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sarU, a *sarA* Homolog, Is Repressed by SarT and Regulates Virulence Genes in *Staphylococcus aureus*

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In searching the *Staphylococcus aureus* genome, we previously identified *sarT*, a homolog of *sarA,* which encodes a repressor for α-hemolysin synthesis. Adjacent but transcribed divergently to *sarT* is *sarU,* which encodes a 247-residue polypeptide, almost twice the length of SarA. Sequence alignment disclosed that SarU, like SarS, which is another SarA homolog, could be envisioned as a molecule with two halves, with each half being homologous to SarA. SarU, as a member of the SarA family proteins, disclosed conservation of basic residues within the helix-turn-helix motif and within the beta hairpin loop, two putative DNA binding domains within this protein family. The transcription of *sarU* is increased in a *sarT* mutant. Gel shift and transcriptional fusion studies revealed that SarT can bind to the *sarU* promoter region, probably acting as a repressor for *sarU* transcription. The expression of RNAII and RNAIII of *agr* is decreased in a *sarU* mutant. As RNAIII expression is up-regulated in a *sarT* mutant, we hypothesize that *sarT* may down regulate *agr* RNAIII expression by repressing *sarU*, a positive activator of *agr* expression. We propose that, in addition to the quorum sensing effect of the autoinducing peptide of *agr*, the *sarT-sarU* pathway may represent a secondary amplification loop whereby the expression of *agr* (e.g., those found in vivo) might repress *sarT*, leading to increased expression of *sarU*. Elevated *sarU* expression would result in additional amplification of the original *agr* signal.

Staphylococcus aureus is an important human pathogen in both the community and hospital settings (33). The spectrum of diseases caused by this organism is extremely broad, ranging from cutaneous to deep-seated infections such as pneumonia, endocarditis, and sepsis (33). Within its arsenal are virulence genes coding for proteins that facilitate tissue colonization, immune evasion, and tissue destruction (33). Superimposed upon these virulence genes is a network of regulatory genes that confer precise gene expression during different stages of infection (2, 6, 33). Expression of the regulatory elements, in turn, exerts transcriptional control of unlinked target genes. During growth in vitro, *S. aureus* expresses a number of cell wall-associated adhesins that are believed to promote host tissue colonization. In transition to the postexponential phase, the expression of cell wall proteins is repressed while the synthesis of exoproteins predominates, presumably to facilitate host cell lysis (33).

Postexponential protein expression in *S. aureus* is generally governed, in part, by global regulatory elements such as *agr* (20), *sae* (15), and *sarA* (10). The *agr* locus, a pleiotropic regulator of exoprotein synthesis in *S. aureus*, comprises two divergent transcripts, RNAII and RNAIII (20, 28, 29, 32), which encode *agrDBCA* and *hld*, respectively. AgrC and AgrA correspond to the sensor and activator of a two-component regulatory system (20). AgrD encodes a 46-residue peptide which, aided by AgrB, undergoes processing and cyclization to yield a quorum-sensing cyclic peptide (17). AgrC is the putative sensor for this cyclic peptide (23) and, upon phosphorylation, will lead to a second phosphorylation step of the activator AgrA. Phosphorylated AgrA is believed to bind to the *agr* promoter to activate transcription of RNAIII, which, as the regulatory molecule, up-modulates the transcription of exoprotein genes and down-modulates the expression of cell wall protein genes during the postexponential phase (32).

Contrary to *agr*, the *sarA* locus up-regulates the expression of many cell wall proteins (e.g., fibronectin binding protein A) and selected exoproteins (e.g., α [*hla*] and β hemolysins) (10) while repressing the transcription of the protein A gene (*spa*) (9). The *sarA* locus comprises a major 372-bp *sarA* open reading frame driven by three distinct promoters (4). DNA binding studies revealed that SarA, the major *sarA* effector molecule, binds to several target gene promoters (e.g., *agr*, *hla,* and *spa*) to modulate gene transcription (12), thus accounting for both *agr*-dependent and *agr*-independent pathways of regulation. As mutations in *sarA* or *agr* have been found to affect the transcription of over 100 genes (14), it is not surprising that other regulatory factors may be at work, in part to control SarA and *agr* expression and also to regulate genes downstream of the *sarA-agr* regulatory cascade. We and others have found several SarA homologs that are involved directly or indirectly in virulence gene regulation. SarR, being a SarA homolog with a molecular mass of 13.6 kDa (26), represses *sar*A (26), *agr*, *hla, hlb,* and *spa* expression during the postexponential phase (unpublished data), presumably by binding to the *sarA* or other target gene promoters. SarS (also called SarH1), a 250-residue SarA homolog (11, 37) repressible by *agr*, likely acts downstream of *agr* to up-regulate *spa* expression. An additional regulatory gene with partial homology to SarA, *rot*, is a repressor of *hla* and probably acts downstream of *agr* (27).

In searching the recently released *S. aureus* genomes (www.ncbi.nlm.nih.gov/genome/ and www.TIGR.org), we found additional SarA homologs. One of these homologs, SarT (a 118-residue protein), is a repressor of *hla* expression and is nega-

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TABLE 1. Strains and plasmids used

Strain or plasmid	Reference or source	Comments
S. aureus		
RN4220	31	Mutant of 8325-4 that accepts foreign DNA
RN6390	31	Laboratory strain that maintains its hemolytic pattern when propagated on sheep erythrocyte agar (parental strain)
RN6911	20	*agr* mutant of RN6390 with an Δ*agr*::*tetM* mutation
ALC1342	Laboratory strain	A *sarA* mutant with deletion of open reading frame 3 and the *sarA* open reading frame and replaced with an *ermC* gene
ALC1713	26	*sarR* mutant of RN6390 with Δ*sarR*::*ermC*
ALC1905	36	*sarT* mutant of RN6390 with *sarT*::*ermC*
ALC2071	36	*sarT* mutant ALC1905 carrying pSK236 with wild-type *sarT* gene
ALC1927	11	*sarS* mutant of RN6390 with *sarS*::*ermC*
ALC2272	This study	*sarU* mutant of RN6390 with a deletion of amino acids 1 to 153 of the N terminus of the *sarU* gene product and its replacement with an *ermC* gene
ALC2380	This study	RN6390 with pALC2360
ALC2381	This study	ALC2272 with pALC2360
ALC2601	This study	RN6390 with pALC2591
ALC2602	This study	RN6390 with pALC2599
ALC2604	This study	RN6911 with pALC2591
ALC2605	This study	RN6911 with pALC2599
ALC2607	This study	ALC1905 with pALC2591
ALC2608	This study	ALC1905 with pALC2599
ALC2609	This study	ALC2272 with pALC2591
ALC2610	This study	ALC2272 with pALC2599
ALC2714	This study	RN6390 with pALC2707
ALC2716	This study	ALC1905 with pALC2707
ALC2717	This study	ALC2272 with pALC2707
E. coli		
XL-1 Blue	25	Host strain for cloning
Topo InvαF′	Invitrogen	Host strain for the TA cloning vector
BL21(DE3)pLysS	Novagen	Host strain for the pET expression system
Plasmids		
pCL52.2	34	Temperature-sensitive *E. coli-S. aureus* shuttle vector
pCR2.1	Invitrogen	*E. coli* cloning vector for direct cloning of PCR products
pET14b	Novagen	Expression vector in *E. coli*
pSK236	19	Shuttle vector containing pUC19 cloned into the *Hin*dIII site of pC194
pUC19	25	*E. coli* cloning vector
pALC1484	18	Modified pSK236 shuttle vector with a promoterless gfp_{uvr} reporter gene preceded by an *S. aureus* ribosome binding site
pALC1740	This study	pALC1484 with the *hla* promoter
pALC1742	36	pALC1484 with the *agr* P2 promoter
pALC1743	18	pALC1484 with the *agr* P3 promoter
pALC1904	This study	pET14b containing the 345-bp *sarT* gene at the *Xho*I/*Bam*HI sites
pALC2208	This study	pCR2.1 containing a 2.3-kb PCR *sarU-sarT* fragment with flanking upstream and downstream sequences
pALC2229	This study	pUC19 containing a 2.3-kb *Eco*RV-*Kpn*I fragment from pALC2208 at the *Hinc*II and *Kpn*I sites
pALC2240	This study	pCL52.2 containing a 2.9-kb DNA fragment, which has a deletion of a 547-bp *Cla*I-*Hinc*II fragment that includes residue 1 to 153 of the N terminus of the *sarU* gene product and an insertion of the 1.2-kb *ermC* gene at the *Eco*RI site
pALC2360	This study	pSK236 with a 1.5-kb DNA fragment containing the *sarU* gene with a 392-bp upstream sequence at the *Eco*RI site
pALC2591	This study	pALC1484 with a 310-bp promoter fragment of the *sarU* gene fused with the gfp_{uvr} reporter gene at the *Eco*RI and *Xba*I sites
pALC2599	This study	pALC1484 with a 147-bp promoter fragment of the *sarU* gene fused with the gfp_{uvr} reporter gene at the *Eco*RI and *Xba*I sites
pALC2707	This study	pALC1484 with a 310-bp promoter fragment of *sarT* fused with the gfp_{uvr} reporter gene at the *Eco*RI site

tively controlled by *sarA* (36). Contiguous to *sarT,* but transcribed in opposite orientation, is *sarU*. The expression of *sarU* is negatively controlled by *sarT*. Phenotypic and transcriptional analysis revealed that *sarU* is a positive regulator of RNAIII and contributes to the expression of virulence genes controlled by *agr*.

MATERIALS AND METHODS

Bacterial strains, plasmids, and growth media. The bacterial strains and plasmids used in this study are listed in Table 1. CYGP, 0.3GL medium (30), and tryptic soy broth were used for the growth of *S. aureus* strains, while Luria-Bertani broth was used to cultivate *Escherichia coli.* Antibiotics were used at the following concentrations: erythromycin at 5 μg/ml, kanamycin at 50 μg/ml, tet-

racycline at 5 μg/ml, and chloramphenicol at 10 μg/ml for *S. aureus*; and ampicillin at 50 μg/ml, chloramphenicol at 30 μg/ml, and spectinomycin at 75 μg/ml for *E. coli.*

Genetic manipulations in *E. coli* and *S. aureus*. Based on homology with SarA, the *sarU* gene product was identified in the *S. aureus* genome database (www.TIGR.org/). To construct a *sarU* mutant, the *sarU* gene together with flanking sequences on both sides was amplified by PCR with the primers 5′-TGACGATTTCGGCTGAACTTC-3′ and 5′-TGGAACACGAAATGGTGAAC-3′, with chromosomal DNA from strain RN6390 being used as the template. The 2.3-kb PCR fragment was cloned into cloning vector pCR2.1 (Invitrogen, San Diego, Calif.) in *E. coli*. The *Kpn*I-*Eco*RV DNA fragment containing the 2.3-kb fragment was then cloned into the *Kpn*I and *Hin*cII sites of pUC19. A 547-bp fragment comprising the N-terminal 153 amino acids of SarU was deleted by restricting with *Hin*cII and *Cla*I and then replaced with an ~1.2-kb *ermC* fragment. The fragment containing an *erm*C insertion into the partially deleted *sarU* gene was cloned into the temperature-sensitive shuttle vector pCL52.2 (22). The recombinant pCL52.2 was transformed into RN4220 by electroporation (35). Plasmid isolated from RN4220 was restriction digested for authenticity and introduced into RN6390 by electroporation. Transformants were selected at 30°C on erythromycin- and tetracycline-containing agar plates. *S. aureus* RN6390, harboring the recombinant pCL52.2 construct, was grown overnight at 30°C in liquid medium in the presence of erythromycin, diluted 1:1,000 in fresh medium, and propagated at 42°C, a nonpermissive temperature for the replication of pCL52.2. This cycle was repeated four times, and the cells were plated onto 03GL plates containing erythromycin and erythromycin-tetracycline to select for tetracycline-sensitive but erythromycin-resistant colonies, representing mutants with double crossovers (a frequency of 10^{-2} after the fourth passage). The mutations were confirmed by PCR and Southern hybridization with *sarU* and *ermC* probes as described previously (25). One clone, designated ALC2272, was selected for further study.

To complement the *sarU* mutation, a 1.5-kb fragment encompassing the *sarU* gene and a 392-bp sequence upstream of the *sarU* transcription start site (see below) was cloned into shuttle plasmid pSK236. The recombinant plasmid was electroporated into RN4220, selecting for chloramphenicol-resistant colonies. The transformant was verified by plasmid restriction analysis. The plasmid from RN4220 was then electroporated into parental strain RN6390 and the *sarU* mutant (ALC2272).

Immunoblot analysis. To assess SarA expression in different *S. aureus* strains, cellular proteins were extracted from RN6390, the *sarU* mutant (ALC2272), and the complemented strains (ALC2380 and ALC2381). In brief, after being pelleted, the cells were suspended in 1 ml of TEG buffer (25 mM Tris-Cl, 5 mM EGTA; pH 8) and cell extracts were prepared from lysostaphin-treated cells as described previously (13). Cell extracts were immunoblotted onto nitrocellulose membranes. For the detection of SarA, monoclonal antibody 1D1 (1:6,000 dilution) was added to the blot and incubated for 3 h, followed by another hour of incubation with a 1:10,000 dilution of goat anti-mouse alkaline phosphatase conjugate (Jackson Immuno Research, West Grove, Pa.). Reactive bands were detected with developing substrates as described previously (5).

Isolation of RNA and Northern blot hybridization. Overnight cultures of *S. aureus* were diluted 1:50 in CYGP or tryptic soy broth and grown to mid-log (optical density at 650 nm [OD_{650}] = 0.7), late log (OD_{650}=1.1), and early postexponential (OD_{650}=1.7) phases. The cells were pelleted and processed with a Trizol isolation kit (Gibco BRL, Gaithersburg, Md.) in combination with 0.1-mm-diameter sirconia-silica beads in a Biospec reciprocating shaker, as described previously (8). Ten or 15 μg of each sample was electrophoresed through a 1.5% agarose–0.66 M formaldehyde gel in morpholinepropanesulfonic acid (MOPS) running buffer (20 mM MOPS, 10 mM sodium acetate, 2 mM EDTA, pH 7.0). Blotting of RNA onto Hybond N^+ membranes (Amersham, Arlington Heights, Ill.) was performed with a Turbo blotter alkaline transfer system (Schleicher & Schuell, Keene, N.H.). For detection of specific transcripts (*agr*, *sarA*, *sarU, sarT*, *spa*, *coa*, *agr,* and *hla*), gel-purified DNA probes were radiolabeled with [α-^{32}P]dCTP with a random-primed DNA labeling kit (Roche Diagnostics GmbH) and hybridized under aqueous-phase conditions at 65°C (7). The blots were subsequently washed, autoradiographed, and developed with Kodak BioMax film or Kodak Blue film.

Primer extension analysis. Mapping of the 5′ end of the *sarU* transcript was performed with the primer 5′-GTTGCTTTAACTCTTGAGTGAG-3′, which is complementary to the coding strand for *sarU* and is located at nucleotide positions 85 to 64 downstream from the putative initiation codon GTG. Primer extension was carried out as described previously (3, 4).

Transcriptional fusion studies of RNAII, RNAIII, *hla,* and *sarU* and *sarT* promoters linked to the gfp_{uvr} reporter gene. A 229-bp fragment in forward (nt 1528 to 1756) and reverse (nt 1756 to 1528) positions (20), representing RNAII and RNAIII promoter fragments, respectively, was amplified by PCR with genomic DNA of *S. aureus* strain RN6390 as the template and cloned into the TA cloning vector pCR2.1. Similarly, a 440-bp *hla* promoter fragment, representing sequence upstream of the ribosomal binding sites of *hla*, was cloned into pCR2.1. Various lengths of the *sarU* promoter region (310 and 148 bp) extending from the sequence upstream of the ribosomal binding sites were amplified by PCR by using chromosomal DNA of *S. aureus* strain RN6390 and primers with flanking *Eco*RI or *Xba*I sites. *Eco*RI and *Xba*I fragments containing RNAII, RNAIII, *hla,* or various lengths of *sarU* promoter fragments were cloned into shuttle plasmid pALC1484 (18), generating transcriptional fusions to the gfp_{uvr} reporter gene as pALC1743, pALC1742, pALC1740, pALC2591, and pALC2599. As *sarT* is transcribed contiguously but divergently from *sarU*, we also cloned the 310-bp *sarU* promoter fragment in reverse orientation to the gfp_{uvr} gene to yield a *sarT* promoter fragment driving the reporter gene (pALC2707). The construction of plasmid pALC1484 and modification of gfp_{uv} (Clontech, Palo Alto, Calif.) to gfp_{uvr} with a S65T mutation to yield a red shift (excitation maxima from 395 to 488 nm) were described earlier (18). The orientation and authenticity of the promoter fragments were confirmed by restriction analysis and DNA sequencing. The recombinant plasmids containing the respective RNAIII, RNAII, and *hla* and *sarU* promoters driving the gfp_{uvr} reporter were first introduced into *S. aureus* strain RN4220 by electroporation (35). Plasmids containing RNAII, RNAIII, and *hla* promoters were electroporated into RN6390 and its isogenic *sarU* and *sarT* mutants, while the *sarU* and *sarT* promoter constructs were introduced into RN6390, the isogenic *agr*, *sarT,* or *sarU* mutant.

After overnight culture, *S. aureus* strains harboring the recombinant plasmids were diluted 1:100 and grown at 37°C with shaking in tryptic soy broth containing chloramphenicol (10 μg/ml). Aliquots (100 μl) were transferred hourly to microtiter plates to assay for cell density (OD_{650}) and fluorescence for 10 h in a FL600 fluorescence reader (BioTek Instrument, Winooski, Vt.). Promoter activation was plotted as mean fluorescence per OD unit over time, using the average values from triplicate readings.

Overexpression and purification of SarT in a pET vector. The 420-bp DNA fragment containing the full-length *sarT* gene was amplified by PCR by using chromosomal DNA from *S. aureus* RN6390 as the template and primers containing flanking restriction sites (*Xho*I and *Bam*HI for *sarT*) to facilitate cloning into the expression vector. The purified PCR product was digested with *Xho*I and *Bam*HI, gel purified, ligated into the expression vector pET14b (Novagen, Madison, Wis.), and transformed into *E. coli* XL-1 Blue. The recombinant plasmid containing a full-length *sarT* coding region was confirmed by restriction digestion and DNA sequencing. The recombinant plasmids were then transformed to *E. coli* BL21(DE3) pLys.S. The resulting plasmid (pALC1904; see Table 1) contained the entire *sarT* coding region in frame with an N-terminal His tag. The expression of recombinant protein was induced by adding isopropyl-1-thio-β-D-galactopyranoside (ITPG; final concentration, 1 mM) to a growing culture (37°C) at an OD_{600} of 0.5. After a 4-h induction, the cells were harvested, resuspended in binding buffer (5 mM imidazole, 0.5 M NaCl, 20 mM Tris-HCl, pH 7.9), frozen by dipping in liquid N_2, and thawed overnight in ice. Cellular debris were removed by centrifugation (TL-100 tabletop ultracentrifuge; Beckman, Palo Alto, Calif.) at 45,000 rpm for 60 min, and the clarified supernatant was applied to a nickel affinity column (Novagen) in accordance with the manufacturer's instruction. The protein was eluted with the elution buffer (1 M imidazole, 0.5 M NaCl, 20 mM Tris-HCl, pH 7.9), followed by dialysis in a buffer containing 20 mM Tris-Cl (pH 8.0), 50 mM NaCl, 1 mM EDTA, 10% glycerol, and 1 mM DTT. The authenticity of the purified SarT protein was confirmed by N-terminal sequencing, and the size and purity of the recombinant protein was verified by sodium dodecyl sulfate gels stained with Coomassie brilliant blue R-250.

Gel shift assays. To determine whether the recombinant SarT protein binds to the *sarU* promoter, a 150-bp fragment representing nucleotide positions 13 to 160 bp upstream of the *sarU* start codon was end labeled with [γ-^{32}P]ATP by using the T4 polynucleotide kinase. Labeled fragment (0.1 ng) was incubated at room temperature for 20 min with various amounts of purified SarT protein in 25 μl of binding buffer (25 mM Tris-Cl, pH 7.5, 0.1 mM EDTA, 75 mM NaCl, 1 mM dithiothreitol, and 10% glycerol) containing 0.5 μg of calf thymus DNA. The reaction mixtures were analyzed in an 8.0% nondenaturing polyacrylamide gel. The band shifts were detected by exposing dried gels to X-ray film.

RESULTS

Identification of the *sarU* gene. In searching for SarA homologs in the *S. aureus* N315 genome (www.ncbi.nlm.nih.gov/genome/staphylococcus), we found at least 12 proteins homol-

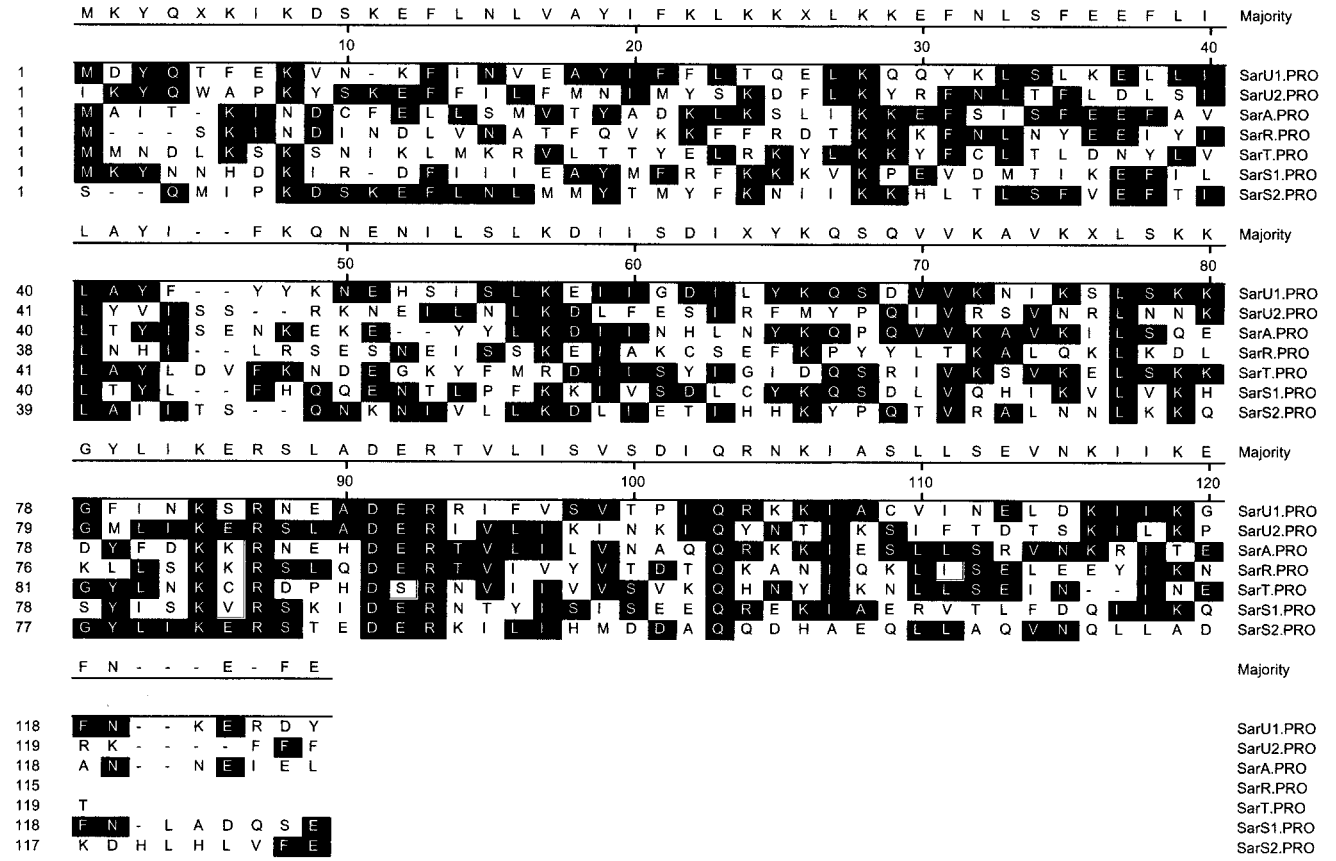

FIG. 1. Sequence alignment of SarU, SarA, SarR, SarT, and SarS. SarU is a 247-residue polypeptide. Based on regional homology, SarU can be divided into two domains, U1 and U2, of 124 and 123 residues, respectively. The homology of SarU with a two-domain protein like SarS is higher, with 39% identity and 64% homology. The conserved residues, seen in the black boxes, are designed as the majority at each position when at least half of the residues have the same amino acid. The alignment was done by the Clustal program of DNASTAR.

ogous to SarA (protein GI 13700508) with a default setting of 30 as the cutoff in the BLAST search (21). Some of these homologs, including SarR (protein GI 13702095, with an *e* value of $9e^{-12}$), SarS (also called SarH1; protein GI 13700028, with an *e* value of $1e^{-16}$), SarT (also called SarH3; protein GI 13702448, with an *e* value of $9e^{-17}$), and Rot (protein GI 13701558, with an *e* value of 0.003 and a 50% homology) (27) have been previously studied. The remaining homologs, many of which we are characterizing, are SarX (protein GI 13700559, with an *e* value of $1e^{-04}$ and a 46% homology), SarV (protein GI 13702067, with an *e* value of $5e^{-04}$ and a 48% homology), SarZ (protein GI 13702335, with an *e* value of 0.002 and a 53% homology), and three proteins of unknown function (proteins GI 13701144, GI 13700062, and GI 13702441, with *e* values of 0.003, 0.030, and 0.066 and homologies of 48, 48, and 45%, respectively). SarR is a 113-residue protein that binds to the *sarA* promoter to down-modulate SarA protein expression (26). SarS, a 250-residue protein whose expression is normally repressed by *sarA* and *agr* (11, 37), acts as an activator of protein A synthesis. SarT, identified initially by its homology to SarA, is a 118-residue protein that functions as a repressor for *hla* transcription (36). Adjacent to *sarT* but transcribed in the opposite direction is a gene, designated *sarU,* that encodes another SarA homolog (protein GI 13702449 with an *e* value of $2e^{-15}$). SarU is the third protein that came up in our BLAST search for SarA homologs, with SarT, SarS, and SarR being the first, second, and fourth hits, respectively. SarU, a 247-residue polypeptide (29,276 Da), is almost twice the size of the smaller SarA homologs (e.g., SarA, SarR, and SarT). Similar to SarS, SarU, which has a 64% homology to SarA, can be considered to be a molecule with two halves (namely, SarU1, consisting of residues 1 to 124, and SarU2, consisting of residues 125 to 247), with each half sharing sequence similarity to the smaller SarA homologs (Fig. 1). SarU1 has a 25% sequence identity to SarU2 and shares 56, 59, and 51% homology to SarA, SarT, and SarR, respectively. Likewise, SarU2 has 56, 53, and 57% homology to these respective proteins. Interestingly, when SarU was analyzed with BLAST against the entire microbial data bank, most of the hits with significant *e* values (e^{-36} to 0.05) were proteins from *S. aureus* strains as well as from *Staphylococcus epidermidis*. Only the MarR protein from *Bacillus subtilis* yielded a significant *e* value of 0.045 (see Discussion, below).

Translational and transcriptional start site and promoter structure of *sarU*. To verify that the start codon of the *sarU* gene is indeed GTG, a valine (V) residue, we resequenced our cloned *sarU* fragment (pALC2208) by using primer 5′-GTTG CTTTAACTCTTGAGTGAG-3′ (nucleotide positions 85 to

64 downstream from the putative start codon GTG). Repeated sequencing confirmed GTG to be the start codon, with a strong ribosome-binding site AGGAGA that is located 7 bp upstream. To determine the transcriptional start site and the promoter sequences, primer extension was performed with an identical primer, and total RNA was isolated from the wild-type strain RN6390 and the isogenic *sarT* mutant (data not shown). The transcriptional start site of *sarU* was mapped, based on data from both strains, to an A residue located 90 bp upstream of the initiation codon. Based upon the transcriptional start site, the predicted promoter boxes are TATAAA (−10)–N_{16}-TTTATA (−35), which has close resemblance to the −10 and −35 consensus sequences of σ^A-dependent promoters (16). The organization of the *sarU-sarT* intergenic region is complex, as the two genes are divergently transcribed and separated by 323 bp, and the −10 region of the *sarU* and *sarT* genes are located 97 and 233 bp upstream of their corresponding start sites, respectively. The putative TATAAT (−10)–N_{24}-AAGACA (−35) promoter region for *sar*T is predicated upon transcript size, and sequence prediction and precise mapping have not been done (36).

Expression of *sarU* in RN6390 and its isogenic *agr*, *sarA*, *sarR*, *sarS*, and *sarT* mutants. To assess the role of *sarU* within the *sarA-agr* regulatory cascade, we constructed a *sarU* mutant by allelic replacement, essentially replacing the *sarU* gene with an *ermC* cassette (see Materials and Methods). A 547-bp region, containing the N-terminal 153 amino acids of SarU and an 88-bp sequence upstream of the translation start, was deleted and replaced with an ~1.2-kb *ermC* fragment. PCR amplification with primers outside the *ermC* insertion yielded a fragment that was 1.2 kb larger than the wild type, consistent with an *ermC* insertion into the *sarU* gene. This result also corroborated Southern hybridization data with selected *ermC* and *sarU* probes (data not shown).

A Northern blot with a *sarU* probe (820 bp) that encompassed the coding region disclosed that the *sarU* gene was poorly transcribed in the parental strain. Interestingly, the *sarU* transcript, consisting of ~1,200 nt, was enhanced in the *sarT* mutant but not in the *sarA*, *sarR*, *sarS,* or *agr* mutants (Fig. 2). Interestingly, the level of the *sarU* transcript was higher during the postexponential phase (OD, 1.7) than in the exponential phase (OD, 0.7). The size of the transcript (650 nm) also hinted at the possible monocistronic nature of the *sarU* transcript. In addition to the 1,200-nt transcript, we also observed an ~1,400-nt transcript that hybridized with the *sarU* probe in the *sarT* mutant. However, upon introducing the complemented plasmid into the *sarU* mutant or the parental strain, we were not able to restore the larger transcript (strains ALC2381 and ALC2380) in repeated attempts. A representative blot is shown in Fig. 2A. This finding suggests that the larger transcript might arise from neighboring genes or a cross-hybridizing band (see Discussion, below). Nevertheless, the possibility of a cryptic promoter in this region cannot be entirely ruled out. Both transcripts were abolished in the complemented *sar*T mutant (ALC2071 in Fig. 2B). The *sarU* transcript was undetectable by Northern blotting in strain SH1000, an *rsbU*$^+$ strain, as well as in RN6390 (data not shown).

Gel shift assays of the SarT protein with *sarU* promoter fragments. Since the transcription of *sarU*, a gene adjacent to but divergently transcribed from *sarT*, is increased in a *sarT* mutant (Fig. 2) but not vice versa (data not shown), we speculated that SarT might bind to the *sarU* promoter to modulate *sarU* expression. Taking advantage of the fact that the coding regions of *sarU* and *sarT* are only 323 bp apart, we amplified by PCR a 148-bp *sarU* promoter fragment directly upstream of the *sarU* ribosomal binding site. The fragment was end labeled with [γ-^{32}P]ATP and used in gel shift assays with increasing concentrations of purified SarT protein (Fig. 3). Retarded protein-DNA complexes can be detected with as little as 0.2 μg of protein (6.8 pM). As the concentrations of SarT increased, multiple retarded DNA-protein complexes were discerned, suggesting that multiple proteins may bind to the *sarU* promoter region. Alternatively, SarT may bind to multiple sites on the *sarU* promoter region. Based on the data, we estimated the dissociation constant (K_d) to be ~102.5 pM.

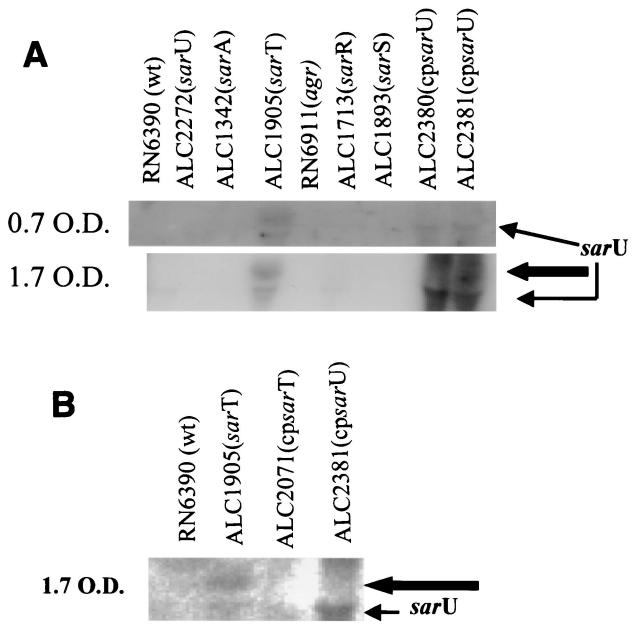

FIG. 2. (A) Northern analysis of the *sarU* transcript in *sarA*, *agr*, *sarT*, *sarR*, and *sarS* mutants and in complemented strains at exponential (OD_{650}, ~0.7) (upper panel) and postexponential (OD_{650}, ~1.7) (lower panel) phases of growth. Expression of the *sarU* transcript appeared to be higher during the postexponential phase than during the exponential phase in the *sarT* mutant, a conclusion drawn from multiple repeated experiments. A total 15 μg of cellular RNA was loaded onto each lane. The blot was probed with an 820-bp *sarU* probe containing the entire open reading frame of the *sarU* gene. The thin arrow points to the ~1,200-nt *sarU* transcript and the thick arrow points to a 1,400-nt transcript (see Discussion). (B) Northern hybridization with the same fragment to the wild-type RN6390, *sarT* mutant (ALC1905), and to ALC1905 complemented with a wild copy of the *sarT* gene (ALC2071) and complemented *sarU* mutant (ALC2381).

***sarU* and *sarT* promoter-*gfp*$_{uvr}$ fusion studies in isogenic *sarT* and *sarU* strains.** To confirm the involvement of *sarT* in *sarU* expression, we constructed transcriptional fusions of two *sarU* promoter fragments, representing 148 and 310 bp upstream of the *sarU* ribosomal binding site, to the *gfp*$_{uvr}$ reporter gene. The constructs, pALC2591 and pALC2599, containing 310- and 148-bp fragments, respectively, were introduced into the wild-type strain RN6390, the *sarT* mutant (ALC1905), the *agr* mutant RN6911, and the *sarU* mutant (ALC2272) and

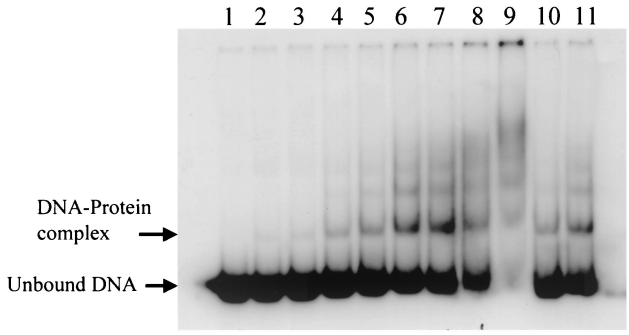

FIG. 3. Autoradiogram of an 8.0% nondenaturing polyacrylamide gel showing a gel shift assay for purified SarT protein with a *sarU* promoter fragment. Purified SarT protein was allowed to bind a 148-bp radiolabeled *sarU* promoter fragment. Lanes 1 to 9, mobility of the 148-bp DNA fragment with 0, 0.2, 0.3, 0.5, 0.75, 1.0, 2.0, 2.5, and 3.0 μg of purified SarT protein, respectively; lanes 10 and 11, mobility of the same fragment in the presence of 1.0 μg of the purified protein and a 40-fold excess (molar ratio) of unlabeled 148-bp *sarU* fragment and a 273-bp nonspecific DNA fragment, respectively.

assayed for green fluorescence emitted from GFP_{uvr}. The results, presented in Fig. 4B, disclosed that there are ~7- to 11-fold increases in mean fluorescences attributable to *sarU* promoter activity in the *sarT* mutant (1,007 ± 95 and 657 ± 65 fluorescence units plus or minus the indicated standard deviations for pALC2599 and pALC2591, respectively) compared to those in the parental strain (89 ± 20 and 95 ± 25 units for pALC2599 and pALC2591, respectively). The levels of *sarU* promoter activity in the *sarU* mutant (140 ± 30 and 124 ± 50 units for pALC2599 and pALC2591, respectively) were not significantly higher than those in the parental strain, consistent with a lack of significant autoregulatory effect. Although the mean fluorescence values in the parental strain are not very high, the level of *sarU* promoter activity in an isogenic *agr* mutant (58 ± 20 and 38 ± 25 units versus 89 ± 20 and 95 ± 25 in the parental strain for pALC2599 and pALC2591, respectively) was even lower. These results were consistent in several repeated experiments. Collectively, these data confirmed the role of *sarT* in repressing *sarU* expression and probably in slightly lowering *sarU* promoter activity in the *agr* mutant relative to the parental strain.

We also attempted to verify whether *sarT* regulates its own expression. For this experiment, we introduced pALC2707, a gfp_{uvr} construct carrying a 310-bp *sarU* promoter fragment with an orientation opposite to that found in pALC2591, thus yielding a *sarT* promoter-gfp_{uvr} construct, into various strains and then subjected them to fluorescence spectrometry. There were only slight differences in GFP expression among the wild-type

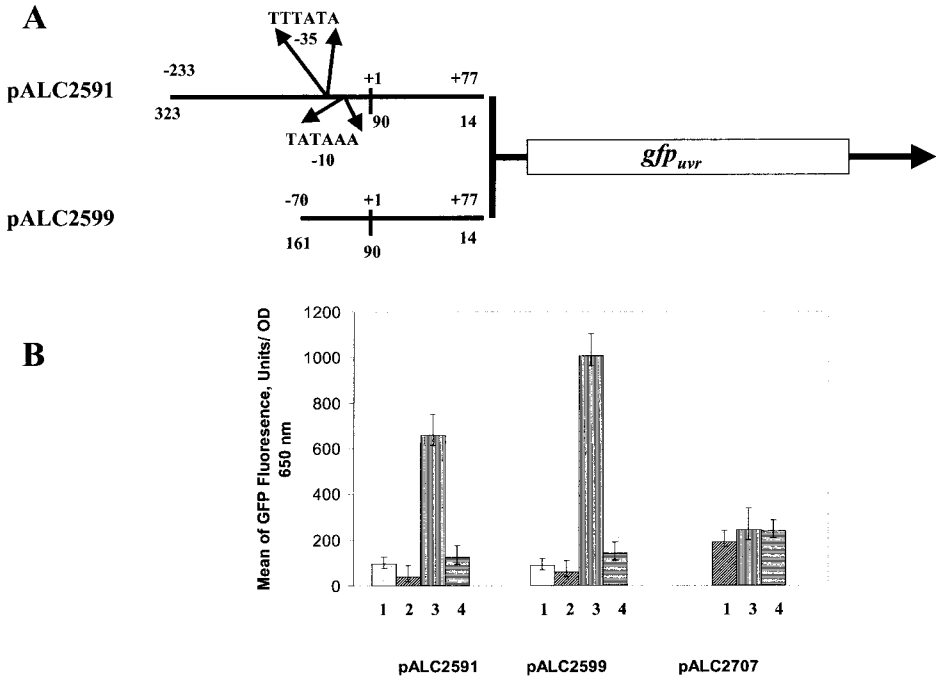

FIG. 4. Promoter activation of the *sarU* promoter fused to a gfp_{uvr} reporter gene, as evaluated with a fluorescence spectrophotometer (FL600; BioTek Instruments). (A) Graphical representation of the 310- and 147-bp DNA fragments of *sarU* promoter region fused to a promoterless gfp_{uvr} gene with an *S. aureus* ribosome-binding site. The transcriptional start site, labeled as +1, was identified by primer extension, and the putative promoter −10 and −35 boxed sequences are also indicated. The numbers at the lines (both top and bottom) are marked according to the transcriptional start site and to the start codon of the *sarU* gene, respectively. (B) Recombinant shuttle constructs pALC2591, pALC2599, and pALCALC2707 containing 310-bp, 148-bp, and reversed-orientation 310-bp *sarU* promoter fragments, respectively, were introduced into the wild type and its isogenic mutant strains, namely, wild-type RN6390 (lanes 1), *agr* mutant RN6911 (lanes 2), *sarT* mutant ALC1905 (lanes 3), and *sarU* mutant ALC2272 (lanes 4). To minimize variations in fluorescence attributable to cell density, the data are presented as the averages of reported fluorescence per OD_{650} unit in triplicate samples obtained during the postexponential phase, when *sarU* promoter activity is expected to be higher (see Fig. 2). The experiment was repeated several times, and one of the typical experiments is shown. A negative control with the shuttle vector pALC1484 alone lacking any promoter fragment was performed and showed no significant background (less than 50 mean fluorescence units) (data not shown).

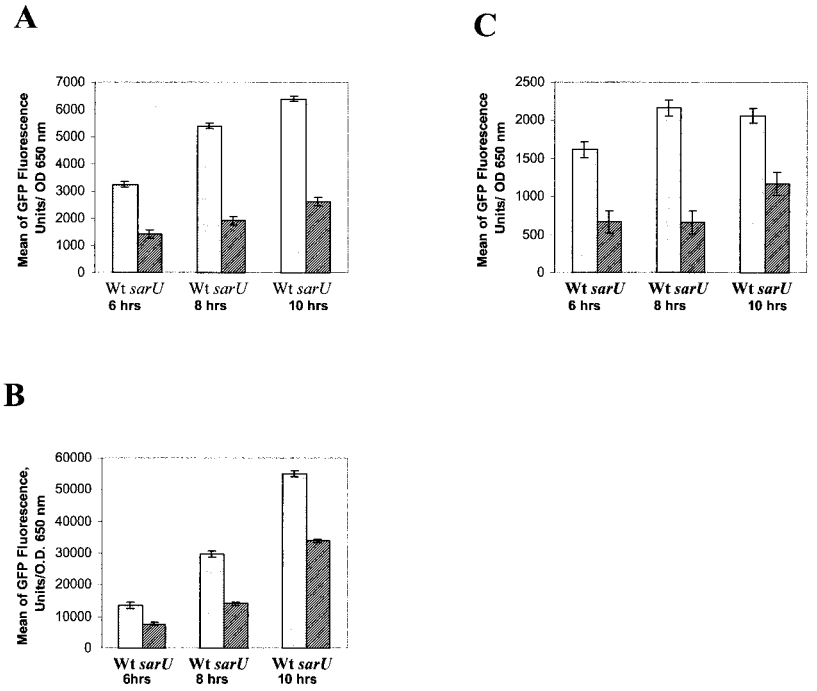

FIG. 5. GFP expression was driven by *agr* RNAII (A), *agr* RNAIII (B), and *hla* (C) promoters in the wild type (Wt) and in an isogenic *sarU* strain of *S. aureus*. Bar diagrams represent various time points of the growth phase after inoculation. Samples obtained at 6 h of growth represent early postexponential phase, a time point at which RNAII, RNAIII and *hla* promoter activities can easily be detected.

RN6390 (190 ± 30 units), *sarU* mutant ALC2272 (236 ± 50 units), and *sarT* mutant ALC1905 (243 ± 45 units), thus confirming an absence of a major autoregulatory effect by *sarT*. In addition, *sarT* regulates *sarU* but not vice versa.

GFP fusion assays for RNAII, RNAIII, and *hla* promoters in a *sarU* mutant. In addition to the regulation of *sarU* by *sarT*, we also found that *sarU* plays a regulatory role in target gene expression. In previous studies, we have found that *sarT* can repress the expression of RNAII and RNAIII (36). Cognizant of the SarT-mediated repression of *sarU* (delineated above), we hypothesized that *sarT* may repress RNAII and RNAIII promoter activities by down-regulating *sarU*. If this hypothesis were correct, one would expect RNAII and RNAIII expression to be decreased in a *sarU* mutant. To verify this possibility, we introduced plasmids containing an RNAII (pALC1742) or RNAIII (pALC1743) promoter linked to gfp_{uvr} into wild-type RN6390 and the isogenic *sarU* mutant (ALC2272). Upon monitoring bacterial growth and serial GFP expression over a 10-h period, we found that the growth rates were comparable between these two constructs, with early stationary phase appearing after ~6 h of growth. However, the expression of RNAII and RNAIII was significantly lower in the *sarU* mutant than in the parental strain (Fig. 5A and B). Recognizing that RNAIII activates *hla* transcription, we also explored the effect of the *sarU* mutation on *hla* promoter activity (Fig. 5C). In parallel with the RNAII and RNAIII data, the GFP-mediated fluorescence attributable to the *hla* promoter activity was lower in the *sarU* mutant than in the parental strain. Northern blotting with respective probes also confirmed the reduction in RNAII, RNAIII, and *hla* expression in the *sarU* mutant relative to that of the wild-type strain (data not shown).

Expression of *sarA, spa,* and *coa* (coagulase) in the *sarU* mutant. The above data indicated that *sarU* positively regulates *agr* RNAII and RNAIII expression. To determine whether *sarU* also controls *sarA*, we assayed for *sarA* transcripts (*sarA* P2, *sarA* P3, and *sarA* P1 transcripts originating from P2, P3, and P1 promoters of the *sarA* locus, respectively) in the *sarU* mutant. Our data indicated that *sarA* P2 and *sarA* P1 transcripts expressed during the log phase were not significantly altered in the *sarU* mutant compared with the parental strain (Fig. 6A). However, the *sarA* P3 transcript appeared to be slightly increased in the *sarU* mutant during the early stationary phase, whereas *sarA* P1 and P3 transcripts were increased and decreased, respectively, in the wild type carrying *sarU* in a shuttle plasmid (ALC2380), and both *sarA* P1 and P3 transcripts were enhanced in the complemented mutant strain (ALC2381). As differential activation of the three *sarA* promoters leads to varying SarA protein levels, we also determined the overall *sarU* mutation effect on the expression of SarA, the regulatory molecule of the *sarA* locus. Cell extracts from parental, mutant, and complemented strains during the stationary phase were obtained, and various amounts of extracts (5, 10, and 20 μg) were analyzed by Western blotting with anti-SarA monoclonal antibody 1D1. Significant alterations in SarA expression were not observed among the wild type (RN6390), *sarU* mutant (ALC2272), and complemented strains (ALC2380 and ALC2381). These data suggest that, despite the mild effect of *sarU* on individual *sarA* promoters, *sarU* does not have a significant effect on the expression of SarA.

The effect of the *sarU* mutation on the expression of protein A was more variable, with a slight decrease in *spa* transcription

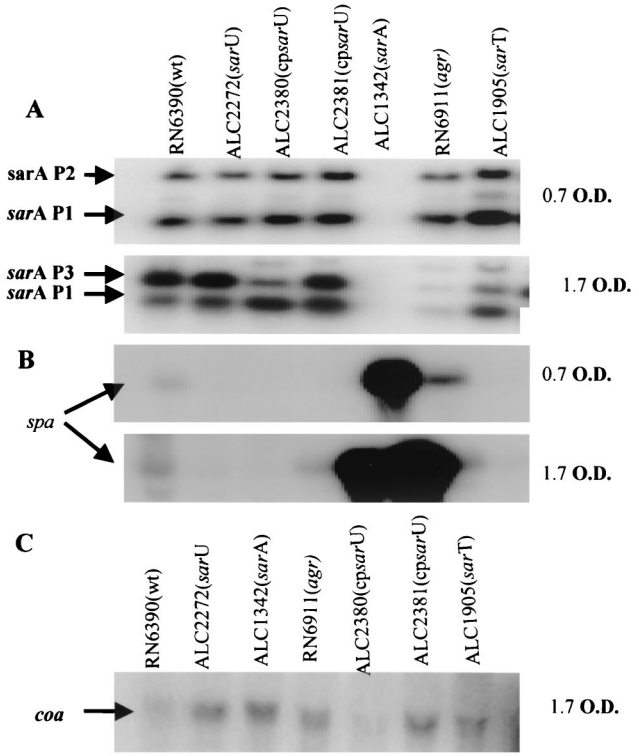

FIG. 6. Northern blots of *sarA*, *spa*, and *coa* transcripts in the wild type, *sarU* mutant, *trans*-complemented strains, *sarA*, *agr,* and *sarT* mutants. A total of 10 μg of RNA was applied to each lane. RNAs extracted from the wild type (RN6390), various mutants, and complemented strains are from the exponential (OD_{650}, 0.7) and postexponential (OD_{650}, 1.7) phases. (A) The blot was probed with a 400-bp fragment containing the *sarA* open reading frame. The transcripts are the larger *sarA* P2 (1.2 kb), *sarA* P3 (0.8 kb), and the smaller *sarA* P1 (0.5 kb) transcripts, respectively (4). (B and C) The blot was hybridized with a 1.4-kb DNA fragment containing the *spa* gene (B) and a labeled 4.5-kb *coa* fragment of *S. aureus* as a probe (C).

in the mutant (Fig. 6B), thus indicating that *sarU*, besides its effect on *agr*, may have a direct effect on the target gene as well. One effect of *agr* is the repression of cell wall-associated protein genes, such as that for coagulase. To ascertain the effect of *sarU* on *coa*, Northern blot analyses were conducted, revealing that the *coa* transcript level was higher in the *sarU* mutant than in the parental strain (Fig. 6C). As a control, we confirmed that *coa* expression was higher in the *agr* mutant than in the parental strain. Upon providing the *sarU* in *trans* in the parental strain (ALC2380), the transcript remained low. Curiously, complementation of the *sarU* mutant in *trans* (ALC2381) did not lead to a reduction in *coa* transcription as anticipated. Conceivably, this may be due to a higher gene dosage or to possible action of SarU in conjunction with other factor(s) that cannot be readily restored with the *sarU* fragment.

DISCUSSION

In prior studies, we have observed that SarT, a SarA homolog, represses *hla* expression in part by down-modulating *agr* RNAIII expression (36). In deciphering the *S. aureus* genomes (www.TIGR.org and www.ncbi.nlm.nih.gov), we found a divergently transcribed gene, *sarU*, adjacent to *sarT*. Remarkably, the transcription of *sarU*, not readily detected in the parental strain, was enhanced in a *sarT* mutant but not vice versa, indicating that *sarT* may repress *sarU* transcription. The gene product of *sarU* is 247 residues in length, with a molecular mass of 29,276 Da. As with many of the SarA homologs, SarU is a basic protein with a pI of 9.81. Similar to SarS (*sar*H1), which is a 250-residue SarA homolog, SarU can be envisioned as a molecule with two halves, with each half sharing sequence similarity to the smaller homologs such as SarA, SarR, and SarT (Fig. 1). SarU has good homology with the MarR protein of *B. subtilis.* The MarR proteins have been implicated in the negative regulation of multiple antibiotic resistances in gram-negative species (1). In particular, the expression of *marAB*, the genes encoding the antibiotic efflux pump itself, is controlled by the MarR repressor. A large number of compounds induce the transcription of the *marAB* operon, presumably by binding to MarR and thus preventing the binding of MarR to the *marAB* promoter (1).

Within the *S. aureus* genome, a family of proteins homologous to SarA can now be identified. We called this group of proteins the SarA protein family. Included in this family are SarA, -R, -S, -T, and -U; we are in the midst of characterizing other mutants within this protein family, including SarV (protein GI 13702067), SarX (protein GI 13700559), SarZ (protein GI 13702335), and SarY (protein GI 13702097)(unpublished data). To understand the mechanism of gene regulation in this protein family, we recently determined the crystal structure of SarR, a member of the SarA protein family (24). SarR is a dimeric structure comprising five α-helices, three β-strands, and several flexible loops (α1α2-β1α3α4-β2β3-α5). The SarR structure can also be envisioned as a three-domain structure, with a central helical core and two winged helix motifs. A short turn between α3 and α4 constitutes a typical helix-turn-helix motif (HTH) within each of the winged helix motifs. Deletion analysis indicated that the HTH motif is essential to the function of SarA, the prototypic member of the SarA protein family (7). In examining the amino acid sequence alignment (Fig. 1), the HTH region corresponds to residues 53 to 76 in the first half and to residues 178 to 201 in the second half of the SarU molecule. In the SarR dimeric structure, this region has been modeled to be the DNA binding domain that maintains contact with the major groove of DNA (24). Interestingly, many basic residues (Lys54, Lys63, and Arg69 in SarU1 and Lys179 and Arg194 in SarU2) within this region are conserved within the SarA protein family. Besides the HTH motif, the SarR structure also predicts the beta hairpin loop (β2-β3) corresponding to residues 81 to 97 in the first half and residues 206 to 212 in the second half to be another DNA-binding domain. In particular, the basic residues Lys82 (residue 207), Arg84 (residue 209), Arg90 (residue 215), and acidic residues Asp88 (residue 213) and Glu89 (residue 214) of SarU within the putative beta hairpin loop are absolutely conserved. These data are consistent with the notion that the DNA-binding domains might be highly conserved within this protein family. The structure of MarR in *E. coli* has recently been described; interestingly, MarR is also a dimeric structure with two winged helix motifs (1).

Despite sequence similarity among members of the SarA family, each member is also unique in its own function. This

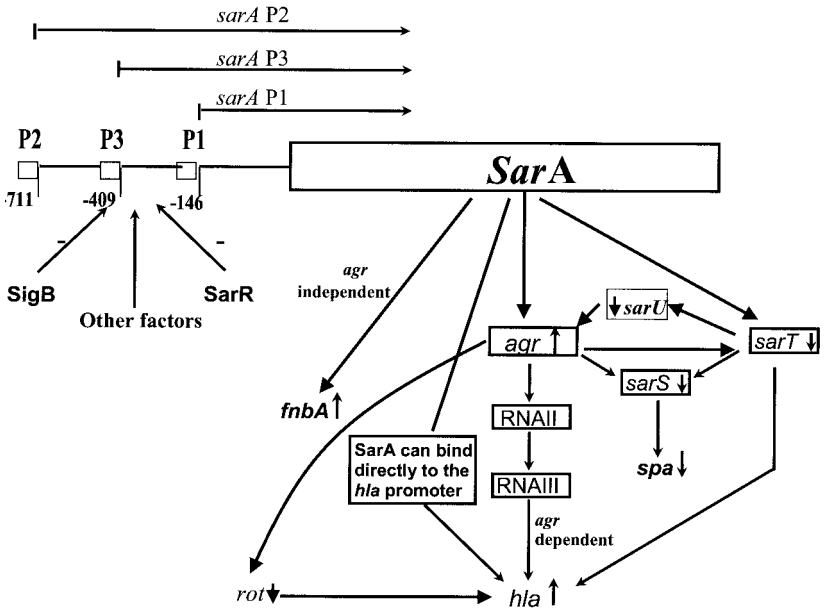

FIG. 7. A model of the *agr-sarT-sarU* pathway for regulating the expression of virulence genes in *S. aureus*. SarA is synthesized during the exponential phase based on differential activation of three *sarA* promoters (*sarA* P2, P3, and P1). SarR and SigB can down-regulate SarA expression, and other factors may up-regulate it. The basal level of RNAII expression, in part in response to SarA, would lead to synthesis of the AIP. Upon bacterial accumulation, the AIP would activate RNAIII transcription via a quorum-sensing mechanism. Besides the quorum-sensing pathway, we also propose that RNAIII activation would lead to repression of SarT. SarT repression would result in elevated *sarU* and decreased *sarS* expression; Rot protein acts as a repressor for *hla* expression (27). As SarU is an activator of RNAIII synthesis, this would provide a secondary amplification of the original *agr* signal. Accordingly, this model predicts a reduction in RNAIII promoter activity in a *sarU* mutant as well as decreased *sarU* promoter activity in an *agr* mutant compared to activities in the parental strain.

view is supported by the lesser degree of conservation within the activation domain among family members, thus accounting for diverse functions of the family members (24). For example, SarS activates the synthesis of protein A, while SarT represses the expression of α-hemolysin (11, 36). Northern and transcriptional analyses revealed that *sarU* likely activates *agr*, as reflected by lower RNAII and RNAIII expression in a *sarU* mutant than in the parental strain. Additionally, the expression of *sarU* was also enhanced during the postexponential phase in the *sarT* mutant, corresponding to a period when *agr* activation is expected to be highest. This effect on *agr* is independent of *sarA,* since the expression of SarA, the *sarA* regulatory molecule, was not significantly altered in the *sarU* mutant compared to that in the parental strain. We also confirmed that the transcription of *hla*, a target gene of *agr*, was diminished in a *sarU* mutant, in concordance with the decreased *agr* transcription in the *sarU* mutant relative to that in the parental strain. Moreover, the expression of one of the cell wall-associated proteins (i.e., coagulase) controlled by *agr* was also increased in the *sarU* mutant relative to the parental strain. However, the effect of the *sarU* mutation on protein A expression is more variable, with slightly lower expression in the *sarU* mutant than in the parental strain, contrary to what one would expect from reduced *agr* expression in a *sarU* mutant. This finding suggests that besides its effect on *agr* and the ensuing target genes, *sarU* may also regulate target genes via an *agr*-independent mechanism, possibly like SarA-dependent regulation of virulence genes (12).

In prior studies, we have made the observation that a mutation in *sarT* has led to an increase in *agr* RNAIII expression compared to that in the parental strain. Paradoxically, *sarT* expression is also elevated in an *agr* mutant (36). Thus, the identification of *sarU*, a gene repressed by *sarT*, has provided the plausible link for a putative pathway whereby a feedback loop on *agr* amplification can be discerned (Fig. 7). In this scheme, activation of *agr* would repress *sarT*, leading to a moderate increase in *sarU* transcription, presumably occurring as a result of decreased SarT binding to the *sarU* promoter. Secondary activation of *sarU* would promote transcription of RNAII and RNAIII promoters of *agr,* leading to a further increase in *agr* transcription. This model also predicts *sarU* promoter activity to be lower in the *agr* mutant than in the parent (Fig. 7). Given the constraint that the *sarU* promoter was not highly active in the parent strain, we have consistently found that the fluorescence attributable to the *sarU* promoter was lower in the *agr* mutant than in the parent (Fig. 4B). Viewed in this context, activation of *agr* via the *sarT-sarU* pathway may represent an alternative mechanism to the primary quorum-sensing mechanism mediated by the cyclic autoinducing peptide (AIP). Although the RNAIII promoter cannot be activated in an *agr* mutant in vitro, Xiong et al. have found in a recent study that the RNAIII promoter can be activated at a significant level in an *agr* mutant in a rabbit endocarditis model (38). Whether the *sarT-sarU* activation mechanism is deployed in vivo in response to host signals in the absence of *agr* is not known. Nevertheless, the identification of the *sarT-sarU* pathway has provided a plausible mechanism to explain *agr* activation in vivo.

The *sarU* and *sarT* genes are divergently transcribed and are separated by a 323-bp intergenic sequence between the two

coding regions. Transcriptional analysis indicated that *sarU* expression is repressed by the *sarT* gene product and not vice versa. Mapping of the transcriptional start site indicated that the start site is located 90 bp upstream of the initiation codon of the *sarU* gene, with typical σ^A-dependent −10 and −35 promoter boxes. In gel shift studies, we found that purified SarT can bind to the *sarU* promoter region with fairly high affinity (K_d, ~102.5 pM). As multiple protein-DNA complexes are discerned, we speculate that multiple SarT dimers may interact with each other to bind to the *sarU* promoter region. This is based on our modeling data showing that three SarA dimers may bind to a single 135-bp *agr* promoter fragment (unpublished data). Nevertheless, we cannot exclude the possibility that a single dimer may bind to multiple sites on a 148-bp *sarU* promoter fragment.

Given that SarT is a repressor of *sarU* promoter activity, we speculated that *sarU* is primarily transcribed in the absence of SarT. Whether other factors are involved in augmenting *sarU* transcription in a *sarT* mutant is not defined in this study. It is curious that two hybridizing bands reacted with a *sarU* probe that encompassed only the coding region in the *sarT* mutant. Complementation of the *sarU* mutant with a 1.5-kb fragment encompassing both the *sarU* gene and an ~392-bp upstream sequence restored only the smaller hybridizing band of ~1,200 nt, presumably representing the monocistronic form of the *sarU* RNA message. Complementation with a 2.3-kb fragment containing 392-bp upstream and 1,158-bp downstream sequences of *sarU* plus the *sarU* coding region did not restore the larger, ~1,400-nt transcript (data not shown). Thus, the origin of the larger hybridizing band remains unclear. The gene immediately downstream of *sarU* encodes a 288-residue polypeptide, representing UTP-glucose-1-phosphate uridyltransferase (*gtaB*). The *gtaB* gene is transcribed divergently from the cDNA strand, with the stop codons of both genes being separated by 210 bp. Northern hybridization with a 2.15-kb DNA probe containing open reading frames of *sarU* and *gtaB* yielded an additional ~1,000-nt transcript (data not shown) distinct from the ~1.2-kb *sarU* RNA message. These data suggested that the *gtaB* gene is likely to be controlled from its own promoter and that the larger, ~1,400-nt transcript that hybridized with the *sarU* probe is not part of the *gta*B RNA message. Upstream of *sarT-sarU* is a gene that encodes a hypothetical protein (SA2285) homologous to these proteins with the serine-aspartic acid repeats (e.g., clumping factors A and B), and this gene is transcribed in the same orientation as *sarT*. Given the size of the transcript (~1,400 nt), this gene is unlikely to be part of that message. Collectively, these data suggested that the larger, ~1,400-nt transcript might be a cross-hybridizing band present in a *sarT* mutant. However, we did not entirely rule out the possibility that a cryptic promoter may originate upstream of *sarU* and that it is transcribed in opposite orientation to *sarT* or other genes. Clearly, the molecular architecture of the *sarT-sarU* loci needs to be further defined before we can clearly assign the origin of the larger transcript.

In sum, the *sarU* gene product participates in the activation of *agr* and some of its target genes (e.g., *hla* and *coa*). Additionally, *sarU* may also regulate target genes via an *agr*-independent pathway. To further define the exact function of the *sarU* gene product, we are in the process of constructing a double knockout of *sarU* and *sarT*; the combination of a *sarU* knockout together with mutations in *sarA*, *sarR*, *sarS*, *agr,* and other regulatory genes may provide a clearer understanding of the mechanism of virulence gene activation by SarU and other members of the SarA protein family.

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