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The ArlR-MgrA regulatory cascade regulates PIA-dependent and protein-mediated biofilm formation in Rbf-dependent and Rbf-independent pathways

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ABSTRACT

The two-component system response regulator ArlR and the global regulator MgrA in *Staphylococcus aureus* participated in numerous biological processes including biofilm formation inhibition. Previous studies have shown that these two regulators could function as a regulatory cascade. Rbf is a positive regulator of biofilm formation enhancing the production of PIA (polysaccharide intercellular adhesin). Here we have demonstrated that both ArlR and MgrA can directly bind to the promoter of *rbf* and repress its expression. ArlR and MgrA can also directly bind to the promoter of *ica* operon and enhance the expression of *icaA* and PIA production, revealing that the ArlR-MgrA regulatory cascade controls PIA-dependent biofilm formation. In addition, we have found that Rbf can directly bind to the *aur* promoter and repress the expression of *aur*, which encodes a protease initiating a protease cascade to inhibit protein-mediated biofilm formation. Moreover, our data indicate that the ArlR-MgrA regulatory cascade can promote the expression of *aur* by directly binding to its promoter and inhibit protein-mediated biofilm formation. These findings shed light on the molecular mechanisms of both PIA-dependent and protein-mediated biofilm formation modulated by the ArlR-MgrA regulatory cascade and the new role of Rbf in protein-mediated biofilm formation, and broaden our understanding of the biofilm formation regulation in *S. aureus*.

1. Introduction

Staphylococcus aureus is a major human pathogen that can cause a wide variety of infectious diseases ranging from minor skin infections to life-threatening septicemia, osteomyelitis, and toxic shock syndrome (Lowy, 1998). Biofilm formation is often involved in *S. aureus* infections such as endocarditis, septic arthritis, osteomyelitis, and infections on indwelling medical devices (Costerton et al., 1999). Biofilms are microbial aggregates that accumulate at a solid-liquid interface and are encased in a matrix of highly hydrated extracellular polymeric substances, including polysaccharides, proteins, nucleic acids, and lipids secreted by biofilm cells (Otto, 2013). Biofilm formation provides a protecting environment enabling the bacteria cells to proliferate by limiting antibiotic access and shielding the bacterial pathogen from host immune defenses (Trotonda et al., 2008).

Exopolysaccharides, predominantly polysaccharide intercellular adhesin (PIA), are the most common constituents of staphylococcal biofilm. The biosynthesis of PIA is mediated by the intercellular adhesin (*ica*) locus, which contains an operon composed of four open reading frames (ORFs) *icaA*, *icaD*, *icaB*, and *icaC*. This *ica* operon is negatively regulated by the transcriptional factor IcaR. The gene *icaR* is also part of the *ica* locus and located in the upstream of the *ica* operon but in reverse direction (Heilmann et al., 1996; Vuong et al., 2004). The decreased PIA production level is considered to be the main cause of the disability of biofilm formation in *S. aureus* strain NCTC8325 (Cramton et al., 1999).

Rbf possesses a consensus region of the AraC/XylS family of transcriptional regulators and can modulate biofilm formation in response to glucose and salt (Lim et al., 2004). This modulation is mediated by the transcriptional regulator IcaR, which binds to the *icaA* promoter region and represses the expression of *ica* operon. The deletion of *icaR* has been shown to significantly increase the expression of the *ica* operon and the production of PIA (Conlon et al., 2002). Recently, studies have revealed that Rbf can bind to the *sarX* promoter and positively

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regulate its expression, then SarX can bind to the upstream sequence of *icaR* within the *icaA* coding region to negatively regulate the expression of *icaR*, thereby activating the *ica* operon (Cue et al., 2013; Cue et al., 2009). LuxS is the enzyme responsible for synthesizing the precursor of autoinducer-2 (AI-2), which is the crucial signal molecule involved in a quorum sensing system shared by Gram-positive and Gram-negative bacteria (Schauder et al., 2001). Our previous work indicated that the LuxS/AI-2 system inhibited PIA-dependent biofilm formation by repressing the expression of *rbf* in *S. aureus* strain NCTC8325 and Rbf could bind to the *rbf* promoter to positively regulate its own expression (Ma et al., 2017; Yu et al., 2012).

ArlR is the response regulator of the two-component system *arlRS* and belongs to the OmpR-PhoB response regulator family. It has been reported to participate in a number of important biological processes in *S. aureus*, such as inhibition of autolysis and α-toxin secretion, enhancement of extracellular proteolytic activity and capsular polysaccharide synthesis (Fournier and Hooper, 2000; Fournier et al., 2001). ArlR also has been reported to play an important role in the regulation of biofilm formation in *S. epidermis* via both *ica*-dependent and protein-dependent pathways (Wu et al., 2014).

MgrA was first identified as a global regulator of *S. aureus*. It can promote the production of capsular polysaccharide 8 and nuclease but repress the production of α-toxin, coagulase, protease and protein A (Luong et al., 2003). MgrA can regulate *hla* and *spa* in both *agr*-dependent and *agr*-independent patterns. In the *agr*-independent pathway, MgrA can directly bind to the promoters of *hla* and *sarS* to modulate α-toxin and protein A expression (Ingavale et al., 2005). As for biofilm formation regulation, the *mgrA* mutants of *S. aureus* strains RN6390, SH1000, and MW2 displayed an enhanced biofilm formation independent of σ^B and *ica* operon (Trotonda et al., 2008). Mutation of *mgrA* enhanced biofilm formation in *S. aureus* strain LAC, but had no impact on protease activity or accumulation of PIA. In *S. aureus* strain UAMS-1, mutation of *mgrA* was correlated with increased production of PIA (Atwood et al., 2015), indicating that the regulatory effects of MgrA on PIA vary in different *S. aureus* strains.

It has been proven that ArlR functions upstream of MgrA with respect to the regulation of capsule production, although ArlR can also regulate capsule independently from MgrA. One study suggested that ArlR could positively regulate capsule polysaccharide production at the transcriptional level mainly through an MgrA-dependent pathway (Luong et al., 2006). A recent research conducted by the Horswill group showed that ArlR and MgrA can constitute a regulatory cascade to control the expression of various genes important for virulence, mainly genes coding for cell wall-associated proteins such as *ebh*, *sraP*, and *sasG* (Crosby et al., 2016).

In this study, we have revealed that ArlR and MgrA can directly repress the expression of *rbf*, while they can also directly promote the expression of *ica* operon to enhance the production of PIA in *S. aureus* strain NCTC8325. Further, our data indicate that Rbf can bind to the *aur* promoter to negatively regulate its expression to increase biofilm formation. In addition, the ArlR-MgrA regulatory cascade can enhance the expression of *aur* both in *rbf*-dependent and *rbf*-independent pathways.

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. *Staphylococcus aureus* strain RN4220 was used for the propagation of plasmids prior to transformation into other *S. aureus* strains. *Escherichia coli* was grown in Luria-Bertani (LB) medium (Oxoid), and *S. aureus* was grown in tryptic soy broth (TSB; BD) medium at 37 °C. When needed, 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*, chloramphenicol at 15 μg/ml.

2.2. DNA manipulation

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

2.3. DNA pulldown assay

DNA pulldown assay was performed as previously described (Jutras et al., 2012; Zhang et al., 2015), with minor modifications. Briefly, the biotin-labeled DNA fragment containing the promoter region of *rbf* was amplified from *S. aureus* strain NCTC8325 genomic DNA using primers p*rbf*-F/p*rbf*-R-biotin (Table 2). The control DNA fragments of the promoter regions of *hla* and *psm* were amplified with primers p*hla*-F/p*hla*-R-biotin and p*psm*-F/p*psm*-R-biotin. Stationary-phase cultures of *S. aureus* strain NCTC8325 were diluted 1:500 into 20 ml TSB. Cells were cultivated for 6 h and then collected and washed twice with lysis buffer (10 mM HEPES, 10 mM $MgCl_2$, 200 mM NaCl, 1 mM DTT, 1% Triton X-100, pH 7.0) and resuspended in 10 ml lysis buffer with lysostaphin (10 mM HEPES, 10 mM $MgCl_2$, 200 mM NaCl, 1 mM DTT, 1% Triton X-100, 40 U/ml lysostaphin, 10 μg/ml DNase I, pH 7.0) under shaking conditions at 37 °C for 15 min until thoroughly lysed. The lysate was centrifuged at 12,000 × *g* at 4 °C for 40 min to remove insoluble debris, and the supernatant was concentrated to 2 ml with a Centrifuge Biomax-5 column (Millipore, Billerica, MA, USA). The prepared biotin-labeled DNA (25 μg) of the *rbf*, *hla* and *psm* promoter DNA was coupled to 0.9 ml of streptavidin-coated magnetic beads (Promega) with the same volume of 2 × B/W buffer (10 mM Tris − HCl, pH 7.5, 1 mM EDTA, 2 M NaCl) at room temperature. After incubation for 1 h, the beads were washed with lysis buffer 3 times to create an atmosphere suitable for protein binding. The 500 μl supernatant with 10 μg/ml of poly(dI-dC) was added to the DNA-coated beads and incubated at 4 °C for 1 h. The combination of supernatant and DNA-coated beads was repeated once to increase the amount of proteins obtained. Beads were washed 5 times with 500 μl lysis buffer containing 10 μg/ml of poly(dI-dC) and twice with 500 μl lysis buffer, and then it was supplemented with 70 μl ddH_2O and incubated at 70 °C for 10 min. Samples were segregated by SDS-PAGE and detected by silver staining. The gel was then excised and subjected to liquid chromatography-tandem mass spectrometry (LC–MS/MS) analysis with an LTQ mass spectrometer (ProteomeX-LTQ; Thermo). Sequence and peptide fingerprint data were analyzed using the NCBI database.

2.4. Expression and purification of ArlR and MgrA

The 6-His-tagged ArlR and MgrA proteins were expressed and purified respectively using standard procedures. The full-length *arlR* and *mgrA* ORF fragments were amplified using PCR with the primers ArlRexp-F/ArlRexp-R and MgrAexp-F/MgrAexp-R from *S. aureus* strain NCTC8325 genomic DNA, cloned into the expression vector pET28a (+) (Novagen, Merck, Darmstadt, Germany), and transformed into *E. coli* BL21 (DE3). The transformant was grown in LB at 37 °C to an OD_{600} of 0.4 and induced with 0.5 mM isopropyl-β-$_D$-1-thiogalactopyranoside (IPTG) at 37 °C for additional 3 h. The cells were harvested and lysed by sonication in a lysis buffer (50 mM Tris-HCl, pH 8.0, 300 mM NaCl). ArlR and MgrA were purified with a nickel-nitrilotriacetic acid agarose solution (Qiagen, Valencia, CA, USA) following the manufacturer's instruction. The bound protein was eluted with an elution buffer (300 mM imidazole, 50 mM Tris-HCl, pH 8.0, 300 mM NaCl). The imidazole in the eluent was removed using a Centrifuge Biomax-5 column (Millipore, Billerica, MA, USA). The purity of the proteins was analyzed

Table 1
Strains and plasmids used in this study.

Strain or plasmid	Description	Reference or source
Strains		
NCTC8325	Wild-type	NARSA[a]
RN4220	8325-4 r^-, initial recipient for modification of plasmids that are introduced into *S. aureus* from *E. coli*	NARSA
WT pLI50	8325 wild-type strain with pLI50	This study
Δ*arlR* pLI50	8325 *arlR* mutant strain with pLI50	This study
Δ*mgrA* pLI50	8325 *mgrA* mutant strain with pLI50	This study
Δ*rbf* pLI50	8325 *rbf* mutant strain with pLI50	(Ma et al., 2017)
Δ*luxS* pLI50	8325 *luxS* mutant strain with pLI50	(Zhao et al., 2010)
c*arlR* pLI50	8325 *arlR* complemented strain with pLI50	This study
c*mgrA* pLI50	8325 *mgrA* complemented strain with pLI50	This study
c*rbf* pLI50	8325 *rbf* complemented strain with pLI50	This study
WT pLI*rsbU*	8325 wild-type strain with pLI*rsbU*	This study
Δ*arlR* pLI*rsbU*	8325 *arlR* mutant strain with pLI*rsbU*	This study
Δ*mgrA* pLI*rsbU*	8325 *mgrA* mutant strain with pLI*rsbU*	This study
Δ*rbf* pLI*rsbU*	8325 *rbf* mutant strain with pLI*rsbU*	This study
WT pOS*rbf*	8325 wild-type strain with pOS*rbf*	This study
Δ*arlR* pOS*rbf*	8325 *arlR* mutant strain with pOS*rbf*	This study
Δ*mgrA* pOS*rbf*	8325 *mgrA* mutant strain with pOS*rbf*	This study
WT pOS*aur*	8325 wild-type strain with pOS*aur*	This study
Δ*arlR* pOS*aur*	8325 *arlR* mutant strain with pOS*aur*	This study
Δ*mgrA* pOS*aur*	8325 *mgrA* mutant strain with pOS*aur*	This study
Δ*rbf* pOS*aur*	8325 *rbf* mutant strain with pOS*aur*	This study
Trans5α	Clone host strain	TransGen
BL21 (DE3)	Expression strain	TransGen
Plasmids		
pBTs	Shuttle vector, temperature sensitive, Ap^r Cm^r [b]	(Liu et al., 2016)
pBT*arlR*	pBTs containing upstream and downstream fragments of *arlR*, for *arlR* mutagenesis, Ap^r Cm^r	This study
pBT*mgrA*	pBTs containing upstream and downstream fragments of *mgrA*, for *mgrA* mutagenesis, Ap^r Cm^r	This study
pBTc*arlR*	pBTs containing the recombinant fragment of *arlR*, for *arlR* chromosomal complementation, Ap^r Cm^r	This study
pBTc*mgrA*	pBTs containing the recombinant fragment of *mgrA*, for *mgrA* chromosomal complementation, Ap^r Cm^r	This study
pBTc*rbf*	pBTs containing the recombinant fragment of *rbf*, for *rbf* complementation, Ap^r Cm^r	This study
pLI50	Shuttle cloning vector, Ap^r Cm^r	Addgene
pET28a (+)	Expression vector with hexahistidine tag, Kan^r	Novagen
pET*arlR*	pET28a (+) with *arlR*, Kan^r	This study
pET*mgrA*	pET28a (+) with *mgrA*, Kan^r	This study
pOS1-*lacZ*	Shuttle vector, containing promoter-less *lacZ* gene, Ap^r Cm^r	(Liu et al., 2011)
pOS*rbf*	*rbf-lacZ* fusion shuttle vector, a derivative of pOS1-*lacZ*	(Ma et al., 2017)
pOS*aur*	*aur-lacZ* fusion shuttle vector, a derivative of pOS1-*lacZ*	This study
pLI*rsbU*	pLI50 with *rsbU* ORF and its promoter, Ap^r Cm^r	(Ma et al., 2017)

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

using SDS-PAGE, and the protein concentrations were determined using the BCA assay with bovine serum albumin as the standard.

2.5. Electrophoretic mobility shift assay

To determine the binding specificity, electrophoretic mobility shift assay (EMSA) was performed using purified ArlR and MgrA proteins and the biotin-labeled DNA fragment containing the promoter region amplified from *S. aureus* strain NCTC8325 genomic DNA. The biotin-labeled probe was incubated at 25 °C for 20 min with various amounts of ArlR or MgrA in 10 μl of incubation buffer (50 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 (Thermo) according to the manufacturer's instructions. The unlabeled fragment of each promoter was added to the labeled fragment at a ratio of approximately 100:1 as a specific competitor. The unlabeled fragment of the *hu* ORF region (100-fold) was added as a nonspecific competitor.

2.6. Construction of S. aureus mutant strains

The *luxS* mutant and the *rbf* mutant from *S. aureus* strain NCTC8325 were constructed previously (Zhao et al., 2010; Ma et al., 2017). To construct the *arlR* and the *mgrA* mutants from *S. aureus* strain NCTC8325, the upstream and downstream fragments of *arlR* and *mgrA* were amplified from *S. aureus* strain NCTC8325 genomic DNA using the *arlR*-up-F/*arlR*-up-R, *arlR*-down-F/*arlR*-down-R, *mgrA*-up-F/*mgrA*-up-R, *mgrA*-down-F/*mgrA*-down-R sets of primers (Table 2). The upstream and downstream regions of each gene were ligated to form an up-down fragment. The resultant fragment was digested with restriction enzymes and then cloned into pBTs. The resulting plasmids, pBT*arlR*, and pBT*mgrA*, were first electroporated into *S. aureus* strain RN4220 for modification and subsequently transformed into *S. aureus* strain NCTC8325. The allelic replacement mutants were selected using a previously described method (Liu et al., 2016) and were further confirmed by PCR and sequencing.

2.7. Complementation of mutants

To construct the *arlR*, the *mgrA* and the *rbf* chromosomal complementation strains, the fragments covering the truncated region in the mutant strains were amplified from *S. aureus* strain NCTC8325 genomic DNA using the c*arlR*-F/c*arlR*-R, c*mgrA*-F/c*mgrA*-R, c*rbf*-F/c*rbf*-R sets of primers (Table 2). The fragments of each gene were digested with restriction enzymes and then cloned into pBTs. The resulting plasmids, pBTc*arlR*, pBTc*mgrA* and pBTc*rbf*, were first electroporated into *S. aureus* strain RN4220 for modification and subsequently transformed into the mutant strains respectively. The allelic replacement complementation strains were selected using the same method described above and were further confirmed by PCR and sequencing.

Table 2
Oligonucleotide primers used in this study.

Primer	Oligonucleotide (5'-3')[a]	Application
RT-*hu*-F	AAAAAGAAGCTGGTTCAGCAGTAG	RT-real-time PCR
RT-*hu*-R	TTTACGTGCAGCACGTTCAC	RT-real-time PCR
RT-*arlR*-F	GCAAGATTTCTTGAATTGG	RT-real-time PCR
RT-*arlR*-R	CGCTTTATCTAAACCGTC	RT-real-time PCR
RT-*mgrA*-F	AACGAATGGAACAAGTAG	RT-real-time PCR
RT-*mgrA*-R	ACCTAATAAGCGATTAAGTT	RT-real-time PCR
RT-*luxS*-F	GCCAAACTGGTTTCTATG	RT-real-time PCR
RT-*luxS*-R	GCACCTTCTAATGAATGAC	RT-real-time PCR
RT-*rbf*-F	CGATATGCGTATTATGGTGATT	RT-real-time PCR
RT-*rbf*-R	AAGTAAGTGGAATTGTGATGAC	RT-real-time PCR
RT-*icaA*-F	GAATATGGCTGGACTCA	RT-real-time PCR
RT-*icaA*-R	ATGCGACAAGAACTACT	RT-real-time PCR
RT-*icaR*-F	ATCTAATACGCCTGAGGA	RT-real-time PCR
RT-*icaR*-R	TTCTTCCACTGCTCCAA	RT-real-time PCR
arlR-up-F	CGgaattcGCCTTGTGTTACAGTATTCTAT	*arlR* deletion
arlR-up-R	CGggatccTGACGGCAACATTAAATCTAA	*arlR* deletion
arlR-down-F	CGggatcc GATATTGAACAAGAAAAGCTGATTTTT	*arlR* deletion
arlR-down-R	GGggtaccATTGTGGCAGTTATAATTGTTG	*arlR* deletion
mgrA-up-F	GCGgtaccATGTCACTTAGTTTCAAC	*mgrA* deletion
mgrA-up-R	CGCggatccGCTGTTCTTTTAAATTATG	*mgrA* deletion
mgrA-down-F	CGCggatccACTTAATCGCTTATTAGGTAA	*mgrA* deletion
mgrA-down-R	GCGgtcgacCAGGGTTATATCAATTAGATAG	*mgrA* deletion
c*arlR*-F	GCgtcgacGAATGCCATTAACTGATTA	*arlR* complementation
c*arlR*-R	GGggtaccTTAACAGGCTTAGAATGAA	*arlR* complementation
c*mgrA*-F	CGgaattcTTAGCATGGCATTGAATC	*mgrA* complementation
c*mgrA*-R	GGggtaccTTAGATGAACCAACAACT	*mgrA* complementation
c*rbf*-F	CCGgaattc AGCAATAAAAATATATTACCTTAATTG	*rbf* complementation
c*rbf*-R	CGGggtacc ATTGCTGGTTTAAGGTAGTTGTCAATTT	*rbf* complementation
ArlRexp-F	GCGcatatgACGCAAATTTTAATAGT	Expression of ArlR
ArlRexp-R	GCGctcgagTCATCGTATCACATACCCAA	Expression of ArlR
MgrAexp-F	GCGcatatgTCTGATCAACATAATTTA	Expression of MgrA
MgrAexp-R	GCGctcgagTTATTTTTCCTTTGTTTCATC	Expression of MgrA
p*rbf*-F	CAGGTGTACTTGCCTTTCTA	EMSA and DNA pulldown
p*rbf*-R-biotin	AAGCATGATTTTGCCATAAC	EMSA and DNA pulldown
p*hla*-F	TTCTTATAATGCCTCTAACT	DNA pulldown
p*hla*-R-biotin	TAGTGTTGTTGTTACTGAG	DNA pulldown
p*psm*-F	TCTGTTCAATTCATCTTCATA	DNA pulldown
p*psm*-R-biotin	GCCAGCGATGATACCCATTAAG	DNA pulldown
p*aur*-F	CTAGAGTGTGAGGAGTGATAC	EMSA
p*aur*-R-biotin	AGGTTAATGCTGCCATACTT	EMSA
p*ica*-F	TGCGTTATCAATAATCTTATCCT	EMSA
p*ica*-R-biotin	GCCTTCTAATTCATCCACATT	EMSA
lacZ-aur-F	CGgaattcCTAGAGTGTGAGGAGTGATAC	*lacZ* report
lacZ-aur-R	CGggatccGGCAAGGTTAATGCTGCCAT	*lacZ* report

[a] Lowercase letters: restriction endonuclease recognition sites.

2.8. Total RNA extraction, cDNA generation, and real-time quantitative reverse transcription-PCR

Overnight cultures of *S. aureus* were diluted 1:100 in TSB and then grown to the certain cell density until being collected (OD_{600} = 2.0). 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, OH, USA), and the residual DNA was removed with RNase-free DNase I (TaKaRa). The concentration of total RNA was adjusted to 100 ng/μl. Reverse transcription was carried out with the PrimeScript 1 st Strand cDNA synthesis kit (Takara) and real-time quantitative reverse transcription-PCR (qRT-PCR) was performed with SYBR Premix Ex Taq (TaKaRa) using a StepOne real-time system (Applied Biosystems). The relative quantity of cDNA measured by real-time PCR was normalized to the average abundance of wild-type (WT) strain samples using *hu* gene as the reference gene (Valihrach and Demnerova, 2012).

2.9. 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 TSB medium containing 1% glucose into each well. The plates were incubated at 37 °C for 24 h, 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 the unbound stain. Then, the plates were dried for 8 h, and the optical density at 560 (OD_{560}) was determined with an enzyme-linked immunosorbent assay reader ELX800 (Bio-Tek) in a 3 × 3 scan model.

2.10. PIA detection

The PIA extracted from *S. aureus* was blotted onto a nitrocellulose membrane (GE Healthcare) according to a method described before (Cramton et al., 2001). Briefly, cell surface extracts were prepared by growing cells to an OD_{600} of 2.0 in TSB medium, the optical density was determined, and an equal number of cells from each culture (typically 2 ml) was resuspended in 50 μl of 0.5 M EDTA (pH 8.0). Cells were then incubated for 5 min at 100 °C and centrifuged, and 40 μl of the supernatant was incubated with 10 μl of 20 mg/ml proteinase K (Sangon Biotech) for 30 min at 37 °C to minimize nonspecific background. Then the extracts were spotted onto a nitrocellulose membrane. 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 PBS 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).

2.11. β-Galactosidase activity assay

To create the pOS*aur* reporter vector, the fragment containing the 5′UTR and the coding sequence of the first 6 amino acids of Aur was amplified by PCR from the *S. aureus* strain NCTC8325 genomic DNA with primers *lacZ-aur*-F/*lacZ-aur*-R. The products and pOS1-lacZ plasmid were digested with EcoRI and BamHI and then ligated together, resulting in the in-frame fusion of *lacZ* to the amplified fragment. The recombinant plasmid was then transformed into experimental strains. The rest procedures were followed as described previously (Ma et al., 2017).

2.12. Biofilm formation mechanism detection

Sodium metaperiodate ($NaIO_4$) and proteinase K were previously used to degrade polysaccharide and protein-mediated biofilms, respectively (Brady et al., 2017; Dakheel et al., 2016). If biofilm formation is mediated by polysaccharide, β-1,6-*N*-acetyl-$_D$-glucosamine, treatment with metaperiodate will lead to biofilm dispersal. On the contrast, if the biofilm formation is mediated by extracellular proteins, treatment with proteinase K will lead to biofilm dispersal.

After incubation for over 24 h, the plates were rinsed three times with 200 μl sterile PBS, and the supernatant was discarded. The plates were then treated as follows: 1) Each cell was filled with 200 μl of a $NaIO_4$ solution (40 mM in 50 mM sodium acetate buffer, pH 5.5) and incubated for further 24 h at 4°C in the darkness. After incubation, the cell was rinsed with PBS three times and dried, and the plates were then

stained with crystal violet and OD_{560} was determined as described above; 2) Each cell was filled with 200 µl of a proteinase K solution (0.1 mg/ml in PBS) and incubated for 2 h at 37°C. After incubation, the cell was rinsed with PBS three times and dried. The plates were then stained with crystal violet and OD_{560} was determined as described above.

2.13. Statistical analysis

All data were analyzed using a non-parametric statistic test: the Mann-Whitney-U-test for direct comparison of two groups and the Kruskal-Wallis test for more than two groups. A value of $P < 0.05$ was considered statistically significant.

3. Results

3.1. Identification of rbf promoter-binding proteins

To identify proteins involved in the transcriptional regulation of *rbf*, we performed DNA affinity pulldown assays using a biotin-labeled fragment from the *rbf* promoter region as the probe. The obtained protein solution was analyzed by SDS-PAGE (Fig. 1A) and the DNA binding proteins identified by mass spectrum were listed in Table 3. In this study, we aimed to identify main transcriptional factors that regulate the expression of *rbf*. We considered ArlR and MgrA as the most promising candidates and performed further study. ArlR and MgrA were overexpressed as recombinant proteins in *E. coli*, and purified using nickel nitrilotriacetic acid agarose (Fig. 1B). EMSA was then performed to confirm the binding ability and specificity. The EMSA results showed that both ArlR and MgrA retarded the mobility of the *rbf* promoter region. The binding can be inhibited with an approximately 100-fold concentration of unlabeled *rbf* promoter fragment, while the same amount of nonspecific unlabeled coding sequence fragment of *hu* did not have the effect (Fig. 1C and 1D). This result indicated that ArlR and MgrA can specifically bind to the *rbf* promoter region.

3.2. ArlR and MgrA repress rbf expression

To determine the regulatory effect of ArlR and MgrA on *rbf*, we constructed the *arlR* mutant strain, the *mgrA* mutant strain and their complemented strains, respectively. The transcript levels of *rbf* were measured by qRT-PCR with RNAs isolated from the WT, the *arlR* mutant, the *arlR* complemented, the *mgrA* mutant, and the *mgrA* complemented strains. The transcript levels of *rbf* increased significantly in the *arlR* mutant and the *mgrA* mutant strains, and the changes could be fully reversed by chromosomal complementation (Fig. 2A). To further confirm the repressive effect of these two regulators, the β-galactosidase activities of the WT and mutant strains containing pOS*rbf* were

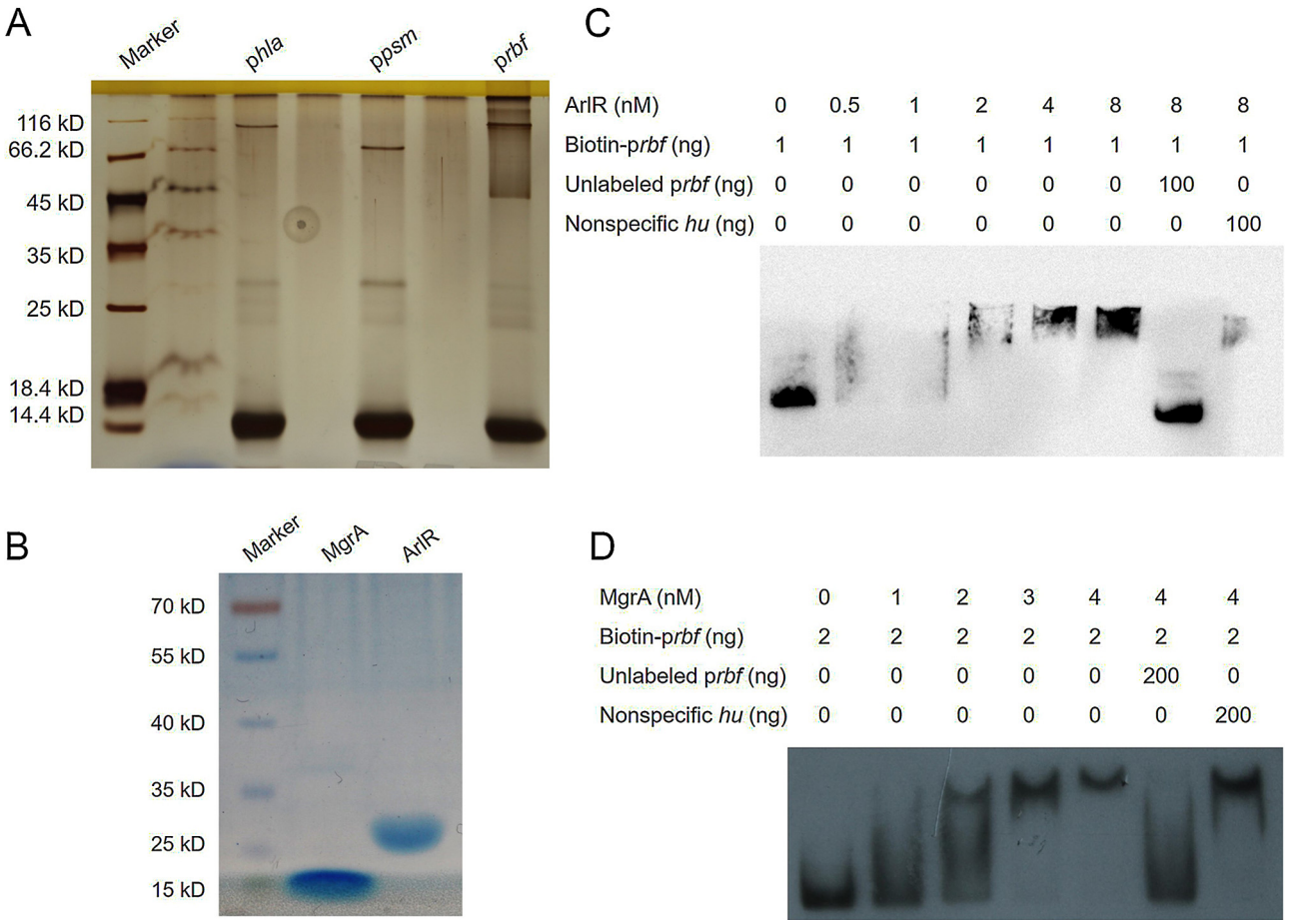

Fig. 1. Identification of the *rbf* promoter-specific binding proteins ArlR and MgrA. (A) SDS-PAGE analysis of proteins binding to *hla* promoter, *psm* promoter or *rbf* promoter. The gel was treated with silver staining, and among the specific bands, proteins ArlR and MgrA were identified by mass spectrometry. (B) SDS-PAGE analysis of ArlR and MgrA purified from the pET28a (+) expression vector. The gel was stained with Coomassie blue. (C) EMSA of the purified ArlR with probe of *rbf* promoter labeled with biotin. Increasing concentrations of purified ArlR and 1 ng of the biotin-labeled probe was used in the reactions. The unlabeled probe was added as a specific competitor, and the unlabeled fragment of the *hu* ORF region was added as a nonspecific competitor. (D) EMSA of the purified MgrA with probe of *rbf* promoter labeled with biotin. Increasing concentrations of purified MgrA and 2 ng of the biotin-labeled probe was used in the reactions. The unlabeled probe was added as a specific competitor, and the unlabeled fragment of the *hu* ORF region was added as a nonspecific competitor.

Table 3
List of the proteins with DNA-binding domains identified by LC–MS/MS.

Gene	Protein	Molecular function	Peptide (Hits)
SAOUHSC_00694	MgrA	Regulatory protein involved in autolytic activity, multidrug resistance and virulence	5
SAOUHSC_01420	ArlR	Member of the two-component regulatory system ArlS/ArlR involved in the regulation of adhesion, autolysis, multidrug resistance and virulence	2
SAOUHSC_00675	Hypothetical protein	Probable transcriptional regulatory protein	5
SAOUHSC_02819	Hypothetical protein	HTH marR-type DNA-binding domain, transcription factor activity, sequence-specific DNA binding	2
SAOUHSC_01797	DNA polymerase I	DNA polymerase I. 3'-5' exonuclease activity; 5'-3' exonuclease activity;	4
SAOUHSC_01222	DNA topoisomerase I	Catalyzes the ATP-dependent breakage of single-stranded DNA followed by passage and rejoining, maintains net negative superhelicity	4

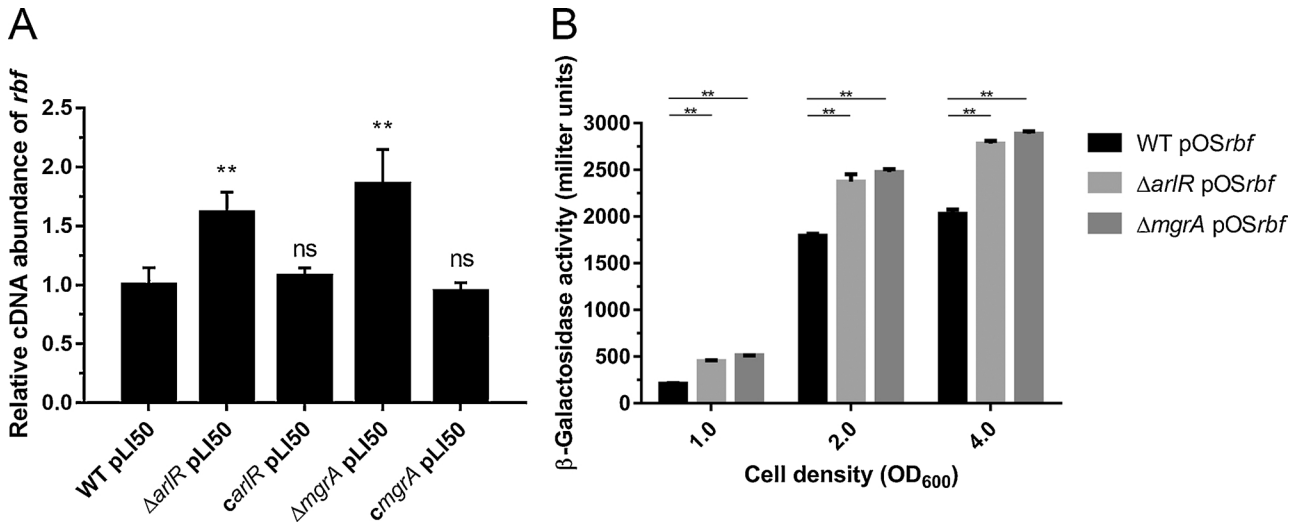

Fig. 2. ArlR and MgrA directly inhibit the expression of *rbf* in *S. aureus* strain NCTC8325. (A) Relative *rbf* transcript levels of the WT strain with a blank plasmid pLI50, the *arlR* mutant strain with a blank plasmid pLI50, the *arlR* complemented strain with a blank plasmid pLI50, the *mgrA* mutant strain with a blank plasmid pLI50, and the *mgrA* complemented strain with a blank plasmid pLI50 were measured by qRT-PCR. (B) β-Galactosidase activity of the *rbf* promoter. β-Galactosidase activities of the WT strain with a plasmid pOS*rbf*, the *arlR* mutant strain with a plasmid pOS*rbf*, and the *mgrA* mutant strain with a plasmid pOS*rbf* were measured at the indicated time points. The error bars represent the standard deviation (SD) of 6 biological replicates. Statistically significant differences between the WT and the other experimental strains calculated by the Mann-Whitney-U-test are indicated: **P < 0.01; ns = not significant.

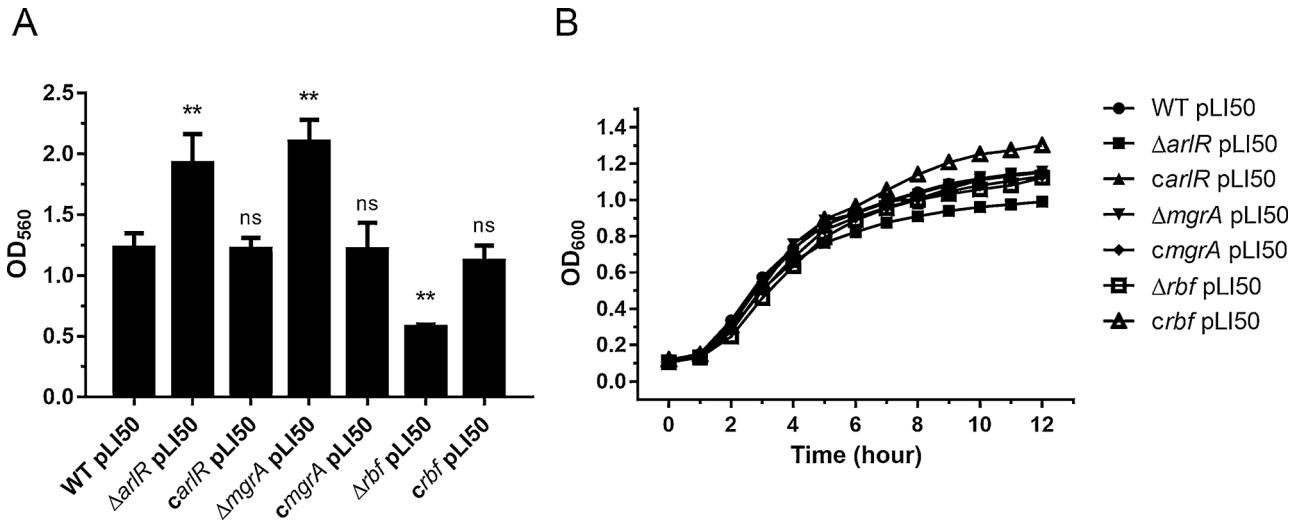

Fig. 3. ArlR and MgrA inhibit biofilm formation. (A) Comparison of biofilm formation of the WT strain with a blank plasmid pLI50, the *arlR* mutant strain with a blank plasmid pLI50, the *arlR* complemented strain with a blank plasmid pLI50, the *mgrA* mutant strain with a blank plasmid pLI50, the *mgrA* complemented strain with a blank plasmid pLI50, the *rbf* mutant strain with a blank plasmid pLI50, and the *rbf* complemented strain with a blank plasmid pLI50 on a polystyrene microtiter plate. (B) Comparison of the growth rates of strains used for biofilm formation detection. The error bars represent the SD of 6 biological replicates. Statistically significant differences between the WT and the other experimental strains calculated by the Mann-Whitney-U-test are indicated: **P < 0.01; ns = not significant. No significant difference of the growth rates between all these experimental strains was detected using the Kruskal-Wallis test.

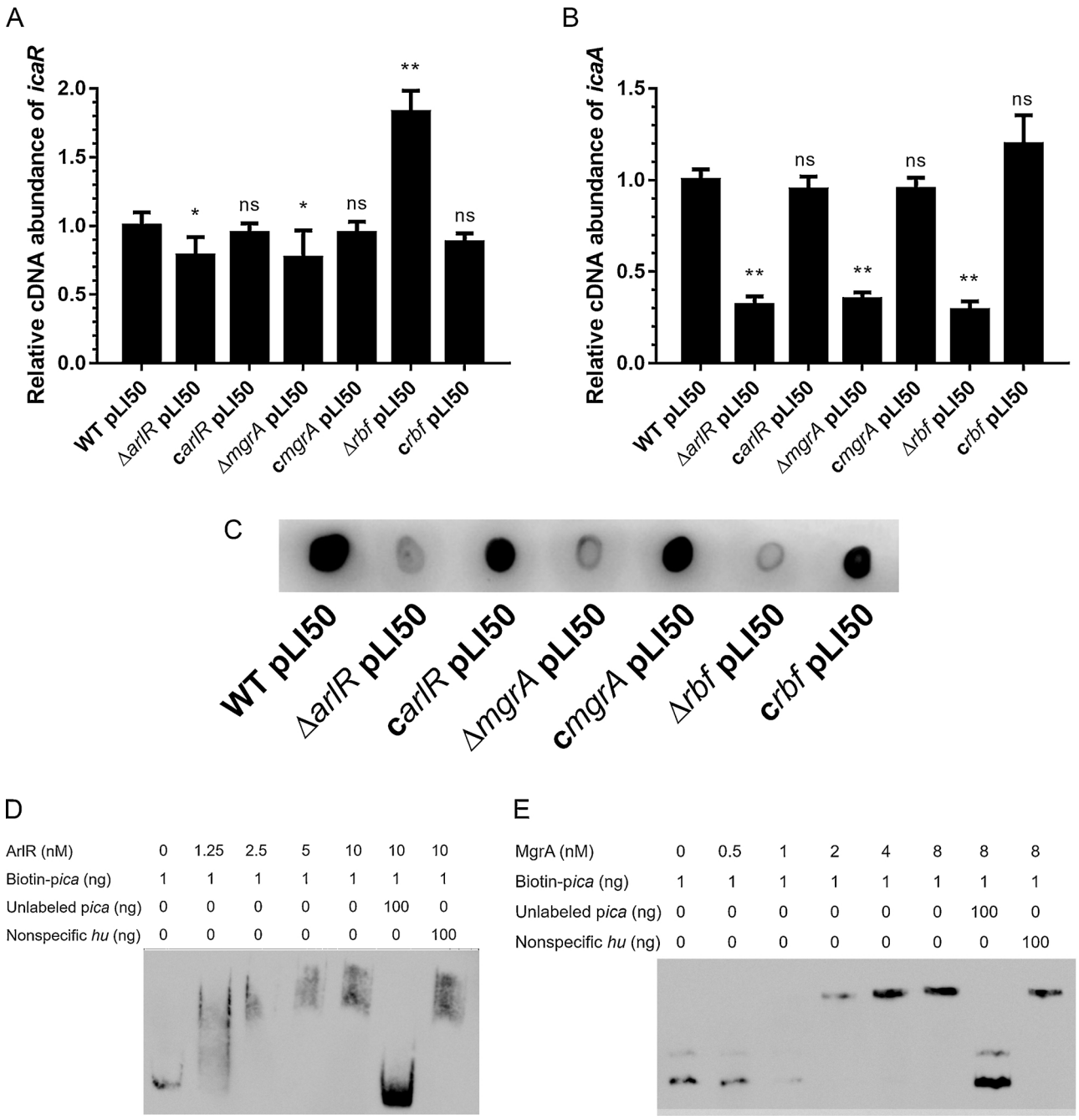

Fig. 4. ArlR and MgrA enhance *icaA* transcription and PIA production. (A) Relative *icaR* transcript levels of the WT strain with a blank plasmid pLI50, the *arlR* mutant strain with a blank plasmid pLI50, the *arlR* complemented strain with a blank plasmid pLI50, the *mgrA* mutant strain with a blank plasmid pLI50, the *mgrA* complemented strain with a blank plasmid pLI50, the *rbf* mutant strain with a blank plasmid pLI50, and the *rbf* complemented strain with a blank plasmid pLI50 were measured by qRT-PCR. (B) Relative *icaA* transcript levels of the WT strain with a blank plasmid pLI50, the *arlR* mutant strain with a blank plasmid pLI50, the *arlR* complemented strain with a blank plasmid pLI50, the *mgrA* mutant strain with a blank plasmid pLI50, the *mgrA* complemented strain with a blank plasmid pLI50, the *rbf* mutant strain with a blank plasmid pLI50, and the *rbf* complemented strain with a blank plasmid pLI50 were measured by qRT-PCR. (C) Quantification of PIA production. PIA was extracted from overnight cultures of each strain and applied to a nitrocellulose membrane after properly diluted. PIA was detected using WGA-biotin. After incubation with HRP-streptavidin, the spots were visualized by chemiluminescence detection. EMSA of the purified ArlR with probe of *ica* promoter labeled with biotin (D) and the purified MgrA with probe of *ica* promoter labeled with biotin (E) was performed. Increasing concentrations of purified ArlR or MgrA and 1 ng of the biotin-labeled probe was used in the reactions. The unlabeled probe was added as a specific competitor, and the unlabeled fragment of the *hu* ORF region was added as a nonspecific competitor. The error bars represent the SD of 6 biological replicates. Statistically significant differences between the WT and the other experimental strains calculated by the Mann-Whitney-U-test are indicated: *P < 0.05; **P < 0.01; ns = not significant.

detected. The results showed that the β-galactosidase activities in both mutant strains were higher than that in the WT strain throughout the growth phases (Fig. 2B). These data suggested that ArlR and MgrA can negatively regulate *rbf* expression by directly binding to its promoter region.

3.3. ArlR and MgrA inhibit biofilm formation

Since ArlR and MgrA can directly repress *rbf* expression, biofilm formation of the WT, the *arlR* mutant, the *mgrA* mutant, the *rbf* mutant, and the complemented strains was monitored. As predicted, the *arlR*

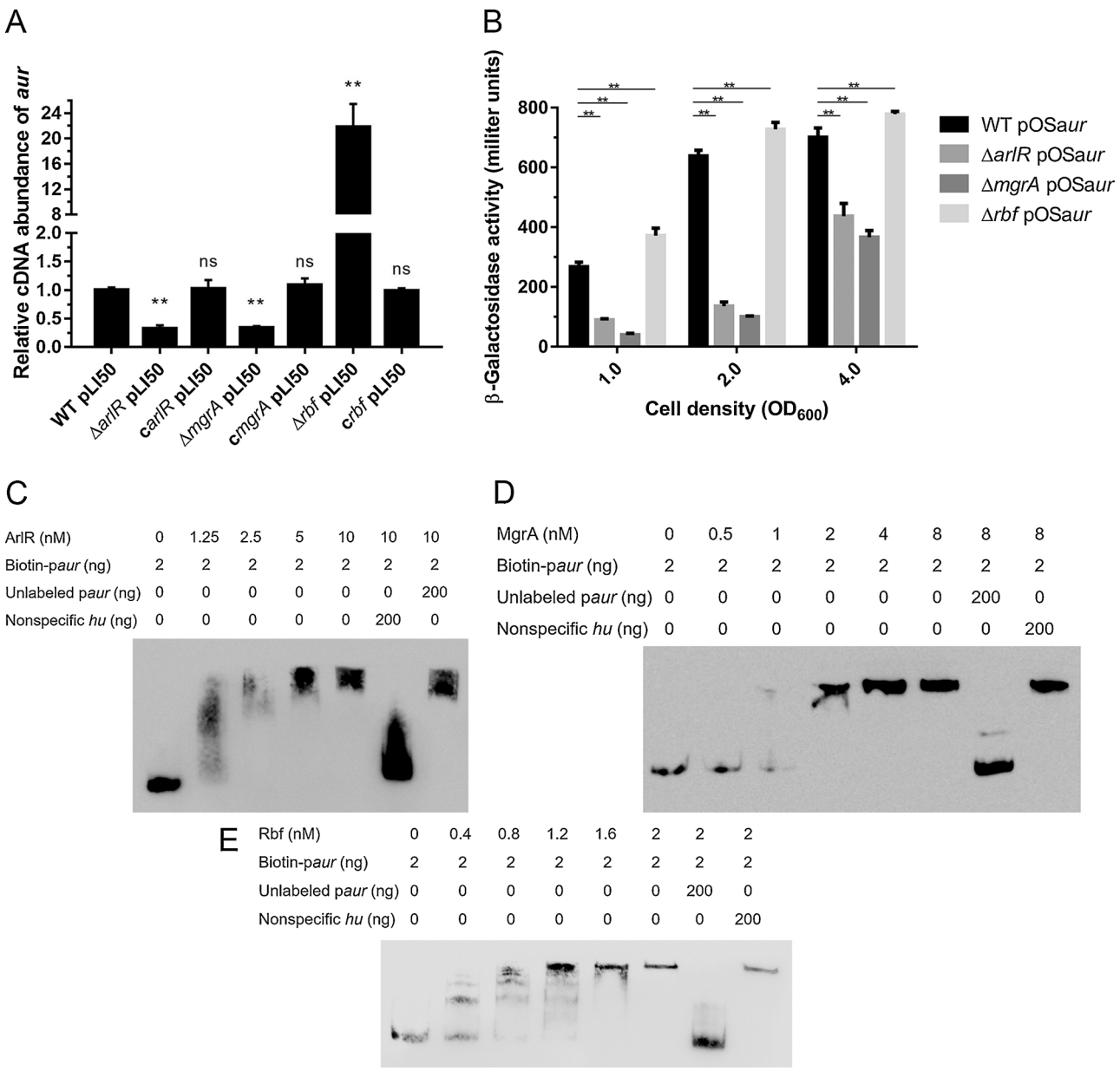

Fig. 5. ArlR and MgrA directly promote *aur* expression while Rbf directly represses *aur* expression. (A) Relative *aur* transcript levels of the WT strain with a blank plasmid pLI50, the *arlR* mutant strain with a blank plasmid pLI50, the *arlR* complemented strain with a blank plasmid pLI50, the *mgrA* mutant strain with a blank plasmid pLI50, the *mgrA* complemented strain with a blank plasmid pLI50, the *rbf* mutant strain with a blank plasmid pLI50, and the *rbf* complemented strain with a blank plasmid pLI50 were measured by qRT-PCR. (B) β-Galactosidase activity of the *aur* promoter. β-Galactosidase activities of the WT strain with a plasmid pOS*aur*, the *arlR* mutant strain with a plasmid pOS*aur*, the *mgrA* mutant strain with a plasmid pOS*aur*, and the *rbf* mutant strain with a plasmid pOS*aur* were measured at the indicated time points. EMSA of the purified ArlR with probe of *aur* promoter labeled with biotin (C), the purified MgrA with probe of *aur* promoter labeled with biotin (D), and the purified Rbf with probe of *aur* promoter labeled with biotin (E) was performed. Increasing concentrations of purified ArlR, MgrA or Rbf and 2 ng of the biotin-labeled probe was used in the reactions. The unlabeled probe was added as a specific competitor, and the unlabeled fragment of the *hu* ORF region was added as a nonspecific competitor. The error bars represent the SD of 6 biological replicates. Statistically significant differences between the WT and the other experimental strains calculated by the Mann-Whitney-U-test are indicated: **P < 0.01.

mutant and the *mgrA* mutant strains displayed significantly increased biofilm formation ability, while the *rbf* mutant strain exhibited decreased biofilm formation (Fig. 3A). Meanwhile, no significant difference of the growth rates between all seven experimental strains was detected using the Kruskal-Wallis test (Fig. 3B).

3.4. ArlR and MgrA directly enhance icaA expression

The higher level of Rbf will result in a lower level of IcaR and then an enhanced biofilm. To determine whether the enhanced biofilm formation ability of the *arlR* mutant and the *mgrA* mutant strains was associated with the level of PIA production, we examined the transcript levels of *icaR* and *icaA*. Contrary to our expectation, the *icaR* transcript level in these two mutant strains decreased slightly compared with the WT strain (Fig. 4A), but the expression of *icaA* decreased significantly in the *arlR* mutant and the *mgrA* mutant strains (Fig. 4B). Moreover, we measured the PIA production in all seven experimental strains. As shown in Fig. 4C, the *arlR* mutant, the *mgrA* mutant, and the *rbf* mutant strains all displayed much weaker PIA production compared with the WT strain, while the complemented strains exhibited restored PIA production. This result was consistent with the qRT-PCR result but contradicted to the phenotype of biofilm formation, suggesting that

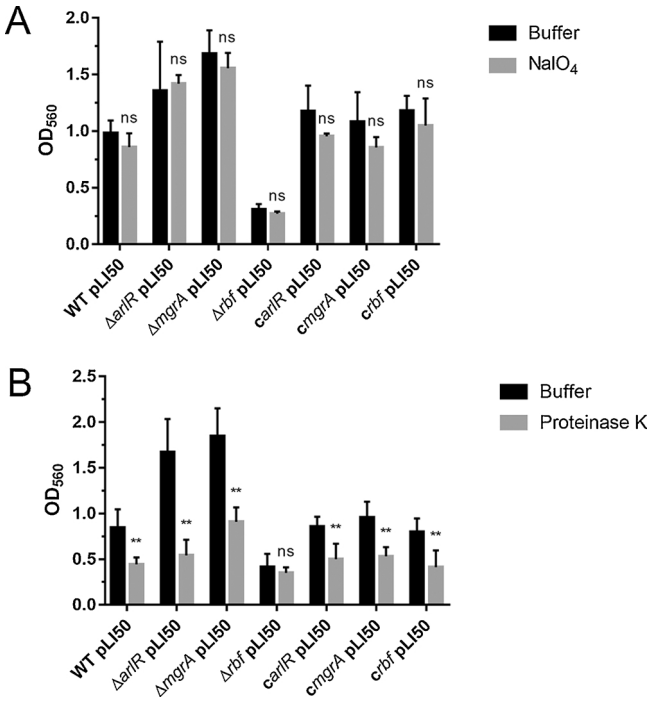

Fig. 6. Differentiation of mechanism of biofilm formation as determined by crystal violet staining. (A) Dispersal of mature biofilms by $NaIO_4$. The experimental strains are indicated on the *x*-axis; the mature biofilms were treated by buffer alone (black bar) or buffer containing 40 mM $NaIO_4$ (grey bar). (B) Dispersal of mature biofilms by proteinase K. The experimental strains are indicated on the *x*-axis; the mature biofilms were treated by buffer alone (black bar) or buffer containing 0.1 mg/ml proteinase K (grey bar). The error bars represent the SD of 6 biological replicates. Statistically significant differences between the treated group and control group calculated by the Mann-Whitney-U-test are indicated: $^{**}P < 0.01$; ns = not significant.

ArlR and MgrA promote the production of PIA.

Since the qRT-PCR result indicated that ArlR and MgrA promoted the expression of *ica* operon, EMSA was performed using purified ArlR and MgrA and probe containing the *ica* operon promoter region. The results showed that both ArlR and MgrA could specifically bind to the *ica* operon promoter and the binding was inhibited with a 100-fold concentration of specific competitors, while the same amount of nonspecific competitors did not have the effect (Fig. 4D and E).

In conclusion, ArlR and MgrA can specifically bind to the *ica* promoter region and positively regulate its transcription.

3.5. ArlR, MgrA, and Rbf directly regulate aur expression

The *aur* gene codes for aureolysin, an extracellular zinc-dependent metalloproteinase in *S. aureus* and a negative factor of biofilm formation. We found that the *aur* transcript levels decreased dramatically in the *arlR* mutant and the *mgrA* mutant strains, while the level of the *rbf* mutant strain was significantly higher (Fig. 5A). So we detected the β-galactosidase activities of all experimental strains containing pOS*aur* to confirm this finding. The result showed that the β-galactosidase activities in the *arlR* mutant and the *mgrA* mutant strains were significantly lower than that in the WT strain throughout the growth phases, and the β-galactosidase activity in *rbf* mutant strain was significantly higher although the folds were less than the qRT-PCR result. We figured that the high folds of RNA coding β-galactosidase might trigger some unknown inhibitory regulatory mechanisms to ease this metabolic burden since the β-galactosidase is not a necessary product for *S. aureus*.

To confirm if the regulatory effects are direct, the EMSA was performed. The result showed ArlR, MgrA, and Rbf all bind to the probe containing the *aur* promoter region, and the protein-DNA complex can be inhibited with a high concentration of a specific competitor but not influenced by the addition of high concentration of the nonspecific competitor *hu* fragment (Fig. 5C, D, and E). These data indicated that ArlR and MgrA can bind to the *aur* promoter to positively regulate its expression, while Rbf can bind to the *aur* promoter to negatively regulate its expression.

3.6. Extracellular proteins are the main components of the biofilm produced by the arlR mutant and the mgrA mutant strains

To determine the crucial components of the biofilm, we examined the mature biofilms of all experimental strains for interaction with $NaIO_4$ (Fig. 6A) and proteinase K (Fig. 6B). After exposure to 40 mM $NaIO_4$ for 24 h at 4°C in the darkness, the WT strain, the *arlR* mutant, and the *mgrA* mutant strains showed no significant reduction in biofilm formation compared to that of control. In contrast, all these three strains exhibited a significant decrease in biofilm formation when treated with 0.1 mg/ml proteinase K for 2 h at 37°C. These data indicated that extracellular proteins instead of polysaccharides constituted main components of the biofilm matrix produced by these strains. In addition, the results suggested that the ArlR-MgrA regulatory cascade inhibits the biofilm formation mainly by enhancing the expression level of *aur*.

3.7. ArlR and MgrA repress the expression of rbf and aur in a σ^B-independent manner

The alternative transcriptional factor σ^B has been recognized to be a global regulator involved in stress response, and to modulate the expression of various genes including *aur* (Bischoff et al., 2004). *S. aureus* strain NCTC8325 displays a reduced σ^B activity due to a deletion in the 5′ region of *rsbU* (Giachino et al., 2001). To investigate whether the regulatory effects described above still exist in *S. aureus* harboring a functional σ^B, we introduced pLI*rsbU* encoding an intact RsbU from *S. aureus* strain SH1000 into our experimental strains to restore the σ^B activity. Biofilm formation of all *rsbU*-complemented strains was monitored and the result showed that the differences between the *rsbU*-complemented *arlR* mutant, the *rsbU*-complemented *mgrA* mutant, the *rsbU*-complemented *rbf* mutant, and the *rsbU*-complemented WT strains remained the same (Fig. 7A). Meanwhile, the increased biofilm formation ability compared to the WT strain with blank pLI50 suggested the restoration of σ^B activity. The growth rates of all *rsbU*-complemented strains and the WT strain with blank pLI50 were examined and no significant difference between all these experimental strains was detected using the Kruskal-Wallis test (Fig. 7B). We also detected the transcript levels of *rbf*, *icaR*, *icaA,* and *aur* in all *rsbU*-complemented strains (Fig. 7C). The result showed that the regulatory effects of ArlR and MgrA on *rbf*, *icaA,* and *aur* remained the same, and *icaR* was still only slightly influenced by these two regulators. At the same time, Rbf still represses the expression of *icaR* and *aur*. These results indicated that all the transcriptional regulatory effects revealed in this study are σ^B-independent.

3.8. The LuxS/AI-2 system has no interaction with the ArlR-MgrA regulatory cascade and also represses aur expression

Our previous study revealed that the LuxS/AI-2 system represses *rbf* expression and biofilm formation in *S. aureus* strain NCTC8325 (Ma et al., 2017; Yu et al., 2012). To investigate whether the LuxS/AI-2 system has an interaction with the ArlR-MgrA regulatory cascade, we examined the transcript levels of *arlR* and *mgrA* in the *luxS* mutant strain (Fig. 8A). The result showed no significant difference between the WT and the *luxS* mutant strains, indicating that the LuxS/AI-2 system doesn't regulate the ArlR-MgrA regulatory cascade. Meanwhile, we examined the transcript level of *luxS* in the *arlR* mutant and the *mgrA* mutant strains, and the result showed no significant difference

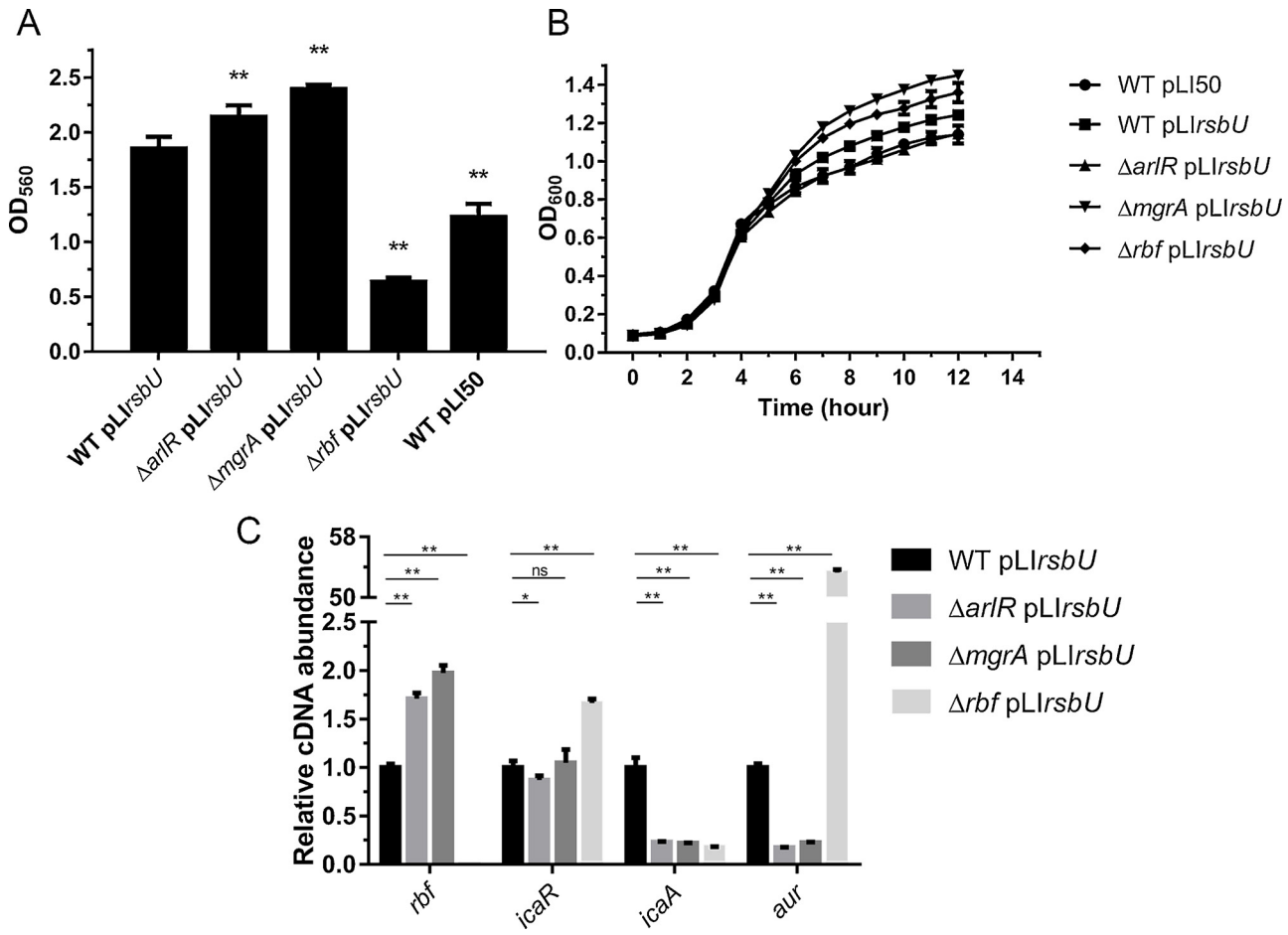

Fig. 7. Biofilm formation and the transcript levels of *rbf*, *icaR*, *icaA,* and *aur* in *rsbU*-complemented strains. (A) Comparison of biofilm formation of the WT strain with a blank plasmid pLI50 and all four *rsbU*-complemented strains on a polystyrene microtiter plate. (B) Comparison of the growth rates of the WT strain with a blank plasmid pLI50 and all four *rsbU*-complemented strains. (C) Relative transcript levels of *rbf*, *icaR*, *icaA,* and *aur* in *rsbU*-complemented strains were measured by qRT-PCR. The error bars represent the SD of 6 biological replicates. Statistically significant differences between the WT and the other experimental strains calculated by the Mann-Whitney-U-test are indicated: **P < 0.01; ns = not significant. No significant difference of the growth rates between all these experimental strains was detected using the Kruskal-Wallis test.

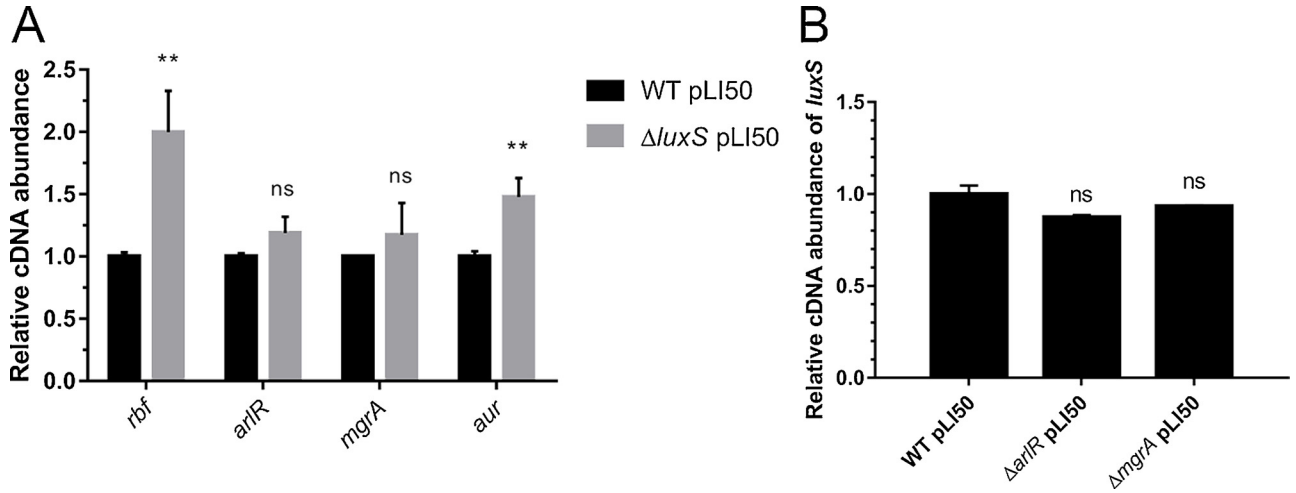

Fig. 8. The LuxS/AI-2 system has no interaction with the ArlR-MgrA regulatory cascade and represses *aur* expression. (A) Relative *rbf*, *arlR*, *mgrA,* and *aur* transcript levels of the WT strain with a blank plasmid pLI50 and the *luxS* mutant strain with a blank plasmid pLI50 were measured by qRT-PCR. (B) Relative *luxS* transcript levels of the WT strain with a blank plasmid pLI50, the *arlR* mutant strain with a blank plasmid pLI50, the *mgrA* mutant strain with a blank plasmid pLI50 were measured by qRT-PCR. The error bars represent the SD of 6 biological replicates. Statistically significant differences between the WT and the other experimental strains calculated by the Mann-Whitney-U-test are indicated: **P < 0.01; ns = not significant.

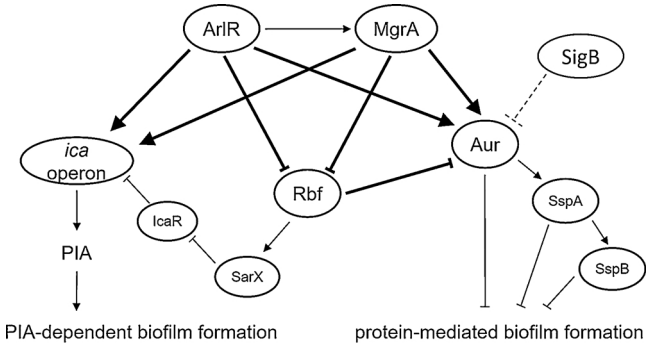

Fig. 9. The regulatory network of ArlR, MgrA, and Rbf on PIA-dependent and protein-mediated biofilm formation. The ArlR-MgrA regulatory cascade directly represses Rbf. Rbf increases PIA production by repressing IcaR via SarX, and represses Aur directly. The ArlR-MgrA regulatory cascade also directly enhances *ica* operon expression and PIA production, and directly enhances *aur* expression. Aur could initiate the downstream protease cascade of SspA and SspB to decrease protein-mediated biofilm formation. The regulation of ArlR and MgrA on PIA-dependent and protein-mediated biofilm formation could be Rbf-dependent or Rbf-independent. The solid lines stand for direct regulation and the dashed line stands for indirect regulation. The new findings in this study are presented in bold lines.

either (Fig. 8B), suggesting that the LuxS/AI-2 system and the ArlR-MgrA regulatory cascade have no interaction with each other. In addition, the transcript levels of *rbf* and *aur* showed that the higher abundance of *rbf* in the *luxS* mutant strain didn't result in a lower *aur* transcript level (Fig. 8A), indicating that the LuxS/AI-2 system might have another Rbf-independent pathway to downregulate the expression of *aur*.

4. Discussion

Rbf was first identified as a biofilm formation regulator. Several studies have demonstrated that Rbf can bind to the *sarX* promoter and upregulate its expression. SarX can bind to a sequence upstream of *icaR* within the *icaA* coding region to downregulate IcaR, which can inhibit biofilm formation by binding to the *icaA* promoter region and repressing the *ica* operon expression and PIA production (Conlon et al., 2002; Cue et al., 2013, 2009). Our previous work indicated the LuxS/AI-2 system can repress PIA-dependent biofilm formation by repressing the expression of *rbf* in *S. aureus* strain NCTC8325 and Rbf can bind to the *rbf* promoter to upregulate its own expression (Ma et al., 2017; Yu et al., 2012). In this study, we identified two novel regulators of *rbf*, ArlR and MgrA. The results of EMSA, qRT-PCR, and β-galactosidase activity assay revealed that both ArlR and MgrA can directly bind to the *rbf* promoter to repress its expression in *S. aureus* strain NCTC8325. We assumed this inhibitory effect of ArlR and MgrA on *rbf* would lead to an increase of biofilm formation in their mutants due to the increased PIA production caused by increased Rbf. Although the phenotype of biofilm formation conformed to our expectation, the transcript levels of *icaR* showed only slightly decrease in the *arlR* mutant and the *mgrA* mutant strains compared to the WT strain. The transcript levels of *icaA* even decreased significantly in the *arlR* mutant and the *mgrA* mutant strains, leading to decreased PIA production. A previous study has shown that ArlR in *S. epidermidis* can bind to the promoter region between *icaR* and *ica* operon, then positively regulate biofilm formation in an *ica*-dependent manner (Wu et al., 2012). Thus, we inferred that ArlR and MgrA in *S. aureus* might have the same direct impact on *ica* operon. The EMSA results confirmed that ArlR and MgrA can bind to the promoter region of *ica* operon, which might explain the decrease of *icaA* transcription and PIA production in the *arlR* mutant and the *mgrA* mutant strains. Taken together, our results suggest that ArlR and MgrA regulate PIA in two manners: ArlR and MgrA can repress the expression of *rbf* and decrease its upregulation on *ica* operon; they also bind to the promoter of *ica* operon to positively regulate the PIA production. We speculated that the positive regulations of ArlR and MgrA take the dominance, since both the mutant strains showed decreased *icaA* transcription and PIA production.

Since the PIA-dependent pathway didn't explain the increased biofilm formation in the *arlR* mutant and the *mgrA* mutant strains, we examined the expression of genes related to protein-mediated biofilm formation. Our data indicated that the transcript level of *aur* changed dramatically in the *arlR* mutant, the *mgrA* mutant, and the *rbf* mutant strains. Aureolysin is an extracellular zinc-dependent metalloproteinase in *S. aureus*. The serine and cysteine proteases SspA and SspB of *S. aureus* are secreted as inactive zymogens zSspA and zSspB. Mature SspA is a trypsin-like glutamyl endopeptidase and is required to activate zSspB. Aur is necessary for the cleavage and activation of SspA to initiate this proteolytic cascade (Nickerson et al., 2007). Although all these three proteases had an inhibitory effect on biofilm formation, Aur plays the most important role due to the position of Aur at the top of the *S. aureus* protease activation cascade (Loughran et al., 2014; Mootz et al., 2013). The results of qRT-PCR, β-galactosidase activity assay, and EMSA evidenced that ArlR and MgrA upregulate the expression of *aur* while Rbf downregulates the expression of *aur* all by directly binding to the *aur* promoter. These new findings were consistent with not only the increased biofilm formation of the *arlR* mutant and the *mgrA* mutant strains but also the decreased biofilm formation of the *rbf* mutant strain. Rbf has been recognized to be a biofilm formation regulator only functioning in PIA-dependent way (Conlon et al., 2002; Cue et al., 2013, 2009). Our research revealed that it also enhances protein-mediated biofilm formation by itself, or functions downstream of the ArlR-MgrA regulatory cascade. Meanwhile, the ArlR-MgrA regulatory cascade also directly represses the expression of *aur* in a Rbf-independent manner to limit biofilm formation. Our results also demonstrated that the main components of the biofilm produced by the *arlR* mutant and the *mgrA* mutant strains are extracellular proteins, suggesting that the increases of biofilm formation of these two mutant strains are mainly due to the decreased expression level of *aur*. Because of the significance of Aur in virulence and immune evasion (Laarman et al., 2011), our data partially help to explain the decreased virulence of the *arlR* mutant and the *mgrA* mutant strains (Crosby et al., 2016; Walker et al., 2013).

The alternative transcriptional factor σ^B has been recognized to be a global regulator involved in stress response. *S. aureus* strain NCTC8325 displays a reduced σ^B activity due to a deletion in gene *rsbU* (Giachino et al., 2001). σ^B was reported to inhibit the expression of *aur* (Bischoff et al., 2004). We examined the promoter sequences of *arlR*, *mgrA*, *rbf*, *icaA*, and *aur*, but found that none contains the nucleotide sequence (GTTATT-$_{14}$-GGGTAT) that matches the proposed σ^B consensus, suggesting that their expression was not directly controlled by σ^B. We also checked the biofilm formation and transcript levels in all four *rsbU*-complemented strains with a functional σ^B, and the results showed the same tendency. These results corroborated the regulatory effects and also proved that they are σ^B-independent.

As mentioned, we previously found that the LuxS/AI-2 system can repress *rbf* expression in *S. aureus* strain NCTC8325 and then inhibits biofilm formation (Ma et al., 2017; Yu et al., 2012). This study suggests that LuxS can slightly repress the expression of *aur* in an unknown Rbf-independent pathway.

In conclusion, we have revealed that the ArlR-MgrA regulatory cascade can directly repress the expression of Rbf, the positive regulator of *ica* operon. We have also unveiled the direct upregulation of the ArlR-MgrA regulatory cascade on *aur*, the inhibitor of protein-mediated biofilm formation, and the novel repressive effect of Rbf on *aur*. All these findings demonstrate a regulatory network linking PIA-dependent biofilm formation and protein-mediated biofilm formation together, with ArlR, MgrA, and Rbf as important nodes (Fig. 9). And how these regulatory pathways take dominance over each other to control biofilm

formation and other physiological features deserves further investigation.

Declarations of interest

none

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