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SsrA (tmRNA) acts as an antisense RNA to regulate *Staphylococcus aureus* pigment synthesis by base pairing with *crtMN* mRNA

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ABSTRACT

SsrA RNA (small stable RNA A), also known as tmRNA and 10Sa RNA, functions both as tRNA and mRNA through its unique structure. The carotenoid pigment is the eponymous feature of human pathogen *Staphylococcus aureus*. Here, we found that the pigment of the mutant strain with ssrA deletion was increased. Furthermore, it was demonstrated that ssrA could act as an antisense RNA aside from its well-known biological function, and *crtMN*, encoding two essential enzymes for the pigment synthesis, was identified as target of ssrA. Further investigation showed ssrA could specifically base pair with the RBS (ribosomal binding site) region of the *crtMN* mRNA. Our results revealed a new mechanism by which ssrA regulated the biosynthesis of pigment in *S. aureus*.

1. Introduction

Staphylococcus aureus is a major human pathogen that can cause many diseases [1]. Its carotenoid pigment is an eponymous feature of *S. aureus*, which is a virulence factor and potential novel target for antimicrobial therapy [2]. The biosynthetic pathway for *S. aureus* carotenoids includes *crtM* and *crtN* genes, which encode dehydrosqualene synthase and dehydrosqualene desaturase, respectively [3]. The *crtM* mutant strain has no golden pigments [2].

SsrA RNA (small stable RNA A), also known as tmRNA and 10Sa RNA, is the central player of a unique quality control system that deals with ribosomes after they are stalled on mRNAs without stop codons [4]. SsrA RNA functions both as transfer RNA and messenger RNA through its unique structure [5]. ssrA acts initially as tRNA, being animoacylated at its 3′ end by alanyl-tRNA synthetase, to add alanine to the stalled polypeptide chain. Then the stalled peptide is added with an 11-amino acids tail encoded by the reading frame of ssrA. The 11-amino acids tag is specifically recognized and degraded by some the energy dependent proteases ClpXP, ClpAP, FtsH and Tsp [6–8]. It is known that SmpB (small protein B) can bind ssrA and is involved in ssrA biological function in *Escherichia coli* [5,9]. The length of bacterial ssrA ranges from ~260 to ~430 nucleotides [10]. It is reported that ssrA is important for the survival, growth under stress and pathogenesis of the different bacteria [11]. However, there are no reports on delicate mechanisms of ssrA functions in *S. aureus.*

There are some regulatory non-coding RNAs encoded in prokaryotic genomes. Most of our present knowledge on regulatory sRNAs derives from detailed studies of some of the ~70 sRNAs identified in *E. coli* [12,13], which can regulate genes expression at the post-transcriptional level by base pairing with complementary sequences in target mRNAs [13]. RNAIII is a well studied regulatory sRNA in *S. aureus*, which can regulate the expression of several mRNA targets at the translational and/or transcriptional level [14–16]. Some additional sRNAs have been indentified in *S. aureus* in recent work [17–20].

In the present study, we tried to investigate the biological function of ssrA in *S. aureus*. Here, we demonstrated that the ssrA could act as an antisense RNA besides its known function and inhibit the synthesis of *S. aureus* pigments by regulating the expression of *crtMN*.

2. Materials and methods

2.1. Bacterial strains and growth conditions

The strains used in this study are listed in Table 1. *S. aureus* strains were routinely grown in BHI and *E. coli* strains were grown

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Table 1
Bacterial strains and plasmids.

Strain or plasmid	Comments	Source or reference
Strain		
S. aureus		
8325-4	Wild-type	[31]
ΔssrA	8325-4 with a ssrA::kan mutation	This study
ΔsmpB	8325-4 with a smpB::kan mutation	This study
ssrAr	the restoration of ssrA activity in *ΔssrA*	This study
ssrAms	the restoration of tag-mutant ssrA in *ΔssrA*	This study
E. coli		
DH5α	A host strain for cloning	Transgene
Plasmids		
pOS1	*E. coli–S. aureus* shuttle vector	[32]
pOS1-lacZ	pOS1 contains a copy of lacZ encoding β-galactosidase without promoter and 5′UTR	This study
pOS1-Ucrt-lacZ	UTR of crt-lacZ fusion(Ucrt::lacZ) shuttle vector, a derivative of pOS1	This study
pOS1-mUcrt-lacZ	Mutant of UTR of crt-lacZ fusion(Ucrt::lacZ) shuttle vector, a derivative of pOS1	This study

in LB medium either with no antibiotics, or with 20 μg ml^{-1} erythromycin, 100 μg ml^{-1} ampicillin, 20 μg ml^{-1} chloromycetin and 100 μg ml^{-1} kanamycin, respectively.

2.2. Construction of ssrA deletion mutant (*ΔssrA*) and smpB inactivation mutant (*ΔsmpB*)

The mutant was constructed using the method described previously [21] with some modifications as follows: two regions of DNA flanking the *ssrA* gene were amplified using the primers (ssrA1/2; ssrA3/4) and two regions flanking the *smpB* gene were amplified using the primers (smpB1/2; smpB3/4) listed in Table 2.

2.3. Oxidant susceptibility assay

The assay to test susceptibility to oxidants was performed as previously described [2].

2.4. Neutrophil intracellular survival assay

Neutrophils for intracellular survival assays were purified from healthy human volunteers as previously described [2].

Table 2
Sequences of forward and reverse primers used in this study.

Primer/ sequence	Oligonucleotide sequence (5′–3′)
R1	5′ACAGCAUUUCCUAUG 3′
16S F	GCCTAATACATGCAAGT
16S R	CATGTTATCCGGCATTAG
crtM F	GACTTGGTGAATCGTTGC
crtM R	CTATGATTGGTGATGCTTC
ssrA1	CATCCGGAATTCATAAAAGTGT GAAAAA
ssrA2	CGTCCGGTACCTCCATGAACGTCCCCGAAAT
ssrA3	ATGGAGGTACCGGACGCGGGTTCAAATCCCG
ssrA4	ACACGCGTCGACGAAGTAGTGTAGTTGC
smpB1	CATCCGGAAT TCATGGCTAA GAAGAAATCA
smpB2	CCAATCTCACGTGTTTGGGTACCCAATTTAATGATTTCAC
smpB3	GTGAAATCATTAAATTGGGTACCCAAACACGTGAGATTGG
smpB4	ACACGCGTCGACaTAACGGGCTT TCATATCGC
UcrtF	GACTACGTGAATTCGCGAGTATTTTAGGG
UcrtR	CATGCGTAGGATCCCCCATATCCATCATTGTCAT
mUcrtR	ATCATCGCGG ATCCCCCATA TCCATCATTG TCATTATTTT ATCTTCTTGT TGAAATGCC
ssrArF	GATATGCATGAATTC TTCGTTCTCG AAAATTATTA
ssrArR	ATCATCGCGG ATCCTGGAGA CGGCGGGATT TGAAC
ssrAmsF	AATTAAGCAG TAGATGACTA ATCGCAC
ssrAmsR	CTGCTTAATT ATTGTTTGAT TAGCCAGTTA TT

2.5. Construction of lacZ reporter vector

The Ucrt and mUcrt regions spanning nucleotides −300 to +18 of the *S. aureus* 8325-4 *crtM* operon were amplified by PCR from *S. aureus* 8325-4 chromosomal DNA with primers UcrtF/UcrtR and UcrtF/mUcrtR (Table 2), respectively. The PCR products were ligated into pOS1-lacZ plasmid DNA, resulting in the Ucrt-lacZ and mUcrt-lacZ fusions (Fig. 2a).

2.6. Real-time quantitative PCR (qPCR)

Total bacterial RNA was extracted from *S. aureus* using Trizol (invitrogen) and qPCR was performed as previously described [22]. The specific primers were listed in Table 2.

2.7. Preparation of RNA substrates

RNA was transcripted in vitro using SP6 RNA polymerase (TAKARA) and purified by denaturing gel electrophoresis according to standard methods.

2.8. Equilibrium gel mobility-shift assays (EMSA)

The EMSA was performed as described previously [23] with some modifications. 1 nM biotinylated R1 and 0 or 5 nM unlabeled 5′ UTR of *crtMN* mRNA were incubated for 20 min at 37 °C. All samples were electrophoretically transferred to nylon membranes and he bands were quantified using a LightShift® Chemiluminescent EMSA Kit (Pierce).

2.9. Statistical analysis

All quantitative data were analyzed using Student *t*-tests. $P < 0.05$ was considered to be statistically significant.

3. Results

3.1. Mutagenesis of ssrA up-regulated the expression of S. aureus pigments

SsrA is highly conserved throughout Eubacteria [24]. The *ΔssrA* was generated by allelic replacement of *ssrA* in *S. aureus* 8325-4. Interestingly, it was found that the pigment of the *ΔssrA* displayed high intensities of golden pigments compared with its parent strain (Fig. 1a) and showed the characteristic spectral profile of carotenoid (Fig. 1b). At the same time, the full length gene of *ssrA* containing its promoter was amplified and cloned into the pOS1 vector to generate plasmid pOS1-ssrA and the plasmid was transferred into the *ΔssrA* to restore the level of ssrA, named as *ssrAr* (Table 1). It was found that the pigment of the *ssrAr* was recovered as the wild type (Fig. 1a and b).

As is known, the SmpB protein is essential for ssrA [9]. So we constructed a *smpB* mutant strain (*ΔsmpB*) to test whether the SmpB was involved in the process of pigment change. Unexpectedly, the color of the *ΔsmpB* was the same as the wild type rather than *ΔssrA* (Fig. 1a and b). These results suggested that SmpB was not necessary for ssrA to regulate the expression of the pigment in *S. aureus,* and ssrA might regulate the pigment by another mechanism which was different from the known way. In order to furtherly test if ssrA regulated the pigment synthesis through being as the tmRNA, we transferred the mutated ssrA with the mutated tag sequence to the *ΔssrA*, named as *ssrAms* (Fig. 1c). Several nucleotides of the tag sequence were replaced and there were two stop codons in the mutated tag sequence. At the same time, the last two alanine residues were replaced with aspartate. Those changes could inhibit

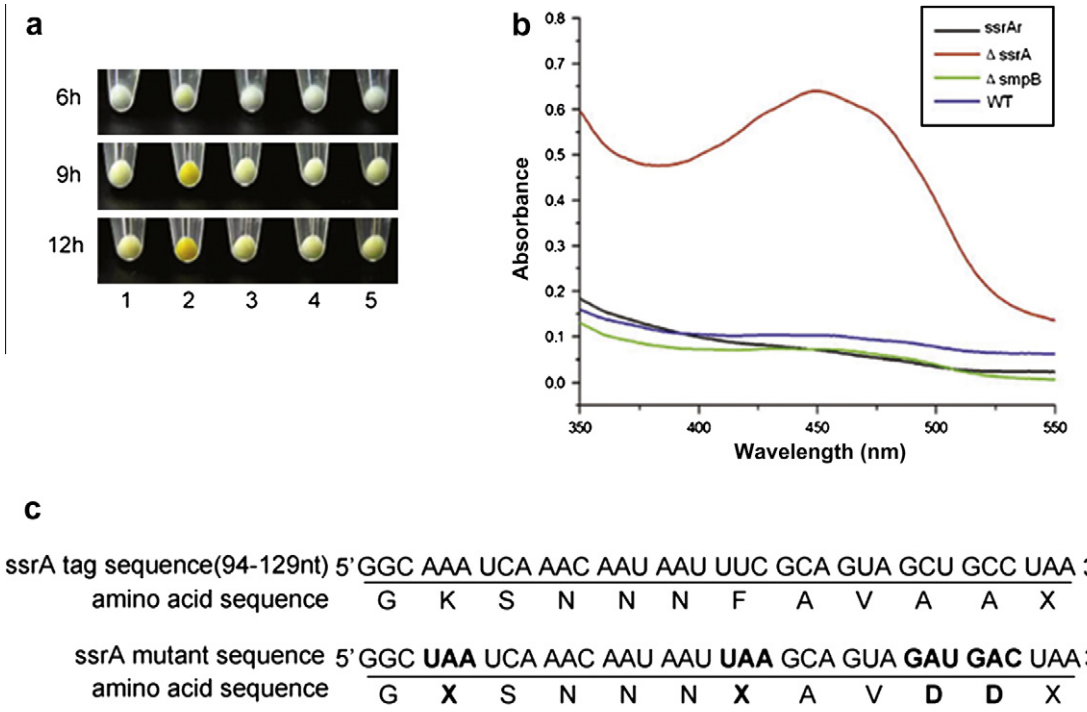

Fig. 1. The pigment of Δ*ssrA* was increased. (a) After the different strains were cultured for 6, 9 and 12 h, cells were collected and the pigment of different strain was tested. 1, WT (8325-4); 2, *ΔssrA*; 3, *ssrAr*; 4, *ssrAms*; 5, *ΔsmpB*. (b) *S. aureus* cells were cultured for 12 h, then subjected to methanol extraction. The absorbance profile of the extracts was measured. (c) Schematic diagram of construction of the mutated tag sequence of ssrA. X means stop codon.

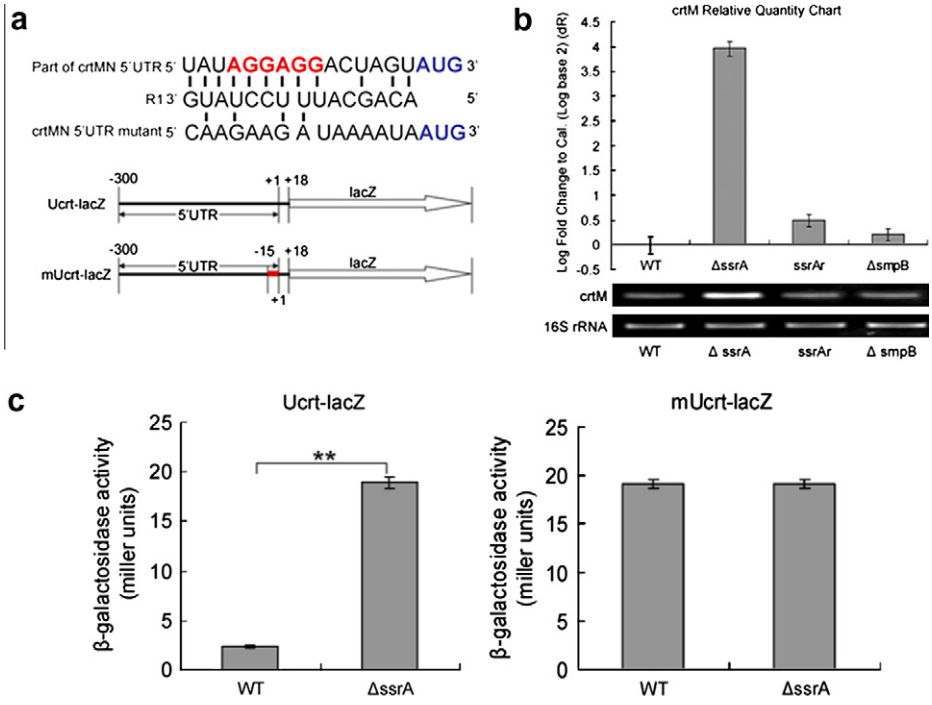

Fig. 2. *ssrA* could regulate the expression of *crtMN*. (a) Sequence alignment and schematic diagram of construction of reporter vectors. The sequence similarities between ssrA and 5′UTR of *crtMN* mRNA was analyzed. At the same time, some nucleotides in 5′UTR of *crtMN* mRNA were replaced to disrupt the formation of duplex between ssrA and *crtMN* mRNA. (The red bold colored fragment was the RBS region and the blue bold fragment referred to the start codon). Then the fragment containing 5′UTR and coding sequence of the first six amino acids of CrtM was fused with lacZ to generate Ucrt-lacZ. The mutant fragment was fused with lacZ to generate mUcrt-lacZ. (b) The expression of *crtM* in the different strains was tested by RT-PCR. (c) Determination of the β-galactosidase activities of Ucrt-lacZ and mUcrt-lacZ reporters in the *ΔssrA* and WT (8325-4) strain. The results represented a mean of three independent experiments ($^{**}P < 0.01$).

the protein degradation by the tag encoded by ssrA. Our data showed that the pigment of *ssrAms* was recovered as the *ssrAr* (Fig. 1a). This result suggested that the regulation on the synthesis of pigment by ssrA is independent on the tmRNA function.

Table 3
ΔssrA was more resistant to oxidant and neutrophil killing.

Strains	Assays		
	Surviving CFU		
	Singlet oxygen ($\times 10^2$)	Hydrogen peroxide ($\times 10^5$)	Human neutrophil killing assay ($\times 10^2$)
WT	6.47 ± 0.09	4.94 ± 0.46	11.67 ± 0.60
ΔssrA	31.68 ± 0.67**	13.24 ± 0.08**	61.20 ± 0.61**
ssrAr	8.06 ± 0.13	7.04 ± 0.21	7.25 ± 0.25
ΔsmpB	6.68 ± 0.18	4.37 ± 0.26	5.18 ± 0.27

The results represented a mean of three independent experiments. The significant difference between the *ΔssrA* and wild type was observed.
** $P < 0.01$.

Phagocytic cell can eliminate pathogens through the release of reactive oxygen species generated by nicotinamide adenine dinucleotide phosphate (NADPH) oxidase [25]. It was reported that *S. aureus* pigments could act as antioxidants against host defense [2]. Our data showed that the *ΔssrA* was more resistant to both hydrogen peroxide and singlet oxygen compared to the wild type (Table 3). In the further investigation, it showed that *ΔssrA* was more resistant to the neutrophil killing than its parent strains (Table 3). However, *ΔsmpB* and *ssrAr* were as sensitive as the wild type (Table 3). These results were in accordance with the intense orange color of the *ΔssrA* strain and also suggested that SmpB was not involved in the process of pigment change.

3.2. The expression level of crtMN was increased in the ΔssrA

CrtM and CrtN, which are transcribed by the same promoter in the genome of *S. aureus* 8325-4, are two key enzymes of the pigment synthesis in *S. aureus*. So we checked if ssrA could regulate the expression level of *crtM* and *crtN*. The bacteria were cultured for 12 h and the total RNAs were extracted. The expression level of *crtM* was tested by RT-PCR. Compared with the wild type strain, the expression levels of *crtM* was increased in the *ΔssrA* and recovered in the *ssrAr*. And there was no significant difference observed between *ΔsmpB*, *ssrAms* and the wild type strain (Fig. 2b).

In the further investigation, the 5′UTR of *crtMN* mRNA was fused with lacZ to generate the reporter vector of Ucrt-lacZ (Fig. 2a) and was transferred to the *ΔssrA* and the wild type. The results of -galactosidase activities showed that the expression of Ucrt-lacZ was significantly increased in the *ΔssrA* (Fig. 2c).

3.3. ssrA could act as antisense RNA to directly bind the 5′UTR of crtMN

As the SmpB protein was not involved in this process, we wondered if ssrA could act as a regulatory RNA to modify mRNA stability or translation by pairing with its targets. By sequence analysis, we found that the region R1 (nucleotides 133–147) of ssrA was complementary to the RBS sequence of *crtMN* (Fig. 2a). Then the biotinylated R1 was synthesized and incubated with 5′UTR of *crtMN* mRNA. The results of EMSA showed that R1 could specifically bind the 5′UTR of *crtMN* mRNA (Fig. 3). These results suggested that ssrA could act as an antisense RNA to pair with *crtMN* mRNA. In the further investigation, the mutated 5′UTR of *crtMN* was fused with lacZ (termed as mUcrt-lacZ) according to the paring sequence between ssrA and *crtMN* 5'UTR (Fig. 2a) to test if ssrA can interact with 5′UTR of *crtMN* directly. Several nucleotides was replaced in the complementary sequence of crtMN and the mutated sequence could not basepair with R1. The results of -galactosidase activities showed that the expression of mUcrt-lacZ was not altered in the *ΔssrA* (Fig. 2c).

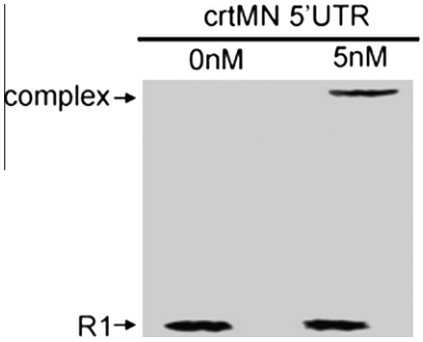

Fig. 3. ssrA could act as antisense RNA to directly bind the 5′UTR of *crtMN* mRNA. The binding between R1 and the 5′UTR of *crtMN* mRNA was determined by EMSA.

4. Discussion

It is known to all that the ssrA can function as a tmRNA. SmpB is required for the all known biological activities of ssrA [24]. In *S. aureus*, it is found that the pigment of the *ΔssrA* is increased comparing with its parent strain, but the color of the bacteria is not changed when the *smpB* gene is inactivated. So SmpB is not involved in this process. At the same time, the mutated ssrA whose tag sequence was mutated could recover the alternation caused by ssrA deletion in *S. aureus*. These results suggest that ssrA maybe have an additional activity except its known function of tmRNA. Although SmpB is not involved in the pigment synthesis regulated by ssrA, we find that the cytotoxicity of smpB mutant and ssrA mutant is decreased compared with their parent strain (data not shown). So it suggests that the tmRNA function of ssrA may also play the important role in the pathogenesis of *S. aureus*. In the further investigation, we find that ssrA can act as an antisense RNA to pair with 5′UTR of *crtMN* mRNA.

There are many sRNAs identified in various organisms, including the pathogenic bacteria *S. aureus*, *Streptococcus pyogenes*, *Clostridium perfringens*, *Vibrio cholerae*, and *Chlamydia trachomatis* [17,26–29]. Most of them can control the gene expression by base pairing with their target mRNAs. The complementary sequences usually locate at 5′UTR of the target mRNA [30]. In *S. aureus*, RNAIII is a well characterized sRNA, which can basepair and regulate several targets expression. The targets of RNAIII include hla, spa, rot etc., [14–16].

We identify here that ssrA can regulate the expression of *crtMN* via specifically binding the 5′UTR of its mRNA. The RBS region of *crtMN* mRNA is found to be located in the complementary sequences. So it suggests that the translation of the CrtM and CrtN is suppressed by ssrA. The CrtM and CrtN are two essential enzymes involved in the process of the pigment synthesis. Mutagenesis of *crtM* can efficiently block the biosynthetic pathway for *S. aureus* carotenoids [2]. So it is not surprising that the pigment of *ΔssrA* is increased. Although the fragment of ssrA (R1) can specifically bind the 5'UTR of *crtMN* mRNA, we do not observe the binding between *crtMN* mRNA and the intact ssrA. We think there should be some chaperone molecules to mediate the interaction between the intact ssrA and *crtMN* mRNA in *S. aureus*.

In a word, our study reveals that ssrA can act as an antisense RNA to regulate the expression of CrtM/N and influence the synthesis of *S. aureus* pigment.

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