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SarT Influences *sarS* Expression in *Staphylococcus aureus*

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***Staphylococcus aureus* is a gram-positive pathogen that is capable of expressing a variety of virulence proteins in response to environmental signals. Virulence protein expression in *S. aureus* is controlled by a network of regulatory loci including *sarA* and *agr*. The *sarA*/*agr* network is associated with the expression of cell wall-associated adhesins during exponential growth and the expression of secreted enzymes and toxins in the transition to post-exponential growth. A number of *sarA* homologs, including *sarT* and *sarS*, have been identified in the *S. aureus* genome. Previous studies have shown that *sarA* influences expression of both *sarT* and *sarS* in the global regulatory network. SarS has been shown to bind to the *spa* promoter to induce expression of protein A. SarT, one of the SarA homologs that represses *hla* expression and is repressible by SarA and *agr*, was found to induce *sarS* expression in this report. Northern blot analysis of *sarS* and *spa* expression in *S. aureus* RN6390, and the isogenic *sarT*, *sarT sarA*, and *sarT agr* mutants showed that while *sarA* regulated *spa* expression directly, the *agr* locus used *sarT* as an intermediary to regulate *sarS*, thus leading to *spa* repression in *agr*-activated cells. Gel shift and footprinting analysis showed that SarT binds to the *sarS* promoter, indicating that the interaction of the *sarT* gene product with the upstream region of *sarS* is likely direct. Induction of *sarS* and *spa* by SarT in *agr*$^{+}$ strains was confirmed by a tetracycline-inducible system to titrate *sarT* expression.**

Throughout in vitro growth, and presumably during an infection, *Staphylococcus aureus* responds to environmental signals by selectively generating specific virulence proteins. During the exponential phase in vitro, *S. aureus* synthesizes surface proteins that mediate attachment, including protein A, fibrinogen, and fibronectin binding proteins (27, 45). In an infection, bacterial attachment would facilitate active colonization and formation of bacterial vegetations within the host (15, 27, 45). As the cells enter the post-exponential phase in vitro, proteins mediating attachment are repressed, while *S. aureus* synthesizes exoproteins (including hemolysins, toxins, proteases, and lipases) capable of causing host cell tissue damage (21, 47). By lysing host cells, proteins expressed in the post-exponential phase likely aid in the acquisition of additional nutrients and facilitate dissemination of *S. aureus* in vivo (45). A complex regulatory network, with many linked components apparently imparts precise and flexible coordination of protein expression in response to the microenvironment (1, 9, 36). The activation, repression, and interactions of the regulatory genes must be understood to develop a comprehensive picture of the infectious process and disease development.

The *sarA* and *agr* loci comprise a primary global regulatory system that coordinates synthesis of cell wall and extracellular virulence proteins during late exponential and post-exponential phases (1) (Fig. 1). Transcriptional profiling studies comparing *sarA* and *agr* mutants with wild-type cells at different growth phases indicate that in addition to the virulence proteins, *sarA* and *agr* regulate expression of a number of genes involved in metabolic processes, and activation of metabolic and virulence genes is associated with the transition from exponential to post-exponential phase of growth (21).

The *agr* locus (Fig. 1) is a pleiotropic regulator for the synthesis of exoproteins as well as a repressor of cell wall synthesis during the post-exponential phase (10, 23, 25, 27, 42, 46). The *agr* locus encodes two divergent transcripts, RNAII and RNAIII, originating from two adjacent promoters, P2 and P3, respectively (27, 41) (Fig. 1). RNAII encodes four genes, *agrB*, *-D*, *-C*, and *-A*, which comprise the genetic elements of a two-component quorum-sensing system (25, 30, 38). The RNAII gene product AgrD encodes a 46-residue peptide that is processed to a cyclic peptide (autoinducing peptide [AIP]) by AgrB and exported (24, 25). AgrC is a transmembrane sensor that is autophosphorylated when activated by a threshold concentration of AIP and, in turn, activates AgrA, the response regulator for transcriptional activation of the *agr* P2 and P3 promoters (30, 41). The P3 promoter mediates transcription of RNAIII, the *agr* regulatory molecule. RNAIII mRNA forms a complex three-dimensional structure containing multiple loops that are believed to control transcription and under certain circumstances, translation of exoprotein genes by binding to specific target sites (5, 23, 37, 42).

The *agr* P2-P3 interpromoter region, in addition to self-activation via AgrA, is also activated by SarA, the *sarA* gene product (16). The *sarA* locus consists of a 372-bp open reading frame driven by three sequential promoters (P2, P3, and P1) (4, 34) (Fig. 1a). Due to their overlapping nature, all three transcripts encode the *sarA* open reading frame. The P2 transcript encodes the entire *sarA* locus, including the triple promoter region (11). The P1 and P2 promoters are active during exponential and late exponential growth, while the P3 promoter is most active during the post-exponential phase and is regulated by σ^{B}, a stress-induced transcription factor (34). Protein-DNA binding studies revealed that SarA binds to a

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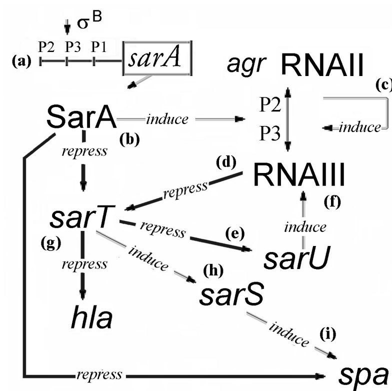


FIG. 1. A simple overview of the predicted *sarA*/*agr* regulatory web involving SarT and SarS. (a) SarA is transcribed from one of three nested promoters (P1, P2, or P3). (b) SarA represses expression of *spa* (protein A) and *sarT* (17, 48, 49) and induces *agr* RNAII (19, 38). (c) *agr* RNAII encodes a two-component quorum-sensing system, which activates expression of *agr* RNAIII, a pleiotropic regulator for expression of proteins associated with virulence (23, 27, 42, 46). (d) *agr* RNAIII represses *sarT*. (e) Increased expression of *sarT* causes repression of *sarU*, particularly during exponential growth (35). (f) *sarU* induces expression of *agr* RNAIII (35). (g) SarT represses expression of *hla* (48). (h) SarT induces expression of *sarS* (this study), and *sarS* induces expression of *spa* (17). The conditions and regulators mediating induction of *sarT* have not yet been determined.

29-bp sequence in the *agr* P2-P3 interpromoter region (Fig. 1c), activating *agr* RNAII and RNAIII transcription (19, 38).

While the *sarA*/*agr* system is the major controlling element for the expression of a variety of virulence proteins during the growth cycle (10, 13, 20), a series of other SarA-like proteins, discovered by promoter trap and genomic scanning, also appear to interact within the *sarA*/*agr* global regulatory network. Examples of these SarA homologs include *sarR*, which appears to repress *sarA* expression by binding to the *sarA* P1 and P3 promoters (33), and *sarS*, which induces *spa* (protein A) transcription (17, 49) (Fig. 1i). *sarT*, which is repressed by *sarA* and *agr* (Fig. 1b and d), has been shown by Northern blot, Western blot, and hemolytic assays to repress *hla*, the gene encoding α-hemolysin (48) (Fig. 1g). Interestingly, even though *sarT* is repressed by *agr*, it was shown both by Northern blotting and by a promoter-reporter system that repression of *sarT* could result in up-regulation of *sarU*, an activator of RNAIII, thus implying a positive-feedback control loop (35, 48) (Fig. 1e and f).

The *sarS* locus, adjacent to the *spa* gene, has been linked with *spa* induction (17, 49) (Fig. 1i). In this study, we showed that expression of SarT was accompanied by increased transcript levels of *sarS* and *spa*. Northern blot analysis of *sarS* and *spa* expression in *S. aureus* RN6390 and the isogenic *sarT*, *sarT sarA*, and *sarT agr* mutants showed that while *sarA* regulates *sarS* and *spa* expression directly, *agr* utilizes *sarT* to down-regulate *sarS* and thereby repress *spa* expression (Fig. 1h). Gel shift and DNA footprinting analysis revealed that purified SarT protein binds specifically to the *sarS* promoter region. Importantly, *sarS* and *spa* expression was induced in a dose-dependent manner when *sarT* expression was driven by a tetracycline-inducible promoter. Taken together, these data suggest that SarT is a positive regulator of *sarS* and the ensuing *spa* expression.

MATERIALS AND METHODS

Bacterial strains, plasmids, and growth media. The bacterial strains and plasmids used in this study are listed in Table 1. Phage 80α (39) was used as a transducing phage. *Escherichia coli* strains were grown in Luria-Bertani (LB) medium (32). *S. aureus* strains were maintained in tryptic soy medium (Difco) and grown in CYGP or 03GL medium (39). Erythromycin (5 μg/ml), chloramphenicol (34 μg/ml in *E. coli* and 10 μg/ml in *S. aureus*), tetracycline (5 μg/ml), ampicillin (50 μg/ml), and kanamycin (50 μg/ml) were used for selection of transformants and transductants.

Generating a *sarT* mutant. The *tetK* gene was cloned from pT181 (26) using the primers 5′-GAT AAA AGA AAT TTC GCC AGT C-3′ and 5′ ACG CGT CGA CAC TCG TTA ATA CGT GTG CTC TG-3′ and ligated into pCR2.1 (Invitrogen, San Diego, Calif.). *tetK* was ligated into a blunted *Nde*I site within the *sarT* coding region of pALC1894 (48), the insertion was confirmed by DNA sequencing, and the resulting 4.9-kb fragment containing *tetK* inserted into the *sarT* region was ligated into pBT2 (7). The resulting plasmid, pALC2229, was electroporated into *S. aureus* RN4220 and propagated through several cycles of alternating the temperature between 30 and 42°C as described elsewhere (8). Chloramphenicol-sensitive, tetracycline-resistant colonies, representing possible double-crossover events, were selected (14) and screened for *tetK* insertion into *sarT* by Southern blotting and by sequencing of the PCR fragment containing the junctional fragment. The *S. aureus* RN4220 *sarT*::*tetK* mutant (ALC2313) was used as the source for transduction of the *sarT*::*tetK* mutation to other *S. aureus* strains.

The *sarT* mutant of *S. aureus* SH1000 was generated by transducing strain SH1000 with an 80α phage lysate of *S. aureus* ALC2313 as previously described (14, 50). To confirm the genotype, chromosomal DNA from *S. aureus* cultures grown overnight was isolated by lysostaphin lysis and phenol extraction as described previously (14). DNA extracts of the putative transductants were digested with restriction enzymes and screened by Southern blotting for the presence of antibiotic resistance genes and shifts in the sizes of restriction digest fragments as previously described (48). Northern blot analysis with relevant DNA probes confirmed the loss of RNA message.

RNA analysis. Cells were grown to exponential (optical density at 650 nm [OD_{650}] of 0.7), late exponential (OD_{650} of 1.1), and postexponential (OD_{650} of 1.7) phases, and RNA was extracted using the Trizol isolation procedure (Gibco BRL, Gaithersburg, Md.) as previously described (18, 28, 48). RNA concentrations in the extracts were determined by absorbance at 260 nm using an Eppendorf BioPhotometer (Brinkmann, Westbury, N.Y.). Part (20 or 30 μg) of each sample was electrophoresed through a 1.5% agarose–0.66 M formaldehyde gel in 4-morpholinepropanesulfonic acid (MOPS) (Roche Diagnostics, Indianapolis, Ind.) buffer (20 mM MOPS, 10 mM sodium acetate, 2 mM EDTA [pH 7.0]) and blotted onto Hybond-N+ membranes (Amersham, Arlington Heights, Ill.) as previously described (16). Prior to blotting, the gel was viewed under UV light to ensure that equivalent amounts of ethidium bromide-stained rRNA bands were present for each sample. After blotting, the gel was viewed under UV light to confirm complete RNA transfer. Northern blots were probed with radiolabeled DNA probes for the detection of *sarT*, *sarS*, RNAIII, *hla*, *spa* and HU (a housekeeping transcript) transcripts as previously described (48). The HU transcript served as an internal gel loading control (20, 27).

Cell wall-associated proteins. Staphylococcal strains were grown overnight in CYGP broth and washed, and the cell density was adjusted to an OD_{650} of 1.0 in PBS. Cell wall-associated proteins were extracted after lysostaphin digestion in a hypertonic buffer (0.05 M Tris, 0.145 M NaCl, 30% raffinose [pH 8.0]) with protease inhibitors as previously described (13). Equivalent amounts of cell wall extracts were separated by electrophoresis on sodium dodecyl sulfate–10% polyacrylamide gels and transferred to nitrocellulose membranes by electroblotting. To detect protein A, nitrocellulose blots that had been incubated overnight in bovine serum albumin (BSA) blocking buffer (19) were incubated with affinity-purified chicken anti-protein A antibody (1:3,000 dilution) (Accurate Chemicals, Westbury, N.J.) and then with an alkaline phosphatase-conjugated F(ab′)2 fragment of sheep anti-chicken immunoglobulin G (Jackson Immunoresearch, West Grove, Pa.). Reactive bands were detected by incubating the blot with Nitro Blue Tetrazolium (NBT)/5-bromo-4-chloro-3-indolylphosphate (BCIP) substrate (Sigma Chemical Co., St. Louis, Mo.). Band intensities were determined by densitometric scanning using SigmaGel software (Jandel Scientific, San Rafael, Calif.), with the data presented as integrated area units (IAU).

Expression and purification of SarT. The 420-bp DNA fragment containing the *sarT* coding region was cloned from *S. aureus* RN6390 chromosomal DNA by

TABLE 1. Bacterial strains and plasmids used in this study

Strain or plasmid	Description	Reference(s)
S. aureus		
8325-4	Prophage-cured strain of NCT8325, harboring an 11-bp deletion in *rsbU*, which regulates *sigB* activity by activating *rsbV*, a factor that competitively binds to the anti-sigma factor RsbW	39, 42
RN4220	Mutant strain of 8325-4 that accepts foreign DNA	39
RN6390	*agr*$^+$ laboratory strain related to 8325-4	39
RN6911	*agr* mutant of RN6390 (Δ*agr*::*tetM*)	42
SH1000	8325-4 with intact *rsbU*	22
ALC1905	*sarT* mutant of RN6390 (*sarT*::*ermC*)	48
ALC1927	*sarS* mutant of RN6390 (*sarS*::*ermC*)	15
ALC2009	ALC1927 with pSK236 containing a 1.2-kb *sarS* coding region fragment	17
ALC2056	*agr sarT* mutant of RN6390	48
ALC2057	RN6390 *sarA*::*kan*	48
ALC2071	ALC1905 with pALC2047	48
ALC2076	ALC2056 with pALC2047	48
ALC2122	ALC2057 with *sarT*::*ermC* (*sarA sarT*)	48
ALC2150	ALC2122 with pALC2047	48
ALC2221	ALC1905 with pALC2217	This study
ALC2235	ALC2056 with pALC2217	This study
ALC2236	ALC2122 with pALC2217	This study
ALC2253	ALC2122 with pALC2073	This study
ALC2254	ALC2056 with pALC2073	This study
ALC2255	ALC1905 with pALC2073	This study
ALC2305	RN4220 with pALC2229	This study
ALC2313	RN4220 *sarT*::*tetK*	This study
ALC2389	*sarT* mutant of SH1000 (*sarT*::*tetK*)	This study
ALC2394	ALC2389 with pALC2047	This study
ALC2398	RN6390 with pALC2047	This study
ALC2399	ALC2389 with pALC2217	This study
ALC2402	ALC2389 with pACL2073	This study
Plasmids		
pALC1894	pUC18 with 3.2-kb fragment containing the *sarT* coding region	48
pALC2047	pSK236::*sarT*	48
pALC2073	pSK236::*xyl*/*tetO* promoter::*tetR*	3
pALC2217	*sarT* ligated into the *Eco*RI and *Sst*I sites of pALC2073	This study
pALC2223	pALC1894 containing the *sarT* mutation (*sarT*::*tetK*)	This study
pALC2229	pBT2 with the *sarT* mutation (*sarT*::*tetK*)	This study
pACL2321	pCR2.1 Topo with the *sarS* promoter fragment	This study

PCR with the primers 5′-GTA AGG GAT GAA CTC GAG ATG AAT GAT TTG AA-3′ and 5′-ACG GGG ATC CAA AAA TAC ATT TAA CTG CAC CAA-3′, ligated into the *Xho*I and *Bam*HI sites of pET14b (Novagen, Madison, Wis.), and transformed into BL21 (Novagen). Proper insertion of the *sarT* gene into the recombinant plasmid was confirmed by restriction digestion and by sequencing (Novagen). Protein expression was induced in broth culture with 1 mM isopropyl-β-D-thiogalactopyranoside (IPTG), the cells were harvested and lysed, and the protein was purified on a His-Tag column following the manufacturer's protocols (Novagen). The purity of SarT was confirmed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis, and the authenticity was verified by microsequencing the first 10 amino acids of the purified, thrombin-cleaved protein (48).

Doxycycline induction. The effects of SarT on target gene expression were evaluated with a tetracycline-inducible promoter system to yield a gradient of *sarT* expression. The *sarT* coding region, together with the upstream ribosomal binding site, was ligated downstream of the *xyl*/*tetO* promoter in pALC2073, a derivative of pSK236, containing the *tetR* repressor (3). Insertion was confirmed by restriction mapping and DNA sequencing. *sarT* mutant strains were transformed with the resulting tetracycline-inducible *sarT*-expressing plasmid (pALC2217) or with pALC2073 as a control. Cultures of the *sarT* mutant strains harboring the plasmids (cultures had been grown overnight in broth) were diluted 1:100 in CYGP medium and incubated with shaking at 37°C. To capture differential expression during the transition from the late exponential phase (OD_{650} of ≅1.4) to the post-exponential phase (OD_{650} of >1.7), the cultures were grown to an OD_{650} of 1.1 and doxycycline was added at concentrations of 0, 35, and 50 ng/ml to induce gene expression. Cultures were harvested for RNA analysis 1 and 2 h after the addition of doxycycline (48). Northern blots of RNA from the above strains were probed with ^{32}P-labeled specific gene fragments as previously described (16) for the detection of *sarT*, *sarS*, and *spa* transcripts.

DNA gel shift assay of SarT with the *sarS* promoter. The binding of SarT to the *sarS* promoter was confirmed by gel shift assays. A DNA fragment encompassing the *sarS* promoter region (49) was cloned using the primers 5′ CCC GGT ACC TAT TAC GCT TAC CTC GCT TTA-3′ and 5′-CCC GGA TCC TTT CAT TGT TTT ATC TC-3′, ligated into pCR2.1 Topo (Invitrogen, Carlsbad, Calif.), and confirmed by sequencing. A plasmid harboring the *sarS* promoter region fragment (pALC2321) was digested with *Sal*I and *Eco*RI to yield a 264-bp fragment (nucleotides [nt] 125169 to 125432) (29) for use in gel shift experiments. The gel-purified fragment was dephosphorylated and then end labeled with [γ-^{32}P]ATP using T4 polynucleotide kinase (U.S. Biochemical Corp, Cleveland, Ohio). The labeled fragment was incubated with various concentrations of purified SarT protein and analyzed by nondenaturing polyacrylamide gel electrophoresis as previously described (33). Controls were BSA, the *sarA* P3 promoter fragment as the nonspecific competitor, and unlabeled *sarS* promoter fragment as the specific competitor.

DNase I footprinting. DNase I footprinting assays with a 287-bp DNA fragment encompassing the promoter region upstream of *sarS* were performed as previously described (20). The top-strand primer 5′-ACA TCT AGA TGT TGT TAT TGT TAA CAA GCG −3′ at positions −288 to −259 from the translational start site (ATG) and the bottom-strand primer 5′-CTG TCC ATG GTT TTA TCT CCT TGT ATA TGC AC-3′ at positions −2 to −33 from the translational start site were used to amplify the *sarS* promoter region. To label the PCR product, only one of the primers was end labeled prior to the PCR. For the assay, the binding reactions were performed in the 100-μl reaction volume containing 20 mM Tris-Cl (pH 8.0), 100 mM NaCl, 5 mM $MgCl_2$, 1 mM $CaCl_2$,

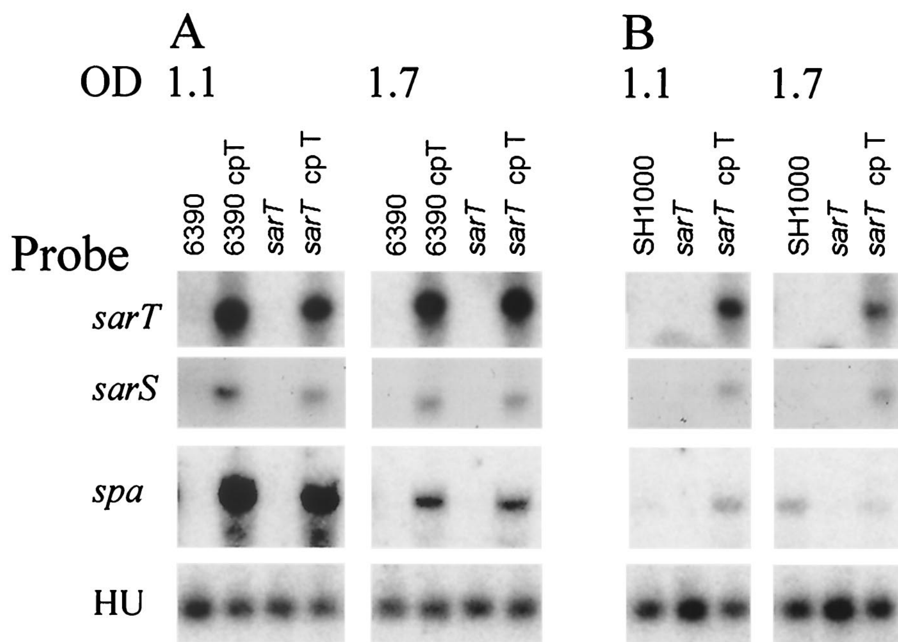


FIG. 2. Effects of *sarT* mutation on expression of *sarS* and *spa*. Northern blots of RNA extracted from *S. aureus* RN6390 (6390) (A) and SH1000 (B) and their isogenic *sarT* mutant and *sarT*-complemented strains were hybridized with *sarT*, *sarS*, *spa*, or HU probes. RNA was extracted during late exponential (OD_{650} of 1.1) and postexponential (OD_{650} of 1.7) phases of growth. Results are representative of multiple Northern blots, utilizing RNA extracts from multiple experimental sets as previously described (48). HU is constitutively expressed and served as an internal loading control (20, 27). Band density measurement for HU had a mean IAU of 3,455 (standard deviation, 498). The presence of pALC2047, a plasmid bearing an intact *sarT*, is indicated by cp T (for complementation in *trans*).

2 mM dithiothreitol, 10 μg of BSA, 0.4 μg of calf thymus DNA, radiolabeled template DNA (10,000 cpm for each reaction), and various amounts of purified SarT at room temperature for 30 min. DNase I (0.02 U) (Boehringer GmbH, Mannheim, Germany) was added and allowed to incubate for 1 min at room temperature. The reaction mixtures were extracted with phenol-chloroform, ethanol precipitated, washed with 70% ethanol, dried, and resuspended in loading buffer (98% deionized formamide, 10 mM EDTA [pH 8.0], 0.025% [wt/vol] xylene cyanol FF, 0.025% [wt/vol] bromophenol blue). The samples were denatured at 95°C for 5 min and analyzed on a 6% denaturing polyacrylamide sequence gel. Positions of the protected region were derived by comparing the footprint with the A+G sequencing ladder of the same fragment (34).

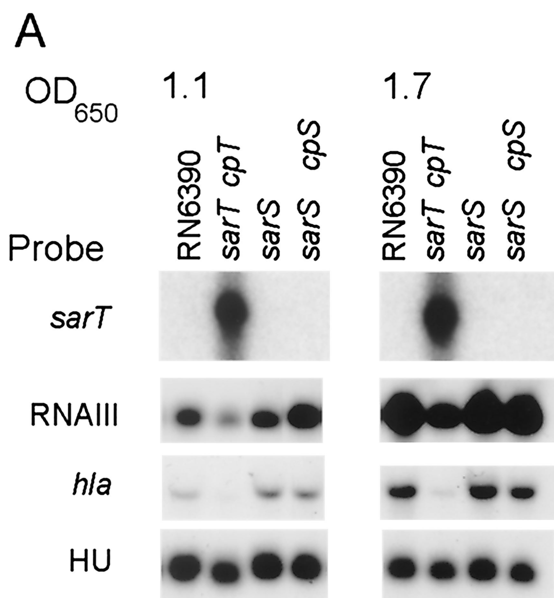
Densitometry. Blots were scanned with an Hewlett-Packard desktop scanner set at 200 dots per inch. Band density was determined from the scan image using the SigmaGel (Jandel Scientific) software. Graphs were drawn using Excel (Microsoft). Blot scans were processed into figures using Adobe Photoshop LE (Adobe Systems Inc., San Jose, Calif.).

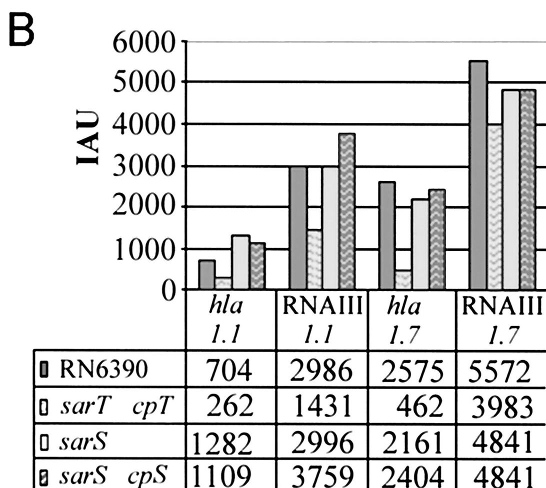
RESULTS

Effects of *sarT* on *sarS* and *spa* expression. In a previous study (48), we generated a *sarT* mutant harboring a plasmid (pALC2047) bearing the *sarT* gene (*sarT* in *trans*) that expressed a high level of SarT. We noted that on a Northern blot, the complemented *sarT* mutant strain (*S. aureus* ALC2071 [*sarT* cpT]) expressed high levels of *sarS* and *spa* messages during late exponential and post-exponential growth phases, concomitant with increased *sarT* expression in these strains (Fig. 2A). The same pattern was obtained when parental strain RN6390 was transformed with pALC2047 (6390 cp T [Fig. 2A]). As has been found previously (12), strain RN6390 was a low protein A producer, as evidenced by our failure to detect a significant level of *spa* transcription in this strain at both growth phases (OD_{650}s of 1.1 and 1.7 [Fig. 2A]).

S. aureus RN6390 harbors an 11-bp deletion in *rsbU*, a gene encoding an anti-sigma factor that, when activated, increases *sigB* expression approximately 50% (43). σ^B has been implicated in regulating expression of *sarA* and *agr* (Fig. 1a) (6, 22). Since *sarA* and *agr* expression influence expression of *sarT* and *sarS* (17, 48) (Fig. 1b and d), we wanted to confirm that the effect of *sarT* on *sarS* in strain RN6390 was not due to the mutation in *rsbU*. Accordingly, a *sarT* mutant was generated by transducing the *sarT* mutation into *S. aureus* SH1000 (*S. aureus* 8325-4 $rsbU^+$ [22], kindly provided by S. J. Foster), using phage 80α.

In Northern blots of late exponential and post-exponential RNA of *S. aureus* SH1000, expression of *sarT* and *sarS* was not detected (Fig. 2B). Interestingly, *spa* expression was detected during the post-exponential phase (OD_{650} of 1.7), but not at the late exponential phase (OD_{650} of 1.1) (Fig. 2B). In the SH1000 *sarT* mutant, neither *sarS* nor *spa* expression was detected (Fig. 2B). Complementation of the *sarT* mutant of SH1000 in *trans* resulted in increased expression of *sarS* and *spa*, particularly during the late exponential phase at an OD_{650} of 1.1 (Fig. 2B). Since *sarS* was expressed at equivalent levels in both the RN6390 and SH1000 *sarT*-complemented mutant strains (the *sarS* band density for RN6390 *sarT*-complemented mutant was 869 IAU versus 898 IAU for the SH1000 *sarT*-complemented mutant at the post-exponential phase) (Fig. 2), σ^B activity does not appear to directly affect the relationship between *sarT* and *sarS*. However, in the complemented *sarT* mutant, the expression of *spa* was lower than in the RN6390 counterpart. Although SarA has been shown to be a direct effector of *spa* expression (Fig. 1b) (12), the discrepancy in *spa*





	hla 1.1	RNAIII 1.1	hla 1.7	RNAIII 1.7
RN6390	704	2986	2575	5572
sarT cpT	262	1431	462	3983
sarS	1282	2996	2161	4841
sarS cpS	1109	3759	2404	4841

FIG. 3. Effects of *sarS* mutation on *sarT*, RNAIII, and *hla* expression. (A) Northern blots of RNA extracted from *S. aureus* RN6390 and isogenic mutants at late exponential (OD_{650} of 1.1) and postexponential (OD_{650} of 1.7) phases of growth and probed with *sarT*, RNAIII, or *hla* probe. Results are representative of Northern blots made from three independently isolated sets of RNA extracts. *cpT*, complemented with *sarT* in *trans*; *cpS*, complemented with *sarS* in *trans*. (B) RNAIII and *spa* signals quantified by densitometry. The mean IAU for the HU bands was 3,849 (standard deviation, 306).

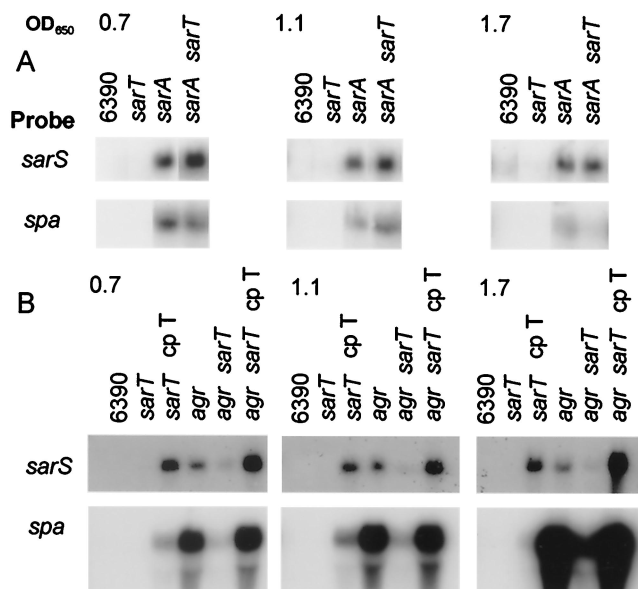


FIG. 4. Expression of *sarS* and *spa* in *agr*, *sarA*, *agr sarT*, and *sarA sarT* mutant strains. RNA extracts from *S. aureus* taken at exponential (OD_{650} of 0.7), late exponential (OD_{650} of 1.1), and postexponential (OD_{650} of 1.7) phases of growth were hybridized with *sarS* and *spa* probes. *sarT* and *sarA* mutant strains (A) and *sarT* and *agr* mutant strains (B) are shown. Results are representative of multiple Northern blots utilizing RNA extracts from multiple experimental sets as previously described. 6390, RN6390; cp T, complemented with *sarT* in *trans*.

expression between RN6390 and SH1000 cannot be attributed to the effect of σ^B on *sarA* expression alone.

Effect of *sarS* on *sarT*. While *sarT* appears to influence *sarS* expression, we wondered if *sarS*, in turn, modulates *sarT*. We thus examined the expression levels of *sarT* and of *agr* RNAIII and *hla*, two targets of *sarT* repression (48), in a *sarS* mutant and its *sarS*-complemented counterpart (*S. aureus* ALC2009) (17). As with *S. aureus* RN6390, *sarT* expression was not detected in either the *sarS* mutant or its isogenic *sarS*-complemented strain (Fig. 3A). Further, the expression levels of *hla* and RNAIII, target genes of *sarT*, did not vary substantially from the RN6390 levels in either the *sarS* mutant or its isogenic complement (Fig. 3). In contrast to RN6390, the parental strain (which has a single copy of *sarT*), the *sarT* mutant expressing *sarT* from a multicopy plasmid in *trans* expressed considerably lower levels of *hla* and RNAIII during the late exponential and post-exponential phases (Fig. 3). These results are consistent with the previous observation that *sarT* is a repressor of *hla* (48) and RNAIII by virtue of a feedback loop involving *sarU* (35) (Fig. 1).

In a previous study (48), expression of *hla* and RNAIII in a *sarT* mutant of *S. aureus* RN6390 was approximately double the levels seen for RN6390. As *agr* RNAIII and *hla* expression levels in the *sarS* mutant and its isogenic complemented strain did not differ substantially from those seen in RN6390 (Fig. 3B) and since any significant alterations in *sarT* expression would be reflected in changes in RNAIII (*sarT* ↑ → RNAIII ↓) and *hla* (*sarT* ↓ → *hla* ↑) expression, these data are consistent with the hypothesis that *sarS* does not exert any great inductive or repressive effect on *sarT*.

Effects of *sarA* and *agr* mutations. In the regulatory pathway (Fig. 1), SarA has been found to repress *sarT* (48), thus accounting for increased transcript levels of *sarT* in the *sarA* mutant compared to the parent (Fig. 4A). To determine whether SarA represses *spa* directly (SarA ↑ → *spa* ↓) or indirectly via *sarT* (SarA ↑ → *sarT* ↓ → *sarS* ↓ → *spa* ↓), a double *sarT sarA* mutant was generated and tested for *sarS* and *spa* expression in *S. aureus* RN6390. Expression levels of *sarS* and *spa* remained high in the *sarA sarT* double mutant compared with the single *sarA* mutant (Fig. 4A). As expected, *sarS* and *spa* expression was not readily detected in the *sarT* mutant. This suggests that *sarA* is repressing *spa* directly, rather than

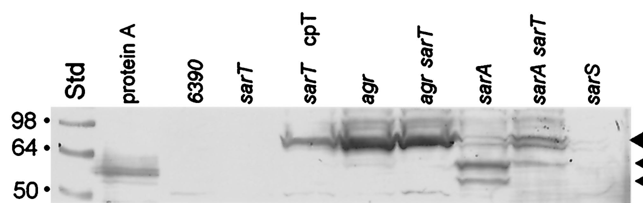


FIG. 5. Western blot of *S. aureus* cell wall-associated protein extracts probed with chicken anti-protein A antibody. Equivalent amounts of cell wall extracts were applied to the lanes, electroblotted, and probed with chicken anti-protein A antibody as described in the text (17, 48). The positions of standard protein molecular mass standards (Std) (in kilodaltons) are shown to the left of the blot. The positions of intact protein A (large arrowhead) and degraded protein A bands (small arrowheads) are shown to the right of the blot. Results are representative of two independent experiments. 6390, RN6390; cpT, complemented with *sarT* in *trans*.

indirectly via *sarT* and *sarS* (Fig. 1), since a *sarA sarS* double mutant has previously been found to express a high level of *spa* despite the absence of *sarS* (17, 49).

Previous studies also showed that transcription levels of *sarS* (17) and *spa* are increased in an *agr* mutant (12, 23). The transcription of *spa* is also reduced in the *agr sarS* double mutant, demonstrating that *agr* represses *spa* expression probably by repressing *sarS*, an activator of *spa* (17). To ascertain whether *sarT* induces *sarS* directly in an *agr* mutant (*agr* ↓ → *sarT* ↑ → *sarS* ↑) (Fig. 1), we generated an *agr sarT* double mutant and compared *sarS* and *spa* expression with expression in the isogenic single *agr* mutant (Fig. 4B). In the *agr sarT* double mutant, *sarS* and *spa* expression levels were reduced from those of the *agr* mutant during both exponential and post-exponential growth. Importantly, the absence of *sarS* in the double mutant correlated with notably reduced *spa* expression. Complementing the double *agr sarT* mutant with *sarT* in *trans* resulted in increased levels of *sarS* and *spa* expression compared to those of the double *agr sarT* mutant (Fig. 4B), particularly in the post-exponential growth phase. The same expression pattern was also seen in *agr sarT* and *sarT*-complemented double mutants of SH1000 (data not shown). Collectively, these data suggest that the increase in *spa* expression seen in an *agr* mutant is likely the result of *sarT* induction of *sarS*. As expression of *sarT* also has a mild repressive effect on *agr* (48), the decreased *sarS* message in an *agr sarT* double mutant also indicated that the high levels of *sarS* message in an *agr* mutant was not due to repression of *agr* RNAIII by SarT (i.e., SarT ↑ → *agr* ↓ → *sarS* ↑).

Cell wall-associated protein A. We also confirmed the Northern blot results by analyzing protein A levels in post-exponential-phase cultures of *S. aureus* RN6390 (cultures grown overnight) with immunoblots (Fig. 5). Consistent with the *spa* message detected in the Northern blots, levels of cell wall-associated protein A were not detected in the parental RN6390 and *sarT* and *sarS* mutant strains. The intensity of the protein A band in the *sarT* mutant strain harboring *sarT* in *trans* (870 IAU) was consistent with the light *spa* band seen on the Northern blot (Fig. 4B). The intensity of the protein A band in the *agr sarT* mutant was lower (1,038 IAU) than that seen in the *agr* mutant strain (1,350 IAU). However, the protein A band of the *agr sarT* mutant on the immunoblot seemed higher than what one would expect from the level of *spa* transcription on the Northern blot (Fig. 4B). This could conceivably be explained by technical differences, since cell wall protein A tends to accumulate throughout the growth cycle, while the Northern blot data are expected to reflect *spa* expression at a single time point during growth (Fig. 4).

As has been noted previously (17, 49), additional lower-molecular-weight protein A-specific reactive bands were present in the *sarA* mutants (Fig. 5). This is presumably due to degradation of protein A as a result of increased proteolytic activity in a *sarA* mutant (17, 49). Interestingly, protein A showed less apparent degradation in the *sarA sarT* double mutant than in the *sarA* mutant. However, the intensities of the four bands seen in the *sarA* mutant (138, 134, 795, and 307 AIU) and the two major bands seen in the *sarA sarT* mutant (1,031 and 188 AIU) added up to 1,374 and 1,219 AIU, respectively, suggesting that protein A expression was likely to be similar in the two strains.

Gel shift and DNase I footprinting. Northern and Western blots of *sarS* and *spa* RNA levels in various mutants led to the hypothesis that *sarT* regulates *sarS* expression. We evaluated the binding of the SarT protein to the region upstream of the *sarS* promoter. A 264-bp region of the *sarS* promoter was cloned and purified. Various concentrations of purified SarT protein (Fig. 6A) were incubated with the ^{32}P-end-labeled fragment encompassing the *sarS* promoter region, and the reaction mixtures were analyzed by gel shift assays (Fig. 6B). The dose-dependent retardation in the mobility of the *sarS* promoter fragment with increasing amounts of SarT (Fig. 6B) indicated that SarT binds to the *sarS* promoter region. As SarA, SarR, and possibly other SarA homologs are believed to form dimeric structures (31), it is likely that the multiple banding pattern (Fig. 6B, third lane from the left) may result from SarT forming dimers and tetramers on the DNA strand, since there were only two binding sites for SarT on the *sarS* promoter (see below).

To confirm that binding was specific, the assay was repeated with unlabeled *sarS* promoter as a competitor (Fig. 6C). The gel shift pattern showed a dose-dependent reduction in the intensity of the SarT-*sarS* promoter complex, while the intensity of the unbound *sarS* promoter fragment increased, indicating that the unlabeled DNA fragment containing the *sarS* promoter is able to bind SarT competitively. No comparable shift in the labeled *sarS* promoter region fragment was noted when the noncompetitive *sarA* P3 promoter was added to the SarT-*sarS* promoter DNA mix.

To verify the specificity and map the probable binding site of SarT on the *sarS* promoter region, DNase I footprinting was performed, using a 287-bp DNA fragment encompassing the *sarS* promoter region (49). DNase I footprinting of the top strand (Fig. 7A and C) showed two major protected regions, one region corresponding to positions 147 to 167 (region I [Fig. 7C]) and one region corresponding to positions 191 to 203 (region II [Fig. 7C]) (139 to 119 bp and 95 to 83 bp upstream of the translation start, ATG, respectively). With the bottom strand, we found binding sites at positions 144 to 164 and 188 to 199 (Fig. 7C) (142 to 121 bp and 98 to 87 bp upstream of the translation start, ATG, respectively) (Fig. 7B and C). The two protected regions occur in corresponding locations on the top

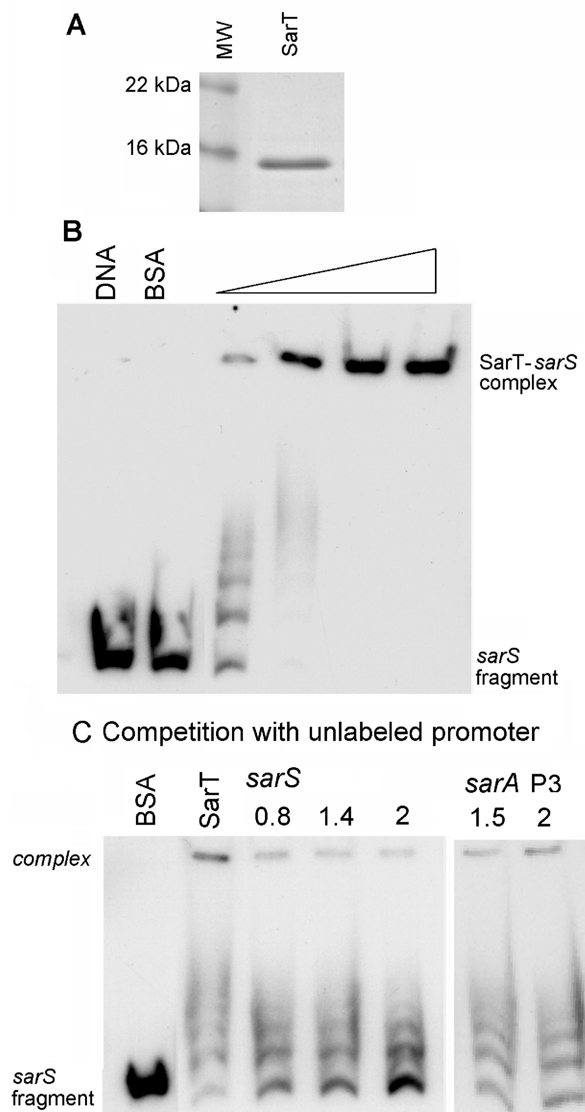


FIG. 6. Gel shift assay of the *sarS* promoter. (A) Coomassie blue-stained sodium dodecyl sulfate-polyacrylamide gel electrophoresis of SarT purified from the pET14b expression vector. MW, molecular mass standards. (B) Gel shift assay showing the effect of increasing concentrations of SarT protein on the mobility of a 264-bp fragment (nt 125169 to 125432) (29). The *sarS* promoter fragment was end labeled with [γ-32P] ATP, and approximately 11,000 cpm was used in each lane. Purified SarT at 0.19, 0.38, 0.56, and 0.75 μg (indicated by the thickness of the triangle over the lanes) were added to the lanes. (C) Competition assays. Increasing amounts of unlabeled *sarS* fragment (specific competitor [in micrograms]) and *sarA* P3 promoter fragment (nonspecific competitor [in micrograms]) (161 bp, nt 365 to 525) (4) were added to a mixture containing *sarS* promoter (10,000 cpm) and 0.45 μg of SarT protein.

and bottom strands and are separated by approximately 23 bp of AT-rich sequence.

A close analysis of the four protected sequences in two regions suggested a putative conserved 12-bp SarT binding sequence with a consensus sequence of AAATG(A/T)CAT TTT, present in both the top and bottom strands of region I and region II (Fig. 7D).

Doxycycline-dependent induction of *sarT*. Dose-dependent induction of *sarS* and *spa* was used to confirm the regulatory control of *sarT* on *sarS*. A shuttle plasmid (pALC2217) was constructed such that *sarT* was placed downstream of the tetracycline-induced *xyl/tetO* promoter (3). *sarT* mutant strains of RN6390 and SH1000 were transformed with pALC2217 or with vector plasmid pALC2073 as a control. Tetracycline was used in initial experiments, but the dose range required for a suitable *sarT* response from the inducible promoter was too high (data not shown). Therefore, we used doxycycline as an alternative to induce a *sarT* response at a low enough dose level to avoid an untoward response from the control strain. Northern blots showed that *sarT* expression from pALC2217 occurred even in the absence of added doxycycline (Fig. 8), but the band intensity for *sarT* increased with increasing amounts of doxycycline, particularly in strain SH1000. *sarS* and *spa* induction roughly mirrored the *sarT* band intensity. Collectively, these data with the tetracycline-inducible promoter system indicated that *sarT* induced *sarS* and the ensuing *spa* expression.

DISCUSSION

S. aureus exhibits complex patterns of protein expression in response to different environmental conditions (1, 36). It is therefore not surprising that *S. aureus* harbors a complex regulatory network to coordinate expression of its array of virulence proteins. The *sarA/agr* interface within the regulatory system controls growth phase-mediated repression of cell wall-associated proteins and induction of late exponential to post-exponential expression of a variety of extracellular toxins and enzymes (1, 10, 14, 17, 19, 21, 23, 27, 39, 40, 46).

SarT (48) and SarS (17, 49), two recently described SarA homologs, have been shown to be modulated by *sarA* and *agr* in the regulatory pathway (Fig. 1). In previous studies, we have shown that *agr* likely represses *spa* expression by down-regulating *sarS* (17), while the *sarA* locus probably activates *hla* expression by down-regulating *sarT* (48). The above findings are of interest because *sarS* and *sarT*, as an activator of *spa* and repressor of *hla*, respectively, participate in divergent pathways in regulating *spa* (*agr* ↓ → *sarS* ↑ → *spa* ↑) and *hla* (*sarA* ↓ → *sarT* ↑ → *hla* ↓), despite the ability of both *sarA* and *agr* to repress *sarS* and *sarT*. In discovering that *sarS* expression could be enhanced in a *sarT*-complemented strain (Fig. 2), we wanted to investigate the broader relationships of *sarT* with *sarS* within the *sarA/agr* regulatory network. *S. aureus* RN6390 was initially chosen for these studies because of its low basal *spa* expression level; hence, any alteration in *sarS* expression attributable to possible *sarT*-mediated induction can be readily detected by examining *spa* expression, the target gene of *sarS* (12). We found that the complemented *sarT* mutant of strain RN6390 had elevated *sarS* expression and was able to express *spa* at a high level (Fig. 2A). Parallel experiments with *S. aureus* SH1000 (22) and its complemented *sarT* mutant also showed expression patterns similar to those of RN6390 (Fig. 2B).

In earlier studies (17, 48), we found that *sarT*, *sarS*, and *spa* expression was elevated in a *sarA* mutant. In this study, we

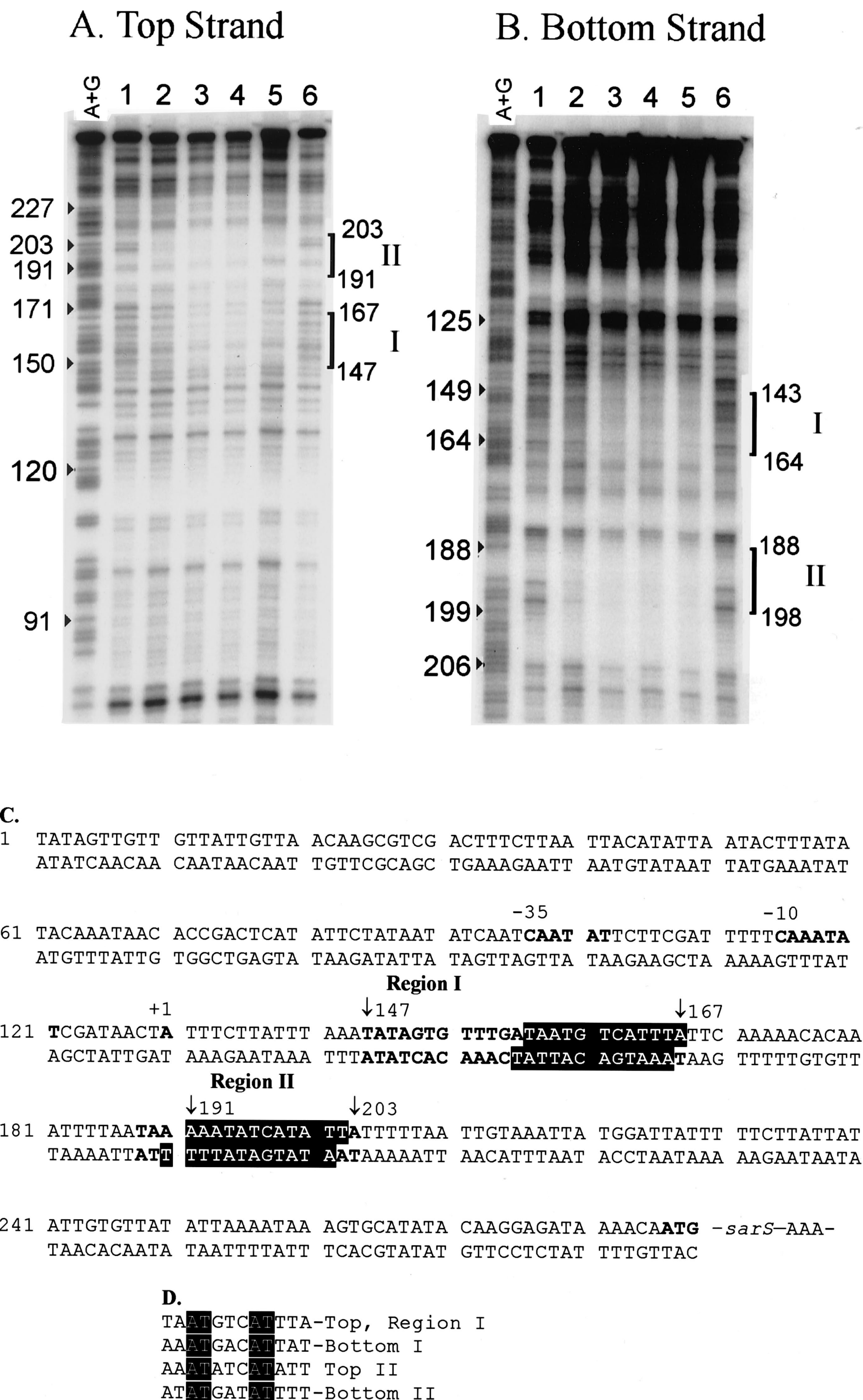


FIG. 7. DNase I footprinting assays of the interaction of SarT with the *sarS* promoter. (A and B) Top-strand (A) and bottom-strand (B) promoter fragments. Lanes: 1 to 6, labeled DNA fragments reacted with 0, 1, 2, 4, 6, and 0 μg of purified SarT protein, respectively, prior to DNase I digestion as described in the text. An A+G ladder was run in parallel to identify the positions of the protected regions. The positions of protected regions I and II are marked with brackets. (C) Sequence of the promoter region upstream of *sarS*, showing the two protected regions (positions 147 to 167 and positions 191 to 203 [shown in bold type]) and showing the SarT binding sites (white letters on a black background) as deduced from the footprinting analysis. (D) Putative 12-bp *sarT* binding sequence deduced from the protected regions on the top and bottom strands. Identical nucleotides are indicated by white lettering on a black background.

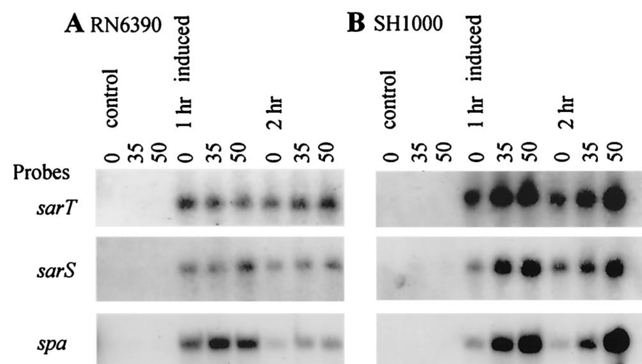


FIG. 8. Doxycycline induction of *sarT*. Northern blots of RNA extracted from *S. aureus sarT* mutants of strain RN6390 (A) or SH1000 (B) harboring either pALC2073 (control; *tet*-inducible promoter) or pALC2217 (*tet*-inducible promoter driving *sarT*) and grown in the presence of 0, 35, or 50 ng of doxycycline per ml. Doxycycline was added to late-exponential-phase cells (OD_{650} of 1.1), and cells were harvested for RNA 1 and 2 h after induction. Strains harboring the control plasmid were harvested only 2 h after the addition of doxycycline. Results are representative of multiple experiments.

found that *sarS* and *spa* expression was elevated in the *sarA sarT* double mutant (Fig. 4A), suggesting that SarA may repress *sarS* rather than *spa*. However, in previous studies, *spa* was found to be repressed in a *sarS* mutant, relative to the *sarS sarA* double mutant, indicating that SarA repression of *spa* is likely independent of *sarS* (17, 49). Taken together, these results indicate that *sarA* repression of *spa* is independent of *sarT* and *sarS*.

We have previously shown that *agr* RNAIII likely regulates *spa* by repressing *sarS*, an activator of *spa* expression (17). However, the positive modulation of *sarS* by SarT (Fig. 2) leads to the question whether RNAIII represses *sarS* transcription directly or indirectly by suppressing *sarT*. A comparison of the *agr* and *sarT* mutants (Fig. 4B) revealed that *sarS* and *spa* expression was induced in an *agr* mutant strain but was almost totally repressed in the *sarT* mutant and *sarT agr* double mutant. Further, when *sarT* was returned in *trans* to the *agr sarT* mutant via a plasmid harboring the *sarT* gene, *sarS* and *spa* expression returned to levels seen in the *agr* mutant strain, suggesting that *sarT* induction of *sarS* may be important for *spa* expression in the *agr* mutant strain.

To further confirm the activation of *sarS* by *sarT*, we took advantage of an inducible promoter system that can be activated by low doses of doxycycline to express *sarT* in a dose-dependent manner. This plasmid, upon introduction into *sarT* mutants of *S. aureus* RN6390 and SH1000, expressed *sarT*, in proportion (approximately) to the doxycycline dose, in particular during the 2-h time points (Fig. 8). At the 1-h time point, the basal level of *sarT* expression (i.e., no doxycycline) remained quite high, especially in RN6390, so the induction phenomenon was less apparent under this condition. Nevertheless, the data on the induction time point at 2 h for both RN6390 and SH1000 indicated that SigB does not play a significant role in this *sarT*-*sarS* induction process.

Gel shift (Fig. 6) and footprinting assays (Fig. 7) confirmed that SarT binds specifically to the *sarS* promoter, presumably leading to *sarS* activation. The presence of multiple bands in gel shift assays indicated that multiple SarT dimers likely bind to the *sarS* promoter. Interestingly, SarT binds to two regions on the *sarS* promoter, separated by an extremely AT-rich 23-bp region (20 of 23 bp or 87% AT). Within the AT-rich region is a short stretch of five adenines on the top strand and four adenines on the bottom strand which potentially may play a role in DNA bending (2, 44), a process that has been recognized to play a role in growth phase-mediated transcriptional regulation (51). Additionally, the binding sites covered in both the plus and minus strands divulged a 12-bp sequence in region I that was repeated in region II (Fig. 7C). We speculate that this 12-bp sequence may represent a conserved binding site for SarT. Nevertheless, additional verification with other SarT target genes should be made to confirm this hypothesis.

In *sarA* or *agr* mutant strains where repression of *sarT* is relieved, *sarT* expression occurs during exponential growth but is highest during the post-exponential phase (48). The repression of *sarT* by *sarA* or *agr*, as demonstrated by the extremely low *sarT* transcriptional levels in *S. aureus* RN6390 (48), suggests that *sarT* induction may require specific environmental activators that in vitro growth conditions replicate poorly or that it is transitory and therefore less likely to be readily detected.

In a transcription profiling study, Dunman et al. (21) have pointed out that the pathogenic process is a complex progression of bacterial and host interactions in a dynamically shifting environment. We have characterized in *sarT* what may be a regulatory subroutine that is activated under a specific environmental stimulus. Further studies are required to discover the specific environmental conditions that induce expression of *sarT* and subsequently *sarS*.

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