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Insights into the regulation of small RNA expression: SarA represses the expression of two sRNAs in *Staphylococcus aureus*

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ABSTRACT

The opportunistic pathogen *Staphylococcus aureus* expresses transcription factors (TFs) and regulatory small RNAs (sRNAs) which are essential for bacterial adaptation and infectivity. Until recently, the study of *S. aureus* sRNA gene expression regulation was under investigated, but it is now an expanding field. Here we address the regulation of Srn_3610_SprC sRNA, an attenuator of *S. aureus* virulence. We demonstrate that SarA TF represses *srn_3610_sprC* transcription. DNase I footprinting and deletion analyses show that the SarA binding site on *srn_3610_sprC* belongs to an essential 22 bp DNA region. Comparative analysis also revealed another possible site, this time in the *srn_9340* promoter. SarA specifically binds these two sRNA promoters with high affinity *in vitro* and also represses their transcription *in vivo*. Chromatin immunoprecipitation (ChIP) assays confirmed SarA attachment to both promoters. ChIP and electrophoretic mobility shift assays targeting σ^A RNA polymerase subunit or using bacterial RNA polymerase holoenzyme suggested that SarA and the σ^A bind *srn_3610_sprC* and *srn_9340* promoters in a mutually exclusive way. Beyond the mechanistic study of SarA repression of these two sRNAs, this work also suggests that some *S. aureus* sRNAs belong to the same regulon and act jointly in responding to environmental changes.

INTRODUCTION

Staphylococcus aureus is an opportunistic pathogen involved in a wide spectrum of diseases, including but not limited to skin infections, pneumonia, endocarditis, osteomyelitis, indwelling device infections, food poisoning and meningitis (1). Approximately 20% of the human population has the bacteria on their skin and nostrils, creating a reservoir of asymptomatic carriers (2). At the same time, infections caused by antibiotic-resistant *S. aureus* strains have severely increased, impacting civil and military healthcare systems worldwide (3). *S. aureus* is remarkable in its aggressiveness and in its resistance potential to many antibiotics. Its versatility depends on its ability to sense and respond to environmental changes by modulating gene expression, using transcription factors (TFs) and regulatory small RNAs (sRNAs). The expression of *S. aureus* virulence factors is tightly controlled by multiple regulators, including TFs, two-component systems and sRNAs (4–6). Many of these regulators are therefore essential for infection (7).

To start bacterial transcription, direct interaction is required between the RNA polymerase's sigma factors and selected gene promoters. Initiation of RNA synthesis is a highly regulated process involving TFs that bind these gene promoters (8). One of the well-studied TFs in *S. aureus* is SarA, a 14.7-kDa DNA-binding protein acting as a dimer. It is one of 11 members of the SarA protein family, with SarR, SarS, SarT, SarU, SarV, SarX, SarY, SarZ, Rot and MgrA. They all have a winged helix motif (9–12) that is required for binding AT-rich double-stranded DNA sequences such as promoters (11,13–15). Directly or indirectly, SarA influences the transcription of at least 120 genes in *S. aureus* (16), and it can either stimulate (with *cna*, *fnbA*, *agr*, *hla*, *fnta*, *fnb* and *sec*) or repress (with *sarV*, *aur*, *sspa*, *spa*, *rot* and *SarS*) target gene expression (9,17–20).

In addition to TFs, *S. aureus* expresses about 160 regulatory RNAs (4,21), all recently compiled into a staphylococcal regulatory RNA database (SRD) (22). sRNAs are expressed from both the core and variable accessory genomes, the latter including pathogenicity islands (PIs) and transposons (23). Among these *S. aureus* sRNAs, only a handful has identified functions including certain ones that influence staphylococcal virulence in animal models of infection (24,25). In *S. aureus*, sRNAs usually pair with target mRNAs for regulation, influencing mRNA expression by modulating its degradation and/or its translation (26). Not only is the *S. aureus* sRNome poorly described, but little is

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known about the regulation of its expression. An exception to this is RNAIII, which is directly regulated by AgrA (27) and indirectly by SarA (28). AgrA also directly represses the expression of ArtR sRNA (29), termed 'Srn_4050' in SRD. Conversely, RNAIII and ArtR negatively regulate the Rot and SarT TFs, respectively (4,30,31), and RNAIII stabilizes *mgrA* mRNA thereby increasing MgrA TF production (32). Since TFs have numerous targets, sRNA regulation of TF expression allows sRNAs to play an important role in adaptation. These few examples highlight the need for investigation into the regulation of *S. aureus* sRNAs.

The small PI rna C ('Srn_3610' in the SRD, and referred to herein as 'Srn_3610_SprC') is located in the νSAβ PI (23) which contains several virulence factors. We recently showed that Srn_3610_SprC attenuates *S. aureus* virulence and spread in an animal model of infection (33). Those observations imply that its expression must decrease during infection. Interestingly, *srn_3610_sprC* expression drastically decreases after host cell internalization. This suggests that the gene's expression is tightly regulated during bacterial infection. Here we therefore investigated the molecular basis of this regulation *in vivo* and *in vitro,* and demonstrated that SarA restrains *srn_3610_sprC* expression through its direct interaction with the sRNA promoter. The DNA sequence required for SarA interaction with the *srn_3610_sprC* promoter was uncovered by DNase I footprinting assays and sequential deletions. The sequence was then used *in silico* to search for similarities in the *S. aureus* genome. This led to the identification of *srn_9340,* a second sRNA gene whose expression is repressed by SarA. Chromatin immunoprecipitation assays (ChIP) demonstrated SarA binding onto *srn_3610_sprC* and *srn_9340* promoters in living bacteria. ChIP experiments targeting σ^A further revealed that SarA prevents σ^A binding onto the *srn_3610_sprC* promoter. Analysis of the *srn_3610_sprC* and *srn_9340*/SarA binding sequences and the electrophoretic mobility shift assay (EMSA) data suggested that SarA transcriptional repression is similar for both, with SarA binding the promoter thus preventing σ^A binding. In this report, we demonstrate that the SarA TF inhibits transcription of two sRNAs located in the same PI. Such results suggest that these sRNAs have similar functions and belong to a common regulatory network. If applied to other sRNAs, our approach could reveal sRNA clusters regulated by dedicated TFs.

While sRNAs are key adaptation modulators, to our knowledge, none of them have been described as essential genes in *S. aureus*. This suggests some redundancy in their actions, or a lack of knowledge about when they might be absolutely essential for the bacteria. Therefore, studying sRNA transcriptional regulation could give clues to these key moments, while allowing for the identification of clusters of sRNAs with similar functions.

MATERIALS AND METHODS

Bacterial strains and culture conditions

Strains, primers and plasmids are listed in Supplementary Tables S1, 2 and 3, respectively. All mutants from the SarA family were obtained through phage transduction from 6390 *S. aureus* deleted strains to strain HG003 (34). *Escherichia coli* strains were grown at 37°C in LB (MoBio), and 50 μg/ml ampicillin or kanamycin antibiotics were added when necessary. *S. aureus* strains were grown at 37°C in either brain-heart infusion (BHI) medium or tryptic soy broth (TSB; both from Oxoid), with antibiotics added when needed (10 μg/ml erythromycin, chloramphenicol, or tetracycline, or else 250 μg/ml kanamycin). All experiments were done on the HG003 *S. aureus* strain (35), using the RN4220 *S. aureus* strain as an intermediate (36).

Genetic manipulation

Bioinformatic analysis. The *S. aureus* NCTC 8325 genome (taxid: 93061) was used for all genetic locations.

Reporter gene experiments. To create pCN41c, the *ermR* erythromycin resistance gene from plasmid pCN41 (37) was replaced by the *cat194* chloramphenicol resistance gene. In pCN41c-P_{sprC}, the *srn_3610_sprC* promoter sequence was amplified from *S. aureus* HG003 genomic DNA using 5′_BamHI_P*sprC* and P*sprC*_EcoRI_3′ primers (Supplementary Table S2) digested and inserted into pCN41c.

Complementation studies. A 1624-bp fragment made up of the SarA endogenous promoter gene and terminator was amplified by polymerase chain reaction (PCR) using 5′_BamHI_P*sarA* and *sarA*_KpnI_3′ primers, then inserted into pCN36 (37).

ChIP experiments. Three PCR steps were required to construct pCN38-SarA6His. The following primers were used: 5′_PstI_PsarA; PsarA_BamHI_3′; 5′_BamHI_sarA; sarA6his_KpnI_3′; 5′_KpnI_TsarA; and TsarA_KpnI_3′. These PCR products were combined to form a 1936-bp fragment containing *sarA* with an in-frame tag made of 6× Histidine (6His) at the 3′ end of the open reading frame (ORF). The final SarA6His is controlled by its endogenous promoter and terminator.

RNA isolation

The protocol for total RNA extraction was adapted from Le Pabic et *al.* (33). Overnight cultures of *S. aureus* were diluted to an $OD_{600\,nm}$ of 0.1 in fresh BHI or TSB broth, then incubated at 37°C and 160 rpm. Hourly, cells were harvested and resuspended in lysis buffer (20 mM sodium acetate, 1 mM ethylenediaminetetraacetic acid (EDTA), 0.5% sodium dodecyl sulphate (SDS), pH 5.5). Cells were broken out using glass beads (Sigma-Aldrich) in a FastPrep 120 cell disrupter (MP Biomedicals). Total RNA were extracted using phenol/chloroform and precipitated in 100% ethanol supplemented with 0.3 M sodium acetate.

Northern blotting

Total RNA were separated on either denaturing polyacrylamide gels or on agarose gels, then transferred onto Zeta-Probe GT (Bio-Rad) or Nytran membranes (Schleicher & Schuell), respectively. All northern blots were revealed using the Digoxigenin method per the manufacturer's instructions (Roche). We used 20 nt probes previously marked with 3′-end DIG labeling. Signal acquisition was done using an

ImageQuant LAS 4000 imager (GE Healthcare). Approximate quantifications were calculated using the ImageQuant software and normalized against transfer-messenger RNA (tmRNA) or 16S ribosomal RNA.

RNA end mapping by RACE

Rapid amplification of cDNA end (RACE) experiments were performed as previously described (38). R1 _srn_9340 primers was used to reverse transcribe or Srn_9340 RNA. Two PCR reactions were performed with Taq polymerase (Invitrogen) using R2-F1 and R2-F2 primer pairs, respectively. PCR products were cloned using a pGEM vector system (Promega), transformed in XL1-Blue *E. coli* and sequenced with M13 reverse primer (Invitrogen, ThermoFisher Scientific) using MegaBACE DNA sequencers (Amersham).

Protein extraction, cell fractionation and western blotting

For protein extraction throughout bacterial growth, cells were resuspended in lysis buffer (50 mM Tris–HCl, pH 7.5, 3 mM $MgCl_2$, 0.1 mg/ml lysostaphin), incubated for 20 min at 37°C, then kept on ice. As measured with Qubit fluorometric quantification (Invitrogen), equal amounts of total proteins were loaded on 15% polyacrylamide gels. Samples were separated on Tris-Glycine sodium dodecyl sulphate-polyacrylamide gel electrophoresis gels and transferred onto Hybond P PVDF membranes (Amersham). SarA proteins were revealed using rabbit anti-SarA antibodies. An ECL Prime Western Blotting Detection kit (Amersham) and an ImageQuant LAS 4000 imager were used to reveal proteins. To ensure that equal protein amounts were loaded on each lane, duplicates of these gels were stained with SYPRO Ruby (Bio-Rad) as per the manufacturer's instructions.

Functional complementation and reporter gene experiments

Overnight cultures were adjusted to an OD_{600nm} of 0.1. For each time point, cells were centrifuged and resuspended in 1× phosphate-buffered saline to obtain an equal cell density throughout the assay. Cell lysis was performed using 0.7 mg/ml lysostaphin, 0.2 U/μl benzonase and 0.1 mM $MgCl_2$ at 37°C for 20 min. After adding 0.25 mg/ml nitrocefin (a ß-lactamase substrate), ß-lactamase activity was quantified on a BioTek instrument every 10 min for 40 min at a wavelength of 492 nm. This activity was normalized against protein quantities as determined by a Bradford assay.

Purification of SarA from *E. coli*

The *sarA* coding sequence was inserted into pET42a in-frame with the 6His N-terminal tag (provided by Marc Hallier) and transformed in BL21 *E. coli*. SarA expression was induced by adding 1 mM isopropyl β-D-1-thiogalactopyranoside (IPTG). Cells were harvested, washed and resuspended in lysis buffer (10 mM HEPES, pH 7.5, 500 mM NaCl). Purification was done as previously described (39).

Electrophoretic mobility shift assays (EMSA)

Probes were amplified from the *S. aureus* HG003 genome by PCR using specific primers ($sprC_{P267}$, $16S_{P267}$, srn_9340_{P225} and $16S_{225}$), or obtained by hybridization of complementary oligonucleotides ($sprC_{47}$, srn_9340_{51}, $sprC_{30left}$, $sprC_{30middle}$, $sprC_{30right}$, srn_9340_{30left}, $srn\_9340_{30middle}$ and $srn\_9340_{30right}$). To construct $sprC_{P267\Delta47}$, $srn\_9340_{P225\Delta51}$, $sprC_{P267\Delta22}$ and $srn\_9340_{P225\Delta23}$, a three-step PCR was necessary. This used the following combinations of four primers: 5′_P267Δ47*sprc,* P267Δ47*sprC*_3′, 5′_P*sprC* and *sprC*_seq_3′ for $sprC_{P267\Delta47}$; 5′_P225Δ51*srn_9340,* P225Δ51*srn_9340*_3′, 5′_Psrn_9340 and *srn_9340*_seq_3′ for $srn\_9340_{P225\Delta51}$; 5′_P267Δ22*sprc,* P267Δ22*sprC*_3′, 5′_P*sprC* and *sprC*_seq_3′ for $sprC_{P267\Delta22}$; and 5′_P225Δ23*srn_9340,* P225Δ23*srn_9340*_3′, 5′_Psrn_9340 and *srn_9340*_seq_3′ for $srn\_9340_{P225\Delta23}$. DNA probes were labeled with [γ-^{32}P] adenosine triphosphate (ATP) using T4 polynucleotide kinase (New England Biolabs). Binding reactions were carried out as previously described (40). Briefly, binding reaction medium (10 fmol DNA template, 20 mM HEPES-KOH, pH 7.9, 20% glycerol, 0.2 mM EDTA, 0.1 M KCl, 0.1 M $MgCl_2$, 5 mM DTT and 0.2 μg poly(dI-dC)), was incubated when necessary with purified SarA or the *E. coli* RNA polymerase holoenzyme (New England Biolabs) for 30 min at 30°C. Samples were loaded on native polyacrylamide gels. Detection was done with a Typhoon FLA 9500 (GE Healthcare).

The dissociation constant (K_d) was determined by comparing the free versus the shifted DNA–protein bands (ImageQuant software), and apparent K_d was derived from the computed concentration at half-maximal binding.

DNase I footprinting assay

DNA probes were radiolabeled at one end. We used the HindIII restriction site, which is present in the *srn_3610_sprC* gene or in the 3′ primer for *srn_9340*. Klenow Fragment (New England Biolabs) filled the restriction site with [α-^{32}P] ATP and other unlabeled nucleotides. Binding reactions were done as previously described, then subjected to 2 or $7.5.10^{-2}$ unit of DNase I (Invitrogen) for 1 min at 30°C after supplementing the protein buffer with 40 mM $CaCl_2$. Reactions were halted by adding a stop buffer (10 μg/ml Invitrogen Proteinase K, 400 mM acetate sodium, 0.2% SDS, 10 Mm EDTA and 50 μg/ml yeast tRNA). After 15 min at 55°C of proteinase K action, the digested DNA samples were extracted by phenol/chloroform and ethanol precipitation. Pellets were resuspended in formamide dye mix and after denaturation were loaded on 8% denaturing polyacrylamide gels.

DNA sequencing

DNA probes used in the footprinting assays were sequenced according to the standard protocol (41). Specific primers were end-labeled with [γ-^{32}P] ATP using T4 polynucleotide kinase (New England Biolabs). The ratio of G, A, T and C nucleotides was adjusted in the sequencing mixture.

Chromatin immunoprecipitation assays

ChIP experiments were adapted from Faith et *al.* (42). HG003Δ*sarA* carrying either pCN38 or pCN38-SarA6His was cultured for 2.5 h at 37°C and 160 rpm, then treated with formaldehyde. Washed cells were thawed, incubated for 30 min at 37°C with 50 μg lysostaphin in a buffer (200 mM Tris, pH 8.0, 600 mM NaCl, 4% Triton X-100 and 1 mM PMSF), then sonicated. Dilution buffer was added to supernatant (see recipe in Supplementary Table S4) and precleared overnight at 4°C with end-over-end rotations with Protein G sepharose beads previously coated with 1% BSA and 0.1 μg salmon sperm. Input, ChIP, DIG, and Pol fractions were separated. The ChIP, DIG, and Pol fractions had 8 μg of antibodies to 6His, digoxigenin, and *E. coli* σ^{70} added, respectively, then they were incubated for 8 h at 4°C with 'end-over-end rotation. Immunoprecipitations were carried out overnight on Protein G magnetic beads previously coated with 1% bovine serum albumin (BSA) and 0.1 μg salmon sperm. Bead washes and DNA elution were also done as described by Faith (42)**.** ChIP analysis was performed by qPCR with comparative enrichment of the P_{sprC}, P_{srn_9340} and P_{hla} promoters. For normalization, 25 ng yeast plasmid DNA was added to all samples before phenol–chloroform extraction.

In vitro transcription assays

$SprC_{456}$ and srn_9340_{337} templates (corresponding to the sequences from promoter to terminator of genes of interest) were amplified from *S. aureus* genome by PCR using specific primers (5′_BamHI_PsprC, 3′_EcoRI_TsprC and 5′_BamHI_Psrn_9340, 3′_EcoRI_Tsrn_9340). *In vitro* transcription was realized adding 100 fmol of *E. coli* RNA polymerase (New England Biolabs) to 10 fmol of DNA template ($sprC_{456}$ or srn_9340_{337}), in 5× *E. coli* RNA polymerase reaction buffer (New England Biolabs) supplementing with nucleoside triphosphate ATP, CTP, GTP (0.75 mM each) and 0.5 μCi of [α-^{32}P] UTP. A total of 5 pmol of purified SarA protein were added to reactions, when needed. Samples were loaded on 8% denaturing polyacrylamide gels. Detection was done with a Typhoon FLA 9500 (GE Healthcare).

Statistical analysis

For statistical analysis, the one-tailed Mann–Whitney test was performed on three independent experiments and used to evaluate the significance of the ChIP assays. The two-tailed Mann–Whitney test was used for reporter gene experiments. Data were expressed as means ± standard deviations.

RESULTS

Srn_3610_SprC attenuates *S. aureus* virulence and host cell uptake. Its expression sharply decreases after *S. aureus* phagocytosis by human macrophages (33). We therefore investigated whether a TF might be involved in controlling *srn_3610_sprC* expression.

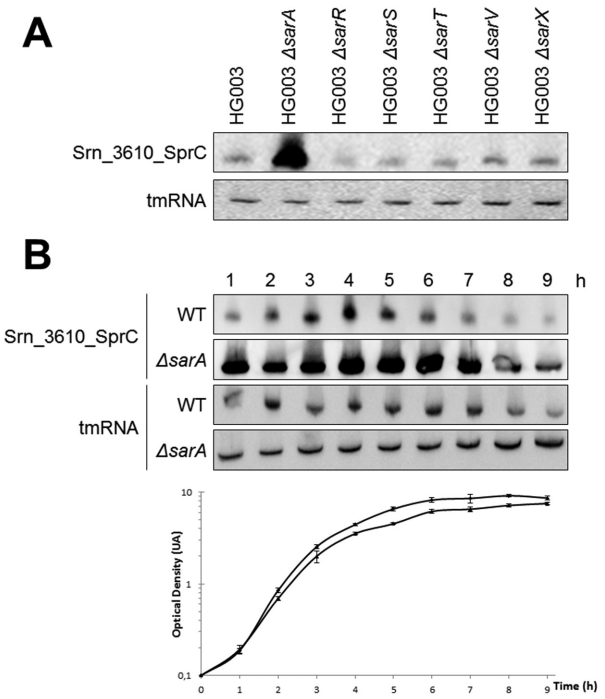

Figure 1. *srn_3610_sprC* expression increases in the absence of SarA. (**A**) *Srn_3610_sprC* expression in different *Staphylococcus aureus* strains lacking transcription factors (TFs). Northern blot analysis of the Srn_3610_sprC and transfer-messenger RNA (tmRNA) transcripts in the *S. aureus* HG003 strain and in isogenic mutants for the SarA protein family. All samples were harvested after 5 h of growth. A total of 10 μg of total RNA were analyzed, and the Digoxigenin method (Roche) was used to reveal Srn_3610_sprC and tmRNA (loading control). (**B**) Northern blot analysis of Srn_3610_SprC and tmRNA transcript expression in *S. aureus* HG003 and isogenic HG003Δ*sarA*. Samples were harvested at the specified time point, and tmRNA was used as a control. In a graph showing optical density as a function of time, the growths of HG003 (diamonds) and HG003Δ*sarA* (triangles) are represented in a semi-logarithmic scale.

The SarA transcription factor reduces levels of Srn_3610_SprC RNA during *S. aureus* growth

To initiate the study of *srn_3610_sprC* transcriptional regulation, we used Northern blotting to monitor RNA levels in six *S. aureus* HG003-derived strains. Each strain was deleted for a member of the sarA family: sarA, sarR, sarS, sarT, sarV or sarX (Supplementary Table S1) (12,19,43,44). The strains were grown in BHI medium, and total RNA extracted after the exponential phase (5 h of growth, OD_{600} ∼6). A DNA probe specific to Srn_3610_SprC was used to compare Srn_3610_SprC RNA levels between the mutants and the parental strain (Figure 1A).

A significant 10-fold increase was detected in the strain deleted for *sarA,* suggesting that SarA is the main repressor of *srn_3610_sprC* transcription. In contrast, a slight (about 30%) decrease was detected in the HG003Δ*sarR* strain, with no significant change observed in the other mutants (Figure 1A). The contradictory effects of SarA and SarR deletion make sense, since SarR represses SarA and because these regulators have antagonistic roles in target gene transcription (28).

To verify the hypothesis that SarA represses *srn_3610_sprC*, we monitored sRNA gene expression all along growth in HG003 and HG003Δ*sarA* strains. The growth of the HG003Δ*sarA* strain was slightly weaker than that of the parental one (Figure 1B), which is consistent with SarA's physiological role in regulating the expression of at least 120 genes in *S. aureus* (16). In the HG003 strain, Srn_3610_SprC levels fluctuate during growth, and after 4–5 h there was a peak corresponding to about a 3-fold increase (Figure 1B). In the *sarA* mutant, *srn_3610_sprC* expression was ∼10× higher at all collected time points, with maximum RNA levels detected after 4–5 h of growth (Figure 1B). Together, these results confirm that SarA has an essential role in *in vivo* regulation of *srn_3610_sprC*. However, these results also imply that other protein(s) than SarA participate(s) to the fluctuation of *srn_3610_sprC* expression during growth.

SarA negatively regulates srn_3610_sprC transcription

To provide further experimental support for a functional link between Srn_3610_SprC and SarA, we (i) did a *trans*-complementation assay and (ii) verified whether SarA acts on *srn_3610_sprC* at the transcriptional level. To these aims, in each of the following experiments, HG003 and HG003Δ*sarA* strains were co-transformed with a combination of two plasmids. The first plasmid carried *sarA* under control of its own promoter (pCN36-SarA), or was left empty (pCN36). The second carried *blaZ,* encoding the ß-lactamase reporter, either promoterless (pCN41c) or under the control of the *srn_3610_sprC* promoter (pCN41c-P_{sprC}). P_{sprC} was defined as a 144 nt-long DNA fragment (SAOUHSC_01956:18611729_186268) ending at the *srn_3610_sprC* 5′ end, as determined by RACE mapping (33). With pCN41c-P_{sprC}, a measure of the ß-lactamase activity in the presence or absence of SarA reflect regulation of this latter on the *srn_3610_sprC* promoter, thus on the transcription level of *srn_3610_sprC*. Total RNAs and proteins were extracted and expression of both endogenous Srn_3610_SprC and endogenous/exogenous SarA revealed by northern and western blots. As shown in Figure 2A, expression of pCN41c-P_{sprC} did not affect Srn_3610_SprC nor SarA endogenous levels (compare lanes 1 and 2 or 3 and 4). Conversely, exogenous expression of *sarA* through pCN36-sarA led to a significant increase in both SarA mRNA and protein levels in HG003 and HG003Δ*sarA* strains (lanes 3 and 4 versus 1 and 2, and 7 and 8 versus 5 and 6). From this result, we infer that both SarA mRNAs and the encoded protein are overexpressed in cells containing 'low copy' plasmid pCN36-SarA.

More importantly, northern blot analyses showed an ∼9-fold increase in Srn_3610_SprC level in the HG003Δ*sarA* strain as compared to HG003 (Figure 2A, lanes 1 and 2 versus 5 and 6), whereas reintroducing *sarA* restored endogenous Srn_3610_SprC level (Figure 2A, lanes 1 and 2 versus 7 and 8), validating that complementation occurred. Interestingly, when *sarA* was overexpressed in HG003 strain, Srn_3610_SprC level slightly decreased (lanes 1 and 2 versus 3 and 4), indicating an inverse correlation between the evolution of SarA and Srn_3610_SprC RNA levels.

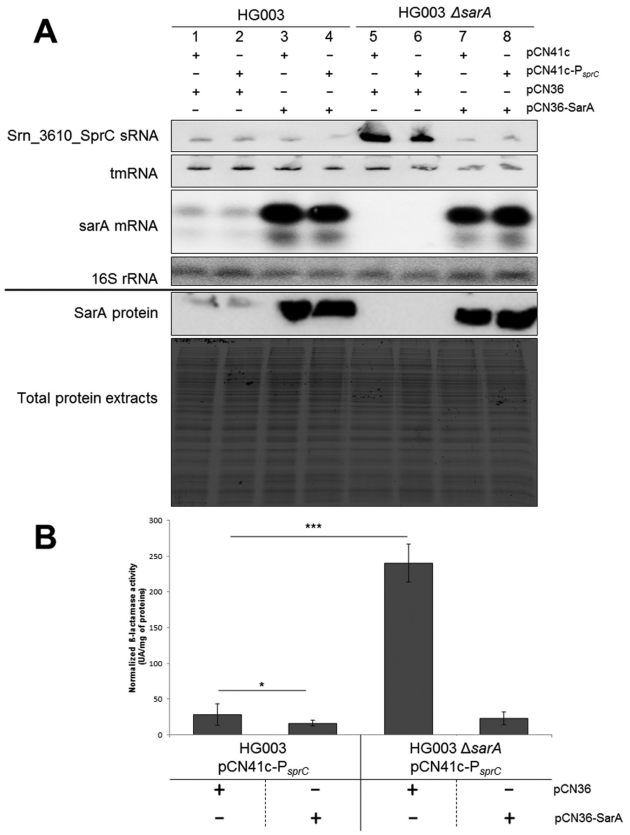

Figure 2. SarA represses *srn_3610_sprC* expression. (**A**) *Staphylococcus aureus* HG003 and HG003Δ*sarA* strains were co-transformed with: pCN36/pCN41c empty vectors (lanes 1 and 5); pCN36/pCN41c-P_{sprC} (lanes 2 and 6); pCN36-SarA/pCN41c (lanes 3 and 7); and pCN36-SarA/pCN41- P_{sprC} (lanes 4 and 8). Srn_3610_SprC and SarA transcripts levels were assessed by northern blot. A 16S rRNA probe was used to reflect total RNA loading for each lane. In parallel, SarA protein levels were checked by western blot (middle panel) and SYPRO Ruby staining of total protein extracts was done to compare loaded protein levels (bottom). (**B**) Effects of SarA on transcriptional activity of the *srn_3610_sprC* promoter (P_{sprC}). *S. aureus* HG003 wild-type strain (HG003) and a strain lacking *sarA* (HG003Δ*sarA*) were co-transformed with pCN41c or pCN41c-P_{sprC} and either pCN36 or pCN36-SarA. P_{sprC} activity was estimated by measuring ß-lactamase substrate hydrolysis. For each lane, indicated ß-lactamase activity was normalized by subtracting the background signal from the same strain where pCN41c-P_{sprC} was replaced by pCN41c empty vector (not shown). Three independent experiments were done, error bars show ± standard deviation. (Mann–Whitney test; $*P < 0.05$; $***P < 0.001$.)

We then measured ß-lactamase activity in all strains. The results for the strains expressing pCN41c-P_{sprC} are depicted in Figure 2B. First, confirming the Northern blot experiment, the ß-lactamase activity underwent a 40% drop when *sarA* was overexpressed (Figure 2B, second bar versus first bar; $P < 0.017$). Second, a 9-fold increase in ß-lactamase activity was measured in HG003Δ*sarA*_pCN36 compared to HG003_pCN36 (third bar versus first bar; $P < 0.0006$), while ß-lactamase activity in HG003Δ*sarA*_pCN36-sarA strain remained at level similar to that of the parental strain (fourth bar versus first bar). All together, these results confirmed that SarA negatively controls Srn_3610_SprC levels, and implied that SarA acts on the *srn_3610_sprC* promoter to reduce RNA expression. The correlation in both

ß-lactamase and Northern blot experiments indicates that SarA's repressive action is restricted to the promoter of the sRNA gene.

SarA interacts with the *srn_3610_sprC* promoter *in vitro*

The above results indicate that SarA probably represses *srn_3610_sprC* expression by acting on the *srn_3610_sprC* promoter. To further test this hypothesis, EMSA studies were done with recombinant SarA and the *srn_3610_sprC* promoter region. For these assays, we used a 6His-tagged full-length SarA with a 267-bp *srn_3610_sprC* DNA region ($sprC_{P267}$) that contains the 144 bp *srn_3610_sprC* promoter region along with 123 bp downstream of the +1 *srn_3610_sprC* transcription start. $sprC_{P267}$forms a complex with purified SarA and has an apparent K_d of 86 ± 3 nM (Figure 3A). A 10-fold excess of unlabeled $sprC_{P267}$ efficiently disrupt SarA from a preformed complex of labeled SarA/$sprC_{P267}$. However, a 100-fold excess of a 267 bp unspecific fragment made up of a section of 16S DNA ($16S_{267}$), was unable to do so (Figure 3B). This demonstrates the specificity of the complex formation between SarA and $sprC_{P267}$. Together, these results indicate that purified SarA directly binds the $sprC_{P267}$ promoter *in vitro* and its binding is specific.

The SarA binding site on the *srn_3610_sprC* promoter extends through the transcribed sequence

To further explore the SarA binding site on the *srn_3610_sprC* promoter, we used 3′-end labeled $sprC_{P267}$ probes with increasing concentrations of purified SarA to perform DNase I footprint assays. As expected, increasing amounts of SarA revealed a region protected from DNAse I cleavage within the *srn_3610_sprC* DNA sequence (Figure 3C).This 47 bp-long sequence is located between positions −32 and +15 in *srn_3610_sprC* (nts 1861616-1861570) and we named it '$sprC_{47}$' (Figure 3D).

To see whether this protected sequence was necessary for SarA binding, we created $sprC_{P267\Delta47}$, a 220 bp DNA fragment made from nucleotides −144 to +123 but with the footprint-identified $sprC_{47}$ sequence deleted (Figure 3D and E). EMSA testing of $sprC_{P267\Delta47}$ showed that SarA could not form a complex with the deleted promoter, even though it could with the native one (Figure 3E). These results confirm the key role of $sprC_{47}$ in SarA/P_{sprC} complex formation.

$sprC_{47}$ is sufficient for SarA binding onto *srn_3610_sprC* promoter

To test whether the 47-bp protected DNA region was sufficient for SarA-specific binding, EMSA was performed using $sprC_{47}$ or $Random_{47}$, a scrambled 47-bp DNA sequence. A 50-fold excess of unlabeled $sprC_{47}$ disrupts the preformed complex, whereas the same amount of $Random_{47}$ does not (Figure 3F), so SarA specifically binds $sprC_{47}$. Therefore, $sprC_{47}$ is necessary and sufficient for SarA binding.

srn_3610_sprC promoter dissection

We divided the $sprC_{47}$ sequence into three overlapping 30 bp sequences and challenged them using EMSA. Two of the overlapping DNA sequences which share 22 bp allowed SarA binding and the third did not (Supplementary Figure S1). We thus created a 245 bp DNA fragment from nts −144 to +123 without the shared $sprC_{22}$ section, resulting in the mutant $sprC_{P267\Delta22}$ (Figure 3D). EMSA was performed between $sprC_{P267}$ and purified SarA protein, and $sprC_{P267\Delta22}$ was used as a competitor to assess the SarA/$sprC_{P267}$ complex formation. The 22 bp sequence is necessary for SarA binding, since a 25-fold excess of unlabeled $sprC_{P267\Delta22}$ did not disrupt the $sprC_{P267}$/SarA complex, whereas the same amount of unlabeled $sprC_{P267}$ did (Figure 3G).

srn_9340 is another sRNA regulated by SarA

Using $sprC_{47}$ as a query, we did comparative sequence analysis on the HG003 strain's genome. This identified a 29 bp sequence sharing 93% identity with $sprC_{47}$ (Figure 4). Interestingly, the sequence is located upstream of *srn_9340* (22), an sRNA gene identified by RNAseq (45) and recently validated by northern blot under the name S774 (46). As for *srn_3610_sprC*, the *srn_9340* (NCTC 8325: 1863905–1863790) is located in the SaPIn3 (vSaß) PI. It is only 2338 bp downstream from *srn_3610_sprC* (1861729-1862268) and both sRNAs are expressed from the same genomic DNA strand (Figure 4, minus strand). Based on the homology between *srn_3610_sprC* and *srn_9340* promoter sequences, we hypothesized that *srn_9340* transcription might also be negatively controlled by SarA.

SarA represses *Srn_9340* transcription

Two ∼110 nt-long transcripts (Figure 5A) were detected in HG003 by northern blots using a *srn_9340*-specific probe. These transcripts were detected throughout bacterial growth (Supplementary Figure S2). In the HG003*ΔsarA* mutant, *srn_9340* expression is about 3.5× higher than in the isogenic HG003 strain (Figure 5A). Thus, *srn_9340* transcription seems to be repressed by SarA, albeit to a lesser extent than SarA represses *srn_3610_sprC*. While *srn_9340* seems to be expressed at lower level, compared to *srn_3610_sprC*, their expression patterns are very similar in HG003. They reach peak expression after 3–5 h of growth and expression levels fluctuate up to 3-fold. These observations suggest that the two sRNAs are analogously controlled. Moreover, these results also imply that other protein(s) than SarA participate(s) to the fluctuation of *srn_9340* expression during growth.

Going further, Srn_9340 levels were monitored by northern blots in the HG003*ΔsarR*, *ΔsarS*, *ΔsarT*, *ΔsarV* and *ΔsarX* strains. We observed a reduction of about 50% of *srn_9340* expression in HG003*ΔsarR,* but the levels were unaffected in the other strains (Supplementary Figure S3). These results are similar to those obtained for Srn_3610_SprC (Figure 1A), confirming the roles of SarA and SarR as antagonists for target gene transcription. We compared the HG003*ΔsarA* strain transformed with pCN36 or complemented by pCN36-*sarA,* and the results showed that the variation in *srn_9340* expression levels is controlled by SarA (Supplementary Figure S4). The Srn_9340 transcript's 5′ ends were mapped by RACE at the same genomic position (1863905), implying that they share

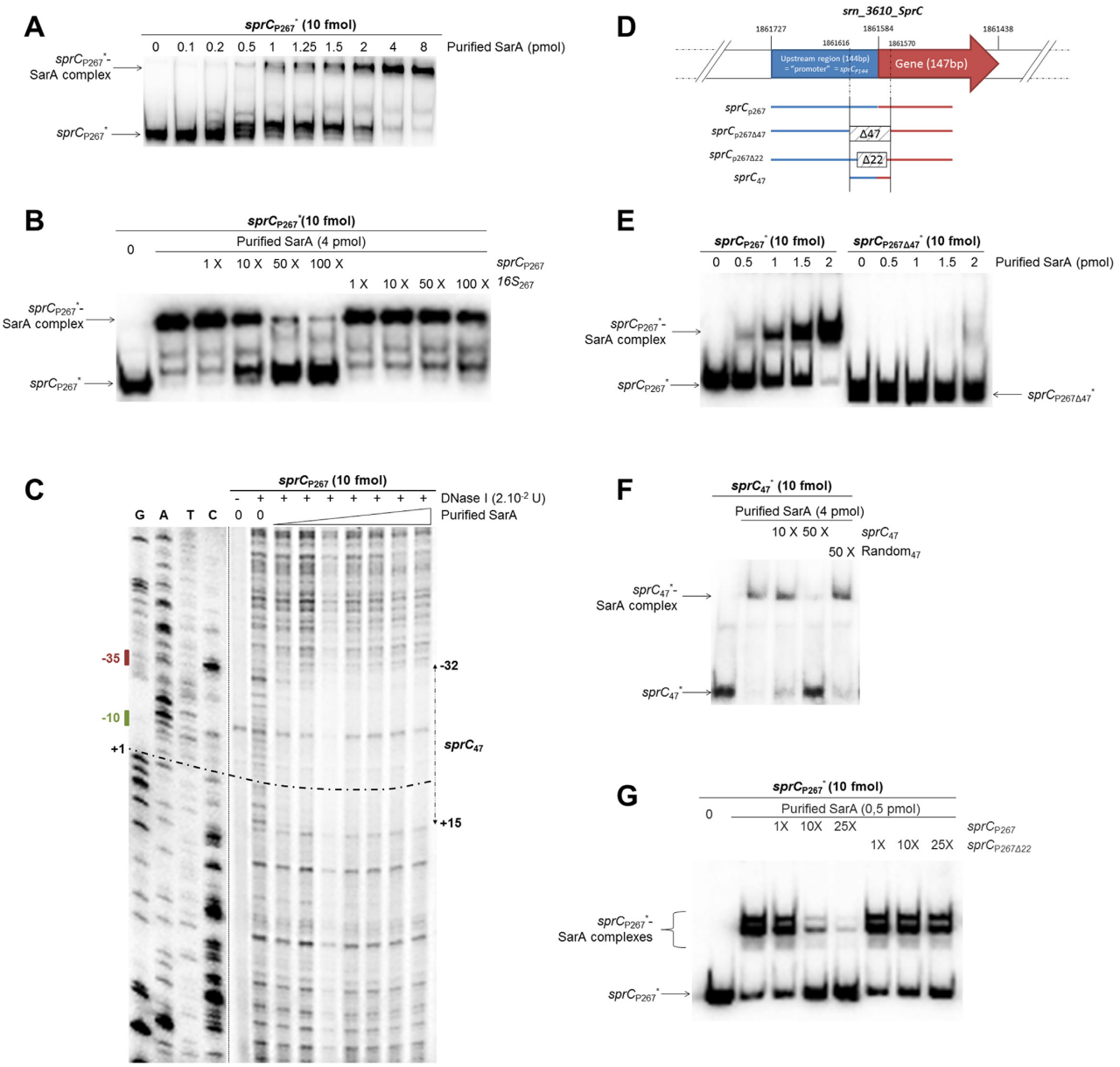

Figure 3. SarA specifically binds a region overlapping the *srn_3610_sprC* promoter and its 5′ end. (**A**) SarA binds the *srn_3610_sprC* promoter *in vitro*. Electrophoretic mobility shift assays (EMSA) were done using 10 fmol $sprC_{P267}$ probe, a ^{32}P-labeled *srn_3610_sprC* promoter fragment and increasing amounts of 0.1 to 8 pmol 6His-tagged SarA. (**B**) SarA specifically binds $sprC_{P267}$. EMSA was performed with 4 pmol of SarA and with increasing amounts of specific (unlabeled $sprC_{P267}$) or non-specific competitors (unlabeled $16S_{267}$). The $sprC_{P267}$/SarA complex is only inhibited in the presence of 50–100× excesses of the specific competitor. (**C**) The SarA binding site overlaps the *srn_3610_sprC* promoter and gene. DNase I footprinting assays were performed in the presence of 10 fmol $sprC_{P267}$, 2.10^{-2} U DNase I, and increasing amounts (0.5–2 pmol) of 6His-SarA. Lanes 1–4 correspond to $sprC_{P267}$ sequencing, and the promoter region is annotated. The vertical dotted arrow indicates the *srn_3610_sprC* region protected from DNase I degradation. The SarA binding site (−32 to +15) was named '$sprC_{47}$.' (**D**) Schematic representation of the different DNA probes used for EMSA studies. (**E**) The 47 bp region protected by SarA is necessary for SarA binding with *srn_3610_sprC*. EMSA were realized as in *A*, using ^{32}P-labeled $sprC_{P267\Delta47}$ (right) or wild-type ^{32}P-labeled $sprC_{P267}$ (left). SarA was unable to form a complex with $sprC_{P267\Delta47}$. (**F**) The 47 bp protected by SarA is sufficient for SarA binding onto *srn_3610_sprC*. EMSA were done using 10 fmol $sprC_{47}$, 4 pmol of 6His-tagged SarA, and increasing amounts of either unlabeled $sprC_{47}$ as a specific competitor or unlabeled 47-bp sequence ($Random_{47}$) as a non-specific competitor. SarA forms a complex with DNA (up-shifted band) that can only be disrupted by an excess of the specific competitor. (**G**) A 22-bp SarA binding site on $sprC_{P267}$ was found with EMSA done in the presence of 0.5 pmol of sarA and with increasing amounts of unlabeled $sprC_{P267}$ or unlabeled $sprC_{P267\Delta22}$.

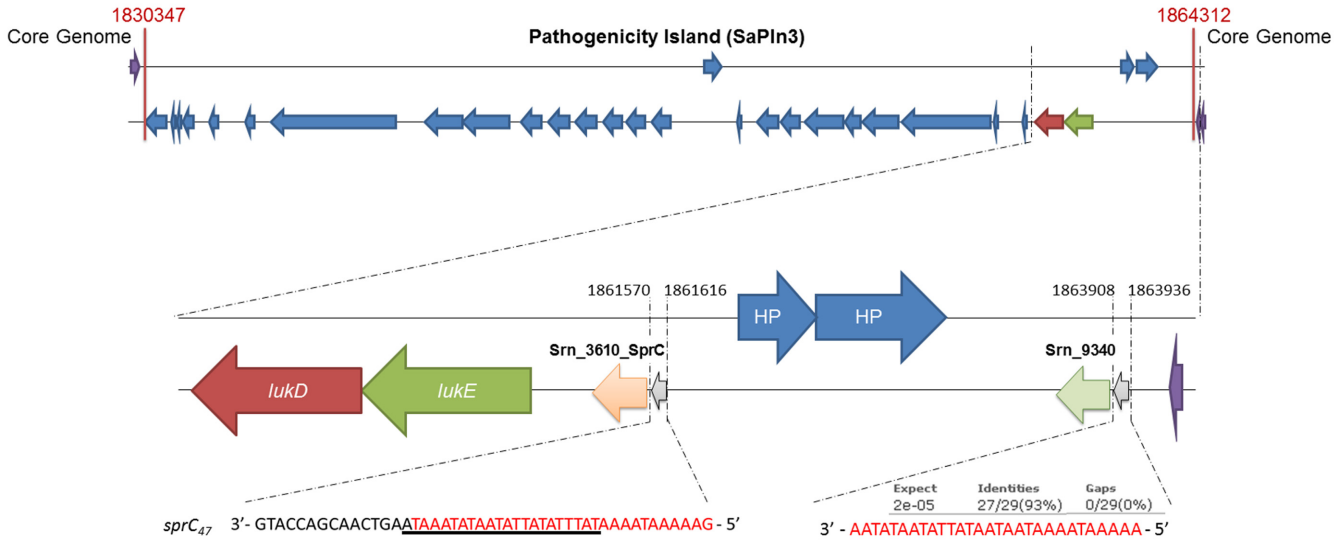

Figure 4. *srn_9340* possesses a putative binding site for SarA. *Srn_3610_sprC* belongs to the pathogenicity island SaPIn3. $SprC_{47}$, which can interact with SarA, possesses a 22-nt palindromic sequence (underlined). Blast analysis using $sprC_{47}$ as the query led to the discovery of another putative SarA binding site within SaPIn3. This sequence was identified as part of the promoter of another small RNA, Srn_9340.

the same transcription start site (TSS). The longer transcript's 3′ end maps at genomic position 1863790, whereas that of the shorter transcript ends at 1863797 (Figure 5B).

Consistent with the northern blot results (Figure 5A), the Srn_9340 transcripts are about 116 and 108 nts long (Figure 5B), with the shorter transcript lacking nucleotides at the 3′ end. No ORF was found in either sequence. The Srn_9340 transcript sizes obtained are slightly different from that of S774 (46). In addition to the size difference, Mäder *et al.* visualized a unique transcript. These differences could have been due to either genetic (HG001 versus HG003) or technical differences (gel resolution) (46).

SarA binds the *Srn_9340* promoter *in vitro*

We used EMSA for binding assays of purified SarA on the *srn_9340* promoter region (Figure 5C). We synthesized a PCR fragment of *srn_9340* nucleotides −165 to +60 (with the coding sequence starting at +1), and named this 'srn_9340_{P225}.' We observed a distinct SarA/srn_9340_{P225} complex with an apparent K_d of 11 ± 0.2 nM (Figure 5C, middle arrow). The complex has an abundance that is positively linked to SarA quantities. A second band shift then appeared which probably correspond to protein aggregation onto the DNA probe (Figure 5C, upper band). A 10-fold excess of srn_9340_{P225}effectively competed with the labeled promoter fragment for SarA binding, whereas a 100-fold excess of $16S_{225}$, an unlabeled non-specific 225 pb 16S DNA fragment, did not displace the labeled one from the complex (Figure 5D). Together, these results indicate that SarA directly and specifically binds the *srn_9340* promoter *in vitro*.

SarA binds the *srn_9340* promoter upstream from the +1 transcription start

We performed DNase I footprinting assays using a 3′-end labeled srn_9340_{P225} probe with increasing concentrations of purified SarA protein. As expected, without SarA, there was no protection against DNase I cleavage. Increasing SarA amounts revealed an *srn_9340* region that became protected from DNase I cleavages (Figure 5E). This 51 bp-long sequence is positioned between −51 and −1 within the *srn_9340* promoter (1863956–1863904), and we named it 'srn_9340_{51}' (Figure 5F).

To test whether this protected sequence is necessary and sufficient for SarA binding, we amplified $srn\_9340_{P225\Delta51}$, a 174 bp DNA fragment (nts −165 to +60) that does not include the srn_9340_{51} sequence (Figure 5F). EMSA performed on $srn\_9340_{P225\Delta51}$ showed that SarA complex formation was greatly reduced as compared to EMSA performed with the native promoter (Figure 5G). To test whether this region is sufficient for SarA binding, EMSA was performed with srn_9340_{51}. Indeed, SarA specifically binds srn_9340_{51}(Figure 5H), therefore, srn_9340_{51} is necessary and sufficient for SarA binding. Furthermore, a 10-fold excess of unlabeled srn_9340_{51} effectively competed with the labeled srn_9340_{P225} for SarA binding, whereas a 50-fold excess of $Random_{51}$, an unlabeled non-specific DNA fragment, did not disrupt the labeled one from the complex (Figure 5I). Considered together, these results confirm the requirement for srn_9340_{51} in complex formation between SarA and the *srn_9340* promoter.

srn_9340 promoter dissection

To further delineate the SarA binding site on the srn_9340 promoter, the srn_9340_{51} sequence was divided into three overlapping 30-bp sequences, then tested for SarA binding by EMSA. The results (see Supplementary Figure S5) clearly indicate the importance of a specific 23 bp sequence. A 25-fold excess of unlabeled $srn\_9340_{P225\Delta23}$ did not disrupt the srn_9340_{P225}/SarA complex, whereas the same amount of unlabeled srn_9340_{P225} did (Figure 5J). Therefore, removing this 23 nt sequence from the 225 nt-long

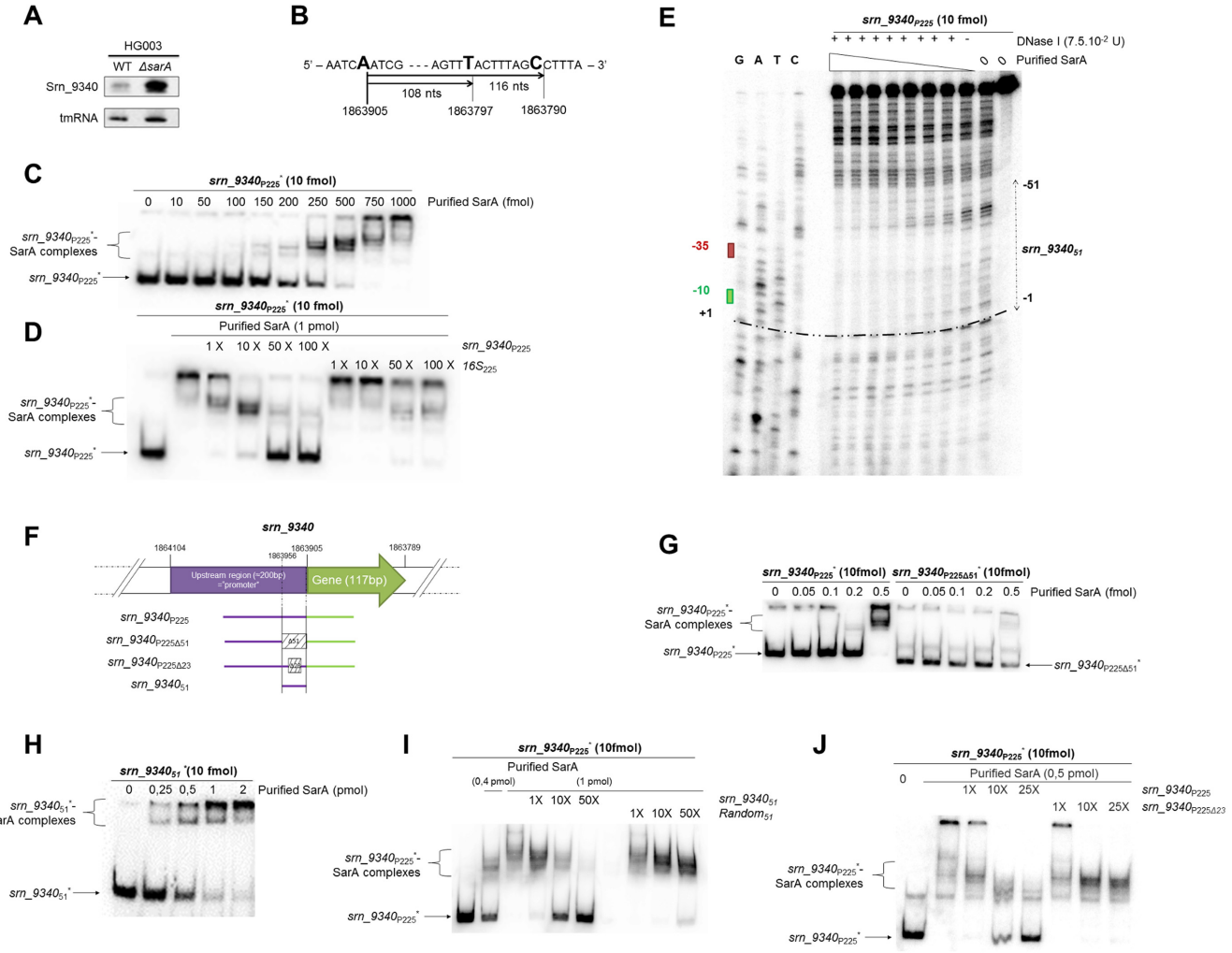

Figure 5. SarA binds *srn_9340* on its promoter and represses transcription. (**A**) SarA represses *srn_9340* expression. After 5 h of growth, 10 μg of total RNA were obtained from *Staphylococcus aureus* HG003 and HG003Δ*sarA*. Srn_9340 expression levels were monitored by northern blot. (**B**) Schematic representation of both the 5′ and 3′ ends of the Srn_9340 transcripts (long and short small RNAs), as determined by rapid amplification of cDNA ends (RACE). (**C**) SarA binds the *srn_9340* promoter *in vitro*. An EMSA was done using 10 fmol of a ^{32}P-labeled *srn_9340* promoter fragment (srn_9340_{P225}) as a probe in the presence of increasing amounts (0.01–1 pmol) of 6His-tagged SarA. Srn_9340_{P225} forms an initial complex with 0.15 pmol of SarA, and a second one at 0.75 pmol. (**D**) SarA specifically binds srn_9340_{P225}*in vitro*. EMSA was done with 10 fmol of ^{32}P-labeled srn_9340_{P225}, 1 pmol of 6His-SarA and increasing amounts of specific (unlabeled srn_9340_{P225}) or non-specific competitors (unlabeled $16S_{225}$). (**E**) DNase I footprinting assays were performed in the presence of 10 fmol srn_9340_{P225}, $7.5.10^{-2}$ U DNase I and increasing amounts (0.1–0.5 pmol) of 6His-SarA. The region protected by SarA is indicated with a vertical dotted arrow, and the numbers indicate relative positions to the previously determined transcription start site (TSS). Lanes G, A, T and C correspond to sequencing. The nucleotides from −51 to −1 (srn_9340_{51}) are protected by SarA against DNase I degradation. (**F**) Schematic representation of the DNA probes used for EMSA studies. (**G**) Deletion of the protected SarA sequence from the *srn_9340* promoter region abolishes SarA's capacity to bind the *srn_9340* promoter. (**H**) A 51 bp protected sequence is sufficient for SarA binding. EMSA were done using 10 fmol srn_9340_{51} in the presence of increasing amounts of 6His-tagged SarA (0.25–2 pmol). (**I**) Srn_9340_{51} competes with $srn_9340_{225}$for SarA binding. EMSA was performed with 10 fmol of ^{32}P-labeled srn_9340_{P225}, 0.4 or 1 pmol of 6His-SarA and increasing amounts of specific (unlabeled srn_9340_{51}) or non-specific competitors (unlabeled $Random_{51}$). (**J**) The 23 bp SarA binding site on $srn_9340_{225}$was confirmed by EMSA done in the presence of 0.5 pmol sarA and with increasing amounts of unlabeled srn_9340_{225}or unlabeled $srn\_9340_{225\Delta 23}$.

DNA promoter prevents it from competing against the preformed SarA/*srn_9340* complex, which implies that this region is mandatory for binding SarA.

SarA binds *srn_3610_sprC* and *srn_9340 in vivo*

In ChIP experiments, cellular macromolecular interactions are frozen and analyzed by protein IP and qPCR. Among other uses, this method allows for the comparison of enriched DNA sequences bound to a protein. We performed ChIP assays on HG003Δ*sarA* carrying either pCN38 or pCN38-SarA6His. We used 6His antibodies to immunoprecipitate SarA6His, with *hla* as a positive control because SarA directly regulates *hla* transcription (18,47). IP induced about a 19-fold enrichment in P_{sprC} (Figure 6A), while in the same extracts the *srn_9340* and *hla* promoters were respectively enriched about 6- and 16-fold. In the presence of SarA (HG003Δ*sarA*_ pCN38-SarA6His), the average P_{sprC} retrievals in the SarA-IP experiments were statistically higher than without it (HG003Δ*sarA*_pCN38). The obtained en-

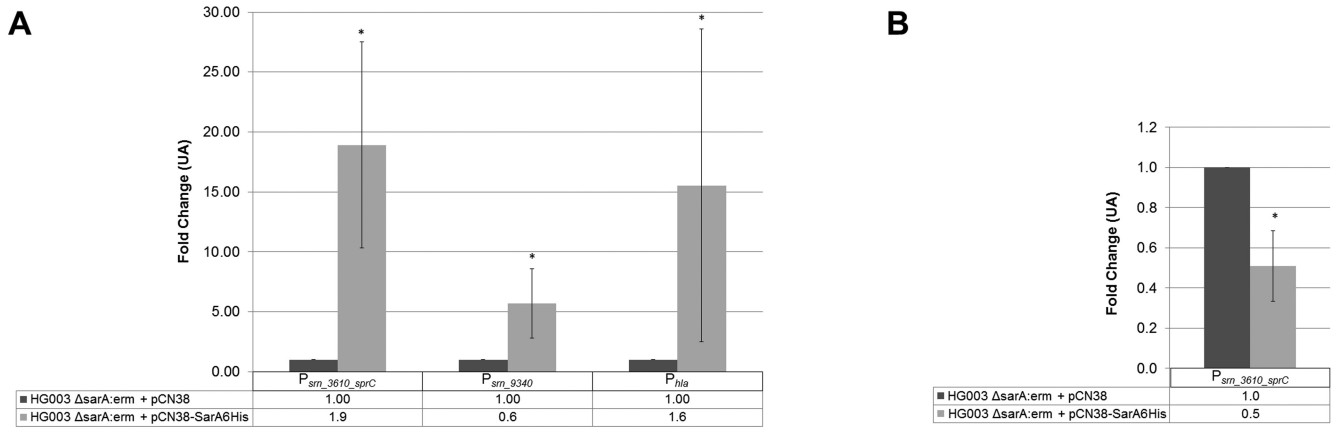

Figure 6. *In vivo* analysis of the srn_3610_sprC and srn_9340 promoters: SarA versus σ^A binding. The *Staphylococcus aureus* HG003Δ*sarA* strain was transformed either with an empty pCN38 vector (dark gray) or with pCN38-SarA6His (light gray). Chromatin immunoprecipitation (ChIP) experiments were conducted using antibodies for 6His-tag (**A**) or for the σ^{70}/σ^A RNA polymerase subunit (**B**). DNA enrichment was assessed by qPCR analysis using specific primers for the promoters of *srn_3610_sprC* (left panel), *srn_9340* (middle) or *hla* (right). Values beneath the bars indicate fold change between specific and non-specific interactions. (Mann–Whitney U test; three independent experiments; $*P < 0.05$). (A) *srn_3610_sprC*, *srn_9340* and *hla* promoters co-immunoprecipitate with SarA6His. (B) SarA6His disturbs σ^A binding on the *srn_3610_sprC* promoter.

richments of P_{hla} and P_{srn_9340} were also statistically significant. These results confirm the presence of SarA on both the *srn_3610_sprC* and *srn_9340* promoters *in vivo*.

In *S. aureus*, σ^A is the functional analog of σ^{70} in *E. coli.* This sigma factor is the RNA polymerase subunit involved in promoter recognition (48). Both *S. aureus* σ^A and *E.* coli σ^{70} RNA polymerase subunits recognize canonical promoters and are interchangeable (49). It has already been reported that *srn_9340* transcription depends on σ^A (46). After promoter sequence analysis, we predicted that the *srn_3610_sprC* promoter must be σ^A-dependent. We used *E. coli* σ^{70} antibodies to immunoprecipitate the *S. aureus* σ^A, investigating its accessibility at P_{sprC} and P_{srn_9340} with and without SarA. $sprC_{47}$ and srn_9340_{51} correspond to the DNA sequences protected by SarA, and they both contain the −10 TATA box and partial or complete −35 box RNA polymerase binding sites. Therefore, SarA should prevent binding of staphylococcal RNA polymerase onto both sRNA promoters. We found that the average amount of *srn_3610_sprC* promoter in the σ^{70}-IP with SarA (HG003*ΔsarA*_pCN38-SarA6His) was statistically lower than that of the deleted strain (HG003*ΔsarA*_pCN38) (Figure 6B). The *srn_3610_sprC* promoter amounts in the ChIP targeting σ^A were also lower in the presence of SarA than without it ($p < 0.05$). Meanwhile, the presence or absence of SarA did not make a statistical difference in the amount of the *srn_9340* promoter recovered after σ^{70}-IP enrichment (HG003*ΔsarA*_ pCN38-SarA6His and HG003*ΔsarA*_pCN38, respectively) (Supplementary Figure S6).

Since in both promoters the SarA binding site includes the RNA polymerase loading location, we expected a similar repression model. We therefore supposed that the σ^{70}-IP ChIP experiment was not useful for the study of P_{srn_9340}. This led us to further explore the genomic environment of *srn_9340*. The highly transcribed gene $tRNA^{ser}$ is 408 bp away from *srn_9340*. Such a close proximity (Supplementary Figure S6) could lead to sonicated DNA fragments containing *srn_9340* and $tRNA^{ser}$, making it difficult to use ChIP data to draw any conclusions. Thereafter, we performed EMSA studies on purified SarA and bacterial RNA polymerase *(E. coli)* together with either *srn_3610_sprC* or the *srn_9340* promoters.

SarA binding onto *srn_3610_sprC* and *srn_9340* promoters prevents RNA polymerase loading

Purified *E. coli* RNA polymerase holoenzyme binds the two $sprC_{P267}$ and srn_9340_{P225}promoters (Figure 7A and B) with about 0.75 and 1.8 nM affinities *in vitro*. To see whether SarA might prevent RNA polymerase binding onto these promoters, another set of SarA EMSA experiments was performed with and without the RNA polymerase (Figure 7C and D). Similar patterns were observed for both promoters in the presence of both SarA and RNA polymerase (Figure 7C and D). Multiple bands corresponding to SarA/DNA low molecular weight complexes appeared, and in the presence of RNA polymerase, high molecular weight complexes emerged. These latter complexes migrate similarly as DNA/polymerase complex (compare lanes 3, 4, 5 with, respectively, lanes 6, 7, 8), allowing the conclusion that these complexes only contain the RNA polymerase and the sRNA promoter. Moreover, using 100 fmol of RNA polymerase in the presence of SarA, we still observed a lower molecular weight sarA/promoter complexes, whereas 50 fmol of RNA polymerase is sufficient to bind nearly all $sprC_{P267}$ and srn_9340_{P225}(Figure 7A and B). However, even when two-fold higher RNA polymerase is added in the presence of SarA, SarA-promoter complexes are still detected (Figure 7C and D). This suggests that DNA/SarA and DNA/RNA polymerase complexes co-exist, implying that when SarA is loaded onto each of the two sRNA promoters, RNA polymerase stops being able to bind. We infer from these results that SarA binding onto $srn_9340_{P225}$and $sprC_{P267}$ promoters hampers RNA polymerase binding. To reinforce that, we performed *in vitro* transcription assay using the *E. coli* RNA polymerase and each of the DNAs en-

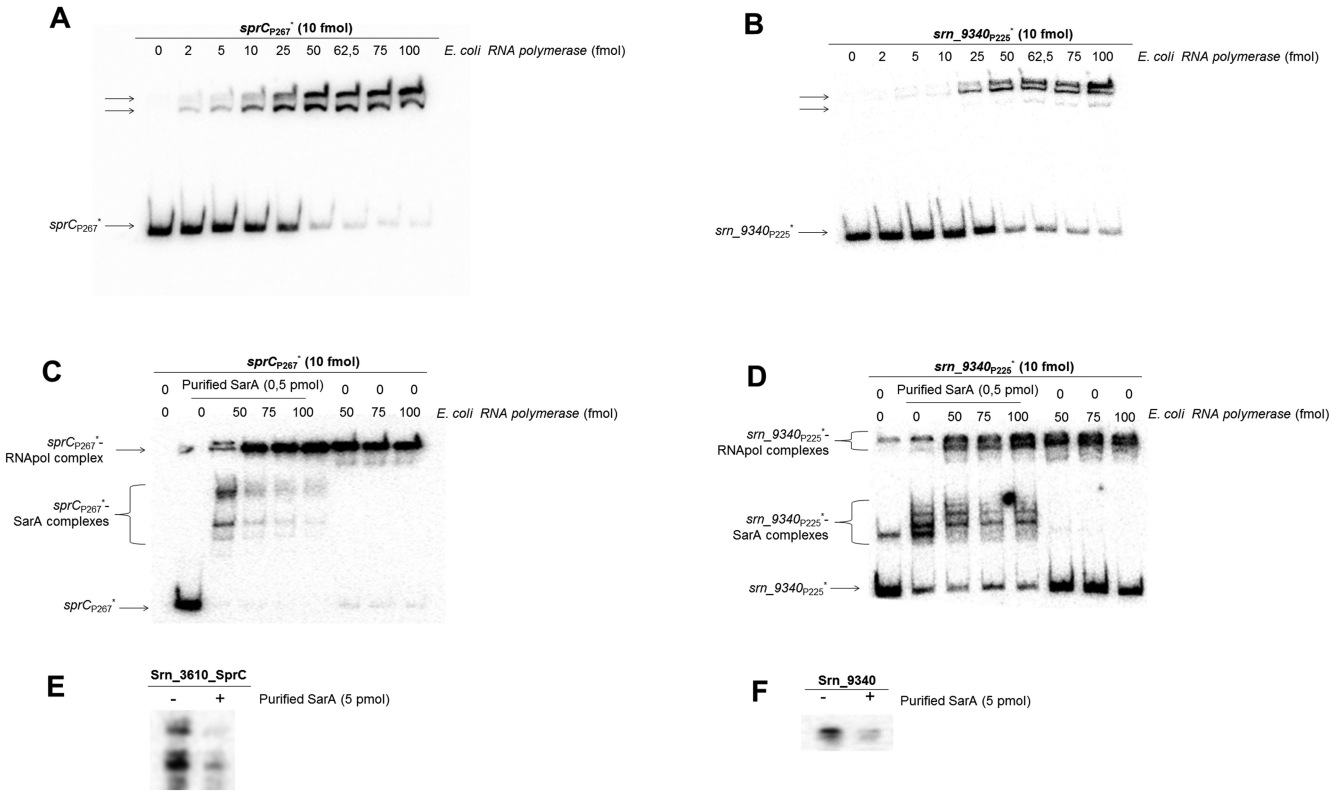

Figure 7. SarA and bacterial RNA polymerase holoenzyme cannot simultaneously bind $sprC_{P267}$ and srn_9340_{P225}. EMSA was done using 10 fmol ^{32}P-labeled $sprC_{P267}$ or srn_9340_{P225} probes with increasing amounts (2–100 fmol) of purified RNA polymerase. The binding capacity of *Escherichia coli* RNA polymerase was confirmed by EMSA experiments using $sprC_{P267}$ (**A**) or srn_9340_{P225} (**B**). To demonstrate their binding exclusivity on $sprC_{P267}$ (**C**) or srn_9340_{P225} (**D**), 0.5 pmol purified SarA and 50–100 fmol *E. coli* RNA polymerase were simultaneously added to EMSA experiments. *In vitro* transcription was realized using 10 fmol of $sprC_{456}$ (**E**) or srn_9340_{337} (**F**) and 100 fmol of *E. coli* RNA polymerase. A total of 5 pmol of the purified TF SarA were added, when indicated.

compassing the sequences from promoter to the sRNA terminators ($sprC_{456}$ and srn_9340_{337}), in the presence/absence of the SarA protein. As shown in panels E and F from Figure 7, the *E. coli* RNA polymerase is able to produce transcripts from *srn_3610_sprC* and *srn_9340* and SarA protein severely impact these transcriptions. Altogether, it is concluded that TF SarA binds *srn_3610_SprC* and *srn_9340* promoters and prevents transcription.

DISCUSSION

How sRNAs are regulated in *S. aureus* remains an essential but unanswered and barely addressed question. Most documented cases of sRNA regulation are in enterobacteria, where sRNAs are transcriptionally controlled by TFs and *vice versa* (50). In *S. aureus*, sRNAs also regulate the expression of TFs. For example, RNAIII represses Rot TF translation (30) and RsaA (Srn_1510) represses MgrA synthesis (25). To learn about sRNA gene regulation in *S. aureus*, we investigated the regulation of *srn_3610_sprC* expression. This sRNA was used as a model since it acts as a virulence attenuator, modulating host phagocytosis, therefore we expected its expression to be tightly controlled. The pleiotropic TF SarA is involved in *S. aureus* biofilm formation and pathogenesis, among other things (51–53). In our study, this TF was identified as a negative regulator of transcription initiation in *srn_3610_sprC* and *srn_9340*. Whereas the absence of SarA correlates with a change in the expression level of at least 120 genes, it has only been shown to directly control the expression of a dozen mRNAs. Here we show that SarA is directly involved in transcriptional regulation of Srn_3610_SprC and Srn_9340 sRNAs. To our knowledge this is the first identification of SarA as a transcriptional repressor of sRNA. Beyond just a mechanistic description of a direct interaction between a TF and an sRNA promoter, our work has identified two sRNAs whose expressions are under control of one general TF. Having arrived with an exogenous PI, these two sRNAs are now negatively regulated by a TF from the core genome. This demonstrates *S. aureus*' ability to take over sRNA regulation. Such a mechanism probably strengthens *S. aureus*' infectivity.

Based on the similarities between SarA repression of *srn_3610_sprC* and *srn_9340* transcription, their comparable expression patterns during bacterial growth, and their close genetic locations, we can guess that these two sRNAs are in part functionally related. Previous studies have shown that *srn_3610_sprC* expression is tightly regulated during host immune cell recognition of the bacteria for clearance during infection (33), while no functions have yet been identified for Srn_9340 (22,46). Our work connects the control of the expression of these two sRNAs to a common TF, sug-

gesting that Srn_9340 might also be involved in virulence and host immune response.

Without SarA, *srn_9340* and *srn_3610_sprC* transcription levels are respectively 3- and 9-folds higher, indicating that their expression should be repressed during bacterial growth. A TF downregulating sRNA gene expression could, upon TF release from the sRNA promoters, allow transient expression of sRNAs. This will enable the bacteria to respond rapidly and efficiently to environmental changes. SarA repression of *srn_9340* and *srn_3610_sprC* was confirmed by *trans*-complementation. In that experiment, higher *sarA* mRNA and protein levels were detected in the HG003*ΔsarA*_pCN36-SarA strain than in the isogenic strain. However, *srn_3610_sprC* and *srn_9340* expression levels did not decline further (Figure 2B and Supplementary Figure S4). This might be because the endogenous levels of SarA are sufficient to repress the transcription of both sRNAs. It also suggests that a phenotype linked to these sRNAs might be detected by stimulating their expression *in vivo* with an SarA-independent promoter.

Our study points out SarA binding differences and similarities for both promoters. Purified SarA protein specifically binds *srn_3610_sprC* and *srn_9340* promoters *in vitro*, with apparent binding constants of about 90 and 10 nM, respectively. These K_ds are within the same range as those previously reported for other SarA mRNA targets (9,54,55). This suggests that, *in vitro*, SarA binds target sRNA and mRNA gene promoters with similar efficiencies. Gel retardation (EMSA) assays between SarA and srn_9340_{P225}revealed multiple retarded bands, as previously observed for SarA and other target mRNA promoters, e.g. on the *tst* toxic shock promoter (10,54). Depending on gel composition and migration conditions, $sprC_{P267}$/SarA complexes appear as either double or single bands (Figures 3E, G and 6C). This suggests the coexistence of multiple conformations of the DNA/SarA complexes, as expected for a dimeric protein with a binding site made up of two anchor points (56).The multiple complexes detected *in vitro* could also be related to several SarA binding sites or SarA could act as an architectural accessory protein rather than as a canonical TF (57). Architectural DNA-binding proteins like these influence genomic DNA superhelicities and modify the number of base pairs per helical turn. Depending on the location of its binding sites, SarA could modify the spacing between the −10 and −35 boxes. This could lead the promoter to either have an optimal conformation for transcription initiation, or, conversely, an unfavorable spacing inducing transcription repression (58,59).

DNase I protection assays between SarA and $sprC_{P267}$ identified a 47-bp protected DNA sequence located at positions −35 to +12. EMSA confirmed that this sequence contains the SarA binding site. When this section was deleted, SarA did not bind to the *srn_3610_sprC* promoter region, while the DNA fragment was sufficient for SarA binding *in vitro*, indicating that it is necessary and sufficient for SarA recruitment. As for SarA binding onto the *srn_9340* promoter, the protected section is a 51 bp sequence located upstream from the +1 transcriptional start site. This sequence is necessary and sufficient for SarA binding onto the *srn_9340* promoter. Previous studies revealed that the SarA region protected against DNAse I cleavages ranges from 31 to 144 bp (10,18,20). Such differences can be excused: some protected sequences corresponded to multiple SarA binding sites, thus the 47/51 bp SarA protected regions are within the ranges previously reported.

The SarA binding sequences were able to be reduced to about 22/23 nts long after EMSA studies using 30-nt overlapping DNA sequences together with longer sequences interrupted by internal deletion (Figures 3G and 5J). Figure 8 sums up the experimental evidence we collected showing SarA TF binding onto the two sRNA promoters, along with the existing information from the literature. The SarA binding site identified on the sarA P3 promoter is 26 bp-long, a similar size to the 22/23 bp sequence identified on the two sRNA promoters. Moreover, the 22/23 nt sequences are similar to a previously reported 26 bp consensus sequence that is required for SarA binding (18). This consensus sequence is not always detected within the promoters of the genes directly regulated by SarA. SarA binding studies used SELEX to identify a 7 bp segment required for SarA binding *in vitro* (56). That same sequence was present (with a mismatch tolerance of one) at least once within the 150 bp upstream from the TSS in 72 of 102 SarA-regulated genes (16). The analysis of the $sprC_{47}$ and the srn_9340_{51} SarA-protected sequences revealed two adjacent palindromes (Figure 8). These palindromes could anchor the SarA dimer onto the *srn_9340* promoter (59). SarA binds DNA as a dimer (20), and although the presence of a palindromic sequence is not a prerequisite for dimer binding, it may facilitate sarA recruitment. In *S. aureus*, the CodY TF acts as a dimer and also binds palindromic sequences (60). Furthermore, the SarA-regulated sRNAs promoters $sprC_{22}$ and srn_9340_{23} each contains part of a palindrome, and their absence in $sprC_{P267\Delta22}$and $srn\_9340_{P225\Delta23}$ prevents SarA from binding *in vitro*. Altogether, our data are consistent with previous studies on SarA binding and provide further evidence that sarA binding requires an ATTTTAT sequence in its target's promoter (56). Moreover, SarA binding covers the TATA box and −35 region of *sarA* mRNA (10), both necessary sequences for σ^A subunit RNA polymerase binding prior to transcription initiation (61). SarA may repress sRNA promoter transcription through its ability to compete with the RNA polymerase for promoter binding, as proposed for mRNAs regulated by SarA (56).

We showed with ChIP that SarA binds the *srn_3610_sprC* and *srn_9340* promoter regions *in vivo* (Figure 6A). These experiments also revealed that when the RNA polymerase subunit σ^A binds the *srn_3610_sprC* promoter, P_{sprC} enrichment with anti σ^{70} antibodies was statistically higher when SarA was deleted. Therefore, SarA and σ^A probably bind P_{sprC} in a mutually exclusive manner. We performed EMSA competition studies with the *srn_3610_sprC* and *srn_9340* promoters, purified bacterial RNA polymerase and SarA. These experiments suggested that RNA polymerase holoenzyme and SarA cannot bind the two promoters simultaneously. This is consistent with the fact that they both bind to the same area within P_{sprC}. ChIP data for the *srn_9340* promoter region using anti-σ^{70} antibodies were variable from one experiment to another (Supplementary Figure S6). The σ^A-ChIP experimental procedure was not useful for the *srn_9340* promoter due to the proximity of an-

Figure 8. Analysis of the SarA-protected sequences on the *srn_3610_sprC* and *srn_9340* promoters. The alignment of the $sprC_{47}$ and srn_9340_{51} sequences was performed using Clustal Omega software (http://www.ebi.ac.uk/Tools/msa/clustalo/). Boxes outline the −35 (yellow) and −10 (red) boxes. The TSS is indicated with a black arrow; the SarA binding site defined by Sterba *et al.* (5′ATTTTAT3′) is shown with dotted arrows (reverse complementary sequences); palindromic sequences are depicted by large arrows; and identical nucleotides are identified with stars. The 22 nt of $sprC_{47}$ and the 23 nt of *srn_9340* that are necessary for SarA binding are bold.

other highly transcribed gene ($tRNA^{ser}$) within the 400 bp surrounding *srn_9340*.Through σ^A binding onto $tRNA^{ser}$, immunoprecipitation with anti-σ^{70} antibodies resulted in a DNA fragment containing *srn_9340*. Therefore, the link between σ^A enrichment of the *srn_9340* promoter and the presence or absence of SarA is not relevant. This highlights the difficulty of ChIP analysis using antibodies directed against housekeeping proteins such as σ factor when done in bacterial genomes with closely located genes. We propose that SarA binds the *srn_3610_sprC* and *srn_9340* promoters and prevents RNA polymerase binding. *In vitro* transcription assays, in the presence/absence of purified SarA, at each sRNA promoter, confirmed that hypothesis since the presence of SarA drastically reduced the synthesis of each sRNA.

We report here that sarA-mediated transcriptional repression of P_{sprC} and P_{srn_9340} is effective throughout bacterial growth. External signals, possibly during infection and phagocytosis, may affect SarA binding onto the two sRNA promoters. This could be through post-translational modifications (62,63) or targeted degradation, with these changes inducing the release of SarA from the sRNA promoters, thus allowing their fast and efficient transcription. In the presence of oxidants, high levels of *srn_3610_sprC* hamper survival (33), implying that the SarA-mediated transcriptional control of this sRNA is essential during oxidative stress. These recent observations suggest that *srn_3610_sprC* expression levels must be tightly regulated, requiring timely transcription derepression for tasks that remain unidentified.

In conclusion, as far as we know these two *S. aureus* sRNAs gene negatively regulated by SarA are the first reported examples of joint transcriptional regulation of sRNAs. This may however represent just the tip of the iceberg, especially since coordinated regulation of several sRNAs by a single TF would be a significant advantage for bacterial fitness. We have already begun investigations aimed at exploring the functioning of Srn_9340 and at uncovering the physiological signals that remove SarA from the sRNA promoters to allow their optimal expression.

SUPPLEMENTARY DATA

Supplementary Data are available at NAR Online.

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