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Staphylococcus aureus autoinducer-2 quorum sensing decreases biofilm formation in an icaR-dependent manner

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Abstract

Background: Staphylococcus aureus is an important pathogen that causes biofilm-associated infection in humans. Autoinducer 2 (AI-2), a quorum-sensing (QS) signal for interspecies communication, has a wide range of regulatory functions in both Gram-positive and Gram-negative bacteria, but its exact role in biofilm formation in S. aureus remains unclear.

Results: Here we demonstrate that mutation of the AI-2 synthase gene luxS in S. aureus RN6390B results in increased biofilm formation compared with the wild-type (WT) strain under static, flowing and anaerobic conditions and in a mouse model. Addition of the chemically synthesized AI-2 precursor in the luxS mutation strain (ΔluxS) restored the WT phenotype. Real-time RT-PCR analysis showed that AI-2 activated the transcription of icaR, a repressor of the ica operon, and subsequently a decreased level of icaA transcription, which was presumably the main reason why luxS mutation influences biofilm formation. Furthermore, we compared the roles of the agr-mediated QS system and the LuxS/AI-2 QS system in the regulation of biofilm formation using the ΔluxS strain, RN6911 and the Δagr ΔluxS strain. Our data indicate a cumulative effect of the two QS systems on the regulation of biofilm formation in S. aureus.

Conclusion: These findings demonstrate that AI-2 can decrease biofilm formation in S. aureus via an icaR-activation pathway. This study may provide clues for therapy in S. aureus biofilm-associated infection.

Background

Staphylococcus aureus is an opportunistic pathogen that can adhere to many tissues and implants in humans to form biofilms causing refractory chronic infections [1,2]. Many therapies have been proposed but the potential efficacy is limited [3]. Given this situation, intensive research into the molecular mechanism of biofilm formation in S. aureus could facilitate the development of novel therapeutic devices.

Biofilms are complex communities of microorganisms encased in slime that can attach to surfaces [4]. Protein, polysaccharide, and extracellular DNA are supposed to be important components of Staphylococcal biofilms [5-7]. Biofilm formation is established using at least two properties: the adherence of cells to a surface and accumulation to form multi-layered cell clusters [8,9]. The latter process is

closely related to polysaccharide intercellular adhesion (PIA), a polysaccharide composed of β-1,6-linked N-acetyl-glucosamine residues in Staphylococci [10]. The intercellular adhesion (ica) locus is composed of four open reading frames (ORFs) icaA, icaD, icaB and icaC in an operon [11,12], and is responsible for generating PIA, which is required for biofilm formation in S. aureus. Moreover, decreased PIA level is considered to be the main factor leading to the destructive ability of biofilm formation in S. aureus RN6390B [13]. In recent years, many factors including glucose, glucosamine, oleic acid, urea, anaerobiosis and iron limitation have been identified as influencing the expression of PIA [12,14-18]. In addition, it has been demonstrated that IcaR represses ica expression by binding to the icaA promoter region [19]. Furthermore, QS has been recently shown to control the expression of the ica operon [20].

Quorum sensing is a widespread system used by bacteria for cell-to-cell communication, which regulates expression

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of multiple genes in a cell density-dependent manner [21,22]. The unique QS system shared by Gram-positive and Gram-negative bacteria is mediated by AI-2 [23], which is a signalling molecule synthesized by the *luxS* gene [24,25]. AI-2 originates from the auto-cyclization of precursor 4, 5-dihydroxy-2, 3-pentanedione (DPD) [26,27], and has been reported to regulate luminescence, motility and virulence [28-30]. Biofilm formation is known as the "bacterial social behaviour", in part owing to an organised mode of growth in a hostile environment. Many studies have described the role of AI-2 in biofilm formation. For example, synthetic AI-2 directly stimulates *Escherichia coli* biofilm formation and controls biofilm architecture by stimulating bacterial motility [31]. Subsequently, several studies also indicated that AI-2 indeed controls biofilm formation [32-34]. In contrast, some researchers reported that addition of AI-2 failed to restore biofilm phenotype of the parental strain [35-40], owing to the central metabolic effect of LuxS or difficulty in complementation of AI-2 [41]. There exists a conserved *luxS* gene in *S. aureus*, and it has been proved to be functional for generating AI-2 [42]. Previous work indicated that AI-2-mediated QS modulated capsular polysaccharide synthesis and virulence in *S. aureus* [43], deletion of the *luxS* gene led to increased biofilm formation in *Staphylococcus epidermis* [20], and biofilm

enhancement due to *luxS* repression was manifested by an increase in PIA [44].

In this study, we provide evidence that *S. aureus* ΔluxS strain formed stronger biofilms than the WT strain RN6390B, and that the *luxS* mutation was complemented by adding chemically synthesized DPD, the exogenous precursor of AI-2. AI-2 activated the transcription of *icaR*, and subsequently led to decreased *icaA* transcription, as determined by real-time RT-PCR analysis. Furthermore, the differences in biofilm-forming ability of *S. aureus* RN6911, ΔluxS strain, and the ΔagrΔluxS strain were also investigated. Our data suggest that AI-2 could inhibit biofilm formation in *S. aureus* RN6390B through the IcaR-dependent regulation of the *ica* operon.

Methods

Bacterial strains, plasmids and DNA manipulations

The bacterial strains and plasmids used in this study are described in Table 1. *E. coli* cells were grown in Luria-Bertani (LB) medium (Oxoid) with appropriate antibiotics for cloning selection. *S. aureus* strain RN4220, a cloning intermediate, was used for propagation of plasmids prior to transformation into other *S. aureus* strains. *S. aureus* cells were grown at 37°C in tryptic soy broth containing 0.25% dextrose (TSBg)

Table 1 Strains and plasmids used in this study

Strain or plasmid	Description	Reference or source
RN6390B	Standard laboratory strain	NARSA[a]
RN4220	8325-4 r⁻	NARSA
ΔluxS	RN6390B *luxS::ermB*	This study
RN6911	RN6390B derivative; agr locus replaced with tetM cassette	NARSA
ΔagrΔluxS	RN6911 *luxS::ermB, agr/luxS* double mutant	This study
ΔluxSpluxS	Complemented strain of ΔluxS; Ap^r Cm^r	This study
RN6390BG	RN6390B/pgfp	This study
ΔluxSG	ΔluxS/pgfp	This study
RN6911G	RN6911/pgfp	This study
ΔagrΔluxSG	ΔagrΔluxS/pgfp	This study
NCTC8325	Standard Laboratory strain	NARSA
NCTC8325ΔluxS	NCTC8325 *luxS::ermB*	60
E. coli strains		
TOP10	Cloning	Invitrogen
Plasmids		
pEASY-Blunt	Clone vector, Kan^r Ap^r	Transgen
pBTluxS	Vector used for *luxS* mutagenesis, Ap^r Cm^r Em^r	60
pLI50	*E. coli-S. aureus* shuttle cloning vector, Ap^r Cm^r	Addgene
pLIluxS	pLI50 with *luxS* and its promoter, Ap^r Cm^r	60
pgfp	*gfp* expression with the promoter of S10 ribosomal gene, Ap^r, Cm^r	

[a] *NARSA*, Network on Antimicrobial Resistance in *Staphylococcus aureus*.

(Difco No. 211825). In the flow cell assay, biofilm bacteria were grown in tryptic soy broth without dextrose (TSB) (Difco No. 286220). Medium was supplemented when appropriate with ampicillin (150 μg/ml), kanamycin (50 μg/ml), erythromycin (2.5 μg/ml) and chloramphenicol (15 μg/ml).

Construction of bacterial strains

To construct the ΔluxS strain from *S. aureus* RN6390B and the Δagr ΔluxS strain from *S. aureus* RN6911, the purified pBTluxS plasmid was used for allele replacement by erythromycin-resistance gene insertional mutagenesis as described previously [45]. Briefly, the appropriate upstream and downstream fragments of *luxS* were amplified from the genome of RN6390B, and the erythromycin-resistance gene was amplified from pEC1 with the relevant primers. The three fragments were ligated with each other with the erythromycin-resistance gene in the middle, and then ligated with the temperature-sensitive shuttle vector pBT2. The resulting plasmid pBTluxS [43] was introduced by electroporation into *S. aureus* strain RN4220 for propagation, and then transformed into *S. aureus* RN6390B for *luxS* mutation and *S. aureus* RN6911 for *agr luxS* double-gene mutation. All primers used in this study are listed in Table 2.

To make the *luxS*-complemented strain, the pLIluxS plasmid, which contains the native promoter of *luxS* and its intact open reading frame, was constructed in our previous work [43]. We purified the pLIluxS plasmid and transformed it into the ΔluxS strain for complementation, thus constructing the ΔluxSpluxS strain. WT and ΔluxS strains were also transformed with the empty

Table 2 Oligonucleotide primers used in this study

Primer	Sequence
rt-16S-f	CGTGGAGGGTCATTGGA
rt-16S-r	CGTTTACGGCGTGGACTA
rt-icaA-f	TTTCGGGTGTCTTCACTCTAT
rt-icaA-r	CGTAGTAATACTTCGTGTCCC
rt-icaR-f	ATCTAATACGCCTGAGGA
rt-icaR-r	TTCTTCCACTGCTCCAA
rt-clfB-f	TTTGGGATAGGCAATCATCA
rt-clfB-r	TCATTTGTTGAAGCTGGCTC
rt-fnbA-f	ATGATCGTTGTTGGGATG
rt-fnbA-r	GCAGTTTGTGGTGCTTGT
rt-fnbB-f	ACAAGTAATGGTGGGTAC
rt-fnbB-r	AATAAGGATAGTATGGGT
rt-map-f	AAACTACCGGCAACTCAA
rt-map-r	TGTTACACCGCGTTCATC
rt-efb-f	TAACATTAGCGGCAATAG
rt-efb-r	CCATATTCGAATGTACCA

plasmid pLI50 constructing strains WTp and ΔluxSp, which were used as the control. These strains transformed with plasmid were cultured in medium with chloramphenicol (15 μg/ml). The AI-2 precursor molecule, DPD, of which the storage concentration is 3.9 mM dissolved in water, was purchased from Omm Scientific Inc., TX, USA.

Biofilm formation and analysis

Biofilm formation under static conditions was determined by the microtitre plate assay based on the method described previously [46]. Briefly, the overnight cultures were made at a 1:100 dilution using fresh TSBg. The diluted cell suspension was inoculated into flat-bottom 24-well polystyrene plates (Costar 3599, Corning Inc., Corning, NY), 1 ml for each well. The plates were incubated at 37°C for different time courses and the wells were rinsed gently with water five times to remove non-adherent cells. Subsequently, the plates were stained with 0.5% crystal violet for 15 m, and then rinsed again with water to remove unbound stain. After that, the plates were dried, and the optical density at 560 nm (OD_{560}) was determined with an enzyme-linked immunosorbent assay reader in a 5×5 scan model. To investigate the effect of AI-2, the medium was supplemented with chemically synthesized DPD with a concentration range of 0.39 nM to 390 nM.

Biofilm formation was also examined in a flow cell (Stovall, Greensboro, USA), which was assembled and prepared according to the manufacturer's instructions. Flow cell experiments and laser scanning confocal microscope (CLSM) were performed as described previously [47]. Overnight cultures of different strains were adjusted to OD_{600} of 6.5 and made at a 1:100 dilution in fresh 2% TSB. Flow cells were inoculated with 4 ml of these culture dilutions and incubated at 37°C for 1 h, and then laminar flow (250 μl/m) was initiated. Biofilms of different strains were cultivated at 37°C in 2% TSB in three individual channels. The strains were transformed with the GFP plasmid for fluorescence detection, thus chloramphenicol was added to the flow cell medium to maintain plasmid selection. CLSM was performed on a Zeiss LSM710 system (Carl Zeiss, Jena, Germany) with a 20×0.8 n.a. apochromatic objective. Z-stacks were collected at 1 μm intervals. Confocal parameters set for WT biofilm detection were taken as standard settings. Selected confocal images stood for similar areas of interest and each confocal experiment was repeated four times. The confocal images were acquired from Zeiss ZEN 2010 software package (Carl Zeiss, Jena, Germany) and the three-dimensional biofilm images were rendered with Imaris 7.0 (Bitplane, Zurich, Switzerland). Biofilm biomass and average thickness were analysed with the

COMSTAT program [48] and were indicated as the mean ± standard deviation calculated from three images obtained from a given biofilm.

Ethical statement

The use and care of mice in this study was performed strictly according to the Institutional Animal Care and Use Committee guideline of University of Science and Technology of China (USTCACUC1101053).

In vivo model of catheter-associated biofilm formation

Biofilm formation was assessed *in vivo* using a murine model of catheter-associated infection [49]. Briefly, male BALB/c mice (6- to 8-weeks old) were obtained from Shanghai Laboratory Animal Centre of Chinese Academy of Sciences (Shanghai, China). The mice were anaesthetised with 1% pentobarbital sodium (0.01 ml/g of body weight) and surgically dissected. Specifically, a 1-cm 18G FEP polymer catheter (Introcan, Melsungen, Germany) was implanted subcutaneously in the dorsal area of the mice. The wound was closed with surgical glue. After incubation of 24 h, 5×10^7 colony-forming units (CFU) of the test strains in a total volume of 100 µl were introduced directly into the lumen of the catheters. Mice were euthanised after 3 days of infection, and then the catheters were removed carefully and washed briefly with phosphate-buffered saline (PBS). Catheters were placed in 1 ml of sterile PBS and sonicated for 30 s to remove the adherent bacteria. The number of bacteria was determined by plating on tryptic soy agar (TSA).

Anaerobic conditions

Biofilm formation was also monitored under anaerobic conditions. The Forma Anaerobic System (Thermo, Waltham, USA) was used to provide strictly anaerobic conditions for bacterial growth and related operations. Overnight cultures were adjusted to OD_{600} of 6.5, and then the bacterial cultures were carried into the anaerobic system for 1:100 dilution and inoculated into 24-well plates. Resazurin, which is used as an indicator for anaerobic conditions, was added to final concentration of 0.0002% (w/v). The plates were incubated at 37°C for 4 h and OD_{560} was determined after crystal violet staining.

A standard anaerobic jar of 120 ml volume was used to monitor the biofilm formation of the WT strain and the mutants under anaerobic conditions. Medium and containers with thorough scavenging were prepared as follows. Water was boiled using a three-necked bottle to degas the water while nitrogen was bubbled into the bottle to keep the contents anaerobic. TSBg medium was prepared with this degassed water. Then each anaerobic jar was dispensed with 50 ml TSBg while nitrogen was gassed into the jar to drive out the oxygen. The rubber

plug was quickly stuffed up following by an aluminium cap added, and then the jar containing TSBg was autoclaved at 121°C, 15 m. After preparation of the medium, biofilm formation under anaerobic conditions was examined and the operations were carried out in the anaerobic system.

Scanning electron microscopy (SEM)

Biofilm bacteria were grown on coverslips for five days, and then the coverslips were cut from the flow-cell settings and immediately fixed with 2.5% (vol/vol) glutaraldehyde in Dulbecco PBS (pH 7.2) overnight. According to the methods described previously [50], the coverslips were rinsed with PBS three times and dehydrated through an ethanol series (30%, 50%, 75%, 85% and 95%). Samples were dried and gold-palladium coated prior to SEM examination and micrographs were made with a XL-30 SEM at × 1500 to × 5000 magnification (FEI, Hillsboro, USA).

RNA isolation and real-time RT-PCR

All the bacteria used for RNA isolation to investigate the expression of genes that affect biofilm formation were those that grew statically in the 24-well plate. Bacteria in the wells of biofilm formation at different time courses (4 h, 8 h, 12 h) were collected and re-suspended in TE (Tris-EDTA) buffer (pH 8.0) containing 10 g/l lysozyme and 40 mg/l lysostaphin. After incubation at 37°C for 8 m, S. aureus cells were prepared for total RNA extraction using the Trizol method (Invitrogen), and the residual DNA was removed with RNase-free DNase I (TaKaRa). The concentration of RNA was adjusted to 100 ng/µl, and the samples were stored at –70°C. cDNA templates were synthesized from 50 ng RNA with PrimeScript™ 1st strand cDNA Synthesis Kit (TaKaRa) and gene-specific primers at 42°C for 15 m, 85°C for 5 s. Real-time PCR was performed with the cDNA and SYBR Premix Ex Taq (TaKaRa) using a StepOne Real-Time PCR System (Applied Biosystems). The quantity of cDNA measured by real-time PCR was normalised to the abundance of 16S cDNA. Real-time RT-PCR was repeated three times in triplicate parallel experiments.

Statistical analysis

The paired t test was used for statistical comparisons between groups. The level of statistical significance was set at a P value of ≤ 0.05.

Results

AI-2 inhibits biofilm formation in a concentration-dependent manner under static conditions

Previous studies showed that biofilm formation was influenced by the LuxS/AI-2 system both in Gram-positive

and Gram-negative bacteria [32,34]. The genome of *S. aureus* encodes a typical *luxS* gene, which plays a role in the regulation of capsular polysaccharide synthesis and virulence [43]. In this study, to investigate whether LuxS/ AI-2 system regulates biofilm formation in *S. aureus*, we monitored the biofilm formation of *S. aureus* WT strain RN6390B and the isogenic derivative ΔluxS strain using a microtitre plate assay. As shown in Figure 1A, the WT strain formed almost no biofilm after 4 h incubation at 37°C. However, the ΔluxS strain formed strong biofilms as measured by quantitative spectrophotometric analysis based on OD_{560} after crystal violet staining (Figure 1A). This discrepancy could be complemented by introducing a plasmid that contains the *luxS* gene (Figure 1B).

The effects of LuxS could be attributed to its central metabolic function or the AI-2-mediated QS regulation, which has been reported to influence biofilm formation in

Figure 1 Biofilm formation under static conditions and chemical complementation by DPD of different concentrations. Biofilm growth of *S. aureus* WT (RN6390B), ΔluxS and ΔluxS complemented with different concentrations of chemically synthesized DPD in 24-well plates for 4 h under aerobic conditions (A1: 0.39 nM, A2: 3.9 nM, A3: 39 nM, A4: 390 nM). The cells that adhered to the plate after staining with crystal violet were measured by OD_{560}.

some strains [32-34]. To determine if AI-2, as a QS signal, regulates biofilm formation in *S. aureus*, the chemically synthesized pre-AI-2 molecule DPD at concentrations from 0.39 nM to 390 nM was used to complement the ΔluxS strain. The resulting data suggested that exogenous AI-2 could decrease biofilm formation of the ΔluxS strain and the effective concentration for complementation was from 3.9 nM to 39 nM DPD (Figure 1A). As expected, these concentrations were within the range that has been reported [51]. The phenomenon that the higher concentration of AI-2 does not take effect on biofilm formation is very interesting, which has also been found in other species [51]. In the previous work [52,53], they indicated that AI-2 activity was associated with cyclic derivatives of this molecule that can be generated spontaneously. Therefore, it is possible that the concentration of effective molecules is different as the DPD concentration changes. These findings indicate that AI-2 could complement the effect of *luxS* mutation on biofilm formation and act in a concentration-dependent manner in *S. aureus*.
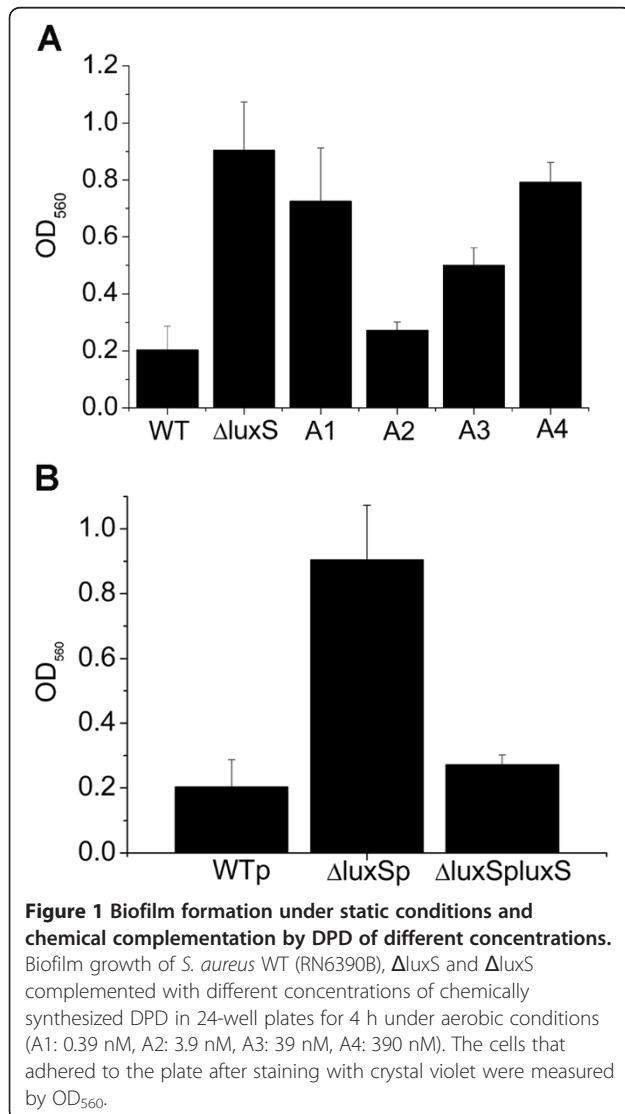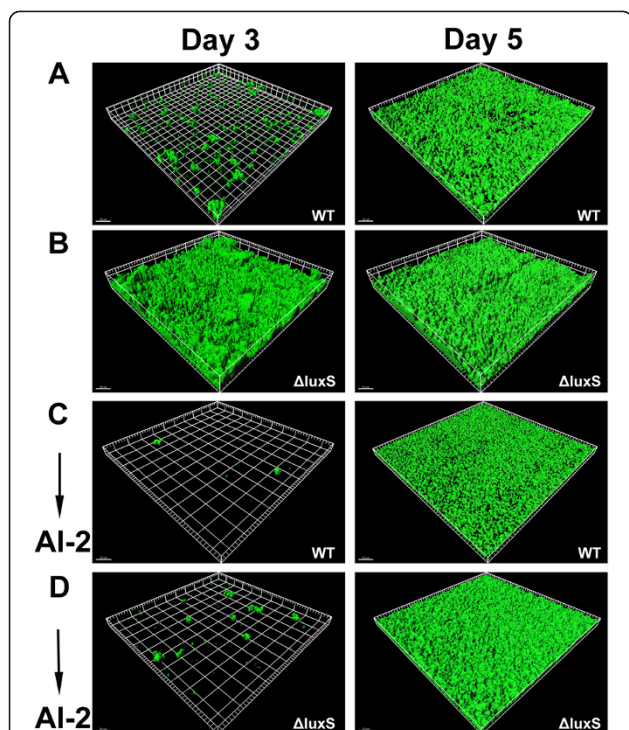
AI-2 inhibits biofilm formation in flow cell

To further compare the different biofilm formation ability owing to *luxS* deletion, biofilm formation of WT and the ΔluxS strains was assessed using a flow-cell assay. After 3 days of incubation, biofilms produced by WT strain were undetectable as monitored by CLSM. In contrast, the ΔluxS strain began to form intact and rough biofilms. At the 5th day, the WT strain produced biofilms similar to that formed by the ΔluxS strain 2 days before; meanwhile, the ΔluxS strain formed thicker and stronger biofilms (Figure 2A and B). Analysis of the biofilms by COMSTAT is shown in Table 3. The ΔluxS strain exhibited significantly increased total biomass and average thickness of biofilms relative to those of the WT strain.

In the flow-cell assay, 3.9 nM DPD was added to the culture medium at the beginning of the experiment. As expected, examination with CLSM showed that the ΔluxS strain complemented with 3.9 nM DPD did not produce biofilms after 3 days of growth in the flow cell, and formed biofilms similar to that of the WT strain at the 5th day (Figure 2C and D). As shown in Table 3, they both formed ~10-μm thick biofilms until the 5th day. These results suggest that AI-2 supplementation decreases biofilm formation under flow conditions.

Inactivation of *luxS* results in increased biofilm formation *in vivo*

To further verify the effect of AI-2 on biofilm formation *in vivo*, a murine model of catheter-associated biofilm formation was used. In this assay, mice were separately infected with 5×10^7 CFU/ml of the WT strain and the ΔluxS strain. After incubation for 3 days, the catheters were taken out and the number of bacteria was counted.

Figure 2 Biofilm formation in flow cell and chemical complementation by DPD. Biofilms of WT (RN6390BG) and ΔluxS (ΔluxSG) were grown in a flow cell in 2% TSB with chloramphenicol (15 μg/ml). Biofilm integrity and GFP fluorescence were monitored at the 3rd day and the 5th day by CLSM. For chemical complementation, 3.9 nM DPD was added to the TSB medium at the beginning of the experiment. CLSM images are representative of two separate experiments and each grid square represents 20 μm (**A**) WT. (**B**) ΔluxS. (**C**) WT supplemented with DPD. (**D**) ΔluxS supplemented with DPD.

Figure 3 Biofilm formation of *S. aureus* in vivo. Biofilm formation was assessed using a murine catheter-associated model of WT (NCTC8325) and ΔluxS (NCTC8325ΔluxS). Overnight culture of 5×10^7 CFU was injected into the catheters, which were implanted subcutaneously in the dorsal area of the mice. Results shown are the number of bacteria counted from the catheters after incubation for 3 days. Each point stands for one independent mouse. *P* value refers to a comparison between WT and ΔluxS and means statistically significant differences (P = 0.001) by Student's *t* test.

As shown in Figure 3, the ΔluxS strain exhibited significantly increased capacity to form biofilms compared to the WT strain (P = 0.001) *in vivo*. These results suggest that LuxS/AI-2 system is involved in the regulation of biofilm formation *in vivo*, which is consistent with the conclusion *in vitro*.
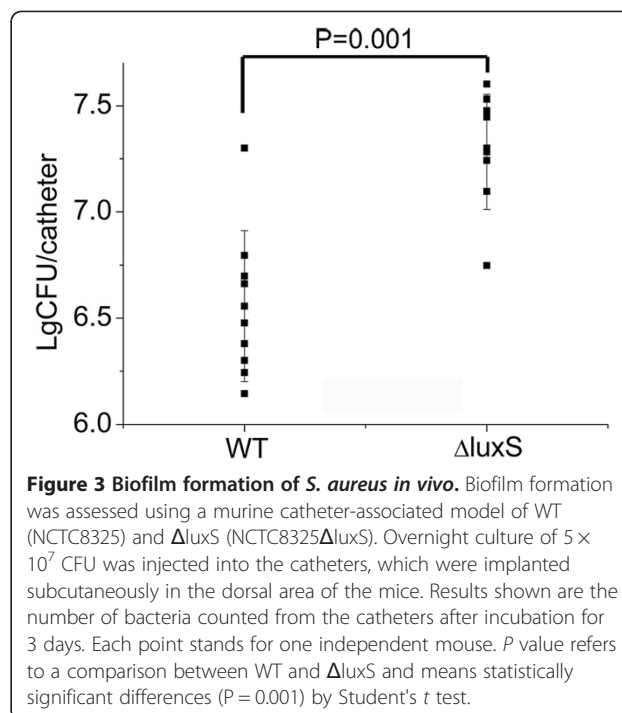
AI-2 represses the transcription of *icaA* via the activation of *icaR*

PIA is considered to be a major factor determining biofilm formation in some bacteria [10,54,55]. To test if AI-2-mediated biofilm reduction is due to a change in PIA expression, the transcription of *icaA* was examined using real-time RT-PCR with RNA isolated from biofilm bacteria at different time points. Transcription of *icaA* reached its peak at 4 h of biofilm formation and the maximum difference between the WT strain and the ΔluxS strain was also highlighted at this time (data not shown). Thus, RNA was isolated from 4 h biofilm bacteria of the WT strain, the ΔluxS strain, and the ΔluxS strain complemented with 3.9 nM DPD. Expression of *icaA* was examined using real-time RT-PCR. The resulting data showed that expression of *icaA* was elevated in the ΔluxS strain, and it could be complemented by 3.9 nM DPD (Figure 4A). As expected, corresponding to the biofilm formation in Figure 1, thicker biofilms were presented owing to the *luxS* mutation while the bacteria within the biofilms also displayed elevated *icaA* transcription. Moreover, we examined the expression of several main adhesion molecules. As shown in Additional file 1: Figure S1, there were no obvious differences between the WT, ΔluxS and ΔluxS transformed with the pLIluxS plasmid for complementation (ΔluxSpluxS). Here, the WT and ΔluxS strains were also transformed with an empty PLI50 plasmid constructing the WTp strain and ΔluxSp strain, which were used as the control. Besides, we added sodium-metapeiodate into the well-developed biofilms and found that biofilms dispersed after 2 h incubation at 37°C. Taken together, our results suggest that PIA is the main factor controlled by AI-2 in the regulation of biofilm formation in *S. aureus*.

Table 3 Biofilm formation of WT and ΔluxS strains

Strains	Biofilm biomass ($μm^3/μm^2$)		Average thickness (μm)	
	Day 3	Day 5	Day 3	Day 5
WT	3.01 ± 0.2	11.71 ± 1.25	3.81 ± 0.35	11.51 ± 0.92
ΔluxS	20.16 ± 1.59*	25.67 ± 1.16*	20.79 ± 1.47*	26.18 ± 0.43*
WT + AI-2	0.11 ± 0.01	10.44 ± 0.51	0.12 ± 0.01	9.45 ± 0.5
ΔluxS + AI-2	0.49 ± 0.018	14.31 ± 0.59	0.59 ± 0.06	13.53 ± 0.5

* Significantly different results compared with WT (P < 0.01).

Aerobic conditions

Figure 4 Transcriptional regulation of *icaA* and *icaR* by AI-2.
Real-time RT-PCR of *icaA* and *icaR* transcription was measured. The
bacteria used for RNA extraction were those that were incubated at
4 h for biofilm formation under aerobic conditions. Error bars
indicate the variation between triplicate samples within the
real-time RT-PCR. The relative cDNA abundance of the WT sample
was assigned a value of 1. (**A**) Relative transcript levels of *icaA* of WT
(RN6390B), ΔluxS and ΔluxS complemented with 3.9 nM DPD under
aerobic conditions. (**B**) Relative transcript levels of *icaR* of WT
(RN6390B), ΔluxS and ΔluxS complemented with 3.9 nM DPD under
aerobic conditions.

It was reported that IcaR is a negative regulator of the
icaA locus [19], and that *icaR* could be regulated by Rbf,
SarA and SigB [56,57]. However, few studies indicate
that the signalling molecule AI-2 could be an activator
of *icaR*. We therefore investigated whether repression of
icaA by AI-2 was mediated by IcaR by examining the
icaR transcription in the biofilm bacteria of the WT
strain, the ΔluxS strain and the ΔluxS strain comple-
mented with 3.9 nM DPD. We found that the ΔluxS
strain displayed decreased transcription of *icaR* com-
pared to WT, and DPD supplementation could comple-
ment the effect of *luxS* mutation (Figure 4B). These data

indicate that the repression of *icaADBC* transcription by
AI-2 is through the activation of *icaR*. These results
allow us to conclude that AI-2 activates *icaR*, which
results in decreased *icaADBC* transcription and subse-
quently decreased biofilm formation.

AI-2 inhibits biofilm formation and represses the transcription of *icaA* under anaerobic conditions
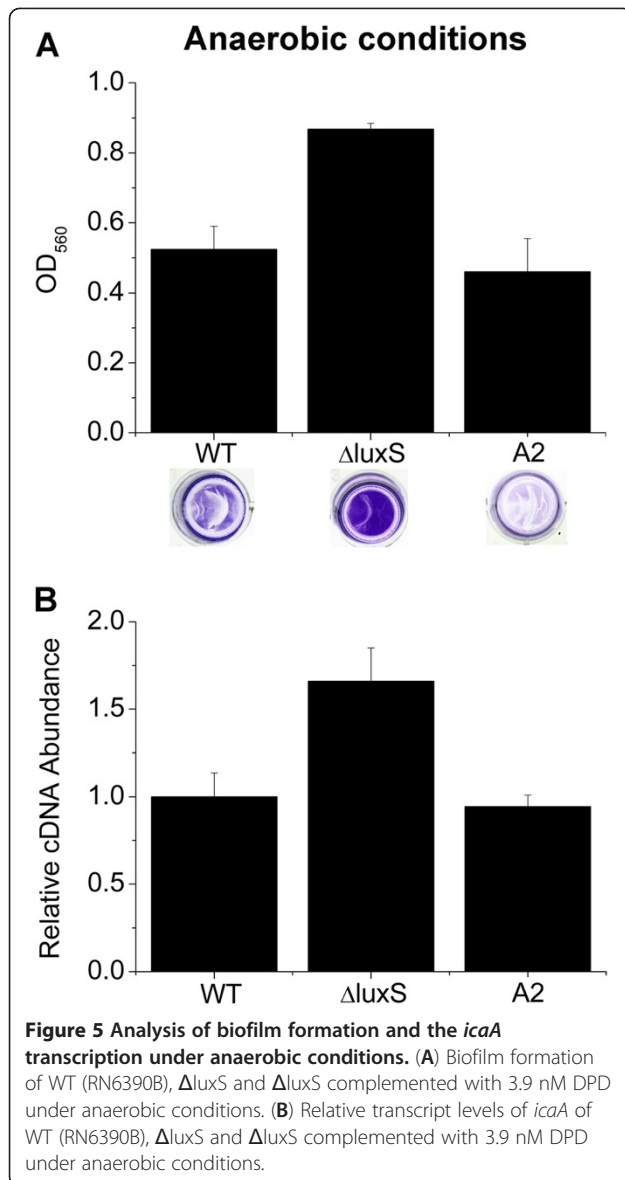
Hypoxia or anaerobic conditions is a common hostile
environment that the biofilm bacteria suffer *in vivo*
[3,58,59]. To determine whether or not AI-2 could also
affect biofilm formation under anaerobic conditions, the
microtitre plate assay was used to examine the biofilm
growth. After incubation of the plate for 4 h under anae-
robic conditions, we found that the ΔluxS strain displayed
increased biofilm formation compared to the WT strain,
and AI-2 supplementation restored the WT phenotype
(Figure 5A). Consistently, AI-2 repressed the transcription
of *icaA* under anaerobic conditions (Figure 5B).

The LuxS/AI-2 QS system and the *agr*-mediated QS system have a cumulative effect on the regulation of biofilm formation

It was reported that the *agr* QS system mediates biofilm
dispersal in *S. aureus* [60]. To determine whether the
LuxS/AI-2 QS system and the *agr*-mediated QS system
have a cumulative or complementary effect on the regula-
tion of biofilm formation, we constructed a Δagr ΔluxS
strain and compared the biofilm formation among the
WT strain and the mutants using different assays, includ-
ing the microtitre plate assay, flow cell, anaerobic jar and
SEM. Consistently, we found that the Δagr ΔluxS strain
displayed the strongest capacity for biofilm formation
among all the stains we investigated.

In the flow-cell assay, as shown in Figure 6A, the Δagr
ΔluxS strain formed stronger biofilms than RN6911, as
shown by CLSM, indicating that mutation of *luxS* indeed
influences biofilm formation and that the two systems seem
to play a cumulative effect. Moreover, similar results were
obtained in the microtitre plate assay and the anaerobic jar
assay under anaerobic conditions (Figure 6B and D).

To accurately describe the distinct biofilm formation
resulting from *luxS* deletion, SEM was used for evaluating
the structure and surface appearance of the mature biofilm.
Therefore, the coverslips of the flow-cell chamber on which
5 days biofilms of WT and the ΔluxS strain grew were cut
out. SEM analysis showed that the ΔluxS strain produced a
compact biofilm structure with increased coverage than
that of the WT strain (Figure 6C). On closer inspection, we
found that the ΔluxS strain displayed stronger intercellular
adhesion and this was also reflected in the Δagr ΔluxS
strain. The Δagr ΔluxS strain showed stronger intercellular
adhesion ability than RN6911 (Figure 6C), indicating a pos-
sible result of elevated expression of PIA. Interestingly,

Figure 5 Analysis of biofilm formation and the _icaA_ transcription under anaerobic conditions. (**A**) Biofilm formation of WT (RN6390B), ΔluxS and ΔluxS complemented with 3.9 nM DPD under anaerobic conditions. (**B**) Relative transcript levels of _icaA_ of WT (RN6390B), ΔluxS and ΔluxS complemented with 3.9 nM DPD under anaerobic conditions.

microscopic analysis of the biofilm structure revealed that the _agr_ mutation led to biofilms that adopted a "ridged" appearance with many channels, rather than the relatively smooth, confluent layer normally detected in the WT and ΔluxS strains, presumably because the thicker biofilms with a dense compact structure restrict the growth of bacteria inside. Based on these results, we speculate that the LuxS/AI-2 QS system and the _agr_-mediated QS system play a cumulative effect on the regulation of biofilm formation in _S. aureus_. It has been reported that induction of the _agr_ system in established _S. aureus_ biofilms detaches cells in an _ica_-independent manner and they also demonstrate that the dispersal mechanism requires extracellular protease activity [60]. Therefore, it seems that the influences of the LuxS/AI-2 QS system and the _agr_-mediated QS system

on biofilm formation are through different pathways in _S. aureus_.

Discussion

Most previous studies of biofilm formation have been performed under one or two conditions to present this phenotype. However, biofilm is a kind of "smart community" that seems able to cope with different environments. Therefore, a single condition may lead to misunderstanding regarding the elaborate function of a specific gene. To provide sufficient and rigorous evidence, we demonstrate that the LuxS/AI-2 system is involved in the regulation of biofilm formation under different conditions. In contrast to the most commonly used microtitre plate assay, flow cell is increasingly used for detecting biofilm growth, of which the dynamic three-dimensional image could be monitored by CLSM dynamically. This setting simulates the environment of flowing surfaces _in vivo_, such as some interfaces between body fluids and implants. The result under this condition may offer more accurate information about flow surroundings _in vivo_. In addition, we also investigated biofilm formation under anaerobic conditions, which the biofilm bacteria undergo. The oxygen partial pressure of air-equilibrated medium is high _in vitro_, whereas hypoxic environments may prevail in body implants and human tissues distant from arterial blood flow [58,61]. Moreover, most locations in which the biofilm bacteria accumulate are usually niches of local low oxygen because of inflammatory cell aggregation [59,62].

The mouse model was used here to compare biofilm formation of the WT and the ΔluxS strains and our data were consistent with the _in vitro_ data. Nevertheless, there are few studies regarding AI-2 complementation in the mouse model to date, and the specific mechanism of these signal molecules _in vivo_ remains elusive. In general, we offer consistent results under different conditions to emphasise that the conclusion drawn is consistent and worthy of reference in most cases.

LuxS and AI-2 affect biofilm development, whereas the results may be different in the same strain due to various factors. Previous work has shown that AI-2 was produced in rich medium under aerobic and anaerobic conditions peaking during the transition to stationary phase, but cultures retained considerable AI-2 activity after entry into the stationary phase under anaerobic conditions. In addition, the _S. aureus_ RN6390BΔluxS strain showed reduction in biofilm formation in TSB containing 1% glucose and 3% sodium chloride under anaerobic conditions [42]. However, in our study, analysis of biofilm growth _in vitro_ and _in vivo_ led to the conclusion that the ΔluxS strain exhibited increased biofilm formation compared to the WT strain. Importantly, the _luxS_ mutation could be complemented by chemically synthesized DPD, indicating the effect of AI-2-mediated QS on biofilm formation in
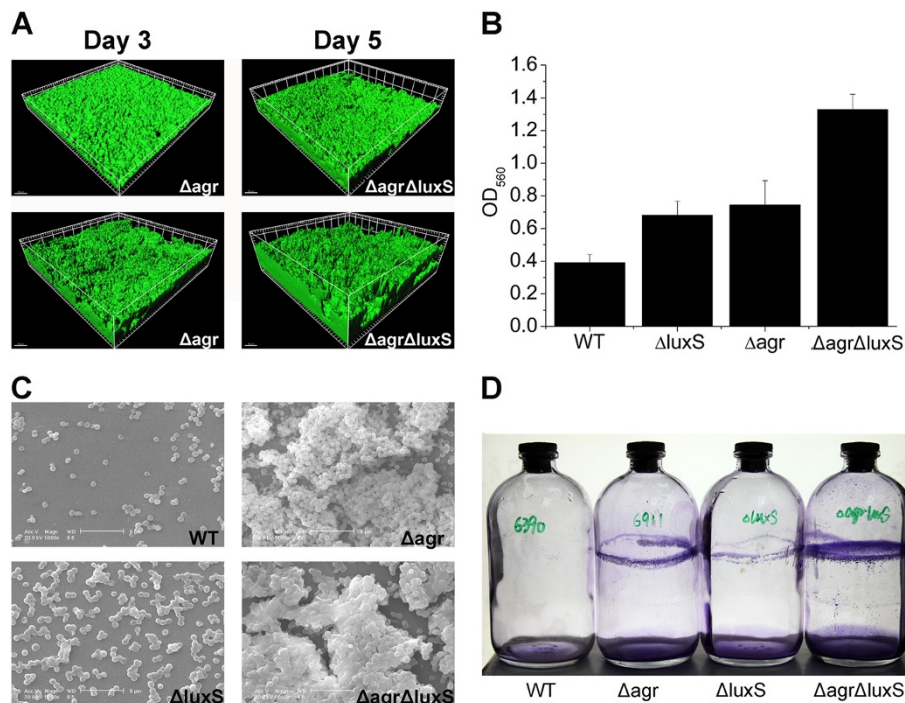
Figure 6 Additive effect played by the LuxS/AI-2 QS system and the *agr*-mediated QS system. (A) The ΔagrΔluxSG and RN6911G grew biofilms in the flow cell, and the representative images were measured by CLSM at the 3rd and 5th day of biofilm formation. Strains are indicated in the figure. **(B)** Overnight cultures of WT (RN6390B), Δagr (RN6911), ΔluxS and Δagr ΔluxS were inoculated in 24-well plate and formed biofilms under anaerobic conditions. **(C)** WT, Δagr, ΔluxS and Δagr ΔluxS formed 5 days biofilms in a flow cell on the upper surface of the coverslips, which were cut and examined by scanning electron microscopy. **(D)** The anaerobic jar was used for monitoring the biofilm formation of the WT, Δagr, ΔluxS and Δagr ΔluxS, OD_{560} was measured after crystal violet staining.

S. aureus. Hardie and Heurlier [41] summarised six main factors that influence the experimental results for doing research on the LuxS/AI-2 system: experimental design; genetic complementation; chemical complementation; conditioned supernatant complementation; and complementation with molecules linked to AI-2 production and that independent of *luxS* status. With detailed analysis, we found that the inconsistency of the results is in part owing to the different growth medium provided to the biofilm bacteria, especially the different concentrations of glucose and sodium chloride, which are both important factors enhancing biofilm formation [63].

In addition to the present evidence of AI-2-regulated biofilm formation in *S. aureus*, we found that the transcription of *icaR* is activated by AI-2, which is barely reported, although we have not yet identified the mechanism of the interaction between them. This is partly because the detailed mechanism of transport and action of AI-2 has only been described in several strains and different mechanisms are applied to different species, although AI-2 has been proven to act as a signalling molecule that could regulate series of gene expression. The first mechanism revealed was in *Vibrio harveyi*, which responds to AI-2 by initiating a phosphorylation cascade [64]. In *Salmonella*

typhimurium [65] and *E. coli* [66,67], AI-2 seems to be taken up by an ABC transporter. However, the mechanism of AI-2 transport and functional performing in *Staphylococci* was still unknown. Therefore, the detailed mechanism through which AI-2 functions in *S. aureus* should be highlighted here, and the interaction between AI-2 and IcaR requires further study.

In addition to PIA, we do not observe any obvious increase of extracellular protein (Additional file 2: Figure S2) or bacterial autolysis in the ΔluxS strain (Additional file 3: Figure S3). Our results showed no significant differences in the transcriptional levels of several main adhesion molecules. Moreover, previous work indicated that *S. aureus* strains 8325-4 and RN4220 formed PIA-dependent biofilms [68]. We thus propose that AI-2 signalling represses the *icaA* expression, and subsequently leads to decreased biofilm formation in *S. aureus*.

More and more studies concerning multispecies biofilms gradually uncover the mechanisms of the interaction and communication of the different species inside the biofilms. One of the most popular approaches of the signalling regulation is directed towards the AI-2-controlled QS system for its extensive use of interspecies. The plaque biofilms on tooth surfaces consist of various oral bacteria

including *S. aureus* and involve complex microbial interactions [69-71]. There is evidence that AI-2-mediated QS may play a significant role in oral biofilm formation [50]. As reported by others, airway infections by *Pseudomonas aeruginosa* afflicting patients with cystic fibrosis (CF) are among the most enigmatic of biofilm diseases [2]. *S. aureus* is also found to be a major pathogen associated with *P. aeruginosa* in CF lung infection [72]. Previous work has shown that PIA is expressed in lungs infected with *S. aureus*, whereas CP8 is not expressed because of limited oxygen [73]. Here, we provide evidence that AI-2 can regulate *icaA* expression under anaerobic conditions, suggesting a potential role of AI-2 in influencing *S. aureus* infection in lungs. However, few studies about biofilm formation cooperated by *S. aureus* and the other species are reported. Therefore, could *S. aureus* and the other species in their focus areas form multispecies biofilms? Could AI-2 play an important role in this process? It is interesting to discuss the actual complex-flora interaction in human and social behaviour of the bacteria. Therefore, revelation of the AI-2-regulated biofilm formation in *S. aureus* possesses instructive meaning for these related studies.

Conclusions

These findings demonstrate that AI-2 can decrease biofilm formation in *S. aureus* via an *icaR*-activation pathway. This study may provide clues for therapy in *S. aureus* biofilm-associated infection.

Additional files

Additional file 1: Relative transcript levels of several adhesions. The levels of transcription of these genes including *map*, *fnbA*, *fnbB*, *clfB*, *efb* were measured by real-time RT-PCR in *S. aureus* WTp and ΔluxSp and ΔluxS complemented with a plasmid containing *luxS* gene for genetic complementation (ΔluxSpluxS). As the control, WT and ΔluxS were transformed with empty plasmid PLI50, constructing WTp and ΔluxSp.

Additional file 2: Extracellular protein loaded on SDS-PAGE. The levels of extracellular-protein expression of biofilm bacteria, which were incubated at 37°C for 4 h and 24 h, were measured.

Additional file 3: Triton X-100-stimulated autolysis. The autolysis of WT, ΔluxS and ΔluxSpluxS induced in 0.05 M Tris–HCl buffer containing 0.05% (vol/vol) Triton X-100 were measured.

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