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AI-2 quorum sensing negatively regulates *rbf* expression and biofilm formation in *Staphylococcus aureus*

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A B S T R A C T

Staphylococcus aureus is an important pathogen that is capable of forming biofilms on biomaterial surfaces to cause biofilm-associated infections. Autoinducer 2 (AI-2), a universal language for interspecies communication, is involved in a variety of physiological activities, although its exact role in Gram-positive bacteria, especially in *S. aureus*, is not yet thoroughly characterized. Herein we demonstrate that inactivation of *luxS*, which encodes AI-2 synthase, resulted in increased biofilm formation and higher polysaccharide intercellular adhesion (PIA) production compared with the wild-type strain in *S. aureus* NCTC8325. The transcript level of *rbf*, a positive regulator of biofilm formation, was significantly increased in the *luxS* mutant. All of the parental phenotypes could be restored by genetic complementation and chemically synthesized 4,5-dihydroxy-2,3-pentanedione, the AI-2 precursor molecule, suggesting that AI-2 has a signaling function to regulate *rbf* transcription and biofilm formation in *S. aureus*. Phenotypic analysis revealed that the *luxS rbf* double mutant produced approximately the same amount of biofilms and PIA as the *rbf* mutant. In addition, real-time quantitative reverse transcription-PCR analysis showed that the *icaA* transcript level of the *rbf* mutant was similar to that of the *luxS rbf* double mutant. These findings demonstrate that the LuxS/AI-2 system regulates PIA-dependent biofilm formation via repression of *rbf* expression in *S. aureus*. Furthermore, we demonstrated that Rbf could bind to the *sarX* and *rbf* promoters to upregulate their expression.

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1. Introduction

Quorum sensing (QS) is a widespread signaling system used by bacteria for cell-to-cell communication. Bacterial QS coordinates social activities and population behaviors, such as bioluminescence, bacterial competence, virulence, antibiotic production, and biofilm formation. QS is accomplished by the production, release, and detection of small diffusible signaling molecules called autoinducers that accumulate in proportion to cell density (Bassler, 1999; Miller and Bassler, 2001; Waters and Bassler, 2005). In Gram-negative bacteria, QS is typically mediated by a LuxI/LuxR-type system in which LuxI-type proteins produce AHL autoinducers, and LuxR-type proteins detect the autoinducers and subsequently regulate the transcription of target genes (Fuqua et al., 1994). In Gram-positive bacteria, QS is typically regulated by the production of and response to processed oligopeptide autoinducers, which act through two-component systems (Kleerebezem et al., 1997).

Studies have indicated that a QS system shared by Gram-positive and Gram-negative bacteria involves the production of autoinducer 2 (AI-2). AI-2 is not a single compound but is a family of interconverting compounds derived from 4,5-dihydroxy-2,3-pentanedione (DPD), which is synthesized by the LuxS enzyme in the activated methyl cycle (Schauder et al., 2001; Surette et al., 1999). The enzymatic activity of LuxS is influenced by its phosphorylation status, which is mediated by the serine/threonine kinase Stk1 (Cluzel et al., 2010). LuxS is widely present in bacteria, leading to the suggestion that AI-2 is a universal language for interspecies communication (Xavier and Bassler, 2003). The LuxS/AI-2 QS system was first identified in *Vibrio harveyi*, where it functions as one part of complex parallel QS systems to regulate bioluminescence (Bassler et al., 1994). In *V. harveyi*, AI-2 is detected and transduced by a two-component system. Borate-AI-2 is recognized by a periplasmic binding protein LuxP that interacts with cytoplasmic response

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regulator proteins, initiating a phosphorylation cascade to finally regulate the expression of the luciferase structural operon (Bassler et al., 1994; Chen et al., 2002). In *Escherichia coli* and *Salmonella typhimurium*, LsrB, a component of the *lsr* transporter, binds to a chemically distinct form of the AI-2 molecule lacking boron (Miller et al., 2004). AI-2 is first taken up by the *lsr* transporter, and then the expression of genes controlled by the QS system is initiated (Taga et al., 2003; Xavier and Bassler, 2005). However, it is not clear how AI-2 is detected or what functions are regulated by this signaling molecule in many *luxS*-containing bacteria, especially in Gram-positive bacteria.

Staphylococcus aureus is a major human pathogen that could cause a wide variety of diseases ranging from minor skin infections to life-threatening syndromes (Archer, 1998; Lowy, 1998). Many *S. aureus* infections are involved in biofilm formation, including endocarditis, septic arthritis, osteomyelitis, and infections associated with implanted medical devices (Costerton et al., 1999; Parsek and Singh, 2003). A biofilm is a surface-associated community of microorganisms encompassed by an extracellular matrix. In addition to assisting the bacterial colonization of surfaces, biofilms are believed to increase the tolerance toward antimicrobial agents and host defenses (Fux et al., 2005; Leid et al., 2002; Otto, 2008). Protein, exopolysaccharide, and extracellular DNA are supposed to be the important components of staphylococcal biofilm (Laverty et al., 2013; Otto, 2008). Exopolysaccharides, predominantly polysaccharide intercellular adhesin (PIA), are the most common constituents of staphylococcal biofilm. The biosynthesis of PIA is mediated by the intercellular adhesion (*ica*) locus, which is composed of the four open reading frames (ORFs) *icaA*, *icaD*, *icaB*, and *icaC* in a single operon (Heilmann et al., 1996; Vuong et al., 2004). Moreover, a decreased PIA level is thought to be the main cause of the disability of biofilm formation in *S. aureus* NCTC8325 (Cramton et al., 1999).

In recent years, many factors including high temperature, osmotic stress, glucose, glucosamine, ethanol, anaerobiosis, and sub-inhibitory concentrations of certain antibiotics have been found to affect PIA production in vitro (Conlon et al., 2002; Cramton et al., 2001; Dobinsky et al., 2003; Knobloch et al., 2001; Rachid et al., 2000b). In addition, several staphylococcal proteins, such as IcaR, SarA, SigB, TcaR, SrrAB, SarX, and Rbf, have been reported to be involved in the transcriptional regulation of the *ica* operon (Cue et al., 2012, 2013; Jefferson et al., 2004; Rachid et al., 2000a; Ulrich et al., 2007; Valle et al., 2003; You et al., 2014). Among these, the most important regulator involved in *ica* operon regulation is IcaR, which binds to the *icaA* promoter region and negatively regulates *ica* operon expression. The deletion of *icaR* has been shown to significantly increase *ica* operon expression and PIA production (Conlon et al., 2002). Recently, the Lee group found that Rbf is required for PIA-dependent biofilm formation in response to glucose and NaCl in *S. aureus*. They demonstrated that Rbf upregulates SarX, which then downregulates IcaR, thereby activating the *ica* operon (Cue et al., 2009, 2013; Lim et al., 2004). Furthermore, QS has been recently shown to regulate the expression of the *ica* operon (Xu et al., 2006). The *luxS* gene has been identified in *S. aureus* and has been shown to function in AI-2 production (Doherty et al., 2006). The function of the LuxS/AI-2 system in *Staphylococcus epidermidis* and *S. aureus* was investigated. The LuxS/AI-2 system regulates biofilm formation via transcriptional regulation of the *ica* locus and controls virulence-related gene expression (Xu et al., 2006; Yu et al., 2012; Zhao et al., 2010). However, no potential AI-2 receptor has been found, and the mechanism by which AI-2 functions has not yet been thoroughly characterized in *S. aureus*.

In this study, we have revealed that AI-2 QS regulated biofilm formation by repressing the expression of Rbf, a positive regulator of biofilm formation. Furthermore, we found that Rbf can bind to the *sarX* and *rbf* promoters to upregulate their expression.

2. Materials and methods

2.1. Bacterial strains, plasmids, and growth conditions

The bacterial strains and plasmids used in this study are described in Table 1. *S. aureus* RN4220 was used for the propagation of plasmids prior to transformation into other *S. aureus* strains. *E. coli* was grown in Luria-Bertani (LB) medium (Oxoid), and *S. aureus* was grown in tryptic soy broth (TSB; BD) medium or tryptic soy broth medium without glucose (TSBg-; BD) at 37 °C. When required, appropriate antibiotics were used for plasmid selection and maintenance at the following concentrations: for *E. coli*, ampicillin at 150 μg/ml and kanamycin at 50 μg/ml; for *S. aureus*, kanamycin at 50 μg/ml, erythromycin at 2.5 μg/ml, and chloramphenicol at 15 μg/ml.

2.2. DNA manipulation

Genomic DNA of *S. aureus* was prepared by a standard protocol for Gram-positive bacteria. Plasmid DNA was extracted with a plasmid purification kit (Promega) according to the manufacturer's instructions. Easy Taq DNA polymerases and PrimeSTAR HS DNA polymerase were purchased from TransGen and TaKaRa, respectively. All plasmids transformed into the target *S. aureus* strains were first introduced into *S. aureus* RN4220 for modification by electroporation, as described previously (Kraemer and Iandolo, 1990).

2.3. Construction of S. aureus mutant strains

The *luxS* mutant from *S. aureus* NCTC8325 was constructed in our previous work (Zhao et al., 2010). To construct the *rbf* mutant from *S. aureus* NCTC8325 and the *luxS rbf* double mutant from the *luxS* mutant, the upstream and downstream fragments of the *rbf* gene were amplified from the genome of NCTC8325 using the up-rbf-f/up-rbf-r and down-rbf-f/down-rbf-r set of primers, and the kanamycin-resistant gene was amplified from the genome of the *Δrnc* strain with the relevant primers (kana-f/kana-r). The three fragments were ligated with each other with the upstream and downstream fragments flanking the kanamycin-resistant gene and then were cloned into the temperature-sensitive shuttle vector pBT2 to create pBT*rbf*. The resulting plasmid was transformed by electroporation into *S. aureus* NCTC8325 and the *luxS* mutant. The mutant strains were screened using a previously described method (Bruckner, 1997). The mutants were verified by PCR and sequencing. All primers used in this study are listed in Table 2.

2.4. Complementation of mutants

The pLI*luxS* plasmid containing the native promoter of *luxS* and its intact ORF was constructed in our previous work (Zhao et al., 2010). For the construction of pLI*rbf*, the *rbf* gene and its native promoter from *S. aureus* NCTC8325 was amplified by PCR with the primers c-rbf-f and c-rbf-r. To construct pLI*rsbU*, the *rsbU* gene and its promoter from *S. aureus* SH1000 was amplified by PCR with the primers c-rsbU-f and c-rsbU-r. The PCR product was cloned into pLI50 (Addgene). The complementary plasmids were transferred into the mutant strains. The wild-type (WT) and the mutant strains were also transformed with the empty plasmid pLI50, which was used as control. The chemically synthesized AI-2 precursor molecule, DPD, of which the storage concentration is 3.9 mM dissolved in water, was purchased from Omm Scientific Inc., Texas, USA.

Table 1
Strains and plasmids used in this study.

Strain or plasmid	Description	Reference or source
Strains		
NCTC8325	WT	NARSA[a]
RN4220	8325-4 r⁻, initial recipient for modification of plasmids that are introduced into *S. aureus* from *E. coli*	NARSA
SH0	8325 pLI50	This study
SH1	8325 *luxS::ermB*	Zhao et al. (2010)
SH2	8325 *luxS::ermB* pLI50	This study
SH3	8325 *luxS::ermB* pLI*luxS*	Zhao et al. (2010)
SH4	8325 *rbf::Kan*	This study
SH5	8325 *rbf::Kan* pLI50	This study
SH6	8325 *rbf::Kan* pLI*rbf*	This study
SH7	8325 *luxS::ermB rbf::Kan*	This study
SH8	8325 *luxS::ermB rbf::Kan* pLI50	This study
SH9	8325 *luxS::ermB rbf::Kan* pLI*rbf*	This study
SH10	8325 *luxS::ermB rbf::Kan* pLI*luxS*	This study
SH11	8325 pOSR*lacZ*	This study
SH12	8325 *rbf::Kan* pOSR*lacZ*	This study
SH13	8325 pGFP	This study
SH14	8325 *luxS::ermB* pGFP	This study
SH15	8325 *rbf::Kan* pGFP	This study
SH16	8325 *luxS::ermB rbf::Kan* pGFP	This study
SH17	8325 pLI*rsbU*	This study
SH18	8325 *luxS::ermB* pLI*rsbU*	This study
SH19	8325 *rbf::Kan* pLI*rsbU*	This study
SH20	8325 *luxS::ermB rbf::Kan* pLI*rsbU*	This study
Trans5α	Clone host strain	TransGen
BL21 (DE3)	Express strain	TransGen
Plasmids		
pEasy-blunt simple	Clone vector, Kan^r [b] Ap^r [b]	TransGen
pEC1	pUC18 derivative, source of the *ermB* gene, Ap^r	Bruckner
pBT2	Shuttle vector, temperature sensitive, Ap^r Cm^r [b]	Bruckner
pBT*rbf*	pBT2 containing upstream and downstream fragments of *rbf* and kanamycin-resistant gene, for *rbf* mutagenesis, Ap^r Cm^r Kan^r	This study
pOS1-*lacZ*	Shuttle vector, containing promoter-less *lacZ* gene, Ap^r Cm^r	Liu et al. (2011)
pOSR*lacZ*	*rbf-lacZ* fusion shuttle vector, a derivative of pOS1-*lacZ*	This study
pLI50	Shuttle cloning vector, Ap^r Cm^r	Addgene
pLI*rbf*	pLI50 with *rbf* ORF and its promoter, Ap^r Cm^r	This study
pLI*luxS*	pLI50 with *luxS* ORF and its promoter, Ap^r Cm^r	Zhao et al. (2010)
pLI*rsbU*	pLI50 with *rsbU* ORF and its promoter, Ap^r Cm^r	This study
pGFP	*gfp* expression with the promoter of S10 ribosomal gene, Ap^r, Cm^r	You et al. (2014)

[a] NARSA, Network on Antimicrobial Resistance in *Staphylococcus aureus*.
[b] Kan^r, kanamycin-resistant; Ap^r, ampicillin-resistant; Cm^r, chloramphenicol-resistant.

2.5. Total RNA isolation, cDNA generation, and real-time quantitative reverse transcription-PCR (qRT-PCR)

Overnight cultures of *S. aureus* were diluted 1:100 in TSB and then grown to the indicated cell density until being collected. The collected cells were processed with 1 ml of RNAiso Plus (TaKaRa) in combination with 0.1-mm-diameter-silica beads in a FastPrep-24 automated system (MP biomedicals Solon, Ohio, USA), and the residual DNA was removed with RNase-free DNase I (TaKaRa). The concentration of total RNA was adjusted to 100 ng/μl, and the samples were stored at −80 °C for later use. For the reverse-transcription, cDNA templates were synthesized from 100 ng total RNA with PrimeScript 1st Strand cDNA Synthesis Kit (TaKaRa). qRT-PCR was performed with SYBR Premix Ex Taq (TaKaRa) using the StepOne Real-Time PCR System (Applied Biosystems). The quantity of cDNA measured by real-time PCR was normalized to the abundance of *hu* cDNA (Valihrach and Demnerova, 2012). qRT-PCR was repeated three times in triplicate parallel experiments.

2.6. Biofilm formation and analysis

Biofilm formation under static conditions was determined by the microtiter plate assay based on the method described previously (Beenken et al., 2003). Briefly, overnight cultures were diluted 1:100 in fresh TSB medium and the diluted cell suspension was inoculated into flat-bottom 96-well polystyrene plates (Costar 3599, Corning Inc.) at 200 μl into each well. The plates were incubated at 37 °C for different time courses, and the wells were rinsed gently with water three times to remove non-adherent cells. The plates were stained with 0.5% crystal violet for 15 min, and then rinsed again with water to remove unbound stain. Then, the plates were dried, and the optical density at 560 (OD_{560}) was determined with an enzyme-linked immunosorbent assay reader in a 3 × 3 scan model. For the assay of the effect of glucose, TSBg-medium was supplemented with glucose in a concentration range of 0–5.0%. To investigate the effect of AI-2, the medium was supplemented with chemically synthesized DPD in a concentration range of 0.39–390 nM.

Biofilm formation was also measured 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 microscopy (CLSM) were performed as described previously (You et al., 2014). Overnight cultures of different strains were adjusted to an OD_{600} of 6.5 and diluted 1:100 in fresh 0.5% TSB. The 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/min) was initiated. Biofilms of different strains, which were transformed with the GFP plasmid for fluorescence detection, were cultivated at 37 °C in 0.5% TSB in three individual channels. 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 for WT biofilm detection were

Table 2
Oligonucleotide primers used in this study.

Primer	Oligonucleotide (5′–3′)[a]	Application
rt-hu-f	AAAAAGAAGCTGGTTCAGCAGTAG	qRT-PCR
rt-hu-r	TTTACGTGCAGCACGTTCAC	qRT-PCR
rt-rbf-f	AACCACCTAACTGATGTTATAC	qRT-PCR
rt-rbf-r	GACAACTTGACTGTTCTTATTC	qRT-PCR
rt-icaA-f	GAATATGGCTGGACTCA	qRT-PCR
rt-icaA-r	ATGCGACAAGAACTACT	qRT-PCR
rt-icaR-f	ATCTAATACGCCTGAGGA	qRT-PCR
rt-icaR-r	TTCTTCCACTGCTCCAA	qRT-PCR
rt-asp23-f	TTCAACAACTTCATCAGAGA	qRT-PCR
rt-asp23-r	ACAAGCATACGACAATCA	qRT-PCR
e-rbf-f	CCGgaattcAGGTGTACTTGCCTTTC	Expression of Rbf
e-rbf-r	GCGggatccTTACTTATCGTCGTCATCCTTGTAATCCTTATCGTCGTCATCCTTGTAATCTTTTTTTCCTATTTTAATTA	Expression of Rbf
p-rbf-f	CCGgaattcCAGGTGTACTTGCCTTTCTA	*lacZ* report
p-rbf-r	CGCggatccCCCAAGCATGATTTTGCCATAAC	*lacZ* report
up-rbf-f	CCGgaattcAGCAATAAAAATATATTACCTTAATTG	*rbf* deletion
up-rbf-r	CCCCatcgatCGTTGTCGCATATTCAT	*rbf* deletion
down-rbf-f	CCCaagcttGATATTGAACAAGAAAAGCTGATTTTT	*rbf* deletion
down-rbf-r	CGCggatccATTGCTGGTTTAAGGTAGTTGTCAATTT	*rbf* deletion
c-rbf-f	CCGgaattcAGGTGTACTTGCCTTTC	*rbf* complementation
c-rbf-r	CGCggatccAGCCATTGTCTTTCTC	*rbf* complementation
c-rsbU-f	GCGgaattcTTAAGATTCATTTCATTACA	*rsbU* complementation
c-rsbU-r	GCGggatccTGAACAAGGGGGAGTCAGAC	*rsbU* complementation
kana-f	CCCaagcttCCATTTGAGGTGATAGGTAA	Antibiotic gene marker
kana-r	CCCCatcgatTTTAGACATCTAAATCTAGG	Antibiotic gene marker
Em-f	CCCatcgatGAAATAGATTTAAAAATTTCGC	Antibiotic gene marker
Em-r	CCCaagcttGATACAAATTCCCCGTAGGC	Antibiotic gene marker
p-rbf-f-biotin	CAGGTGTACTTGCCTTTCTA	EMSA
p-rbf-r	CAAGCATGATTTTGCCATAAC	EMSA
p-sarX-f-biotin	GGAACCCTAGTTTACTATCT	EMSA
p-sarX-r	TCTTAAATTTTTCAGCCATTGT	EMSA

[a] The sequences in lowercase letters refer to the restriction endonuclease recognition sites.

taken as the standard settings. Selected confocal images represented 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).

2.7. Detection of PIA

The PIA extracted from *S. aureus* was blotted onto a nitrocellulose membrane (GE Healthcare) using a 96-well dot-blot apparatus according to a method described elsewhere (Lin et al., 2012). After blotting, the membrane was dried and soaked in a solution containing 3% bovine serum albumin and 0.05% Tween-20 in phosphate buffered saline (PBS). The membrane was then incubated at room temperature for 1 h in solution containing 0.8 mg/ml wheat germ agglutinin conjugated with biotin (WGA–biotin) (Sigma–Aldrich). After washing four times with PBS, PIA was detected using horseradish peroxidase-conjugated streptavidin followed by chemiluminescence detection (Thermo).

2.8. β-Galactosidase activity assay

To create the pOSRlacZ reporter vector, the fragment containing the 5′UTR and coding sequence of the first 6 amino acids of Rbf was amplified by PCR from the *S. aureus* NCTC8325 genome with the primers p-rbf-f and p-rbf-r. The products and pOS1-*lacZ* plasmid were digested with EcoRI and BamHI, and ligated together, resulting in the in-frame fusion of *lacZ* to the amplified fragments. The recombinant plasmid was transformed into *E. coli* Trans5α and transformed into *S. aureus* RN4220 and subsequently transformed into NCTC8325 and the *rbf* mutant. The cells were grown for different time courses and collected for the assay as described previously with some modification (Liu et al., 2011). Briefly, overnight cultures of SH11 and SH12 were diluted 1:100 into 20 ml of TSB with 15 μg/ml chloramphenicol and grown for the specified times. A sample (200 μl) of the culture was centrifuged, and the pellet was washed with PBS. The cells were pelleted and resuspended in 100 μl lysis buffer (60 mM K_2HPO_4, 40 mM KH_2PO_4, 100 mM NaCl, 0.1% Triton X-100) containing 20 μg/ml lysostaphin and incubated at 37 °C with gentle shaking for 30 min. Lysis buffer (100 μl) and 100 μl AB buffer (60 mM K_2HPO_4, 40 mM KH_2PO_4, 100 mM NaCl, 4 mg/ml o-NPG) were added to initiate the reaction. After incubation at 37 °C for the proper time (0–2 h), the reactions were terminated by the addition of l ml of 1 M Na_2CO_3. The OD_{420} was determined with an enzyme-linked immunosorbent assay reader. The β-galactosidase activity was expressed in Miller units (Zhang and Bremer, 1995).

2.9. Expression and purification of Rbf

The 2×FLAG-tagged Rbf was expressed and purified using the following procedures. The fragment of the full-length Rbf ORF and its native promoter was amplified by PCR with the e-rbf-f and e-rbf-r primers, cloned into pLI50, and transformed into *S. aureus* RN4220. The transformant was grown in TSB at 37 °C to an OD_{600} of 6.0. Approximately 100 g of cells was collected, and the whole-cell extract was prepared as previously described (Cai et al., 2009). Briefly, the lysed cells were re-suspended in extraction buffer (50 mM HEPES, pH 7.6, 300 mM KOAc, 0.5 mM EDTA, 5 mM β-mercaptoethanol, 10% (v/v) glycerol, 0.1% (v/v) NP-40 and protease inhibitors). After removing the DNA pellet with 0.1% polyethylenimine, the supernatant was selectively precipitated in 30–55% ammonium sulfate and resuspended in 1× TEZ buffer (50 mM Tris–HCl [pH 7.5], 1 mM EDTA, 10 mM $ZnCl_2$, 5 mM β-mercaptoethanol, and protease inhibitors). Then, the suspension was clarified by centrifugation and incubated for 2 h at 4 °C with 1 ml of a 50% slurry of ANTI-FLAG M2 Affinity Gel (Sigma–Aldrich) that had been pre-equilibrated with 1× TEZ. Then, the beads were washed with 40 ml of 1× TEZ plus 500 mM ammonium sulfate,

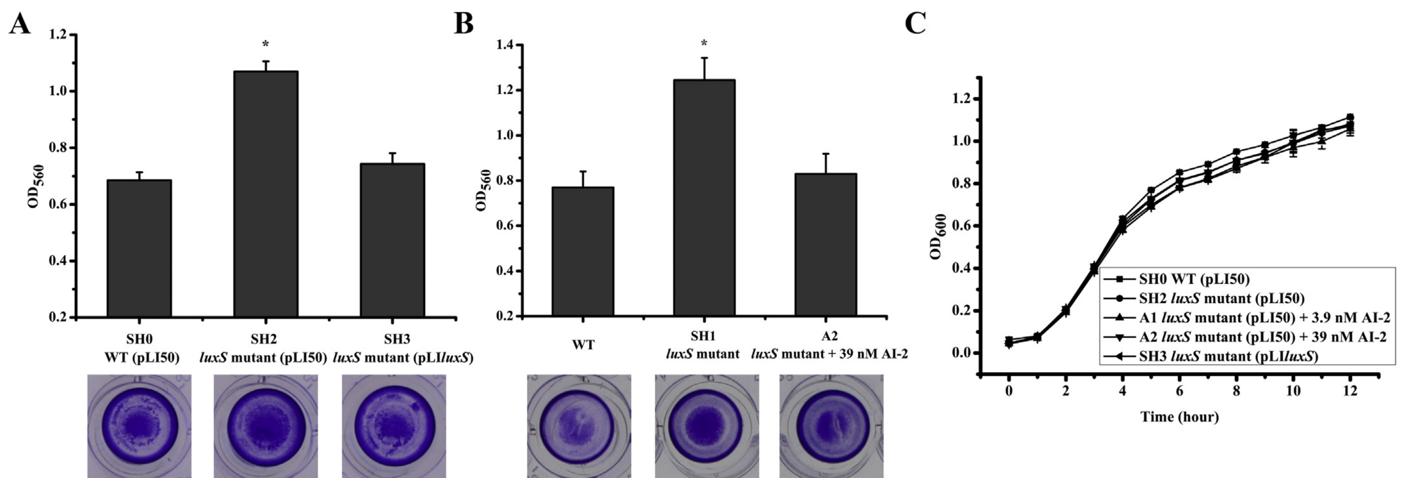

Fig. 1. LuxS/AI-2 regulates biofilm formation under static conditions. (A) Comparison of biofilm formation of the WT with a blank plasmid pLI50 (SH0), the *luxS* mutant with a blank plasmid pLI50 (SH2), and the *luxS* mutant with a plasmid pLI*luxS* encoding LuxS (SH3). (B) Comparison of biofilm formation of the WT, the *luxS* mutant (SH1), and the *luxS* mutant with 39 nM AI-2 for complementation (A2). After incubation for 6 h in 96-well plates under static conditions, the cells that adhered to the plate after staining with crystal violet were measured by OD_{560}. (C) Comparison of the growth rates of strains SH0, SH2, SH3, A1 (the *luxS* mutant with 3.9 nM AI-2 for complementation) and A2. Error bars represent the standard deviation (SD) ($n = 3$). Statistically significant differences calculated by the unpaired two-tailed Student's *t*-test are indicated: $^{*}P < 0.05$; $^{**}P < 0.01$.

followed by a second wash with 40 ml of 1× TEZ plus 50 mM ammonium sulfate. After equilibration of the column with 1× TEZ plus 100 mM ammonium sulfate, 3 ng of 3× Peptide (Sigma–Aldrich) was added to the resin beads and incubated for 1 h at 4 °C. The protein was then eluted with three column volumes of 1× TEZ plus 100 mM ammonium sulfate, 10% glycerol was added, and the resulting aliquot was snap-frozen in liquid nitrogen and temporarily stored at −80 °C.

2.10. Electrophoretic mobility shift assay (EMSA)

The biotin-labeled DNA fragments containing the promoter region were amplified from the *S. aureus* genomic DNA. The biotin-labeled DNA fragment was incubated at 25 °C for 15 min with various amounts of Rbf in 10 μl of incubation buffer (10 mM Tris–HCl, pH 8.0, 100 mM NaCl, 1 mM EDTA). After incubation, the mixtures were added with 2 μl of gel loading buffer and then electrophoresed in a 5% native polyacrylamide gel in 1× Tris–borate–EDTA buffer. The band shifts were detected and analyzed with the Chemiluminescent Nucleic Acid Detection Module (Pierce) according to the manufacturer's instructions. The unlabeled fragments of each promoter were added to the labeled fragments at a ratio of approximately 100:1 as specific competitors. The unlabeled fragment of the *pta* ORF region (100-fold) was added as a non-specific competitor.

3. Results

3.1. The LuxS/AI-2 system inhibits biofilm formation via the production of PIA

Several studies showed that the LuxS/AI-2 system could affect biofilm formation in Gram-positive and Gram-negative bacteria (Auger et al., 2006; Shao et al., 2007; Vidal et al., 2011). The LuxS/AI-2 system is present in staphylococci and plays a role in the regulation of biofilm formation (Doherty et al., 2006; Xu et al., 2006). To investigate whether the LuxS/AI-2 system modulates biofilm formation in *S. aureus* NCTC8325, we monitored the biofilm formation of the WT strain, the *luxS* mutant, and the *luxS*-complemented strain using microtiter plate assays. As shown in Fig. 1A, the *luxS* mutant resulted in increased biofilm formation ability compared with the WT strain. This alteration could be complemented by a complementary plasmid containing *luxS*. To further examine whether the increased biofilm formation ability of the *luxS* mutant was achieved by AI-2 signaling, the pre-AI-2 molecule DPD was used to complement the *luxS* mutant at a concentration of 39 nM within a range of reported concentrations (Rickard et al., 2006; Zhao et al., 2010). The resulting data showed that the biofilm formation of the *luxS* mutant could be restored by exogenous AI-2 (Fig. 1B). In addition, we compared the growth rates of the WT strain, the *luxS* mutant, the *luxS*-complemented strain, and *luxS* mutants complemented with 3.9 nM and 39 nM DPD. The results showed no significant differences between these strains (Fig. 1C). These findings indicate that the *luxS* gene could repress biofilm formation through a signaling mechanism based on AI-2 production, and these results are consistent with those of previous studies of biofilm formation in staphylococci (Xu et al., 2006; Yu et al., 2012).

Production of exopolysaccharide PIA is a major factor determining biofilm formation in some bacteria and is essential for biofilm formation in *S. aureus* NCTC8325 (Cramton et al., 1999; Heilmann et al., 1996; Mack et al., 1996). To detect whether the LuxS/AI-2 system inhibits biofilm formation through regulating the transcription of the *ica* operon and subsequent PIA production, we measured the transcript levels of *icaR* and *icaA* using qRT-PCR and PIA production assay. The transcript level of *icaR* was significantly decreased in the *luxS* mutant (Fig. 2A), whereas the expression of *icaA* was significantly increased in the *luxS* mutant (Fig. 2B), and these effects could be restored in the *luxS* mutant complemented with 39 nM DPD, suggesting that AI-2 signaling activates *icaR*, which subsequently decreases *ica* operon transcription. These data were in accordance with our previous study in *S. aureus* RN6390B (Yu et al., 2012). As shown in Fig. 2C, PIA production was consistent with the biofilm formation ability (Fig. 1B). Taken together, these results indicate that the LuxS/AI-2 system inhibits biofilm formation via PIA production in *S. aureus* NCTC8325.

3.2. The LuxS/AI-2 system represses rbf expression in an AI-2 dose-dependent manner

To investigate whether the LuxS/AI-2 system regulates the transcription of *rbf*, the transcript level of *rbf* was measured using qRT-PCR with RNA isolated from NCTC8325 and its isogenic *luxS*

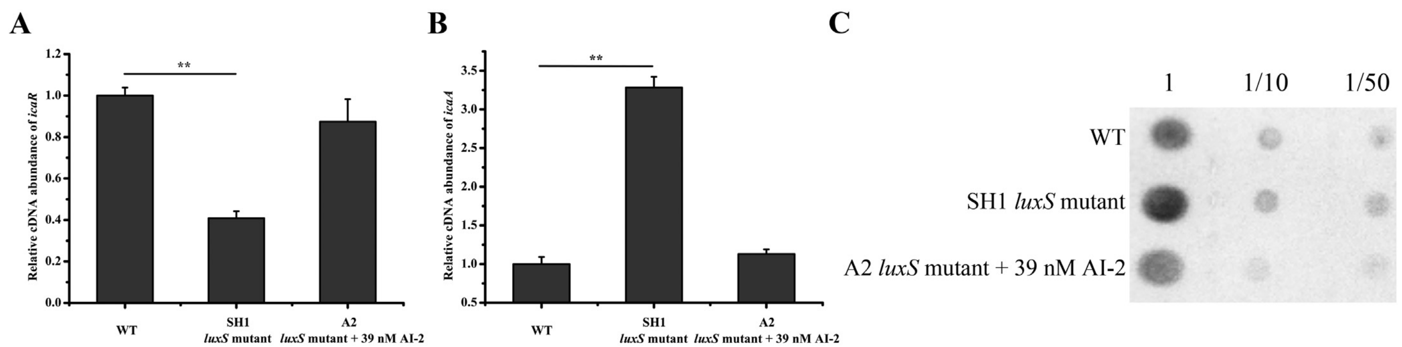

Fig. 2. LuxS/AI-2 modulates the transcription of *icaR* and *icaA* and the production of PIA. Relative *icaR* (A) and *icaA* (B) transcript levels of the WT, the *luxS* mutant (SH1), and the *luxS* mutant with 39 nM AI-2 for complementation (A2) were measured by qRT-PCR. (C) Quantification of PIA of WT, SH1, and A2. PIA was extracted from overnight cultures of each strain, serially diluted, and applied to a nitrocellulose membrane. PIA was detected using WGA-biotin. After incubation with HRP-streptavidin, the spots were visualized by chemiluminescence detection. The numbers at the top of the figure indicate the PIA dilutions. Error bars represent the SD ($n = 3$). Statistically significant differences calculated by the unpaired two-tailed Student's *t*-test are indicated: $^{*}P < 0.05$; $^{**}P < 0.01$.

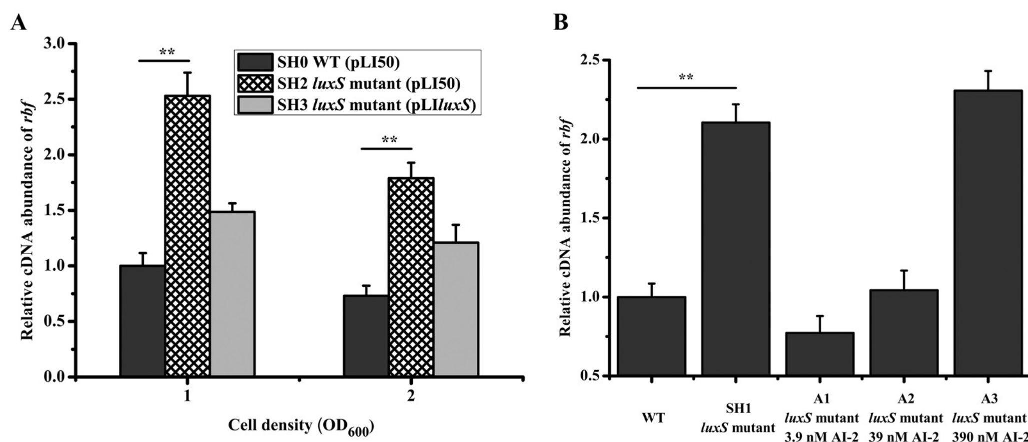

Fig. 3. Transcriptional regulation of *rbf* by LuxS/AI-2. (A) The relative transcript level of *rbf* was measured by qRT-PCR in the WT with a blank plasmid pLI50 (SH0), the *luxS* mutant with a blank plasmid pLI50 (SH2), and the *luxS* mutant with a plasmid pLI*luxS* encoding LuxS (SH3). All of the strains were grown in TSB medium to an OD_{600} of 1.0 and 2.0. (B) The relative transcript levels of *rbf* in the WT, *the luxS* mutant (SH1), and the *luxS* mutant with 3.9–390 nM AI-2 for complementation (A1, 3.9 nM; A2, 39 nM; A3, 390 nM). The error bars represent the SD ($n = 3$). Statistically significant differences calculated by the unpaired two-tailed Student's *t*-test are indicated: $^{*}P < 0.05$; $^{**}P < 0.01$.

mutant at different growth phases. The transcript level of *rbf* reached its peak at an optical density at 600 nm (OD_{600}) of 1.0, and the difference between the WT strain and the *luxS* mutant was significant at an OD_{600} of 1.0 and 2.0 (Figure S1). As shown in Fig. 3A, the transcript level of *rbf* in the *luxS* mutant increased compared with that in the WT strain, and the level could be complemented by a complementary plasmid containing *luxS*. To further explore if this alteration was achieved by AI-2 signaling, the pre-AI-2 molecule DPD was used to complement the *luxS* mutant with a concentration ranging from 3.9 nM to 390 nM. The transcription of *rbf* was restored in the *luxS* mutant under the concentration of 3.9 nM and 39 nM (Fig. 3B). The higher concentration of DPD did not restore the phenotype in *S. aureus*, which is consistent with other findings of AI-2 (Ahmed et al., 2007; Rickard et al., 2006). These results strongly indicated that the repression of the *rbf* expression in the WT strain was a consequence of AI-2-mediated signaling.

3.3. The LuxS/AI-2 system inhibits biofilm formation by repressing rbf *expression*

A series of studies conducted by the Lee group showed that Rbf can enhance biofilm formation in strains 8325-4, Newman, and UAMS-1 through regulating the expression of the *icaR* gene and the *ica* operon (Cue et al., 2009; Luong et al., 2009). To determine if Rbf is involved in biofilm formation in *S. aureus* NCTC8325, we constructed the *rbf* mutant and the *rbf*-complemented strain. As expected, the *rbf* mutant resulted in significantly decreased biofilm formation ability and showed a decrease in glucose-induced biofilm formation at all glucose concentrations tested. The biofilm formation ability of the *rbf* mutant could be complemented by the complementary plasmid containing *rbf* (Figure S2A, S2B).

To determine if the LuxS/AI-2 system could inhibit biofilm formation by repressing *rbf* expression, we constructed a *luxS rbf* double mutant and performed the biofilm formation assays with the *rbf* mutant and the *luxS rbf* double mutant. As shown in Fig. 4A, the *luxS rbf* double mutant and the *rbf* mutant resulted in significantly decreased biofilm formation. The amount of biofilm formed by the *luxS rbf* double mutant was approximately the same as that formed by the *rbf* mutant. We also performed complementation of the *luxS rbf* double mutant with the complementary plasmid pLI*rbf* or pLI*luxS*. The results showed that transformation of the double mutant with pLI*rbf* restored the biofilm formation and that pLI*luxS* did not affect biofilm formation relative to the *luxS rbf* double mutant (Fig. 4A). In addition, we compared the growth rates of these strains, and the results indicated no significant difference (Fig. 4D).

To confirm if the phenotype of biofilm formation was associated with the level of PIA production, we examined the transcript level of *icaA* (Fig. 4B). In the *luxS rbf* double mutant and the *rbf* mutant, the expression of *icaA* was significantly decreased. The *icaA* transcription of the double mutant was similar to that of the *rbf* mutant. Introduction of pLI*rbf* into the *rbf* mutant and the *luxS rbf* double

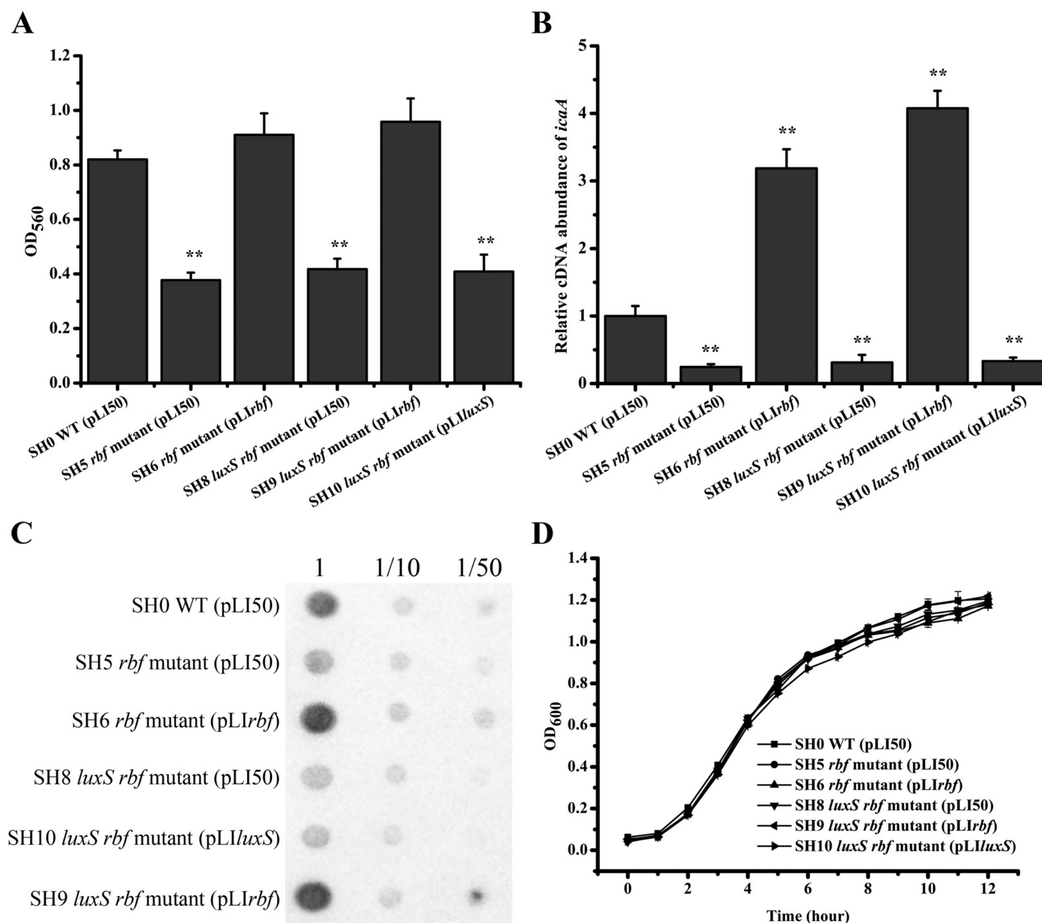

Fig. 4. *luxS* regulates biofilm formation through *rbf* in *S. aureus* NCTC8325. (A) Comparison of biofilm formation of the WT with a blank plasmid pLI50 (SH0), the *rbf* mutant with a blank plasmid pLI50 (SH5), the *rbf* mutant with a plasmid pLI*rbf* encoding Rbf (SH6), the *luxS rbf* double mutant with a blank plasmid pLI50 (SH8), the *luxS rbf* double mutant with a plasmid pLI*rbf* encoding Rbf (SH9), and the *luxS rbf* double mutant with a plasmid pLI*luxS* encoding LuxS (SH10) on a polystyrene microtiter plate. (B) Relative *icaA* transcript levels of strains SH0, SH5, SH6, SH8, SH9, and SH10 were measured by qRT-PCR. (C) Quantification of PIA of strains SH0, SH5, SH6, SH8, SH9, and SH10. The numbers at the top of the figure indicate the PIA dilutions. (D) Comparison of the growth rates of strains SH0, SH5, SH6, SH8, SH9, and SH10. The error bars represent the SD ($n = 3$). Statistically significant differences calculated by the unpaired two-tailed Student's *t*-test are indicated: $^{*}P < 0.05$; $^{**}P < 0.01$.

mutant restored the *icaA* expression to the WT level. In contrast, complementation of the *luxS rbf* double mutant with pLI*luxS* did not affect *icaA* transcription relative to the double mutant. Moreover, we measured the PIA production in these strains (Fig. 4C) and the results showed that the *luxS rbf* double mutant and double mutant complemented with pLI*luxS* produced approximately the same amount of PIA as the *rbf* mutant. Transformation of the *rbf* mutant and the double mutant with pLI*rbf* could restore PIA production. These results were consistent with the phenotype of biofilm formation (Fig. 4A), indicating that the LuxS/AI-2 system inhibits biofilm formation by repressing the *rbf* expression.

It has been recognized that σ^B plays a role in staphylococcal biofilm formation (Rachid et al., 2000a) and seems to interfere with *sarX* transcription (Ballal and Manna, 2009). A deletion in the 5′ region of *rsbU* in 8325 derivatives leads to the loss of σ^B activity (Giachino et al., 2001). *S. aureus* SH1000, a *sigB*-positive strain, is an *ica*-independent biofilm development strain (Boles and Horswill, 2008), and the *sigB*-positive strains Newman and MW2 are weak biofilm producers (Luong et al., 2009). To investigate whether LuxS/AI-2/Rbf regulatory cascade could modulate biofilm formation in *S. aureus* harboring a functional *sigB* operon, we first restored the σ^B activity by introducing pLI*rsbU* encoding an intact RsbU from *S. aureus* SH1000 into our experimental strains. We used alkaline shock protein gene *asp23* as a positive marker in the *rsbU*-complemented strains, since the transcription of *asp23* is highly dependent on σ^B (Gertz et al., 1999). As shown in Fig. 5A, the transcript level of *asp23* was significantly increased, indicating the restoration of σ^B activity. Then the biofilm formation assays was performed. The *rsbU*-complemented WT strain showed increased biofilm formation compared with the WT strain containing a blank plasmid pLI50 (Figure S3), which is consistent with previous studies (Cerca et al., 2008; Knobloch et al., 2004). We further monitored the biofilm formation and the transcript level of *icaA* in these *rsbU*-complemented strains. The *rsbU*-complemented *luxS* mutant exhibited an increased biofilm formation ability, and the *rsbU*-complemented *rbf* mutant and the *luxS rbf* mutant showed a similarly decreased biofilm formation (Fig. 5B). Moreover, the *icaA* transcription was consistent with the phenotype of biofilm formation (Fig. 5C), suggesting that LuxS/AI-2/Rbf regulatory cascade can modulate biofilm formation in *S. aureus* harboring a functional *sigB* operon.

3.4. Regulation of biofilm formation by the LuxS/AI-2 system and Rbf in a flow-cell system

To compare the biofilm formation abilities in different strains in the dynamic state, biofilm formation of the WT strain, the *luxS* mutant, the *rbf* mutant, and the *luxS rbf* double mutant was assessed using a flow-cell system. After incubation for 22 h, biofilms produced by these strains were examined by CLSM. Consistent with the

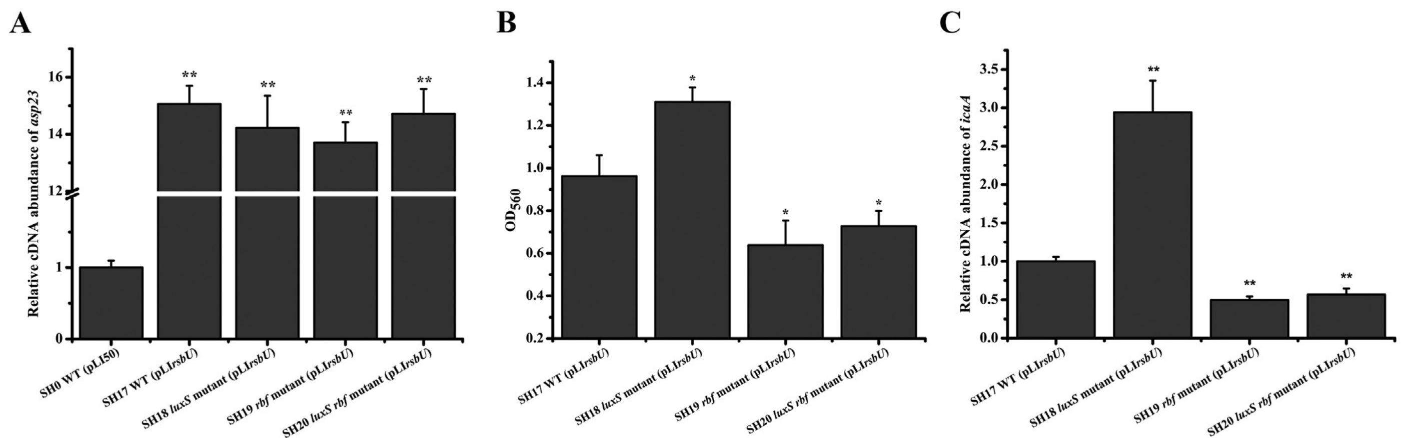

Fig. 5. Biofilm formation and *icaA* transcription in *rsbU*-complemented strains. (A) The relative transcript level of *asp23* was measured by qRT-PCR in the WT with a blank plasmid pLI50 (SH0), the WT with a plasmid pLI*rsbU* (SH17), the *luxS* mutant with a plasmid pLI*rsbU* (SH18), the *rbf* mutant with a plasmid pLI*rsbU* (SH19), and the *luxS rbf* mutant with a plasmid pLI*rsbU* (SH20). All of the strains were grown in TSB medium to an OD_{600} of 2.0. (B) Comparison of biofilm formation of strains SH17, SH18, SH19, and SH20. After incubation for 6 h in 96-well plates under static conditions, the cells that adhered to the plate after staining with crystal violet were measured by OD_{560}. (C) The relative transcript levels of *icaA* was measured by qRT-PCR in strains SH17, SH18, SH19, and SH20. All of the strains were grown in TSB medium to an OD_{600} of 2.0. The error bars represent the SD ($n = 3$). Statistically significant differences calculated by the unpaired two-tailed Student's *t*-test are indicated: $^{*}P < 0.05$; $^{**}P < 0.01$.

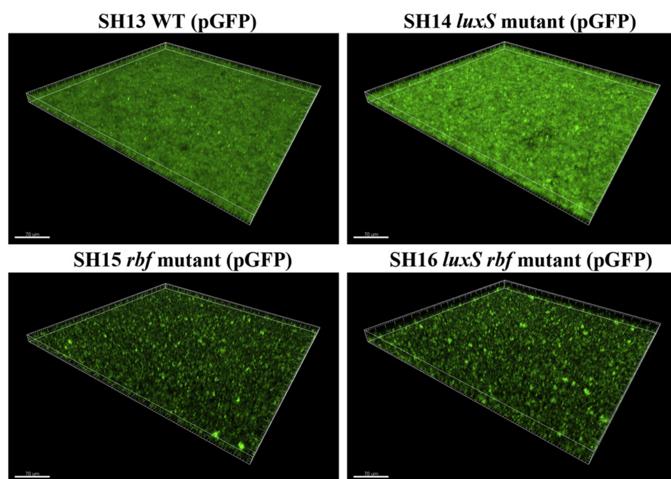

Fig. 6. Biofilm formation in a flow cell system. Biofilms of the WT with pGFP (SH13), the *luxS* mutant with pGFP (SH14), the *rbf* mutant with pGFP (SH15), and the *luxS rbf* double mutant with pGFP (SH16) were grown in a flow cell in 0.5% TSB with chloramphenicol (15 μg/ml). The biofilm integrity and GFP fluorescence were monitored after incubation for 22 h by CLSM.

static biofilm assays, the *luxS* mutant formed a stronger biofilm than the WT strain, and the *rbf* mutant exhibited decreased biofilm formation. The biofilm formation ability of the *luxS rbf* double mutant was the same as that of the *rbf* mutant (Fig. 6). These results indicate that the LuxS/AI-2 system and Rbf have similar effects in the regulation of biofilm formation in the static or dynamic states.

3.5. *Rbf binds to the* sarX *and* rbf *promoters to upregulate their expression*

Rbf has been revealed to promote transcription from the *sarX* promoter, with deletion of *rbf* resulting in significantly decreased transcription of *sarX* in *S. aureus* (Cue et al., 2013). Because Rbf is a member of the AraC/XylS family of proteins containing a predicted helix-turn-helix DNA-binding domain, Rbf has been suggested to upregulate the transcription of *sarX* by directly binding to the *sarX* promoter. To determine if Rbf could bind to the *sarX* promoter region in vitro, we purified a FLAG-tagged Rbf to perform EMSA assay. DNA probes containing the putative promoter regions were amplified. As shown in Fig. 7A, a clearly shifted band of DNA was visible after incubation of Rbf with the DNA probes containing the *sarX* promoter. The shifted band disappeared in the presence of an approximately 100-fold excess of unlabeled *sarX* promoter fragment but not in the presence of 100-fold excess of unlabeled coding sequence fragment of *pta*. This result indicated that Rbf could specifically bind to the *sarX* promoter region. We also found that Rbf could specifically bind to its own promoter region (Fig. 7B). To investigate if Rbf regulates its own expression, a β-galactosidase assay was also performed. The 5′UTR and coding sequence of the first six amino acids of *rbf* was fused with *lacZ*, and the fusion plasmid was transformed into the WT strain and the *rbf* mutant. In the *rbf* mutant, the β-galactosidase activity was significantly decreased throughout the growth phases (Fig. 7C). Taken together, our data suggest that Rbf could bind to the *sarX* and *rbf* promoters to upregulate their expression.

4. Discussion

Many bacteria use QS systems to coordinate gene expression in response to cell density. The only quorum-sensing mechanism shared by both Gram-negative and Gram-positive bacteria involves the production of AI-2 molecule. This system is widely present in bacteria, suggesting that the AI-2 molecule serves as a universal language for interspecies communication (Xavier and Bassler, 2003). However, LuxS functions as an integral component of the activated methyl cycle, which raises the question whether AI-2 serves as a QS signaling molecule in Gram-positive bacteria (Winzer et al., 2003). A previous work showed that the *luxS* gene was transcribed throughout growth under a variety of conditions and that AI-2 was produced in rich media under aerobic and anaerobic conditions, peaking during the transition to the stationary phase (Doherty et al., 2006). Moreover, the *luxS* mutants displayed a growth defect when grown in a sulfur-limited defined medium. Virulent phenotypic differences were observed between the WT strain and the *luxS* mutant in the RN6390B and NCTC8325-4 backgrounds, with a general reduction of biofilm formation upon loss of the *luxS* gene. In addition, the parental phenotype was not restored by complementary plasmid or in vitro-synthesized AI-2. Therefore, the researchers concluded that this phenotype was due to second-site mutations and that LuxS played a role in metabolism but not QS (Doherty et al., 2006). However, the research by Xu et al. showed that the *luxS* mutant formed thicker biofilm than WT strains of *S. epidermidis* and that the effect could be restored by exogenous AI-2. Thus they con-

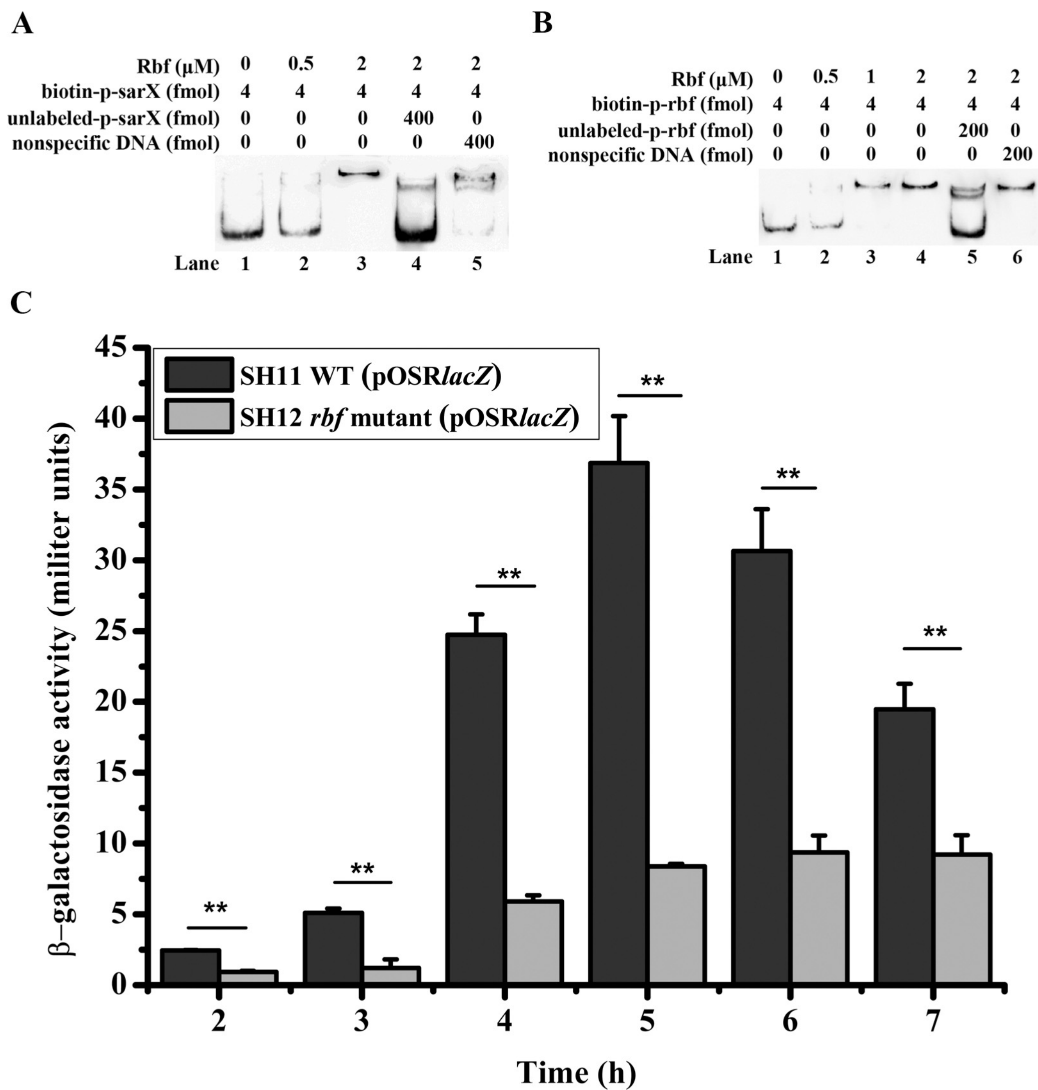

Fig. 7. Rbf binds to the *sarX* and *rbf* promoters to upregulate their expression. EMSA assays demonstrate that Rbf can bind to *sarX* promoter (A) and *rbf* promoter (B). The increasing amounts of Rbf was incubated with biotin-labeled probes. The unlabeled probes were added as specific competitors, and the unlabeled fragment of the *pta* ORF region was added as a non-specific competitor. (C) β-Galactosidase activity of the *rbf* promoter. β-Galactosidase activities of the WT with a plasmid pOSR*lacZ* (SH11) and the *rbf* mutant with a plasmid pOSR*lacZ* (SH12) were measured at the indicated time points. The error bars represent the SD ($n = 3$). Statistically significant differences calculated by the unpaired two-tailed Student's *t*-test are indicated: $^{*}P < 0.05$; $^{**}P < 0.01$.

cluded that the *luxS* gene of *S. epidermidis* is involved in repressing biofilm formation through a cell-cell signaling mechanism based on AI-2 secretion rather than cellular metabolic processes, as there were no noticeable differences in the growth patterns of the *luxS* mutant and the WT strain (Xu et al., 2006). Our present work demonstrated that the *luxS* mutant formed a stronger biofilm compared with the wild type strain NCTC8325. In addition, We found that deletion of *luxS* resulted in enhancement of the transcript level of the *rbf* gene. Of importance, the effects could be complemented by the effective concentrations of exogenous AI-2. These results suggest that the involvement of LuxS in biofilm formation and *rbf* expression could be attributed to a signaling mechanism associated with the AI-2 molecule rather than metabolic processes in *S. aureus*.

Although the LuxS/AI-2 system regulates many cellular functions in a variety of bacteria, the detailed mechanism by which AI-2 functions has only been investigated in several bacterial species. In *V. harveyi*, AI-2 is detected and transduced by a two-component system to finally regulate the expression of the luciferase structural operon (Bassler et al., 1994; Lilley and Bassler, 2000). In *E. coli* and *S. typhimurium*, AI-2 is first imported into the cell by an *lsr* transporter, and then the expression of target genes controlled by the QS system is initiated (Taga et al., 2003; Xavier and Bassler, 2005). To date, no potential homologues of the known AI-2 receptor have been identified in Gram-positive bacteria, especially in *S. aureus*. Therefore, if AI-2 functions as an extracellular signal by *S. aureus*, it must operate via a novel mechanism. Our previous study (Yu et al., 2012) showed that the LuxS/AI-2 system could repress the transcription of *icaA* via the activation of *icaR* in *S. aureus*. However, the detailed mechanisms whereby the signaling molecule AI-2 activates *icaR* still remain unknown. Herein our study demonstrated that the LuxS/AI-2 system could regulate PIA-dependent biofilm formation through the repression of Rbf, which was reported to downregulate IcaR (Cue et al., 2009). The deletion of *luxS* results in a significant increase in *rbf* transcription, and the alteration could be complemented by exogenous AI-2. Meanwhile, *S. aureus* NCTC8325 is *sigB* deficient and σ^B plays a role in staphylococcal biofilm formation (Laverty et al., 2013). We restored the σ^B activity in our experimental strains by complementation of pLI*rsbU* and demonstrated that LuxS/AI-2/Rbf regulatory cascade can modulate biofilm formation in *S. aureus* harboring a functional *sigB* operon. Rbf contains a

region of sequence significantly homologous to the 99-amino-acid consensus sequence of the AraC/XylS family of transcriptional regulators (Lim et al., 2004), which generally contain a ligand domain for binding chemical molecules (Gallegos et al., 1997). Bioinformatics prediction by Protein Homology/analogY Recognition Engine v2.0 (http://www.sbg.bio.ic.ac.uk/phyre2) (Kelley et al., 2015) revealed that Rbf might contain a domain functioning as a xylosidase in the C terminus. Given that xylose, a pentose, has a similar structure with the pre-AI-2 molecule DPD, we speculated that AI-2 could interact with the Rbf protein to modulate the expression of target genes. However, our EMSA data showed that addition of chemically synthesized AI-2 could not affect the binding of Rbf to the promoters of target genes (Figure S4). Therefore, the detailed mechanism by which LuxS/AI-2 QS system functions in *S. aureus* should be highlighted, and the interaction between Rbf and AI-2 molecule requires further study.

Previous studies showed that Rbf plays a significant role in the positive transcriptional regulation of *ica* operon and biofilm formation in *S. aureus* (Cue et al., 2009; Lim et al., 2004). Further study revealed that Rbf upregulates SarX, which then downregulates IcaR, thereby activating *ica* operon (Cue et al., 2013). Cross-linking and immunoprecipitation experiments suggested that Rbf selectively binds to the *sarX* promoter in *S. aureus*. However, the in vitro binding of Rbf with the *sarX* promoter region is nonspecific (Cue et al., 2013). In this study, we have provided direct evidence for the specific binding of Rbf to the *sarX* promoter in vitro. Some factors summarized by Cue et al. could lead to the different results (Cue et al., 2013). First, protein solubility and stability may be relevant to binding of Rbf to DNA. We could not isolate soluble and stable recombinant Rbf from *E. coli*. Second, it is possible that Rbf undergoes some form of posttranslational modification in vivo, which is important to maintain its native conformation. To perform EMSA experiment, we successfully purified a FLAG-tagged Rbf directly from *S. aureus*. Furthermore, we found that Rbf enhanced the transcription of itself by directly binding to its promoter region.

In conclusion, our data demonstrate that *S. aureus* LuxS/AI-2 QS system can regulate PIA-dependent biofilm formation via the repression of the *rbf* expression, and that Rbf can directly bind to the *sarX* and *rbf* promoters to upregulate their expression. These findings reveal that Rbf is a central player linking AI-2 signaling and biofilm formation regulation in Staphylococci.

Acknowledgements

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.ijmm.2017.03.003.

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