INTRODUCTION

*Staphylococcus epidermidis* has now joined its more virulent counterpart *Staphylococcus aureus* as a leading cause of healthcare-associated infections involving implanted medical devices. This opportunistic pathogen does not produce the numerous virulence determinants associated with *S. aureus* (Gill et al., 2005) and owes its virulence almost exclusively to its capacity to form biofilms (Otto, 2009). Production of the icaADBC operon-encoded polysaccharide intercellular adhesin (PIA) or by surface and extracellular proteins. Deletion of the *Staphylococcus* accessory regulator sarX significantly reduced biofilm-forming capacity in *Staphylococcus epidermidis* CSF41498, whereas multicopy sarX complemented the sarX mutant and increased wild-type biofilm production. In *Staphylococcus aureus*, SarX negatively regulates the accessory gene regulator (Agr) system, which in turn has strain-specific effects on biofilm regulation. Here we found that purified *S. epidermidis* SarX protein bound specifically to the agr P3 promoter. However RT-PCR analysis revealed that both mutation of sarX and multicopy sarX activated RNAIII transcription, making it difficult to correlate sarX-mediated biofilm regulation with altered agr activity. In contrast, RT-PCR and immunoblot analysis revealed that icaA transcription and PIA expression were decreased in the sarX mutant, whereas multicopy sarX increased ica and PIA expression. Furthermore, multicopy sarX did not promote biofilms in an icaC mutant. Finally, purified SarX protein bound specifically to the ica operon promoter. Taken together, these data reveal 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.

Biofilm production by staphylococci is an important virulence determinant mediated by the icaADBC-encoded polysaccharide intercellular adhesin (PIA) or by surface and extracellular proteins. Deletion of the *Staphylococcus* accessory regulator sarX significantly reduced biofilm-forming capacity in *Staphylococcus epidermidis* CSF41498, whereas multicopy sarX complemented the sarX mutant and increased wild-type biofilm production. In *Staphylococcus aureus*, SarX negatively regulates the accessory gene regulator (Agr) system, which in turn has strain-specific effects on biofilm regulation. Here we found that purified *S. epidermidis* SarX protein bound specifically to the agr P3 promoter. However RT-PCR analysis revealed that both mutation of sarX and multicopy sarX activated RNAIII transcription, making it difficult to correlate sarX-mediated biofilm regulation with altered agr activity. In contrast, RT-PCR and immunoblot analysis revealed that icaA transcription and PIA expression were decreased in the sarX mutant, whereas multicopy sarX increased ica and PIA expression. Furthermore, multicopy sarX did not promote biofilms in an icaC mutant. Finally, purified SarX protein bound specifically to the ica operon promoter. Taken together, these data reveal 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.

Abbreviations: AIP, autoinducing peptide; EMSA, electrophoretic mobility shift assay; MBP, maltose-binding protein; PAS, polyanethole sodium sulphate; PIA, polysaccharide intercellular adhesin.

A novel role for SarX in *Staphylococcus epidermidis* biofilm regulation

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*Staphylococcus epidermidis* has now joined its more virulent counterpart *Staphylococcus aureus* as a leading cause of healthcare-associated infections involving implanted medical devices. This opportunistic pathogen does not produce the numerous virulence determinants associated with *S. aureus* (Gill et al., 2005) and owes its virulence almost exclusively to its capacity to form biofilms (Otto, 2009). Production of the icaADBC operon-encoded polysaccharide intercellular adhesin (PIA) or by surface and extracellular proteins. Deletion of the *Staphylococcus* accessory regulator sarX significantly reduced biofilm-forming capacity in *Staphylococcus epidermidis* CSF41498, whereas multicopy sarX complemented the sarX mutant and increased wild-type biofilm production. In *Staphylococcus aureus*, SarX negatively regulates the accessory gene regulator (Agr) system, which in turn has strain-specific effects on biofilm regulation. Here we found that purified *S. epidermidis* SarX protein bound specifically to the agr P3 promoter. However RT-PCR analysis revealed that both mutation of sarX and multicopy sarX activated RNAIII transcription, making it difficult to correlate sarX-mediated biofilm regulation with altered agr activity. In contrast, RT-PCR and immunoblot analysis revealed that icaA transcription and PIA expression were decreased in the sarX mutant, whereas multicopy sarX increased ica and PIA expression. Furthermore, multicopy sarX did not promote biofilms in an icaC mutant. Finally, purified SarX protein bound specifically to the ica operon promoter. Taken together, these data reveal 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.

62 kDa Ami with C-terminal R2 and R3 repeats, and the 51 kDa GL with an N-terminal R3 repeat (R3-GL) (Heilmann et al., 1997; Oshida et al., 1995; Sugai et al., 1989). Disruption of *atlE* abolishes initial attachment to hydrophobic polystyrene, while attachment to more hydrophilic glass remains unaffected (Heilmann et al., 1997). Autolysin-mediated DNA release has recently been implicated in the *S. epidermidis* biofilm phenotype, and DNase I significantly reduces rates of primary attachment to surfaces (Qin et al., 2007).

The accessory gene regulator (agr) system controls quorum sensing, and is known to influence the biofilm phenotype of staphylococci in a strain-dependent manner (Beanken et al., 2003, 2010; Boles & Horswill, 2008; Coelho et al., 2008; O’Neill et al., 2007; Regassa et al., 1992; Vuong et al., 2000a, b, 2003, 2004). The agr locus encodes two divergently transcribed mRNA transcripts, RNAII and RNAIII (Morfeldt et al., 1995; Novick & Geisinger, 2008). RNAII encodes an 8 aa autoinducing peptide (AIP), which is processed to generate the AIP signalling molecule. When AIP reaches a critical concentration, it facilitates activation of the RNAIII P3 promoter (Novick et al., 1993). RNAIII is a regulatory RNA molecule, and controls the expression of several hundred exoprotein genes, including proteases,
haemolysins and toxins. The RNAIII transcript also encodes the δ-toxin, which reduces cell attachment to polystyrene (Vuong et al., 2000a, 2003).

A number of studies have implicated the agr system in regulating biofilm detachment. Indeed, the agr locus is repressed in biofilms (Yarwood et al., 2004), and agr-deficient mutants can produce thicker biofilms from which detachment is impaired (Beenken et al., 2003; Vuong et al., 2003, 2004; Yarwood & Schlievert, 2000). Boles & Horswill (2008) demonstrated that adding exogenous AIP or depleting glucose activates the agr system and the production of extracellular proteases necessary for bacterial detachment (Boles & Horswill, 2008). Thus, excess glucose leads to an acidification of the culture medium which can repress the agr system and promote biofilm production, suggesting that acidic niches colonized by S. aureus may activate biofilm production in this manner (Boles & Horswill, 2008). In S. epidermidis, rates of primary attachment and biofilm-forming capacity are increased in an agr mutant (Vuong et al., 2003). Consistent with this, Agr has been shown to negatively regulate atlE transcription, while immunoblots have revealed increased AtlE levels in an agr mutant (Vuong et al., 2003).

SarX, a SarA family protein, was first identified in S. aureus by Manna & Cheung (2006). Transcription of sarX in S. aureus has been shown to be temporal and expressed maximally in stationary phase (Manna & Cheung, 2006). Significantly, expression of the agr transcripts, RNAII and RNAIII, is increased in a sarX mutant, and SarX has been found to bind to the agr promoter to repress agr expression and exoproduct synthesis (Manna & Cheung, 2006). SarX shares 66% identity and 88% similarity with its S. epidermidis homologue (Manna & Cheung, 2006), and here we investigated the role of sarX in S. epidermidis. The impact of a sarX deletion mutation on agr, icaADBC, PIA and biofilm regulation was examined. The interaction of recombinant SarX protein with the agr and icaADBC promoters was also investigated. Our data reveal a novel role for SarX in the PIA-dependent S. epidermidis biofilm phenotype.

METHODS

Bacterial strains, plasmids, media and growth conditions. The bacterial strains and plasmids used in this study are listed in Table 1. Escherichia coli strains were grown at 37 °C in Luria–Bertani (LB) medium (Sigma) supplemented when required with kanamycin (Kan) (50 μg ml⁻¹) or carbenicillin (Car) (50 μg ml⁻¹), with the exception of E. coli strain Rosetta when used for protein expression, which was grown in overnight expression medium (Novagen) supplemented with chloramphenicol (Cam) (34 μg ml⁻¹) and Car (50 μg ml⁻¹).

S. epidermidis and S. aureus strains were routinely grown at 30 or 37 °C on brain heart infusion (BHI) medium (Oxoid). When required, BHI was supplemented with Cam (10 μg ml⁻¹) or tetracycline (Tc) (5 μg ml⁻¹). BHI was also supplemented with 1% glucose and 0.04% polyanethole sodium sulphate (PAS), as required.

Construction of an S. epidermidis sarX deletion mutant. The sarX deletion mutant strain SARX1 was constructed as follows. A 1760 bp fragment containing the sarX gene was amplified by PCR using Phusion High-Fidelity DNA polymerase (New England Biolabs; NEB) from CSF41498 genomic DNA using primers SEsarX_flank1 and SEsarX_flank2. Oligonucleotide primers used in this study are listed in Table 2. The blunt PCR product was cloned into plasmid pCR-Blunt II-TOPO (Invitrogen) to create pSEXT8. The Tc' gene from plasmid pT181 was digested with HindIII on a 2236 bp fragment, which was subcloned into pBluescript (Stratagene) to create pBlue-tet. A 359 bp fragment containing the sarX gene was subsequently deleted from pSEXT8 by inverse PCR using Phusion High-Fidelity DNA polymerase and primers SEinvPCRsarX1 and SEinvPCRsarX2, which had Stul enzyme sites incorporated at the 5' ends (Table 2). The resulting PCR fragment was purified and digested with Stul, followed by religation to create pSXDEL8. The 2336 bp Tc' gene was cloned from pBlue-tet on an Swa1–Swal fragment and ligated into the Stul site of pSXDEL8, yielding pSXDEL8-tet. A 3734 bp BamHI–XbaI fragment containing the mutant allele from pSXDEL8-tet was ligated into pRT2 digested with BamHI/XbaI to create pBSXDEL8. The temperature-sensitive pBSXDEL8 was electroporated into RN4220 and subsequently into CSF41498. Allele replacement of the temperature-sensitive pBSXDEL8 in CSF41498 was achieved by growing at 30 °C in the presence of 10 μg Cam ml⁻¹ and 5 μg Tet ml⁻¹ (three subcultures), followed by repeated subculture (three times) at 42 °C without antibiotic selection, and finally selection of Tc' colonies on BHI agar plates. Tc' colonies were then screened for sensitivity to Cam to confirm plasmid loss, and PCR analysis was used to verify the presence of the sarX::Tc' deletion allele on the chromosome using primers SEsarX_flank1 and SEsarX_flank2.

To complement the sarX mutation a 538 bp fragment containing the sarX gene and its upstream promoter region was amplified using primers SEsarX1 and SEsarXprot2 and cloned into pCR-Blunt II-TOPO to create pTSEXS5. From pTSEXS5, a BamHI–XbaI fragment containing sarX was cloned in pL50 digested with BamHI/XbaI to create pSESAX6, which was ultimately electroporated into CSF41498 and SARX1.

Biofilm assays. Semiquantitative measurements of biofilm formation under static conditions were determined using Nunclon tissue culture-treated (ΔSurface) 96-well polystyrene plates (Nunc), as described previously (Conlon et al., 2002a). Each strain was tested at least three times and mean results are presented. A biofilm-positive phenotype was defined as an A₅₉₀ ≥ 0.17.

Primary attachment assays. Attachment assays using Nunclon tissue culture-treated (ΔSurface) 96-well polystyrene plates (Nunc) were carried out using overnight cultures in BHI adjusted to OD₅₉₀ 1.0. A 200 μl volume of each suspension was used to inoculate the microtitre plate wells prior to incubation at 37 °C for 1 h. Following incubation, the wells were washed gently three times with PBS, dried at 65 °C for 1 h and stained for 5 min with 0.4% crystal violet. After staining, the plates were washed gently three times with distilled water and the remaining crystal violet was solubilized using 100 μl 5% acetic acid. The A₅₉₀ of the solubilized crystal violet was measured. Each experiment was carried out at least three times.

Autolysis assays. The Triton X-100-induced autolysis assay was performed essentially as described by Mani et al. (1993). Overnight cultures of S. aureus were subcultured in BHI and incubated at 37 °C with shaking (200 r.p.m.) to OD₅₉₀ 1.0. Cells were then pelleted and washed twice with ice-cold PBS, and subsequently resuspended in PBS containing 0.02% Triton X-100. The cell suspensions were then incubated with shaking (200 r.p.m.) at 37 °C. OD₅₉₀ readings were taken at 0 and 10 min, and then at 30 min intervals. Triton X-100-induced
Construction of a maltose-binding protein (MBP)–SarX fusion
RNA purification and real-time RT-PCR. RNA was extracted from
cultures grown in BHI glucose to OD_{600} 1.0 or 4.0, as previously
described (Conlon et al., 2002a, b). RNA concentration and integrity
were determined using a Nanodrop spectrophotometer. RT-PCR was
performed on a Roche LightCycler using the RNA amplification kit
Invitrogen and analysed on a 10 % SDS-polyacrylamide gel. The concentration
of purified protein was measured using a Nanodrop spectrophotometer.

Electrophoretic mobility shift assay (EMSA). The agr P3 and ica
promoter regions were amplified from S. epidermidis CSF4198
genomic DNA using Phusion High Fidelity polymerase with primers
S3icaProm1 and S3icaProm2 (for P_{ica}) and S3agrProm1 and
S3agrProm2 (for P_{agr}). The resultant PCR products were first purified
from a 2 % agarose gel and analysed on a 10 % SDS-polyacrylamide gel. The DNA concentration was determined using a Nanodrop spectrophotometer.

autolysis was measured as a percentage of the initial OD_{600}. Each
experiment was repeated three times.

RNA purification and real-time RT-PCR. RNA was extracted from
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Role of sarX in S. epidermidis biofilm regulation

**RESULTS AND DISCUSSION**

**Mutation of S. epidermidis sarX impairs biofilm-forming capacity**

A sarX deletion mutant, designated SARX1, was constructed in the biofilm-forming clinical isolate CSF41498. Semiquantitative biofilm assays revealed a 50% decrease in biofilm production by SARX1 in BHI and BHI glucose compared with the wild-type strain (Fig. 1a). This diminished biofilm-forming capacity was not due to altered growth of the SARX1 mutant (data not shown). Biofilm formation by SARX1 was restored to wild-type levels by multicopy sarX (pSESAX6), whereas overexpression of sarX in CSF41498 and SARX1 increased biofilm-forming capacity (Fig. 1a). Primary attachment rates were similar in SARX1 and CSF41498 (P>0.05), but were significantly increased in CSF41498 pSESAX6 compared with CSF41498 (P<0.05) (Fig. 1b). These data reveal a positive role for sarX in S. epidermidis biofilm regulation, and suggest that although sarX can influence primary attachment it is primarily involved in regulating the accumulation phase of biofilm production.

**SarX regulates the agr system in S. epidermidis**

The S. aureus SarX protein has been implicated in the regulation of the agr system (Manna & Cheung, 2006), which is known to regulate biofilms in a strain-dependent manner (Beenken et al., 2003, 2010; Boles & Horswill, 2008; Coelho et al., 2008; O’Neill et al., 2007; Regassa et al., 1992; Vuong et al., 2000a, b, 2003, 2004). Here, real-time RT-PCR was employed to compare RNAIII transcription in overnight BHI glucose cultures of CSF41498 pLI50, CSF41498 pSESAX6, SARX1 pLI50 and SARX1 pSESAX6. These experiments revealed a fivefold increase in RNAIII transcription in SARX1 compared with CSF41498 (Fig. 2a). Interestingly, multicopy sarX also increased RNAIII transcription in CSF41498 (Fig. 2a), indicating that SarX can positively and negatively influence agr transcriptional activity in a concentration-dependent manner. EMSAs revealed a concentration-dependent binding of purified recombinant SarX protein to a biotinylated P<sub>agr</sub> probe, which resulted in multiple protein–DNA complexes, as evidenced by a laddering of bands on the gel (Fig. 2b). This binding was disrupted by a 100-fold excess of specific unlabelled probe, whereas non-specific DNA had no effect (Fig. 2b), indicating that the interaction was specific. DNA bending by SarA-family proteins has been proposed to shorten the spacing between the −10 and −35 promoter regions, turning repression into activation (Morfeldt et al.,

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**Table 2. Oligonucleotide primers used in this study**

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<tr>
<td></td>
<td>SEagrProm2</td>
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Biotinylated promoter probes (4.5 ng) were added to increasing concentrations of MBP fusion protein in a 20 μl binding reaction containing 0.2 μg poly(dI-dC), 5% (v/v) glycerol, 5 mM MgCl<sub>2</sub>, 10 mM Tris, 50 mM KCl and 1 mM DTT at pH 7.5. The reaction was incubated at room temperature for 20 min, and loaded onto a 5% non-denaturing polyacrylamide gel and electrophoresed at 100 V for 60 min using a Bio-Rad electrophoresis apparatus, and cross-linked to the membrane under UV light for 10 min. The biotinylated probes were detected using a LightShift Chemiluminescent EMSA kit (Pierce Chemicals) and a FluorChem FC2 imaging system (Alpha Innotech).

**PIA assays.** PIA assays were performed based on the method of Cramton et al. (1999), as described previously (Holland et al., 2008).

**Statistical analysis.** Two-tailed, two-sample equal-variance Student’s t tests (Microsoft Excel 2007) were used to determine statistically significant differences in assays performed during this study. A significant difference was indicated as a P value of <0.05.

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**Oligonucleotide primers used in this study**

- **gyrB**
- **icaA**
- **RNAIII**
- **sarX**
- **P<sub>ica</sub>**
- **P<sub>agr</sub>**

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If a similar mechanism was involved in SarX-mediated agr regulation, it would explain why overexpression of SarX causes it to act as an activator of the agr system instead of a repressor. These data suggest that increased RNAIII expression may contribute to impaired biofilm production by SARX1. However, because both multicopy sarX and mutation of sarX activate RNAIII transcription but have different effects on biofilm production, it seems unlikely that SarX-mediated biofilm regulation in S. epidermidis CSF41498 is mediated exclusively via changes in agr activity.

Earlier reports have indicated that Agr controls expression of the major autolysin gene atlE (Vuong et al., 2003), which has been implicated in primary attachment and biofilm formation in S. epidermidis (Heilmann et al., 1997). More recently, a role for AtlE-mediated autolysis and extracellular DNA (eDNA) release in the early stages of S. epidermidis CSF41498 has also been reported (Heilmann et al., 1997). SarX-mediated regulation of the agr locus in S. epidermidis CSF41498 (a) Comparative measurement of RNAIII transcription by real-time RT-PCR in strains CSF41498 and SARX1 complemented with pLI50 (control) and pSESAX6 (multicopy sarX). Total RNA was extracted from cultures grown at 37 °C to OD600 4.0. RelQuant software (Roche) was used to measure the relative expression of RNAIII against that of the constitutively expressed gyrB gene. RNAIII transcript levels in all strains were then compared with CSF41498 pLI50, which was assigned a value of 1. Data presented are the mean of three independent experiments; error bars, SD. (b) Recombinant SarX binding to the agr promoter. Increasing concentrations (0.2–1.6 µM) of recombinant SarX protein were added to a biotinylated oligonucleotide Pagr probe before being separated on a 5% polyacrylamide gel. The protein–DNA interactions were competed with 100× specific or non-specific competitor DNA.

Fig. 1. Contribution of sarX to the biofilm and primary attachment phenotypes of S. epidermidis CSF41498. (a) Comparative biofilm phenotypes of strains CSF41498 and SARX1 harbouring plasmids pLI50 (control) and pSESAX6 (multicopy sarX). Semiquantitative measurements of biofilm formation under static conditions were performed in tissue culture-treated 96-well plates. All strains were grown at 37 °C in BHI and BHI glucose (BHI Glu) for 24 h. (b) Primary attachment phenotypes of strains CSF41498 and SARX1 harbouring plasmids pLI50 and pSESAX6 grown in BHI. Each strain was tested at least three times and mean results are presented. Error bars, SD; asterisks denote significant differences (P<0.05).

Fig. 2. SarX-mediated regulation of the agr locus in S. epidermidis CSF41498.
epidermidis biofilm formation has been reported (Qin et al., 2007). Here we investigated whether sarX-mediated agr regulation influenced autolytic activity in CSF41498 as well as the biofilm phenotype of this strain.

Triton X-100-induced autolysis of CSF41498 pLI50, CSF41498 pSESAX6, SARX1 pLI50 and SARX1 pSESAX6 cultures grown in BHI and BHI glucose was measured. Multicopy sarX significantly increased Triton X-100-induced autolysis in strains grown in BHI (Fig. 3a). Interestingly, all strains grown in BHI glucose displayed significantly reduced autolytic activity (Fig. 3b), and although multicopy sarX increased autolytic activity in BHI glucose cultures, the effect was not significant (Fig. 3b). Thus, repression of autolysis in all strains in BHI glucose was also associated with a decrease in biofilm production compared with BHI (Fig. 1a). However, overexpression of sarX in CSF41498 or SARX1 enhanced biofilm-forming capacity in BHI glucose (Fig. 1a) but had no significant effect on autolytic activity (Fig. 3a, b). Biofilm assays revealed that PAS, which is known to block autolytic activity without affecting viability or growth (Wecke et al., 1986; Yabu & Kaneda, 1995), increased biofilm production in all strains (Fig. 3c), further suggesting that autolytic activity is not required for CSF41498 biofilm development and that biofilms are actually enhanced in this strain when autolytic activity is impaired. Taken together, these data do not support a correlation between sarX-activated biofilm production and sarX-induced autolytic activity.

Contribution of sarX to ica-dependent biofilm formation

To investigate whether sarX-induced biofilm production was dependent on icaADBC-encoded PIA, we introduced pSESAX6 into the icaC mutant CSF-2 (Hennig et al., 2007). Biofilm assays in BHI glucose revealed that multicopy sarX increased biofilm formation in CSF41498 but not in CSF-2, indicating that sarX regulates biofilm formation in an ica-dependent manner (Fig. 4a). Real-time RT-PCR performed on CSF41498 and SARX1 harbouring plasmids pLI50 and pSESAX6 grown in BHI glucose revealed that deletion of sarX was associated with a 2.6-fold reduction in icaA transcription, which was complemented by multicopy sarX (Fig. 4b). Transcription of icaR was unaffected in SARX1, indicating that SarX activates the ica operon independently of icaR (Fig. 4c). Immunoblots revealed a small but measurable decrease in PIA production in SARX1 compared with CSF41498, which was complemented by pSESAX6 (Fig. 4d). In addition, PIA production was increased in CSF41498 pSESAX6 (Fig. 4d). EMSAs revealed that SarX bound specifically to the ica promoter (Fig. 4e), resulting in multiple protein–DNA complexes (Fig. 4e). Thus, as observed with the agr promoter, the ica operon promoter may bind dimers/higher-order multimers of SarX or may contain more than one SarX-binding site.
Taken together, these data reveal that SarX binds to and regulates the agr and icaADBC promoters in *S. epidermidis*. However, SarX-promoted biofilm production is mediated primarily through increased *icaADBC* operon expression and PIA production.

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