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ORIGINAL INVESTIGATION

ArtR, a novel sRNA of *Staphylococcus aureus*, regulates α-toxin expression by targeting the 5′ UTR of *sarT* mRNA

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Abstract Recent studies point to the importance of small-noncoding RNAs (sRNAs) in bacterial virulence control. In *Staphylococcus aureus*, functional dissections of sRNAs are limited to RNA III, SprD, RsaE, SprA1, and SSR42 only. Here, we report the identification and functional analyses of a novel sRNA, which we have designated ArtR. Our data show that the AgrA protein can bind to the *artR* promoter and repress *artR* transcription, suggesting that, after RNA III, ArtR is the second sRNA regulated by AgrA. Furthermore, ArtR is unique in *S. aureus* and involved in virulence regulation by activating α-toxin expression. ArtR promotes the degradation of *sarT* mRNA by RNase III and arrests the translation of SarT by direct binding to the 5′ untranslated region of the *sarT* mRNA, suggesting that the activation of ArtR on the α-toxin expression was through SarT. This study reveals another kind of staphylococcal regulatory small RNA that plays a role in virulence control. It also indicates the diversity of small RNA-target mRNA interactions and how these multiple interactions can mediate virulence regulation in this pathogen.

Keywords *Staphylococcus aureus* · sRNA · AgrA · α-Toxin · SarT

Ting Xue and Xu Zhang have contributed equally to this study.

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Introduction

Over the past 10 years, small, noncoding RNA (sRNA) encoded on bacterial chromosomes has become very attractive to researchers. Hundreds of candidate sRNA genes in various bacteria have been identified by computational prediction, oligonucleotide microarrays, and deep sequencing [1–5]. Some sRNAs can bind to proteins and then modulate their activity, or act by direct base pairing to their target mRNA. Base pairing between sRNA and target RNA can lead to translation blocking, mRNA translation activation, or specific cleavage of the complex by RNase [6, 7]. Through these mechanisms, sRNA can repress transposition in *Salmonella enterica* [8] and *Listeria monocytogenes* [9], regulate the synthesis of some transcription regulators in *Escherichia coli* [10], and influence some metabolic and virulence factors [11–13]. Recent studies performed on sRNA in some pathogens point to the importance of sRNAs in virulence control [9, 14–18].

Staphylococcus aureus is a human and animal pathogen that can cause multiple diseases, ranging from localized skin infections to life-threatening systemic infections [19, 20]. The high infection ability of *S. aureus* depends on the production of many virulence factors and regulatory elements, including some transcriptional regulator proteins, two-component systems, and other regulators [21–23]. One of the most important regulators is the Agr/RNA III system. It is a global regulon composed of two divergent transcripts called RNA II and RNA III. The RNA II transcript contains a density-sensing cassette Agr D/B and a two-component system AgrC/A, while RNA III is a multifunctional sRNA and the best characterized sRNA in *S. aureus*. RNA III regulates the expression of many virulence genes as well as some transcription factors at the post-transcriptional level. Binding between RNA III and its

targets (*hla, spa, rot, coa*) can lead to translation activation, translation blocking, or unstable mRNA [24–27].

Research into RNA III has been conducted for several years, and an increasing focus has been placed on the search for additional sRNAs in *S. aureus*. Close to one hundred novel sRNAs have been found in *S. aureus* in recent years by computational or experimental analysis [18, 28–32]. Most of them were predicted based on the search for intergenic regions (IGRs) with a rho-independent terminator, conserved structural motifs, and the absence of open-reading frame (ORF) [28], by which the transcripts encoded in genes or those overlapped by genes will not be found. As experimental approaches have improved, more powerful sRNA detection methods have been developed, including direct labeling, microarrays, and sequencing [30, 31, 33, 34]. Nevertheless, only a few cis-encoded sRNAs have been found because the data analysis was associated with the IGRs [31]. Besides this, although more and more sRNAs have been identified in *S. aureus* and *S. aureus* has emerged as a model organism for the study of bacterial sRNA [35], functional studies of these sRNAs are behind and are irregular in their prediction. Detailed functional analysis is limited to RNA III, SprD, RsaE, SprA1, and SSR42 only [29, 36–38].

We show here that a novel sRNA overlapped by *luxS* was discovered and studied. This sRNA was named ArtR (AgrA-repressed, toxin-regulating sRNA) and found to be repressed by the transcriptional regulator AgrA. Our gel-shift assays showed that AgrA can bind to the promoter region of *artR,* and RT-PCR confirmed that AgrA can repress *artR* transcription. The deletion of *hfq* did not affect *artR* transcription, and the Hfq protein could not bind to the ArtR transcript in vitro, indicating that Hfq has no influence on the stability or functioning of ArtR. In addition, our results also showed that ArtR is involved in staphylococcal virulence regulation. The deletion of *artR* led to a decrease in α-toxin, and we further proved that the activation of *hla* occurred through the transcriptional regulator SarT. Direct binding between ArtR and the *sarT* transcript was observed, and the binding domain was located at the 5′ UTR region of *sarT* mRNA. Binding between ArtR and the 5′ UTR of *sarT* mRNA provides digestion sites for endoribonuclease III (RNase III), promoting the degradation of *sarT* mRNA and activation of the expression of *hla*.

Materials and methods

Strains and plasmids

Escherichia coli strains were grown at 37 °C in Luria–Bertani (LB) medium with a suitable antibiotic: ampicillin (100 μg/mL) or kanamycin (50 μg/mL). *S. aureus* strains were grown at 37 °C in either B-medium (1 % peptone, 0.5 % yeast extract, 0.1 % glucose, 0.5 % NaCl, 0.1 % K_2HPO_4) or tryptic soy broth (TSB, Oxiod) with a suitable antibiotic: erythromycin (2.5 μg/mL) or chloramphenicol (15 μg/mL), as appropriate. The media were solidified with 1.5 % (wt/vol) agar as needed. Chromosomal DNA from *S. aureus* was prepared according to the standard procedures for Gram-positive bacteria [39]. Plasmids from *E. coli* were extracted using a plasmid purification kit (Promega); plasmid extracts from *S. aureus* cells were pre-digested with a digestion buffer containing 40 U/mL lysostaphin, 10 mg/mL lysozyme, and 10 % (v/v) glycerol. The plasmids were transformed into RN4220 and then NCTC8325 by electroporation, as previously described. The bacterial strains and plasmids used in this study are described in Table 1.

Construction of the mutant and complementing strains

The genes were inactivated using the shuttle plasmid pBT2 as described [40]. Target genes were replaced by the erythromycin-resistant gene (*ermB*), and all mutants were confirmed by PCR and sequencing. In order to construct the mutant complement, genes with their native promoters were amplified and ligated into the shuttle plasmid pLI50 and transformed into the relevant mutant strains. The primers used in this study are listed in Supplemental Table S1.

RNA isolation, Northern blot analysis, RACE, cDNA generation, and RT-PCR

For RNA extraction, overnight cultures of *S. aureus* were diluted 1:100 in LB medium and grown to the appropriate phase. RNA was extracted as Wolz et al. [41] described with some modifications. *S. aureus* cells were pelleted and lysed in 1 mL of RNAiso (TaKaRa) with 0.7 g of zirconia-silica beads (0.1 mm in diameter) in a high-speed homogenizer (IKA® T25 digital UlTRATURRAX®). Residual DNA was degraded by treatment with 10 U of DNaseI (Takara) at 37 °C for 1 h. The 5′ and 3′ ends of ArtR were determined by RACE as described [42], using the 3′-Full RACE Core Set Ver.2.0 and 5′-Full RACE Kit (Takara). Reverse transcription was carried out with the PrimeScript 1st Strand cDNA synthesis kit and real-time PCR was performed with SYBR Premix Ex Taq (TaKaRa) using a StepOne real-time system (Applied Biosystems). The quantity of cDNA was normalized to the abundance of 16S cDNA. For Northern blot analysis, 10 or 50 μg of total RNA was separated in 3.5 % polyacrylamide-7 M urea gel electrophoresis in 1 × TBE and then electro-transferred to a charged nylon membrane (Millipore) in 0.5 × TBE. Biotin-labeled primer probes complementary

Table 1 Bacterial strains and plasmids used in this study

Strain or plasmid	Relevant genotype	Reference or source
Strains		
S. aureus		
NCTC8325	Wild type	NARSA
RN4200	8325-4, restriction-negative strain	NARSA
MW2	USA400	NARSA
Mu50	Vancomycin-intermediate strain isolated in 1996	NARSA
N315	Methicillin-resistant strain isolated in 1982	NARSA
COL	Clinical isolate sequenced at TIGR	NARSA
Sanger 252	Epidemic MRSA strain sequenced at Sanger Centre	NARSA
Sanger 476	Hyper-virulent community-acquired MSSA sequenced at Sanger Centre, UK	NARSA
SXZ01	8325-4 *rnc*::*kan*	[65]
SXZ02	8325 *artR*::*ermB*	This study
SXZ03	8325 *artR*::*ermB* pLIartR	This study
SXZ04	8325 *artR*::*ermB* pOS1sarT	This study
SXZ05	8325 *hfq*::*ermB*	This study
SXZ06	8325 *hfq*::*ermB* pLIHfq	This study
SX15	8325 *agr*::*ermB*	[66]
SX16	8325 *agr*::*ermB* pLIagr	[66]
SX17	8325 *RNA III*::*ermB*	[66]
SX18	8325 *RNA III*::*ermB* pLIRNA III	[66]
S. epidermidis		
RP62a	Slime positive and prototype biofilm producer strain	NARSA
E. coli		
DH5α	Clone host strain, *supE44* Δ*lacU169* (80d*lacZ* ΔM15) *hsdR17 recA1 endA1 gyrA96 thi-1 relA1*	Laboratory stock
BL21 (DE3)	F^{-} *ompThsd*S_B (r_B^- m_B^-) *gal dcm* (DE3)	Laboratory stock
Plasmids		
pEASY TB	Clone vector, Kan^r Ap^r	Transgen
pET28a (+)	Expression vector with hexahistidine tag, Kan^r	Novagen
pQE30	Expression vector with hexahistidine tag, Ap^r	QIAGEN
pEC1	pBluescript derivative. Source of *ermB* gene. Ap^r	[40]
pBT2	Shuttle vector, temp sensitive, Ap^r Cm^r	[40]
pLI50	Shuttle cloning vector, Ap^r Cm^r	Addgene
pSXZ02	pBT2 containing upstream and downstream fragments of *artR* and *ermB* gene, for ArtR mutagenesis, Ap^r Cm^r Em^r	This study
pSXZ03	pLI50 with *artR* and its promoter, Ap^r Cm^r	This study
pSXZ04	LacZ-sarT reporter	This study
pSXZ05	pEASY TB with *artR* fragment for in vitro transcription	This study
pSXZ06	pEASY TB with *RNA III* fragment for in vitro transcription	This study
pSXZ07	pEASY TB with *hla* fragment for in vitro transcription	This study
pSXZ08	pEASY TB with *sarT* fragment for in vitro transcription	This study
pSXZ09	pEASY TB with *sarT* 5′ UTR fragment for in vitro transcription	This study
pSXZ10	pEASY TB with *sarT* CDS fragment for in vitro transcription	This study
pSXZ11	pEASY TB with *sarT* 3′ UTR fragment for in vitro transcription	This study
pSXZ12	pEASY TB with 40 bp-deleted *sarT* fragment for in vitro transcription	This study

NARSA network on antimicrobial resistance in *Staphylococcus aureus*

to ArtR were chemically synthesized (Invitrogen). Hybridization and luminescence detection were performed using the North2South Chemiluminescent Hybridization and Detection Kit (Pierce). The size of the transcripts was estimated by comparing them with RNA molecular standards (Takara).

Western blot of the α-toxin and SarT

Western blot was performed as previously described [43]. For the α-toxin Western blot assay, stationary-phase supernatant was collected and concentrated by 10 kD ultra-filtration (Minipole). After heated for 10 min at 95 °C, the samples were separated by 12 % SDS-PAGE and then electrotransferred onto a polyvinylidene difluoride membrane (GE). The protein was detected by a rabbit anti-α-toxin antibody (Sigma) followed by horseradish peroxidase-conjugated sheep anti-rabbit antibodies (Pierce). For the SarT protein, bacterial cells at early log phase ($OD_{600} = 1.0$) was collected and lysed for 30 min at 37 °C by lysostaphin and then heated for 10 min at 95 °C as the sample for electrophoresis. Polyclonal antibody against SarT was acquired from Abgent Biotechnology (SuZhou, China), using peptide "PKWLAVYSEADSSKDHC" as the antigen.

Protein overexpression of AgrA DNA-binding domain, RNase III, and Hfq

The His-tagged AgrA DNA-binding domain was cloned and purified using standard procedures. The fragment of DNA-binding domain of *agrA* was amplified from *S. aureus* NCTC8325 genomic DNA, inserted into pET28a (+), and then transformed into *E. coli* BL21 (DE3). The strain was grown at 37 °C to an OD_{600} of 0.4; the temperature was reduced to 16 °C, and the cells were induced overnight with 1 mM IPTG. The method for Hfq expression was similar to AgrA. Expression of the RNase III was induced as described [44], and the sequence was cloned into pQE30 and then transformed into *E. coli* M15 (pREP-4). The strain was incubated at 37 °C for 4 h with 1 mM IPTG. The fusion proteins were purified by Ni–NTA resin, eluted, passed through an ultrafiltration system to remove imidazole, and then stored in 10 % glycerol at −80 °C until use. Protein purity and concentration were determined by SDS-PAGE and the Bradford assay.

RNA preparation and labeling

The RNAs were produced by in vitro transcription as described [27] using a RiboMAX Large Scale RNA Production Systems-T7 (Promega). Full-length labeled RNAs were produced using the Digoxigenin (DIG) RNA labeling Mix (Roche) or Biotin RNA labeling Mix (Roche). End-labeling RNAs were produced using a DIG gel-shift kit (second generation; Roche). The RNAs were purified from 6 % denaturing polyacrylamide gels and stored at −80 °C until use.

Gel-shift assay

The gel-shift assay between AgrA and the promoter DNA was performed as previously described [45–47]. The *artR* promoter was amplified from NCTC8325 genomic DNA and labeled with DIG by using a DIG gel-shift kit (Roche). The binding reaction was performed by incubating the labeled probe with increasing amounts of AgrA at 25 °C for 20 min. After incubation, the mixtures were electrophoresed in 4 % native polyacrylamide gel in a 0.5 × TBE buffer. The band shifts were detected and analyzed according to the manufacturer's instructions.

Determination of the ArtR complex with its target mRNAs

Gel-shift assays between ArtR and the mRNAs were performed as described [27]. The binding reactions were performed by mixing the labeled ArtR with increasing amounts of the target mRNAs (RNA III, *hla*, *sarT*, *sarT* 5′ UTR, *sarT* CDS, and *sarT* 3′ UTR) in TMN buffer (20 mM Tris–acetate at pH 7.5, 10 mM magnesium acetate, 150 mM sodium acetate, 1 mM DTT). The mixture was then denatured at 85 °C for 2 min, followed by refolding at 37 °C for 10 min. The mixture was added to RNA loading buffer and electrophoresed in 4 % native PAGE in 1 × TBE, electrotransferred and detected according to the manufacturer's instructions.

Cleavage assays of the RNA complex by RNase III

Cleavage of labeled RNA was performed as previously described [25, 44]. The DIG-labeled free RNA or the RNA–RNA complex was mixed in Tris-NaCl buffer (pH 8.5) containing 100 mM $MgCl_2$. Cleavage was carried out at 37 °C for 30 min with 2 pmol RNase III, and then the mixtures were added to RNA loading buffer, electrophoresed in 4 % native PAGE in 1 × TBE, electrotransferred and detected according to the manufacturer's instructions.

Statistical analysis

All quantitative data were analyzed using Student's *t* tests. $P < 0.05$ was considered to be statistically significant.

Results

Identification of *artR* locus

The discovery of ArtR is from our previous study on the function of *luxS* in *S. aureus* [45]. We found that deletion of the whole coding sequence of *luxS* led to the decrease in *hla* expression. However, the complementing plasmid of *luxS* can not restore the phenotype. Therefore, we constructed a series of complementing plasmids (Fig. 1a) and found that the sequence that was overlapped with the 3′

region of *luxS* can restore the phenotype (Fig. 1b), suggesting that a hypothetical functional gene may exist in the region. By RT-PCR, Northern blot, and RACE analysis, we determined the existence and the coding sequence of ArtR. In addition, we constructed the *luxS* single mutant, in which a 15-bp sequence in the *luxS* 5′ region was only deleted as shown in Fig. 1c. The transcription of *hla* in this mutant did not change (Fig. 1d), suggesting that ArtR, not LuxS, is the regulator of *hla*.

Northern blot, RACE, and bioinformatic analyses of ArtR

The existence of the *artR* gene was first verified in the *S. aureus* NCTC8325 by RT-PCR. In order to determine the 5′ and 3′ ends of *artR*, we performed primer extension and rapid amplification of the cDNA ends (RACE) analysis to confirm the full transcript of ArtR. Sequencing analysis indicated that the *artR* gene contains a 345-bp-long sequence located between the *luxS* gene and SAOHSC_02376, flanked by two ORFs (Fig. 2a). Furthermore, we confirmed the existence and length of ArtR by performing the Northern blot assays (Fig. 2b). Hence, an alignment was run between the closely related sequences in several *S. aureus* strains and the *Staphylococcus epidermidis* strain rp62a. As shown in Fig. S1, the 3′ region of *artR*, which overlapped with the *luxS* gene, was highly conserved in all strains, whereas the sequence in the 5′ region of *artR* and in the intergenic region was only conserved in *S. aureus* strains. Because the 5′ region is involved in transcription initiation, this alignment made us wonder whether or not the ArtR transcript exists in *S. epidermidis*. Therefore, RT-PCR was designed and performed on these eight strains according to our RACE

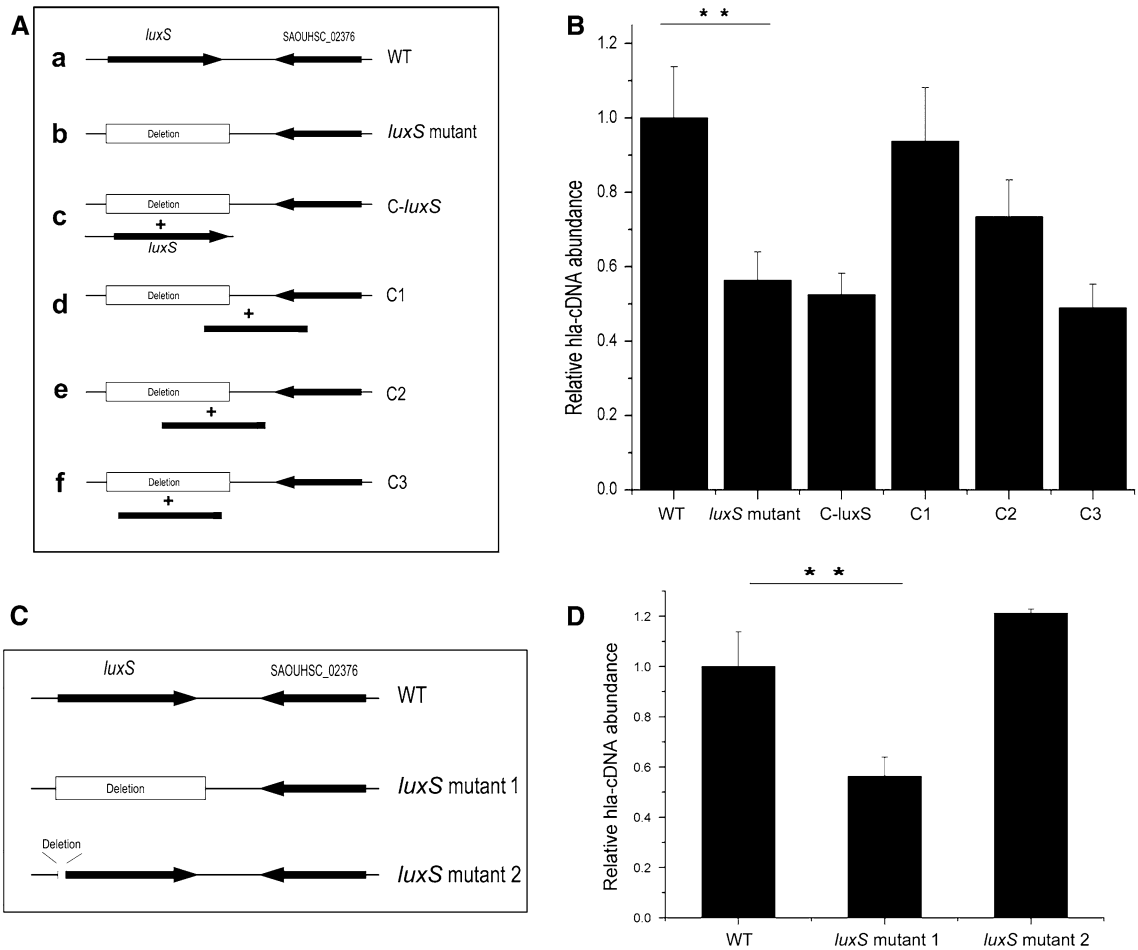

Fig. 1 Identification of the *artR* gene. **a** The maps of the chromosomal organization of the *artR*-surrounding region, the mutational region, and the complementary regions. (*a*) The map of the *artR*-surrounding region in the wild type (*WT*). (*b*) The map of the *artR*-surrounding region in the *luxS* mutant. (*c*) The map of the *artR*-surrounding region in the *luxS* complementary strain. (*d–f*) The maps of the *artR*-surrounding region in the different complementary strains (*C1*, *C2*, and *C3*). **b** RT-PCR analysis of the *hla* transcription in strain NCTC8325 and its corresponding strains. All of the strains were grown in LB medium to cell densities of OD_{600} of 2.0. **c** The maps of the chromosomal organization of the *luxS* region and its mutational regions. **d** RT-PCR analysis of the *hla* transcription in strain NCTC8325 and its *luxS* mutants. The results represented a mean of three independent experiments (**$P < 0.01$)

results, and the length of the PCR products was 330-bp long in the 345-bp ArtR-coding gene. As shown in Fig. 1c, the ArtR transcript was amplified in all *S. aureus* strains that we selected, but not in *S. epidermidis* rp62a. Considering that the *artR*-coding sequence in *S. epidermidis* is most closely related to that in *S. aureus* compared to the other Gram-positive strains, we conclude that ArtR might be a unique sRNA that only exists in *S. aureus*. In addition, we performed RT-PCR assays to test the transcription of *artR* in these staphylococcal strains, and our results showed that the transcript levels of *artR* in these strains were similar (Fig. S2).

AgrA can repress the transcription of *artR*

The Agr system is autoinduced by an extracellular ligand, AIP, which represents a sensor of population density. Activation of the sensor protein AgrC results in the phosphorylation of AgrA, the response regulator, which then binds to the *agr* promoter to initiate RNA II and RNA III transcription [48]. Since the Agr system in *S. aureus* NCTC8325 is generally acknowledged to be indispensable for the regulation of a great amount of genes, we performed RT-PCR assays to measure *artR* transcription in this strain and its *agr* mutant. As shown in Fig. 3a, the *artR* transcription level of the *agr* mutant was always higher than that of the wild type, indicating that Agr might be a repressor of *artR*. Because RNA III is the main effector molecule of the Agr system, we initially speculated that RNA III might regulate *artR* transcription. However, our RT-PCR results showed that inactivation of RNA III did not influence *artR* transcription (Fig. 3b). We also transcribed RNA III and ArtR in vitro and performed RNA–RNA gel-shift assays between them. As shown in Fig. 3c, RNA III easily bound to the 5′ UTR region of *hla* mRNA and this result was consistent with previous work [24]. However, we did not observe the formation of the RNA III–ArtR complex at all. With these in mind, we concluded that the Agr system repressed *artR* transcription through a RNA III-independent pathway. Previous study verified that purified AgrA shows high-affinity binding to the RNA III-*agrCDAB* intergenic region [46]. By DNase I protection assays, they determined the specific binding sequences of AgrA in the P2 and P3 promoter regions of the *agr* locus (Fig. 3d). And, as shown in Fig. 3e, we also found a highly conserved AgrA-binding site that was near the −35 region of the *artR* promoter. According to this, we over-expressed the DNA-binding domain of the AgrA protein and performed gel-shift assays. The results confirmed that AgrA also exhibited high-affinity binding to the *artR* promoter (Fig. 3f), demonstrating that the Agr system represses *artR* transcription through AgrA, but not RNA III.

ArtR activates α-toxin expression through an indirect pathway

Previous studies have indicated that sRNA can mediate virulence regulation in some pathogens [18], and our results also showed that ArtR might be involved in the regulation of the α-toxin expression. As shown in Fig. 4a, the transcript level of *hla* in the *artR* mutant decreased 60 % compare with that in the wild type when the bacteria grew to the post-exponential phase. We also measured the

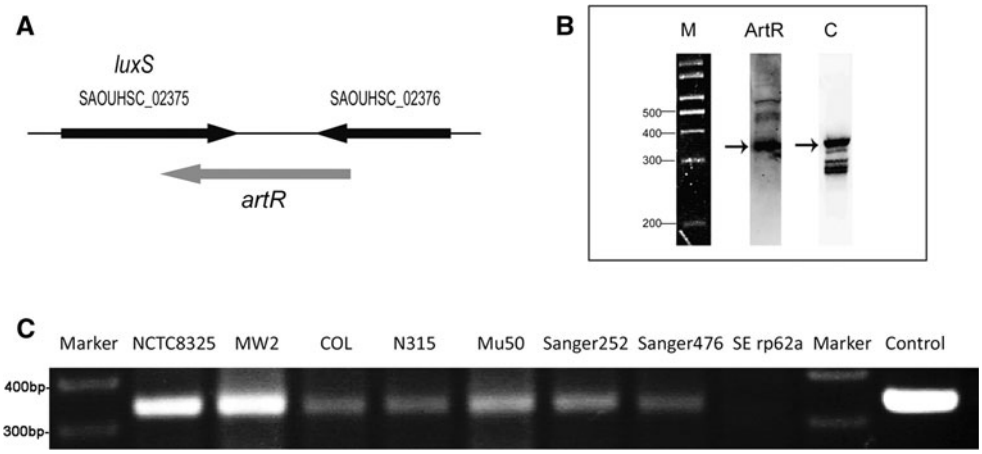

Fig. 2 Bioinformatics identification of the *artR* gene. **a** Chromosomal organization of the *artR* gene and its surrounding region. The encoding sequence of *artR* is overlapped by *luxS* (SAOUSHC_02375) in reverse. The 5′ end of this sequence is overlapped by the 3′ end of the SAOUSHC_02376 gene, and 153 bp of the 3′ end is overlapped by the 3′ end of *luxS*, while the rest is located in the intergenic regions between SAOUSHC_02376 and *luxS*. **b** Northern blot assays of ArtR in *S. aureus* strain NCTC8325. In vitro transcribed ArtR was used as positive control. **c** RT-PCR analysis of the ArtR transcript. RT-PCR was designed and performed according to the RACE results, and the length of the PCR product was 345 bp

transcription of *hla* in a different growth phase in these strains and the results were consistent with the above data. Western blot assays showed that, when the growth of the cells became stationary, the expression of α-toxin also decreased in the *artR* mutant (Fig. 4b), further demonstrating that ArtR is an activator of the α-toxin expression. According to previous studies, ArtR might regulate *hla* expression through two possible pathways: a direct interaction with *hla* mRNA or an indirect interaction through some intermediates. By using bioinformatics methods, we aligned the sequences of ArtR and *hla* mRNA and tried to explore whether or not ArtR could bind to *hla* mRNA as RNA III does. According to the alignments, there are several possible binding sites with low energy in the 5′ UTR region of *hla* mRNA. Therefore, we performed the RNA–RNA binding experiment between ArtR and the 5′ UTR of *hla* mRNA and the *hla* full-length mRNA. As shown in Fig. 4c, no binding complex was observed between ArtR and *hla* mRNA or its 5′ UTR, suggesting that ArtR may activate the *hla* transcription through an indirect pathway.

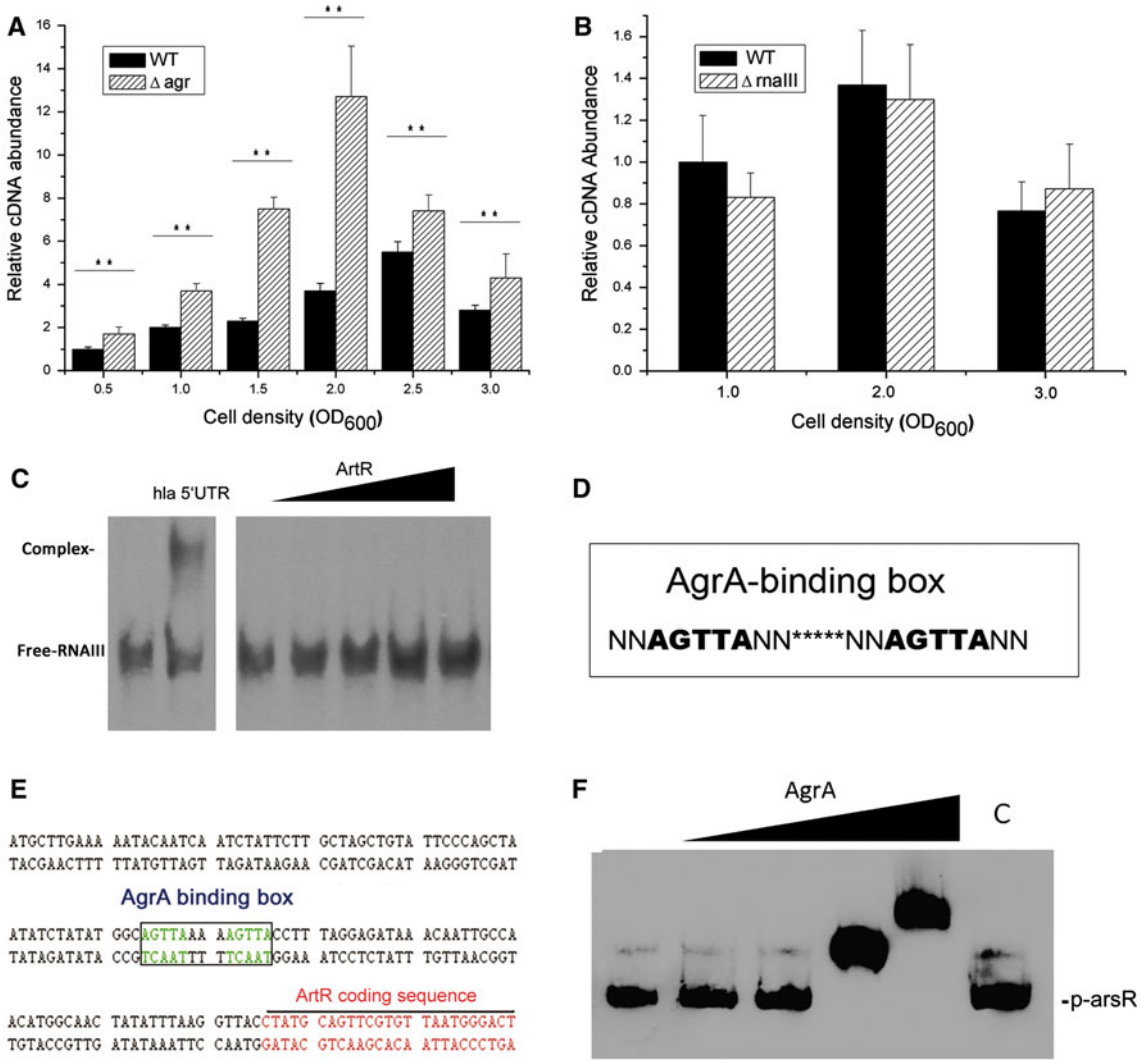

Fig. 3 Influence of the Agr system on *artR* transcription. **a**, **b** Comparative measurements of *artR* transcripts by RT-PCR in *S. aureus* NCTC8325 (WT), the *agr* mutant (Δagr), and the *RNA III* mutant (ΔRNA III). All of the strains were grown in LB medium to corresponding cell densities. **c** The binding ability of RNA III to ArtR was determined by RNA–RNA gel-shift assays. The RNA III and *hla* transcript 5′ UTR complex was used as a positive control. DIG-labeled RNA III probes (100 fmol) were used in all reactions. Increasing amounts of ArtR (0, 225, 450, 900, and 1,800 fmol) were incubated with excess DIG-labeled RNA III probes. **d** Precise binding box of the AgrA protein. **e** Sequence analysis of the promoter region of *artR*. **f** The binding ability of the AgrA DNA-binding domain to the *artR* promoter was determined by gel-shift assays. Increasing amounts of the AgrA DNA-binding domain were incubated with excessive DIG-labeled *artR* promoters. In each panel, from Lanes 1 to 6, the amounts of the AgrA DNA-binding domain were 0, 0.5, 1, 2, 4 and 4 pmol, respectively; there were 50 fmol of all of the DIG-labeled probes. In Lane 6, except for the labeled probes, 1 pmol of unlabeled probes was added as the competitive control. The results represented a mean of three independent experiments (**$P < 0.01$)

ArtR can activate *hla* expression through *sarT* by direct binding to *sarT* mRNA

Since there are many regulators that can modulate *hla* expression, we measured the transcript levels of the regulators *agrA*, *sarA*, *sae*, *rot*, *arl*, *clpP,* and *sarT* [49–52] by RT-PCR. Our results showed that only the transcription of *sarT* was changed in the *artR* mutant compared to that of the wild type. As shown in Fig. 5a, the transcript level of *sarT* was 1.85-fold higher in the *artR* mutant than that in the wild type. Moreover, we compared the protein levels of SarT in the *artR* mutant and the wild type by Western blot assays. The results confirmed that the SarT expression level is higher in *artR* mutant than that in the wild type (Fig. 5b), indicating that SarT might be the mediator linking ArtR regulation to *hla* expression. According to the alignments, we assumed that there is a 40-nt-long possible binding site of ArtR with low energy in the 5′ UTR region of *sarT* mRNA (Fig. 5c). Therefore, we carried out RNA–RNA gel-shift assay between ArtR and the *sarT* mRNA or the different parts of the *sarT* mRNA (5′ UTR, CDS, and 3′ UTR) to investigate whether ArtR directly regulates *sarT* or not. The *luxS* mRNA was chosen for the positive control because there is perfect pairing between *luxS* and *artR*. As shown in Fig. 5d, the *sarT* mRNA or the 5′ UTR of *sarT* mRNA can bind to ArtR, suggesting that ArtR may affect the stability of the *sarT* mRNA through a direct interaction. By using the mutational *sarT* mRNA (*sarT* mRNA with 40-nt-long binding sequence deletion) as the negative control, we further confirmed that the 40-nt-long binding sequence in the 5′ UTR of *sarT* mRNA is essential for the interaction of ArtR with *sarT* mRNA (Fig. S3).

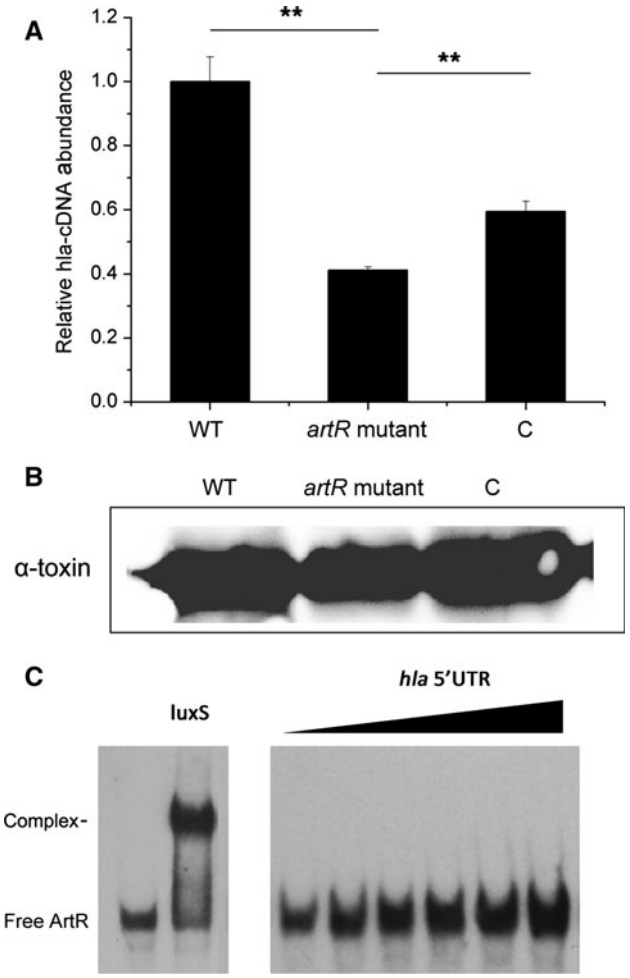

Fig. 4 Regulatory effect of ArtR on *hla* expression. **a** Comparative measurements of the *hla* transcripts by RT-PCR in *S. aureus* NCTC8325 (*WT*), the *artR* mutant, and the *artR* mutant with the complementing plasmid (**c**). All of the strains were grown in LB medium to an OD_{600} of 2.0. **b** Western blot analysis of Hla in *S. aureus* NCTC8325 (*WT*), the *artR* mutant, and the *artR* mutant with the complementing plasmid (**c**). All of the strains were grown in LB medium to an OD_{600} of 5.0. (**c**) The binding ability of ArtR to *hla* mRNA was determined by RNA–RNA gel-shift assays. The ArtR and *luxS* mRNA complex was used as a positive control. There were 180 fmol of all of the DIG-labeled ArtR probes. Increasing amounts of the 5′ UTR of *hla* (0, 0.5, 1, 2, 4, and 8 pmol) were incubated with excessive DIG-labeled ArtR probes. The results represented a mean of three independent experiments (**$P < 0.01$)

ArtR promotes the degradation of *sarT* mRNA by RNase III

As declared before, RNase III contributes a lot to the regulation of sRNA in *S. aureus* [25–27, 53]; thus, we also investigated the influence of RNase III on ArtR-*sarT* regulation. To provide the direct evidence that RNase III is involved in *sarT* regulation by ArtR, a digestion experiment was carried out. As shown in Fig. 6, with the increasing addition of ArtR, a greater digestion of the *sarT* 5′ UTR was observed. These results indicate that ArtR can form a stable complex with the 5′ UTR of *sarT* mRNA and decrease the level of *sarT* mRNA through RNase III-mediated degradation.

Hfq does not bind to ArtR in vitro and is not involved in the stability of ArtR

According to previous studies, Hfq is an RNA binding protein that interacts with both sRNAs and mRNAs to post-transcriptionally modulate gene expression. In *E. coli*, Hfq can affect the stability of several sRNAs and mRNAs [54] and target them for degradation by increasing polyadenylation [55, 56] or interfering with ribosome binding and translation [57]. In *S. aureus*, whether or not Hfq can function as a sRNA chaperone still remains disputable. In order to answer this question, we constructed the *hfq* mutant and measured the transcript level of *artR* by RT-PCR. As shown in Fig. S4A, the transcript level of *artR* in the *hfq* mutant was similar to that in the wild type throughout the whole growth phase. Our protein-RNA gel-shift assays also

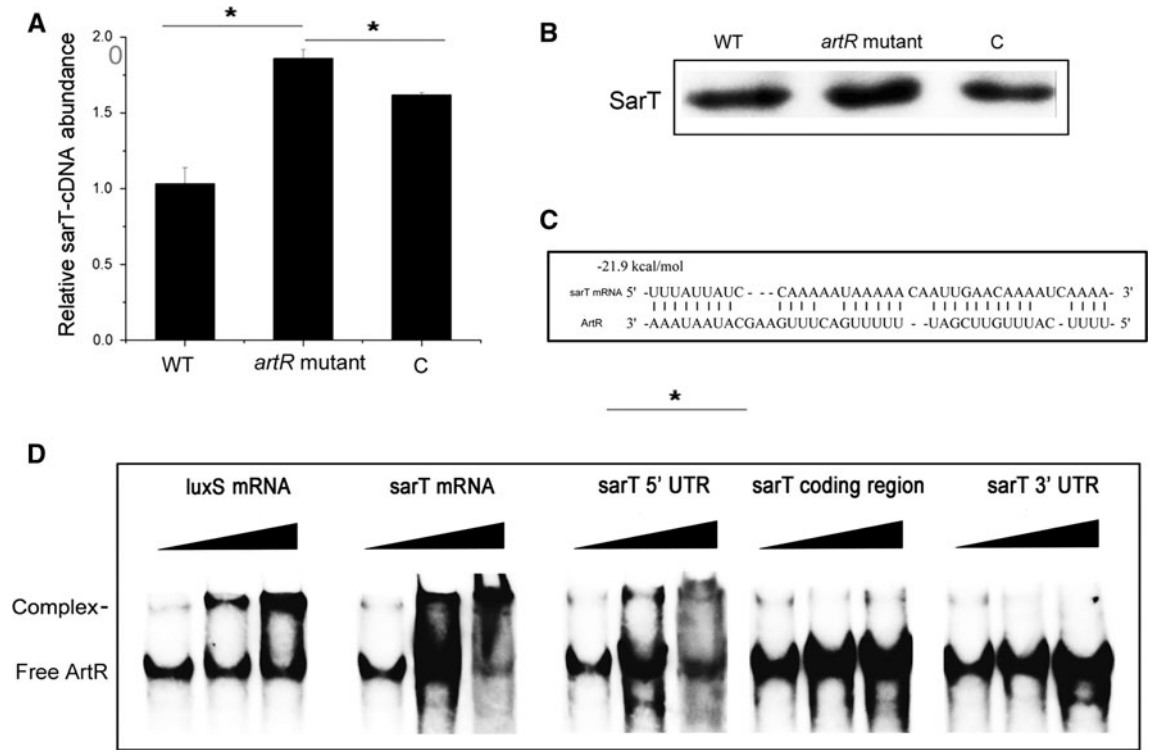

Fig. 5 The regulation of SarT by ArtR involves a direct interaction between ArtR and the *sarT* mRNA. **a** Comparative measurements of *sarT* transcripts by RT-PCR in *S. aureus* NCTC8325 (*WT*), the *artR* mutant, and the *artR* mutant with the complementing plasmid (*C*). All of the strains were grown in LB medium to an OD_{600} of 2.0. **b** Western blot analysis of SarT in *S. aureus* NCTC8325 (*WT*), the *artR* mutant, and the *artR* mutant with the complementing plasmid (*C*). **c** Possible binding sites with low energy between ArtR and the 5′ UTR region of *sarT* mRNA. Secondary structure and minimum free energy between the two RNAs was calculated by RNA hybrid (http://bibiserv.techfak.uni-bielefeld.de/rnahybrid/webservice.html). **d** The binding ability of ArtR to *sarT* mRNA or the different part of *sarT* mRNA was determined by RNA–RNA gel-shift assays. The ArtR and *luxS* mRNA complex was used as a positive control. DIG-labeled ArtR probes (180 fmol) were used in all reactions. Increasing amounts of *sarT* mRNA (0, 0.9, and 3.6 pmol), 5′ UTR (0, 0.45, and 1.8 pmol), CDS (0, 0.45, and 1.8 pmol) and 3′ UTR (0, 0.9, and 3.6 pmol) were incubated with excess DIG-labeled ArtR probes, respectively. The results represented a mean of three independent experiments (*$P < 0.05$)

confirmed that Hfq could not bind to the ArtR transcript in vitro (Fig. S4B). These data indicate that Hfq does not interact with ArtR or affect its stability. In addition, we measured the transcript levels of *sarT* and *hla*, which might be the target mRNAs of ArtR. The results showed that the inactivation of *hfq* did not affect the transcription of the two genes either (Fig. S5), indicating that Hfq might not be required for ArtR-dependent regulation.

Discussion

In recent years, an increasing amount of research has focused on bacterial sRNAs, which are emerging as regulators that enable bacterial pathogens to express virulence factors when required during infection. Roles of numerous sRNAs in the pathogenesis have been identified in many Gram-positive and Gram-negative bacteria. However, the progress of sRNA research in *S. aureus* is relatively slow. A detailed functional analysis has only been performed for four sRNAs: RNA III, SprD, SprA1, and RsaE. In this work, we identified a novel sRNA, ArtR. The 5′ end of *artR* sequence is overlapped with the 3′ end of the SAOUSHC_02376 gene, and 153 bp of the 3′ end is overlapped with the 3′ end of *luxS*, while the rest is located in the intergenic regions between SAOUSHC_02376 and *luxS*. Because of the limitations in the methodology, this kind of genetic constitution of sRNA has not been recognized before in *S. aureus*. Besides this, bioinformatic analysis indicated that the coding sequence of ArtR is extremely conserved among *S. aureus*. RT-PCR data showed that there were obvious ArtR transcripts in seven *S. aureus* strains (Fig. 2c), whereas no ArtR transcript was observed in *S. epidermidis*. Furthermore, considering that the virulence factor α-toxin, which is regulated by ArtR, does not exist in *S. epidermidis*, we suggest that ArtR might be a virulence-associated regulatory sRNA particular to *S. aureus*.

In *S. aureus*, RNA III is the model of RNA-controlled virulence gene expression, being the effector of the Agr

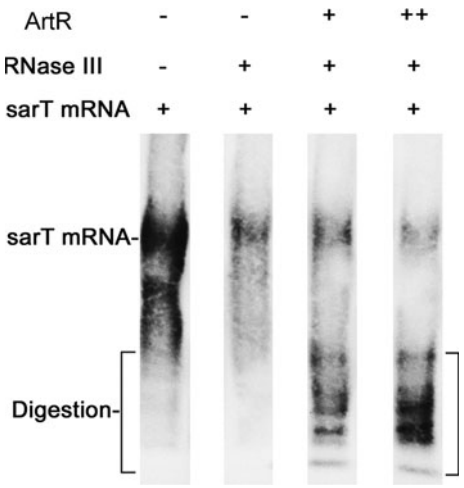

Fig. 6 Regulatory effect of ArtR on *sarT* expression. DIG-labeled 5′ UTR of *sarT* mRNA (90 fmol) was digested by RNase III (2 pmol) with increasing amount of ArtR (0, 1.35, and 2.7 pmol)

system. When bacteria grow to the post-exponential phase, phosphorylation-activated AgrA can bind to the promoter region of *RNA III* and activate its transcription. A previous study reported that phenol-soluble modulins expression was regulated by direct binding of the AgrA, suggesting that AgrA may play a more important role in virulence regulation by binding to other potential targets except RNA III [58]. Interestingly, we found that there is also a highly conserved AgrA-binding box in the promoter region of *artR* by sequence alignment. Our gel-shift data confirmed the high-affinity binding of AgrA to the promoter region of *artR*. Apart from that, the RT-PCR data showed that inactivation of Agr resulted in an apparent increase in the transcript level of *artR*. These results suggest that, after RNA III, ArtR is the second sRNA that can be regulated by the transcriptional regulator AgrA. However, unlike RNA III, AgrA always repressed the transcription of *artR* regardless of any bacterial growth phase under our experimental conditions (Fig. 3a). We also performed gel-shift experiments by adding acetyl phosphate to AgrA, and the results indicated that exogenous acetyl phosphate did not influence the binding ability of AgrA to the *artR* promoter (data not shown). Moreover, as shown in Fig. 3e, the binding box of AgrA on the *artR* promoter is adjacent to the −35 region, suggesting that this binding may cause the interference of transcription initiation.

As a SarA homolog, the transcriptional regulator SarT can repress *hla* expression [51], and this gene exists in *S. aureus* strains COL, RN6390, Newman, and DB and *S. saprophyticus*, but not in *S. epidermidis* or *S. haemolyticus* [59]. Our gel-shift assays indicated that the SarT protein exhibits high-affinity binding to the *hla* promoter region in vitro (Fig. S6). In this study, we show that ArtR can repress α-toxin expression by binding to the *sarT* mRNA and promoting its degradation. The regulatory behavior of ArtR on SarT is very similar to that of RNA III on Rot [26, 60], suggesting that the regulation of the transcription factor by regulatory sRNAs via an antisense mechanism is ubiquitous in bacteria. In addition, as reported before, the regulation by sRNA may enable fine-tuning of target gene expression level [61, 62], implying that in a complex regulatory network, the influence of the sRNA on target gene expression may be limited.

The Hfq protein has generally been recognized as the small RNA chaperone in Gram-negative bacteria. The Hfq protein of *E. coli* has been directly linked to the action of several small regulatory RNAs that use base pairing to regulate the expression of target mRNAs [63, 64]. In *S. aureus*, whether or not Sm-like Hfq can function as a small RNA chaperone still remains unknown. In a previous study using gel-shift experiments, *S. aureus* Hfq was shown to bind to RNA III with a higher affinity than the regulatory region of the *spa* mRNA. However, the addition of increasing concentrations of Hfq had no significant effect on the formation of the RNA III-spa mRNA complex [25]. Despite the fact that Hfq binds to RNA III, the observation that the deletion of *hfq* does not exhibit severe phenotypic defects rules out the possibility of direct involvement of Hfq in regard to RNA III-mediated regulation in *S. aureus* [25]. Another previous work reported that SprA1 steady-state levels were unaffected by the presence or absence of Hfq in vivo, suggesting that the protein is dispensable for the interaction between $SprA1_{AS}$ and SprA1 [37]. Our study showed that the inactivation of *hfq* did not affect the stability of ArtR (Fig. S4) or the transcription of *sarT* and *hla* (Fig. S5). The RNA–RNA gel-shift experiments also showed no binding effects between Hfq and ArtR. These findings led us to suggest that the *S. aureus* Hfq protein may not be required for small RNA-dependent regulation.

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