td>24 h control (without antibiotics)</td>
<td>714 (25.6)</td>
<td>689 (27.6)</td>
</tr>
<tr>
<td>ERY (0.02 mg/L)</td>
<td>501 (29.0)</td>
<td>211 (25.5) a</td>
</tr>
<tr>
<td>ERY (0.06 mg/L)</td>
<td>457 (62.2)</td>
<td>23 (10.0) a</td>
</tr>
<tr>
<td>AMP (0.05 mg/L)</td>
<td>394 (51.7)</td>
<td>180 (38.9) a</td>
</tr>
<tr>
<td>AMP (0.08 mg/L)</td>
<td>277 (23.6)</td>
<td>32 (5.4) a</td>
</tr>
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</table>

*Significantly different from S. anginosus WT (P ≤ 0.05).

To provide evidence for the involvement of AI-2 in antibiotic susceptibility of S. anginosus, the pre-AI-2 molecule DPD was used to complement SA001. SA001 complementation was reached at DPD threshold concentrations between 1.5 and 1.8 nM. At these DPD concentrations, SA001 viable counts from mid-exponential (6 h) and overnight growth (24 h) significantly increased in the presence of erythromycin or ampicillin (Figures 3 and 4). DPD values below 1.5 and above 8 nM did not show any significant effect on antibiotic susceptibility (data not shown). These findings strongly indicate that the lower susceptibility to antibiotics in WT was a consequence of AI-2-mediated signalling. DPD displayed no effect on the growth of either S. anginosus WT or SA001 (data not shown).

Figure 1. Effect of (a) erythromycin (ERY) and (b) ampicillin (AMP) on growth of S. anginosus WT NCTC 10713 (filled circles) and its isogenic luxS mutant SA001 (open circles). Optical density measurements were taken at mid-exponential phase (6 h) (broken lines) and after overnight growth (24 h) (continuous lines). Data points represent mean values (n = 12) and the bars represent the SEM.

Figure 2. Overnight growth of luxS mutant SA001 complemented with culture supernatants in the presence of 0.06 mg/L erythromycin (ERY) and 0.05 mg/L ampicillin (AMP). Black bars represent the luxS mutant SA001 (160 μL) with upper compartment filter inserts containing S. anginosus WT (60 μL) and white bars represent SA001 (160 μL) with upper compartment filter inserts containing SA001 luxS mutant (60 μL). Bars represent mean values with SEM (n = 6). *Significantly different from S. anginosus WT supernatant (P ≤ 0.05).
Discussion

In the current study, we have examined the association between AI-2 intercellular communication and antibiotic susceptibility in S. anginosus by comparing WT and its isogenic luxS mutant, deficient in AI-2 production. To investigate whether the role of AI-2 QS was dependent on the antibiotic mode of action, two groups of antibiotics with different antibacterial mechanisms were studied. Erythromycin is a macrolide antibiotic that acts intracellularly by dissociation of peptidyl tRNA from the bacterial ribosome during the elongation phase of protein synthesis. Ampicillin, in contrast, is a β-lactam antibiotic that inhibits peptidoglycan cross-linking and subsequently cell wall synthesis by interacting with the penicillin-binding protein located on the outer region of the cytoplasmic membrane. The AI-2-deficient SA001 displayed increased susceptibility to both antibiotics. Notably, S. anginosus WT and SA001 showed similar growth profiles in the absence of antibiotics. Thus, the higher susceptibility of SA001 to antibiotics could not be ascribed to growth alterations caused by luxS inactivation.

Previous studies have shown an association between AI-2 QS and the ability to cope with stress, supporting a role of AI-2 in adaptation to environmental challenges. Thus, inactivation of luxS in S. anginosus and the consequent inability to communicate could possibly affect their response to antimicrobial stress.

The luxS-based communication system could function as a global regulatory mechanism that triggers a cascade of events involving up- or down-regulation of several genes. Microarray analyses indicate, for instance, that AI-2 QS regulates up to 10% of the Escherichia coli genes and more than 70 genes in Vibrio cholerae, including basic physiological and virulence genes. In Pseudomonas aeruginosa, microarray analysis shows that expression of multidrug-resistant pumps may be regulated by the AI-1 QS used in intraspecies communication. Whether regulation of antibiotic efflux systems is also associated with AI-2 QS in S. anginosus remains to be determined. Reductions in antibiotic susceptibility have also been attributed to the dormant status of some bacterial cells, referred to as persisters. Experiments have not yet revealed an association between persister cells and QS. Future studies to characterize the regulatory response involved in AI-2 communication may shed further light into the mechanism of increased antibiotic susceptibility (e.g. persister cell regulation).

In the activated methyl cycle, S-adenosylmethionine is used as a methyl donor resulting in the accumulation of the toxic intermediate S-adenosylhomocysteine (SAH). The LuxS enzyme functions in detoxification of SAH to homocysteine and DPD. DPD is a highly active pre-AI-2 molecule. Disruption of the activated methyl cycle through inactivation of luxS could possibly result in the modification of a range of chemical reactions. Thus, the conditioned media prepared from the luxS mutant strains might differ in many aspects other than AI-2 content. Therefore, even though supernatant complementation from WT to SA001 increased growth in the presence of antibiotics, it may not necessarily reveal a direct link to the role of AI-2. We therefore conducted further complementation studies using synthetic DPD. Our results displayed an association between threshold DPD concentrations and antibiotic susceptibility in S. anginosus. These results are consistent with other DPD and AI-2 studies, showing the significance of reaching an appropriate AI-2 threshold level in a bacterial population.

The concern over rising antibiotic resistance necessitates exploration of alternative approaches in antimicrobial therapy. The widespread AI-2 communication among bacteria renders it a possible therapeutic target. It is therefore important to understand the role of AI-2 QS in antibiotic susceptibility. Targeting AI-2 communication in bacteria could represent a novel antimicrobial strategy.

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Transparency declarations

None to declare.

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