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ORIGINAL ARTICLE

The Functional Study of Response Regulator ArlR Mutants in *Staphylococcus Aureus*

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Abstract

Staphylococcus aureus is a major cause of hospital-associated infections worldwide. The organism's ability to form biofilms has led to resistance against current treatment options such as beta-lactams, glycopeptides, and daptomycin. The ArlRS two-component system is a crucial regulatory system necessary for *S. aureus* autolysis, biofilm formation, capsule synthesis, and virulence. This study aims to investigate the role of the arlR deletion mutant in the detection and activation of *S. aureus*. We created an *arlR* deleted mutant and complementary strains and characterized their impact on the strains using partial growth measurement. The quantitative real-time PCR was performed to determine the expression of *icaA,* and the microscopic images of adherent cells were captured at the optical density of 600 to determine the primary bacterial adhesion. The biofilm formation assay was utilized to investigate the number of adherent cells using crystal violet staining. Eventually, the Triton X-100 autolysis assay was used to determine the influence of *arlR* on the cell autolytic activities. Our findings indicate that the deletion of *arlR* reduced the transcriptional expression of *icaA* but not *icaR* in the *ica* operon, leading to decrease in polysaccharide intercellular adhesin (PIA) synthesis. Compared to the wild-type and the complementary mutants, the *arlR* mutant exhibited decreased in biofilm production but increased autolysis. It concluded that the *S. aureus* response regulatory *ArlR* influences biofilm formation, agglutination, and autolysis. This work has significantly expanded our knowledge of the *ArlRS* two-component regulatory system and could aid in the development of novel antimicrobial strategies against *S. aureus*.

Keywords *Staphylococcus aureus* · Response regulator ArlR · Biofilm formation · Adherence

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Introduction

Staphylococcus aureus, also known as golden staph, is a gram-positive bacterium categorized as one of the ESKAPE pathogens, the highly virulent antibiotic-resistant bacterial strains, resulting in significant morbidity and mortality rates worldwide [1]. *S. aureus* can cause a variety of infections ranging from mild to severe, including blood septicemia, thrombophlebitis, infectious endocarditis [2–4] osteomyelitis, or device-related infections (DRI) [5], such as intravascular catheters, prosthetic joints, vascular grafts, and pacemakers [6].

Staphylococcus aureus has emerged as a leading cause of hospital-associated infections and the most life-threatening multidrug-resistant opportunistic pathogens worldwide [7, 8]. *Methicillin-resistant S. aureus* (MRSA), also known as oxacillin-resistant *S. aureus*, is the most virulent form of *S. aureus*, which has reached a particular epidemic and healthcare threat globally [9, 10]. MRSA is defined as an oxacillin minimum inhibitory concentration (MIC) greater than or equal to 4 mg/L [11]. MRSA has developed resistance to current treatment options such as -lactams, glycopeptides, and daptomycin due to the organism's capacity to produce biofilms [12, 13], commonly known as the two-component regulatory system (TCS), which functions as a sensory system for *S. aureus* [14]. The success of *S. aureus* in invading humans highly depends on various virulence factors, including exoenzymes, toxins, and adhesions that make the bacterial adhere to the mucous membranes or invade the immune system and adapt to dynamic environmental stimuli to survive [15, 16]. Multiple virulence factors are controlled by the two-component regulatory system (TCS). Most of the *S. aureus* strains possess 16 TCSs, one of which is vital for bacterial viability [17, 18].

The ArlRS TCS has been involved in various pathogenic processes, such as autolysis, biofilm formation, virulence, and capsule synthesis, which plays a vital role in the regulation of clumping and adherence [19, 20]. ArlRS consists of a membrane-bound histidine kinase ArlS that responds to different environmental signals, auto-phosphorylates a conserved histidine residue, and a cognate response regulator ArlR which its aspartate residue receives the phosphate and affects the transcription of a subset of target genes [21, 22]. The *arlR* was found to activate the production of MgrA responsible to controls the virulence factors and the intracellular adhesion gene cluster (*ica* operon) [23, 24]. Additionally, it serves as a direct activator for abcA, a gene that encodes an ATP-dependent transporter associated with cell wall autolysis and -lactam antibiotics resistance [25]. ArlR-MgrA directly controls the cascade that represses the biofilm formation regulator Rbf and upregulates SarX, a Sar transcriptional regulatory protein family member that regulates *ica* expression [26]. It has been reported that MgrA knockout in USA300 resulted in just a slight reduction of MIC for oxacillin (from 64 to 32 mg/L), but arlRS knockout in USA300 results in a significantly decreased MIC value (4 mg/L) [10]. Moreover, the *arlR* mutant has an essential clumping defect for staphylococcal infection. Polysaccharide intercellular adhesion (PIA) is a common element of staphylococcal biofilm [27, 28]. The ArlR plays a vital role in regulating biofilm formation, which can directly bind to the promoter region between *icaR* and *icaADBC*, enhancing the expression of *icaA* and PIA production [29].

The synthesis of PIA is performed via the *ica* locus, which has an operon composed of four open frames named *icaA*, *icaD*, *icaB*, and *icaC*. The *icaR* is likewise a component of the *ica* operon and is located upstream but in the opposite location [30, 31]. Our previous study showed structural insights into the activation and recognition of ArlR, and ArlR recognizes a 20 bp imperfect inverted repeat sequence located at the *ica* operon [32].

Nonetheless, the downstream gene regulatory mechanisms of *arlR* in *S. aureus* are poorly studied. In this research, we have introduced a novel approach to investigate the role of the arlR deletion mutant in detecting and activating *S. aureus*. We have created the strains mutant and complementary mutant, evaluated the effects of the *arlR* in the growth measurement, determined the influence of *arlR* on the cell autolytic activities, and assessed the intracellular adhesion cells agglutination. This study provides an in-depth understanding of the ArlR response regulator and its functions, offering unique opportunities to combat antibiotic resistance and directly target pathogen virulence. The outcomes of this study may also pave the way for further research into targeting the ArlR regulatory system as a novel target for fighting *S. aureus*.

Materials and Methods

Bacterial Strains and Primers

The bacterial strains used in this study are listed in Table 1. *S.aureus* were cultured in Trypticase soy broth (TSB) (Oxoid) at 37 °C. *Escherichia coli* cells were cultured in Luria-Bertani (LB) (Oxoid) medium. Cloning of *S. aureus* chromosomal DNA fragments was performed in *Escherichia coli* stains DH5α using plasmid $PKOR_1$ and vector pLi50 [33].

Construction of the arlR Mutant Strains

A 1000 bp upstream and downstream region of the *arlR* were amplified by homologous recombinant form *S. aureus* genome DNA using BIO-RAD C1000 Touch PCR thermal cycler, ligated to temperature-sensitive shuttle plasmid PKOR1 through fusion PCR and Gateway cloning technology using primers. The recombinant plasmid was extracted and transformed into a restriction system-defective *S. aureus* RN4220 by electroporation. The target strain was electrophoresed with a modified plasmid. Positive colonies were broth

Table 1 Strains and primers

Strains and primers	Description	Source
S. aureus NCTC8325	NCTC 8325 = AS 1.2132	NCTC
S. aureus RN4220	Restriction deficient cloning host	ATCC
E.coli. DH5α	Host for plasmid construction	Thermo Scientific™
attB1-UP-F	GGGGACAAGTTTGTACAAAAAAGCAGGCTGCAAAGACAATATGATACT TAC	
UP-overlap-R	GCGCAATTTACGTTTTGTTGTACACCTCATATTACGAC	
Down-overlap-F	GTCGTAATATGAGGTGTACAACAAAACGTAAATTGCGC	
attB2-Down-R	GGGGACCACTTTGTACAAGAAAGCTGGGTAGGATGCAATTGTTTTAATG	
pArlR-Kpn1-F	GGGGTACCATATTGCGGTAAGGCCTTGTGTTACAG	
ArlR-HindIII-R	CCCAAGCTTTCATCGTATCACATACCCAACGC	

incubated overnight at 30 °C with 2 mL TSB containing 10 μg/mL chloramphenicol (Sigma). The strains were diluted by a ratio of 1:100 into a total volume of 50 mL TSB containing 10 μg/mL chloramphenicol and incubated overnight at 42 °C. The cultivation process was repeated twice. The cultures were diluted before plating 100 μL on TSB plate (10 μg/mL chloramphenicol) by incubating overnight at 42 °C. A single colony was resuspended with 2 mL TSB and incubated overnight at 30 °C without any additive antibiotics to facilitate plasmid excision. This culture's 100 folded serial dilutions were spread on the TSB containing 1 μg/mL tetracycline (Sigma) and incubated for 24 h at 37 °C. The transformants were screened for chloramphenicol-sensitive colonies, which grown on the TSB plates not on TSB plate containing 10 μg/mL tetracycline. A single colony was isolated and re-cultured on the TSB plates and then inoculated into 5 mL TSB and incubated overnight with continuous shaking at 37 °C. The deleted mutants were identified by PCR using the primers $attB_1$-UP-F and $attB_2$-Down-R.

Creation of the arlR Complement

The *arlR* gene pair and its native promoter were amplified with the primer pairs of p*arlR*-Kpn1-F and p*arlR*-HindIII-R using *S. aureus* genome DNA. The PCR product was gel-purified and digested by KpnI and HindIII at 37 °C overnight. The digested fragment was cloned into vector pLi50 and then introduced into *E.coli* DH5α to generate the plasmid pLi50-*arlR*. The restriction defective RN4220 was electroporated by plasmid after genetic modification in RN4220. The recombinant plasmid was transduced into the *S. aureus* Δ*arlR*.

Quantitative PCR

For qPCR experiments, *arlR* deleted mutant and complementary strains were activated using 5 mL TSB and incubating overnight at 37 °C. Following incubation, 50 μL cultures were transferred to 50 mL TSB and incubated at 37 °C for 12 h then lysed for 30 min at 37 °C with 100 μL lysostaphin (Sigma). The total RNA was extracted by Qiagen RNeasy Mini kit, and 300 ng of RNA was verified by the A260/A280 ratio of 2.0–2.1. Then the high-capacity cDNA reverse transcription kit (Bio-Rad) was used for the first-strand cDNA synthesis. The qPCR experiments were performed using the Power SYBR Green PCR Master Mix (Bio-Rad) to determine the expression of *icaA* via Bio-Rad CFX Connect Real-Time PCR Detection System by following conditions: 95 °C for 10 min, 95 °C for 10 s, 60 °C for 15 s, and 72 °C for 20 s with 39 cycles. All qRT-PCR primers used for the qRT-PCR (Table 2) were verified using normal PCR. 16 S-RNA was used as a reference gene to standardize all the data.

Growth Curves of arlR Wild-Type, Delete Mutant, and Complementation Strains

The strains were incubated overnight with 5 mL of TSB at 37 °C before being diluted 1:100. Using the TSB medium to adjust an OD_{600} to 0.15, cultures were grown at 37 °C with 200 rpm of continuous shaking. The optical density was determined by measuring the OD_{600} every 30 min for 10 h.