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 (pPHPS9sarA) 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.

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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 relevant characteristics of *S. aureus* strains. Luria-Bertani (LB) was used for relevant characteristics of *S. aureus* strains. Relevant characteristics of *S. aureus* strains.

**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', Ery', Ap' | ATCC                 |
| *S. epidermidis* a sedA deletion on the chromosome, Tet', sarA::tet | This study            |
| *S. epidermidis* RP62A (pHP89sarA) | Restriction-negative, intermediate host for plasmid transfer from *E. coli* to *S. epidermidis* | This study            |
| *S. aureus* RN4220   |                         | [14]                 |

**Plasmids**

| Plasmids | Relevant characteristics | Sources or references |
|----------|-------------------------|----------------------|
| pBR322   | Donor of tet gene (Tet'). Ap' Tet' | [15]                 |
| pBluescriptSK | E. coli cloning vector, Ap' | [11]                 |
| pBT       | Temperature-sensitive shuttle vector. Ap' (E. coli) Cm' (Staphylococcus) | [16]                 |
| pBT::sarA | Integration vector for homologous recombination of theΔsarA gene in *S. epidermidis*; tet inserted into sarA locus as resistance selection marker | This study            |
| pHP59    | Expression shuttle vector | [17]                 |
| pHP89sarA | pHP89 inserted sarA gene | This study            |

**Constructions 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 TGTAAACATCT AGTGCAAA-3' and (2) 5' -AGCAGCTGAC TTTAATCTGTC AGCATAGTG-3' with HindIII and PstI respectively. The downstream of 857 bp, fragment 2, was amplified using primer pairs (3) 5' -ACGAAGCTGA TTATAACACCA CTCAAGTTG-3' and (4) 5' -AGGGAATTTC GGGCATATTG CCAGTGAAC-3' with PstI and EcoRI respectively. The two fragments were cloned into the multicloning region of temperature-sensitive *E. coli*-Staphylococcus shuttle vector pBT2 [19], 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' -CGCGCGGCCGC TTCTCTCATGT TTGACAGCTT-3' and (6) 5' -CGGAGATCTT CAGGTCAGG TGGGCC-3'. The *tet* gene was inserted into the vector pBT2-1, resulting in plasmid, pBT2ΔsarA. Following passage through the restriction-negative strain *S. aureus* RN4220, pBT2ΔsarA 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 ΔsarA following a temperature shift to the nonpermissive temperature (42°C) of the shuttle vector [19]. 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' -CACCCTGACG ACCGCCAGAT A-3'; for icaA transcript, (9) 5' -AACAAGTTGA AGGCATCCTC-3' and (10) 5' -GATGCTTTGG TTGATCCT-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.
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'-AGGAAATTCG GGCATCATTG CGAGTGA-3' (in the central part of the tet gene) (Figure 1); SA2: 5' -AGGAAATTCG GGCATCATTG CGAGTGA-3' (next to the downstream of sarA 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 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 icaA was normalized to gyrB expression\(^\text{[19]}\). 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 (pHPS9sarA), the icaA transcription was activated (Figure 3, lane 3). Consistent with this, at the phenotypic level, the capacity of RP62A△sarA (pHPS9sarA) 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

Quantitative determination of biofilm formation

Strains were cultivated overnight (16 h) in 96-well flat-bottomed tissue culture plates at 37°C in TSB growth medium. Based on the optical densities (OD\(_{570}\)) of the biofilm, the strains were classified as non-adherent strains (OD\(_{700} \leq 0.120\)).

Determination of antibiotic susceptibilities

Three antibiotics were used to determine the impact of sarA gene 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 \(\mu\)g/mL\(^\text{[20,21]}\). 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°C, these inocula were determined with spectrophotometer at 570 nm.

RESULTS

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

Figure 1 Disruption and detection of chromosomal sarA locus in S. epidermidis.
1. Insertion of tet gene into sarA locus to form recombinant plasmid pBTΔsarA. 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 icaA and gyrB transcription in S. epidermidis by RT-PCR analysis. 1. S. epidermidis RP62A; 2. S. epidermidis RP62A△sarA; 3. S. epidermidis RP62A△sarA (pHPS9sarA).
RP62AΔsarA (pHPS9sarA) 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 RP62AΔsarA (pHPS9sarA) 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* RP62AΔsarA insertion mutant and *S. epidermidis* ATCC12228 failed to produce any detectable biofilm. In the case of *S. epidermidis* RP62AΔsarA (pHPS9sarA), in which the deleted chromosomal locus of sarA was complemented by a plasmid carrying sarA, biofilm formation was restored.

**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, RP62AΔsarA did not grow, while RP62A survived at concentrations over 800 µg/mL (Figure 6A). RP62A appeared at all kanamycin concentrations investigated, whereas RP62AΔsarA 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,[23,24] 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 SarA 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 SarA 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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