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SarS, a SarA Homolog Repressible by *agr*, Is an Activator of Protein A Synthesis in *Staphylococcus aureus*

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The expression of protein A (*spa*) is repressed by global regulatory loci *sarA* and *agr*. Although SarA may directly bind to the *spa* promoter to downregulate *spa* expression, the mechanism by which *agr* represses *spa* expression is not clearly understood. In searching for SarA homologs in the partially released genome, we found a SarA homolog, encoding a 250-amino-acid protein designated SarS, upstream of the *spa* gene. The expression of *sarS* was almost undetectable in parental strain RN6390 but was highly expressed in *agr* and *sarA* mutants, strains normally expressing high level of protein A. Interestingly, protein A expression was decreased in a *sarS* mutant as detected in an immunoblot but returned to near-parental levels in a complemented *sarS* mutant. Transcriptional fusion studies with a 158- and a 491-bp *spa* promoter fragment linked to the *xylE* reporter gene disclosed that the transcription of the *spa* promoter was also downregulated in the *sarS* mutant compared with the parental strain. Interestingly, the enhancement in *spa* expression in an *agr* mutant returned to a near-parental level in the *agr sarS* double mutant but not in the *sarA sarS* double mutant. Correlating with this divergent finding is the observation that enhanced *sarS* expression in an *agr* mutant was repressed by the *sarA* locus supplied in *trans* but not in a *sarA* mutant expressing RNAIII from a plasmid. Gel shift studies also revealed the specific binding of SarS to the 158-bp *spa* promoter. Taken together, these data indicated that the *agr* locus probably mediates *spa* repression by suppressing the transcription of *sarS*, an activator of *spa* expression. However, the pathway by which the *sarA* locus downregulates *spa* expression is *sarS* independent.

Staphylococcus aureus is a versatile human pathogen that can cause a variety of infections ranging from minor wound infections, pneumonia, and endocarditis to sepsis (3). The ability of *S. aureus* to cause a multitude of diseases has been ascribed to the array of extracellular and cell wall virulence determinants produced by this microorganism (27). The regulation of many of these virulence determinants is controlled by global regulatory loci such as *sarA* (previously designated as *sar*), *agr, sae*, and *rot* (8, 13–16, 23). These regulatory elements, in turn, exert transcriptional control of target virulence genes.

The global regulatory locus *agr* encodes a two-component quorum-sensing system that originates from the generation of two divergent transcripts, RNAII and RNAIII. RNAIII is the effector molecule of the *agr* response, which entails upregulation of extracellular protein production (e.g., alpha-toxin) and downregulation of cell wall-associated protein synthesis (e.g., protein A and fibronectin-binding proteins) during the postexponential phase (16). The RNAII transcript encodes a four-gene operon, *agrBDCA*, with AgrC and AgrA corresponding to the sensor and the activator proteins of a two-component regulatory system (16). Additionally, AgrD encodes a 46-residue peptide which undergoes processing to form a quorum-sensing cyclic octapeptide, probably with the aid of the *agrB* gene product. Upon extracellular accumulation of a critical concentration of the cyclic octapeptide, the sensor protein AgrC will become phosphorylated (19), thus leading to a second phosphorylation step of AgrA. Phosphorylated AgrA will activate the transcription of RNAIII, the *agr* regulatory molecule, to modulate target gene transcription (15, 23, 26).

In contrast to *agr*, the *sarA* locus upregulates the synthesis of selected extracellular (e.g., α and β hemolysins) and cell wall proteins (e.g., fibronectin-binding protein A). Like the *agr* locus, the *sarA* locus also represses the transcription of the protein A gene (*spa*) (7). The *sarA* locus, contained within a 1.2-kb fragment, is composed of three overlapping transcripts, all encoding the major 372-bp *sarA* gene (1). DNA-binding studies revealed that SarA, the major *sarA* regulatory molecule, binds to several target gene promoters, including those of *agr, hla* (α hemolysin gene), and *spa*. Accordingly, the binding of SarA to a conserved binding site present in many target gene promoters leads to an upregulation in *agr* and *hla* transcription, as well as to a downregulation in *spa* transcription, thus implicating SarA to be a regulatory molecule that modulates target genes via both *agr*-dependent and *agr*-independent pathways (9).

Considering the fact that both *sarA* and *agr* repress *spa* transcription, it seems reasonable to predict the existence of regulatory element(s) that counteracts this mode of regulation (i.e., activating *spa*). In searching for SarA homolog(s) in the *S. aureus* genome (The Institute for Genome Research [TIGR]), we came upon an open reading frame (ORF) upstream of the *spa* gene that shares homology with SarA. Transcriptional analysis indicated that the expression of this gene, designated *sarS* for a gene supplemental to SarA, is enhanced in *sarA* and *agr* mutants, while the transcription of *sarA* and *agr* loci is unaltered in a *sarS* mutant. Inactivation of this gene leads to a decrease in protein A expression on immunoblots. Transcriptional analyses of *sarA sarS* and *agr sarS* double mutants indicated that the *agr* locus likely downregulates *spa* transcription by repressing *sarS* expression, whereas the *sarA* locus probably

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TABLE 1. Strains and plasmids used in this study

Strain or plasmid	Source or reference	Comments
S. aureus		
RN4220	25	A mutant of 8325-4 that accepts foreign DNA
RN6390	25	Laboratory strain that maintains its hemolytic pattern when propagated on sheep erythrocyte agar (parental strain)
RN6911	26	An *agr* mutant of RN6390 with a Δ*agr*::*tetM* mutation
PC1839	4	8325-4 with a *sarA*::*kan* mutation
ALC184	7	*sarA* mutant of RN6390 with pRN6735 and pI524
ALC865	7	RN6911 (*agr* mutant) with pALC862
ALC1016	This study	RN6390 with pALC1014
ALC1342	This study	A *sarA* mutant in which the *sarA* gene (nt 586 to 1107) (1) has been replaced by an *ermC* gene
ALC1794	9	RN6390 with pALC1639
ALC1927	This study	A *sarS* mutant of RN6390 with an *ermC* gene into the *Eco*RI site of the *sarS* gene
ALC2009	This study	ALC1927 complemented with pALC2010
ALC2033	This study	RN6390 with Δ*agr*::*tetM* and *sarS*::*ermC* mutations
ALC2034	This study	ALC1927 (*sarS* mutant) with pALC1639
ALC2057	This study	RN6390 with a *sarA*::*kan* mutation
ALC2067	This study	RN6390 with *sarS*::*ermC* and *sarA*::*kan* mutations
ALC2115	This study	ALC1927 (*sarS* mutant) with pALC1014
E. coli		
XL1-Blue	21	A host strain for cloning
BL21	21	A host strain for the pET14b expression vector
Plasmids		
pCR2.1	Invitrogen	*E. coli* cloning vector for direct cloning of PCR products
pUC18	21	*E. coli* cloning vector
pCL52.2	17	A temperature-sensitive *E. coli-S. aureus* shuttle vector
pET14b	Novagen	Expression vector for *E. coli*
pI524	16	*S. aureus* plasmid containing a β-lactamase repressor
pLC4	31	A shuttle plasmid containing a promoterless *xylE* reporter gene
pRN6735	16	A derivative of pC194 containing the *bla* promoter and two-thirds of the *blaZ* gene followed by a 1.5-kb RNAIII fragment lacking its promoter
pSK236	12	A shuttle vector containing pUC19 at the *Hin*dIII site of pC194
pALC672	This study	pCR2.1 with a 161-bp *sarA* P3 promoter fragment
pALC862	7	pSK236 containing the entire *sarA* locus with the *sarA* ORF and the triple promoter system
pALC1014	This study	pLC4 containing a 158-bp *spa* promoter fragment (nt 17 to 174) (20)
pALC1639	9	pLC4 (transcriptional fusion vector) with a 491-bp *spa* promoter fragment (nt 1 to 174 plus 319 bp upstream) (20)
pALC1883	This study	pUC18 containing a 1.8-kb *sarS* fragment (nt 2925 to 1082 in contig 6207)
pALC1889	This study	Temperature-sensitive shuttle plasmid pCL52.2 containing the *ermC* gene at the *Eco*RI site (nt 2616 to 2621) of the 1.8-kb *sarS* fragment
pALC2010	This study	Shuttle plasmid pSK236 containing a 1.2-kb *sarS* fragment (nt 3459 to 2189 of contig 6207)
pALC2040	This study	pCR2.1 with a 1,562-bp *spa* structural gene (nt 219 to 1780) (20).
pALC2043	This study	pET14b containing the 750-bp *sarS* gene at the *Xho*I/*Bam*HI site

suppresses protein A expression via a different mechanism. Gel shift analysis revealed that purified SarS binds to the *spa* promoter in a dose-dependent fashion. In contrast to the suppressive effect of *sarA* and *agr*, these data suggested that *sarS* activates protein A synthesis. The fact that *sarS* is repressible by *agr* and not vice versa hints at the possibility that *agr* may exert its effect on *spa* by repressing *sarS* expression.

MATERIALS AND METHODS

Bacterial strains, plasmids, and growth media. The bacterial strains and plasmids used in this study are listed in Table 1. CYGP, O3GL media (25), and tryptic soy broth were used for the growth of *S. aureus* strains, while Luria-Bertani medium was used to cultivate *Escherichia coli*. Antibiotics were used at the following concentrations: erythromycin at 5 μg/ml, kanamycin at 75 μg/ml, tetracycline at 5 μg/ml, and ampicillin at 50 μg/ml.

Genetic manipulations in *E. coli* and *S. aureus*. Based on homology with *sarA*, the *sarS* gene was identified in contig 6207 in the TIGR *S. aureus* genome database (www.TIGR.org). To construct a *sarS* mutant, part of the *sarS* gene, together with a part of flanking sequence, was amplified by PCR with the primers 5′-AGTTTTATGTTATAAACAATCGGA-3′ and 5′-GTTGTTTCTT GTTATTTTACGAA-3′, using chromosomal DNA from strain RN6390 as the template. The 1.8-kb PCR fragment (nucleotides [nt] 2925 to 1082 in contig 6207) was cloned into pUC18 in *E. coli*. Taking advantage of an internal *Eco*RI site (nt 2616 to 2621) in the middle of the *sarS* coding region (nt 3098 to 2346), we cloned a ~1.4-kb *ermC* fragment into this site. The fragment containing an *ermC* insertion into the *sarS* gene was cloned into the temperature-sensitive shuttle vector pCL52.2 (18), which was then transformed into RN4220 by electroporation (28), followed by transduction into RN6390 with phage ϕ11 as described elsewhere (8). Transductants were selected at 30°C on erythromycin- and tetracycline-containing plates.

S. aureus RN6390 harboring the recombinant pCL52.2 was grown overnight at 30°C in liquid medium in the presence of erythromycin, diluted 1:1,000 in fresh media, and propagated at 42°C, a nonpermissive temperature for the replication of pCL52.2. This cycle was repeated four times, and the cells were replicate plated onto O3GL plates containing erythromycin and erythromycin-tetracycline to select for tetracycline-sensitive but erythromycin-resistant colonies, representing mutants with double-crossovers. The mutations were confirmed by Southern hybridization with *sarS* and *ermC* probes. One clone, designated ALC1927, was selected for further study.

To complement the *sarS* mutation in ALC1927, we introduced a 1.2-kb PCR fragment (nt 2925 to 1082 in contig 6207) encompassing the *sarS* gene into the shuttle plasmid pSK236. The recombinant shuttle plasmid was first electroporated into RN4220 and then into the *sarS* mutant ALC1927 (8). The presence of the recombinant plasmid was confirmed by restriction mapping. The presence of the *sarS* transcript in the complemented mutant was confirmed by Northern blots with a *sarS* probe.

For the construction of the *sarA sarS* double mutant, we introduced the *sarA*::*kan* mutation into *sarS* mutant ALC1927 via a 80α lysate of a *sarA* insertion mutant PC1839 (with a *sarA*::*kan* mutation). As an additional control, we used a *sarA* deletion mutant (ALC1342) in which the *sarA* gene has been replaced by the *ermC* gene. Because of the *ermC* insertion, we were not able to construct a *sarA sarS* mutation in the ALC1342 background. Likewise, an *agr sarS* mutant was constructed by infecting the *sarS* mutant with a φ11 lysate of the *agr* mutant RN6911. The authenticity of these double mutants was confirmed by Southern and Northern blots with *sarA* and *agr* probes (data not shown).

Analysis of *hla* and *spa* expression in the *sarS* mutant and its isogenic parents. To assess the phenotypes of the *sarS* mutant, we first evaluated the expression of α hemolysin and protein A, two well-known virulence determinants in *S. aureus*. To determine α-hemolysin expression, equivalent amounts of extracellular proteins that had been harvested at stationary phase and concentrated by 10% trichloroacetic acid precipitation were blotted onto nitrocellulose, probed with rabbit anti-α-hemolysin antibody (a gift from B. Menzies, Nashville, Tenn.) diluted 1:2,000, and then treated with the $F(ab)_2$ fragment of goat anti-rabbit alkaline phosphatase conjugate (Jackson Immunoresearch, West Grove, Pa.) as described previously (5). Reactive bands were visualized as described by Blake et al. (2).

To evaluate protein A production, cell wall-associated proteins were extracted from an equivalent number of *S. aureus* cells (from overnight cultures) with lysostaphin in a hypertonic medium (30% raffinose) to stabilize the protoplasts as described previously (7). Equivalent volumes (1 to 2 μl each) of cell wall protein extracts from 25 ml of cells (10^9 CFU/ml) were resolved on 10% sodium dodecyl sulfate-polyacrylamide gels, blotted onto nitrocellulose, and probed with chicken anti-staphylococcal protein A antibody (Accurate Chemicals, Westbury, N.Y.) at a 1:3,000 dilution. Bound antibody was detected with a 1:5,000 dilution of $F(ab)_2$ fragment of rabbit anti-chicken immunoglobulin G conjugated to alkaline phosphatase (Jackson Immunoresearch), followed by the addition of developing substrates (2). The intensity of the protein A band was quantitated by densitometric software (SigmaGel; Jandel Scientific). The data are presented as densitometric units.

Isolation of RNA and Northern blot hybridization. Overnight cultures of *S. aureus* were diluted 1:50 in CYGP 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 FastRNA isolation kit (Bio 101, Vista, Calif.) in combination with 0.1-mm-diameter zirconia-silica beads in a FastPrep reciprocating shaker (Bio 101) as described earlier (6). Ten or twenty micrograms 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 the Turboblotter alkaline transfer system (Schleicher & Schuell, Keene, N.H.). For the detection of specific transcripts (*agr, sarA, sarS, spa*, and *hla*), gel-purified DNA probes were radiolabeled with [α-^{32}P]dCTP by the random-primed method (Ready-To-Go Labeling Kit; Pharmacia) and hybridized under high-stringency conditions (5). The blots were subsequently washed and autoradiographed.

Preparation of cell extracts for detection of SarA. Cell extracts were prepared for strains RN6390 and the corresponding *sarS* mutant. After pelleting, the cells were resuspended in 1 ml of TEG buffer (25 mM Tris, 5 mM EGTA; pH 8), and cell extracts were prepared from lysostaphin-treated cells as described earlier (11). Cell extracts were immunoblotted onto nitrocellulose membranes as described above. For the detection of SarA, monoclonal antibody 1D1 (1:2,500 dilution) was incubated with the immunoblot for 3 h, followed by another h of incubation with a 1:10,000 dilution of goat anti-mouse alkaline phosphatase conjugate (Jackson Immunoresearch). Reactive bands were detected by developing substrates as described previously (2).

Transcriptional fusion studies of *spa* promoter linked to the *xylE* reporter gene. A 158-bp (nt 17 to 174) (20) and a 491-bp *spa* promoter fragment (9) with flanking *Eco*RI and *Hin*dIII sites were amplified by PCR using genomic DNA of *S. aureus* RN6390 as the template and cloned into the TA cloning vector pCR2.1 (Invitrogen, San Diego, Calif.). The *Eco*RI-*Hin*dIII fragments containing the *spa* promoter were then cloned into shuttle plasmid pLC4 (31), generating transcriptional fusions to the *xylE* reporter gene. The orientation and authenticity of the promoter fragments were confirmed by restriction analysis and DNA sequencing. The recombinant plasmids were first introduced into *S. aureus* RN4220 by electroporation, according to the protocol of Schenk and Laddaga (28). Plasmids purified from RN4220 transformants were then electroporated into RN6390 and its isogenic *sarS* mutant.

For enzymatic assays of the *xylE* gene product, overnight cultures were diluted 1:50 or 1:100 in 250 ml of TSB containing appropriate antibiotics and shaken at 37°C and 200 rpm. Starting after 3 h of growth, 10 to 50 ml of cell culture corresponding to different OD_{600} values was serially removed, centrifuged, and washed twice with 1 ml of ice-cold 20 mM potassium phosphate buffer (pH 7.2). The pellets were resuspended in 500 μl of 100 mM potassium phosphate buffer (pH 8.0) containing 10% acetone and 25 μg of lysostaphin per ml, incubated for 15 min at 37°C, and then kept on ice for 5 min. Extracts were centrifuged at 20,000 × *g* for 50 min at 4°C to pellet cellular debris. The XylE (catechol 2,3-dioxygenase) assays were determined spectrophotometrically at 30°C in a total volume of 3 ml of 100 mM potassium phosphate buffer (pH 8.0) containing 100 μl of cell extract and 0.2 mM catechol as described earlier (31). The reactions were allowed to proceed for 25 min with an OD_{375} reading taken at the 25-min time point. One milliunit is equivalent to the formation of 1.0 nmol of 2-hydroxymuconic semialdehyde per min at 30°C. The specific activity is defined as a milliunit per milligram of cellular protein (31).

Overexpression and purification of SarS in a pET vector. The 750-bp *sarS* gene was amplified by PCR using the following oligonucleotides: 5′-GCCG(CTCGAG)ATGAAATATAATAACCA-3′ and 5′-GCACTTTA(GGATCC)AGCACAC-3′. The PCR product was digested with *Xho*I and *Bam*HI (restriction sites are indicated in parentheses), ligated into the expression vector pET14b (Novagen, Madison, Wis.), and transformed into the *E. coli* BL21(DE3).pLys.S. The resulting plasmid (pALC2043; see Table 1) contained the entire *sarS* coding region in frame with a N-terminal His tag. Recombinant protein expression was induced by adding ITPG (isopropyl-β-D-thiogalactopyranoside; final concentration, 1 mM) to a growing culture (30°C) at OD_{600} of 0.5. At 3 h after induction, the cells were harvested, resuspended in binding buffer (5 mM imidazole, 0.5 M NaCl, 20 mM Tris-HCl; pH 7.9), and sonicated on ice. Cellular debris was removed by centrifugation at 15,000 × *g* for 15 min, and the clarified supernatant was purified on a nickel affinity column (Novagen) according to the manufacturer's instructions. 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 the same buffer lacking the imidazole. The authenticity of the purified SarS protein was confirmed by N-terminal sequencing, and the size of the recombinant protein was verified by sodium dodecyl sulfate-gels stained with Coomassie blue.

Gel shift assays. To determine if the recombinant SarS protein binds to the *spa* promoter, DNA fragment (158 bp) was end labeled with [γ-^{32}P]ATP by using T4 polynucleotide kinase. Labeled fragments were incubated at room temperature for 15 min with the indicated amount of purified protein in 25 μl of binding buffer (25 mM Tris-HCl, pH 7.5; 0.1 mM EDTA; 75 mM NaCl; 1 mM dithiothreitol; 10% glycerol) containing 0.5 μg of calf thymus DNA. The reaction mixtures were analyzed by nondenaturing polyacrylamide gel electrophoresis. The band shifts were detected by exposing dried gels to film.

RESULTS

Identification of the *sarS* gene. Predicated upon the SarA protein sequence, we ran the BlastP program against the TIGR *S. aureus* genomic database. One of the matches is located upstream of the *spa* gene (contig 6207, with the coding region from nt 3098 to 2349). An identical gene, designated *sarS*, was also found in contig 773 at the University of Oklahoma genome database. This gene, preceded by a typical Shine-Dalgarno sequence (AGGAGA) located 7 bp upstream of the initiation codon, contains a 750-bp ORF encoding a 29.9-kDa protein with a deduced pI of 9.36. A putative transcription terminal signal corresponding to a 13-bp inverted repeats (nt 2345 to 2301 in contig 6207) is located 10 bp downstream of the TAA stop codon. About 33.2% of the residues are charged. Like that of SarA, the relatively small size, a predominance of charged residues, and a basic pI of SarS are features consistent with regulatory proteins in prokaryotes (29). An alignment of SarS with SarA revealed that SarS has two regions of identity with SarA, with the first region (residues 1 to 125) having 28.3% identity and the second region having 34.5% identity (Fig. 1). The extent of homology is relatively global in nature. A survey of the GenBank database indicated that SarS is identical to the SarHI homolog recently reported by Tegmark et al. (30). Interestingly, SarS is also homologous to SarR (22), a

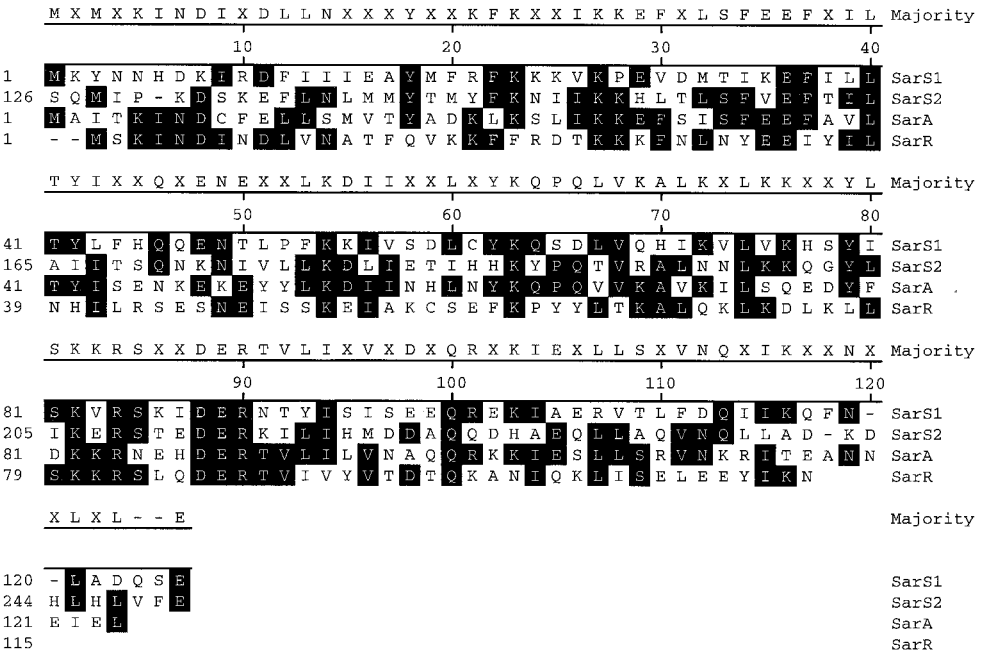

FIG. 1. Sequence alignment of SarS, SarA, and SarR. SarS is 250 residues long. Based on regional homology, SarS can be divided into two domains (S1 and S2) of 125 residues each. The homology with SarA is higher with the C-terminal domain (34.5%) than with the N-terminal domain (28.3%). The consensus residues are in black boxes. The consensus residue, designated as the majority at each position, is assigned when at least half of the residues have the same amino acid.

recently described SarA homolog that downregulates SarA protein expression.

Expression of *sarS* in RN6390 and its isogenic *sarA* and *agr* mutants. To assess the role of *sarS* within the *sarA*/*agr* regulatory cascade and its mode of control on virulence gene expression, we proceeded to construct a *sarS* mutant by inserting an *ermC* gene into the *Eco*RI site within the *sarS* gene in strain RN6390 (see Materials and Methods), thus resulting in a truncation of 91 residues from the C terminus. PCR with an *ermC* (5′-ATGGTCTATTTCAATGGCAGTTAC) primer and a *sarS* primer (5′-AGGCTTTGGATGAAGCCGTTAC) outside the construct yielded a fragment consistent with the insertion of *ermC* into the *sarS* gene. Subsequent sequencing of the PCR product has verified the disruption of the *sarS* gene in the mutant. This was also corroborated with Southern blots with selected *ermC* and *sarS* probes (data not shown).

A Northern blot with a *sarS* probe (nt 3098 to 2349 in contig 6207), encompassing only the *sarS* coding region, revealed that the *sarS* gene is poorly transcribed in the parental strain RN6390, thus rendering the absence of the *sarS* gene difficult to decipher in the *sarS* mutant. Interestingly, the transcription of the *sarS* gene, sizing at 930 nt, was more prominent in both *agr* and *sarA* mutants of RN6390 (Fig. 2A). In particular, in the *sarA* mutant, the *sarS* transcript was detected at late exponential phase (OD_{650} = 1.1 using an 18-mm borosilicate glass tube) and was maximally transcribed during the postexponential phase (OD_{650} = 1.7). The transcription of *sarS* was also increased in the *agr* mutant, but the magnitude of the increase was less than that of the *sarA* mutant (Fig. 2A). In contrast to the *sarA* mutant, the *agr* mutant expressed the *sarS* transcript maximally during the late exponential phase. To assess the relative contributions of *sarA* and *agr* to *sarS* repression, we assayed the *sarS* transcript level in an *agr* mutant complemented with a plasmid carrying the entire *sarA* locus (5), as well as in a *sarA* mutant complemented with a fragment encoding RNAIII, the *agr* regulatory molecule. Remarkably, the transcription of *sarS*, augmented in an *agr* mutant, was repressed in the *agr* mutant clone expressing *sarA* in *trans* (Fig. 2B). However, we were not able to detect transcriptional repression of *sarS* in a *sarA* mutant expressing RNAIII of *agr*, thus implying a differential role for *sarA* and *agr* in repressing *sarS* transcription.

We also examined the transcription of *sarA* and *agr* loci in the *sarS* mutant. Northern analysis of RNAII and RNAIII did not reveal any differences between the parental strain and the isogenic *sarS* mutant (data not shown). Likewise, the expression of three *sarA* transcripts (designated *sarA* P1, P3, and P2 transcripts) was similar between the two isogenic strains. Since SarA is encoded by these transcripts (1), we also probed for SarA expression in an immunoblot of the cell extracts of the isogenic pair (25 μg of protein in each lane) with 1D1 anti-SarA monoclonal antibody (10). Our data showed that the expression of SarA was comparable between RN6390 and its isogenic *sarS* mutant (data not shown). Collectively, these data implied that the transcription of *sarS* is repressed by the *sarA* and *agr* gene products and not vice versa.

Assessment of *hla* and *spa* in a *sarS* mutant of *S. aureus*. Cognizant of the fact that both α-hemolysin and protein A are regulated by *sarA* and *agr*, two regulatory loci capable of repressing *sarS* expression, we proceeded to evaluate *hla* and *spa* expression in the *sarS* mutant. In an immunoblot in which equivalent amounts of extracellular proteins were blotted onto nitrocellulose and probed with rabbit anti-α-hemolysin antibody (1:2,500 dilution), we found that α-hemolysin was syn-

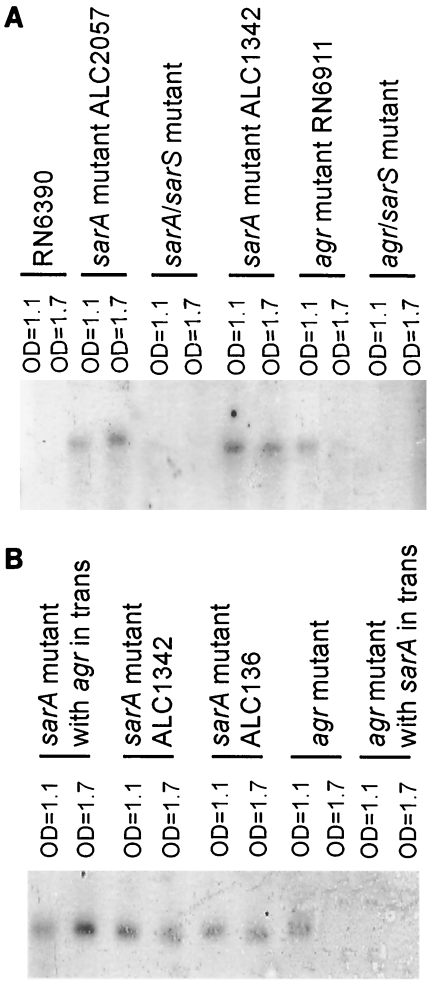

FIG. 2. (A) Northern blot of the *sarS* transcripts in *sarA, agr, sarA sarS*, and *agr sarS* mutants. A total of 20 μg of cellular RNA was loaded onto each lane. The intensity of the 16S and 23S RNA band was found to be equivalent among lanes prior to transfer to Hybond N^+ membrane. The blot was probed with a 750-bp *sarS* fragment (nt 2349 to 3098 in contig 6207) labeled with [α-^{32}P]dCTP, washed, and autoradiographed. Both *sarA* and *sarA sarS* mutants contained the *sarA*::*kan* mutation. The *sarA* deletion mutant ALC1342 in which the *sarA* gene has been replaced by an *ermC* gene was used as an additional control. (B) Northern blot of the *sarS* transcript in *sarA* mutant (ALC136) and *agr* mutant with *agr* and *sarA* provided in *trans*, respectively. The *sarA* mutant was complemented with pRN6735, yielding ALC184. The plasmid pRN6735 was basally transcribed, yielding a low level of RNAIII transcript (data not shown) even in the presence of a repressor plasmid pI524. The *agr* mutant RN6911 was complemented with pALC862, a recombinant pSK236 containing the entire *sarA* locus.

thesized in the *sarS* mutant at a level similar to that of the parental strain. Northern blotting with an *hla* probe also confirmed comparable levels of gene expression between the two strains (data not shown).

To ascertain the effect of a *sarS* mutation on *spa* expression, we first assayed for transcriptional activity of 491-bp (9) and 158-bp (20) *spa* promoter fragments linked to the *xylE* reporter gene in the isogenic *sarS* strains. Based on XylE assays, the activity of the 491-bp *spa* promoter fragment was lower in the *sarS* mutant (29.6 ± 0.06 and 24.0 ± 0.28 mU /mg of cellular proteins at OD_{650} values of 1.1 and 1.7, respectively) than in its isogenic parent (53.4 ± 0.06 and 74 ± 1.3 MU/mg of cellular proteins for OD_{650} values of 1.1 and 1.7, respectively). A similar expression pattern was also observed with the 158-bp *spa* promoter fragment, but the magnitude of the XylE activity was much less in both isogenic strains (data not shown). We next probed an immunoblot, containing equivalent amounts of cell wall protein extracts of the mutant and complemented mutant, with affinity-purified chicken anti-protein A antibody (1:3,000 dilution). As displayed in Fig. 3, the expression of protein A was higher in the parental strain than in the *sarS* mutant. However, upon complementation with a plasmid expressing the *sarS* gene, the expression of protein A was increased to near parental level. These data implicated *sarS* to be involved in the upregulation of *spa* expression in *S. aureus*.

Analysis of *spa* transcription in *agr, sarA, agr sarS*, and *sarA sarS* mutants. Since both *sarA* and *agr* repress *sarS* (see above) and *spa* transcription (7), we wanted to assess the relative contribution of *sarS*, as mediated by *sarA* and *agr*, in *spa* repression. For this purpose, we compared *spa* transcription of the *sarA sarS* and *agr sarS* double mutants to single *sarA* and *agr* mutants in the RN6390 background. In a previous study of the effect of *agr* and *sarA* on *spa* transcription (7), we chose strain RN6390 since this strain has a low basal level of *spa* transcription that can be accentuated by selective mutations. As shown in Fig. 4A, the transcription of *spa*, while enhanced in an *agr* mutant, was significantly reduced in the *agr sarS* double mutant, thus demonstrating that *agr* likely mediates *spa* repression by downregulating *sarS*. On the contrary, the upregulation in *spa* expression in a *sarA* mutant (*sarA*::*kan*) was maintained in the *sarS-sarA*::*kan* double mutant. As an additional control, the *sarA* deletion mutant ALC1342 also expressed a high level of *spa* transcription. A similar expression pattern was also observed in an immunoblot of cell wall protein A for these strains (Fig. 4B), demonstrating repression in pro-

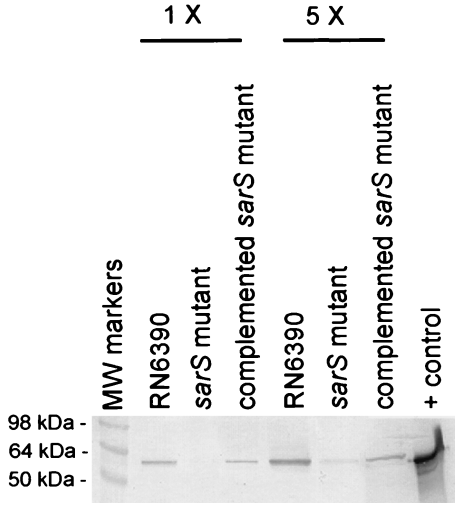

FIG. 3. Immunoblot of equivalent amounts of cell wall extracts of RN6390, *sarS* mutant, and complemented mutant. The blot was probed with affinity-purified chicken anti-protein A antibody at a 1:3,000 dilution, followed by the addition of the appropriate conjugate and substrate. The portion of the blot labeled "5X" represents five times as much cell wall extracts as the lanes labeled as "1X." Purified protein A (0.1 μg) was used as a positive control.

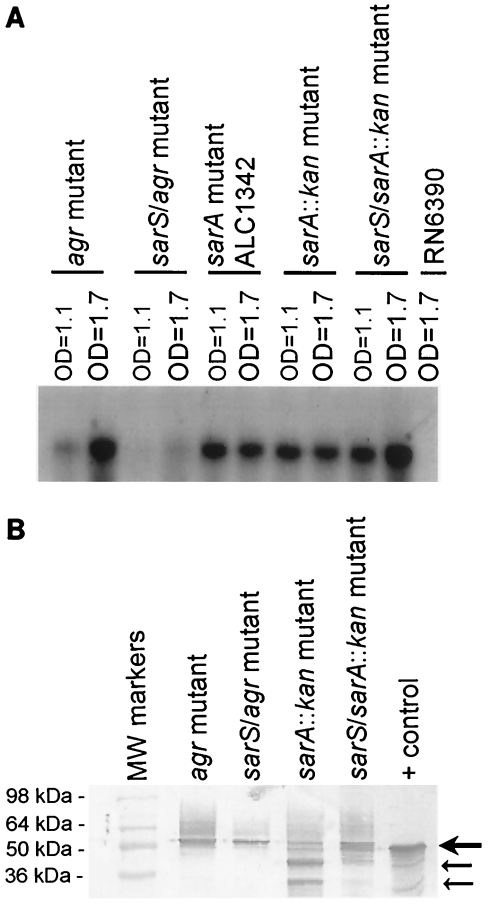

FIG. 4. (A) Northern blot of the *spa* transcript in *agr, sarA, sarS agr*, and *sarS sarA* mutants. The *sarA* and the *sarS sarA* mutants had the *sarA*::*kan* mutation. A total of 10 μg of RNA was applied to each lane. The blot was probed with a 1,562-bp *spa* fragment (nt 219 to 1780) (20). The parental strain RN6390 was a low protein A producer, with a very reduced level of *spa* transcription (7). The *sarA* deletion mutant ALC1342 served as a positive control. (B) Immunoblot of cell wall extracts of *agr, sarA, sarS agr*, and *sarS sarA* mutants probed with chicken anti-protein A antibody. Equivalent amount of cell wall extracts was applied to each lane. The positive control is purified protein A (0.1 μg). The big arrow points to intact protein A, while the two smaller arrows highlight degraded protein A fragments in the *sarA*::*kan* mutant (ALC2057).

tein A expression in the *agr sarS* mutant (175 densitometric units) compared with the single *agr* mutant (264 densitometric units). However, the contribution of *sarS* to the *sarA* mutant (i.e., the *sarA sarS* double mutant) was more difficult to decipher since there were two major protein A bands of lower molecular size in the *sarA* mutant (corresponding to densitometric units of 314 and 131 for the upper and lower bands, respectively) compared with the *sarS sarA* double mutant (346 densitometric units). The lower protein A bands may have been attributable to enhanced proteolytic activity in the *sarA* mutant, as has been previously reported (4, 8). Nevertheless, in comparing the intensity of the protein A band between the double *sarS sarA* mutant (346 densitometric U) and the *agr* mutant (264 densitometric U), we surmised that the expression of protein A in the double mutant was not significantly lower than in the single *sarA* mutant (two bands at 314 and 131 densitometric U). Unlike the single *sarA* mutant, the *sarS sarA* double mutant did not exhibit a protein A band of smaller molecular size. Whether *sarS* plays a role in modulating proteolytic activity in the *sarA* mutant remains to be determined. Nevertheless, these data collectively supported the notion that the *agr* locus, in distinction to the *sarA* locus, likely mediates *spa* repression via a *sarS*-dependent pathway.

To corroborate the view that *agr* and *sarA* mediate *spa* repression via different pathways, we determined *spa* transcription in an *agr* mutant complemented with a shuttle plasmid carrying the entire *sarA* locus in *trans*, as well as in a *sarA* mutant with a plasmid expressing RNAIII. Although this approach represented a higher gene dosage than at the physiologic level, the result of this experiment, coupled with those of *sarS* expression, provide additional evidence for the regulatory linkage between *agr* and *spa*, using SarS as an intermediary. Accordingly, the transcription of *spa* in an *agr* mutant with *sarA* provided in *trans* (Fig. 5), as with the expression of *sarS* (Fig. 2B), was repressed compared to the *agr* mutant control. In contrast, a *sarA* mutant with *agr* expressed in *trans*, while maintaining an elevated level of *sarS* transcription compared with the parent (Fig. 2B), was still able to repress *spa* transcription (Fig. 5). Taken together, our results clearly indicated that *sarA* and *agr* repress *spa* transcription via divergent pathways, with *agr* being dependent on *sarS*, while the effect of *sarA* on *spa* is *sarS* independent.

Gel shift assay of SarS with the *spa* promoter fragment. Recognizing that the *sarS* gene product may be an activator of protein A synthesis, we proceeded to evaluate the binding of the SarS protein to the *spa* promoter. For this experiment, we cloned the *sarS* gene into the pET14b expression vector (Novagen, Madison, Wis.) in *E. coli* BL21. SarS was then overexpressed in inducing conditions with 1 mM IPTG and purified with a nickel affinity column from the crude cell lysate according to the manufacturer's instructions. SarS, as eluted from the column, was essentially homogeneous (>95%) (Fig. 6A). Using purified SarS protein, we conducted gel shift assays of SarS with a 158-bp *spa* promoter fragment (nt 17 to 174) (20). As

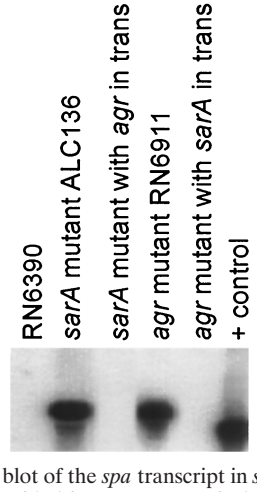

FIG. 5. Northern blot of the *spa* transcript in *sarA* and *agr* mutants, with *agr* and *sarA* provided in *trans*, respectively. The strains used in this blot are identical to those in Fig. 2B. The positive control is a plasmid (pCR2.1) carrying a 1,562-bp *spa* fragment (nt 219 to 1780) (20).

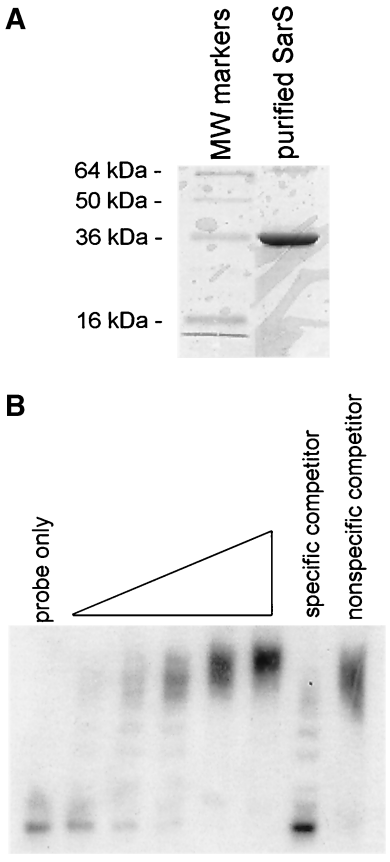

FIG. 6. (A) Sodium dodecyl sulfate-polyacrylamide gel electrophoresis of SarS purified from the pET14b expression vector. About 5 μg of purified SarS protein was applied to the lane. The gel was stained with Coomassie blue. (B) Gel shift assay of purified SarS with a 158-bp *spa* promoter fragment (nt 17 to 174) (20). The *spa* promoter fragment was end labeled with [γ-^{32}P]ATP. About 25,000 cpm was used in each lane. Increasing concentrations of purified SarS (0.125, 0.25, 0.5, 1, and 2 μg of SarS) were used in the lanes. The specific competitor was the cold unlabeled 158-bp *spa* promoter. The nonspecific competitor was a 161-bp *sarA* P3 promoter fragment (nt 365 to 525) (1).

displayed in Fig. 6B, SarS was able to retard the mobility of the *spa* promoter fragment in a dose-dependent fashion. Notably, the laddering pattern in the gel shift assay is consistent with either multimers of SarS binding to the *spa* promoter or multiple binding sites on the *spa* promoter or both. In competition assays with unlabeled *spa* promoter fragment, the gel retarding activity of SarS was abolished, thus demonstrating the specificity of the binding.

DISCUSSION

In prior studies with *sarA* and *agr* in *S. aureus*, it was observed that both of these regulatory loci play important roles in repressing *spa* transcription during the postexponential phase. Thus, the synthesis of protein A occurs primarily during the exponential phase and is repressed postexponentially. Predicated upon this observation, it seems reasonable to hypothesize that an activator of protein A synthesis likely exists within the staphylococcal genome. In searching the partially released genome for homologs of SarA, the major *sarA* regulatory molecule, we found an ORF encoding a SarA homolog (SarS) upstream of *spa*. In contrast to SarA (124 residues), the longer SarS protein (250 residues) can be divided into two SarA-homologous domains of 125 residues each. The C-terminal domain of SarS appears to share a high degree of similarity with SarA (34.5 identity versus 28.3% for the N-terminal domain). The SarS protein, like SarA, has a basic pI and a high percentage of charged residues (33.2 versus 33% for SarA), features consistent with DNA-binding proteins in prokaryotes. Indeed, gel shift studies of purified SarS with a 158-bp *spa* promoter fragment supported the notion that SarS is likely a DNA-binding protein, modulating the transcription of the *spa* gene.

In searching the literature, we found that SarS is identical to SarH1 recently reported by Tegmark et al. (30). Using a fragment encompassing only the 750-bp *sarS* coding region as a probe, we were only able to detect a single 930-nt *sarS* transcript as opposed to the three transcriptional units (1.0, 1.5, and 2.9 kb) described in those earlier studies. This transcript likely corresponds to the most prominent transcript reported by Tegmark et al. (30). It is not immediately apparent why such differences in transcription exist between our studies and theirs. We surmise that the hybridization (65°C) and washing (60°C with 0.1× SSC [1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate]) conditions in our Northern blot studies might be more stringent. It is also plausible that the larger but weaker bands in their study may be cross-hybridizing bands. Alternatively, the smaller 1.5-kb band may be a processed product from the larger 2.9-kb transcript. In any event, in the absence of additional primer extension data (for the 2.9-kb transcript) and transcriptional fusion studies of the putative promoters for the 1.5- and 2.9-kb transcripts, it is not certain if other promoters are part of the *sarS* operon.

Despite differences in *sarS* transcriptional patterns between the two studies, we confirmed that *sarS* is repressed by *sarA* and *agr*. However, the mode of *sarS* repression, as revealed by our data, differed between the two global regulatory loci. More specifically, inactivation of *sarA* yielded a higher level of *sarS* expression on Northern blots than that of the *agr* mutant (Fig. 2A). Additional Northern blot studies divulged that the elevated *sarS* level in an *agr* mutant can be repressed by a plasmid supplying *sarA* in *trans*. In contrast, the *sarS* level remained high in a *sarA* mutant even when a plasmid encoding RNAIII was present. More importantly, despite a dissimilarity in the *sarS* level, *spa* transcription was repressed in the *agr* mutant with *sarA* supplied in *trans* as well as in the *sarA* mutant expressing RNAIII from a plasmid source. Although the gene dosage (i.e., *sarA* or RNAIII) in these studies is not provided at the single-copy level, we contend that the expression of *sarA* or *agr* from multiple-copy plasmid would still permit us to decipher the putative interactive pathway, in particular in a situation where the putative gene (e.g., *sarS*) is expressed at such a low level that it may easily be missed by routine Northern blot analysis. Thus, a persistently high *sarS* level in the *sarA* mutant expressing RNAIII in *trans* (Fig. 2B), coupled with an effective repression of *spa* (Fig. 5), hinted at the differential roles in *spa* regulation between *sarA* and *agr*.

Several lines of experimental evidence suggested a role for *sarS* as an activator of protein A synthesis. First, in complementation studies of the *sarS* mutant, we showed by immuno-

blots that the diminution in protein A synthesis was restored by a shuttle plasmid carrying *sarS*. Second, we confirmed by transcriptional fusion studies of a *spa* promoter linked to the *xylE* reporter gene that *spa* promoter activity was indeed reduced in a *sarS* mutant. Third, gel shift studies have validated the notion that SarS can bind directly to the *spa* promoter in a dose-dependent fashion. Fourth, we extended our observation by Northern analyses that the upregulation in *spa* transcription in an *agr* mutant, presumably mediated by a derepression of *sarS*, was abolished in an *agr sarS* double mutant, thus implicating the role of *sarS* in activating *spa* transcription in an *agr* mutant. However, contrary to the data of Tegmark et al., we found that *spa* transcription in a *sarA sarS* double mutant, as with a single *sarA* mutant, remained elevated. Thus, despite the experimental observation that *sarS* is derepressed in a *sarA* mutant, the continued augmentation in *spa* transcription in a *sarA sarS* double mutant implied that the *sarA* locus likely represses protein A synthesis via a SarS-independent pathway. In this regard, our recent finding that SarA, the major *sarA* regulatory molecule, can directly bind to a consensus recognition sequence upstream of *spa* promoter to downregulate *spa* transcription would provide an explanation for an alternative mechanism for direct SarA-mediated *spa* repression (9). Alternatively, other intermediate factor(s) controlled by *sarA* or other factors that act in conjunction with SarS may play a role in *sarA*-mediated *spa* repression. It is also plausible that these "controlling factors" may be mediated via *agr*, since RNAIII supplied in *trans* in a significant gene dosage in a *sarA* mutant could also suppress *spa* transcription (Fig. 5). Nonetheless, we are left to offer an explanation for the high level of *sarS* expression in a *sarA* mutant. Perhaps, it may be reasonable to interpret the upregulation in *sarS* in terms of *hla* repression, since Tegmark et al. found that a *sarA sarH1* (i.e., *sarA sarS*) mutant, as opposed to a single *sarA* mutant, exhibited an upregulation in *hla* transcription.

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REFERENCES

1. **Bayer, M. G., J. H. Heinrichs, and A. L. Cheung.** 1996. The molecular architecture of the *sar* locus in *Staphylococcus aureus*. J. Bacteriol. **178:**4563–4570.
2. **Blake, M. S., K. H. Johnston, G. J. Russell-Jones, and E. C. Gotschlich.** 1984. A rapid sensitive method for detection of alkaline phosphatase-conjugated anti-antibody on Western blots. Anal. Biochem. **136:**175–179.
3. **Boyce, J. M.** 1997. Epidemiology and prevention of nosocomial infections, p. 309–329. *In* K. B. Crossley and G. L. Archer (ed.), The staphylococci in human disease. Churchill Livingstone, New York, N.Y.
4. **Chan, P. F., and S. J. Foster.** 1998. Role of SarA in virulence determinant production and environmental signal transduction in *Staphylococcus aureus*. J. Bacteriol. **180:**6232–6241.
5. **Cheung, A. L., M. G. Bayer, and J. H. Heinrichs.** 1997. *sar* genetic determinants necessary for transcription of RNAII and RNAIII in the *agr* locus of *Staphylococcus aureus*. J. Bacteriol. **179:**3963–3971.
6. **Cheung, A. L., K. Eberhardt, and V. A. Fischetti.** 1994. A method to isolate RNA from gram-positive bacteria and mycobacteria. Anal. Biochem. **222:**511–514.
7. **Cheung, A. L., K. Eberhardt, and J. H. Heinrichs.** 1997. Regulation of protein A synthesis by the *sar* and *agr* loci of *Staphylococcus aureus*. Infect. Immun. 2243–2249.
8. **Cheung, A. L., J. M. Koomey, C. A. Butler, S. J. Projan, and V. A. Fischetti.** 1992. Regulation of exoprotein expression in *Staphylococcus aureus* by a locus (*sar*) distinct from *agr*. Proc. Natl. Acad. Sci. USA **89:**6462–6466.
9. **Chien, C.-T., A. C. Manna, S. J. Projan, and A. L. Cheung.** 1999. SarA, a global regulator of virulence determinants in *Staphylococcus aureus*, binds to a conserved motif essential for *sar* dependent gene regulation. J. Biol. Chem. **274:**37169–37176.
10. **Chien, Y. T., A. C. Manna, and A. L. Cheung.** 1998. SarA level is a determinant of *agr* activation in *Staphylococcus aureus*. Mol. Microbiol. **31:**991–1001.
11. **Chien, Y., and A. L. Cheung.** 1998. Molecular interactions between two global regulators, *sar* and *agr*, in *Staphylococcus aureus*. J. Biol. Chem. **237:**2645–2652.
12. **Compagnone-Post, P., U. Malyankar, and S. A. Khan.** 1991. Role of host factors in the regulation of the enterotoxin B gene. J. Bacteriol. **173:**1827–1830.
13. **Giraudo, A. T., A. L. Cheung, and R. Nagel.** 1997. The *sae* locus of *Staphylococcus aureus* controls exoprotein synthesis at the transcriptional level. Arch. Microbiol. **168:**53–58.
14. **Giraudo, A. T., C. G. Raspanti, A. Calzolari, and R. Nagel.** 1996. Characterization of a Tn*551* mutant of *Staphylococcus aureus* defective in the production of several exoproteins. Can. J. Microbiol. **40:**677–681.
15. **Janzon, L., and S. Arvidson.** 1990. The role of the δ-hemolysin gene (*hld*) in the regulation of virulence genes by the accessory gene regulator (*agr*) in *Staphylococcus aureus*. EMBO J. **9:**1391–1399.
16. **Kornblum, J., B. Kreiswirth, S. J. Projan, H. Ross, and R. P. Novick.** 1990. *Agr*: a polycistronic locus regulating exoprotein synthesis in *Staphylococcus aureus*, p. 373–402. *In* R. P. Novick (ed.), Molecular biology of the staphylococci. VCH Publishers, New York, N.Y.
17. **Lee, C. Y.** 1992. Cloning of genes affecting capsule expression in *Staphylococcus aureus* strain M. Mol. Microbiol. **6:**1515–1522.
18. **Lin, W. S., T. Cunneen, and C. Y. Lee.** 1994. Sequence analysis and molecular characterization of genes required for the biosynthesis of type 1 capsular polysaccharide in *Staphylococcus aureus*. J. Bacteriol. **176:**7005–7016.
19. **Lina, G., S. Jarraud, G. Ji, T. Greenland, A. Pedraza, J. Etienne, R. P. Novick, and F. Vandenesch.** 1998. Transmembrane topology and histidine protein kinase activity of AgrC, the *agr* signal receptor in *Staphylococcus aureus*. Mol. Microbiol. **28:**655–662.
20. **Löfdahl, S., B. Guss, M. Uhlen, L. Philipson, and M. Lindberg.** 1983. Gene for staphylococcal protein A. Proc. Natl. Acad. Sci. USA **80:**697–701.
21. **Maniatis, T., E. F. Fritsch, and J. Sambrook.** 1989. Molecular cloning: a laboratory manual, 2nd ed. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
22. **Manna, A., and A. L. Cheung.** 2001. Characterization of *sarR*, a modulator of *sar* expression in *Staphylococcus aureus*. Infect. Immun. **69:**885–896.
23. **McNamara, P. J., K. C. Milligan-Monroe, S. Khalili, and R. A. Proctor.** 2000. Identification, cloning, and initial characterization of *rot*, a locus encoding a regulator of virulence factor expression in *Staphylococcus aureus*. J. Bacteriol. **182:**3197–3203.
24. **Novick, R. P., S. J. Projan, J. Kornblum, H. F. Ross, G. Ji, B. Kreiswirth, F. Vandenesch, and S. Moghazeh.** 1995. The *agr* P2 operon: an autocatalytic sensory transduction system in *Staphylococcus aureus*. Mol. Gen. Genet. **248:**446–458.
25. **Novick, R. P.** 1990. The staphylococcus as a molecular genetic system, p. 1–40. *In* R. P. Novick (ed.), Molecular biology of the staphylococci. VCH Publishers, New York, N.Y.
26. **Novick, R. P., H. F. Ross, S. J. Projan, J. Kornblum, B. Kreiswirth, and S. Moghazeh.** 1993. Synthesis of staphylococcal virulence factors is controlled by a regulatory RNA molecule. EMBO J. **12:**3967–3977.
27. **Projan, S. J., and R. P. Novick.** 1997. The molecular basis of pathogenicity, p. 55–81. *In* K. B. Crossley and G. L. Archer (ed.), The staphylococci in human disease. Churchill Livingstone, New York, N.Y.
28. **Schenk, S., and R. A. Laddaga.** 1992. Improved method for electroporation of *Staphylococcus aureus*. FEMS Microbiol. Lett. **94:**133–138.
29. **Smith, I.** 1993. Regulatory proteins that control late-growth development, p. 785–800. *In* A. L. Sonenshein, J. A. Hoch, and R. Losick (ed.), *Bacillus subtilis* and other gram-positive bacteria. ASM Press, Washington, D.C.
30. **Tegmark, K., A. Karlsson, and S. Arvidson.** 2000. Identification and characterization of SarH1, a new global regulator of virulence gene expression in *Staphylococcus aureus*. Mol. Microbiol. **37:**398–409.
31. **Zukowski, M. M., D. G. Gaffney, D. Speck, M. Kauffman, A. Findeli, A. Wisecup, and J. P. Lecocq.** 1983. Chromogenic identification of genetic regulatory signals in *Bacillus subtilis* based on expression of a cloned *Pseudomonas* gene. Proc. Natl. Acad. Sci. USA **80:**1101–1105.

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