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SarT, a Repressor of α-Hemolysin in *Staphylococcus aureus*

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In searching the *Staphylococcus aureus* genome, we found several homologs to SarA. One of these genes, *sarT*, codes for a basic protein with 118 residues and a predicted molecular size of 16,096 Da. Northern blot analysis revealed that the expression of *sarT* was repressed by *sarA* and *agr*. An insertion *sarT* mutant generated in *S. aureus* RN6390 and 8325-4 backgrounds revealed minimal effect on the expression of *sarR* and *sarA*. The RNAIII level was notably increased in the *sarT* mutant, particularly in postexponential-phase cells, while the augmentative effect on RNAII was less. SarT repressed the expression of α-hemolysin, as determined by Northern blotting, Western blotting, and a rabbit erythrocyte hemolytic assay. This repression was relieved upon complementation. Similar to *agr* and *sarA* mutants, which predictably displayed a reduction in *hla* expression, the *agr sarT* mutant exhibited a lower level of *hla* transcription than the *sarT* mutant. In contrast, *hla* transcription was enhanced in the *sarA sarT* mutant compared with the single *sarA* mutant. Collectively, these results indicated that the *sarA* locus, contrary to the regulatory action of *agr*, induced α-hemolysin production by repressing *sarT*, a repressor of *hla* transcription.

Staphylococcus aureus is an important human pathogen. Within its arsenal are genes coding for virulence proteins with activities ranging from quorum sensing, tissue colonization, and immune evasion to tissue destruction (39). Superimposed upon these virulence genes is a network of regulatory genes (global regulatory network) that allow exquisite and precise coordination of protein expression during different stages of infection (4, 11, 13, 17, 38). Presumably, the regulatory network permits the bacteria to respond to environmental cues and hence allows the pathogen to thrive in diverse host microenvironments, e.g., blood, heart, lung, kidney, and spleen (39).

During growth in vitro, *S. aureus* expresses a number of cell wall-associated adhesions (fibronectin and fibrinogen binding proteins) that are believed to support adherence and colonization of host tissues (9). In transition to the postexponential phase, the expression of adhesion proteins is repressed, while the synthesis of exoproteins with enzymatic activity (e.g., hemolysins, toxins, proteases, and lipase) predominates. By virtue of their proteolytic enzyme activities (e.g., V8 protease) as well as direct toxin effects on host cells (e.g., α-toxin), these exoproteins likely facilitate dissemination of the organism in vivo (39).

Postexponential protein expression in *S. aureus* is controlled by global regulatory systems such as *sarA* and *agr* (4, 17, 27). The *sarA* locus encodes a 372-bp open reading frame with three upstream promoters (P2, P3, and P1) that initiate overlapping transcripts, each coding for the 14.5-kDa SarA protein (6, 33). The *sarA* P1 and P2 promoters, most active during the exponential phase, are SigA dependent, while the P3 promoter is primarily active during the postexponential phase and is SigB dependent (5, 33). Phenotypically, the *sarA* locus activates the synthesis of fibronectin and fibrinogen binding proteins (for adhesion), as well as that of α-, β-, and δ-hemolysins (for tissue spread) (40). Protein-DNA binding studies revealed that SarA binds to a 29-bp recognition sequence within the P2-P3 intergenic region of *agr* (16, 36), thus playing a role in activating *agr* transcription. As confirmation, a *sarA* mutant also displayed reduced levels of RNAII and RNAIII transcription of *agr* when compared to the parental strain in vitro (12).

The *agr* locus, a well-described pleiotropic regulator of exoproteins synthesis in *S. aureus* (25, 27, 40), comprises two divergent transcripts: RNAII, which encodes *agrDBCA*, and RNAIII, encoding *hld*. AgrC and AgrA are thought to be the sensor and activator of a two-component regulatory system. AgrB and AgrD participate in the synthesis of a cyclic octapeptide, which acts as a quorum-sensing molecule (25). The secreted octapeptide activates the transmembrane sensor AgrC (30), leading to phosphorylation of AgrC and a second step phosphorylation of AgrA, the activator. Phosphorylated AgrA has been postulated to bind to the *agr* promoter region to activate RNAII and RNAIII promoters, leading to the expression of RNAIII, the regulatory molecule that is responsible for the *agr* phenotype (induction of exoproteins and repression of fibrinogen, fibronectin binding proteins, and protein A).

While mutations in *sarA* and *agr* have been shown to reduce virulence in several animal model studies, these mutations did not render the bacteria avirulent (1, 8, 22, 24), suggesting that other regulatory factors may be at work. With the partial release of the *S. aureus* genome, additional genes with homology to *sarA* could be identified. For example, *sarR* encodes a 115-residue protein that represses SarA expression during the postexponential phase, presumably by down-modulating *sarA* P1 transcription (32). In contrast, SarS (also called SarH1) acts downstream of *sarA* and *agr* to activate the transcription of *spa* (protein A) (13, 42). An additional regulatory gene, *rot*, has been defined as a repressor of alpha-toxin synthesis (35).

In searching the *S. aureus* genome (at www.TIGR.org), we found an additional gene with homology to *sarA*. We report here this *sarA* homolog, designated *sarT*, the expression of

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which is negatively controlled by *sarA* and *agr*. SarT represses the expression of *hla*. Surprisingly, RNAIII of the *agr* locus was induced in a *sarT* mutant. Additional transcriptional analysis with *sarA sarT* and *agr sarT* double mutants disclosed that *sarA*, but not *agr*, activates the synthesis of α-hemolysin by repressing *sarT* expression.

MATERIALS AND METHODS

Bacterial strains, plasmids, and growth media. The bacterial strains and plasmids used to generate the data in this study are listed in Table 1. Phage 80α (37) was used as a transducing phage. *Escherichia coli* strains were grown in Luria-Bertani medium (31). *S. aureus* strains were maintained with tryptic soy medium (Difco) and grown in CYGP or 03GL medium (37). Erythromycin (5 μg/ml), chloramphenicol (34 μg/ml for *E. coli* and 10 μg/ml for *S. aureus*), tetracycline (5 μg/ml), ampicillin (50 μg/ml), and kanamycin (50 μg/ml) were used for selection of transformants and transductants.

DNA isolation. Chromosomal DNA was isolated from overnight broth cultures of *S. aureus* by lysostaphin lysis and phenol extraction as described elsewhere (11). Plasmid DNA was isolated from *E. coli* strains by using a Qiagen plasmid mini kit. Plasmid DNA was extracted from *S. aureus* strains by a modification of the Qiagen plasmid mini kit in which cells collected from overnight culture were resuspended in the Qiagen P1 buffer with lysostaphin (100 μg/ml) and incubated at 37°C for 1 h.

Southern blot hybridization. Restriction endonuclease-digested staphylococcal chromosomal DNA was resolved by overnight electrophoresis at 20 V in 0.8% agarose as described elsewhere (31). The DNA was transferred to Hybond-N^+ nylon membrane by alkaline blotting (Amersham, Pharmacia Biotech UK). Target genes were detected by hybridization with gel-purified DNA probes radiolabeled with [α^{32}P]dCTP (Amersham, Pharmacia Biotech) using a Ready-To-Go labeling kit (Amersham, Pharmacia Biotech) or Random Primer kit (Roche).

Cloning *sarT* and generating a *sarT* mutant. To clone the *sarT* gene, primers based on flanking sequences (TIGR *S. aureus* contig 8076 [COL], nucleotides [nt] 1417 to 4061) were synthesized. A 3.2-kb fragment was amplified by PCR from *S. aureus* RN6390 chromosomal DNA with primers 1003 and 1004 (Table 2), digested with *Bam*HI, ligated into the *Bam*HI site of pUC18 (to make pALC1894), and transformed into *E. coli* XL1 Blue. Plasmids extracted from ampicillin-resistant colonies were screened for *sarT* fragment insertion by restriction endonuclease mapping and confirmed by DNA sequencing. To generate a *sarT* mutant, *ermC* (20) was ligated into a blunted *Nde*I site within the putative *sarT* coding region (nt 3093 to 3098). The resultant 4.4-kb *sarT*::*ermC* *Bam*HI fragment was confirmed by DNA sequencing, gel purified, ligated into the temperature-sensitive shuttle plasmid pCL52.2 (to yield pALC1898), and electroporated into RN4220 as previously described (37) to generate transformants. Putative transformants were confirmed by restriction mapping. Electrocompetent RN6390 was subsequently transformed with pALC1898 isolated from RN4220 (11, 43). Colonies isolated at 30°C and resistant to erythromycin and tetracycline were screened for the presence of plasmid by restriction mapping.

RN6390 harboring pALC1898 was grown in 03GL broth with erythromycin (5 μg/ml) at 30°C, diluted 1:1,000 in fresh medium, and propagated through several cycles of alternating 30 and 42°C as described elsewhere (3). Erythromycin-resistant, tetracycline-sensitive colonies, representing possible double-crossover events, were selected (11) and screened for *ermC* insertion into *sarT* by Southern blotting, PCR, and sequencing of the PCR fragment containing the junctional fragment. One putative *sarT* mutant (ALC1905) was selected for further study.

To generate ALC2031, a *sarT* mutant of 8325-4, an 80α phage lysate of ALC1905 was used to infect *S. aureus* strain 8325-4 as previously described (11, 43). The *sarA sarT* double mutants derived from strains RN6390 and 8325-4 (ALC2122 and ALC2050) were generated by transducing ALC2057 and ALC2031, respectively, with an 80α phage lysate of the *sarT* mutant (ALC1905). An 80α phage lysate of RN6911, an *agr* mutant of RN6390 (ALC134), was used to infect the *sarT* mutant (ALC1905) to yield the *agr sarT* double mutant (ALC2056). To confirm the genotypes, DNA extracts of putative transductants were digested with restriction enzymes and screened by Southern blot hybridization for the presence of *erm*C genes and a shift in the size of the restriction digest fragment hybridizing with a *sarT*-specific probe. Interruption of the desired gene was also confirmed by PCR followed by sequencing of the PCR fragment.

Complementation. The *sarT* transcript as derived from the *sarT* mRNA on a Northern blot was estimated to be ~800 nt long. In examining the *sarT* sequence (Fig. 1B), a putative transcriptional termination signal could be identified. Based on these data, we amplified by PCR an 1,196-bp fragment with genomic DNA from RN6390, using primers 1035 and 1036 (nt 2469 to 3665). The PCR fragment was ligated into pCR2.1 and transformed into *E. coli* InvαF′ (Invitrogen) to generate pALC2046. The correct insert was confirmed by DNA sequencing. The inserted fragment in pALC2046 was then cleaved with *Eco*RI, ligated into pSK236, and transformed into *E. coli* XL1 Blue. RN4220 was electroporated with the recombinant plasmid containing *sarT* (37, 41), and transformants selected on tryptic soy agar with chloramphenicol. Recombinant plasmid was purified from RN4220 transformants and electroporated into the RN6390 mutants ALC1905 (*sarT* mutant), ALC 2122 (*sarA sarT* mutant), and ALC2056 (*agr sarT* mutant) and the 8325-4 mutant strains ALC2060 (*sarT* mutant of 8325-4) and ALC2050 (*sarA sarT* mutant of 8325-4). Putative transformants containing the plasmid were verified by restriction mapping. The presence of a *sarT* transcript in the transformants was confirmed by Northern blots.

RNA analysis. To minimize variations from environmental factors, all of the strains in an experimental set were grown up within the same week, in the same incubator, using the same batch lot of CYGP broth. Results were obtained from at least two complete experimental sets, using RNA from cells grown and extracted at different times. In brief, overnight cultures were diluted to an optical density at 650 nm (OD_{650}) of 0.1 (using an 18-mm borosilicate glass tube) in CYGP broth with appropriate antibiotics and grown at 37°C with shaking. At exponential (OD_{650} = 0.7), late exponential (OD_{650} = 1.1), and postexponential (OD_{650} = 1.7) phases, RNA was extracted with a reciprocating shaking device (BIO 101, Vista, Calif.) and precipitated with 2-propanol as previously described (14, 28) and then resuspended in 0.5% sodium dodecyl sulfate (SDS); the RNA concentration was determined by absorbance at 260 nm.

Twenty micrograms of total RNA was electrophoresed through a 1.5% agarose–0.66 M formaldehyde gel in morpholine propane sulfonic acid and blotted onto Hybond-N^+ membranes as previously described (12). 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 again under UV light to confirm complete RNA transfer.

Gel-purified DNA probes were radiolabeled with [α^{32}P]dCTP as described above for the detection of specific transcripts (*sarR, sarT, sarA, hla, agr*RNAII, and *agr* RNAIII). Blots were hybridized under high-stringency conditions, washed, and autoradiographed with Kodak X-Omat film.

RNAII and RNAIII promoter activation. Plasmids pALC1742 and pALC1743, derivatives of shuttle plasmid pSK236 (26) containing the green fluorescent protein (GFP_{uvr}) gene under the control of the *agr* P2 and P3 promoters, respectively, were electroporated into *S. aureus* strains ALC1905 (*sarT* mutant), ALC 2057 (*sarA* mutant), ALC2122 (*sarA sarT* mutant), and ALC2056 (*agr sarT* mutant). The resulting strains harboring the plasmids were grown with shaking in tryptic soy broth at 37°C. Aliquots were removed to microtiter plates, and the cell density (OD_{650}) and degree of fluorescence were read hourly for 10 h in an FL600 fluorescence microplate reader (BioTek Instruments, Winooski, V.). Promoter activation was plotted as the ratio of fluorescence/optical density versus optical density, using the average values from triplicate readings.

Phenotypic characterization. Extracellular proteins were precipitated from supernatants of overnight cultures with trichloroacetic acid as described previously (10, 40). Proteins were separated by electrophoresis on SDS–12% polyacrylamide gels (44) and electroblotted onto nitrocellulose (Osmonics, Westborough, Mass.). The blots were blocked overnight in blocking buffer (0.1 M Tris–0.5 M NaCl [pH 8.2] with 2% bovine serum albumin and 1% Tween 20) and probed with sheep antibody specific for α-hemolysin (1:2,000 dilution) (Toxin Technology, Sarasota, Fla.). Antibody binding was detected with alkaline phosphatase-labeled secondary antibody (Jackson ImmunoResearch Laboratories) and nitroblue tetrazolium–5-bromo-4-chloro-3-indolylphosphate substrate (Sigma) as described previously (6). Band intensities for the Northern blots were determined by densitometric scanning using SigmaGel software (Jandel Scientific, San Rafael, Calif.), with the data presented as integrated area units.

Hemolysin assays. The spent supernatant from overnight cultures was assayed for α-hemolysin production using 4% defibrinated rabbit blood in triplicate in a microtiter assay as previously described (18). The positive control for lysis was 1% SDS. Titers were expressed as the reciprocal of the highest dilution showing 50% of the mean of the value for SDS hemolysis after 2 h of incubation at 37°C.

RESULTS

In searching for SarA homologs in the *S. aureus* genome, we found three homologous proteins, SarR, SarS (also called SarH1), and SarT (Fig. 1A). SarR is a 113-residue protein that binds to the *sarA* promoter to down-modulate SarA expression (32). SarS, a 250-residue protein that is identical to SarH1

TABLE 1. Bacterial strains and plasmids used for this study

Strain or plasmid	Comment	Reference or source
S. aureus		
RN4220	Mutant strain of 8325-4 that accepts foreign DNA	37
RN6390	*agr*$^+$ laboratory strain related to 8325-4, maintains hemolytic pattern when propagated on sheep erythrocytes	37 38
8325-4	Prophage-cured strain of NCTC8325 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	37 21
COL	Methicillin-resistant laboratory strain	37
DB	Clinical blood isolate previously used in adhesion and endocarditis studies	9
Newman	Laboratory strain	34
S. epidermidis	From the Ultrecht University Hospital	
S. haemolyticus	From the Ultrecht University Hospital	
S. saprophyticus	From the Ultrecht University Hospital	
ALC133	RN 6112; RN6390 with *agrA*::*ermC*	40
RN6911	*agr* mutant of RN6390 (Δ*agr*::*tetM*)	38
ALC135	*agr sarA* double mutant of RN6390	2
ALC1342	*sarA* deletion mutant in which *sarA* (nt 586–1107) has been replaced by *ermC*	13
ALC1905	*sarT* mutant of RN6390 (*sarT*::*ermC*)	This study
ALC2031	RN8325-4 with a *sarA*::*kan* mutation	4
ALC2050	*sarA sarT* mutant of 8325-4	This study
ALC2056	*agr sarT* mutant of RN6390	This study
ALC2057	RN6390 with a *sarA*::*kan* mutation	13
ALC2060	*sarT* mutant of 8325-4 (*sarT*::*ermC*)	This study
ALC2063	RN4220 with pALC2047	This study
ALC2071	ALC1905 with pALC2047	This study
ALC2072	ALC2050 with pALC2047	This study
ALC2075	ALC2060 with pALC2047	This study
ALC2076	ALC2056 with pALC2047	This study
ALC2122	*sarA sarT* mutant of RN6390	This study
ALC2150	ALC2122 with pALC2047	This study
E. coli		
XL1 Blue	General-purpose host strain for cloning	31
InvαF′	Host strain for the TA cloning vector (pCRII)	Invitrogen
BL21(DE3)pLysS	Host strain for expression vector pET14b	Novagen
ALC1904	BL21(DE3)pLysS containing pET14b::*sarT*	This study
Plasmids		
pUC18	*E. coli* cloning vector	31
pCL52.2	Temperature-sensitive *E. coli-S. aureus* shuttle vector	29
pSK236	Shuttle vector containing pUC19 cloned into the *Hin*dIII site of pC194	19
pCR2.1	*E. coli* PCR cloning vector	Invitrogen
pET14b	Expression vector	Novagen
pALC1894	pUC18 with a 3.2-kb fragment containing the *sarT* coding region	This study
pALC1896	pUC18 with a 4.4-kb fragment containing the *sarT*::*ermC* mutation	This study
pALC1898	pCL52.2 with a 4.4-kb fragment containing the *sarT*::*ermC* mutation ligated at the *Bam*HI site	This study
pALC1904	pET14b with the *sarT* coding region at the *Xho*I-*Bam*HI site	This study
pALC2046	pCR2::*sarT*	This study
pALC2047	pSK236::*sarT*	This study
pALC1742	pSK236 (gfp_{uvr} with *agr* P2 promoter)	This study
pALC1743	PSK236 (gfp_{uvr} with *agr* P3 promoter)	26

recently reported by Tegmark et al. (42), is normally repressed by *sarA* and *agr* (13). Contrary to SarA, SarS is an activator of protein A synthesis (13). An additional putative regulator, SarT, was also identified by its homology with SarA in the *S. aureus* genome database (TIGR contig 8076). A six-frame translation of the sequence revealed a putative protein of 118 amino acids (Fig. 1A). Lying 7 bp upstream of the predicted translation start is a ribosomal binding site, followed by typical initiation (ATG) and termination (TAA) codons (Fig. 1B). The SarT protein has a predicted molecular mass of 16,096 Da, a high percentage of charged residues (43%), and homology with SarA (35%) and SarR (20%).

The gene was expressed by cloning the putative *sarT* coding region (primers 1005 and 1006) into pET14b, a His-tag (Invitrogen) expression vector. After induction with isopropyl-β-D-thiogalactopyranoside and purification on a nickel affinity column, we isolated a protein of ~16 kDa after thrombin digestion. This protein, upon N-terminal microsequencing,

TABLE 2. Primers used for this study

Primer	Primer sequence	Comment
1003	5′-ACGGGGATCCTTATGACGTTGGAGAAAA	Upstream of *sarT, Bam*HI site added (underlined)
1004	5′-AGCGGGGATCCCAAGTTTTACCAGCATA	Downstream reverse primer, *sarT*
1005	5′-GTAAGGGATGAACTCGAGATGA**ATG**ATT	Start of *sarT* (bold), added *Xho*I site (underlined)
1006	5′-ACGGGGATCCAAAAATACATTTAACTGC	Reverse primer, downstream of *sarT*, *Bam*HI site added (underlined)
1013	5′-ATGGTCTATTTCAATGGCAGTTAC-3′	Internal reverse primer, *ermC*
1017	5′-GATGCGATTGAACGTATGAATAATGAT-3′	Upstream of primer 1003
1035	5′-GCGAATTCTACCGGTCCTTTCTTATCTCT	Downstream of *sarT* coding for complete transcript, *Eco*RI site added (underlined)
1036	5′-GCGAATTCCAGATTGTTTGTAAAGTATGT	Upstream *sarT* complete transcript, *Eco*RI site added

showed agreement with the nine N-terminal amino acids of the predicted sequence (data not shown).

Characterizing the *sarT* gene in staphylococcal strains and in *sarA* and *arg* mutants. Previous studies (32) have shown that *sarR*, a gene homologous with *sarA*, was present in *S. aureus* and *S. saprophyticus* but not in *S. epidermidis* or *S. haemolyticus* when hybridized under high-stringency conditions. To determine the distribution of *sarT* in staphylococci, a 0.4-kb fragment encompassing the putative *sarT* gene was used to probe genomic DNA from several staphylcoccal species. The *sarT* probe hybridized with *S. aureus* strains COL, RN6390, Newman, and DB and *S. saprophyticus*, but not with *S. epidermidis* or *S. haemolyticus*, on a Southern blot of *Hin*dIII-digested genomic DNA (Fig. 2A). As with *sarR* (32), the failure of the *sarT* probe to hybridize with *S. epidermidis* or *S. haemolyticus* genomic DNA may be a result of either the absence of *sarT* or genetic divergence.

The *sarT* message (Fig. 2B) was found to be ~800 bp long when calculated from a plot of relative migration distance versus RNA size markers in a *sarA* mutant (Fig. 2B, fourth panel from the left). As a putative transcriptional termination signal was found downstream of the stop codon (Fig. 1B), we surmise that the *sarT* transcript is likely monocistronic.

Northern blots of wild-type, *agr, sarA*, and *agr sarA* mutant strains of RN6390 were probed with a ^{32}P-labeled *sarT* fragment to ascertain *sarT* expression in these genetic backgrounds (Fig. 2B). In *sarA, agr*, and *sarA agr* mutants of RN6390, expression of *sarT* was significantly higher than in the parental strain at all time points during growth. Notably, *sarT* expression in these mutants was maximal during the postexponential phase (OD_{650} = 1.7), at a time when the secretion of exoproteins is generally the highest in the parental strain. In addition, the expression of *sarT* was higher in the *sarA* and *agr sarA* double mutants than the *agr* mutant. These data suggest that *sarT* transcription is repressed by *sarA* and *agr*, particularly in the postexponential phase.

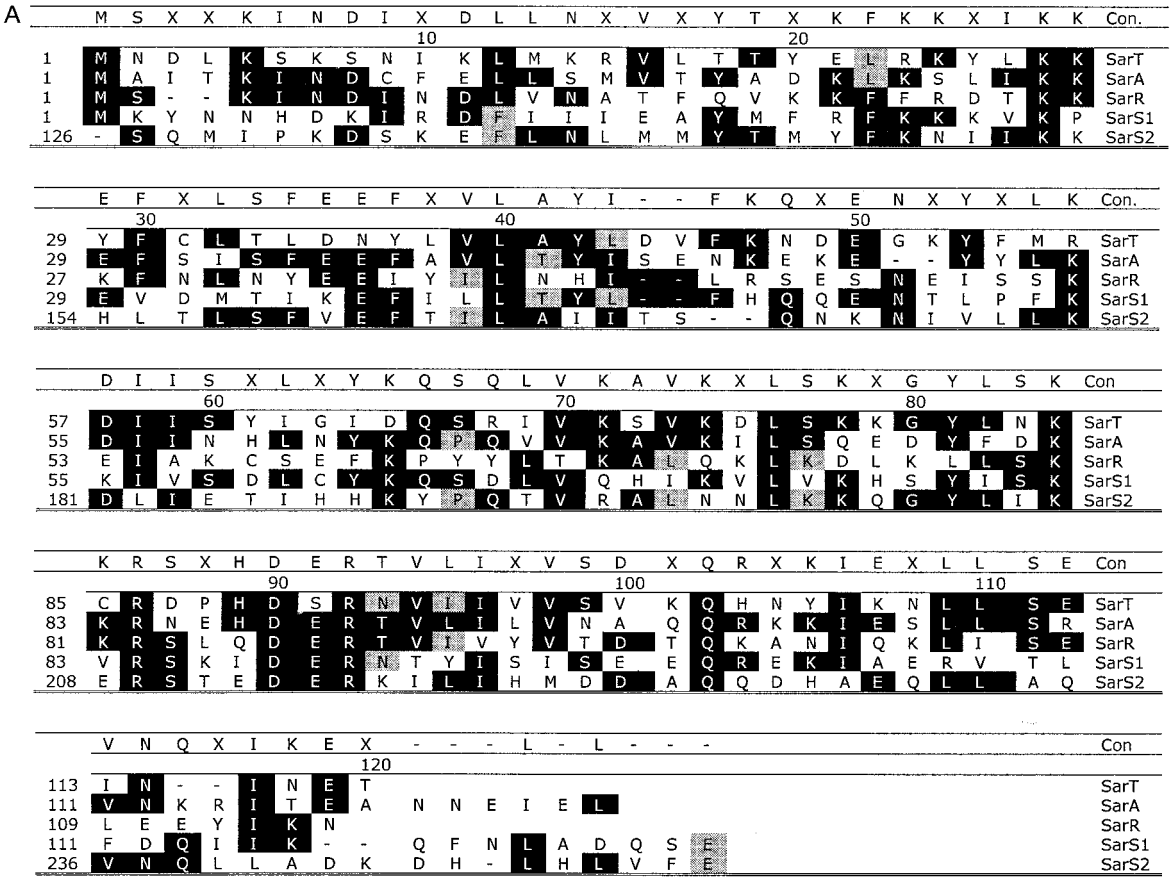
Construction of *sarT* and *sarA sarT* and *agr sarT* double mutants. Since *sarT* has homology with *sarA* and other genes in the SarA family, we surmise that SarT may participate as an additional regulator downstream of *sarA* and *agr* in the regulatory cascade. To address this possibility, we generated the *sarT* mutant ALC1905 by transforming RN6390, a prototypic *S. aureus* strain, with a temperature-sensitive plasmid (pALC1898) that contained an *ermC* cassette within the *sarT* coding region, and selecting recombinants by antibiotic sensitivity. Successful generation of the *sarT* mutant in *S. aureus* was confirmed by probing Southern blots of *Cla*I or *Xmn*I chromosomal digests with ^{32}P-labeled fragments of *ermC* and *sarT* (Fig. 3A).

Northern blotting revealed that there was no detectable *sarT* message in the mutant strain (ALC1905) (Fig. 2B). For additional confirmation, we generated a PCR fragment by using a primer specific for *ermC* (primer 1013) and another primer from the chromosomal region outside the original *sarT* construct (primer 1017). The size of the PCR fragment as well as direct sequencing of the PCR fragment confirmed that a double-crossover event had taken place between the plasmid and the chromosome.

We recognized the possibility that the observed phenotypes might be strain dependent. To evaluate this, we generated another *sarT* mutant in strain 8325-4 by using an 80α lysate of ALC1905. Putative transductants were confirmed by Southern blot analysis (Fig. 3B) with *ermC* and *sarT* probes as described. The results from the RN6390 mutant strains were compared with results in corresponding 8325-4 mutant strains.

We also generated additional mutant strains harboring *sarA sarT* and *agr sarT* mutations to explore the effect of *sarT* on the *sarA* and *agr* mutant phenotypes. Accordingly, an 80α lysate of RN6911 (Δ*agr*::*tetM*) was used to transduce the *agr* mutation into ALC1905 to generate an *agr sarT* double mutant. Similarly, a *sarA sarT* mutant was constructed by transducing ALC2057 (RN6390 with a *sarA*::*kan* mutation), and ALC2031 (8325-4 with a *sarA*::*kan* mutation) with an 80α lysate of ALC1905. To ensure that the observed effect of the above strains was attributable to the *sarT* mutation, the double mutant was also complemented with a recombinant shuttle plasmid (pSK236) carrying a 1.2-kb *sarT* fragment (pALC2047).

To ascertain the effect of the *sarT* mutation on the transcription of *sarR* and *sarA*, Northern blots of the wild-type strains (RN6390 and 8325-4), *sarT* mutants and complemented strains were probed with gel-purified ^{32}P-labeled DNA fragments of *sarR* and *sarA* (Fig. 4). In blots probed with *sarR*, the *sarT* mutant strains showed a very slight increase in *sarR* transcription that appeared to be reversed by complementation in both RN6390 and 8325-4 backgrounds (Fig. 4). Interestingly, the *sarA* transcript level (i.e., P1, P3, and P2 transcripts) was not significantly altered among any of the *sarT* mutants or complemented strains compared with parental strains. Notably in strains RN6390 and 8325-4, the insertion of the kanamycin cassette (*kan*) within the *sarA* gene led to *sarA* transcripts of higher molecular size (Fig. 4). However, these altered transcripts did not result in synthesis of SarA, the *sarA* regulatory molecule, as determined by probing an immunoblot of cell extracts of *sarA*::*kan* strains with anti-SarA antibody (data not



B

TTATATTATT TAAAGTAATA TAAAAGCAA TCTCTTTTGA AGACATGTTA

−35

AACCTAAATA TTTTTATATA TAATATATAT AAAATATAAA TAACTAAAAA

−10

TCACAGAATA ACAAATGACA AATTCATTTA TTATCCAAAA ATAAAAACAA

TTGAACAAAA TCAAAATATA GTGTTATGAT TCATTTGTGT TTATAGCTAG

CACCACAAAC TAAATATATT TGGAGTTATT ACTAGTTAAA GACAAATATT

ATTTGACGTG CAATTAAAAG TAAGGGATGA AGACTTG**ATG**-***sarT***-

TAAAGTATGT TTCGAGATTT TAATATCTTG GTGCAGTTAA ATGTATTTTT

AAATGCGTTA AGGTATTTTT TATTTTAGAA ATTCAATGCT ATTGAGTTGA

TGGGTTTTTC CAACAGATAA GAATGAATTA TTCATTGAAA ACTAAAAATA

ATAATTTTAT TTGTAGATTG TTTTAAGTGC GTCAATATAT AAAATAATTT

TATAAAGATA AGTGTTATAT TCCATTGTGC AACTCCTATT GTATTTGTAA

AATTACATGT AAATTCACTG TAAGTAAAGT GGAAAATATG GAAAAAGGAG

TATGCAAATG AGAGATAAGA AAGGACCGGT AAATAAAAGA GTAGATTTTC

TATCAAATAA ATTGAATAAA *TATTCAAT*AA GAAAATTTAC AGTTGGAACA

FIG. 1. Amino acid sequences for the SarA family of proteins. (A) Comparison of SarT with SarA, SarR (32), and SarS (13, 42). Con, consensus (shaded with black or gray). SarS, a 250-residue protein, has two 125-residue SarA-like modules; the C-terminal half (SarS2, 126 to 250 amino acids) shows homology with the N-terminal half (SarS1) and with other SarA homologs. (B) Promoter and termination regions of *sarT*. The putative −35 and −10 promoter recognition sites are underlined. The ribosomal binding site 7 bp upstream of the predicted translation start is underlined, and typical start (ATG) and termination (TAA) codons are bold. The putative terminator region consists of a T-rich region containing two potential base-paired stem-loop sequences (underlined).

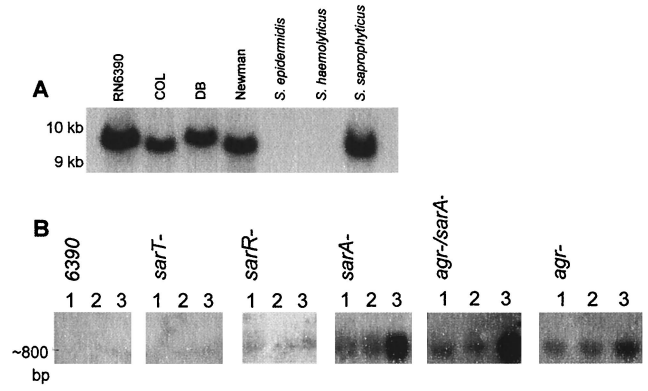


FIG. 2. *sarT* genes and expression. (A) Southern blot. Genomic DNAs extracted from a collection of *S. aureus* and other staphylococcal species were digested with *Hin*dIII (expected fragment size, 9,470 bp) and probed with a 0.4-kb fragment encompassing the putative *sarT*. The *sarT* probe did not hybridize with chromosomal DNA from *S. epidermidis* or *S. haemolyticus*. (B) Northern blots to determine if the *sarT* message is influenced by the *sarA/agr* regulatory system. RNA extracted from wild-type (RN6390) and mutant strains of *S. aureus* was probed with ^{32}P-labeled *sarT* at exponential phase (OD_{650} = 0.7) (lane 1), late exponential phase (OD_{650} = 1.1) (lane 2), and postexponential phase (OD_{650} = 1.7) (lane 3).

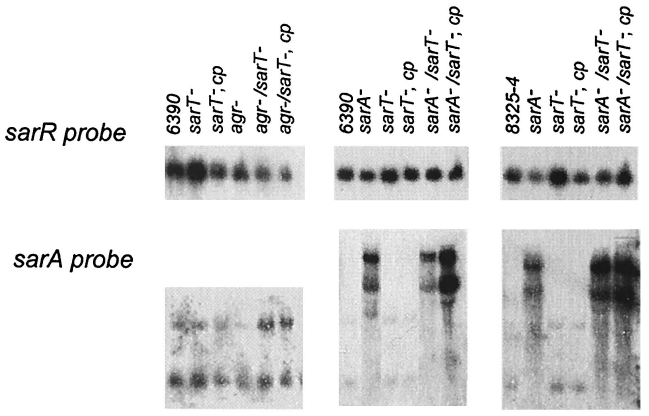


FIG. 4. Northern blots of RNA extracted from *S. aureus* strains at the postexponential phase of growth (OD_{650} = 1.7) and probed with *sarR* or *sarA*. cp, complemented with *sarT* in *trans*. The *sarA* mutant strains express an RNA message that is larger than the wild-type message, but it is apparently not translated, since SarA protein cannot be detected by a monoclonal antibody on a Western blot of whole-cell extracts.

shown). Additionally, mutation in *agr* in a *sarT* mutant also did not markedly modify *sarA* transcription. Collectively, the data indicate that the effect of the *sarT* mutant on the expression of *sarA* is minimal and that *sarT* likely lies downstream of *sarA*.

Northern blots (Fig. 5) showed that the RNAII levels were slightly higher in the *sarT* mutant than with the parental strains (RN6390 and 8325-4) but returned to near parental levels with *sarT* complementation. In contrast, the *agr* RNAIII message increased markedly in *sarT* mutants and was reduced to near parental levels in complemented *sarT* mutant strains in both RN6390 and 8325-4 backgrounds (Fig. 5). Thus, despite the repressive effect of *sarA* and *agr* on *sarT* expression, these data suggested that *sarT* might have a significant down-modulating effect on RNAIII transcription, while the effect on RNAII is much less.

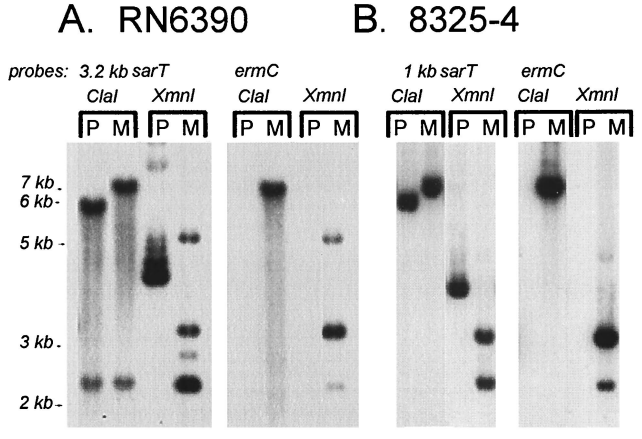


FIG. 3. Southern blot of restriction digests of genomic DNA to demonstrate a change in the band size of the putative insertion mutation relative to the parental type, indicative of insertion of *ermC* into *sarT*. P, parental; M, *sarT* mutant. *Cla*I cleaves *sarT* but not *ermC*; the 3.2-kb *sarT* DNA probe hybridizes with a 5.6- and a 2.2-kb fragment, while the 1-kb *sarT* DNA probe hybridizes only with the 5.6-kb fragment. Insertion of *ermC* increases the larger fragment to 6.9 kb. There is a single *Xmn*I site within *ermC*. *Xmn*I yields a 4.0-kb fragment encompassing *sarT*. With the *ermC* insert, expected fragment sizes are 2.2 and 3.1 kb. The 2.6- and 5-kb fragments seen in panel A are consistent with incomplete enzyme digestion.

To further confirm the differential effects of the *sarT* mutation on *agr* expression, RN6390 and the isogenic *sarT* mutant strains were transformed with shuttle plasmid pSK236, harboring the GFP_{uvr} gene driven by the RNAII or RNAIII promoter (pALC1742 or pALC1743, respectively). Levels of GFP expression in the mutants paralleled the RNA blot data (Fig. 6). With this assay, RNAII expression levels were slightly increased and RNAIII levels were significantly elevated when *sarT* was inactivated by mutation, particularly in postexponential-phase cells (Fig. 6). The *sarA sarT, agr*, and *agr sarT* mutants expressed GFP reporter activities for RNAII and RNAIII promoters at levels comparable to those for the *sarA* mutant (Fig. 6).

Characterization of the *sarT* mutant phenotype. The *sarA agr* global regulatory network has been shown to activate the expression of a number of exoproteins with toxin and enzymatic activities (e.g., hemolysins, toxins, proteases, and lipase) during the postexponential phase. As a putative regulatory component downstream of *sarA* and possibly *agr* in the regulatory cascade, we hypothesize that *sarT* could function as an intermediary to repress exoprotein synthesis, particularly in light of the observation that *sarT* transcription was elevated in *sarA* and *agr* mutants and that *sarT* was maximally expressed during the postexponential phase.

To ascertain the effect of *sarT* on the expression of α-hemolysin, an important extracellular virulence determinant of *S. aureus*, we probed the parental, mutant, and complemented strains for *hla* expression on Northern blots (Fig. 7). Remarkably, the level of message for *hla* was higher in *sarT* mutants than in parental strains for both RN6390 and 8325-4 (Fig. 7, P versus *sarT*- lanes). However, upon complementation, the level

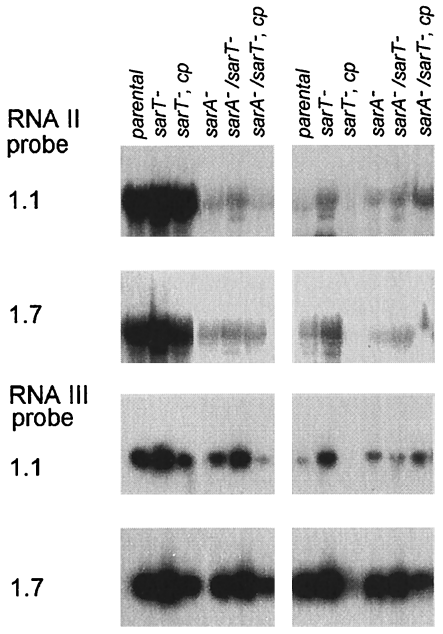


FIG. 5. Effects of *sarT* mutation on expression of RNAII and RNAIII. Northern blots of RNA extracted from *S. aureus* strains RN6390 (left) and 8325-4 (right) at late exponential (OD_{650} = 1.1) and postexponential (OD_{650} = 1.7) phases of growth were hybridized with *agr* RNAII or *agr* RNAIII probes. cp, complemented with *sarT* in *trans*.

of *hla* expression was reduced to very low levels (*sarT- cp* lane in each panel), presumably due to enhanced repression from increased *sarT* gene dosage. As predicted from the *agr* phenotype, *hla* transcription was markedly diminished in the *agr* mutant (Fig. 7A, lane m). Contrary to the *sarT* mutant, which displayed augmented *hla* transcription relative to the parental strain, the *agr sarT* double mutant did not display a higher level of *hla* transcription than the *agr* single mutant (Fig. 7A, lane *m*/*sarT*-), thus implying that *sarT* is not the primary intermediary target of *agr* that mediates enhanced *hla* expression.

Contrary to the *agr sarT* mutant, *hla* transcription was enhanced in the *sarA sarT* mutant compared with the *sarA* single mutant (Fig. 7B, lane *m*/*sarT*- versus lane *m*). Complementation of the *sarA sarT* mutant with a recombinant shuttle plasmid carrying *sarT* repressed *hla* expression to a certain extent, but not always to parental levels, particularly during the postexponential phase (Fig. 7B, lane *m*/*sarT-cp*). This finding would be consistent with the presence of a SarA-independent activator of *hla* that can overcome the suppressive effect of *sarT* on *hla* transcription.

Western blot analysis. Western blots of extracellular protein from wild-type, mutant, and complemented mutant strains were probed with sheep antibody specific for α-hemolysin (Fig. 8A and B). α-Hemolysin, normally expressed maximally during the postexponential phase, was produced in higher quantities in the *sarT* mutant and returned to a very low level in the *sarT* complemented strains (Fig. 8A and B). Although the *sarA* mutant expressed very little α-hemolysin, the *sarA sarT* double mutant exhibited detectable levels of α-hemolysin production (Fig. 8A). In contrast, the *agr sarT* mutant did not produce a detectable level of α-hemolysin (Fig. 8B). The titers from the rabbit erythrocyte hemolysis assay (Fig. 8C) are comparable

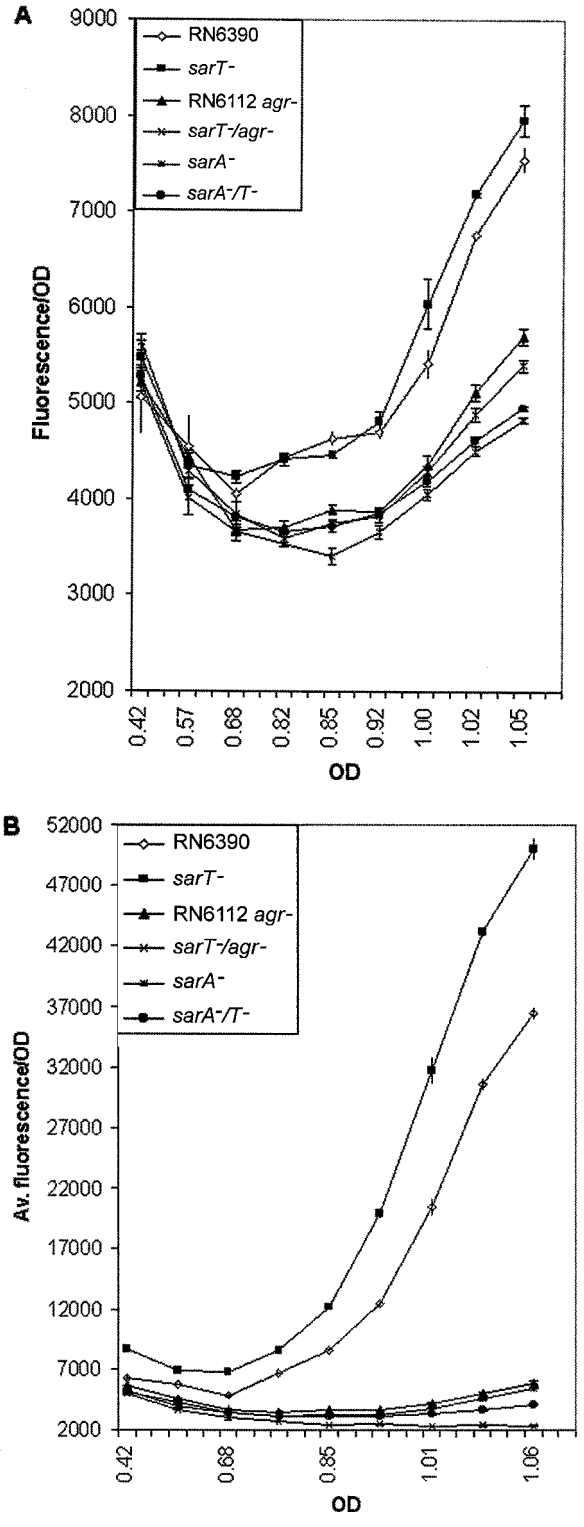


FIG. 6. GFP expression driven by the *agr* RNAII (A) and *agr* RNAIII (B) promoters.

with the Western blot results with respect to relative activity levels for various strains. The 24-h broths showed an increase in α-hemolysin relative to the 12-h broths, possibly due to accumulation of α-hemolysin with time.

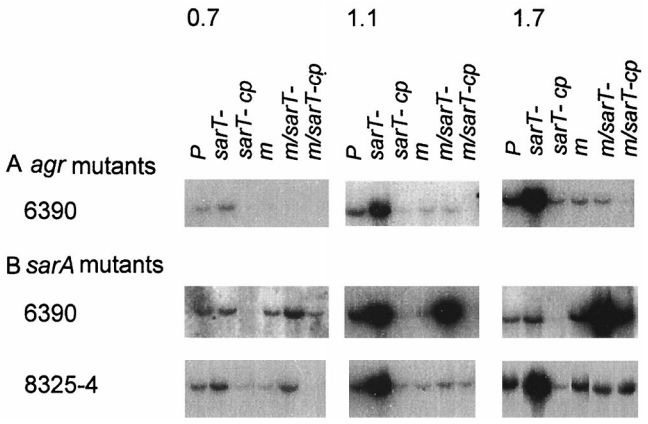


FIG. 7. Comparison of *hla* expression in *agr* (A) and *sarA* (B) mutants. Shown are Northern blots of RNA extracted from *S. aureus* strains at mid-exponential (OD_{650} = 0.7), late exponential (1.1), and postexponential (1.7) phases of growth. *P*, parental strain, either 6390 or 8325-4; *m*, mutation, either *agr* or *sarA*; *cp*, complemented with *sarT* in *trans*. The various *sarA* and *sarT* mutants show that *hla* transcription is repressed by *sarT*. However, *hla* expression is also influence by the strain background.

DISCUSSION

The *sarA agr* regulatory system is a major controlling element for the expression of a number of virulence determinants during the growth cycle (4, 10, 17). In addition to modulating the expression of a number of cell wall proteins (e.g., fibronectin binding proteins) during the exponential phase, the *sarA*/*agr* regulatory system also plays a major role in regulating toxin synthesis (e.g., α-hemolysin) during the postexponential phase. Because of the complexity and the growth phase dependency of the *sarA*/*agr* regulatory system, it has been speculated that other regulatory elements may be involved in the precise downstream control of virulence determinants during the transition from one growth phase to another.

Synthesis of α-hemolysin occurs primarily in transition from late exponential phase to postexponential phase. This suggests a requirement for the activation of additional genes or the suppression of preexisting repressor gene products. *sarT*, discovered by virtue of its homology to *sarA*, appears to be an intermediary gene that functions downstream of *sarA*. Evidence from our data (Fig. 2) indicates that *sarT* is induced during the exponential-postexponential transition and that *sarA* acts as a major *sarT* repressor, since *sarT* levels are significantly elevated in *sarA* mutants. As *sarR* and *sarA* expression was not significantly altered in *sarT* mutants relative to the parental strain, *sarT* is likely downstream of *sarA* in the regulatory cascade (Fig. 9).

Although *sarT* is repressed by *agr* (Fig. 2), our data also indicated that *sarT* significantly down-modulates the expression of RNAIII of the *agr* locus. This effect on RNAIII was reversible upon complementation. It is possible that *sarT* also has a slight effect on *agr* RNAII, since levels were slightly elevated in *sarT* mutants. This effect is *sarA* independent, since there are no major differences in RNAII and RNAIII expression levels in the *sarA* and *sarA sarT* mutants.

Based on the finding that *sarT* may be a repressor of *hla* expression, it is logical to assume that repression of *sarT* by both *sarA* and *agr* may activate *hla* expression. However, our data clearly demonstrated that only *sarA* activates *hla* transcription by repressing *sarT*, since a *sarA sarT* double mutant was able to augment *hla* expression to a level higher than that

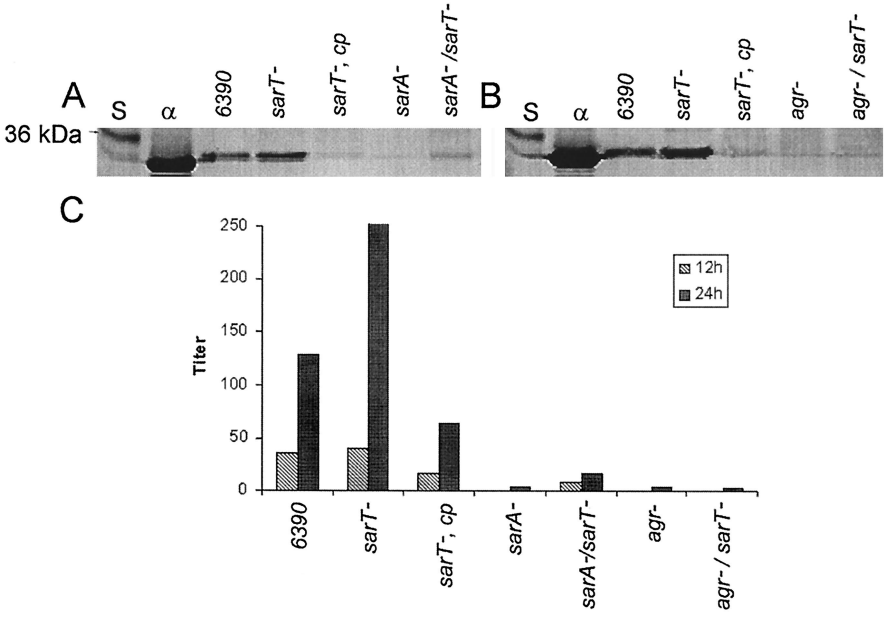


FIG. 8. Western blot and α-hemolysin assay of *sarT* mutants. (A and B) Extracellular protein probed with sheep polyclonal antibody to α-hemolysin; (C) α-hemolysin-induced rabbit erythrocyte hemolysis assay. S, protein molecular weight standards; α, α-hemolysin control; *cp*, complemented with *sarT* in *trans*: (A) RN6390 parental, 452 integrated area units determined by densitometric scanning (IAU); *sarT*, 600 IAU; *sarA*/*sarT*, 83 IAU; *sarA* mutant, undetectable level. (B) RN6390 parental, 207 IAU; *sarT*, 765 IAU. Titers are expressed as the reciprocal of the highest dilution showing 50% hemolysis.

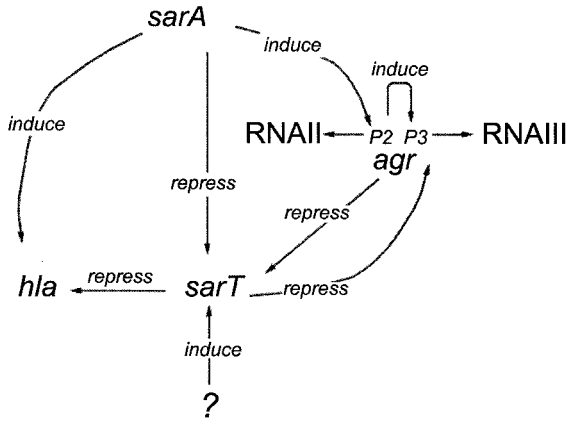


FIG. 9. Regulation of *sarT*. The most probable interaction of the *sarA*/*agr*/*sarT* regulatory network as it is currently understood. SarA induces the P2 promoter of RNAII, which in turn induces expression of RNAIII (15). SarA and *agr* both act to repress expression of *sarT*; SarT in turn appears to repress expression of *agr* RNAIII and *hla*. An unknown element induces *sarT*, and *sarA* induces *hla* via an *agr*-independent pathway (7). It is possible that *hla* induction by SarA is via SarT.

in the *sarA* mutant. In contrast, the *agr* locus did not utilize this pathway because *hla* expression in the *agr sarT* double mutant remained depressed to a level similar to that of the *agr* mutant. Collectively, these data indicate that *sarA* likely activates *hla* expression by repressing *sarT*.

The effect of *sarT* on *hla* expression is complex. While a *sarT* mutation resulted in an increase in *hla* transcription, the mutant also exhibited an increase in RNAIII transcription, as verified by Northern blot and transcriptional fusion data (Fig. 5 and 6). This finding for *sarT* thus hinted at the complexity of *hla* regulation by the *sarA* locus. In the presence of an intact *sarA*, the expression of *sarT* is repressed, leading to elevated *hla* transcription. However, the relative contribution of the effect of SarT on the expression of *hla*, as mediated via RNAIII, versus that which occurs as a result of direct interaction of *sarT* with the *hla* promoter is not clear. In addition, we have previously reported that SarA, the major *sarA* regulatory molecule, can up-regulate *hla* expression via both RNAIII-independent and RNAIII-dependent pathways. With the RNAIII-independent pathway, SarA binds directly to a recognition sequence in the *hla* promoter to activate transcription (17). With the RNAIII-dependent pathway, SarA binds to the conserved sequence upstream of the *agr* promoter to stimulate RNAII and RNAIII transcription (37) and possibly transcription and translation of *hla* (36). Collectively, these data hint at the complexity of the pathways by which *hla* expression is activated.

Our data also seems to suggest complex interactions between *sarT* and *agr* (Fig 9). On one hand, we recognize that the transcription of *sarT* is increased in *agr* mutants. On the other hand, RNAIII expression is also increased in a *sarT* mutant. Thus, there appears to be an inverse relationship (or possibly a negative feedback loop) between the presence of *sarT* and the expression of RNAIII. This putative feedback loop may conceivably lie downstream of *sarA*. This mode of regulatory hierarchy may explain (i) increased *hla* transcription in the *sarA sarT* double mutant by virtue of increasing RNAIII expression (Fig. 7B, lane 5; Fig. 5, lane 5) and (ii) a failure to increase *hla* transcription in an *agr sarT* double mutant compared with the *agr* single mutant (Fig. 7A, lane 5 versus lane 4).

Although *sarA* likely mediates *hla* expression by repressing *sarT*, RNA complementation data disclosed that the regulation of *hla* by the *sarA* locus, particularly during the postexponential phase, likely involves additional regulatory factors. This notion is supported by the observation that complementation of the *sarA sarT* mutant with *sarT* in *trans* could suppress *hla* expression in the mutant strain only during the exponential phase (OD_{650} of 0.7 and 1.1) but not during the postexponential phase (OD_{650} = 1.7) (Fig. 7B and C). Additionally, RNAIII repression in a complemented *sarA sarT* mutant was highly successful during exponential growth but not postexponentially (Fig. 5). These data are consistent with the observation of Vandenesch et al. (45) that a separate postexponential phase signal other than *agr* is also needed for activating *hla* transcription.

The large number of regulatory proteins recently described in *S. aureus* as a result of genomic advances (4, 11, 23), coupled with the elucidation of their regulatory controls on target genes, suggests that virulence gene regulation in *S. aureus* entails a complex network of regulatory genes. Some of these gene products (e.g., SigB and SarR) control the expression of SarA, while others such as SarH1 (also called SarS), Rot, and SarT may act as intermediaries between the regulatory elements (*sarA*/*agr*) and target genes (e.g., *hla* and *spa*). Clearly, additional regulatory factors will be discovered as the *S. aureus* genome is completed.

ACKNOWLEDGMENTS

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