

# Deletion of *SarX* Decreases Biofilm Formation of *Staphylococcus aureus* in a Polysaccharide Intercellular Adhesin (PIA)-Dependent Manner by Downregulating *spa*

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**Introduction:** Biofilm formation by *Staphylococcus aureus* is an important virulence determinant mediated by the polysaccharide intercellular adhesin (PIA) encoded by the *ica* operon or mediated by surface and extracellular proteins. SarX is a 250-residue two-domain SarA homolog that activates *spa* transcription. Previous studies demonstrated that *Staphylococcus epidermidis* SarX protein regulated the transcriptional activity of the *agr* and *ica* loci and controlled the biofilm phenotype, primarily by regulating *icaADBC* transcription and PIA production.

**Results:** In this study, biofilm formation and detachment of the clinical isolate *S. aureus* SA75 were significantly decreased in the *sarX* mutant strain. The effect of *sarX* mutation on *S. aureus* biofilm formation was related to the production of PIA and not to that of eDNA. Deletion of *sarX* was associated with a 1.8-fold reduction in *spa* transcription as determined by RT-PCR analysis, and this reduction could be restored by chromosomal complementation of *sarX*. Expression of Spa protein was also decreased in the *S. aureus sarX* mutant.

**Conclusion:** *sarX* promoted biofilm production of *S. aureus* that may primarily be mediated through increasing *ica* operon expression and PIA production. Furthermore, deletion of *sarX* reduced *S. aureus* biofilm formation by downregulating *spa*.

**Keywords:** *Staphylococcus aureus*, *ica*, biofilm formation, *sarX*, *spa*

## Background

*Staphylococcus aureus* is a human pathogen responsible for a variety of community- and hospital-acquired infections, including relatively benign illnesses and fatal systemic disease (pneumonia, endocarditis, mastitis, osteomyelitis, etc.).<sup>1,2</sup> *S. aureus* can form biofilms on host tissues and medical devices,<sup>3</sup> leading to chronic infections; up to 80% of chronic bacterial infections are associated with biofilms.<sup>4</sup> *S. aureus* infections associated with biofilm formation are difficult to treat. By forming a biofilm, *S. aureus* can escape the immune defense of the host and the attack of multiple immune factors. Dense biofilm can also prevent or delay the infiltration of antibiotic drugs, allowing bacteria to produce drug resistance genes that reduce the sensitivity of bacteria to antibiotics.<sup>5</sup> Detailed evaluation of the biofilm formation process of *S. aureus* will likely contribute to our understanding of the infection process for this pathogen.

Biofilm development and formation involve initial adhesion, proliferation, maturation, and diffusion.<sup>6</sup> *Staphylococcus* biofilm formation is modulated by transcriptional regulators (SarA, MgrA, and Rbf) and various regulatory systems (*agr* quorum sensing system) that control the production of biofilm-formation-associated factors (surface proteins, polysaccharide intercellular adhesin (PIA), eDNA, and other extracellular components). PIA and poly-*NN*-acetylglucosamine (the product of *icaA* gene) are the most common constituents of staphylococcal biofilm and were first reported in *Staphylococcus epidermidis*.<sup>7</sup> Biosynthesis of PIA is mediated by the *ica* operon, which contains four open reading frames (*icaA*, *icaD*, *icaB*, and *icaC*) and one regulatory gene (*icaR*). This operon encodes the IcaA protein with *N*-acetamide glucanotransferase activity that can synthesize UDP-*N*-acetylglucosamine into an oligomer structure with a chain length of 20 residues. The *icaADBC* operon in *S. aureus* was negatively controlled by IcaR and the teicoplanin-associated locus regulator TcaR.<sup>8</sup> The transcription regulator TcaR, a member of the multiple antibiotic resistance regulator (MarR) family, is involved in teicoplanin and methicillin resistance in staphylococci.<sup>9</sup> Members of the SarA family of transcriptional regulators in *S. aureus* share homology with each other as well as with the MarR family. SarX, a member of the SarA/MarR family, was first identified in *S. aureus* by Manna and Cheung.<sup>10</sup> Transcription of *sarX* in *S. aureus* is temporal, with maximal expression in stationary phase. Inactivation of *sarX* does not affect the expression of regulatory genes in the *sarA* family or *saeRS*, but does have a significant negative effect on the transcription of *agr*. In *S. epidermidis*, the SarX protein regulated transcriptional activity of the *agr*

and *ica* loci and controlled the biofilm phenotype, primarily by regulating *icaADBC* transcription and PIA production.<sup>11</sup> In addition to the SarA/MarA family members, there are various cell wall-anchored proteins that participate in biofilm formation, including biofilm-associated protein (Bap), clumping factor B (ClfB), fibronectin-binding proteins (FnBPs), *Staphylococcus aureus* surface protein C (SasC), *Staphylococcus aureus* surface protein G (SasG), and *Staphylococcus* protein A (Spa).<sup>12</sup> Spa, the first-identified surface protein of *S. aureus*, was established as a model for the characterization of the covalent anchorage of the LPXTG domain proteins by sortase A to the bacterial cell surface.<sup>13</sup> Spa has been shown to comprise 7% of the cell wall. Spa is ubiquitous in *S. aureus* and is often used in strain typing on the basis of variation in the DNA sequence encoding the X region of the protein.<sup>14</sup> Spa binds the Fc fragment of immunoglobulins from several mammalian species and may be important in phagocytosis avoidance.<sup>15</sup>

The purpose of this study was to investigate the impact of a *sarX* deletion mutation on *ica*, PIA, Spa, and biofilm regulation in *S. aureus*.

## Methods

### Bacterial Strains, Plasmids, and Culture Conditions

Bacterial strains and plasmids used in this study are listed in Table 1. *S. aureus* strain SA75 was isolated from a patient with a purulent skin infection at the First Affiliated Hospital of Wenzhou Medical University (Wenzhou, China). Identification of the isolates was carried out using a VITEK-2 microbiology analyzer according to the manufacturer's instructions (bioMérieux, Marcy

**Table 1** Bacterial Strains and Plasmids Used in This Study

Strains and Plasmids	Description	Source
<b>Strains</b>		
<i>S. aureus</i> SA75	Wild type, clinical MRSA strain	The First Affiliated Hospital of Wenzhou Medical University
SA75Δ <i>SarX</i>	Isogenic <i>SarX</i> deletion mutant in SA75	This study
SA75Δ <i>SarX</i> -C	<i>SarX</i> mutant complemented with pRBSarX	This study
DH5α	<i>Escherichia coli</i> isolates, clone host strain	Laboratory stock
DC10B	<i>Escherichia coli</i> isolates, clone host strain	Laboratory stock
<b>Plasmids</b>		
pKORI	Shuttle cloning vector; temp sensitive (Cm <sup>R</sup> , Amp <sup>R</sup> )	Laboratory stock
pRB473	Shuttle cloning vector (Cm <sup>R</sup> )	Laboratory stock

**Note:** Italic font represents the name of the bacteria.

**Abbreviations:** *S. aureus*, *Staphylococcus aureus*; MRSA, methicillin resistant *Staphylococcus aureus*; Cm<sup>R</sup>, chloramphenicol resistance; Amp<sup>R</sup>, ampicillin resistance.

l'Etoile, France). *S. aureus* SA75 and *S. aureus sarX* mutant were grown in tryptic soy broth (TSB, BD) medium and complemented strain was grown in tryptic soy broth (TSB, BD) medium containing 10 mg/l chloramphenicol at 37°C with shaking at 220 rpm. *Escherichia coli* was cultured in Luria broth (LB, Oxoid) medium with appropriate antibiotics (ampicillin at 100 mg/l and anhydrotetracycline at 50 mg/l).

## Construction of *S. aureus sarX* Mutant (SA75 $\Delta$ *sarX*) and Complemented Strain (SA75 $\Delta$ *sarX*-C)

The *sarX* deletion mutant of *S. aureus* strain SA75 was constructed by allelic replacement using the temperature-sensitive plasmid pKOR1. Upstream and downstream fragments of *sarX* were amplified from genomic DNA of *S. aureus* SA75 using the primer sets *sarX*-UP-F/*sarX*-UP-R and *sarX*-DOWN-F/*sarX*-DOWN-R (Table 2). The resulting amplicons were digested with Kpn I and then ligated with T4 DNA ligase to yield the homologous arm fragment with the deletion of the *sarX* gene, and this fragment was then cloned into pKOR1. The recombinant plasmid pKOR1- $\Delta$ *sarX* was successively transferred into

*E. coli* DH5 $\alpha$  and DC10B competent cells, then ultimately electroporated into *S. aureus* SA75 competent cells. Allelic replacement mutants were selected as previously described<sup>16</sup> and were further confirmed by PCR and sequencing.

To construct the *sarX* chromosomal complementation strain, fragments covering the truncated region in the mutant strains were amplified from genomic DNA of *S. aureus* SA75 using the primer set *sarX*-C-F/*sarX*-C-R (Table 2). The gene fragments were digested with restriction enzymes and then cloned into PRB473. The resulting plasmid, PRB473-*sarX*, was electroporated into the mutant strain. Allelic replacement complementation strains were selected using the method described above and were further confirmed by PCR and sequencing.

## RNA Isolation and Quantitative Real-Time RT-PCR Analysis

RNA isolation was performed as previously described.<sup>17</sup> Briefly, overnight cultures were inoculated into fresh TSB medium to an optical density of 0.01. Total RNA was isolated and purified using a PureLink RNA Mini Kit (Invitrogen, Carlsbad, CA, USA). The resulting RNA

**Table 2** Primers Used in This Study

Primer	Primer Sequence (5'-3')	Note
<i>sarX</i> -F	AAGATAATAATGACAGAAGC	
<i>sarX</i> -R	AAAATTGTTCTACATCTTCA	
<i>sarX</i> -UP-F	GGGGACAAGTTTGTACAAAAAGCAGGCTAGCGACTTAAATTCGATTCGTTA	attB1
<i>sarX</i> -UP-R	CGGGGTACCGTCTTTCTCATTGTGTTTTAATACG	KpnI
<i>sarX</i> -DOWN-F	CGGGGTACCTTGAAATTGAGTGTGAAAGCATAG	KpnI
<i>sarX</i> -DOWN-R	GGGGACCACTTTGTACAAGAAAGCTGG GTTCCATTGTTCTGCTGATT	attB2
<i>sarX</i> -RT-F	AACATTGCTTGGCTTCTAT	
<i>sarX</i> -RT-R	AATCTAGCTCATCCATTGC	
<i>sarX</i> -C-F	CGGAATTCCG ACACCTTGATATGTATTGCA	EcoRI
<i>sarX</i> -C-R	CGGGATCCCG CATTATTGAACTACGATTCC	BamHI
<i>icaA</i> -RT-F	CTTGATGCAGATACTATCG	
<i>icaA</i> -RT-R	GCGTTGCTTCCAAAGACCTC	
<i>spa</i> -RT-F	CAATTCTAGCTATTATCACTT	
<i>spa</i> -RT-R	ATTAATACCCCCTGTATGTA	

**Note:** Italics represent gene names.

was then reverse transcribed into cDNA using a PrimeScript RT reagent kit (TaKaRa, Tokyo, Japan) according to the manufacturer's protocol and using the oligonucleotides (*sarX*-RT-F/*sarX*-RT-R, *icaA*-RT-F/*icaA*-RT-R, *spa*-RT-F/*spa*-RT-R) shown in Table 2. The real-time PCR was performed using a SsoFas EvaGreen Supermix kit (Bio-Rad, USA) with the Bio-Rad CFX96 Manager software. *S. aureus* SA75 wild-type was used as a control (relative expression = 1), and *gyrB* was used as a reference gene to investigate genes of interest. RNA transcript levels were calculated by the  $\Delta\Delta C_t$  method.<sup>18</sup> Data analysis was conducted using Bio-Rad CFX software. Each reaction was performed in triplicate.

## Biofilm Formation and Analysis

Biofilm formation was determined by the microtiter plate assay based on a previously described method.<sup>19</sup> Briefly, overnight cultures of bacterial strains were diluted 1:200 in fresh TSB. Two hundred microliters of the diluted cultures were pipetted into sterile 96-well polystyrene plates (BD Biosciences) with three attached wells of each bacteria, and incubated overnight at 37°C without shaking. The wells were then washed gently three times with phosphate-buffered saline (PBS) (to remove nonadherent cells). Methanol (99.5%) was used to stabilize the biofilms. The wells were stained with 200  $\mu$ L of 1% (w/v) crystal violet (Sigma) for 10 min before being washed three times with water. After air drying, 30% glacial acetic acid was used to release the biofilm into solution and the optical density at 600 nm ( $OD_{600}$ ) of each well was recorded. TSB medium was used as a blank control. *Staphylococcus epidermidis* ATCC 12228 was used as negative control, and *Staphylococcus aureus* ATCC 29213 positive control, respectively.

## Scanning Electron Microscopy

Biofilms formed on glass coverslips (10 mm diameter) were observed by scanning electron microscopy (SEM) as previously described.<sup>20</sup> Briefly, overnight bacterial cultures were diluted 1:200 with TSB. A glass coverslip was placed in advance in the bottom of sterile flat-bottomed polystyrene plates (Costar 3524; Corning, NY, USA) and 1 mL of the bacterial cultures were added to each well and incubated statically at 37°C for 24 h. Each well was rinsed three times for 10 min with sterile PBS. Biofilms formed on the glass coverslips were fixed with 2.5% glutaraldehyde in 0.1 M sodium cacodylate buffer (pH 7.4) by incubation for 2 h at room temperature. Next, the

coverslips were washed three times with sterile PBS for 15 min and then 1% osmium tetroxide was applied for a post-fixation step for 2 h at 4°C, followed by washing three times for 10 min with distilled water. The samples were then dehydrated in a series of ascending ethanol baths (25, 50, 75, 95, and 100%) for 10 min each. The coverslips were placed in a freeze-dried apparatus for 5 h, and the gold spray was taken out for 3 min. Biofilm observations were performed with a scanning electron microscope (FEI Quanta 200; USA). The experiment was repeated three times.

## Triton X-100 Induced Autolysis

Autolysis assays were performed as described by Brunskill and Bayles.<sup>21</sup> Briefly, overnight cultures of bacteria were cultured in 50 mL TSB containing 1 M NaCl until they reached the early exponential phase. Following centrifugation (4000 g, 20 min), the bacteria were washed twice with 50 mL ice-cold water and resuspended in 50 mL Tris-HCl (pH 7.2) containing 0.05% (vol./vol.) TritonX-100. The  $OD_{600}$  was adjusted to 1.0, followed by shaking of the culture at 37°C for 3 h, measuring the  $OD_{600}$  at 30 min intervals. The experiment was repeated three times. *Staphylococcus epidermidis* 1457 $\Delta$ atlE was used as the control strain.

## Western Blot Analysis

Spa protein expression was determined by Western blot analysis as previously described.<sup>22</sup> Bacterial culture supernatants were collected and washed twice with sterile PBS. The supernatants were then suspended in 100  $\mu$ L PBS, incubated at 37°C for 2 h with 3  $\mu$ L lysostaphin (1 mg/mL), and centrifuged at 8000 g, 4°C for 30 min. The protein concentration of each sample was determined by Bradford dye binding method. The samples were separated by 12% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and transferred onto a polyvinylidene difluoride (PVDF) membrane. After blocking with 5% nonfat milk in PBST buffer at room temperature for 1 h, the membrane was incubated with HRP-conjugated anti-Protein A antibody at a 1:5000 dilution. The protein was visualized using the ECL Western blotting assay kit (GE Healthcare) and the Chemi doc TM XRS system (Bio-Rad).

## PIA/PNAG Detection

PIA extracted from *S. aureus* was detected by dot blot assay with a wheat germ agglutinin horseradish peroxidase

(WGA-HRP) conjugate according to a previously described protocol.<sup>23</sup> Briefly, overnight bacterial cultures were diluted to  $10^7$  colony-forming units (CFU) and added to 2 mL TSB at a ratio of 1:100 in six-well polystyrene plates. An equal number of cells from each culture were resuspended in 0.5 M EDTA (pH 8.0) and incubated for 5 min at 100°C. The culture was then centrifuged and 40 µL of the supernatant was incubated with 10 µL proteinase K (20 mg/mL) (Sangon Biotech) at 37°C for 2 h to minimize nonspecific background. Ten microliters of the extracted PIA sample was pipetted onto a PVDF membrane after formaldehyde treatment. The membrane was kept moist during the spotting process and was dried at room temperature after spotting. After drying, the membrane was blocked with 3.5% bovine serum albumin (BSA) in PBS with 0.1% Tween 20, and was incubated in a dish containing WGA-HRP for 1 h at 37°C. Protein detection was then performed using the Pierce enhanced chemiluminescence (ECL) Western blotting substrate (Thermo Scientific, Rockford, IL, USA).

## Statistical Analyses

All experiments were performed in biological triplicate. Statistical analyses were performed using SPSS statistical software (version 23; IBM SPSS Statistic) and GraphPad Prism 8 (version 8.00, La Jolla, CA, USA). For the comparison of two groups, an unpaired *t*-test was used; for three or more groups, one-way or two-way analysis of variance (ANOVA) was used as appropriate. A value of *P* < 0.05 was considered statistically significant.

## Results

### The *sarX* Mutation Reduces *S. aureus* Biofilm Formation

The effect of *SarX* on the biofilm formation capacity of *S. aureus* was evaluated by comparing the *S. aureus* SA75  $\Delta$ *sarX* mutant (SA75- $\Delta$ *sarX*) with the complemented and wild-type SA75 strains (SA75 $\Delta$ *sarX*-C and SA75, respectively). Compared with the wild-type strain, SA75- $\Delta$ *sarX* displayed a marked reduction in biofilm formation (Figure 1A). In contrast, SA75- $\Delta$ *sarX*-C formed stronger biofilms than that of SA75 when the strains were incubated under the same conditions. The biofilms were semi-quantified (Figure 1B), and the biofilm of SA75- $\Delta$ *sarX* was significantly increased compared to that of the SA75 strain (3.31-fold, *P* < 0.05). However, the biofilm formation of SA75- $\Delta$ *sarX*-C was stronger than that of the wild-type

strain. The decreased biofilm formation of the *sarX* mutant strain was not due to a growth defect in the strain. There were no significant differences in growth rates among SA75, SA75- $\Delta$ *sarX*, and SA75- $\Delta$ *sarX*-C under the same inoculation and culture conditions (Figure 1C).

### Scanning Electron Microscopy of Biofilms

Scanning electron microscopy can be used to observe the formation of biofilm. During process of biofilm formation, bacteria cluster together and adhere to each other to form a compact biofilm structure. As shown in Figure 2, SA75 and SA75- $\Delta$ *sarX*-C could be seen to accumulate and form obvious biofilm structures. However, in SA75- $\Delta$ *sarX*, the than that of SA75. In agreement with the biofilm formation results determined by microtiter assay (Figure 1A), biofilm formation of SA75- $\Delta$ *sarX*-C observed by scanning electron microscopy was similar to that of SA75. However, there was no adhesion between the cells of SA75- $\Delta$ *sarX*-C and the profile of bacterial cells was evident on the surface of the agglomerated biofilm.

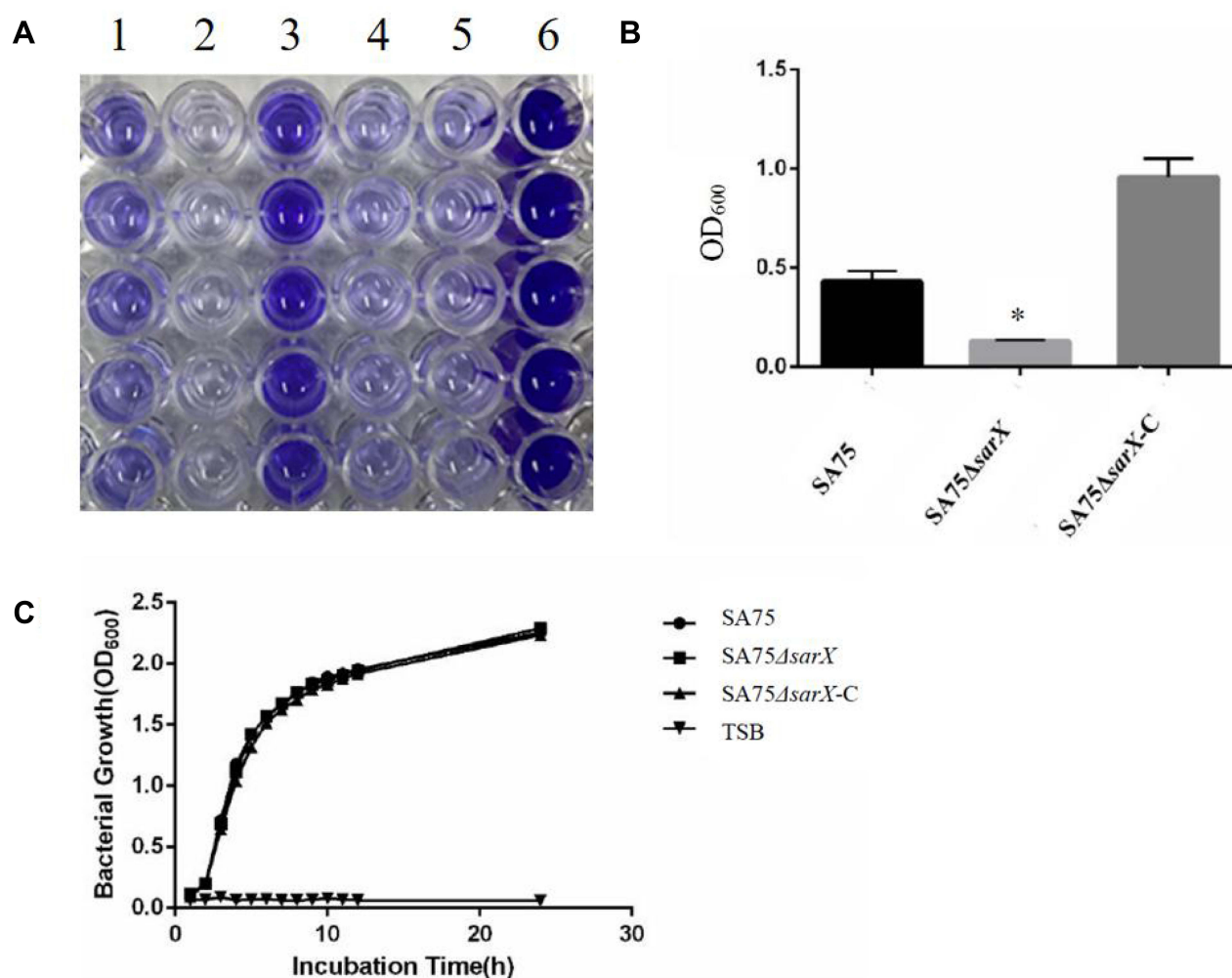
### The Reduced Biofilm Formation of the *sarX* Mutant Strain is PIA Dependent

To investigate the effect of *sarX* on biofilm matrix production, transcription of the *icaADBC* operon and release of PIA in the wild-type SA75, SA75- $\Delta$ *sarX*, and SA75- $\Delta$ *sarX*-C strains was evaluated. PIA production was determined semi-quantitatively with a wheat germ agglutinin-horse radish peroxidase (WGA-HRP) conjugate using a dot blot 96 system (Figure 3A). The *sarX* mutant strain displayed much weaker PIA production compared with the wild-type strain, while complementation of *sarX* restored PIA production. Consistent with these results, expression of *icaA* in SA75- $\Delta$ *sarX* was significantly reduced 2.04-fold in real-time RT-PCR assays (Figure 3B). This indicated that knockout of *sarX* inhibited the expression of *icaA*. Expression of *icaA* in SA75- $\Delta$ *sarX*-C was restored to the wild-type level.

### Autolysis Capacity of the *sarX* Mutant Strain

Autolysis of cells results in the release of intracellular eDNA, which can promote adhesion and aggregation between cells and stimulate the formation of biofilms. We want to investigate that the SA75 $\Delta$ *sarX* biofilm formation was whether related to the production of eDNA or to that of PIA. To ascertain whether the decreased biofilm





**Figure 1** *sarX* mutation reduces *S. aureus* biofilm formation. **(A)** Biofilm formation by SA75 wild-type (column 1),  $\Delta sarX$  mutant (SA75 $\Delta sarX$ ; column 2), and chromosomal complemented strain (SA75 $\Delta sarX$ -C; column 3) in microtiter plates. Columns 4, 5, and 6 of the plates harbor blank, negative (*Staphylococcus epidermidis* ATCC 12228), and positive (*Staphylococcus aureus* ATCC 29213) controls, respectively. **(B)** Quantification of biofilms. The optical density at 600 nm (OD<sub>600</sub>) of each well was recorded. \* $P < 0.05$ . **(C)** Growth curves of the SA75 wild-type, SA75 $\Delta sarX$ , and SA75 $\Delta sarX$ -C strains. TSB liquid medium was used as a control.

formation of the *S. aureus* SA75  $\Delta sarX$  strain was associated with autolysis, Triton X-100 induction was used to assess the autolytic capacity of SA75, SA75- $\Delta sarX$ , and SA75- $\Delta sarX$ -C. *Staphylococcus epidermidis* 1457 $\Delta atlE$  was used as the control strain. No differences were observed between SA75- $\Delta sarX$  and its parent strain in the Triton X-100 induced autolysis assay (Figure 3C).

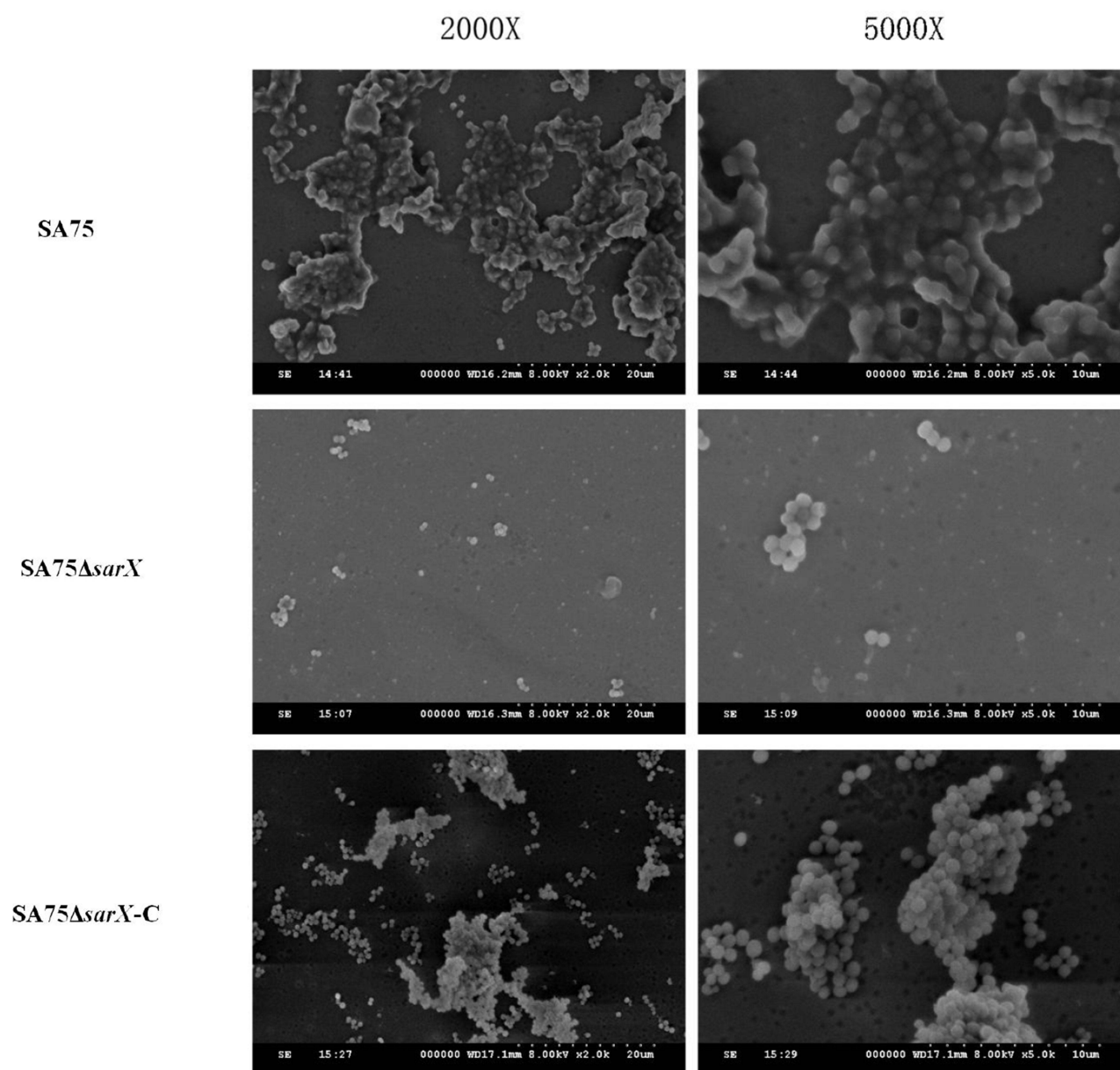
### Effect of *sarX* on Spa in *S. aureus*

Spa is an important adhesion protein of *S. aureus*, enhancing adhesion between bacteria and promoting the formation of biofilms. To investigate the effect of *sarX* on this adhesion protein, Spa expression in SA75, SA75- $\Delta sarX$ , and SA75- $\Delta sarX$ -C was determined by Western blotting,

keeping the total amount of protein loaded for SDS-PAGE consistent in the three strains. Spa production in the *sarX* mutant strain was much lower than in SA75 and SA75- $\Delta sarX$ -C (Figure 4A). RT-PCR showed that *spa* gene expression in SA75- $\Delta sarX$  was also significantly reduced (Figure 4B), indicating that knockout of *sarX* inhibited the expression of *spa*. Expression of *spa* was restored to the wild-type level in SA75- $\Delta sarX$ -C.

### Discussion

Production of PIA is an important contributing factor to biofilm formation by staphylococci. The *icaADBC* operon encoding PIA is subject to regulation by numerous factors, including positive transcriptional regulation by SarA. In

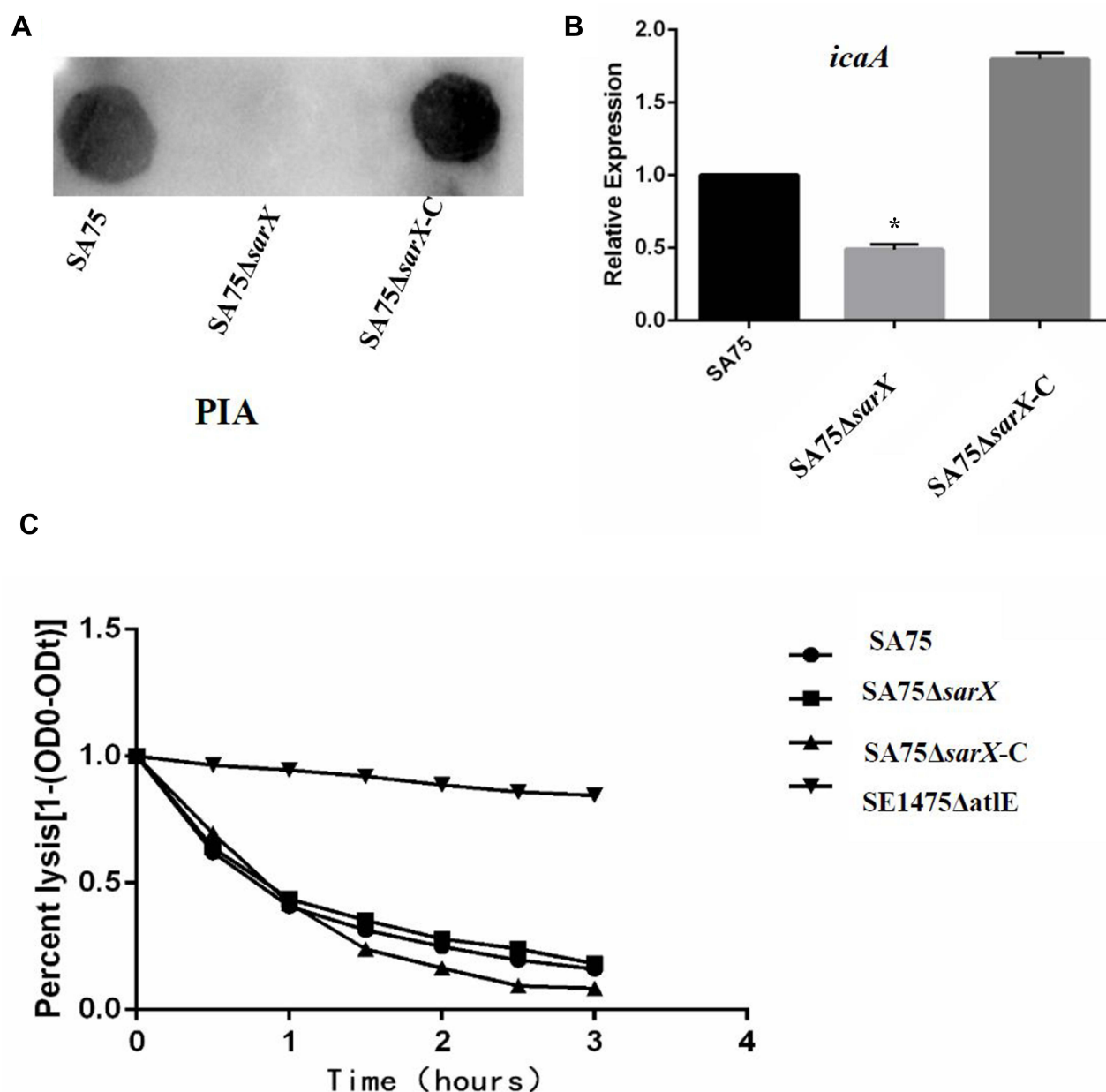


**Figure 2** Scanning electron microscopy of biofilms of *S. aureus* SA75 and  $\Delta sarX$  mutant. Transmission electron micrographs of mature biofilms of wild-type (SA75), isogenic *sarX* mutant (SA75 $\Delta sarX$ ), and chromosomally complemented (SA75 $\Delta sarX$ -C) strains of *S. aureus* SA75. Images on the left and right were magnified 2000 (2000X) and 5000 (5000X) times, respectively.

previous reports, SarA increased biofilm development by suppressing the transcription of either a protein involved in the turnover of PIA/PNAG or a repressor of its synthesis. The Sar family of proteins comprises at least 11 different proteins. Manna and Cheung demonstrated that *sarX* affected biofilm formation in *S. aureus* 8325–4 but not in *S. aureus* RN6390.<sup>10</sup> These two strains are closely related and are derived from the same parent strain (NCTC8325); thus, the reasons underlying the difference in the effect of

*sarX* mutation on biofilm formation of strains RN6390 and 8325–4 have yet to be elucidated.

In the present study on the clinical isolate *S. aureus* SA75, deletion of *sarX* failed to stimulate biofilm formation, while the complemented *sarX* mutant exhibited the stronger levels of biofilm formation than that of the wild-type strain. By colorimetric assay, it is possible to see that it overstimulates biofilm production. We reasoned that this was due to over expression of *sarX* in the SA75- $\Delta sarX$



**Figure 3** The reduced biofilm formation of the *sarX* mutant is polysaccharide intercellular adhesin (PIA) dependent. **(A)** PIA biosynthesis was semi-quantified using a dot blot assay with WGA. **(B)** RT-PCR quantification of the effect of *sarX* mutant on *icaA* gene expression by the SA75 wild-type, SA75Δ*sarX*, and SA75Δ*sarX*-C strains. \*P<0.05. **(C)** Comparison of the autolytic abilities of SA75 wild-type, SA75Δ*sarX*, and SA75Δ*sarX*-C strains.

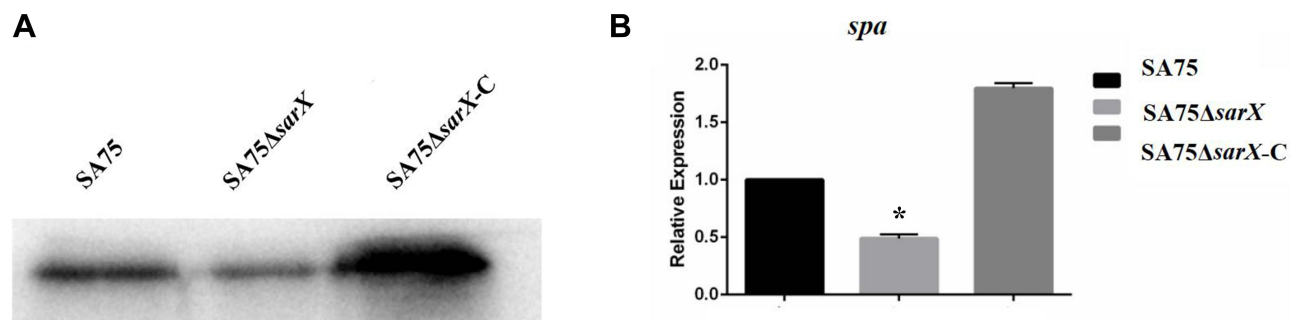
**Abbreviation:** SE1457Δ*atlE*, *Staphylococcus epidermidis* 1457Δ*atlE*.

isolate, and the PRB473 plasmid we used was a high-copy plasmid. These data suggested a positive role for *sarX* in *S. aureus* biofilm regulation. In addition, RT-PCR analysis demonstrated that deletion of *sarX* was associated with a twofold reduction in *icaA* transcription, which was also complemented by *sarX*. PIA production was decreased in the SA75-Δ*sarX* strain. The results indicate that the effect of the *sarX* mutant in *S. aureus* biofilm formation was related to the production of PIA and may not to that of

eDNA. Similarly, Cue et al demonstrated that Rbf and SarX represent a regulatory cascade that promotes PIA-dependent biofilm formation in *S. aureus*.<sup>24</sup> Rbf, a member of the AraC/XylS family of transcriptional regulators, increases *icaADBC* expression by upregulating transcription of *sarX*. In turn, SarX activates *icaADBC* expression.<sup>24</sup>

The *spa* gene encodes Spa, a multifunctional cell-wall protein that binds immunoglobulins, thereby inhibiting





**Figure 4** *Staphylococcus* protein A (Spa) protein expression and *spa* gene expression in *sarX* mutant of *S. aureus* SA75. **(A)** To investigate the effect of *sarX* on this adhesion protein, Spa expression in SA75, SA75-Δ*sarX*, and SA75-Δ*sarX*-C was determined by Western blotting, keeping the total amount of protein loaded for SDS-PAGE consistent in the three strains. **(B)** RT-PCR quantification of the effect of *sarX* mutant on *spa* gene expression. \* $P < 0.05$ .

opsonophagocytosis. Studies have shown that production of Spa in *S. aureus* is controlled by several global regulators including *agr*, *sarA*, *sarS*, *sarT*, *rot* and *mgrA*, which appear to form a regulatory network.<sup>25</sup> SarS, which seems to be a key regulator in this network, is a 250-residue two-domain SarA homolog that activates *spa* transcription.<sup>26</sup> SarX is a 141-residue single-domain SarA homolog. We speculate that SarX also may active *spa* transcription.<sup>26</sup> In the present study, deletion of *sarX* was associated with a 1.8-fold reduction in *spa* transcription as determined by RT-PCR, and this reduction could be complemented by *sarX*. Expression of Spa was also decreased in the *sarX* mutant strain. A previous investigation into protein-dependent biofilm production reported a function for Spa in the proportion of cell-to-cell interactions and biofilm formation.<sup>12</sup> Therefore, we speculate that biofilm formation in *S. aureus* may be associated with *spa*. However, it is not yet known what signals induce *spa* expression, how Spa regulates *sarX* expression, or precisely how SarX promotes *spa* transcription, and these questions could form the basis of future studies.

In conclusion, *sarX* promoted biofilm production that may primarily be mediated through increased *ica* operon expression and PIA production. Furthermore, deletion of *sarX* reduced *S. aureus* biofilm formation by downregulating *spa*.

## Abbreviations

PIA, polysaccharide intercellular adhere; MarR, multiple antibiotic resistance regulator; Bap, biofilm-associated protein; SA75, *Staphylococcus aureus* strain SA75; TSB, tryptic soy broth; LB, Luria broth; SEM, scanning electron microscopy; Spa, *Staphylococcus* protein A; OD, optical density; SDS-PAGE, sodium dodecyl sulfate polyacrylamide gel;

PVDF, polyvinylidene difluoride; CFU, colony-forming units; BSA, bovine serum albumin.

## Ethics Statement

As the *Staphylococcus aureus* clinical isolates in this study were a part of the routine hospital laboratory procedure, the Ethics Committee of Shanghai Pulmonary Hospital, Tongji University School of Medicine Academy of Sciences exempted this research for review.

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## Author Contributions

All authors made a significant contribution to the work reported, whether that is in the conception, study design, execution, acquisition of data, analysis and interpretation, or in all these areas; took part in drafting, revising or critically reviewing the article; gave final approval of the version to be published; have agreed on the journal to which the article has been submitted; and agree to be accountable for all aspects of the work.

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## Disclosure

The authors declare that they have no conflicts of interest.

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