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# Activation of *sarX* by Rbf Is Required for Biofilm Formation and *icaADBC* Expression in *Staphylococcus aureus*

**David Cue, Mei G. Lei, Chia Y. Lee**

Department of Microbiology and Immunology, University of Arkansas for Medical Sciences, Little Rock, Arkansas, USA

**A major constituent of many *Staphylococcus aureus* biofilms is a polysaccharide known as the polysaccharide intercellular adhesin, or poly *N*-acetylglucosamine (PIA/PNAG). PIA/PNAG is synthesized by the 4 gene products of the *icaADBC* operon, which is negatively regulated by the divergently transcribed *icaR* gene. We previously reported the identification of a gene, *rbf*, involved in the positive transcriptional regulation of *icaADBC* transcription by repressing *icaR* in *S. aureus* strain 8325-4. However, we were unable to show binding of Rbf to DNA upstream of *icaR* or *icaA*, suggesting that Rbf may control expression of an unknown factor(s) that, in turn, regulates *ica* expression. Here we report that the unknown factor is SarX protein. Results from epistasis assays and genetic complementation analyses suggest that Rbf upregulates SarX, which then downregulates IcaR, thereby activating *icaADBC*. Electrophoretic mobility shift assays revealed that SarX protein bound to a sequence upstream of *icaR* within the *icaA* coding region. Cross-linking and immunoprecipitation experiments further suggested that Rbf binds to the *sarX* promoter in *S. aureus*. These results demonstrate that Rbf and SarX represent a regulatory cascade that promotes PIA-dependent biofilm formation in *S. aureus*.**

*Staphylococcus aureus* is a major human pathogen causing a diverse array of nosocomial and community-acquired infections. Staphylococcal infections range from superficial infections of the skin and mucosa to highly invasive and potentially lethal infections. Some *S. aureus* infections, such as endocarditis, osteomyelitis, and infections associated with indwelling medical devices, are associated with the formation of bacterial biofilms. Bacterial biofilms are complex communities of organisms containing layers of bacteria within a glycocalyx composed of polysaccharides, DNA, and/or proteins. In addition to aiding bacterial colonization of surfaces, biofilms are believed to confer resistance to antibiotics and immune defenses (1–3).

The major exopolysaccharide in *S. aureus* biofilms is referred to as the polysaccharide intercellular adhesin (PIA), also known as poly-*N*-acetylglucosamine (PNAG) (4, 5). The synthesis of PIA/PNAG is accomplished by four proteins, IcaA, IcaD, IcaB, and IcaC, encoded by the *ica* operon (5). Production of PIA/PNAG is tightly regulated, but the signals that are responsible for induction of PIA/PNAG synthesis *in vivo* are unknown. A variety of environmental conditions have been shown to affect *icaADBC* expression under laboratory conditions. High temperature, high osmolarity, glucose, ethanol, anaerobiosis, and subinhibitory concentrations of certain antibiotics have all been found to induce PIA/PNAG production *in vitro*. There is, however, significant strain-to-strain variation regarding what factors affect expression.

Several different *S. aureus* proteins have been shown to be involved in the transcriptional regulation of *icaADBC*. These factors include global regulatory proteins, such as SarA and $\sigma^B$, as well as factors like IcaR and TcaR, which seem to regulate relatively few genes (6–11). Some factors regulate *icaADBC* expression directly (e.g., IcaR), whereas regulation by other proteins seems to be indirect (e.g., $\sigma^B$). IcaR is arguably the most important factor involved in *icaADBC* regulation. The *icaR* gene is located immediately upstream of and is divergently transcribed from *icaADBC*. IcaR binds to the *icaADBC* promoter and represses transcription (10). Deletion of *icaR* has been shown to dramatically increase *icaADBC* expression and PNAG production (10, 12). Some regulatory proteins appear to upregulate *icaADBC* expression by inhibiting expression of *icaR*. IcaR also plays an important role in *icaADBC* expression in *Staphylococcus epidermidis* (13).

We have previously described a gene, *rbf*, which regulates expression of *icaADBC* and PIA/PNAG production in *S. aureus* strain 8325-4 (12, 14). Rbf is a member of the AraC/XylS family of transcriptional regulators, a family in which all members bear a highly conserved 100-amino-acid region forming a dual, helix-turn-helix DNA binding motif (15, 16). Rbf is a positive regulator of biofilm (12, 14). Extensive macrocellular aggregation was observed when Rbf was expressed from a multicopy plasmid in *S. aureus* or *S. epidermidis* (14, 17). Overexpression of Rbf significantly increased *icaA* transcription and PIA/PNAG production in both wild-type and *rbf* mutant strains of *S. aureus* 8325-4 and UAMS-1 (12). The gene was also found to play a significant role in *S. aureus* virulence (18).

Microarray experiments revealed that *rbf* was able to reduce *icaR* transcription in a clinical isolate of *S. aureus*, strain UAMS-1 (12). This finding was confirmed by quantitative reverse transcription-PCR (qRT-PCR) experiments for both UAMS-1 and 8325-4 (12). Thus, it appears that *rbf* activates *icaADBC* expression, at least in part, by inhibiting expression of *icaR*. AraC/XylS proteins typically act as activators of transcription, but at least some, such as AraC, can also function as repressors (15, 16). These data suggested that Rbf might bind directly to the *icaR-icaA* promoter region. Experiments to test for binding of recombinant Rbf to the *ica* promoter yielded only negative results, however. These results suggested that Rbf may regulate *ica* expression through another factor (12).

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Address correspondence to Chia Y. Lee, clee2@uams.edu.

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Microarray experiments also revealed that Rbf regulates several genes that encode potential transcriptional regulatory proteins, including SarX, a member of the Sar family of transcriptional regulatory proteins (19). The *sarX* gene is positioned immediately downstream of *rbf* in the *S. aureus* chromosome (20). Overexpression of Rbf in the *S. aureus* clinical isolate UAMS-1 increased *sarX* expression by over 50-fold. SarX has also been shown to promote biofilm formation and *icaADBC* expression in *S. epidermidis* (21). Thus, *sarX* appeared to be a likely transcription factor through which Rbf may regulate *ica* expression. Here we report that SarX is an activator of *icaADBC* transcription and is required for biofilm formation in *S. aureus*. Additionally, we show that SarX binds with high affinity to *icaA* DNA. We also show that transcription of the *sarX* gene is dependent upon Rbf and provide evidence that Rbf binds to the *sarX* promoter *in vivo*.

## MATERIALS AND METHODS

**Bacterial strains and culture media.** The bacterial strains used in this study are listed in Table 1. Staphylococci were cultured in tryptic soy broth (TSB) (Difco Laboratories, Detroit, MI) or tryptic soy agar (TSA). In some experiments, growth medium was supplemented with glucose to a final concentration of 0.75% and NaCl to a final concentration of 3.5%, as described below. Antibiotics were added to *S. aureus* culture media, as appropriate, at final concentrations of 10 μg per ml chloramphenicol (Cm), 3 μg per ml tetracycline (Tc), 50 μg per ml kanamycin (Kn), and 150 ng per ml anhydrotetracycline (aTc). *Escherichia coli* strains DH5α and XL1-Blue were used for plasmid construction and maintenance. *E. coli* BL21 (λDE3) (plysS) was used for expression of recombinant Rbf and SarX. *E. coli* was cultivated in Luria-Bertani broth or agar (Difco) supplemented with, as appropriate, 100 μg per ml penicillin (Pen), 34 μg per ml Cm, or 50 μg per ml Kn.

**Plasmid and strain construction.** To construct a *sarX* deletion mutant of 8325-4, PCR primer set attB1-sarX-KO1 and sarX-KO2 and primer set attB2-sarX-KO4 and sarX-KO3 (Table 2) were used to amplify the upstream (1.2-kb) and downstream (0.93-kb) fragments of the *sarX* gene, respectively. Advantage High-Fidelity 2 polymerase was used for amplification (Clontech, Mountain View, CA). The fragments were cloned into plasmid pKOR1 (22) using Gateway BP Clonase II enzyme mix (Invitrogen) and transformed into *E. coli* DH5α. The resulting plasmid, pML3792, was first transformed into *S. aureus* RN4220 by electroporation (23). Cm was used for selection of transformants. The plasmid was then transduced into 8325-4 using phage 52A. The *sarX* mutant was selected by using aTc as described by Bae and Schneewind (22). Allelic replacement was confirmed by PCR.

The *sarX* complementation plasmid pML3793 (pLI50-*sarX*) was constructed by PCR amplification of the 8325-4 *sarX* gene using primers sarXP1 and sarXP2. The amplified fragment was cut with BamHI and EcoRI and cloned into plasmid pLI50. The aTc-inducible *sarX* expression plasmid was similarly constructed. Primers sarX5 and sarX6 were used to PCR amplify *sarX*, and the resulting DNA fragment was cloned into pGEM-T Easy (Promega Corp., Madison, WI), resulting in plasmid pAG4084. Plasmid pAG4084 was digested with EcoRI, and the *sarX*-bearing DNA fragment was cloned into pML100 to create pAG4031 (pML100-*sarX*) and pAG4032 (pML100->*sarX*). Plasmids were transduced into the *S. aureus* strains listed in Table 1 using phage 80α or phage 52A.

Plasmid pTL3664 was constructed by PCR amplification of the *rbf* gene of 8325-4 using primers rbf39 and rbf41 (Table 2) and cloning it into the expression vector pET15b. Plasmid pAG3919 was constructed for expression of SarX in *E. coli*. A synthetic *sarX* gene, with codons that would be efficiently recognized in *E. coli*, was synthesized by EZBiolab Inc. (Carmel, IN). Based upon the studies of Manna and Cheung (20), we placed the *sarX* start codon at bp 664,327 of the 8325-4 genome (GenBank accession no. NC_007795) (24). The open reading frame of the synthetic gene

**TABLE 1** Strains and plasmids

| Strain or plasmid | Description | Source or reference |
|---|---|---|
| Strains | | |
| *S. aureus* | | |
| 8325-4 | Prophage-free laboratory strain | J. Iandolo |
| RN4220 | Restriction-negative laboratory strain | J. Iandolo |
| SH1000 | *rsbU*-positive 8325-4 | 47 |
| *S. aureus* 8325-4 derivatives | | |
| CYL1112 | Wild type (pLI50) | 14 |
| CYL6968 | *rbf* (pLI50) | 14 |
| CYL6973 | Wild type (pYL8565) | 14 |
| CYL6974 | *rbf* (pYL8565) | 14 |
| CYL11688 | *icaR* (pLI50) | 12 |
| CYL11699 | *icaR* (pML3796) | This study |
| CYL11551 | *sarX* (pLI50) | This study |
| CYL11552 | *sarX* (pYL8565) | This study |
| CYL11580 | Wild type (pML3793) | This study |
| CYL11514 | *rbf* (pML3793) | This study |
| CYL11555 | *sarX* (pML3793) | This study |
| CYL11696 | *icaADBC* | 12 |
| CYL12512 | Wild type (pML100) | 27 |
| CYL12513 | Wild type (pAG4031) | This study |
| CYL12514 | Wild type (pAG4032) | This study |
| CYL12515 | *rbf* (pML100) | This study |
| CYL12516 | *rbf* (pAG4031) | This study |
| CYL12517 | *rbf* (pAG4032) | This study |
| CYL12518 | *sarX* (pML100) | This study |
| CYL12519 | *sarX* (pAG4031) | This study |
| CYL12520 | *sarX* (pAG4032) | This study |
| CYL12653 | *sarX icaR* (pLI50) | This study |
| CYL12654 | *sarX icaR* (pML3793) | This study |
| CYL12655 | *sarX icaR* (pML3796) | This study |
| CYL12656 | *icaR* (pML3793) | This study |
| CYL12657 | *sarX* (pML3796) | This study |
| CYL12642 | SH1000 (pLI50) | This study |
| CYL12646 | SH1000 *rbf*::Tn*917* (pML4068) | This study |
| *E. coli* | | |
| DH5α | Host strain for plasmids | Invitrogen |
| XL1-Blue | Host strain for plasmids | Stratagene |
| BL21(λDE3)pLysS | Host for recombinant protein production | Novagen |
| Plasmids | | |
| *E. coli* | | |
| pET15b | Expression vector | Novagen |
| pET28a | Expression vector | Novagen |
| pAG3919 | His-SarX expression plasmid | This study |
| pTL3664 | His-Rbf expression plasmid | This study |
| *S. aureus* | | |
| pLI50 | *E. coli*-*S. aureus* shuttle vector | 48 |
| pML100 | aTc-inducible expression vector | 27 |
| pML3793 | pLI50 with *sarX* | This study |
| pYL8565 | pLI50 with *rbf* | 14 |
| pML3796 | pLI50 with *icaR* | 12 |
| pKOR1 | Vector for allele replacement | 22 |
| pAG4031 | pML100 with *sarX* | This study |
| pAG4032 | pML100 with *sarX* in antisense orientation | This study |
| pML4068 | *S. aureus* His-Rbf expression plasmid | This study |

**TABLE 2** Oligonucleotide primers used in this study

| Primer purpose and name | Sequence |
|---|---|
| Plasmid and strain construction | |
| attB1-sarX-KO1 | GGGGACAAGTTTGTACAAAAAAGCAGGCTCACTAGTATCACAAATAAAGCG |
| sarX-KO2 | CTATGCTTTCGACACTCAATTTCAATTACTAATTTCTCAGTATTCAAAATGTTGC |
| sarX-KO3 | GCAACATTTTGAATACTGAGAAATTAGTAATTGAAATTGAGTGTCGAAAGCATAG |
| attB2-sarX-KO4 | GGGGACCACTTTGTACAAGAAAGCTGGGTCTTTCAATACTTCTCGAATTGTC |
| sarXP1 | GAATTCCACCTTGATATGTATTGC |
| sarXP2 | GGATCCGCCGATAAGCAAATTCTATGC |
| Rbf-hisF | GGATCCGTCATACGCAACGTTTACCACG |
| Rbf-hisR | TTAGTGGTGATGGTGATGGTGTCCACCTTTTTTTCCTATTTTAATTATGTATAACGC |
| Pcp1rbf1 | CAGGTCGACTCTAGAGGATCCGTTTGCAAAATATACAGGGGATTATATATAATGGAAAAC |
| Pcp1rbf2 | TATACAGGGGATTATATATAATGGAAAACAAGAAAGGAAAATAGGAGGTTTATATGGC |
| pcp1rbf3 | ATATTCATTATTAGTAAGTATATGCAAGCATGATTTTGCCATATAAACCTCCTATTTTCC |
| pcp1rbf4 | GACTATGCCATCTTGGCAACGCGTTGTCGCATATTCATTATTAGTAAGTATATGCAAGC |
| rbf39 | ATACATATGGCAAAATCATGCTTGCATATAC |
| rbf41 | ATAGGATCCTTTTGCAATACATATCAAGGTG |
| sarX5 | ACGGCATATGAGAAAGACAATGGCAAAAAAATT |
| sarX6 | TACGGGATCCTCAAATATTTAAAAATTGTTCTACATCTTCAAATAAAGC |
| sarX7 | CATATGAACACCGAAAAACTGGAAACCCTG |
| sarX8 | GGATCCTTAGATGTTCAGGAACTGTTCAACGTCTTC |
| qRT-PCR | |
| sgSarx1 | TGTCCTACTTAAATCTAGCTCATCCATTGCAGTT |
| sgSarx2 | TGAATACTGAGAAATTAGAAACATTGCTTGGCTTCTATAAACA |
| sgicaA2 | GACCTCCCAATGTTTCTGGAACCAACATCC |
| icaA3 | GTCAGACGTTGGCTACTGGGATACTGATATG |
| icaRfor | TACGTTCAATTATCTAATACGCCTGAGGAATTTTCTGGAA |
| icaRrev | AGGATGCTTTCAAATACCAACTTTCAAGAAACAGCAAATATT |
| delta rbf for | ACGCGTTGCCAAGATGGCATAGTCTT |
| delta rbf rev | AGCCTAATTCCGCAAACCAATCGCTA |
| sggyrB3 | GGAATCGGTGGCGACTTTGATCTAGCGAAA |
| sggyrB4 | CGCTCCATCCACATCGGCATCAGTCATAAT |
| EMSAs and immunoprecipitation assays | |
| sgfnbAF2 | GAATATTTGCAAGGGTCAGATCAGGTTAATTTTAGAACTG |
| sgfnbAR2 | CTGTGTGGTAATCAATGTCAAGCGGTGTATTG |
| icaRP1 | CTGCAGGAATTTCTTTACCTACCTTTCGTTAG |
| icaRP2 | GGATCCAACATTTAACACTTTGTTCGTA |
| icaRP3 | GGATCCTCTTGTATTTGTCCGTAAATATTTCCAGAAAATTCC |
| icaAlong 1 | GGATCCGTGTCCCCCTTGAGCCCATC |
| icaAlong 2 | CTGCAGCTTATCCTTCAATTTTTATAACCCCCTACTG |
| icaAdc1 | CTGCAGCCATATGGCTTACAACCTAACTAACGAAAGGTAG |
| icaAdc2 | GGATCCGAAATAGTATTGACTGCGCCAGC |
| icaAdc3 | GGATCCGTGCATCTTGATCAACGATAGTATCTGCATC |
| icaAdc4 | GGATCCTTTCTTCTCGTATTTGAGTGCAAGAACATTAGACA |
| icaAdc5 | GGATCCACATTTATGTCAGGCTTCTTGTTCAATGAATATC |
| icaAdc6 | TTGCAATTTTTTAACTTTTTGCTTTTTTATCCTGTATTTATGTC |
| icaAdc7 | AGATATTCATTGAACAAGAAGCCTGACATAAATG |
| SAO0009F3 | AAGGTGCGCAATTAGAGCGTGCT |
| SAO0009R3 | TCTGCGTTCACAAGCTGTGGTACC |
| sarXdc7 | GAATTCGTTTATAGAAGCCAAGCAATGTTTCTAATTTCTCA |
| sarXdc8 | GGATCCGGAAAAAAATAACACCTTGATATGTATTGCA |

was amplified using primers sarX7 and sarX8 and cloned into pET28a to form pAG3919.

Plasmid pML4068 (pLI50-His-Rbf) was constructed for constitutive expression of His-tagged Rbf in *S. aureus* by first ligating the PCR fragment containing the *rbf* gene with its promoter (primers rbf-hisF and rbf-hisR) to pLI50 at the EcoRI and BamHI sites. The *rbf* promoter in the resulting plasmid was then replaced with Pcap1 (25) by the sequence- and ligation-independent cloning (SLIC) method (26) using primers Pcp1rbf1, Pcp1rbf2, Pcp1rbf3, and Pcp1rbf4. The DNA insert was confirmed by sequencing.

**Assays for biofilm and PIA/PNAG production.** Biofilm assays were performed in 96-well microtiter plates as described previously (14, 27). Assays for PNAG were performed as previously described (12).

**RNA isolation and quantitative real-time PCR.** RNA was isolated as described previously (27). Quantitative real-time RT-PCR was performed as previously described (27) using the primers listed in Table 2. RNA from at least 2 cultures of each strain was analyzed.

**Purification of recombinant SarX.** Expression of histidine-tagged SarX was done in *E. coli* BL21 (λDE3) (plysS). Expression was induced in log-phase cultures by the addition of 1 mM IPTG (isopropyl-β-D-thioga-

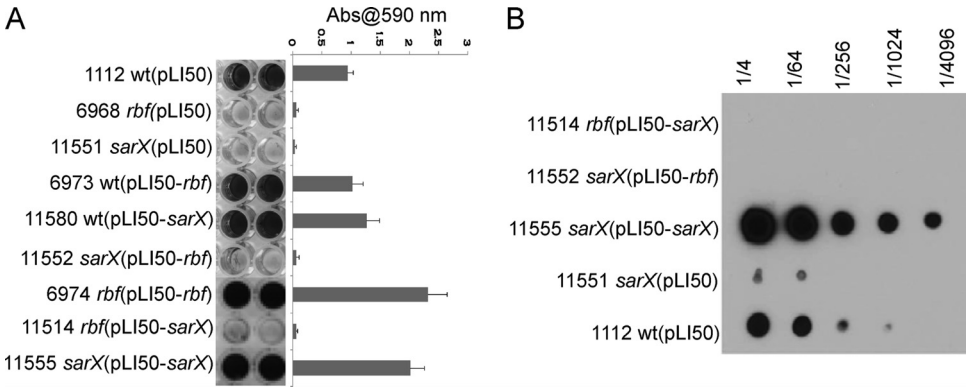


**FIG 1** Biofilm formation requires *sarX*. (A) Biofilm formation under static incubation conditions in 96-well plates inoculated with each of the 8325-4 derivatives listed to the left of the image. Following 24 h of incubation, wells were washed and biofilms were stained with crystal violet. Quantitation of biofilms is shown on the right side. Each assay was performed a minimum of 2 times. Error bars indicate standard deviations. (B) Regulation of PIA/PNAG production by *sarX*. PIA/PNAG was extracted from overnight cultures of each strain, serially diluted, and applied to a membrane. PNAG was detected by incubating the membrane, successively, with rabbit anti-PNAG serum, goat anti-rabbit horseradish peroxidase (HRP), and a chemiluminescent substrate. Numbers at the top of the figure indicate PNAG dilutions.

lactopyranoside). Three hours after induction, bacterial cells were harvested by centrifugation, subjected to osmotic shock (28), and then stored at −80°C. The cells were thawed, incubated with 400 μg per ml lysozyme (Sigma), and sonicated, and the resulting lysate was clarified by centrifugation. His-tagged SarX was purified from clarified lysates by metal affinity chromatography using reagents purchased from EMD Chemicals, San Diego, CA. Following chromatography, SarX protein was dialyzed against buffer containing 25 mM Tris-Cl (pH 7.5), 300 mM NaCl, and 1 mM EDTA. Dithiothreitol (DTT) (1 mM) was then added to the dialyzed protein, aliquoted, and stored at −80°C.

**Electrophoretic mobility shift assays (EMSAs).** DNA fragments were generated by PCR amplification of 8325-4 DNA using the oligonucleotide primers listed in Table 2. The DNA fragments were end labeled with digoxigenin-dUTP using reagents purchased from Roche Applied Sciences, Indianapolis, IN. Binding reactions were performed in 20 μl of 25 mM Tris-Cl (pH 7.5), 1 mM EDTA, 75 mM NaCl, 1 mM DTT, 5% glycerol, and 200 ng poly-dIdC. Reaction mixtures were incubated for 15 min at room temperature and then electrophoresed through 4.0% or 5.0% polyacrylamide gels, buffered with [1/2] × Tris-borate-EDTA (44.5 mM Tris base, 44.5 mM boric acid, 1 mM EDTA, pH 8.0) at 5°C. The DNA fragments were then electroblotted onto nylon membrane (Applied Biosystems, Austin, TX). The digoxigenin-labeled DNA was detected using reagents purchased from Roche Applied Sciences. Negative-control (nonspecific) competitor DNAs were amplified with primer pair sarX7 and sarX8 or sgfnbAF2 and sgfnbAR2, with either pAG3919 or *S. aureus* genomic DNA, respectively, as the template.

**Immunoprecipitation of His-Rbf DNA complexes.** The assay described by Benson et al. (29) was utilized to recover His-Rbf-DNA complexes from *S. aureus* CYL12646 and the negative-control strain CYL12642. Cultures were treated with 1% formaldehyde for 20 min at room temperature. After neutralization with 0.5 M glycine, the cells were washed twice with Tris-buffered saline (TBS) and stored at −80°C. Cells were thawed and incubated with lysostaphin in 10 mM Tris (pH 8.0), 20% sucrose, and 50 mM NaCl. Protoplasts were diluted with immunoprecipitation (IP) buffer (50 mM HEPES-KOH [pH 7.5], 150 mM NaCl, 1% Triton X-100, 0.1% sodium deoxycholate, 0.1% SDS) and sonicated. The lysates were filtered through 0.45-μm filters and then incubated with magnetic beads covalently coated with mouse monoclonal antibody against polyhistidine tags (GenScript, Piscataway, NJ). Mock incubations, containing lysates but no magnetic beads, were conducted in parallel. After incubation at room temperature for 2 h with end-over-end rotation, beads were washed three times with IP buffer, twice with wash buffer (10 mM Tris [pH 8.0], 250 mM LiCl, 0.5% Nonidet-P40, 0.5% sodium deoxycholate), and once with 10 mM Tris (pH 7.5). Bound material was then eluted by incubation at 65°C for 10 min in 50 mM Tris (pH 7.5) and 1% SDS. The eluted material was treated with RNase A and then incubated overnight at 65°C with proteinase K. DNA was recovered by extraction with phenol-chloroform and ethanol precipitation. Five or 10 ng of the recovered DNA was subjected to PCR using (separately) primer pair sarXdc7 and sarXdc8 (to amplify the *sarX* promoter region DNA), icaAP1 and icaAdc4 (to amplify the *icaR-icaA* intergenic region), or SAO0009F3 and SAO0009R3 (to amplify an irrelevant DNA region encoding seryl-tRNA synthetase [24]). PCR products were electrophoresed through 6% polyacrylamide gels. The immunoprecipitation experiment was performed 3 times with similar results.

## RESULTS

**Effects of SarX on biofilm formation, PIA/PNAG production, and *icaADBC* expression in *S. aureus* 8325-4.** We previously reported that Rbf modulates expression of the *icaR* gene and the *icaADBC* operon in strain 8325-4 but that we were unable to detect binding of purified Rbf to the *icaR* or *icaA* promoter region (12). This suggested that the effect of Rbf on *ica* expression might be manifest through activation of another transcription factor. A likely candidate transcription factor is SarX. Transcription of the *sarX* gene is highly activated by Rbf (12), and SarX has recently been shown to affect biofilm formation by *S. epidermidis* (21). Therefore, we introduced an internal deletion of *sarX* in the 8325-4 chromosome. The resulting strain was unable to form a biofilm (Fig. 1A). The *sarX* mutation was able to be complemented with a multicopy plasmid (pLI50-*sarX*) carrying the wild-type *sarX* gene. Additionally, pLI50-*sarX* enhanced biofilm formation in the wild-type strain (Fig. 1A). Notably, a plasmid carrying the wild-type *rbf* gene, pLI50-*rbf*, suppressed the *rbf* mutation and enhanced biofilm formation in 8325-4 but had no significant effect on biofilm formation by the *sarX* mutant.

In order to determine whether SarX affects *icaADBC* expression, we measured *icaA* expression by qRT-PCR and PIA/PNAG production in a set of isogenic strains with mutations in *sarX* or *rbf*. The results in Table 3 and Fig. 1B suggest that the effect of SarX on biofilm formation is due largely to its effect on expression of the *icaADBC* genes and subsequent PIA/PNAG production. The above-described results also showed that although *sarX* was acti-

**TABLE 3** qRT-PCR assays to determine the effects of *sarX* on expression of *icaA* and *icaR*

| Strain [description] | Relative gene expression[a] | | | |
|---|---|---|---|---|
| | *icaA* | *icaR* | *rbf* | *sarX* |
| 6973 [wt (pLI50-*rbf*)] | 10.65 ± 0.94 | 0.67 ± 0.07 | 6.52 ± 2.70 | 5.24 ± 0.50 |
| 11551 [*sarX* (pLI50)] | 0.27 ± 0.13 | 1.05 ± 0.04 | ND | <0.001 |
| 11552 [*sarX* (pLI50-*rbf*)] | 0.14 ± 0.08 | 1.01 ± 0.21 | 8.00 ± 5.3 | <0.001 |
| 11580 [wt (pLI50-*sarX*)] | 142 ± 23.8 | 0.39 ± 0.25 | 1.79 ± 0.13 | 9.13 ± 4.64 |
| 11514 [*rbf* (pLI50-*sarX*)] | 0.14 ± 0.03 | 1.02 ± 0.06 | <0.001 | 0.053 ± 0.01 |
| 11555 [*sarX* (pLI50-*sarX*)] | 152 ± 25.6 | 0.40 ± 0.07 | ND | 19.2 ± 1.41 |
| 11696 [*icaADBC*] | <0.01 | ND | ND | ND |
| 11688 [*icaR* (pLI50)] | ND | <0.001 | ND | ND |
| 6968 [*rbf* (pLI50)] | 0.26 ± 0.01 | ND | ND | 0.01 ± 0.002 |

[a] The levels of mRNA (means and standard deviations, in arbitrary units) for each of the indicated genes are expressed relative to expression in the wild-type strain 1112 [wt (pLI50)]. ND, not done.

vated by Rbf, *rbf* was not activated by SarX (Table 3). These results are consistent with a model wherein Rbf affects biofilm, at least in part, by upregulating expression of *sarX*. SarX, in turn, activates *ica* and possibly other biofilm-related genes. However, pLI50-*sarX* was not able to suppress an *rbf* mutation at the level of *icaADBC* transcription, PIA/PNAG production, or biofilm formation, suggesting interdependency between Rbf and SarX. We reasoned either that this was due to poor expression of *sarX* in the *rbf* mutant or that Rbf affects biofilm formation by both SarX-dependent and SarX-independent mechanisms.

**Suppression of an *rbf* mutation by *sarX*.** To decipher the Rbf-SarX interdependency, we tested the hypothesis that Rbf and SarX are part of a regulatory cascade wherein SarX is the primary effector molecule that acts at *ica*, and possibly other biofilm-related genes, and expression of *sarX* is highly dependent on Rbf. To this end, we placed *sarX* under the control of an anhydrotetracycline (aTc)-inducible promoter, P*xyl/tetO*, in plasmid pML100 (27). Plasmids pML100-*sarX* and pML100->*sarX* are pML100 derivatives that carry the *sarX* gene in sense and antisense orientations, respectively. We found that pML100-*sarX* can promote biofilm formation in cultures grown with 150 ng per ml of aTc but not in cultures lacking aTc (data not shown). As shown in Fig. 2,

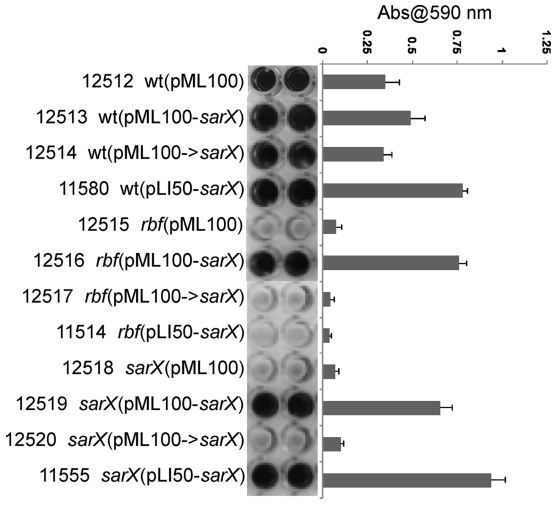


**FIG 2** Suppression of the *rbf* mutation by *sarX*. Biofilm formation under static incubation conditions. Assays were performed as described in the legend of Fig. 1 except that cultures contained 150 ng per ml anhydrotetracycline.

**TABLE 4** Expression of *sarX* and *icaA* in pML100-*sarX* transformants

| Strain [description] | Relative gene expression[a] | |
|---|---|---|
| | *sarX* | *icaA* |
| 12515 [*rbf* (pML100)] | 0.01 ± 0.0 | 0.42 ± 0.14 |
| 12516 [*rbf*(pML100-*sarX*)] | 6.55 ± 0.91 | 18.06 ± 1.62 |
| 12518 [*sarX* (pML100)] | 0.001 | 0.23 ± 0.03 |
| 12519 [*sarX* (pML100-*sarX*)] | 6.94 ± 0.76 | 7.19 ± 0.38 |

[a] Numbers represent levels of mRNA (means and standard deviations, in arbitrary units), as measured by qRT-PCR assays, for each of the indicated genes, relative to expression by strain 12512 [8325-4 (pML100)]. Cultures were grown in the presence of 150 ng per ml anhydrotetracycline.

pML100-*sarX* increased biofilm formation in the wild-type, *rbf*, and *sarX* strains (strains 12513, 12516, and 12519, respectively). Neither pML100 nor pML100->*sarX* significantly affected biofilm formation in any strain. These results indicated that the reason pLI50-*sarX* did not suppress the *rbf* mutation was due to poor expression of the plasmid-encoded, *rbf*-regulated *sarX* gene. Transcription of *icaA* was increased in both the *rbf* and *sarX* mutants carrying pML100-*sarX*, demonstrating that SarX is capable of modulating *icaA* expression in the complete absence of Rbf (Table 4). Thus, taken together, the above-described results suggest that Rbf promotes *icaADBC* expression and biofilm formation by activating *sarX* expression.

**Binding of recombinant SarX protein to *ica* DNA.** The *S. epidermidis* SarX protein has been shown to bind specifically to the *icaA* promoter and activates transcription of *icaADBC* (21). It seemed likely that SarX from *S. aureus* would display similar activity. To verify this, electromobility shift assays (EMSAs) were performed to measure binding of His-tagged SarX protein to the *icaA* promoter region. We first used the 178-bp PCR DNA fragment generated using oligonucleotide primers IcaAP1 and IcaRP1 (Fig. 3A), which extended from the fifth codon of *icaR* to the start codon of *icaA* and thus encompassed the entire 164-bp *icaR-icaA* intergenic region, including the putative *icaA* and *icaR* transcription start sites (7, 30). Surprisingly, although SarX bound the DNA fragment, binding appeared to be nonspecific, as it was able to be competed out with several different nonspecific competitor DNAs (Fig. 3B). Similar results were obtained using a labeled 330-bp DNA fragment (generated using primers icaRP2and icaRP1) that included an additional 152 bp of *icaR* DNA (data not shown).

In further experiments, we found that SarX did bind specifically to a labeled 991-bp DNA fragment that extended 824 bp into the *icaA* open reading frame (ORF) (Fig. 3C). The affinity of SarX for this fragment was relatively high, with most of the labeled DNA being bound at a SarX concentration of approximately 40 nM. The SarX-DNA complex formed was able to be competed out with an unlabeled, specific competitor DNA fragment (Fig. 3C, lane 9) but not by a 50 molar excess of unlabeled, nonspecific competitor DNA (Fig. 3C, lane 10). Moreover, SarX binding occurred in the presence of 50 μg per ml of poly dIdC DNA or 200 μg per ml of herring sperm DNA (data not shown). Binding was inhibited by poly dAdT DNA at concentrations over 10 μg per ml, however, indicating that SarX may have a relatively high affinity for A-T-rich DNA sequences (data not shown). Importantly, the unlabeled *ica* promoter DNA fragment (used in Fig. 3B) did not significantly affect SarX binding to the 991-bp DNA fragment containing the promoter region and the *icaA* ORF (data not shown).

In order to further define the *sarX* binding region, we tested for

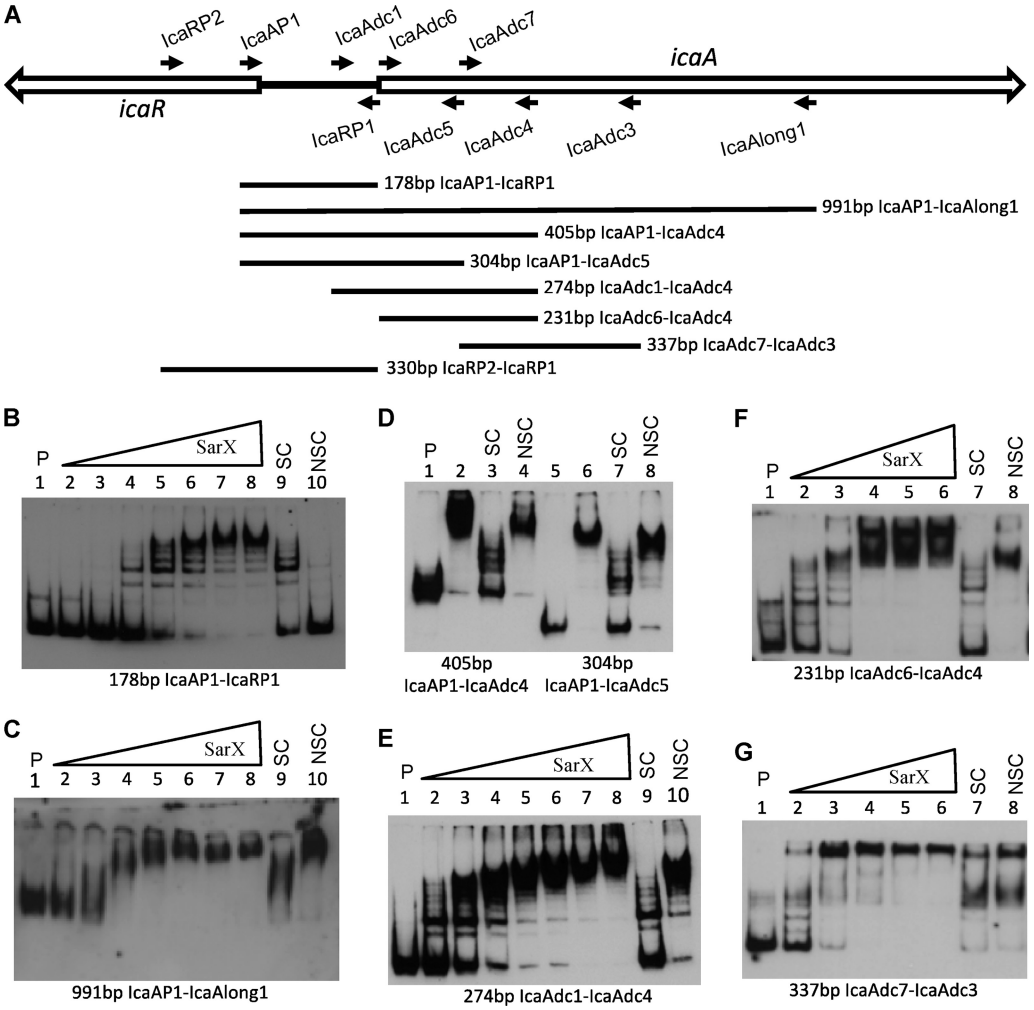


**FIG 3** SarX binding to *ica* DNA and localization of the SarX binding region. (A) Locations of *ica* primers. Forward primers are listed above the genetic map, and reverse primers are listed below the map. PCR DNA fragments for use in EMSAs are shown in the lower portion of the figure. (B to G) EMSAs, with each of the different labeled PCR fragment probes indicated below the figure. P indicates labeled probe without SarX; SC and NSC indicate specific and nonspecific competitors, respectively, each at 50 molar excess. All assays were run on 5% acrylamide gels except that shown in panel C, which was on a 4% gel. The amounts of SarX in each assay are shown below. (B and C) Lanes 2 to 10 were 5.2, 15.3, 45.9, 138, 407, 814, 1,628, 407, and 407 nM, respectively. (D) Lanes 2 to 4 and 6 to 8 were all at 407 nM. (E) Lanes 2 to 10 were 25.4, 50.9, 102, 204, 407, 814, 1,628, 407, and 407 nM, respectively. (F and G) Lanes 2 to 8 were 52.0, 153, 407, 814, 1,628, 407, and 407 nM, respectively.

binding to several different PCR fragments representing overlapping sequences of the *ica* promoter and *icaA* coding region. We first deleted DNA from the 3′ end of the *icaA* coding region. We found that SarX bound specifically to the 405-bp icaAP1-to-icaAdc4 and 304-bp icaAP1-to-icaAdc5 PCR products (Fig. 3D). We then deleted DNA from upstream of the *icaA* coding region. We found that SarX bound specifically to a 274-bp PCR fragment (generated using primers icaAdc1 and icaAdc4) which carried the putative *icaA* −10 promoter element and the *icaA* transcription start site but lacked a −35 promoter region (Fig. 3E). Interestingly, we found that SarX bound specifically to a 231-bp PCR fragment, generated using the icaAdc6 to icaAdc4 primers, which contained no sequence upstream of the *icaA* coding region (Fig. 3F). Further deletion of the *icaA* coding region, in the 337-bp icaAdc7-to-icaAdc3 PCR product, abolished the specific binding of SarX (Fig. 3G). Taken together, these results argue that DNA sequences 5′ to the *icaA* start codon are not required for SarX binding and that there is a high-affinity SarX binding site within the first 129 bp of the *icaA* coding region.

**Binding of Rbf to the *sarX* promoter.** Due to the fact that Rbf increases the level of *sarX* transcripts, it seemed reasonable to expect that Rbf can bind to the *sarX* promoter region. However, we were unable to demonstrate sequence-specific binding of either His-Rbf or a maltose-binding Rbf fusion protein to the *sarX* promoter (data not shown). These results prompted us to see if we could detect Rbf binding to the *sarX* promoter *in vivo*. To accomplish this, we constructed an *S. aureus* strain (CYL12646) that expresses a His-tagged Rbf protein. The strain used in this experiment was chosen because we found a relatively high level of *rbf* expression in this strain (data not shown), a property we felt would increase the likelihood of detecting His-Rbf interaction with DNA. Cultures of strain 12646 were treated with formaldehyde to promote cross-linking of proteins to DNA. Cells were harvested from these cultures, incubated with lysostaphin, and

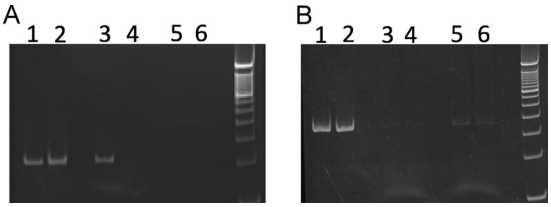


**FIG 4** Immunoprecipitation of His-Rbf bound to the *sarX* promoter. Purified DNA recovered from CYL12646 (His-Rbf expression strain) and CYL12642 (negative-control strain) was PCR amplified with primers sarXdc7 and sarXdc8 (A) or SAO0009F3 and SAO0009R3 (B). Lanes 1, 3, and 4 are from 12646, and lanes 2, 5, and 6 are from 12642. DNA recovered from cell lysates prior to incubation with magnetic beads was used for the reactions shown in lanes 1 and 2. DNA for the reactions shown in lanes 3 and 5 was recovered from incubation of magnetic beads with extracts. Material recovered from mock incubations (extracts without beads) was used for reactions in lanes 4 and 6.

lysed by sonication. The resulting cell-free lysates were incubated with magnetic beads coated with an antibody that recognizes His tags. Parallel mock incubations in which magnetic beads were omitted from the incubation were performed. The beads were washed, and bound proteins were eluted. DNA was recovered from the eluted samples and used in PCRs. As an additional negative control, cultures of CYL12642, which does not express any His-tagged protein, were subjected to the identical regimen.

To determine if Rbf-*sarX* promoter complexes were recovered, PCRs were performed with the recovered DNA using primers that are specific for the *sarX* promoter region. As shown in Fig. 4A, lane 3, the *sarX* promoter DNA fragment was able to be amplified from the DNA obtained from beads incubated with the strain 12646 lysate. This fragment was not amplified from the mock (i.e., without beads) incubation of the same lysate (lane 4). Moreover, the promoter fragment was not amplified from either of the 12642 strain samples (lanes 5 and 6). These results are consistent with the selective enrichment of His-Rbf-*sarX* promoter complexes from the strain 12646 lysate.

As an additional control, the same DNA templates used for Fig. 4A were subjected to PCR using primers specific for an irrelevant DNA sequence encoding seryl-tRNA synthetase (SAOUHSC_00009). We observed no enrichment of DNA in the experiment (Fig. 4B). Collectively, these results are consistent with the idea that Rbf binds to the *sarX* promoter *in vivo*. We were unable to detect enrichment of *icaA* DNA in these same experiments (data not shown).

**Regulation of biofilm formation by *icaR* and *sarX*.** We have shown that Rbf affects transcription of the *icaADBC* genes by downregulating *icaR* transcription and thereby upregulating transcription of *icaADBC* (12). These results, in conjunction with the findings reported above, strongly suggest that SarX may enhance PIA/PNAG production and biofilm formation by repressing transcription of *icaR*, which, in turn, would increase *icaADBC* transcription. To test this, we first performed biofilm assays with *icaR* and *sarX* mutants and the *icaR sarX* double mutant. As shown in Fig. 5A, inactivation of *icaR* (strain 11688) resulted in derepression of biofilm formation and repression was restored by transformation of the mutant with an *icaR*-bearing plasmid, pLI50-*icaR* (strain 11699). In the *icaR sarX* double mutant, biofilm formation was similar to that of the *icaR* mutant. These results indicate that the effect of *icaR* is epistatic to *sarX*, evidence that *icaR* acts downstream of *sarX* in biofilm regulation. We also performed complementation of the *icaR sarX* double mutant with either the *sarX*-bearing plasmid, pLI50-*sarX*, or the *icaR*-bearing plasmid, pLI50-*icaR* (strains 12654 and 12655, respectively). We found that transformation of the double mutant with pLI50-*icaR* repressed biofilm formation. In contrast, pLI50-*sarX* appeared not to affect biofilm formation relative to the double mutant strain.

To confirm that increased biofilm formation was associated with increased PNAG levels, immunoassays for PNAG were performed (Fig. 5B). The results showed that the *icaR sarX* double mutant produced approximately the same amount of PIA/PNAG as the *icaR* mutant. Transformation of the *icaR* or *icaR sarX* mutant with pLI50-*icaR* (carrying *icaR*) decreased PNAG synthesis to less than the wild-type level. Carriage of pLI50-*sarX* had no significant effect on PIA/PNAG production in the double mutant. These results confirm that *icaR* functions downstream of *sarX*.

To test whether SarX affects *icaR* expression, we performed qRT-PCR in the *sarX* mutant and complemented strains. The results in Table 3 indicate that the *sarX* mutation did not appreciably affect *icaR* expression (strain 11551). However, overexpression of *sarX* resulted in repression of *icaR* (see strains 11580 and 11555). These results indicate that SarX significantly represses *icaR* transcription only when SarX is overproduced.

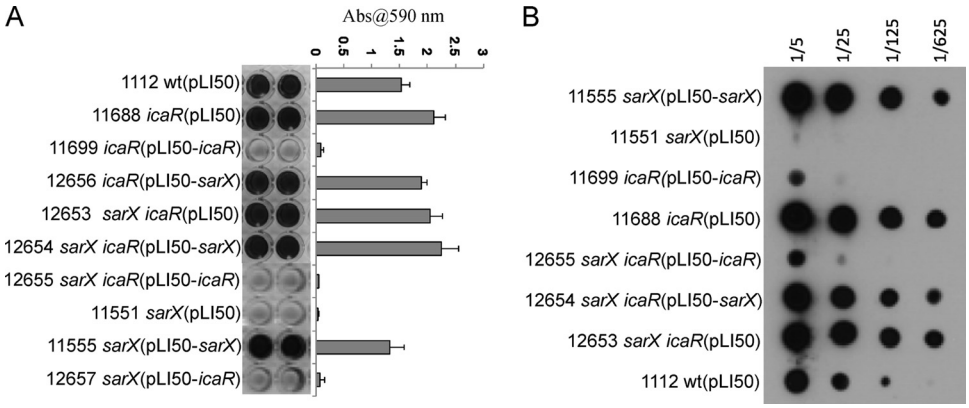


**FIG 5** *icaR* is epistatic to *sarX*. (A) Biofilm assays and quantitation were performed as described in the legend of Fig. 1. (B) PIA/PNAG production. Assays were performed as described in the legend of Fig. 1.

## DISCUSSION

The production of PIA/PNAG is an important contributing factor to biofilm formation by staphylococci. The genes encoding PIA/PNAG biosynthetic proteins, *icaADBC*, are subject to regulation by numerous factors. One factor that plays an important role in activating *icaADBC* expression is Rbf (12). Because Rbf is a member of the AraC/XylS family of proteins, many of which are known transcriptional activators, we anticipated that Rbf would bind directly to the *icaA-icaR* intergenic region. This appears not to be the case, however, as we have been unable to detect specific binding of recombinant Rbf protein to *ica* DNA. Instead, Rbf seems to increase *icaADBC* expression by upregulating transcription of *sarX*. SarX, in turn, activates *icaADBC* expression. In support of this proposal, a mutation in *rbf* reduced transcription of *sarX* by approximately 4-fold and overexpression of Rbf enhanced *sarX* expression by 5- to-6-fold. Although we have been able to demonstrate binding of recombinant Rbf to a DNA fragment encompassing the putative *sarX* promoter *in vitro*, several different "nonspecific" DNA fragments readily compete for Rbf binding. These results suggest that the *in vitro* interaction of Rbf with the *sarX* promoter is nonspecific. In immunoprecipitation experiments using antibody against histidine-tagged proteins, we were able to enrich for *sarX* promoter DNA cross-linked to His-Rbf. On the other hand, no amplification was detected in negative-control experiments in which either antibody was omitted from the immunoprecipitation reaction or when the immunoprecipitation was performed with a strain that did not express His-Rbf. In addition, neither an irrelevant DNA sequence encoding seryl-tRNA synthetase nor even the *icaA* promoter region was enriched. Collectively, these results support the argument that Rbf selectively binds the *sarX* promoter *in vivo*.

The failure to detect specific binding of Rbf *in vitro* may be due to a number of factors. First, some AraC-like proteins, including AraC (15, 16, 31, 32), have been implicated in DNA looping, which requires protein interaction with multiple binding sites. DNA binding by some members of the AraC/XylS family involves protein binding to sites up to several hundred bp upstream or downstream of a regulated promoter. Thus, it is possible that Rbf binds to DNA sites distal to the *sarX* promoter or requires binding to multiple sites to form a stable complex with DNA. Second, protein solubility is another factor that may be relevant to binding of recombinant Rbf to DNA. AraC-like proteins are notoriously insoluble. We have been able to isolate relatively small amounts of recombinant Rbf from *E. coli*, but the vast amount of the protein is insoluble. We cannot be certain that the soluble fraction of recombinant Rbf, which was used in our experiments, is in its native conformation. Third, it is possible that Rbf undergoes some form of posttranslational modification *in vivo* that affects its interaction with DNA. Alternatively, Rbf binding may be influenced by a cofactor, such as a low-molecular-weight molecule, that is present in *S. aureus*. In this regard, we have isolated His-tagged Rbf directly from *S. aureus* and showed that the protein bound to *sarX* promoter but nonspecifically (data not shown), resembling the results using recombinant protein isolated from *E. coli*. These results suggest that a cofactor(s) that may be required for specific binding is (are) absent in our *in vitro* EMSA experiments.

The Sar family of proteins is composed of at least 11 different proteins, some of which are found in both *S. aureus* and *S. epidermidis*. The various Sar proteins have been categorized as fitting into one of three subfamilies (19). The proteins in one subfamily, which includes SarA and SarX, are generally small, about 15-kDa, basic proteins with a single DNA binding domain that probably bind DNA as homodimers. SarA has a central core region comprised of a winged-helix DNA binding domain in which the helix-turn-helix domain recognizes the major groove and the winged region interacts with the minor groove. SarA has a conserved α-helical region near the N terminus of the protein that mediates protein dimerization (19, 33–35). These structural elements appear to be conserved in SarX, suggesting that SarA and SarX may affect transcription by a similar mechanism. Although SarA has been characterized as a DNA binding protein, no true consensus sequence of a SarA binding site, other than that the protein has a high propensity for binding A-T-rich DNA, has emerged. SarX binding to DNA is inhibited by poly-dAdT, suggesting that SarX also has a propensity for binding A-T-rich DNA. Recently, Morrison et al. (36) reported that SarA binds a variety of mRNA molecules and protects them from degradation. It is possible that SarX may affect the steady-state level of *icaADBC* RNA by a similar mechanism. To date, we have not determined the effect of SarX on the half-life of *icaADBC* RNA. In *S. epidermidis* CSF41498, SarX was found to upregulate biofilm formation in an *ica*-dependent manner (21). In that study, expression of *S. epidermidis sarX* on a multicopy plasmid not only complemented a *sarX* mutation but also enhanced biofilm formation by the wild-type strain. *S. epidermidis SarX* has also been shown to bind *ica* DNA (21). Thus, SarX appears to function similarly in *S. aureus* and *S. epidermidis*. However, Manna and Cheung (20) reported that *sarX* did not affect biofilm formation in *S. aureus* RN6390. It is unclear why the difference exists between RN6390 and 8325-4, since they are closely related strains derived from the same parent strain, NCTC8325.

We have clearly shown that SarX can bind specifically to *ica* DNA with high affinity *in vitro*. Surprisingly, however, we found that the SarX binding site was within the *icaA* coding region. While this is an unusual finding, it is not without precedent. The *E. coli* Rns protein is an activator of its own transcription that has a binding site within the *rns* open reading frame (31), and the *S. aureus* SarA protein has a binding site located downstream of its own promoter (34). However, our epistasis assays showed that SarX activation of *icaADBC* is through *icaR*, suggesting that the binding of SarX interferes with IcaR repression (Fig. 5). It is still unknown how IcaR represses *icaADBC* transcription, but it is most likely by blocking RNA polymerase binding to the *icaA* promoter. It has been argued that SarA is a histone-like architectural protein that modifies DNA topology. In fact, it has been shown that SarA can partially substitute for the bacteriophage λ Xis protein in integrase-mediated excision of λ from an *att* site (37). SarA binding has also been shown to cause DNA bending (38). Thus, it is possible that SarX binding within the *icaA* coding region may alter DNA topology such that the affinity of IcaR for the *icaADBC* promoter is decreased and/or the affinity of RNA polymerase is increased. Alternatively, effective repression of *icaR* may require binding to an additional site within the *icaA* coding region, exemplified by *lacI* repression of the *lac* operon in *E. coli* (39).

Our qRT-PCR results showed that deletion of *sarX* did not affect *icaR* transcription but profoundly affected *icaA* transcription and PIA/PNAG production (Table 3 and Fig. 5). The qRT-PCR data also showed that repression of *icaR* transcription was observed only with a multiple-copy plasmid carrying the *sarX* gene. These results suggest that a relatively low level of SarX does

not affect *icaR* expression but can partially block IcaR repression of *icaADBC* whereas a high level of SarX not only affects *icaR* repression of *icaADBC* but also inhibits transcription of *icaR*. How can SarX function differently depending on its concentration? One clue may come from the EMSA results (Fig. 3), which showed that SarX bound specifically to the *icaA* coding region upstream of the *icaR* binding site and may also bind, nonspecifically, to the intergenic region containing the *icaR* and *icaA* promoters. These results suggest that when the concentration of SarX is low, the protein binds to the specific site within the *icaA* coding region. At a high concentration, SarX also binds to the intergenic region containing the *icaR* promoter. Binding to the *icaA* coding region may interfere with IcaR repression as discussed above, but binding may be too far upstream to affect *icaR* transcription whereas binding to the intergenic region would most likely inhibit *icaR* transcription. SarX binding to multiple sites in *ica* DNA probably accounts for the ladder of SarX-DNA complexes observed in EMSAs (Fig. 3) (20). We have recently started to map the transcription initiation sites of *icaR*. Our preliminary results suggest that the *icaR* promoter is located close to the *icaADBC* promoter in the central portion of the 164-bp intergenic region. Thus, binding of SarX in this region, though nonspecific, would be expected to block *icaR* transcription. Additionally, SarX binding in the intergenic region may promote RNA polymerase binding to the *icaA* promoter, which may sterically hinder polymerase binding to the *icaR* promoter.

Based on the above-described interpretation of our results, we propose the following model. At a low level of expression, SarX binds to the specific SarX binding site within the *icaA* coding region with high affinity, which interferes with IcaR repression of *icaADBC* but does not significantly affect *icaR* transcription. Under this condition, IcaR can still repress *icaADBC* but not fully. At a higher level of expression, in addition to binding in the *icaA* coding region, SarX binds to the *icaR*/*icaA* promoter region. This would stabilize RNA polymerase binding to the *icaADBC* promoter and effectively reduce *icaR* transcription. Under this condition, *icaADBC* is fully expressed. We also hypothesize that the level of SarX is likely to be controlled by Rbf, whose activity can be modulated in response to stimuli, such as a cofactor discussed above.

It has been demonstrated that the SarX protein of *S. aureus* RN6390 binds to the *agr* promoter, repressing synthesis of RNAII and RNAIII and thereby indirectly repressing exoprotein synthesis (20). SarX from *S. epidermidis* strain CSF41498 also binds its cognate *agr* promoter and represses *agr* transcription (21). Agr is a negative regulator of biofilm formation; thus, *agr* repression by SarX would be predicted to enhance biofilm formation (40–42). The *agr* effect on biofilm is *ica* independent, so *agr* repression is a second mechanism by which *sarX* can regulate biofilm production.

It is important to note that there are some apparent discrepancies regarding biofilm formation by 8325-4 and related strains. Although several laboratories have found that 8325-4 and its parent strain RN1 can form biofilms under laboratory conditions (12, 17, 43, 44), others report that this strain is a poor former of biofilms. For example, it has been reported that 8325-4 does not form a biofilm whereas strain SA113, also of the 8325 lineage, forms an *ica*-dependent biofilm (1, 5). *S. aureus* SH1000, a SigB-positive ($SigB^+$) derivative of 8325-4, has been reported to form a PIA/PNAG-independent biofilm, but *rsbU* mutants of SH1000 (which have a SigB-negative [$SigB^-$] phenotype) do not form biofilms (45). These results are at odds with our findings, as the major genetic difference between 8325-4 and SH1000 is a functional *rsbU* gene in the latter strain (5). 8325-4 and SH1000 also differ by 3 single nucleotide changes and a deletion upstream of *spa* in SH1000 (46). It is possible that these mutations account for the difference in biofilm formation by 8325-4 and SH1000 *rsbU*. Alternatively, strains derived from NCTC8325 may have acquired mutations after they were segregated into different laboratories. In this regard, we have checked our laboratory 8325-4 strain by a blood agar method and found that the Agr activity was reduced compared to that of RN6390, a strain derived from 8325-4 (43), suggesting that *agr* expression in our 8325-4 isolate may be reduced relative to that in some other closely related strains, an alteration that may affect biofilm formation.

The findings presented here have increased our understanding of how Rbf increases expression of *icaADBC*. Many questions remain, however, as we do not completely understand what signals induce *rbf* expression *in vivo*, how Rbf upregulates *sarX* expression, or precisely how SarX promotes *icaADBC* transcription. Further study of the interplay between Rbf, SarX, and *ica* will undoubtedly uncover novel methods of gene regulation.

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