



Regulation of *saeRS*, *agrA* and *sarA* on *sasX* Expression in *Staphylococcus aureus*

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Abstract

Background: The spread of *Staphylococcus aureus* and the types of infection caused by *S. aureus* are closely related to the secretion of a variety of adhesion proteins, which could be controlled by a variety of regulatory systems. However, for the newly discovered adhesion protein SasX, the regulatory mechanism is not completely clear.

Objectives: The current study aimed at investigating the regulation of Staphylococcal accessory gene regulator A (*agrA*), Staphylococcal accessory regulator A (*sarA*), and two-component signal transduction system (*saeRS*) on the adhesion protein SasX.

Methods: In this research, a *saeRS* mutant strain, a *sarA* mutant strain, and a *agrA* mutant strain were constructed by allelic replacement. In this study mRNA and protein expression levels of *sasX* in wild-type HS770 and knockout strains were studied to investigate the effects of regulatory factor *saeRS*, *agrA*, and *sarA* on adhesion protein SasX.

Results: In contrast with the wild strain HS770, the transcriptional expression of *sasX* was highest at on the sixth hour time point in HS770 Δ *agrA* and at nine and twelve hours in HS770 Δ *sarA*. However, the *sasX* transcription level in HS770 Δ *saeRS* mutant strains had little change at different time points. Western-blot results suggested that the *sasX* expression level of wild strains was the highest at 6 hours; HS770 Δ *saeRS* mutation strains had no expression peak at 6 hours. The expression level of HS770 Δ *agrA* mutant strains decreased at 6 hours of expression, however, increased at 9 hours and 12 hours; the expression level of HS770 Δ *sarA* mutation knockout increased at three, six, nine, and twelve hours.

Conclusions: All the results showed that *agrA* and *sarA* have negative regulation on *sasX*, but *saeRS* may not regulate *sasX*.

Keywords: *Staphylococcus aureus*, *saeRS*, *agrA*, *sarA*, *sasX*

1. Background

Staphylococcus aureus is a major global source of morbidity and mortality (1), and methicillin resistant *Staphylococcus aureus* (MRSA) have shown high virulence and carry multiple resistance genes, and could cause hospital acquired infections (HA-MRSA) and community acquired infections (CA-MRSA), and even lead to outbreaks of nosocomial infections (2). The HA-MRSA global epidemiological survey showed that in European countries, the prevalence of HA-MRSA had stabilized, yet in eastern Asia, the prevalence rate was still high, especially in Sri Lanka (86.5%), South Korea (77.6%), Taiwan China (65%), Hong Kong (56.8%), and Chinese Mainland, close to 50% (2, 3).

Staphylococcus aureus, which were regarded as impor-

tant pathogenic bacteria could become a threat to human health; there are two main factors in this regard as follows: 1, It can continuously evolve new clones by horizontal transfer of genes, which are more virulent and more resistant than the previous ones; 2, *Staphylococcus aureus* parasites on human skin and nasal mucosa can express a variety of adhesion proteins and virulence factors, and severe infections would occur in case of immune system dysfunction. The spread of *S. aureus* and the types of infection caused by *S. aureus* are closely related to the secretion of a variety of adhesion proteins. These adhesion proteins can also promote the formation of biofilms in bacteria, thereby forming chronic infections and helping bacteria escape antibiotic killing.

The predominant lineage causing hospital-associated

infections in Asia is sequence type 239 (ST239) lineage of MRSA isolates (4). In 2010, Holden et al. isolated a strain of ST239 in hospital *S. aureus* infection outbreak in the United Kingdom during year 2008, and sequencing and analysis showed that it contained a new protein of unknown function; the analysis results showed that the C-terminal of the protein contained an LPXTG surface anchoring motif sequence, named *sasX* (2, 3, 5). Research on its function showed that the SasX protein could enhance the adhesion of *S. aureus*, immune escape ability, and virulence, making it easier for bacteria to colonize in the human nasal area, more likely to cause infection (4, 6). It has been suggested that the adhesion protein SasX is associated with the prevalence of ST239 MRSA at the hospital (6). The epidemiology and function of major adhesion genes has been reported, however, the specific regulatory mechanisms are not fully understood. Previous studies have shown that *S. aureus* has two major regulatory systems: 1, DNA binding proteins, such as the SarA family; and 2, Two-component signal transduction systems (TCSs). Among them, *saeRS*, *agrAC*, and *sarA* mainly regulate the expression of a variety of secreted proteins, including adhesion and virulence proteins.

2. Objectives

This study aimed at clarifying the regulatory mechanism of *saeRS*, *agrA*, and *sarA* on adhesion genes *sasX* of *S. aureus*, and provides a theoretical basis for the prevention and treatment of *S. aureus* infection.

3. Methods

3.1. Bacterial Strains, Plasmids and Growth Conditions

The bacterial strains and plasmids used in this study are listed in Table 1. *Staphylococcus aureus* clinical isolates (HS770) was offered by Li Min, from Renji hospital, affiliated to Shanghai Jiao Tong University; it had been identified as *S. aureus* by automatic microorganism analyzer, Gram staining, and the coagulase test. Furthermore, the *sasX*, *sarA*, *saeRS*, and *agrA* gene were confirmed positive by the polymerase chain reaction (PCR) and gene sequencing (Sangon Biotech, China). *Escherichia coli* strains were cultured in Luria-Bertani medium, and *S. aureus* strains were grown in Tryptic Soy Broth (TSB, BD, USA) medium at 37°C with shaking at 220 rpm, overnight.

3.2. DNA Manipulations

Genomic DNA extraction from *S. aureus* strains was performed, according to the manufacturer's instructions (TIANGEN, Beijing, China). Plasmid DNA extracted from *E.*

coli strains and *S. aureus* strains were performed according to the guidelines with slightly modification (Axygen, Union City, CA, USA).

3.3. Construction of *Staphylococcus aureus agrA*, *saeRS*, and *sarA* Mutant Strains by Allelic Replacement

The upstream and downstream fragments of *saeRS*, *agrA*, and *sarA* gene were amplified, respectively, by PCR with the genomic DNA of *S. aureus* as the template (the primers listed in Table 2). Polymerase Chain Reaction production was purified, then digested with KpnI (Thermo, USA) and ligated with T4 DNA ligase (Thermo, USA) to yield an upstream and downstream homologous arm fragment, with *saeRS*, *agrA*, and *sarA* gene deletion. As the upstream and downstream homologous arm fragment with the attB sites at the 5' and 3' ends, then recombined with temperature-sensitive shuttle plasmid pKOR1 to generate recombinant plasmid, resulting in the plasmid pKsaeRS, pKsarA and pKagrA. The recombinant plasmid was transferred first to *E. coli* DH5 α and then to *E. coli* DC10B for modification, then electroporated to *S. aureus* HS770. The mutant strains were selected via changing temperature and anhydrotetracycline (ATc) induction expressing *ccdB* and antisense *secY* RNA, and evaluated with PCR, RT-PCR, and gene sequencing.

3.4. Construction of Complemented Strains

The *agrA* and *sarA* genes and their own upstream promoter and ribosome binding region were amplified by PCR with Iproof High-Fidelity DNA polymerase (the primers listed in Table 2). The PCR products were digested with BamHI (Thermo, USA) and KpnI, and the fragments were ligated to shuttle plasmid pRB473 by the T4 DNA ligase to generate plasmid pRBsarA and pRBagrA, respectively. These recombination plasmids were transferred to *Escherichia coli* DH5 α firstly, and then transferred to DC10B for modification, and finally electroporated to mutant strains HS770 Δ sarA and HS770 Δ agrA, respectively. These complemented strains were confirmed by restriction mapping and sequencing of PCR fragments. The *saeRS* gene-complemented strains was not constructed due to no regulation on *sasX*.

3.5. Growth Curve

Staphylococcus aureus wild type strain HS770, mutant strains HS770 Δ saeRS, HS770 Δ sarA, and HS770 Δ agrA were incubated overnight in 5 mL of TSB at 37°C with shaking at 220 rpm. The cultures were diluted in 50 mL of TSB at a proportion of 1:200 to obtain the same starting optical density (OD) at 600 nm. The growth condition of each strain was detected by Microplate Manager 6 (Bio-Rad, USA) software at one-hour intervals for 12 hours.

Table 1. Bacterial Strains and Plasmids Used in This Study

Strains and Plasmids	Description	Source
Strains		
<i>S. aureus</i> HS770	Wild type, MRSA, ST239	Donated by Limin professor
HS770Δ <i>sarA</i>	Isogenic <i>sarA</i> deletion mutant in HS770	In the study
HS770Δ <i>sarA</i> -C	<i>sarA</i> mutant complemented with pRB <i>sarA</i>	In the study
HS770Δ <i>agrA</i>	Isogenic <i>agrA</i> deletion mutant in HS770	In the study
HS770Δ <i>agrA</i> -C	<i>agrA</i> mutant complemented with pRB <i>agrA</i>	In the study
HS770Δ <i>saeRS</i>	Isogenic <i>saeRS</i> deletion mutant in HS770	In the study
E. coli		
DH5α	Clone host strain	Laboratory stock
DC10B	dam+1dcM ⁻ Δ <i>hsdRMS</i> endA1 <i>recA1</i> ; clone host strain	Laboratory stock
Plasmids		
pKORi	Shuttle cloning vector, temp sensitive (Cm ^r Amp ^r) ^a	Laboratory stock
pK <i>sarA</i>	pKORi containing fragments 1000-bp, upstream and 1000-bp downstream of <i>sarA</i> gene, for <i>sarA</i> mutagenesis, (Cm ^r Amp ^r)	In the study
pK <i>agrA</i>	pKORi containing fragments 1000-bp, upstream and 1000-bp downstream of <i>agrA</i> gene, for <i>agrA</i> mutagenesis, (Cm ^r Amp ^r)	In the study
pK <i>saeRS</i>	pKORi containing fragments 1000-bp, upstream and 1000-bp downstream of <i>saeRS</i> gene, for <i>saeR</i> mutagenesis, (Cm ^r Amp ^r)	In the study
pRB473	Shuttle cloning vector (Cm ^r)	Laboratory stock
pRB <i>sarA</i>	pRB473 with <i>sarA</i> and its promoter (Cm ^r)	In the study
pRB <i>agrA</i>	pRB473 with <i>agrA</i> and its promoter (Cm ^r)	In the study

^aCm^r Amp^r, chloramphenicol and ampicillin resistance.

Table 2. Primers Used in This Study

Primer	Primer Sequence (5' - 3')	Note ^b
<i>sarA</i> us-F	GGGGACAAGTTTGACAAAAAGCAGGCTTTGAAGGTAAGGGGATCC	<i>attB1</i>
<i>sarA</i> us-R	GGGGTACCGAACTATAATTTGTTTAGCG	KpnI
<i>sarA</i> ds-F	GGGGTACCAATGCCATGTTAAAACTC	KpnI
<i>sarA</i> ds-R	GGGGACCACTTTGTACAAGAAAGCTGGGTCTATTATGATTTTGACAGGCA	<i>attB2</i>
<i>agrA</i> us-F	GGGGACAAGTTTGACAAAAAGCAGGCTATAACAATTCACACAGCGT	<i>attB1</i>
<i>agrA</i> us-R	GGGGTACCAATCACATCCTTATGGCTAG	KpnI
<i>agrA</i> ds-F	GGGGTACCTAAGATAATAAAGTCAGTTAAGC	KpnI
<i>agrA</i> ds-R	GGGGACCACTTTGTACAAGAAAGCTGGGTAAAGCGGGGAGCGAGATT	<i>attB2</i>
<i>saeRS</i> us-F	GGGGACAAGTTTGACAAAAAGCAGGCTATAAAAACAAAACACCTAACAGGT	<i>attB1</i>
<i>saeRS</i> us-R	GGGGTACC GTGGTCTATCTATTTTTTCA	KpnI
<i>saeRS</i> ds-F	GGGGTACCACGTCATAATCCGATTTATTTA	KpnI
<i>saeRS</i> ds-R	GGGGACCACTTTGTACAAGAAAGCTGGGTACATTCAAAATCTTTTAAATAAAAAG	<i>attB2</i>
<i>agrA</i> -C-F	CGCGGATCCCTACTAAAGGTGAAGTTCGT	BamHI
<i>agrA</i> -C-R	GGGGTACCTTATCTTTGTTAAAATCCAACAAG	KpnI
<i>sarA</i> -C-F	CGCGGATCCTTGCGCTAAATCGTTTCATTA	BamHI
<i>sarA</i> -C-R	GGGGTACCCTATCAAACTTCACCAAAATG	KpnI
<i>gyrB</i> -F	ACATTACAGCAGCGTATTAG	
<i>gyrB</i> -R	CTCATAGTGATAGGAGTCTTCT	
<i>sasX</i> -RT-F	GCTGCTAATAATACTGAAG	
<i>sasX</i> -RT-R	TGCTACAACCTGATAACAA	

^bRestriction endonuclease site and *attB* sequence.

3.6. RNA Extraction, cDNA Synthesis and Quantitative Reverse Transcription-PCR (qRT-PCR)

The quantitative Real-Time PCR was performed according to the MIQE guidelines. For RNA isolation, *S. aureus*

strains were incubated overnight in 5 mL of TSB at 37°C with shaking at 220 rpm, then the cultures were diluted at 1:200 in TSB and grown to post-exponential phase at 37°C with shaking at 220 rpm. RNA Extraction (Qiagen, USA),

cDNA synthesis (Takara, Japan), and Quantitative Reverse Transcription-PCR (Takara, Japan) were performed as described previously (7) (the primers listed in Table 2).

3.7. Western Blotting

Analysis of the SasX protein products was by Western blotting, as previously described (8). The overnight cultures of HS770, HS770Δ*saeRS*, HS770Δ*sarA*, and HS770Δ*agrA* were inoculated to 8-mL TSB with 37°C, 220 rpm. The cells of different bacterial cultures were harvested by centrifugation at three, six, nine, and twelve hours, respectively. Then the cells were washed two times with PBS buffer, suspended within 500 uL PBS buffer, and 20 uL lysostaphin (1 mg/mL) was added to lytic bacteria, followed by incubation for one hour at 37°C. Then, the cells were fractured by sonication with the samples on ice. The supernatant obtained by centrifugation was considered as bacterial protein samples. The obtained proteins were electrophoretically separated on a 12-% sodium dodecyl sulphate polyacrylamide gel and subsequently transferred onto a PVDF membrane. The primary antibody (1:1000 dilution of rabbit anti-SasX polyclonal antibodies in TTBS) was added to the membranes, which were left overnight, followed by horseradish peroxidase-conjugated anti-rabbit IgGs as secondary antibodies (1:2000 dilution) for two hours. Finally, the presence of SasX was visualized using ECL Western blotting detection kit (GE Healthcare) in combination with a Chemi-Doc™ XRS + System (Bio-Rad).

3.8. Statistical Analysis

Relative expression levels resulting from qRT-PCR were analyzed using GraphPad Prism 5.0 software. Results were considered statistically significant if P values were < 0.05.

4. Results

4.1. Deletion of *sarA*, *saeRS* and *agrA* in *Staphylococcus aureus* HS770

The agarose gel electrophoresis of PCR products showed that the bands size of mutant strains was shorter than those of wild plants, which were the exact the corresponding gene size. Fluorescent quantitative PCR results reflected the relative expression of *saeRS*, *sarA*, and *agrA* in mutant strains HS770Δ*saeRS*, HS770Δ*sarA*, and HS770Δ*agrA*, and were dramatically decreased compared to that of wild type HS770 (Figure 1).

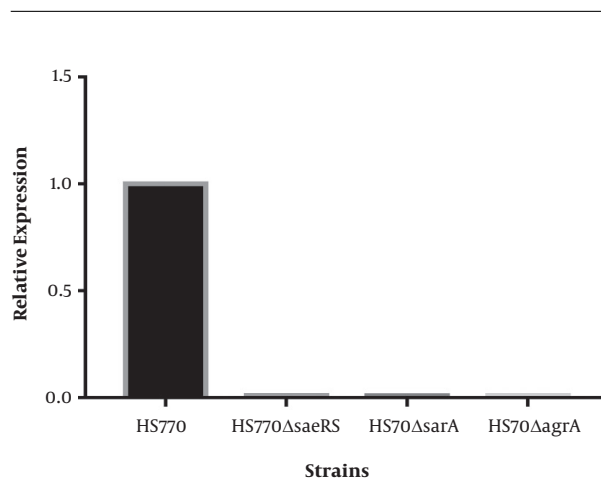


Figure 1. Transcript levels of *saeRS*, *sarA*, *agrA* genes in *S. aureus* HS770, HS770Δ*saeRS*, HS770Δ*sarA*, HS770Δ*agrA* deletion mutant. The total RNA samples were extracted from 6 h cultures of HS770, HS770Δ*saeRS*, HS770Δ*sarA* and HS770Δ*agrA* deletion mutant. The housekeeping gene *gyrB* was used as the reference gene. The expression of *saeRS*, *sarA* and *agrA* in *S. aureus* HS770 was regarded as 1, respectively. Transcript levels of *saeRS* in HS770Δ*saeRS* and *sarA* in HS770Δ*sarA* and *agrA* in HS770Δ*agrA* deletion mutant were detected by qRT-PCR.

4.2. Growth Curve Analysis

In order to eliminate the influence of gene knockout on the growth rate of bacteria, the growth curve of HS770, HS770Δ*saeRS*, HS770Δ*sarA*, and HS770Δ*agrA* was drawn. The sterile TSB medium was used as a blank control. There were no significant changes in the growth curve of the bacteria under the same inoculation and growth conditions. The growth of HS770Δ*agrA* mutant strains was higher than that of other strains in the late stage of platform. The following experiments were carried out on the basis of no obvious effect on bacterial growth (Figure 2).

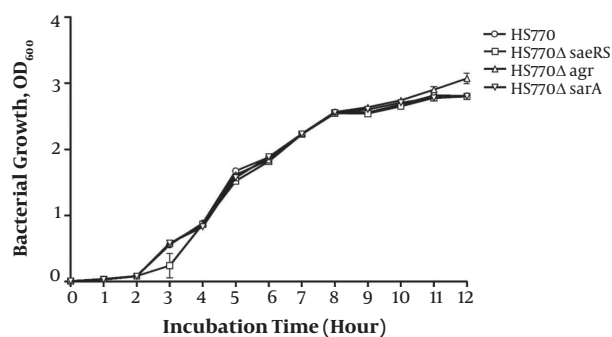


Figure 2. Growth curve of HS770 and HS770Δ*saeRS*, HS770Δ*sarA*, HS770Δ*agrA* deletion mutants. Bacterial growth was monitored by measuring the OD600_{nm} at one-hour intervals for twelve hours. Measurements were made in three independent experiments and the representative result was shown.

4.3. Transcript Level of *sasX* in Wild-Type and Knockout Strains

The total RNA of wild strain, mutant knockout strains, and the complementary expression strains were extracted at three, six, nine, and twelve hours, and were reverse transcribed into cDNA, respectively. The effect of *sarA*, *agrA*, and *saeRS* on expression of *sasX* was assessed by qRT-PCR. Here, the *gyrB* was used as an internal control. In contrast with the wild strain HS770, the *sasX* transcription level in HS770 Δ *agrA* knockout strains were increased by five folds, seven folds, two folds, and two folds at three, six, nine, and twelve hours, respectively (Figure 3A); the *sasX* gene transcription level in HS770 Δ *sarA* mutant strains was increased by five, three, seven, and seven folds at three, six, nine, and twelve hours, respectively (Figure 3B). The difference in expression level of *sasX* gene between wild strain HS770 and mutant strains was statistically significant ($P < 0.05$). However, the *sasX* transcription level in HS770 Δ *saeRS* mutant strains had little change at each of the time points (Figure 3C). The complementary expression strains restored to the expression level of wild strain. All the results showed that *agrA* and *sarA* had negative regulation on *sasX*, yet *saeRS* may not regulate *sasX*.

4.4. Detection of *sasX* Expression

The SasX protein is one of the cell wall surface proteins, thus, the researchers collected the cells of wild type strain HS770 and these mutant strains (HS770 Δ *saeRS*, HS770 Δ *sarA* and HS770 Δ *agrA*) at three, six, nine, and twelve hours. The bacteria were broken by the lysostaphin and ultrasonic, and subsequently, bacterial protein was extracted. After measuring the protein concentration, the same concentration of protein was carried out for 12% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). The expression of SasX protein was detected by Western-blot. It was suggested that the *sasX* expression level of wild strains was the highest at six hours; HS770 Δ *saeRS* mutation strains had no expression peak at six hours. The expression level of HS770 Δ *agrA* mutant strains decreased at six hours of expression, however, increased at nine and twelve hours; the expression level of HS770 Δ *sarA* mutation knockout all increased at three, six, nine, and twelve hours (Figure 4).

5. Discussion

Limiting the virulence factors is critical for treating bacterial infections. The targets can be the virulence genes or the regulation genes of virulence. Limiting SasX expression and secretion is a particularly challenging task for *S. aureus*. This is because SasX facilitates bacterial aggregates and biofilm formation. It is also a virulence factor during

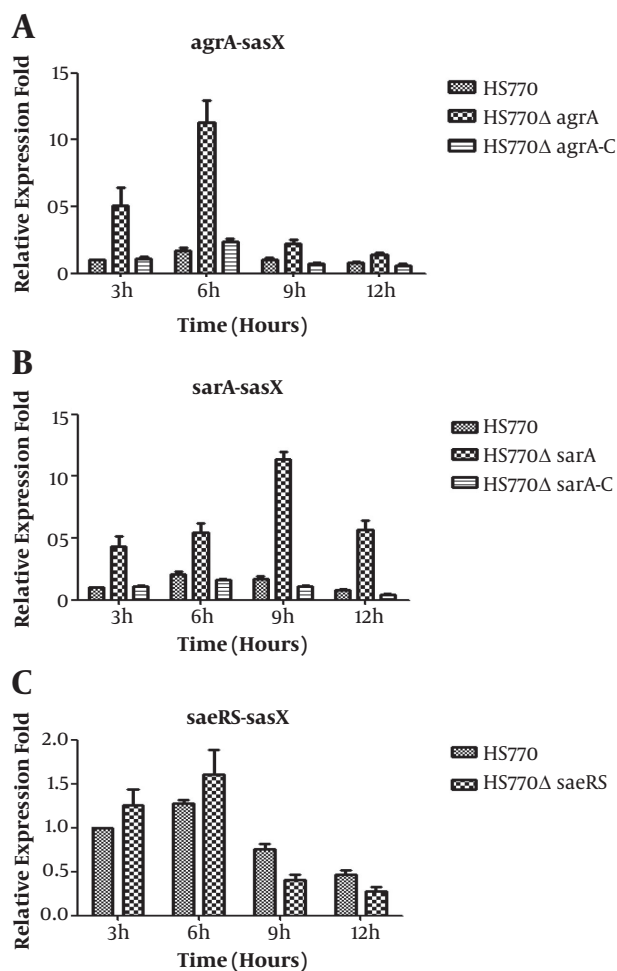


Figure 3. Transcript levels of *sasX* genes in *S. aureus* HS770, HS770 Δ *saeRS*, HS770 Δ *sarA* and HS770 Δ *agrA* deletion mutant. The total RNA samples were extracted from 3 hours, 6 hours, 9 hours, 12 hours cultures of HS770. Transcript levels of *sasX* in *S. aureus* HS770, HS770 Δ *saeRS*, HS770 Δ *sarA* and HS770 Δ *agrA* deletion mutant were detected by qRT-PCR, and the housekeeping gene *gyrB* was used as an internal control. Error bars indicate standard errors for data from three experiments.

S. aureus skin and lung infection models (9). Anti-SasX IgG reduced *S. aureus* colonization and infection (10). However, the regulation mechanism of *sasX* is not clear.

The expression of adhesion and virulence factors is a complex process during different stages of infection. This is controlled by global regulatory elements including two component regulatory systems, and the SarA protein family (11, 12). Furthermore, AgrAC is a density-dependent TCS. The *agr* locus is activated in post-exponential growth. Activated *agr* locus has down-regulative adhesion factors and up-regulative virulence factors, such as the Pantone-Valentine Leukocidin (PVL), enterotoxins, and encoding alpha-hemolysin (Hla). SarA controls regulation of certain virulence factors directly, such as protein A (Spa), Hla, and

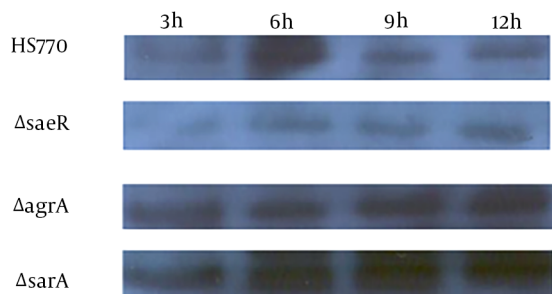


Figure 4. Expression of *sasX* in *S. aureus* clinical isolates and deletion mutant. *sasX* gene expression in *S. aureus* HS770, HS770 Δ *saeRS*, HS770 Δ *sarA* and HS770 Δ *agrA* deletion mutant in different growth time points was detected using Western blot with anti-*sasX* antibody (1:1000).

agr. Furthermore, SarA also binding to *agr* promoter elements indirectly regulates virulence factors (13). The two-component system SaeRS activates several virulence factors, such as serine protease (SspA), thermonuclease (Nuc), coagulase (Coa), Hla, beta-hemolysin (Hlb), and Spa (11).

The researchers constructed the *agrA*, *saeRS*, and *sarA* gene knockout strains and the corresponding complementary expression strains. The growth curve showed that *saeRS* and *sarA* mutation did not affect the growth of *S. aureus*, and *agrA* mutation was slightly faster than that of the wild strain at the station phase, which were consistent with the reports (2, 14). This suggests the regulation of *sasX* by *agr*, *saeRS*, and *sarA* has nothing to do with the growth. This study found that *agr* and *sarA* negatively regulates *sasX* from the results of qRT-PCR and Western-blot, however, *saeRS* may not regulate *sasX*. Since *agr* and *sarA* have the interaction effects, the specific regulation mechanism of *agr* and *sarA* is unknown. This potential mechanism needs to be further explored in detail.

6. Conclusions

In conclusion, the current study showed that *agr* and *sarA* negatively regulate *sasX*. The antimicrobial drugs targeting *agr* Quorum Sensing or SarA protein also limit *sasX*, which is critical for bacterial infections (15, 16).

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Footnote

Authors' Contribution: Yongpeng Shang and Xiuqin Qi contributed equally to this work. Yongpeng Shang, Xiuqin Qi, Shanshan Wang, Yinjuan Guo and Jingnan Lv performed the laboratory measurements. Fangyou Yu and Liangxing Wang made substantial contributions to the conception and design. Fangyou Yu revised the manuscript critically for important intellectual content. Yongpeng Shang and Xiuqin Qi participated in the experimental design and data analysis. Fangyou Yu drafted the manuscript. All authors read and approved the final manuscript.

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