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BASIC RESEARCH

Depression of biofilm formation and antibiotic resistance by *sarA* disruption in *Staphylococcus epidermidis*

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Abstract

AIM: To study the effects of disruption of *sarA* gene on biofilm formation and antibiotic resistance of *Staphylococcus epidermidis* (*S. epidermidis*).

METHODS: In order to disrupt *sarA* gene, the double-crossover homologous recombination was applied in *S. epidermidis* RP62A, and tetracycline resistance gene (*tet*) was used as the selective marker which was amplified by PCR from the pBR322 and inserted into the locus between *sarA* upstream and downstream, resulting in pBT2△*sarA*. By electroporation, the plasmid pBT2△*sarA* was transformed into *S. epidermidis*. Gene transcription was detected by real-time reverse transcription-PCR (RT-PCR). Determination of biofilm was performed in 96-well flat-bottomed culture plates, and antibiotic resistance was analyzed with test tube culture by spectrophotometry at 570 nm respectively.

RESULTS: A *sarA* disrupted strain named *S. epidermidis* RP62A△*sarA* was constructed, which was completely defective in biofilm formation, while the *sarA* complement strain RP62A△*sarA* (pHPS9*sarA*) restored the biofilm formation phenotype. Additionally, the knockout of *sarA* resulted in decreased erythromycin and kanamycin resistance of *S. epidermidis* RP62A. Compared to the original strain, *S. epidermidis* RP62A△*sarA* had an increase of the sensitivity to erythromycin at 200-400 μg/mL and kanamycin at 200-800 μg/mL respectively.

CONCLUSION: The knockout of *sarA* can result in the defect in biofilm formation and the decreased erythromycin and kanamycin resistance in *S. epidermidis* RP62A.

Key words: *sarA*; Biofilm; Antibiotic resistance; Staphylococcus epidermidis

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INTRODUCTION

Staphylococcus epidermidis (*S. epidermidis*), a normal inhabitant of human skin and mucous membranes, is the predominant cause of foreign-body-associated infections. The pathogenesis of *S. epidermidis* infections is correlated with its ability to form biofilms on polymer surfaces[1,2]. Biofilm formation proceeds in two phases[3,4]. Primary attachment of bacterial cells to a polymer surface is a complex process influenced by a variety of factors, including hydrophobic interactions, presence of host proteins, and specific bacterial proteins and polysaccharides like the capsular polysaccharide adhesin, the autolysin AtlE, and other staphylococcal surface proteins[5,6]. This is followed by the second phase leading to accumulation of bacteria in a multilayered biofilm embedded in an amorphous glycocalyx. This phase is a multifactorial process that is influenced by a number of factors. Among the most important of these is polysaccharide intercellular adhesion (PIA). Synthesis of polysaccharide intercellular adhesin is essential for bacterial cell accumulation because it mediates cell-to-cell adhesion of proliferating cells[7,8]. PIA consists of two polysaccharide species which are composed of β-1, 6-linked 2-deoxy-2-amino-D-glucopyranosyl residues containing non-N-acetylated amino groups, phosphate, and succinate. The enzymes required for polysaccharide intercellular adhesin synthesis are encoded within the *icaADBC* operon, mutation of which results in a reduced capacity to form a biofilm in *S. epidermidis*[7]. *SarA*, a central regulatory element that controls the production of *Staphylococcus aureus* (*S. aureus*) virulence factors, is essential for the synthesis of PIA and the ensuing biofilm development in this species[9,10]. Based on the presence of a *sarA* homolog, we hypothesized that SarA could also be involved in the regulation of the biofilm formation process in *S. epidermidis* To elucidate the possible role of SarA in biofilm formation, we used

Table 1 Strains and plasmids used in the study

Strains or plasmids	Relevant characteristics	Sources or references
Strains		
S. epidermidis ATCC12228	Biofilm negative	ATCC
S. epidermidis RP62A (ATCC 35984)	Biofilm positive Kan^r, Ery^r, Ap^r	ATCC
S. epidermidis RP62A△*sarA*	*sarA* deletion on the chromosome, Tet^r, *sarA*::*tet*	This study
S. epidermidis RP62A (pHPS9*sar*A)	*S. epidermidis* RP62A△*sarA* harboring pHPS9*sarA*	This study
S. aureus RN4220	Restriction-negative, intermediate host for plasmid transfer from *E. coli* to *S. epidermidis*	[14]
E. coli DH5α	ϕ80d*lacZ*△M15 *rec*A1 *end*A1 *gyr*A96 *thi*-1 *hsd*R17 (r_K^- m_K^+) *sup*E44 *rel*A1 *deo*R △(*lac*ZYA-*arg*F) *U*169	Promega Co.
Plasmids		
pBR322	Donator of *tet* gene (Tet^r). Ap^r Tet^r	[15]
pBluescriptSK	*E. coli* cloning vector.Ap^r	[11]
pBT2	Temperature-sensitive shuttle vector. Ap^r (*E. coli*) Cm^r (*Staphylococcus*)	[16]
pBT△*sarA*	Integration vector for homologous recombination of the△*sarA* gene in *S. epidermidis*; *tet* inserted into *sarA* locus as resistance selection marker	This study
pHPS9	Expression shuttle vector	[17]
pHPS9*sarA*	pHPS9 inserted *sarA* gene	This study

tet^r, resistance to tetracycline; kan^r, resistance to kanamycin; ery^r, resistance to erythromycin; Ap^r, resistance to ampicillin; Cm^r, resistance to chloramphenicol.

a genetic approach and constructed an *S. epidermidis sarA::tet* knockout mutant of the biofilm-forming strain *S. epidermidis* RP62A. Biofilm formation and *ica* expression of the mutant were compared with the phenotypes of the corresponding wild-type strain and a complemented strain that carried a *sarA* copy in an expression vector.

We were interested in the potential role of SarA in the response of *S. epidermidis* to antimicrobial agents. Therefore, we used this *sarA* knockout mutant and determined its influence on erythromycin and kanamycin resistances in *S. epidermidis* RP62A (i.e. ATCC35984).

MATERIALS AND METHODS

Bacterial strains, plasmids, and growth medium

The bacterial strains and plasmids used in this study are listed in Table 1. Tryptic soy broth (TSB) was used to grow *Staphylococcus* strains. Luria-Bertani (LB) was used for *E coli*. Chloramphenicol was used at 15 μg/mL, ampicillin at 100 μg/mL and tetracycline at 10 μg/mL. Bacteria were grown on Congo red agar (CRA) plates, which were composed of TSB agar supplemented with 5% sucrose (A.R.) and 0.8 mg of Congo red/L (A.R.). All DNA manipulations and handling of *E coli* were performed in accordance with standard protocols[11]. Manipulations with *Staphylococcus* were performed as described previously[12,13].

Construction of plasmids and sarA::tet allele replacement

In order to analyze its function in *S. epidermidis, sarA* was replaced by a tetracycline resistance gene (*tet*) by homologous recombination. The upstream of 836 bp, fragment 1, was amplified using primer pairs (1) 5'-ACGAAGCTTC TGTAACATCT AGTGACAA-3' and (2) 5'-ACGCTGCAGT TTAATCTGTC AGCATAAGTG-3' with *Hin*dIII and *Pst*I respectively. The downstream of 857 bp, fragment 2, was amplified using primer pairs (3) 5'-ACGCTGCAGA TTATAAACAA CCTCAAGTTG-3' and (4) 5'-ACGGAATTCG GGCATCATTG CGAGTGA-3' with *Pst*I and *Eco*RI respectively. The two fragments were cloned into the multi-cloning region of temperature-sensitive *E. coli-Staphylococcus* shuttle vector pBT2[16], resulting in plasmid pBT2-1. A fragment of 1276 bp containing the entire *tet* gene was amplified by PCR from the pBR322, using the primers (5) 5'-CGCGCGGCCG CTTCTCATGT TTGACAGCTT-3' and (6) 5'-GCGAGATCTT CAGGTCGAGG TGGCC-3'. The *tet* gene was inserted into the vector pBT2-1, resulting in plasmid, pBT2△*sarA*. Following passage through the restriction-negative strain *S.aureus* RN4220, pBT2△*sarA* was reisolated and transformed into *S. epidermidis* RP62A by electroporation. Replacement of the chromosomal *S. epidermidis* RP62A *sarA* wild-type gene was obtained by double-crossover integration of the *sarA::tet* insert of pBT2 △*sarA* following a temperature shift to the nonpermissive temperature (42℃) of the shuttle vector[18]. Tetracycline-resistant and chloramphenicol-sensitive colonies were isolated. The *sarA*::*tet* integrations were confirmed by PCR detection (Figure 1 step 4) and the nucleotide sequencing was carried out by Shanghai Bioasia.

RNA purification and RT-PCR

RNA purification, real-time reverse transcription-PCR (RT-PCR) and analysis of RT-PCR data were performed as previously described[19], with the following oligonucleotide primer pairs: for *gyrB* transcript, (7) 5'-TTATGGTGCT GGACAGATAC A-3' and (8) 5'-CACCGTGAAG ACCGCCAGAT A-3'; for *icaA* transcript, (9) 5'-AACAAGTTGA AGGCATCTCC-3' and (10) 5'-GATGCTTGTT TGATTCCCT-3'. The *gyrB* gene was compositively expressed in *S. epidermidis* and thus used as an internal standard in these RT-PCR experiments[19].

Phenotypic assay on biofilm production

The biofilm production assay was performed by cultivation of the *S. aureus* and *S. epidermidis* strains on CRA plates as described by Freeman *et al*[14]. The black, rough and dry colonies on CRA plates indicated the biofilm production. In contrast, the biofilm-negative strains formed red, smooth colonies.

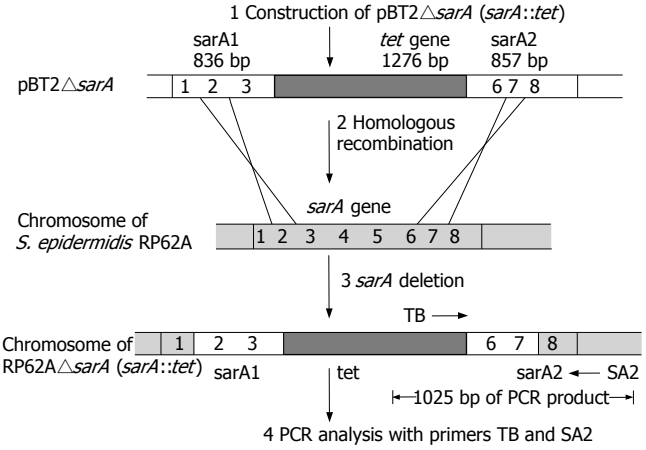

Figure 1 Disruption and detection of chromosomal *sarA* locus in *S. epidermidis*. 1. Insertion of *tet* gene into *sarA* locus to form recombinant plasmid pBT△*sarA*. 2. Double-exchange homologous recombination between chromosomal DNA and plasmid DNA. 3. Locus *sarA* of chromosome was destroyed by DNA recombination. 4. Detection of recombination by PCR amplification with primers TB and SA2.

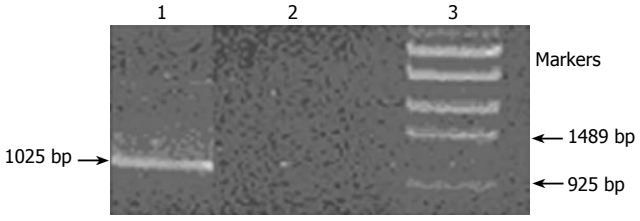

Figure 2 Detection of *sarA* gene locus in chromosome DNA by PCR with primers SA2 and TB. Lane 1, The product was amplified from *S. epidermidis* RP62A△*sarA* with tet insertion; lane 2, No product was amplified from *S. epidermidis* RP62A without tet insertion; lane 3, DNA markers.

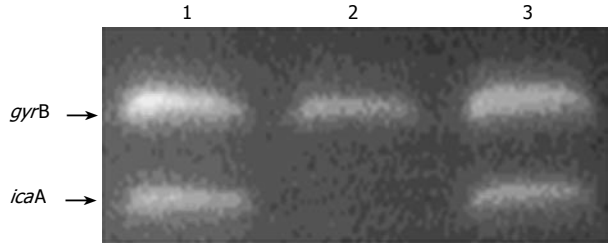

Figure 3 Identification of the genes *ica*A and *gyr*B transcription in *S. epidermidis* by RT-PCR analysis. 1. *S. epidermidis* RP62A; 2. *S. epidermidis* RP62A△*sarA*; 3. *S. epidermidis* RP62A△*sarA* (pHPS9*sarA*).

Quantitative determination of biofilm formation

Strains were cultivated overnight (16 h) in 96-well flat-bottomed tissue culture plates at 37℃ in TSB growth medium. Based on the optical densities (OD_{570}) of the biofilm, the strains were classified as non-adherent strains ($OD_{570} \leqslant 0.120$).

Determination of antibiotic susceptibilities

Three antibiotics were used to determine the impact of *sarA* mutant on *S. epidermidis* resistance to erythromycin or kanamycin. Erythromycin or kanamycin was added into test tubes with a twofold serial dilution scheme (0, 25, 50, 100, 200, 400, 800 μg/mL)[20,21]. The inoculum was derived from overnight liquid cultures in TSB and was inoculated into shaking liquid cultures containing erythromycin or kanamycin. After overnight incubation (16 h) at 37℃, these inocula were determined with spectrophotometer at 570 nm.

RESULTS

Disruption of sarA gene on the chromosome DNA of S. epidermidis RP62A

A strain termed *S. epidermidis* RP62A△*sarA* (*sarA*::*tet*) derived from RP62A with an allele replacement of the *sarA* gene was obtained (Figure 1).

PCR analysis of DNA from strains RP62A and RP62A △*sarA* was performed with two primers as shown below: TB: 5'-ACGGAGCTCA AGCCTATGCC TACAGCA-3' (in the central part of the *tet* gene) (Figure 1); SA2: 5' -ACGGAATTCG GGCATCATTG CGAGTGA-3' (next to the downstream of *sarA*2 fragment) (Figure 1). The fragment was obtained as shown in Figure 2, which indicated that allelic replacement had taken place. The PCR-amplified fragment was further demonstrated by DNA sequencing, and the result revealed that a 1025 bp fragment was composed of part of *tet* gene and chromosomal DNA (the datum of DNA sequence not shown).

Repression of icaA transcription in S. epidermidis RP62A by disruption of sarA

To investigate whether the transcription of *ica* operon expression was altered in the *sarA* mutant strain, RT-PCR was used to measure *icaA* transcription in variants grown in TSB. Total RNA of the original RP62A strain, its *sarA* mutant and corresponding complementary strain were isolated at early exponential and mid-log exponential phases, as the expression of *ica* operon was at maximum during this period. After treatment with DNase to remove contaminant DNA, RNA was reverse transcribed in the presence and absence of reverse transcriptase. The level of expression of *ica*A was normalized to *gyrB* expression[19]. Our results showed that the level of *ica* operon transcription was apparently reduced in the *sarA* mutation compared to that of the wild-type strain at exponential and mid-log exponential phases (Figure 3, Lane 2). Interestingly, in the *sarA* complementation strain, designated as RP62A△*sarA* (pHPS9*sarA*), the *icaA* transcription was activated (Figure 3, lane 3). Consistent with this, at the phenotypic level, the capacity of RP62A △*sarA* (pHPS9*sarA*) to form biofilm was restored in TSB. These data suggested that the gene of *sarA* in the strain of RP62A is responsible for activating *ica* operon expression.

Depression of biofilm formation of S. epidermidis RP62A by inactivation of sarA

Phenotypic assay on biofilm production: The *S. epidermidis* RP62A strain formed typical black, rough colonies after 24 h of incubation. The non-slime producing *S. epidermidis* ATCC 12228 formed smooth, red colonies. RP62A△*sarA* strain produced smooth, red colonies after 24 h, demonstrating the mutant was biofilm-negative. As to the

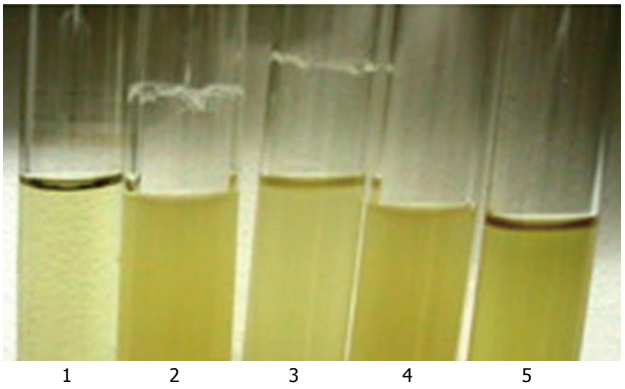

Figure 4 Biofilm formation on the glass surface in the tube culturing *S. epidermidis*. 1. TSB medium not inoculated; 2. Biofilm-positive strain RP62A (biofilm-forming); 3. *sarA* complemented strain RP62A△*sarA* (pHPS9*sarA*) (biofilm-forming); 4. *sarA* deletion strain RP62A△*sarA* (non-biofilm-forming); 5. Biofilm-negative strain ATCC12228 (non-biofilm-forming).

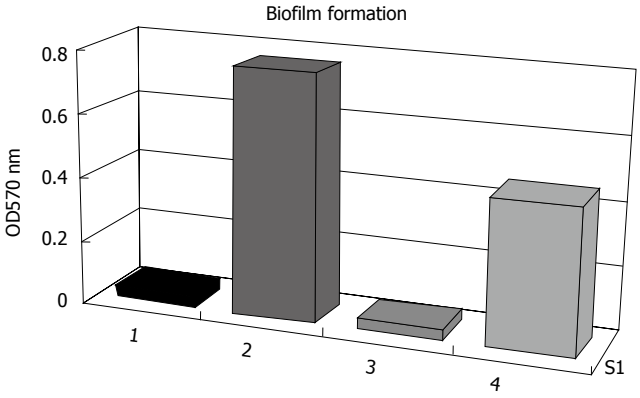

Figure 5 Biofilm formation on polystyrene tissue culture plates of *S. epidermidis* in TSB medium. 1. biofilm-negative strain *S. epidermidis* ATCC12228; 2. original strain *S. epidermidis* RP62A; 3. *sarA*::*tet* insertion mutant *S. epidermidis* RP62A △*sarA*; 4. complemented strain *S. epidermidis* RP62A △*sarA* (pHPS9*sarA*).

RP62A△*sar*A (pHPS9*sarA*) strain, black, rough colonies phenotype was restored.

Biofilm formation on a glass surface of the shaking tube with overnight culture: The *sarA* mutant showed loss of the ability to produce a ring of biofilm adherent to the glass wall at the air-liquid interface (Figure 4, Lane 2). While the complementary strain RP62A△*sarA* (pHPS9*sarA*) displayed a biofilm positive phenotype similar to that of the wild-type strain (Figure 4, Lane 3).

Quantitative determination of biofilm formation: All strains were then tested of their ability to form biofilms on polystyrene surfaces. The isolates were grown overnight in 96-well flat-bottomed tissue culture using TSB as growth medium. As shown in Figure 5, the biofilm formation of *S. epidermidis* RP62A was biofilm-positive when the strain was grown in TSB. In contrast, the *S. epidermidis* RP62A △*sarA* insertion mutant and *S. epidermidis* ATCC12228 failed to produce any detectable biofilm. In the case of *S. epidermidis* RP62A△*sarA* (pHPS9*sarA*), in which the deleted chromosomal locus of *sarA* was complemented by a plasmid carrying *sarA*, biofilm formation was restored.

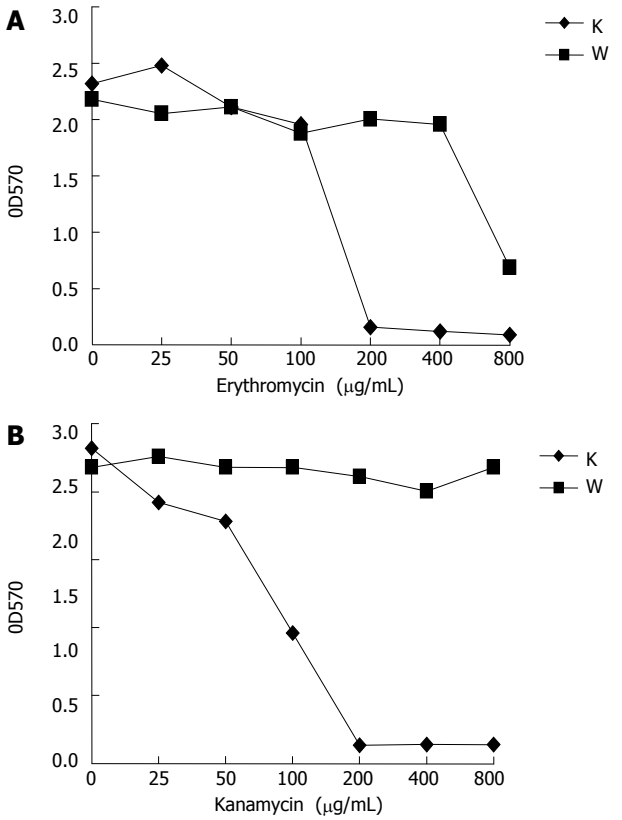

Figure 6 Response of RP62A (original) and RP62A△*sarA* (mutant) to two antibiotics in shaking tube cultures. The cultures of the wild type RP62A (W) and the mutant RP62A△*sarA* (K) were incubated in a shaking incubator (250 r/min) at 37℃ for 24 h, and diluted in fresh TSB medium. Determination of optical density (OD) values was carried out at 570 nm.

Increase in some antibiotic sensitivity of S. epidermidis RP62A by deletion of sarA

The *sarA* mutation had a dramatic effect on the antibiotics resistance of strain RP62A. Two antibiotics, erythromycin and kanamycin, were investigated. The *sarA*::*tet* mutant demonstrated a significant increase of susceptibility along with the increasing concentration of erythromycin and kanamycin compared to its parental strain (Figure 6). At erythromycin concentrations above 200 μg/mL, RP62A△*sarA* did not grow, while RP62A survived at concentrations over 800 μg/mL (Figure 6A). RP62A appeared at all kanamycin concentrations investigated, whereas RP62A △*sarA* did not survive at concentrations over 200 μg/mL (Figure 6B).

DISCUSSION

To investigate the impact of *sarA* on biofilm formation, we constructed a *sarA* mutant of *S. epidermidis* RP62A. At the phenotypic level, the *sarA* mutant revealed a biofilm-negative phenotype, and the capacity of biofilm formation was restored when *sarA* mutant strains were complemented by a plasmid carrying *sarA*. RT-PCR was used to measure the transcription of *icaA* in variants grown in TSB. Consistent with the biofilm-negative phenotype, the results showed that the *sarA* mutation caused a significant

repression of the *ica* operon transcription compared with that of the wild-type strains. The absence of an identifiable *sigB*-consensus binding site on the upstream of the *ica* operon in *S. aureus* has suggested that *sigB* may not regulate *ica* operon directly[22], and the foundation of the SarA-consensus binding site implied that SarA controls directly the transcription of *ica* operon and subsequently regulates biofilm formation[23,24]. However, it is possible that both SigB and SarA are also involved in the posttranscriptional regulation of PIA synthesis in *S. epidermidis* according to previous reports.

The knockout of *sarA* resulted in decreased erythromycin and kanamycin resistance in *S. epidermidis* RP62A. This implies that a protein(s) whose production is controlled by SarA is involved in resistance to these antibiotics. However, the exact role that SarA plays in the response of *S. epidermidis* to multiple antibiotics deserves further attention.

In conclusion, the current studies demonstrate that S*ar*A is typically associated with the transcription of the *ica* operon, the capacity of biofilm-formation and antibiotic resistance of *S. epidermidis*. To answer the question of whether S*ar*A is directly or indirectly involved in those processes, more experimental work, including primer extension analyses under different growth conditions, is needed.

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