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Short communication

# LuxS/AI-2 system is involved in antibiotic susceptibility and autolysis in *Staphylococcus aureus* NCTC 8325

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ABSTRACT

Current treatment for *Staphylococcus aureus* infections relies heavily upon the cell wall synthesis inhibitor antibiotics such as penicillin, oxacillin, vancomycin and teicoplanin. Increasing antibiotic resistance requires the development of new approaches to combating infection. Autoinducer-2 (AI-2) exists widely both in Gram-negative and Gram-positive pathogens and is suggested as a universal language for intraspecies and interspecies communication. This study demonstrates the association between AI-2 signalling and cell wall synthesis inhibitor antibiotic susceptibility in *S. aureus*. In addition, a *luxS* mutant exhibited decreased autolysis and upregulated vancomycin resistance-associated VraRS two-component regulatory system. This finding may provide novel clues for antimicrobial therapy in *S. aureus* infection.


## 1. Introduction

*Staphylococcus aureus*, a leading cause of nosocomial infections worldwide, is the aetiological agent of a wide range of diseases, from relatively benign skin infections to potentially fatal systemic disorders such as endocarditis, osteomyelitis and foreign-body-related infections. The cell wall synthesis inhibitors such as penicillin and the β-lactam groups of antibiotics have been the drugs of choice for treatment, and glycopeptides are considered to be the ultimate weapon against the worst hospital-acquired infections. However, the emergence of multidrug-resistant isolates of meticillin-resistant *S. aureus* also exhibiting decreased susceptibility to glycopeptides [glycopeptide-intermediate *S. aureus* (GISA)] represents a critical challenge for antimicrobial therapy [1,2].

The mechanisms responsible for decreased susceptibility without acquirement of resistance elements, like vancomycin resistance in enterococci, are still poorly understood [1]. It is thought that the phenotypic characteristics of many GISA strains result from global metabolic changes affecting cell wall synthesis and structure. Both clinical GISA strains and GISA strains due to genetic alterations selected in laboratories show multiple changes in the cell wall composition and structure, such as increased synthesis of peptidoglycan, thickening of the cell wall, reduced cross-linking of the cell wall peptidoglycan, reduced muropeptide amidation, decreased levels of autolytic enzymes involved in cell wall turnover, increased levels of penicillin-binding protein 2 (PBP2) production, and decreased levels of PBP4 activity [3–5]. A large number of genes, including regulators and encoding proteins involved in cell wall metabolism (e.g. *pbpB*, *murZ*, *mgtB* and *vraS*), are upregulated in vancomycin-intermediate *S. aureus* (VISA) strains. VraS and VraR are the vancomycin resistance-associated sensor and regulator respectively, and are co-transcribed in *S. aureus*. The two-component system VraSR was remarkably upregulated in VISA and vancomycin-resistant *S. aureus* strains compared with vancomycin-susceptible *S. aureus* strains [6].

Many bacteria, including Gram-negative and Gram-positive pathogens, co-ordinate expression of genes crucial for virulence and survival by producing, secreting and detecting small diffusible signal molecules in a process termed ‘quorum sensing’. Autoinducer-2 (AI-2), considered to be a universal language for interspecies communication, is synthesised by the LuxS enzyme, which is highly conserved and widespread in diverse bacteria [7]. AI-2 signalling has been reported to affect antibiotic susceptibility in *Streptococcus anginosus*. Inactivation of *luxS* in *S. anginosus* resulted in reduced biofilm formation and increased susceptibility to erythromycin and ampicillin [7,8]. Here we show that an *S. aureus luxS* mutant results in decreased susceptibility to cell wall synthesis inhibitor antibiotics accompanied by decreased autolysis and upregulated *vraRS*, and susceptibility was restored by either genetic complementation or addition of AI-2, suggesting that the LuxS/AI-2 system is associated with antibiotic susceptibility in *S. aureus*.

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**Table 1**
Strains, plasmids and primers used in the study.

| Strain or plasmid | Relevant genotype | Reference or source |
|---|---|---|
| Strains | | |
| *Staphylococcus aureus* | | |
| RN4220 | 8325-4 r⁻ | NARSA |
| NCTC 8325 | Wild-type | NARSA |
| SX1 | 8325 *luxS*::*ermB* | This study |
| SX2 | 8325 *luxS*::*ermB* pLIluxS | This study |
| *Escherichia coli* | | |
| DH5α | Clone host strain | Laboratory stock |
| Plasmids | | |
| pEASY-TB | Clone vector; $Kan^r$, $Ap^r$ | TransGen |
| pEC2 | A vector containing a 1.45-kb *ClaI*–*ermB* fragment of Tn*551* cloned into the *ClaI* restriction site of pBluescript | Bruckner |
| pBT2 | Shuttle vector, temperature-sensitive; $Ap^r$, $Cm^r$ | Bruckner |
| pBTluxS | pBT2 containing 500-bp upstream and downstream fragments of *luxS* and *ermB* genes, for *luxS* mutagenesis; $Ap^r$, $Cm^r$, $Emr^r$ | This study |
| pLI50 | Shuttle cloning vector; $Ap^r$, $Cm^r$ | Add gene |
| pLIluxS | pLI50-*luxS* and its promoter, $Ap^r$ | This study |
| Primer | Oligonucleotide sequence | |
| up-luxS-f-BamHI | GCGggatccTGAATCATATGTCGCTATAA | |
| up-luxS-r-HindIII | GCGaagcttCTACTCAAAACTATAAAACG | |
| down-luxS-f-XbaI | ATTtctagaCAAACGAAACGAATGGCATG | |
| down-luxS-r-SalI | GCGgtcgacTGTCGTTCTCGTGTAAAATG | |
| Em-f-XbaI | GCGtctagaGATACAAATTCCCCGTAGGC | |
| Em-r-HindIII | GCGaagcttGAAATAGATTTAAAAATTTCGC | |
| c-luxS-f-HindIII | GCGaagcttGTTAGAAACACAACTTACAACAA | |
| c-luxS-r-EcoRI | GCGgaattcTTATTTTCCTGTACCGAAAA | |
| rt-vraS-f | CAATGGAAGGCGAAACAGT | |
| rt-vraS-r | TCGTGAAGTTCTCGTGCTA | |

NARSA, Network on Antimicrobial Resistance in *Staphylococcus aureus*; $Kan^r$, kanamycin-resistant; $Ap^r$, ampicillin-resistant; $Cm^r$, chloramphenicol-resistant; $Emr^r$, erythromycin-resistant.

## 2. Materials and methods

### 2.1. Bacterial strains, plasmids and DNA manipulations

Bacterial strains and plasmids used in this study are described in Table 1. Genomic DNA and plasmid DNA of *S. aureus* NCTC 8325 were prepared by standard protocols for Gram-positive bacteria. *Staphylococcus aureus* was transformed by electroporation as described previously [9].

### 2.2. Construction of a Staphylococcus aureus luxS mutant strain and complementation of the luxS mutant

The allelic replacement method was performed to construct the *luxS* mutant as described previously [10], and the *luxS* gene was replaced with the erythromycin resistance gene *ermB*. PCR and sequencing were performed to confirm that the desired gene inactivation had occurred by double-crossover recombination. For complementation of the *luxS* mutant, the *luxS* gene and its promoter from *S. aureus* NCTC 8325 were amplified by PCR and the PCR products were then cloned into pLI50 to create plasmid pLIluxS. The primers used in this study are described in Table 1.

### 2.3. Antibiotic susceptibility assay

Antibiotic susceptibility of the *S. aureus* strains was determined for cell wall synthesis inhibitor antibiotics including vancomycin, teicoplanin, penicillin G and oxacillin (Sigma-Aldrich, St Louis, MO). Overnight cultures were initiated by touching a sterile wire loop to three individual colonies on every plate and inoculating into 5 mL of Luria–Bertani (LB) medium (BD, Franklin Lakes, NJ). The inoculum was cultured to a final concentration of $10^6$ CFU/mL in fresh Mueller–Hinton broth (MHB) (BD, Franklin Lakes, NJ) and was dispensed into 96-well plates (Corning, Steuben, NY) containing serial dilutions of antibiotics. To verify the association between AI-2 signalling and altered antibiotic susceptibility, the pre-AI-2 molecule 4,5-dihydroxy-2,3-pentanedione (DPD) (Omm Scientific Inc., Dallas, TX) was used to complement the *luxS* mutant. To determine the appropriate concentration, DPD was added in the range 3.9 nM to 39 μM at time 0 in MHB containing vancomycin (1 μg/mL). Plates were incubated at 37 °C for 24 h and then ten-fold serial dilutions of cultures were performed by successive transfer (0.1 mL) through seven microfuge tubes containing 0.9 mL of MHB. Colony counting (CFU/mL) was performed by the microdilution plating method [11]. Four appropriate dilutions (10 μL each) were dropped on LB agar plates and viable colonies were counted after incubation at 37 °C for 24 h. Experiments were repeated three times with two parallels.

### 2.4. Determination of Triton X-100-induced autolysis

Strains grown overnight in PYK medium [5.0 g/L Bacto peptone, 5.0 g/L yeast extract and 3.0 g/L $K_2HPO_4$ (pH 7.2)] were diluted and grown to mid-exponential phase [absorbance at 600 nm ($A_{600}$) = 0.7] at 30 °C with shaking. Cells were washed twice in cold water and were re-suspended to $A_{600}$ = 0.7 in 50 mM Tris–HCl (pH 7.5) containing 0.05% Triton X-100 (Sigma-Aldrich). Cells were incubated with shaking (150 rpm) at 30 °C and the $A_{570}$ was measured using an ELx800™ Microplate Reader (Bio-Tek, Winooski, VT) every 30 min. Each data point represents the mean and standard deviation from three independent experiments.

### 2.5. RNA isolation and quantification by real-time reverse transcription PCR (RT-PCR)

Overnight cultures of *S. aureus* were diluted 1:100 in tryptic soy broth (BD, Franklin Lakes, NJ) and were grown to exponential phase ($A_{600}$ = 2.1). Cells were collected by centrifugation and re-suspended in Tris–ethylene diamine tetra-acetic acid (TE) buffer (pH 8.0) (Sigma-Aldrich) containing 10 mg/mL lysozyme and 50 μg/mL lysostaphin (both from Sigma-Aldrich). Following incubation at 37 °C for 0.5 h, strains were prepared for total RNA

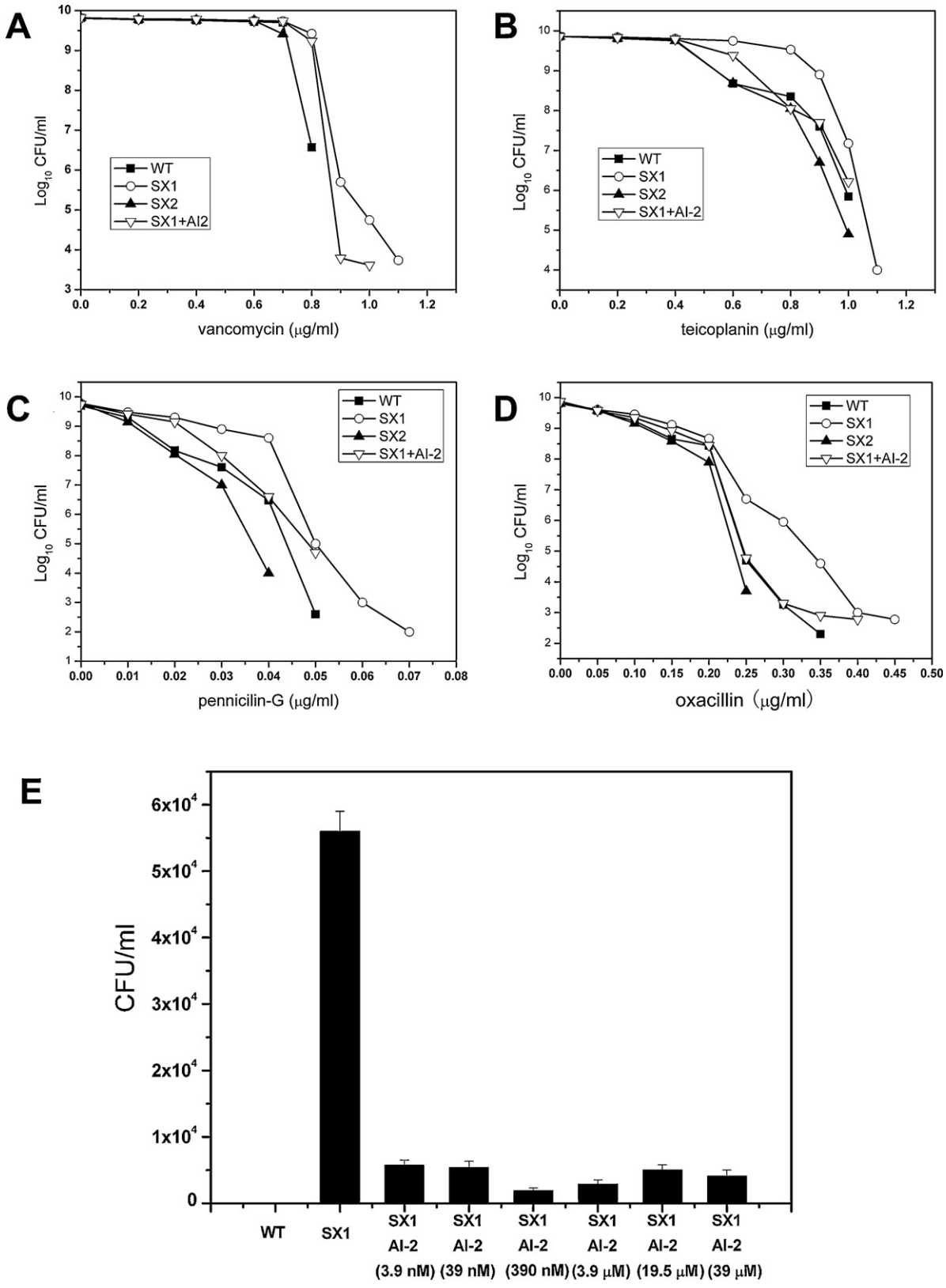


**Fig. 1.** (A–D) Colony counts (CFU/mL) of *Staphylococcus aureus* NCTC 8325 wild-type (WT), the *luxS* mutant (SX1), the *luxS* mutant with plasmid containing *luxS* complementation (SX2) and the *luxS* mutant with 3.9 μM autoinducer-2 (AI-2) complementation (SX1 + AI-2) following 24 h of incubation at 37 °C with or without specific concentrations of vancomycin (A), teicoplanin (B), penicillin G (C) and oxacillin (D). (E) Colony counts (CFU/mL) of *S. aureus* NCTC 8325 WT, the *luxS* mutant (SX1) and the *luxS* mutant with 3.9 nM to 39 μM AI-2 complementation following 24 h of incubation at 37 °C in the presence of 1 μg/mL vancomycin.

extraction using the TRIzol method, and residual DNA was removed with DNase (TaKaRa, Kyoto, Japan). Reverse transcription was conducted using DNase-treated RNA (250 ng) with the primer rt-vraS-r and a PrimeScript™ 1st Strand cDNA Synthesis Kit (TaKaRa) according to the manufacturer's instructions. Quantification of cDNA levels was performed with the primers rt-vraS-f and rt-vraS-r following the instructions of the SYBR® Premix Ex Taq™ (TaKaRa) on an ABI7000 system (Applied Biosystems, Carlsbad, CA). The quantity of *vraS* cDNA measured by real-time PCR was normalised to the 16S cDNA abundance. Analysis was performed using the relative quantification method.

## 3. Results

### 3.1. The LuxS/AI-2 system is involved in susceptibility to cell wall synthesis inhibitor antibiotics

Fig. 1 shows the viable counts of strains after exposure to MHB with or without antibiotics for 24 h at 37 °C. In the absence of antibiotics, *S. aureus* NCTC 8325 wild-type, the *luxS* mutant, the *luxS* mutant with plasmid containing the *luxS* gene, and the *luxS* mutant with exogenous AI-2 showed similar growth after 24 h of incubation, indicating that inactivation of *luxS* or addition of AI-2 had no effect on growth. However, following exposure to cell wall synthesis inhibitor antibiotics such as vancomycin (Fig. 1A), teicoplanin (Fig. 1B), penicillin G (Fig. 1C) and oxacillin (Fig. 1D) for 24 h, the *S. aureus luxS* mutant displayed higher viable counts than the wild-type. Viable counts were almost 1000 times higher in the *luxS* mutant in the presence of 0.8 μg/mL vancomycin compared with the wild-type. No viable counts were detected in the wild-type and the *luxS* mutant with plasmid containing *luxS* gene complementation in the presence of 1.0 μg/mL vancomycin, whereas 5% and 0.5% *luxS* mutant cells survived when exposed to 1.0 μg/mL and 1.1 μg/mL vancomycin, respectively.

AI-2 was used to complement the *luxS* mutant to provide evidence for the involvement of AI-2 signalling in decreased antibiotic susceptibility. To determine the appropriate concentration, AI-2 was added in a range of 3.9 nM to 39 μM in the presence of 1.0 μg/mL vancomycin (Fig. 1E). Viable counts were significantly decreased in the *luxS* mutant complemented with exogenous AI-2 (3.9 nM to 39 μM) compared with that with no AI-2 complementation.

### 3.2. Inactivation of luxS resulted in decreased autolysis induced by Trion X-100

A previous study showed that both clinical and laboratory-altered *S. aureus* strains exhibiting decreased susceptibility to vancomycin have reduced levels of autolysis. To help define the mechanism of *luxS*-based alteration in antibiotic susceptibility, the autolytic activity of these strains was determined. As shown in Fig. 2A, the *luxS* mutant exhibited decreased autolysis rates compared with the wild-type, and it can be complemented with a plasmid containing the *luxS* gene or exogenous AI-2. A previous study showed that autolysis played a role in vancomycin-mediated killing in *S. aureus*, and that a strain with decreased autolytic capacity could evade the lysis-inducing effect of vancomycin at an early stage [12]. The decreased autolytic capacity of the *luxS* mutant was in accordance with its decreased susceptibility to cell wall synthesis inhibitor antibiotics.

### 3.3. Inactivation of luxS resulted in increased transcriptional levels of vraS

Early microarray analysis indicated that many genes, including *vra*, involved in cell wall metabolism were upregulated in the

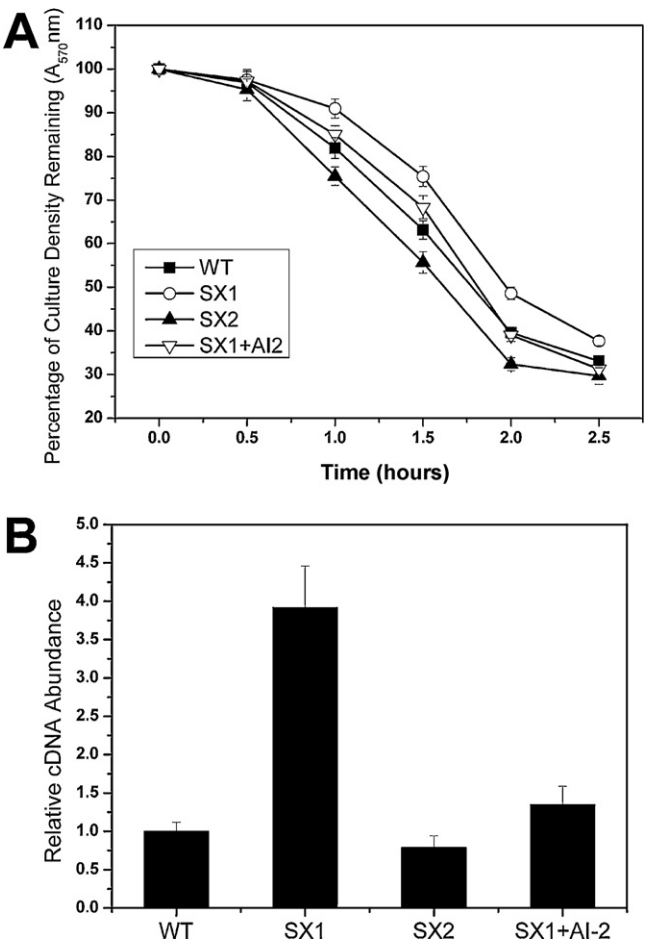


**Fig. 2.** (A) Autolysis of *Staphylococcus aureus* NCTC 8325 wild-type (WT), the *luxS* mutant (SX1), the *luxS* mutant with plasmid containing *luxS* complementation (SX2) and the *luxS* mutant with 3.9 μM autoinducer-2 (AI-2) complementation in the presence of Tris–HCl buffer containing 0.05% Triton X-100 at 30 °C. (B) Relative transcription levels of *vraS* in *S. aureus* NCTC 8325 WT, the *luxS* mutant (SX1), the *luxS* mutant with plasmid containing *luxS* complementation (SX2) and the *luxS* mutant with 3.9 μM AI-2 complementation (SX1 + AI-2) with shaking at 37 °C until absorbance at 600 nm ($A_{600}$) = 2.1.

*luxS* mutant (data not shown). To further determine whether the decreased susceptibility to antibiotics is associated with the *vra* gene in the *luxS* mutant, real-time RT-PCR was used to quantify *vraS* expression at the transcriptional level. As shown in Fig. 2B, transcription of *vraS* was upregulated approximately four-fold in the *luxS* mutant compared with the wild-type. In contrast, there were only slight changes between the wild-type and *luxS* mutant complementation with either plasmid or adding AI-2. This suggests that the LuxS/AI-2 signalling might be associated with the regulation of *vraRS* transcription in *S. aureus*.

## 4. Discussion

According to a previous report, AI-2 in *S. aureus* is synthesised by LuxS and is released in the surroundings during the exponential phase. Subsequently, extracellular AI-2 activity is dramatically reduced, suggesting that it might be imported into the cell and co-ordinates gene expression just like in Gram-negative bacteria [13]. Our previous work also suggested that AI-2 is deployed as an extracellular signalling molecule in *S. aureus* [14]. In this work, we demonstrate that LuxS is involved in antibiotic susceptibility in *S. aureus* and this involvement is probably mainly due to AI-2 signalling. Moreover, as a two-component regulatory system,

VraSR is capable of detecting conditions that threaten to interrupt the synthesis of the bacterial cell wall and regulating the cell wall biosynthesis pathway [15]. The higher VraSR level in the *luxS* mutant suggested that when exposed to cell wall synthesis inhibitor antibiotics, cells can respond to damage of the cell wall structure more rapidly than the wild-type. Accordingly, the *luxS* mutant exhibited decreased susceptibility to cell wall synthesis inhibitor antibiotics and to Trion X-100-induced autolysis. It is proposed that the LuxS/AI-2 system may affect bacterial susceptibility to cell wall synthesis inhibitor antibiotics via VraSR. Moreover, we observed that the *luxS* mutant exhibited a stronger biofilm than the wild-type (data not shown), suggesting that the stronger biofilm formed by the *luxS* mutant might also contribute to its decreased susceptibility to cell wall synthesis inhibitor antibiotics. Considering that biofilms are secreted via efflux pumps (e.g. the NorA system), it may well be that compounds that inhibit the quorum-sensing system also inhibit the efflux pumps system via VraRS or other potential pathways. This finding may provide valuable clues for antimicrobial therapy in *S. aureus* infection.

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