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Characterization of the *sar* Locus and Its Interaction with *agr* in *Staphylococcus aureus*

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The expression of cell wall and extracellular proteins in *Staphylococcus aureus* is controlled by global regulatory systems, including *sar* and *agr*. We have previously shown that a transposon insertion into the 372-bp *sarA* gene within the *sar* locus resulted in decreased expression of several extracellular and cell wall proteins (A. L. Cheung and S. J. Projan, J. Bacteriol. 176:4168–4172, 1994). In this study, Northern (RNA blot) analysis with a 732-bp *sarA* probe indicated that two major transcripts (0.56 and 1.2 kb) were absent in the *sar* mutant compared with the parental strain RN6390. Additional transcriptional studies revealed that the *sarA* gene is encoded within the 0.56-kb transcript. Notably, a plasmid carrying the *sarA* gene together with a 1.2-kb upstream fragment (1.7 kb total) was able to reestablish the 1.2-kb transcript in the mutant. Although reconstitution of the parental phenotype by the *sarA* gene was incomplete, the introduction of a plasmid carrying the 1.7-kb fragment to the mutant restored the parental phenotype. Transcription of RNAII and RNAIII, which encode the structural and regulatory genes of *agr*, respectively, was diminished in the mutant but restored to wild-type levels by complementation with the 1.7-kb fragment. In gel shift assays, cell extracts of this clone were able to retard the mobility of a labeled RNAII promoter probe but not an RNAIII promoter element. These data suggest that *sarA* and the adjacent upstream DNA are essential to the expression of a DNA-binding protein(s) with specificity for the RNAII promoter, thereby controlling *agr*-related transcription.

The emergence of antibiotic-resistant strains of *Staphylococcus aureus* has raised concern because of the organism's ability to cause a wide range of diseases. *S. aureus* has the capacity to synthesize a large number of extracellular and cell wall-associated proteins, some of which are involved in pathogenesis (9, 23). Many of the exoproteins (e.g., α-hemolysin and toxic shock syndrome toxin) are secreted during the postexponential growth phase (9), while certain surface proteins (e.g., protein A and fibrinogen and fibronectin-binding proteins) are repressed postexponentially (9, 12). Temporal expression of many of the proteins involved in virulence has been shown to be under the control of global regulatory systems, in which a common regulator directs the expression of multiple genes.

In *S. aureus*, at least three global regulatory systems (*agr*, *xpr*, and *sar*) have been shown to play a role in the production of surface and extracellular proteins (6, 12, 21). Among these, the *agr* locus is the best characterized and has been shown to act at the transcriptional level to control the synthesis of both extracellular and cell wall proteins (12). Transposon-mediated inactivation of the *agr* locus leads to decreased production of exoproteins, while synthesis of some surface proteins is increased (19). The *agr* locus consists of at least five genes (*agrA, B, C,* and *D* and the δ-hemolysin gene). Analysis of the *agr* sequence revealed features suggestive of a two-component regulatory system. In particular, *agrC* may act as a signaling component, while *agrA* is the transcription activation element (18). The *agr* locus is composed of two divergent transcripts, designated RNAII and RNAIII, initiated from the P2 and P3 promoters, respectively. RNAIII, which encodes the δ-hemolysin gene, has been proposed to be directly responsible for *agr*-mediated regulation (18).

We previously described (6) a second regulatory locus, *sar*, which is also involved in the expression of extracellular and cell wall proteins. One of the genes (*sarA*) within the *sar* locus has recently been cloned and contains an open reading frame of 372 bp (7). Transcriptional studies indicated that the *sarA* gene is necessary for the optimal transcription of RNAIII (7), suggesting that *sar* is essential for *agr*-dependent regulation. However, complementation of *sar* mutants with a 732-bp DNA fragment encoding the *sarA* gene resulted in only partial restoration of the parental phenotype.

We report here the cloning of a DNA fragment located upstream of *sarA* that, when combined with *sarA*, restored the production of exoproteins by a *sar* mutant to parental levels. More important, cell extracts of this complemented mutant were found to bind to the P2 promoter region of the *agr* locus, which controls RNAII transcription. As RNAII promotes the transcription of the *agr* regulatory molecule (i.e., RNAIII), these data suggest that the *sar* locus may control exoprotein synthesis by binding to the *agr* P2 promoter region, thereby regulating the *agr*-mediated pathway of exoprotein production.

MATERIALS AND METHODS

Media and antibiotics. CYGP and 0.3GL media (17) were used for the growth of *S. aureus*, while Luria-Bertani broth (LB) was used for growing *Escherichia coli*. Antibiotics were used at the following concentrations: erythromycin, 10 μg/ml; tetracycline, 5 μg/ml; and ampicillin, 50 μg/ml.

Bacteria, plasmids, and phage. The bacterial strains and plasmids used in this study are listed in Table 1. Phage φ11 was used as the transducing phage for *S. aureus* strains.

Cloning strategies. DNA fragments containing regions of the *sar* locus were amplified by PCR with genomic DNA of *S. aureus* RN6390 as the template and cloned into the TA cloning vector pCRII (Invitrogen, San Diego, Calif.). The authenticity of the amplified fragments was confirmed by sequencing with ^{35}S sequencing mix and Sequenase (US Biochemicals, Cleveland, Ohio) (20). The PCR fragments were cleaved from pCRII, ligated to the shuttle vector pSPT181, and transformed into *E. coli* XL1-Blue. The presence of the correct inserts in the plasmids was confirmed by restriction mapping.

Transformation of *S. aureus*. Protoplast transformation of *S. aureus* RN4220 was performed as previously described (2). Transformants were selected at 32°C on DM3 agar containing tetracycline.

Transduction. Phage φ11 was used to produce a phage lysate of strain RN4220

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TABLE 1. Bacterial strains and plasmids

Strain or plasmid	Reference or source	Comments
S. aureus		
RN4220	16	Mutant of strain 8325-4 that accepts foreign DNA
RN6390	16	Laboratory strain that maintains its hemolytic pattern when propagated on sheep erythrocytes
ALC136	7	Isogenic mutant of RN6390 carrying a *sar*::Tn*917*LTV1 mutation
ALC70	This work	ALC136 with pALC70
ALC103	This work	ALC136 with pALC103
ALC352	This work	ALC136 with pALC352
ALC406	This work	ALC136 with pALC406
ALC409	This work	ALC136 with pALC409
E. coli		
XL1-Blue	15	Highly transformable strain
InvαF′	Invitrogen	Host strain for the TA cloning vector (pCRII)
Plasmids		
pCRII	Invitrogen	*E. coli* cloning vector for direct cloning of PCR fragments
pSPT181	11	Shuttle vector
pALC70	This work	pSPT181 with *sar* 266–68
pALC103	This work	pSPT181 with *sar* 67–68
pALC352	This work	pSPT181 with *sar* 280–67
pALC406	This work	pSPT181 with *sar* 266–331
pALC409	This work	pSPT181 with *sar* 280–353

containing the plasmid pSPT181 with the amplified *sar* DNA. The phage lysate was then used to infect the *sar* transposon mutant ALC136 (previously designated mutant R) at a low multiplicity of infection (6, 8). Transductants were selected at 32°C on 0.3GL agar with tetracycline.

Isolation of RNA and Northern (RNA blot) analysis. To isolate RNA from *S. aureus* cells, an overnight culture of *S. aureus* cells was diluted 1:100 in 10 ml of fresh CYGP broth with the appropriate antibiotics and grown at 33.5°C. This temperature was chosen to accommodate the temperature-sensitive replicon of the shuttle plasmid. Previous studies indicated that the production of hemolysins and *agr*-related transcripts at this temperature did not differ significantly from that at 37°C. Cells were pelleted (2,500 × *g* for 10 min at 4°C), and total cellular RNA was isolated with the FastPrep system (Bio 101, Vista, Calif.) as previously described (3). *E. coli* RNA was similarly isolated from cells grown overnight at 37°C in LB containing ampicillin. Ten micrograms of RNA was electrophoresed through a 1.5% agarose–0.66 M formaldehyde gel in MOPS running buffer (20 mM MOPS [morpholinepropaneethanesulfonic acid], 10 mM sodium acetate, 2 mM EDTA [pH 7.0]). RNA was transferred onto a Hybond N membrane (Amersham, Arlington Heights, Ill.) under mild alkaline conditions by using a Turboblotter system (Schleicher and Schuell, Keene, N.H.) as described by the manufacturer. RNA was fixed to the membrane by baking (80°C for 1 h), hybridized under aqueous conditions at 65°C with gel-purified DNA fragments that had been labeled with ^{32}P (15), washed, and autoradiographed as described before (8). Band intensities were quantitated by densitometric scanning with SigmaGel software (Jandel Scientific, San Rafael, Calif.); these values are presented as integrated area units.

Phenotypic characterization. Strains were tested in duplicate for the production of hemolysins on plain and cross-streaked sheep and rabbit erythrocyte agar, using specific indicator strains as standards as previously described (18). The production of lipase was assayed by measuring zones of clearing on Tween 20 agar (6) and confirmed by streaking colonies on egg yolk agar (13).

To determine levels of protein A production, cell wall-associated proteins were extracted from overnight cultures of *S. aureus* with lysostaphin in a hypertonic medium (30% raffinose) as previously described (5). Equivalent volumes (1 μl each) of extracts of cell wall proteins were separated on sodium dodecyl sulfate–10% polyacrylamide gels. Separated proteins were electrophoretically blotted to 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 rabbit anti-chicken immunoglobulin G (IgG) conjugated to alkaline phosphatase (Jackson Immunoresearch, West Grove, Pa.) (1:5,000 dilution) and visualized as described by Blake et al. (1).

The ability of whole cells to bind to ^{125}I-labeled fibronectin was assayed as described by Fröman et al. (10). The Student *t* test was used to compare the binding of radiolabeled fibronectin to different clones. *P* values of ≤0.05 were considered significant.

Production of cell extracts and gel shift analysis. Cell extracts were prepared from strain RN6390 and the isogenic *sar* transposon mutant ALC136 as well as the mutant complemented with plasmid pSPT181 carrying various PCR-generated *sar* fragments. Cells were grown overnight in 200 ml of CYGP broth supplemented with the appropriate antibiotics. After pelleting, the cells were resuspended in 1 ml of TEG buffer (25 mM Tris, 5 mM EGTA [ethylene glycol tetraacetic acid] [pH 8]), and cell extracts were prepared from lysostaphin-treated cells as described by Mahmood and Khan (14). We followed a similar procedure to harvest *E. coli* cell extracts except that the cells were grown overnight in LB containing ampicillin and the cell pellet was resuspended in 1 ml of buffer containing 100 mM Tris (pH 8.0), 5 mM EDTA, and 2 mg of lysozyme (Boehringer Mannheim Biochemicals, Indianapolis, Ind.) per ml.

For the gel shift assay, various volumes of cell extracts were added to reaction mixtures containing 10 mM Tris HCl (pH 7.5) with EDTA (1 mM), dithiothreitol (1 mM), NaCl (50 mM), glycerol (5%), and 1 μg of poly(dI-dC) to a final volume of 25 μl. Approximately 1×10^4 to 2×10^4 cpm of the ^{32}P-end-labeled P2 (nucleotides [nt] 1603 to 1773) or P3 (nt 1539 to 1660 in the published sequence [12]) promoter probe was then added. In some assays, unlabeled P2 promoter DNA was used as a specific competitor, while a 210-bp fragment upstream of the *sar* locus or a 163-bp PCR fragment of the α-hemolysin gene was used as a nonspecific competitor. The reaction mixtures were incubated at room temperature for 5 min and then on ice for an additional 5 min and electrophoresed on a 6% polyacrylamide gel in 0.25× TBE (Tris-borate-EDTA) for 2 h at 200 V. Following electrophoresis, the gels were dried and exposed to film.

RESULTS

Molecular cloning of the *sar* locus. In a previous study, we defined the original *sar* mutation as a Tn*917*LTV1 insertion 5 bp downstream from the translation start site of the *sarA* gene (7). However, attempts to complement this mutation with a plasmid carrying the *sarA* gene alone partially restored the defect in exoprotein production (7) but did not correct the alteration in the expression of cell wall proteins (e.g., fibronectin-binding protein). To evaluate the contributory role of genes adjacent to *sarA* on extracellular and cell wall protein production, DNA fragments encompassing the region of the *sar* locus were amplified by PCR (see Fig. 1) and cloned initially into the vector pCRII and then into the shuttle vector pSPT181. These shuttle plasmids were transformed into strain RN4220 by protoplast transformation, and the plasmids were then transduced into the *sar* mutant by using phage ϕ11. Five clones containing plasmid pSPT181, carrying different DNA fragments that encompass the *sar* region (Fig. 1), were constructed to evaluate their abilities to restore the parental phenotype to the transposon-mediated *sar* mutant ALC136.

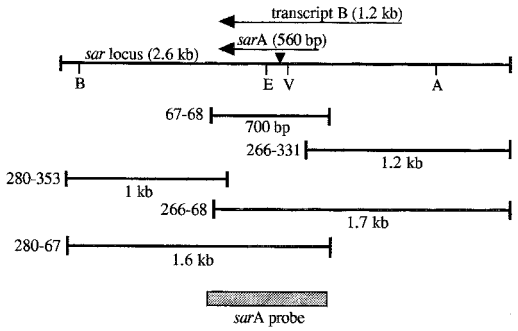

FIG. 1. Construction of clones containing PCR fragments encompassing the *sar* locus. Restriction sites: B, *Bam*HI; E, *Eco*RI; V, *Eco*RV; A, *Acc*I. The transposon insertion site is represented by a triangle. *sar* mutant strains containing these fragments are designated as follows: fragment 67-68, ALC103; fragment 266-331, ALC406; fragment 280-353, ALC409; fragment 266-68, ALC70; and fragment 280-67, ALC352.

Northern analysis of *sar* transcription. To detect the presence of *sar*-related transcripts, we probed a Northern blot of strain RN6390 and its isogenic *sar* mutant ALC136 with a radiolabeled 732-bp *sarA* probe. This 732-bp fragment encompassed the *sarA* gene together with its putative promoter (unpublished observation). More important, it contained no other open reading frames except that of the *sarA* gene. Our results revealed that the transposon insertion near the translation start site of the *sarA* gene abolished two major transcripts, designated *sarA* (0.56 kb) and transcript B (1.2 kb) (Fig. 1 and 2). A shuttle plasmid carrying a cloned 732-bp PCR fragment (clone ALC103), upon introduction into *sar* mutant ALC136, was capable of reestablishing the 560-bp transcript, as detected on a Northern blot probed with the 732-bp *sarA* probe (Fig. 2). However, a clone carrying a plasmid that contained the *sarA* gene together with an adjacent 1.2-kb upstream fragment (ALC70) restored both *sar* transcripts to *sar* mutant ALC136 (Fig. 2). Notably, a ^{32}P-labeled PCR fragment that comprises the 1.2-kb upstream fragment alone (see clone ALC406 in Fig. 1) hybridized only with transcript B in parental strain RN6390 as well as in ALC70 (data not shown). In contrast, a PCR fragment located downstream from the *sarA* gene (see clone ALC409) did not hybridize with either transcript.

Phenotypic characterization. The phenotypic alterations in the *sar* mutant resulting from the introduction of plasmids carrying various PCR fragments of the *sar* region are shown in Table 2. Levels of α and δ hemolysin production, which were reduced in the *sar* mutant ALC136, were restored to nearly parental levels in clones ALC103 and ALC70 but not in ALC406 or ALC409. Interestingly, the production of lipase, which was increased in ALC136, did not return to parental levels except in clones containing the DNA fragment that includes transcript B (ALC70).

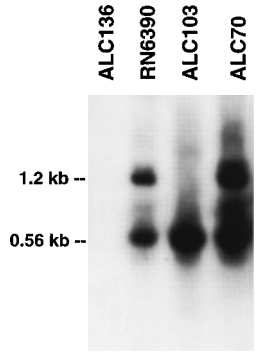

FIG. 2. Northern blot analysis of *sar* transcripts. Ten micrograms of RNA was applied to each lane and probed with a ^{32}P-labeled 732-bp *sarA* fragment. Two major transcripts, designated *sarA* (0.56 kb) and transcript B (1.2 kb), were detected in the parental strain RN6390.

TABLE 2. Phenotypic characterization of *sar* clones

Strain	Hemolysin production[a]		Lipase production[a]	Protein A[b] (integrated area units)	Fibronectin-binding protein[c] (mean cpm of ^{125}I/10^9 CFU ± SEM)
	α	δ			
RN6390	+	±	+	220	28,120 ± 3,766
ALC136	−	−	++	3,719	15,670 ± 1,476
ALC103	+	±	++	250	21,370 ± 4,818
ALC70	+	±	+	403	91,310 ± 12,757
ALC406	−	−	++	3,924	19,800 ± 4,069
ALC409	−	−	++	4,439	23,060 ± 667

[a] Assayed by measuring the zones of hemolysis on cross-streaked rabbit erythrocyte agar plates (hemolysis) or zones of clearing on Tween 20 agar (lipase). ++, strong producer; +, moderate producer; ±, very weak producer; −, nonproducer.

[b] The presence of protein A in cell wall extracts was assayed on immunoblots with affinity-purified chicken anti-protein A antibody. Band intensities were quantitated densitometrically and are presented as integrated area units. Similar results were obtained when we assayed for relative extracellular protein A levels among various clones.

[c] Data presented as ^{125}I-fibronectin bound to 10^9 CFU. ALC136 bound less radiolabeled fibronectin than the parent ($P \leq 0.02$). However, ALC70 bound significantly more fibronectin than *sar* mutant ALC136 alone ($P \leq 0.03$).

Immunoblots were performed to determine the effect of the *sar* locus on cell wall protein A production (Table 2). A low level of protein A was produced by the parental strain (RN6390). However, a mutation in the *sar* locus (ALC136) caused a significant upregulation in protein A expression. Protein A production returned to parental levels in clones containing plasmids carrying either *sarA* alone (ALC103) or *sarA* together with the upstream fragment (ALC70). Similar results were obtained when we assayed for the relative extracellular protein A levels in the culture supernatants. Additional studies indicated that the effect of *sar* on protein A production occurred at the transcriptional level (unpublished data).

The binding of whole cells to ^{125}I-labeled fibronectin was slightly diminished in *sar* mutant ALC136 compared with parental strain RN6390. However, complementation of a *sar* mutant with a plasmid carrying a DNA fragment encompassing both *sar*-related transcripts (0.56 and 1.2 kb) (ALC70) produced a dramatic increase in fibronectin binding (Table 2). In contrast, the transposon mutant complemented with *sarA* alone (ALC103) or other fragments did not exhibit a similar increase in fibronectin-binding capacity.

Northern blot analysis of RNAII and RNAIII in *sar*-containing clones. To determine the effects of the *sar* locus on the production of RNAII (encoding *agrA, B, C,* and *D*) and RNAIII (the *agr* regulatory molecule), Northern blot analysis was performed on RNA extracted from mutants containing various *sar* DNA fragments. Using an *agrA* probe to detect RNAII transcription, we demonstrated (Fig. 3B) that the RNAII level was greatly reduced in the *sar* mutant ALC136 in comparison to the parent (band intensities of 3,029 and 8,331 integrated area units for ALC136 and RN6390, respectively). Although clone ALC103, containing *sarA* alone, was able to increase the RNAII mRNA level in the *sar* mutant (intensity, 6,189 units), the addition of an upstream DNA fragment

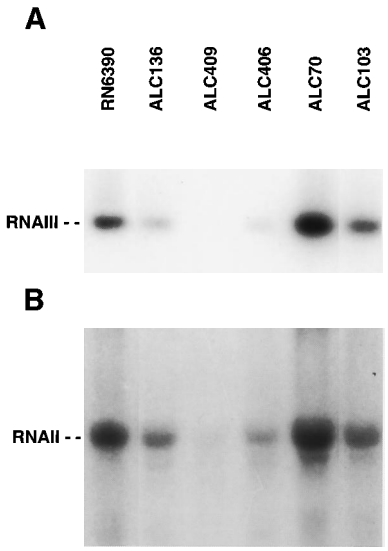

FIG. 3. (A) Northern analysis of RNAIII transcripts in *sar* clones. Ten micrograms of RNA was applied to each lane. The probe was a ^{32}P-labeled 512-bp fragment encoding RNAIII (nt 999 to 1510 [12]). (B) Northern blot analysis of RNAII transcripts in *sar* clones. Ten micrograms of RNA isolated from clones at late log phase was applied to each lane and probed with a ^{32}P-labeled 513-bp *agrA* probe (nt 3830 to 4342 [12]). The parental strain RN6390 served as a positive control.

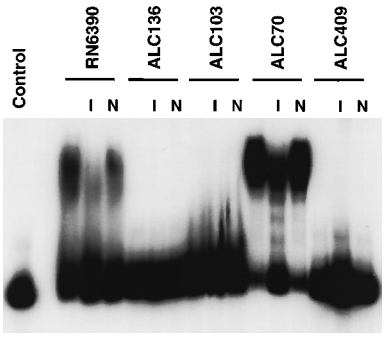

FIG. 4. Effect of cell extracts of *sar* clones on the mobility of a P2 promoter fragment. The P2 promoter region (positions 1603 to 1773 [12]) was amplified by PCR and end labeled with ^{32}P. The cell extract from ALC103 was concentrated approximately 10-fold prior to testing. Eight microliters of extracts was assayed except for ALC103 (15 μl) and ALC70 (5 μl). Control, P2 fragment without cell extract; I, specific inhibitor (≈100 ng of unlabeled P2 fragment); N, nonspecific inhibitor (≈100 ng of a 210-bp PCR fragment upstream from the region encoding transcript B). Extract from parental strain RN6390 was used as a positive control.

(ALC70) resulted in a level of RNAII (intensity, 12,800 units) that was higher than that of the parent. The *sar* mutant complemented with a plasmid containing DNA fragments located either upstream (ALC406) or downstream (ALC409) of *sarA* but not containing the *sarA* gene itself produced very low levels of RNAII.

We also examined the effect of these *sar* fragments on RNAIII, the putative *agr* regulatory molecule. Similar to RNAII, the RNAIII level was greatly diminished in the *sar* mutant ALC136 (band intensities of 999 and 6,867 units for the mutant and parental strains, respectively) but was restored by the introduction of a plasmid containing the *sarA* gene (ALC103; intensity, 6,976 units) (Fig. 3A). However, additional augmentation in the RNAIII level was demonstrated in the clone containing *sarA* plus the upstream 1.2-kb DNA fragment (ALC70; intensity, 16,750 units). The strains containing fragments either upstream (ALC406) or downstream (ALC409) of *sarA* produced very low levels of RNAIII, similar to that seen in *sar* mutant ALC136.

Gel mobility of the P2 promoter region. The P2 promoter in the *agr* operon initiates the transcription of RNAII, which in turn activates RNAIII transcription from the P3 promoter. The effect of the *sar* locus on RNAII and RNAIII transcription suggested that the gene product(s) of *sar* may interact with one of these promoters to regulate the *agr* response. To analyze this interaction, cell extracts prepared from *S. aureus* clones with plasmids containing various *sar* fragments were evaluated for their ability to retard the mobility of radiolabeled P2 and P3 PCR fragments in gel shift assays (Fig. 4). Retardation of the 171-bp P2 promoter probe (nt 1603 to 1773 according to the published sequence [12]) occurred with cell extract from parental strain RN6390 but not from *sar* mutant ALC136. Notably, the extract derived from the *sar* mutant carrying a plasmid with the *sarA* gene and a 1.2-kb upstream fragment (ALC70) was able to shift the mobility of the 171-bp P2 promoter fragment. The effect of this cell extract on the mobility of the P2 promoter fragment appeared to be dose dependent (data not shown). The interaction between this cell extract and the P2 promoter fragment was highly specific, as it could be effectively competed away with an unlabeled P2 fragment but not with a nonspecific competitor, such as a 210-bp PCR fragment located upstream of the *sar* locus. Addition of RNases to the cell extract of ALC70 did not change the extract's ability to impede the mobility of the P2 promoter. Furthermore, a mixture of cell extracts from individual clones complemented with plasmids containing *sarA* (ALC103) and upstream sequence (ALC406) did not alter the mobility of the P2 promoter fragment. The *sar* mutant transformed with a plasmid containing only the downstream region (ALC409) (data not shown) or *sarA* plus the downstream region (ALC352) did not change the mobility of the P2 fragment at any concentration tested. Surprisingly, none of the *sar* clones (including ALC70) altered the mobility of a 122-bp P3 promoter fragment (nt 1539 to 1660 [12]) (results not shown).

To examine the effect of the *sar* gene products in a nonstaphylococcal environment, cell extracts were produced from *E. coli* carrying plasmids with the same PCR fragments that were introduced into *S. aureus*. Only cell extracts from a clone

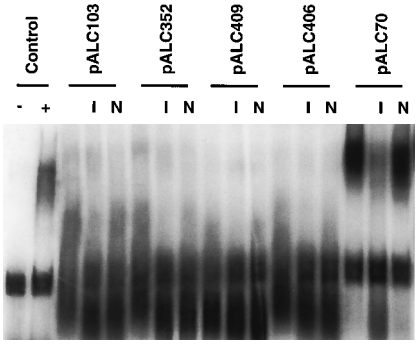

FIG. 5. Effect of cell extracts from *E. coli* clones on the mobility of a P2 promoter fragment. The P2 promoter fragment was end labeled with ^{32}P and added to 8 μl of cell extracts prepared from *E. coli* clones. Controls: −, P2 promoter without cell extract; +, P2 promoter with extract from *S. aureus* ALC70. I, specific inhibitor (≈100 ng of unlabeled P2 DNA); N, nonspecific inhibitor (≈100 ng of a 163-bp PCR fragment of the *S. aureus* α-hemolysin structural gene).

containing the *sarA* gene and the upstream 1.2-kb DNA fragment (ALC70) were able to affect the mobility of the P2 promoter fragment in a gel shift assay (Fig. 5).

DISCUSSION

From Northern blot analysis of strain RN6390 and its isogenic *sar* mutant with a 732-bp *sarA* probe, we determined that a transposon insertion within the *sarA* gene abolished two major transcripts in the *sar* locus. The smaller 0.56-kb transcript is apparently encoded by the *sarA* gene (7), but the function of the larger 1.2-kb transcript (transcript B) has not previously been defined. Although complementation of the mutant with a plasmid containing *sarA* alone (ALC103) reconstituted the 0.56-kb transcript, it did not reestablish the larger 1.2-kb transcript, nor was this clone able to completely restore the parental phenotype to the mutant (e.g., cell wall proteins). Remarkably, clones complemented with a plasmid containing the *sarA* gene together with a 1.2-kb upstream fragment (ALC70) were able to restore both *sar* transcripts (Fig. 2). Ongoing hybridization studies with selected PCR probes (≈200 bp each) as well as primer extension data involving the *sar* locus revealed that both *sar* transcripts are likely to overlap and are probably transcribed in the same orientation (Fig. 1) (unpublished data).

In contrast to clones containing *sarA* alone, a *sar* mutant clone complemented with a plasmid encompassing both *sar* transcript sequences (ALC70) was capable of restoring levels of α and δ hemolysins, lipase, and protein A and fibronectin-binding capacity to near parental levels. Thus, both *sar* transcriptional elements are necessary for the normal expression of *sar*-related phenotypes in both extracellular and cell wall proteins in *S. aureus.*

In analyzing the phenotypic profiles among *sar* clones derived from ALC136, it is evident that the *sar* genetic requirement for synthesis of integral cell wall protein (e.g., fibronectin-binding protein) differs from that of exoproteins (4). For instance, *sarA* without an intact transcript B is capable of restoring hemolysin production in the mutant (ALC103) to nearly parental levels. Like the *agr* locus, the *sarA* gene alone suppresses the synthesis of cell surface molecules such as protein A, which also has an extracellular component (Table 2). In contrast, the augmentation of fibronectin-binding activity occurs only in the mutant complemented with both *sarA* and the 1.2-kb transcriptional elements (ALC70). This observation suggests that the production of integral surface protein (e.g., fibronectin-binding protein) may be controlled by a distinct mode of regulation with different genetic requirements from that of protein A. A comparison of these modes of regulation with those of *agr* is of interest. For cell wall protein A, it appears that both the *agr* and *sar* gene products behave as repressors (12, 19). In the case of an integral cell wall protein such as fibronectin-binding protein, however, it is likely that the *agr* and *sar* loci act diametrically as the repressor and activator, respectively (4).

In previous studies, we demonstrated that the production of exoproteins and RNAIII (the *agr* regulatory molecule) is related to the presence of an intact *sarA* gene (7). However, restoration of RNAIII transcription in *sar* mutants complemented with a plasmid carrying the *sarA* gene was incomplete in some of the strains we studied (7). Interestingly, a *sar* mutant complemented with a DNA fragment that encompassed both the *sarA* and the larger transcriptional elements was found to exhibit a higher level of RNAIII than the parent. As RNAIII transcription is activated by RNAII (12), we also confirmed that the RNAII transcript level was reduced in the *sar* mutant but was partially restored in a mutant clone complemented with *sarA* (Fig. 3B). By introducing both *sar* transcriptional elements into the *sar* mutant, the level of RNAII was fully restored. Hence, our data suggest that both *sar* transcriptional elements are required for optimal *agr* interaction in exoprotein synthesis.

In addition to *sar*-mediated control of RNAII and RNAIII transcription, two other lines of evidence also indicate that *sar* is likely to be a regulator of *agr*, especially in the control of exoprotein synthesis (7). In particular, hemolysin production, which is diminished in *sar* mutants, can be restored to almost parental levels by supplying a plasmid (pRN6735) which carries RNAIII under a controllable β-lactamase promoter (7). In addition, Northern analysis of the mRNA levels of exoprotein genes in *sar* mutants revealed that the *sar* locus probably acts in *trans* at the transcriptional level during the late log to post-exponential phase (8). This mode of regulatory control in exoprotein synthesis is similar to that of *agr*. However, the exact mechanism by which *sar* controls *agr* in exoprotein synthesis has not been previously established.

In this study, we provide evidence from gel shift analysis that the *sar* gene product(s) may activate RNAII transcription by binding to the P2 promoter region. More specifically, cell extracts from *sar* mutant clones carrying plasmids containing both the *sarA* gene and the 1.2-kb upstream sequence (ALC70) were able to retard the mobility of a ^{32}P-labeled DNA fragment containing the P2 promoter region. This interaction is highly specific, as this cell extract did not bind to the P3 promoter contiguous to the P2 promoter region. Additionally, cell extracts from mutants complemented with *sarA* (ALC103) and the 1.2-kb upstream fragment (ALC406) either alone or in combination did not alter the mobility of the P2 promoter fragment. From these data, it is likely that the *sarA* gene product, probably in combination with that of transcript B, is required for optimal binding to the P2 promoter region. Because the introduction of the *sar* locus on a plasmid may cause an artificially high concentration of the gene product(s) within the cell, we are currently investigating this effect by cloning the gene in an integration plasmid for introduction into the chromosome of the mutant.

The fact that cell extracts of an *E. coli* clone containing a DNA fragment that comprises both *sarA* and the upstream region can bind to the P2 promoter and retard its mobility indicates that both *sar* transcripts or their protein products are solely responsible for this DNA interaction. This finding also implies that the *sar* transcripts are operational in an *E. coli* background and are not activating another *S. aureus* protein(s), which then interacts with the P2 promoter. Preliminary sequence analysis of the region encompassing transcript B revealed the *sarA* gene and several smaller open reading frames located upstream. The exact role of these open reading frames within the region encoding transcript B in *agr* regulation is not clear. However, we recognize that the cloned 1.7-kb fragment is larger than transcript B (1.2 kb), and the region upstream of transcript B (0.5 kb) may conceivably be involved in *agr* interaction. Nevertheless, preliminary analysis of this region did not reveal any potential open reading frames. Additional deletion studies will be necessary to rule out the contribution of this 0.5-kb region to *agr* interaction.

Independent investigations by Janzon and Arvidson (11) and Novick and coworkers (18) have shown that the synthesis of exoproteins is controlled by the RNAIII molecule itself rather than any translated products, because a nonsense mutation in the 5′ end of the *hld* gene contained within the RNAIII transcript abolished δ hemolysin production but not the Agr$^+$ phenotype. Whether this unique feature of the *agr* regulatory

system exists in the *sar* locus is not known. However, preliminary mutational analysis within the *sarA* gene indicated that the *sarA* protein itself is required for *agr*-mediated exoprotein control. Regardless of the type of *sar* molecule involved, the *sar* regulation of exoprotein synthesis is mediated by an interaction between a *sar*-encoded molecule(s) and the *agr* P2 promoter, thereby activating P2 transcription. The RNAII transcript, in turn, promotes the transcription of RNAIII, which is ultimately responsible for *agr*-mediated control of exoprotein synthesis (18). Nevertheless, this regulation of *agr* by *sar* occurs primarily during the mid- and late exponential phases and may differ from the *agr*-independent signal that is required for exoprotein gene transcription during postexponential growth (22).

The regulation of extracellular and cell wall protein synthesis by *S. aureus* is a complicated and carefully coordinated process (6, 12, 22). During the early exponential phase, a low basal level of RNAII transcription occurs (12). By the mid- to late exponential phase, the transcription of RNAII initiated from the P2 promoter is greatly increased (12). Our data suggest that the *sar* locus likely triggers this activation by binding to the P2 promoter region of *agr*. The P2 transcript, or RNAII, subsequently activates RNAIII transcription, resulting in modulation of exoprotein target gene transcription.

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