Deletion of *SarX* decreases biofilm formation of *Staphylococcus aureus* in a polysaccharide intercellular adhesin (PIA) dependent manner

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Abstract

Background: Biofilm formation by *Staphylococcus aureus* is an important virulence determinant mediated by the polysaccharide intercellular adhere (PIA) encoded by *ica* operon or by surface and extracellular proteins. Previous studies have shown that *S. epidermidis* SarX protein regulated the transcriptional activity of the *agr* and *ica* loci and controled the biofilm phenotype, primarily by regulating *icaADBC* transcription and PIA production.

Results: In this study, our results indicated that biofilm formation and detachment of *S. aureus* were significantly decreased in the *sarX* mutant strain. *sarX* mutant in *S. aureus* biofilm formation was related to the production of PIA and not to that of eDNA. RT-PCR results showed that deletion of *sarX* was associated with a 1.8-fold reduction in *spa* transcription, which was complemented by *sarX*. Expression of Spa protein was decreased in *sarX* mutant strain.

Conclusions: *sarX* promoted biofilm production of *S. aureus* may mediated primarily through increasing *ica* operon expression and PIA production. Deletion of *sarX* was associated with reduction in *spa* transcription.

Introduction

Staphylococcus aureus is a human pathogen responsible for a wide diversity of community- and hospital-acquired infections, including relatively benign and fatal systemic disease (pneumonia, endocarditis, mastitis, osteomyelitis, etc.) [1, 2]. *S. aureus* can develop biofilms on host tissues and medical devices[3], leading to chronic infections, with up to 80% of chronic bacterial infections associated with biofilms[4]. Biofilms formation during *S. aureus* infection are difficult to treat. *S. aureus* can escape the immune defense of the dormitory and the attack of multiple immune factors by forming the biofilm. The dense biofilm can also prevent or delay the infiltration of antibiotic drugs, leading to bacteria at the bottom of the biofilm to produce drug resistance genes, reducing the sensitivity of bacteria to antibiotic drugs[5]. An evaluation of the biofilm formation process of *S. aureus* will likely contribute to our understanding of the infectious process.

Biofilm development and formation generally involve initial adhesion, proliferation, maturation, and diffusion [6]. Biofilm formation is modulated by transcriptional regulators (SarA, MgrA, and Rbf) and various regulatory systems (*agr* quorum sensing system) by regulating the production of biofilm formation associated factors (surface proteins, polysaccharide intercellular adhesin (PIA), eDNA, and other extracellular components). PIA, poly-NN-acetylglucosamine, were the most common constituents of staphylococcal biofilm, first found in *Staphylococcus epidermidis*[7]. The 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*). *ica* operon encodes the IcaA protein, which has N-acetamide glucanotransferase activity, which can synthesize UDP-N-acetylglucosamine into an oligomer structure with a chain length of 20 residues. *icaADBC* operon was negatively controlled by the IcaR and the teicoplanin-associated locus

regulator TcaR[8]. The transcription regulator TcaR, a member of the multiple antibiotic resistance regulator (MarR) family, is involved in teicoplanin and methicillin resistance in staphylococci[9]. Members of the SarA protein family in *S. aureus* share homology with each other as well as with the MarR family. SarX, a member of the SarA/MarR family of transcriptional regulators, was first identified in *S. aureus* by Manna and Cheung[10]. Transcription of *sarX* in *S. aureus* has been shown to be temporal and expressed maximally in stationary phase. Inactivation of *sarX* did not affect the expression of regulatory genes in the *sarA* family or *saeRS*, but it did have a significant negative effect on the transcription of *agr*. A study revealed that the *S. epidermidis* SarX protein regulates the transcriptional activity of the *agr* and *ica* loci and controls the biofilm phenotype, primarily by regulating *icaADBC* transcription and PIA production[11].

In addition, the cell wall-anchored proteins that participate in biofilm formation are biofilm-associated protein (Bap), ClfB, FnBPs, SasC, SasG and protein A (Spa)[12]. 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[13]. Spa, the first surface protein of *S. aureus*, was identified as a model for the characterization of the covalent anchorage of the LPXTG domain proteins by sortase A to the bacterial cell surface[14].

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

Materials And Methods

Bacterial strains, plasmid, and grow conditions.

The bacterial strains and plasmids used in this study were listed in Table 1. *Staphylococcus 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 l'Etoile, France). *S. aureus* and its derivative strains were grown in tryptic soy broth (TSB, BD) medium with 10 mg/l chloramphenicol at 37℃ with shaking at 220 rpm. *Escherichia coli* was grown in Luria broth (LB, Oxoid) medium with appropriate antibiotics (ampicillin at 100 mg/l and anhydrotetracycline at 50 ng/ml).

Construction of *S. aureus sarX* Mutant (SA75Δ*sarX*) and Complemented strain (SA75Δ*sarX*-C)

The *sarX* deletion mutant of the SA75 strain was constructed by allelic replacement using the temperature-sensitive plasmid pKOR1. The upstream and downstream fragments of sar*X* were amplified from SA75 genomic DNA using the *sarX*-UP-F/*sarX*-UP-R and *sarX*-DOWN-F/*sarX*-DOWN-R sets of primers (Table 2). The amplified products were digested with Kpn and then ligated with T4 DNA ligase to yield the homologous arm fragment with the deletion of *sarX* gene and then cloned into pKOR1. The recombinant plasmid pKOR1-Δ*sarX* was successively transferred into *E. coli* DH5α and DC10B, ultimately electroporated into the *S. aureus* SA75 competent cells. The allelic replacement mutants were selected using a previously described method and were further confirmed by PCR and sequencing [15].

To construct the *sarX* chromosomal complementation strain, the fragments covering the truncated region in the mutant strains were amplified from *S. aureus* strain SA75 genomic DNA using the *sarX*-F/*sarX*-R sets of primers (Table 2). The fragments of each gene were digested with restriction enzymes and then cloned into PRB473. The resulting plasmids, PRB473-*sarX* were electroporated into the mutant strain. The allelic replacement complementation strains were selected using the same 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[16]. Overnight cultures were inoculated to an optical density of 0.01 into fresh TSB medium. Total RNA was isolated and purified using a PureLink RNA Mini Kit (Invitrogen, Carlsbad, CA, United States); then was reverse transcribed into cDNA using a PrimeScript RT reagent kit (TaKaRa, Tokyo, Japan), according to the manufacturer's protocol using the oligonucleotides shown in Table 2. The real-time PCR was carried out using a SsoFas EvaGreen Supermix kit (Bio-Rad, United States) with the Bio-Rad CFX96 Manager software. SA75 wild type strain 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 method of delta delta Ct (ΔΔCt)[17]. Data analysis was carried out 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[18]. The overnight cultures were diluted 1:200 in fresh TSB. Two hundred microliter of the diluted cultures of different bacteria were pipetted into sterile 96-well polystyrene plates (BD Biosciences) with three attached wells of each bacteria, then incubated overnight at 37℃ without shaking. Then, the wells were washed gently three times with phosphate-buffered saline (PBS) (to remove nonadherent cells). Methanol (99.5%) was used to stabilized biofilms. The wells were stained with 200 μl of a 1% (w/v) crystal violet (Sigma) for 10 min, and then again washed three times with water. After drying, 30% glacial acetic acid was used to release the biofilm into the solution. The optical density at 600 (OD_{600}) was recorded.

Scanning Electron Microscopy

Biofilm formed on glass coverslips (10 mm in diameter) was observed by scanning electron microscopy (SEM) as previously described[19]. Briefly, overnight cultures were diluted 1:200 with TSB. A glass disk was put in advance into the bottom of the sterile flat bottomed polystyrene plates (Costar 3524; Corning, NY, United States) and 1 ml of the cell suspensions was added into the wells, with static culture at 37℃ for 24 h. The bacteria were cultured under the same conditions for colony counting to ensure that the amount of bacteria was consistent and the next experiment was carried out. Each well was rinsed three times with sterile PBS, each time for 10 min with slight shaking. Biofilms formed on the glasses 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. Thereafter, 1%

osmium tetroxide was used for a post-fixation step for 2 h at 4℃, followed by three times washed with distilled water for 10 min each, and 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. The observations were usually performed with a scanning electron microscope (FEI Quanta 200; United States). The experiment was repeated three times.

Triton X-100 Induced Autolysis

Autolysis assays were performed as described by Brunskill and Bayles[20]. The overnight cultures were cultured in 50 ml TSB containing 1 M NaCl until they reached the early exponential phase. Following centrifugation (4000g, 20min), the cells were washed twice with 50 ml of ice-cold water and resuspended into 50 ml of Tris-HCl (pH 7.2) containing 0.05% (vol/vol) TritonX-100. The OD_{600} value was adjusted to 1.0, followed by shaking of the culture at 37 ℃ for 3 h and was measured at 30 min intervals. The experiment was repeated three times.

Western Blot analysis

The protein expression level of *Staphylococcus* protein A (SPA) was determined by western blot analysis as previously described[21]. Culture supernatant was collected and washed twice with sterile PBS. Then, supernatant was suspended in 100 μl of PBS incubation at 37℃ for 2 h with 3 μl of lysostaphin (1 mg/ml) and centrifuged (8000 g, 30 min, 4 ℃). The protein concentration of each sample was determined by Bradford dye binding method. The samples were separated by 12% sodium dodecyl sulphate polyacrylamide gel (SDS-PAGE) and electrotransferred 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 Anti-Protein A (HRP) antibody at a 1/5000 dilution. The image was visualized using the ECL Western blotting assay kit (GE Healthcare) and the Chemi doc TM XRS system (Bio rad, USA).

PIA/PNAG detection

Polysaccharide intercellular adhesion (PIA) extracted from *S. aureus* was detected by a dot blot assay with a germ agglutinin horseradish peroxidase (WGA-HRP) conjugate according to a previously described protocol[22]. Briefly, overnight cultures were diluted to 10^7 colony-forming units (CFU) and added to 2 ml TSB at a ratio of 1:100 in the six-well polystyrene plates. An equal number of cells from each culture was resuspended in 0.5 M EDTA (pH 8.0) and incubated for 5 min at 100°C and centrifuged, and 40μl of the supernatant was incubated with 10μl of proteinase K (20 mg/ml) (Sangon Biotech) at 37°C for 2 h to minimize nonspecific background. Pipette 10 μl of the extracted PIA sample onto the PVDF membrane after formaldehyde treatment, keep the PVDF membrane moist during the spotting process, and dry the membrane at room temperature after spotting. The membrane was blocked with 3.5% bovine serum albumin (BSA) in PBS with 0.1% Tween 20. The membrane was placed in a dish containing wheat germ agglutinin conjugated to WGA-HRP, and incubated at 37 °C for 1 h. Then color developed with Pierce enhanced chemiluminescence (ECL) Western blotting substrate (Thermo Scientific, Rockford, IL).

Statistical analyses

All experiments were performed in biological triplicate. Statistical analysis was performed using SPSS statistical software (version23; IBM SPSS Statistic) and GraphPad Prism 8 (version 8.00, La Jolla, CA, United States). For the comparison of two groups, unpaired t test was used; for three or more groups, 1-way or 2-way analysis of variance was used, as appropriate. A value of $P < 0.05$ was considered statistically significant.

Results

The *sarX* mutation reduces *S. aureus* biofilm formation

We evaluated the effect of SarX on the biofilm formation capacity of *S. aureus* by comparing the *ΔsarX* mutant with SA75Δ*sarX*-C and SA75 strains. The decreased biofilm formation of the *sarX* mutant strain could not be the consequence of the growth defect. There was no significant difference of the growth rates among SA75, SA75-*ΔsarX*, and SA75-Δ*sarX*-C strains under the same inoculation and growth conditions (Fig.1c). In comparison with SA75, SA75-Δ*sarX* displayed a marked reduction in biofilm formation (Fig. 1a). In contrast, SA75-Δ*sarX*-C formed biofilms similar to the SA75 strain when the strains were incubated under the same conditions. The semi quantification result for biofilm was shown in Fig.1b. The mature biofilm of the SA75-Δ*sarX* strain was significantly increased compared to that of SA75 strain (3.31-fold, $P<0.05$). However, the density of the mature biofilm of SA75-Δ*sarX*-C was similar to the wild type strain.

Scanning Electron Microscopy

Scanning electron microscopy can be used to observe the formation of biofilm. As shown in Fig. 3, SA75 and SA75-Δ*sarX*-C could be seen to accumulate and formed biofilm obviously, the bacteria clustered together, and the bacteria and bacteria adhere to each other to form a compact biofilm structure. However, the thickness of mature biofilms decreased in SA75- Δ*sarX* strain. The biofilm formed by strain of SA75- Δ*sarX* was significantly thinner than SA75. Similar to the biofilm formation results, the biofilm formation of SA75-Δ*sarX*-C was similar to that of SA75, but there was no adhesion between the colonies, and the profile of a single colony can be clearly observed on the surface of the agglomerated biofilm.

The reduced biofilm formation of the *sarX* mutant strain is PIA dependent

The PIA production was determined semi quantitatively with WGA-HRP conjugate sing a dot blot 96 system (Figure 2a). Consistent with the result of RT-PCR, the *sarX* mutant strain displayed much weaker PIA production compared with the wild type strain, while the complemented strain exhibited restored PIA production. In order to investigate the effect of gene *sarX* on biofilm matrix production, the transcription *icaADBC* operon in the wild type SA75, SA75-Δ*sarX* and SA75-Δ*sarX*-C strains using real-time RT-PCR, and the release of PIA was determined. As shown in Fig. 2b, the expression of *icaA* in SA75-Δ*sarX* was

significantly reduced, indicating that knockout of *sarX* would inhibit the expression of *icaA*. The expression level of SA75-Δ*sarX*-C can be restored to the wild level.

The autolysis capacity

Autolysis of cells resulted in the release of intracellular eDNA, which can promote adhesion and aggregation between cells and promote the formation of biofilms. We want to know whether the decreased biofilm formation of *sarX* strain was associated with autolysis or not. The autolytic capacity of SA75, SA75-Δ*sarX* and SA75-Δ*sarX*-C were assessed by TritonX-100 introduction. *Staphylococcus epidermidis* 1457Δ*atlE* was used as the control strain. As shown in Fig. 2c, no difference was found between SA75-Δ*sarX* and its parent strains in the TritonX-100 induced autolysis.

Detection of *Staphylococcus* protein A (SPA) in *S. aureus*

Staphylococcus protein A (SPA) is an important adhesion protein of *S. aureus*. It can enhance the adhesion between bacteria and promote the formation of biofilms. As shown in figure 4a, we extracted the SPA of three strains of bacteria (SA75, SA75-Δ*sarX* and SA75-Δ*sarX*-C), and carried out while keeping the total amount of protein loaded SDS-PAGE, Western Blot method was used to detect the protein expression of SPA. The production level of SPA in *sarX* mutant strain was much lower than the SA75 strain and *sarX* complemented strains. The results showed (Figure 4b) that the expression of *spa* in SA75-Δ*sarX* was significantly reduced, indicating that knockout of *sarX* would inhibit the expression of *spa*. The expression level of SA75-Δ*sarX*-C can be restored to the wild level.

Discussion

The production of PIA is an important contributing factor to biofilm formation by staphylococci*. icaADBC* encoding PIA are subject to regulation by numerous factors. Transcription of *icaADBC* is also subject to positive regulation by SarA. 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 is composed of at least 11 different proteins. Manna and Cheung found that *sarX* affected biofilm formation in *S. aureus* 8325-4, while did not in *S. aureus* RN6390 [10]. It is still unknown that reasons of the difference exists between RN6390 and 8325-4. Because they are closely related strains derived from the same parent strain (NCTC8325). In present study, we found that deletion of *sarX* failed to stimulate biofilm formation, while complement *sarX* mutant isolate exhibited the same levels of biofilm formation as the clinical isolate *S. aureus* SA75. In brief, our results revealed a positive role for *sarX* in *S. aureus* biofilm regulation. In addition, RT-PCR results revealed that deletion of *sarX* was associated with a 2-fold reduction in *icaA* transcription, which was complemented by *sarX*. PIA production was increased in SA75-Δ*sarX* strain. The results revealed here that *sarX* mutant in *S. aureus* biofilm formation was related to the production of PIA and not to that of eDNA. Similarly, David Cue et al. demonstrate that Rbf and SarX represent a regulatory cascade that promotes PIA-dependent biofilm formation in *S. aureus* [23]. Rbf, member of the AraC/XylS family of transcriptional regulators, increase *icaADBC* expression by upregulating transcription of *sarX*. SarX, in turn, activates *icaADBC* expression[23].

The *spa* gene encodes protein A, a multifunctional cell wall protein that binds immunoglobulins, thereby inhibiting opsonophagocytosis. In present study, RT-PCR results showed that deletion of *sarX* was associated with a 1.8-fold reduction in *spa* transcription, which was complemented by *sarX*. Expression of Spa protein was decreased in *sarX* mutant strain. Previous study found that function for Spa in the proportion of cell to cell interactions and biofilm formation by investigating the protein dependent biofilm production[12]. Therefore, we speculated biofilm formation in *S. aureus* may associated with *spa*. However, many questions remain as we do not completely understand what signals induce *spa* expression, how Spa regulates *sarX* expression, or precisely how SarX promotes *spa* transcription.

In conclusion, *sarX* promoted biofilm production may mediated primarily through increased *ica* operon expression and PIA production. Deletion of *sarX* was associated with reduction in *spa* transcription.

Declarations

Ethics approval and consent to participate

This study was approved by the Ethics Committee of the first Affiliated Hospital of Wenzhou Medical University. All methods were carried out in accordance with relevant guidelines and regulations. All experimental protocols were approved by a named institutional and/or licensing committee. Informed consent was obtained from all subjects.

Consent for publication

Not applicable

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Authors' contributions

ZH, YG, JY, LR, QZ and YX performed the laboratory measurements. BW and XW made substantial contributions to conception and design. ZH drafted the manuscript. YG and FY revised the manuscript critically for important intellectual content. All authors read and approved the final manuscript.

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the manuscript.

Availability of data and materials

The datasets used during the current study are available from the corresponding author upon reasonable request. All the data is included in this published article.

Consent for publication

Not applicable.

Competing interests

The authors declare they have no competing interests.

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 sulphate polyacrylamide gel

PVDF: polyvinylidene difluoride

CFU: colony-forming units

BSA: ovine serum albumin

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Tables

Table 1. Bacterial strains and plasmids used in this study

Strains and Plasmids	Description [a]	Source
Strains		
S. aureus SA75	Wild type, clinical MRSA strain	The First Affiliated Hospital of Wenzhou Medical University
SA75Δ*SARX*	Isogenic *SARX* deletion mutant in SA75	This study
SA75Δ*SARX*-C	*SARX* mutant complemented with pRB*SARX*	This study
DH5	*Escherichia coli* isolates, clone host strain	Laboratory stock
DC10B	*Escherichia coli* isolates, dam+Δdcm$^-$hsdRMS endA1 recA1; clone host strain	Laboratory stock
Plasmids		
pKOR1	Shuttle cloning vector, temp sensitive (Cm^R, Amp^R)	Laboratory stock
pRB473	Shuttle cloning vector (Cm^R)	Laboratory stock

[a] Cm^R, chloramphenicol resistance; Amp^R, ampicillin resistance.

Table 2. Primers used in this study

Primer	Primer sequence (5’-3’)	Note
sarX-F	AAGATAATAATGACAGAAGC	
sarX-R	AAAATTGTTCTACATCTTCA	
sarX-UP-F	GGGGACAAGTTTGTACAAAAAAGCAGGCTAGCGACTTAAATTCGATTCGTTA	attB1
sarX-UP-R	CGGGGTACCGTCTTTCTCATTTGTTTTTAATACG	KpnI
sarX-DOWN-F	CGGGGTACCTTGAAATTGAGTGTCGAAAGCATAG	KpnI
sarX-DOWN-R	GGGGACCACTTTGTACAAGAAAGCTGG GTTCCATTGTTCTGCTGATT	attB2
sarX-RT-F	AACATTGCTTGGCTTCTAT	
sarX-RT-R	AATCTAGCTCATCCATTGC	
sarX-C-F	CGGAATTCCG ACACCTTGATATGTATTGCA	EcoRI
sarX-C-R	CGGGATCCCG CATTATTGAACTACGATTCC	BamHI
icaA-RT-F	CTTGGATGCAGATACTATCG	
icaA-RT-R	GCGTTGCTTCCAAAGACCTC	
spa-RT-F	CAATTCTAGCTATTATCACTT	
spa-RT-R	ATTAATACCCCCTGTATGTA	

Figures

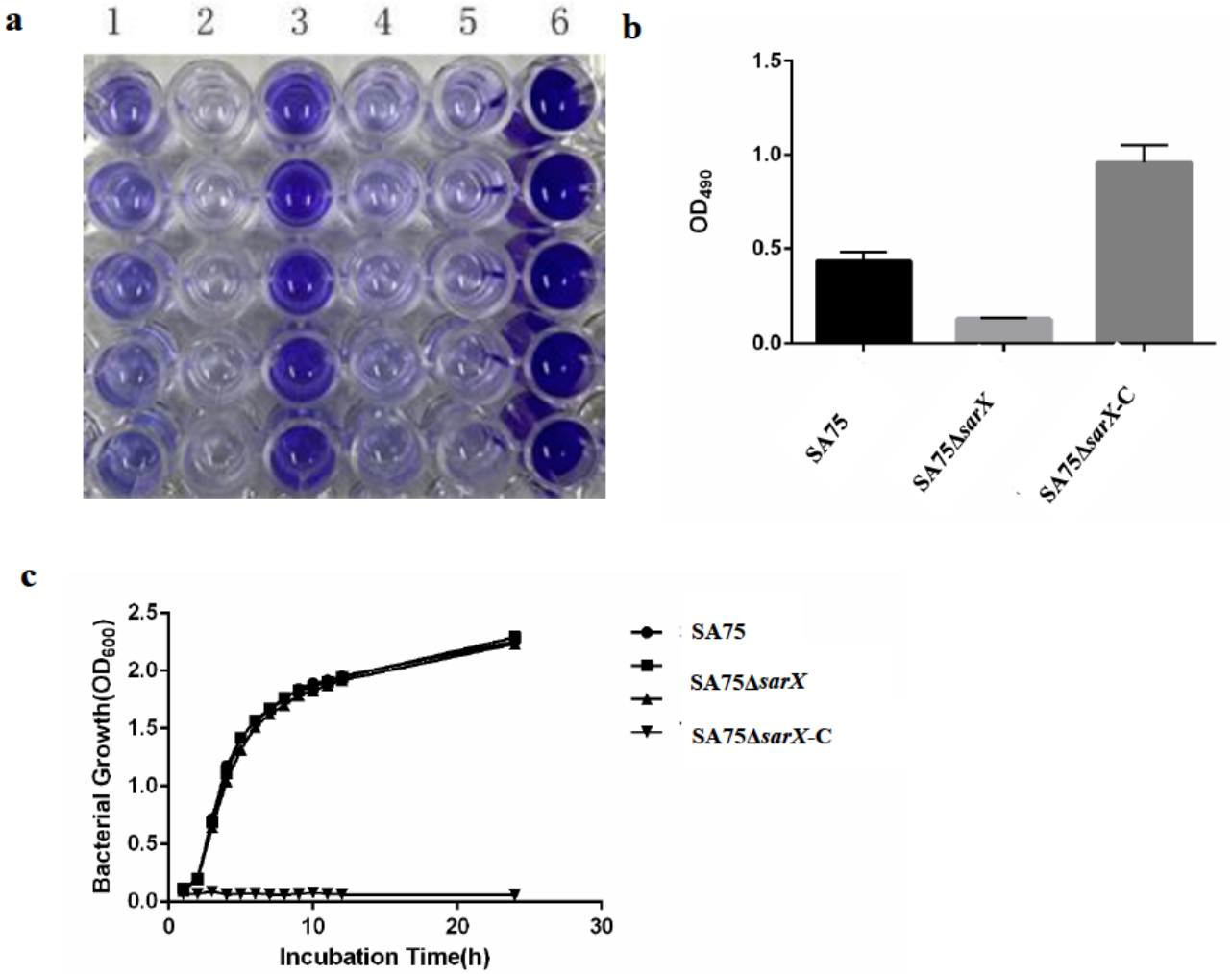


Figure 1

The sarX mutation reduces S. aureus biofilm formation. (a): Biofilm formation by SA75 wild-type (1), SA75ΔsarX (2) and SA75ΔsarX-C (3) strains on microtiter plates. 4: blank control. 5: negative control. 6: positive control. (b): Biofilm quantification. Glacial acetic acid (33%) was used to release the biofilms into solution. Each experiment was repeated three times, and the data represent means + standard deviations. (c): Growth of the SA75 wild-type, ΔsarX mutant and chromosomal complemented strains.

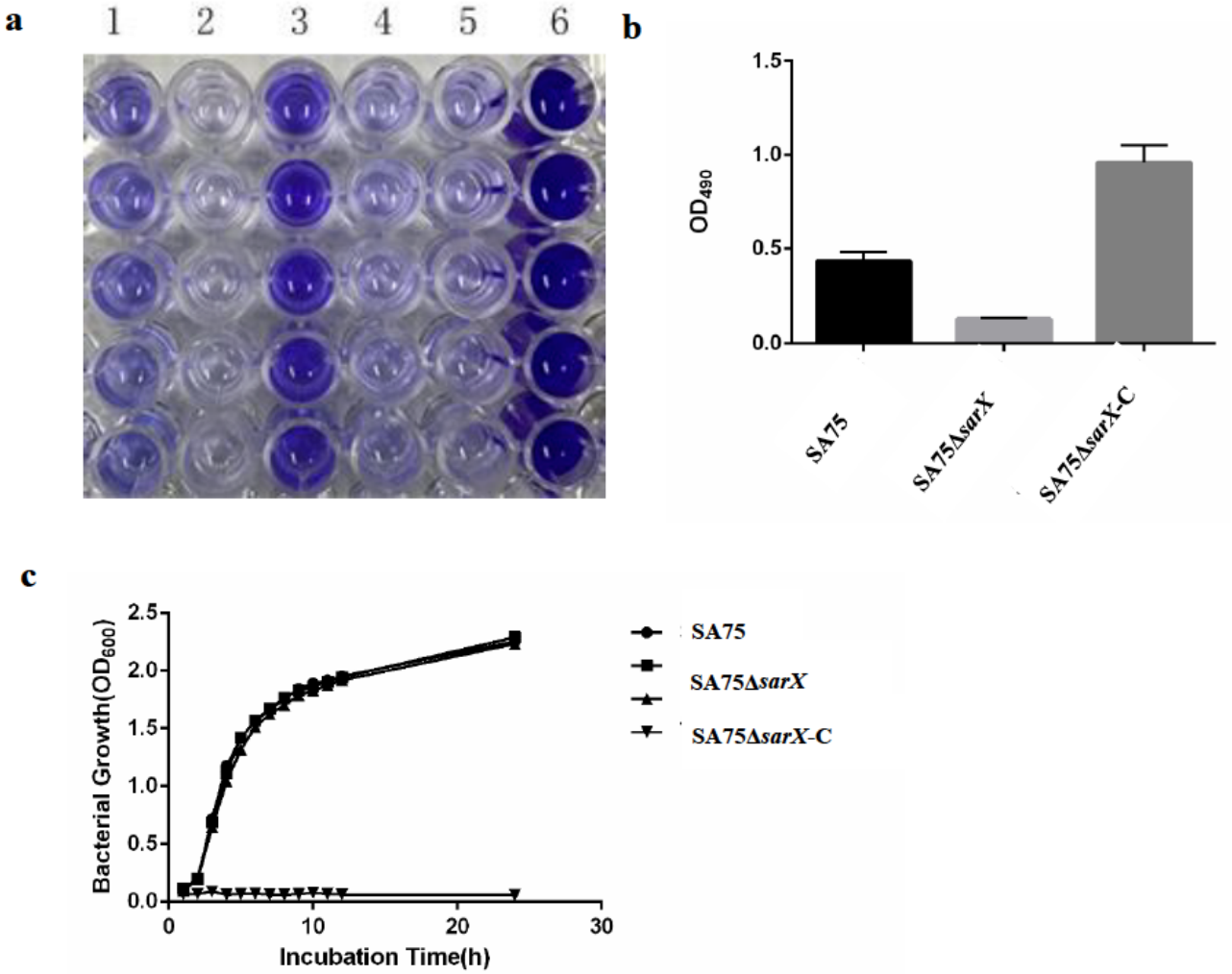


Figure 1

The sarX mutation reduces S. aureus biofilm formation. (a): Biofilm formation by SA75 wild-type (1), SA75ΔsarX (2) and SA75ΔsarX-C (3) strains on microtiter plates. 4: blank control. 5: negative control. 6: positive control. (b): Biofilm quantification. Glacial acetic acid (33%) was used to release the biofilms into solution. Each experiment was repeated three times, and the data represent means + standard deviations. (c): Growth of the SA75 wild-type, ΔsarX mutant and chromosomal complemented strains.

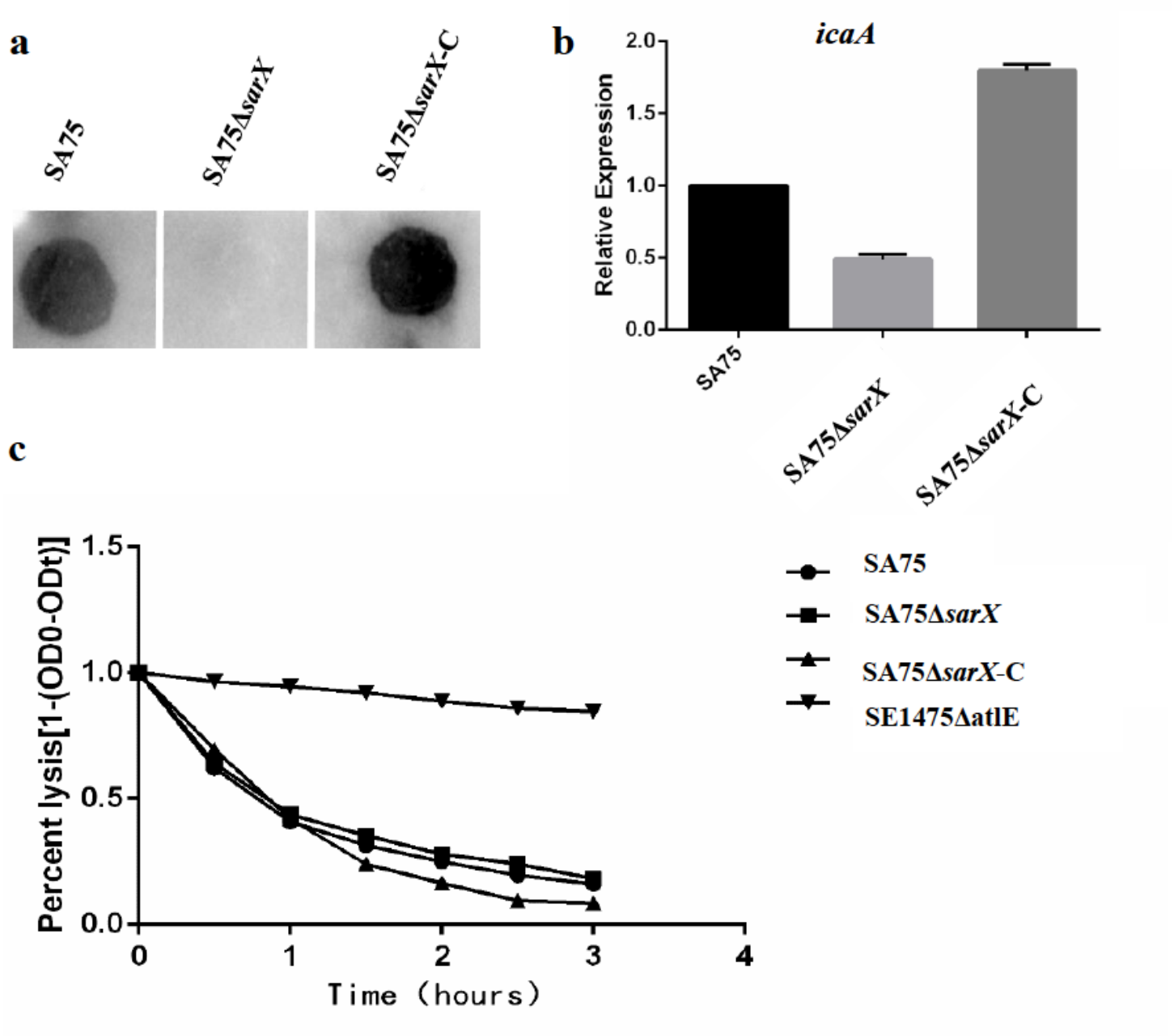
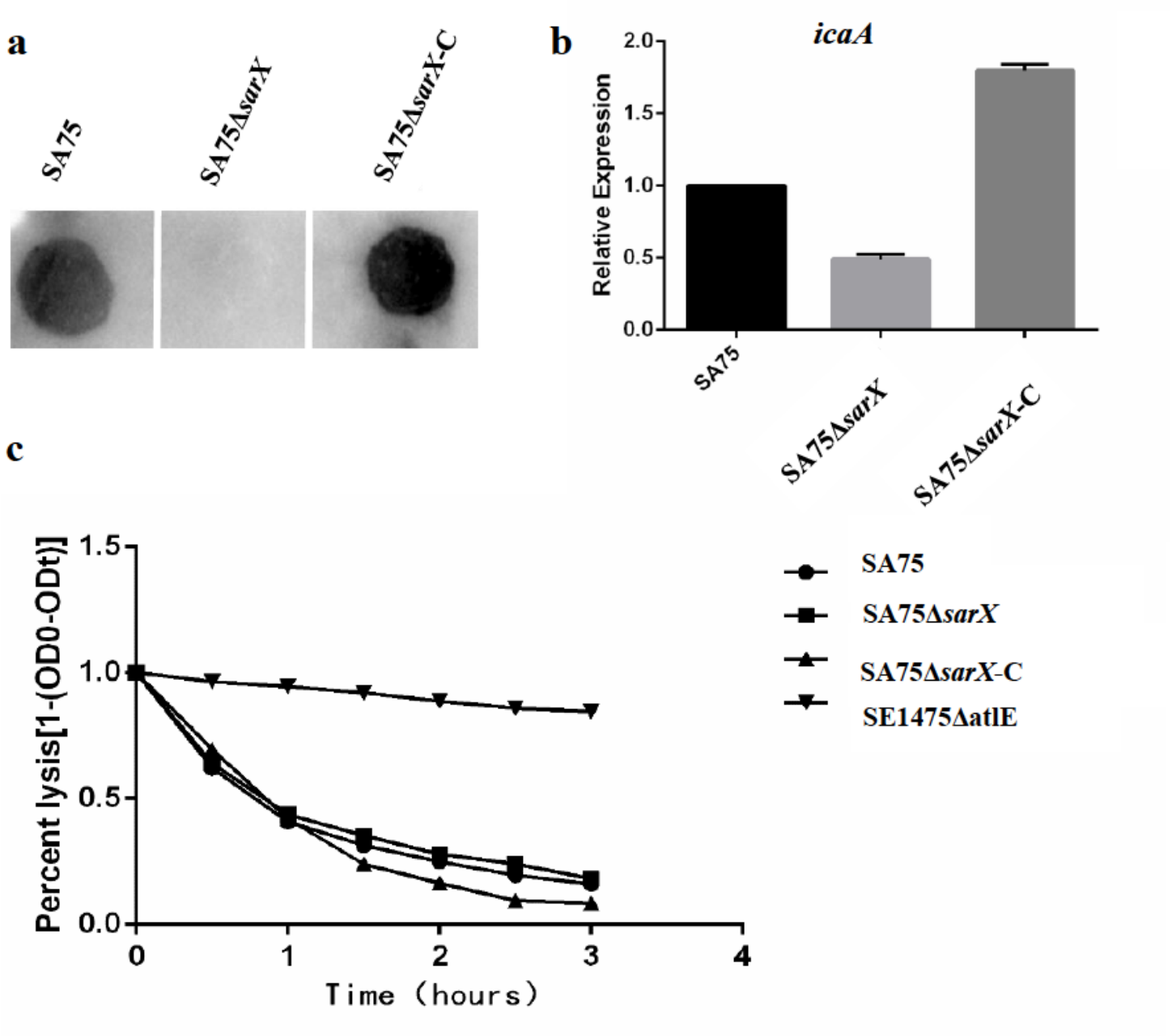


Figure 2

The reduced biofilm formation of the sarX mutant is PIA dependent. (a) PIA biosynthesis was semi quantified using a dot blot assay with WGA. (b) Reverse transcription-PCR (RT-PCR) quantification of the effect of sarX mutant on icaA gene expression by the SA75 strain. Comparison of the autolytic abilities of SA75 wild-type, SA75ΔsarX and SA75ΔsarX-C strains. TritonX-100 induced



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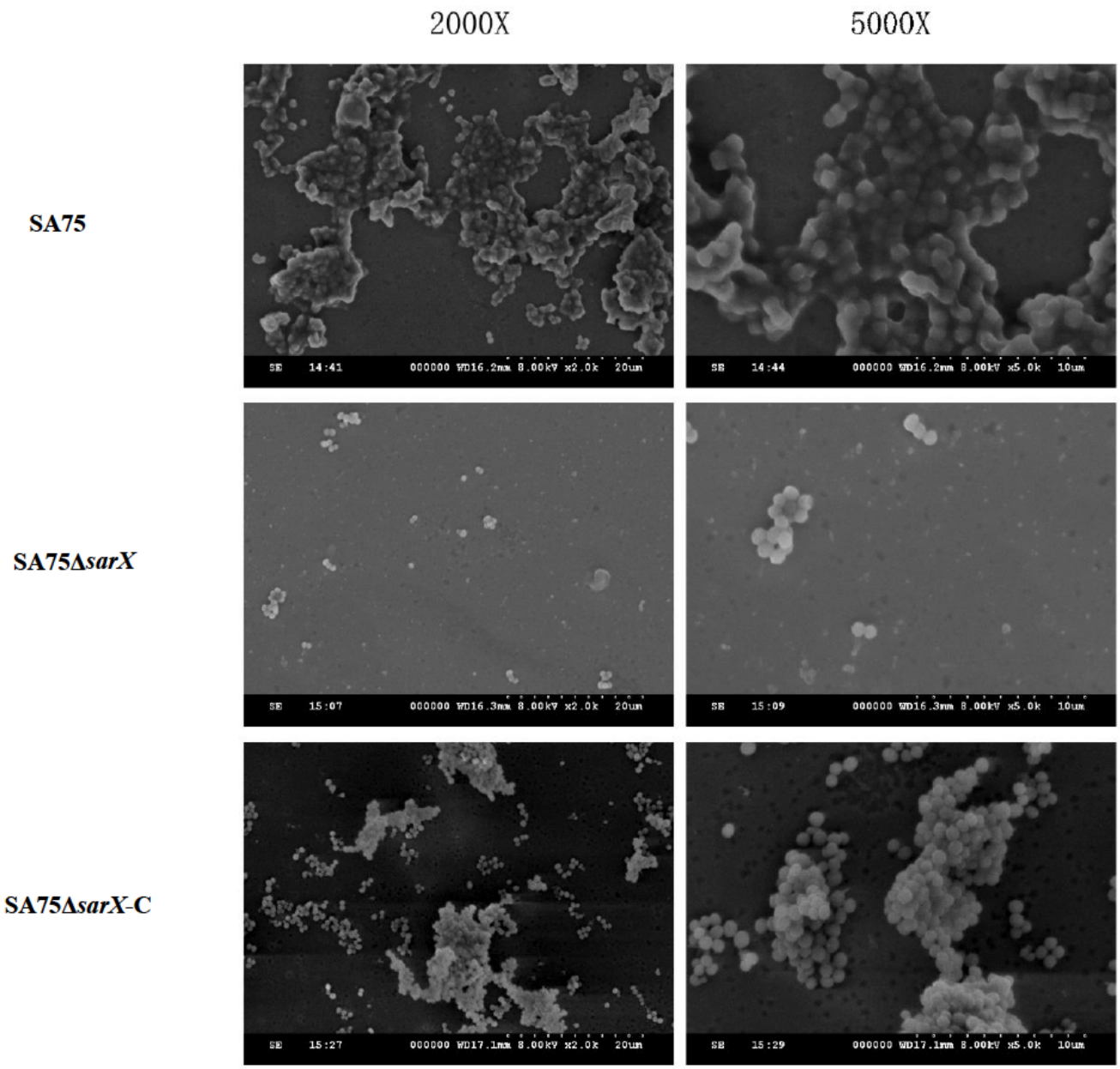
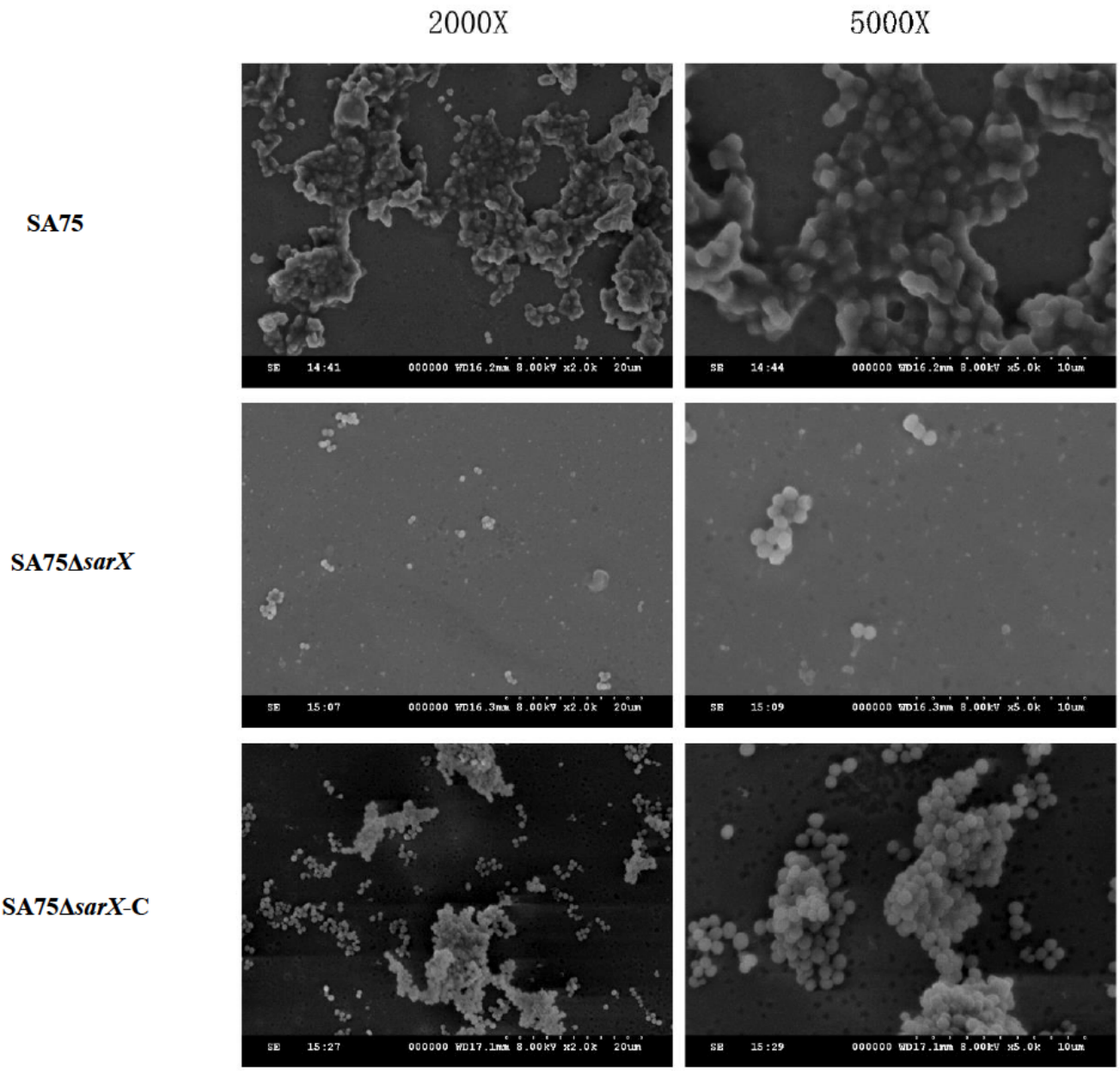


Figure 3

Scanning electron microscope study. Transmission electron micrograph of biofilm in mature stage of wild type (SA75), isogenic sarX mutant, and SA75ΔsarX-C strains. The images on the left side were magnified 2,000 times, and images on the right sides were magnified 5,000 times.



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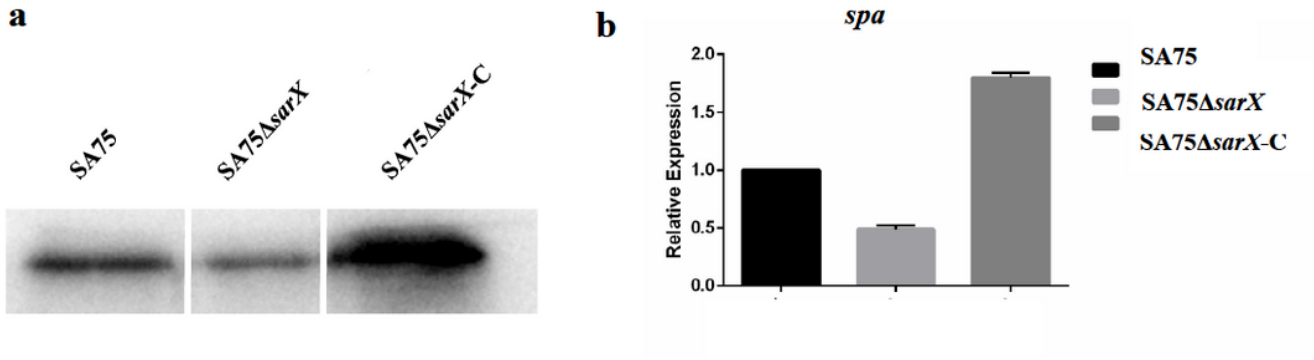
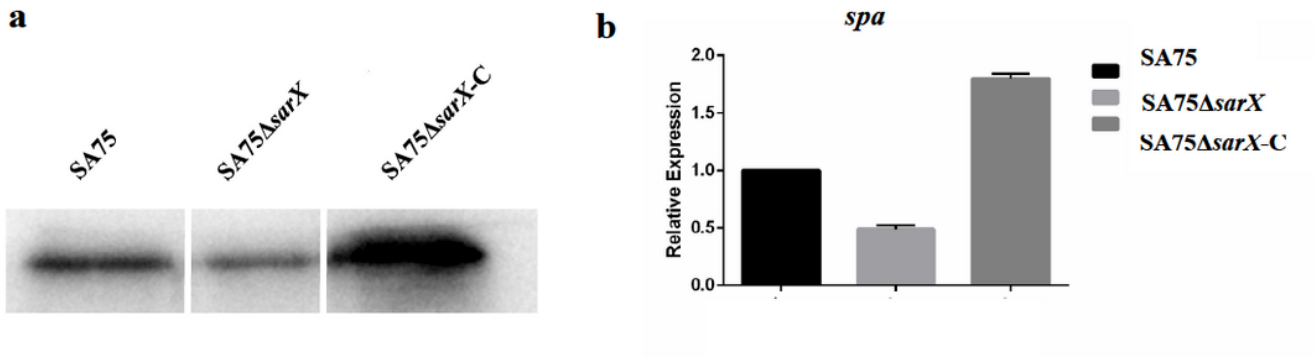


Figure 4

SPA expression levels of SA75, SA75-ΔsarX, and SA75-ΔsarX-C RT-PCR results of spa



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SPA expression levels of SA75, SA75-ΔsarX, and SA75-ΔsarX-C RT-PCR results of spa

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