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Transcriptional Analysis of Different Promoters in the *sar* Locus in *Staphylococcus aureus*

ADHAR C. MANNA, MANFRED G. BAYER, AND AMBROSE L. CHEUNG*

Laboratory of Bacterial Pathogenesis and Immunology, The Rockefeller University, New York, New York 10021

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The expression of extracellular virulence determinants in *Staphylococcus aureus* is controlled by a 510-nucleotide RNA molecule (RNAIII) which is a part of the *agr* system. The *agr* operon, which encodes a multicomponent signal transduction system, is partially under the influence of an unlinked regulatory locus called *sar*. The *sar* locus is composed of three overlapping transcripts, designated *sarA* (0.56 kb), *sarC* (0.8 kb), and *sarB* (1.2 kb), originating from the P1, P3, and P2 promoters, respectively. In this study, we analyzed the differential expression of these promoters by using transcriptional fusion with the *xylE* reporter gene to study the activation of the *sar* locus. The data confirm the existence of three independent promoters with different promoter activities. Maximal promoter activity was observed with the combined fusion of P_2-P_3-P_1 promoters. Expression studies with a *sigB* mutant revealed that the P3 promoter is SigB dependent. Analysis of these transcriptional fusions in a *sarA* mutant and in complemented strains with each of the *sar* transcriptional units revealed that the *sar* locus is autoregulatory, with SarA acting as a positive regulator. From various transcriptional fusion studies of the upstream region of the P1 promoter, we have localized a 34-bp sequence which seems to play a role in down-modulating P1 transcription. Using heparin-Sepharose and DNA-specific columns, we partially purified a 12-kDa protein, possibly a repressor, which binds to the promoter regions upstream of P2 and P1 and which also binds to the 34-bp sequence. These data indicated that the regulation of the *sar* locus is complex and may involve the *sar* gene product(s) and other regulatory protein(s).

Staphylococcus aureus is an important human pathogen (25). It causes a variety of infections in humans ranging from localized skin suppuration to life-threatening septicemia. *S. aureus* produces a plethora of exotoxins, including hemolysins, enterotoxins, and toxic shock syndrome toxin 1 (TSST-1). The latter toxins are the causative agents for food poisoning and toxic shock syndrome, respectively (24). The pathogenesis of *S. aureus* is complex and probably involves the synthesis of cell wall-associated adhesins and the secretion of extracellular toxins with damaging effect on host cells, including those within the immune system.

Many of these extracellular virulence determinants are regulated by pleiotropic regulatory elements such as *sar* and *agr* (2, 12, 13, 20). The *agr* locus has been shown to be an activator for the expression of extracellular virulence genes (i.e., α-toxin, β-hemolysin, TSST-1, enterotoxins, etc.), while it negatively regulates the synthesis of cell surface proteins, such as protein A and fibronectin-binding proteins (12, 13, 15). The *agr* locus is composed of two divergent transcripts, RNAII and RNAIII, with sizes of 3.0 and 0.5 kb, respectively. The transcript RNAII initiating from the P2 promoter contains *agrA*, *-B*, *-C*, and *-D*, all of which are required for the activation of P2 and the ensuing RNAIII transcription. RNAIII, which also contains the δ-hemolysin gene, is the *agr* effector molecule ultimately responsible for the control of extracellular and cell surface protein synthesis (13, 16, 19).

In addition to *agr*, staphylococcal accessory regulator (*sar*) and the exoprotein gene regulator (*sae*) have been recently identified as two distinct global regulatory elements that are also involved in the expression of extracellular and cell surface proteins (4, 5, 9, 24). The *sar* locus is composed of three overlapping transcripts, designated *sarA* (0.56 kb), *sarC* (0.8 kb), and *sarB* (1.2 kb) originating from three distinct promoters, P1, P3, and P2, respectively. The expression of each of the three transcripts varies during the growth cycle, with *sarA* and *sarB* being maximal at the exponential phase and *sarC* peaking during the postexponential phase (1). Sequence analysis revealed that the *sarA* transcript codes for a 124-amino-acid polypeptide (SarA), while the transcript *sarC* encodes SarA and a putative 39-amino-acid open reading frame (ORF3) (1). Molecular analysis indicated that the larger *sarB* transcript, encoding SarA, ORF3, and an additional 18-amino-acid ORF (ORF4), is essential for full expression of RNAII and RNAIII in *S. aureus*, while the shorter *sarA* and *sarC* transcripts only partially restored *agr*-related transcription. It is likely that *agr* activation is partially mediated by the binding of *sar* gene product(s) to the *agr* promoter (2, 7, 10). Accordingly, the mechanism by which *sar* is activated from its own promoter has bearing on *agr* expression.

In this paper, we examined the regulation of *sar* expression in a pair of isogenic *sar* strains of *S. aureus* by using transcriptional fusion with the *xylE* reporter gene. Expression studies suggested that the P1 promoter is the strongest promoter compared with the P2 and P3 promoters in the parental strain. In assaying transcriptional activity in an isogenic *sigB* mutant, we confirmed our previous speculation that the central promoter (P3) of *sar* is σ^B dependent. Transcriptional fusion studies with the wild type and its isogenic *sar* mutant indicated that the expression of the *sar* locus is partially dependent on its own gene product. We have also identified a binding site for a putative repressor protein upstream of the P1 promoter. In gel shift assays, the partially purified 12-kDa protein binds to the *sar* P2 promoter region as well as to a 34-bp sequence upstream of P1. Therefore, we propose that the partially purified protein may act as a repressor for down-regulating *sar* expres-

* Corresponding author. Mailing address: Laboratory of Bacterial Pathogenesis and Immunology, The Rockefeller University, 1230 York Ave., New York, NY 10021. Phone: (212) 327-8163. Fax: (212) 327-7584. E-mail: cheunga@rockvax.rockefeller.edu.

TABLE 1. *S. aureus* strains and plasmids used in this study

Strain or plasmid	Description	Source or reference
Strains		
RN4220	Mutant of strain 8325-4 that accepts foreign DNA	17
RN6390	Parental strain	17
RUSA168	*sigB*::Tn*551* mutant of strain COL	27
ALC488	*sarA*::*ermC* mutant of RN6390	3
ALC812	ALC488 with *sarB* (nt 1–1349 plus 180 bp of upstream sequence) integrated into the *geh* locus on the host chromosome	2
ALC996	ALC488 with *sarA* (nt 530–1349) integrated into the chromosomal lipase gene (*geh*) via the integration vector pCL84	2
ALC997	ALC488 with *sarC* (nt 364–1349) integrated into the lipase gene (*geh*)	2
ALC1001	*sigB*::Tn*551* mutant of RN6390	This study
CYL316	Derivative of RN4220 supplying the integrase gene in *trans* (the recipient strain for pCL84)	14
COL	Methicillin-resistant strain	27
Plasmids		
pCRII	*E. coli* cloning vector for direct cloning of PCR product	Invitrogen
pLC4	Transcriptional fusion vector with a promoterless *xylE* gene	21
pSL24	Derivative of pLC4 with a pUC18 multiple cloning site	23
pRN6735	Derivative of pC194 containing pI258 *bla* promoter and 2/3 of the *blaZ* gene followed by the 1,566-bp *Mbo*I fragment of RNAIII lacking its promoter	17
pCL84	*S. aureus* integration vector that inserts into the lipase gene (*geh*) of the host chromosome	14
pALC686	pLC4 containing a 209-bp fragment upstream of *sarA* from nt 651–859; P_{11}	This study
pALC690	pLC4 containing a 329-bp fragment upstream of *sarA* from nt 531–859; P_{14}	This study
pALC695	pLC4 containing a 162-bp fragment upstream of *sarC* from nt 364–525; P_3	This study
pALC698	pLC4 containing a 196-bp fragment upstream of *sarB* from nt 1–196; P_{22}	This study
ALC706	pLC4 containing a 496-bp fragment upstream of *sarA* from nt 364–859; P_3-P_1	This study
pALC707	pLC4 containing a 1,039-bp fragment upstream of *sarA* from nt 1–859 plus 180 bp further upstream; P_2-P_3-P_1	This study
pALC926	pUC18 containing a 49-bp fragment of the upstream P2 promoter region (nt 71–119) (1) at the *Bam*HI site	This study
pALC932	pLC4 containing a 148-bp fragment upstream of *sarB* from nt 49–196; P_{21}	This study
pALC936	pLC4 containing a 376-bp fragment upstream of *sarB* from nt 1–196 plus 180 bp further upstream; P_{23}	This study
pALC1030	pSL24 containing a 183-bp *bla* promoter fragment from pRN6735	This study
pALC1050	pLC4 containing a 258-bp fragment upstream of *sarA* from nt 601–859; P_{12}	This study
pALC1167	pLC4 containing a 299-bp fragment upstream of *sarA* from nt 561–859; P_{13}	This study
pALC1227	pLC4 containing a 277-bp fragment upstream of *sarA* from nt 567–859; P_{15}	This study
pALC1229	pUC18 containing a 34-bp fragment of the upstream of P1 promoter from nt 567–600 (1) at the *Bam*HI site	This study
pALC1362	pCL84 containing the P_{14} promoter upstream of *xylE* reporter gene	This study

sion, whereas *sar* gene product(s) may act as an activator of its own gene's expression.

MATERIALS AND METHODS

Bacterial strains, plasmids, and growth media. The bacterial strains and plasmids used in this study are listed in Table 1. Phage ϕ11 was used as the transducing phage for *S. aureus* strains. CYGP, 0.3GL medium (17, 18), and tryptic soy broth were used for the growth of *S. aureus* strains, while Luria-Bertani medium was used for growing *Escherichia coli*. Antibiotics were used at the following concentrations: erythromycin, 5 μg/ml; chloramphenicol, 10 μg/ml; tetracycline, 5 μg/ml; and ampicillin, 50 μg/ml.

Construction of transcriptional fusions. DNA fragments encompassing various *sar* promoter regions (Fig. 1) were amplified by PCR by using genomic DNA of *S. aureus* RN6390 as the template and cloned into the TA cloning vector pCRII (Invitrogen, San Diego, Calif.). The *Eco*RI fragment containing the promoter region was cleaved from pCRII and cloned into plasmid pLC4 (21), generating transcriptional fusion to the *xylE* reporter gene. The orientation and authenticity of the cloned promoter fragments were confirmed by restriction analysis and DNA sequencing. As a positive control, a 183-bp *Hin*dIII fragment containing the *bla* promoter of *S. aureus* from plasmid pRN6735 (13) was cloned into plasmid pSL24 (23) to form a transcriptional fusion with the *xylE* reporter gene. For gel shift assays with the putative repressor protein, the 51-bp DNA fragment (nucleotides [nt] 71 to 119 with flanking *Bam*HI sites) and the 34-bp region encompassing the repressor protein binding site (nt 567 to 600) (1) were synthesized chemically and cloned into the *Bam*HI site of pUC18. All transcriptional fusions and relevant constructs in different mutants are described in Table 1.

Genetic manipulations in *S. aureus*. Different transcriptional fusions and other constructs were first transformed by electroporation to *S. aureus* RN4220, a restriction-deficient derivative of strain 8325-4 (17). Transformants were selected on NYE agar (14) containing 10 μg of chloramphenicol per ml. For transduction, phage ϕ11 was used to produce a phase lysate of strain RN4220 containing various *sar* transcriptional fusions. The phage lysate was then used to infect the recipient strain of *S. aureus* as described previously (4). The presence of the correct plasmids was confirmed by restriction mapping.

Single copies of specific *sar* fragments were introduced into the chromosome of *sar* mutant ALC488 as previously described (2). In brief, a specific *sar* fragment was cleaved from pCRII and cloned into the polyclonal site of the integration vector pCL84 (14). Upon transformation into strain CYL316, a derivative of RN4220 containing the integrase gene in *trans*, this vector inserts preferentially into the lipase gene (*geh*) of the host chromosome, resulting in tetracycline-resistant integrants with a loss of lipase activity. The integrated fragment was transduced into the *sar* mutant ALC488 as described previously (2, 3). Correct integration was verified by Southern blotting with lipase- and *sar*-specific probes.

A *sigB* mutant of RN6390 was constructed as described previously (4) by transducing the parental strain with a phage lysate of strain RUSA168 carrying the *sigB* mutation (27).

Catechol 2,3-dioxygenase assays. For enzymatic assays, overnight cultures were diluted 1:50 in 250 ml of tryptic soy broth containing the 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 optical densities at 600 nm (OD_{600}) was serially removed and centrifuged. The cells were washed twice with 1 ml of ice-cold 20 mM potassium phosphate buffer (pH 7.2). Pellets were resuspended in 500 μl of 100 mM potassium phosphate buffer (pH 8.0) contain-

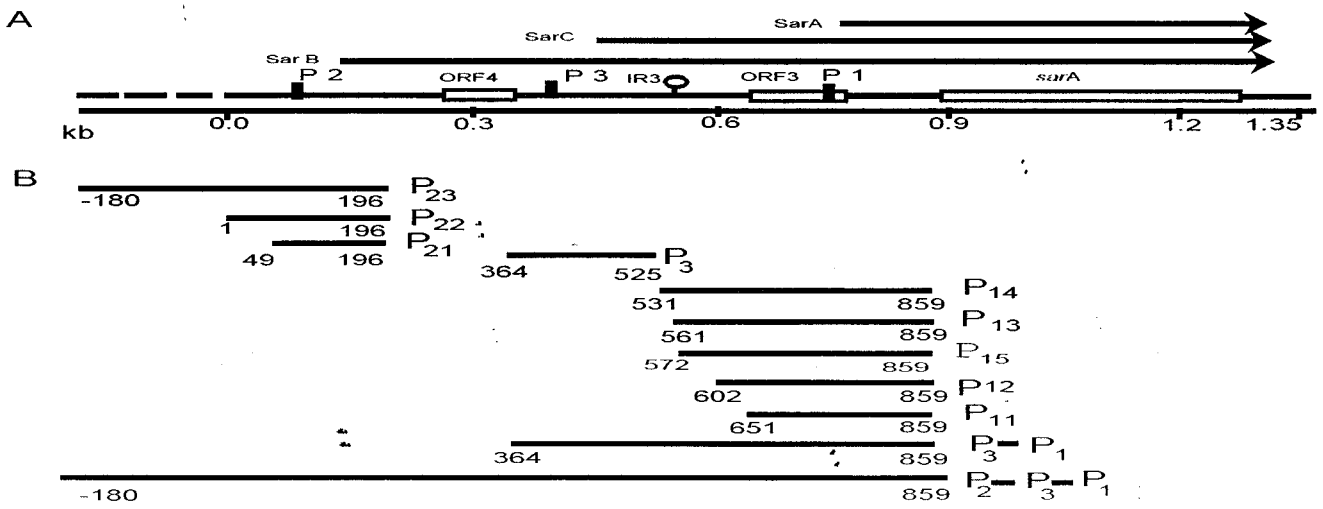

FIG. 1. Organization of the *sar* locus. (A) Schematic representation of the *sar* locus showing various transcripts, *sarA*, *sarC*, and *sarB* that originate from the P1, P3, and P2 promoters (arrows), respectively. The ORFs are indicated by boxes, promoters are indicated by vertical solid bars, and an inverted repeat (IR3) is indicated by a hairpin loop structure. The DNA sequence of the 1.35-kb *sar* locus region has been published (1), as indicated by a solid line, whereas the broken line region (180 bp) is unpublished. (B) Different promoter fragments of the *sar* locus were used to construct the transcriptional fusions to the *xylE* reporter gene. The numbers are the nucleotide positions from the published sequence (1).

ing 10% acetone and 25 μg of lysostaphin per ml and incubated for 15 min at 37°C and then iced for 5 min. Extracts were centrifuged at 20,000 × *g* for 50 min at 4°C to pellet cell 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 previously (28). The reactions were allowed to proceed for 25 min, with OD_{375} readings taken at the 2-, 5-, 15-, and 25-min time points. One milliunit is equivalent to the formation of 1.0 nmol of 2-hydroxymuconic semialdehyde per min at 30°C. Specific activity is defined as milliunits per milligram of cellular protein (28).

Purification of the 12-kDa protein. The cell extract of *S. aureus* RN6390 was used to purify protein with binding activity to the *sar* promoter. The culture was grown overnight in 1 liter of CYGP medium and harvested by centrifugation. Cells were washed with 20 mM potassium phosphate (pH 7.5) and resuspended in buffer (100 mM Tris-HCl [pH 7.5], 100 mM KCl, 5 mM $MgCl_2$, 1 mM EDTA, 1 mM dithiothreitol [DTT]). The cells were lysed by incubation with lysostaphin (25 μg/ml) for 30 min at 10°C and then frozen at −70°C. After repeating freezing and thawing twice, the lysate was centrifuged at 35,000 rpm (TLA100.4 rotor in an Optima TL ultracentrifuge; Beckman Instruments, Fullerton, Calif.) for 40 min to remove cellular debris and dialyzed against buffer A (25 mM Tris-HCl [pH 7.5], 100 mM NaCl, 1 mM EDTA, 5 mM $MgCl_2$, 1 mM DTT, 1 mM phenylmethylsulfonyl fluoride, 10% glycerol). The protein solution was applied to a 10-ml heparin-Sepharose column preequilibrated with buffer A. The column was then washed with five column volumes of buffer A and eluted with a continuous gradient of buffer A and buffer B (buffer A containing 1.5 M NaCl). Fractions were assayed for DNA binding activity by gel shift assays with an γ-^{32}P-labeled 51-bp *sar* promoter fragment which encompasses the sequence upstream of the *sar* P2 promoter (nt 71 to 119) (1). This fragment (nt 71 to 119) (1) was originally synthesized and cloned into the *Bam*HI site of pUC18. The 51-bp *Bam*HI fragment for gel shift assays was gel purified. Fractions containing DNA binding activity were pooled, dialyzed against buffer A, and loaded onto a preequilibrated 5-ml DNA-specific column containing the 51-bp DNA fragment covalently linked to Sepharose as described by Hughes et al. (11). The column was washed with buffer A and eluted with a linear gradient of buffer A to buffer B. Fractions with DNA binding activity as determined by gel shift assays were pooled, dialyzed against buffer A with 50% glycerol, and stored at −70°C. Protein concentration was estimated with the Bio-Rad Protein Assay with bovine serum albumin as the standard. The apparent molecular weight of the putative protein was assessed by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis.

Gel shift assays. To determine if the 12-kDa DNA binding protein interacts with the *sar* promoter fragments, DNA fragments were end labeled with [γ-^{32}P]ATP by using 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 DTT, 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

Rationale for the construction of various transcriptional fusions of the *sar* locus. In previous studies, we demonstrated that the expression of *sar* transcripts varies during the growth cycle, with *sarA* and *sarB* being most abundant in the exponential phase and with *sarC* being maximally expressed toward the postexponential phase (1). Our speculation is that these three transcripts reflect the activities of three different *sar* promoters and that one or more *trans*-acting regulatory elements may control the expression of different *sar* promoters by binding to the respective upstream region. To confirm the existence and the strength of these *sar* promoters and to detect possible regulatory regions within *sar*, a series of XylE transcriptional fusions with various lengths of different *sar* promoters were constructed (Table 1 and Fig. 1). Three fusions of the P2 promoter, P_{21}, P_{22}, and P_{23}, containing 70, 120, and 300 bp upstream of the deduced −35 promoter box, respectively, were prepared. With the P3 promoter region relatively short, we constructed a single fusion with a 57-bp fragment upstream of the −35 promoter box. We speculated that an inverted repeat (IR3 [nt 553 to 593]) upstream of P1 may play an important role in down-regulating *sarA* transcription. To investigate the regulatory function of this region, we constructed five transcriptional fusions, P_{11}, P_{12}, P_{15}, P_{13}, and P_{14}, to include sequences 35, 85, 119, 125, and 155 bp upstream of the P1 −35 promoter box, respectively (Fig. 1 and 2). All of these constructs were introduced into the wild type and an assortment of *S. aureus* mutant strains and assayed for the activity of catechol 2,3-dioxygenase, an enzyme which is the gene product of the *xylE* reporter gene.

Transcriptional fusion studies of the wild type and *sar* mutant strains of *S. aureus*. To determine the relative strength of these promoters and the regulatory region within IR3 upstream of the P1 promoter, the transcriptional activity of all three *sar* promoters was analyzed with the parental strain and an isogenic *sarA* mutant of *S. aureus* (Table 2). Based on XylE activity, the P1 promoter (P_{11} and P_{12}) was the strongest, with ≈50-fold-more activity than the homologous P2 and P3 pro-

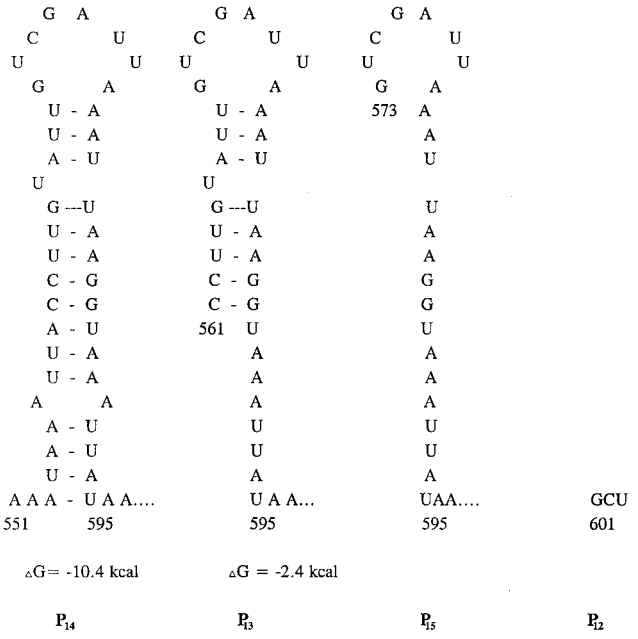

FIG. 2. Proposed stem-loop structure for the inverted repeat region (IR3) upstream of the P1 promoter of the *sar* locus. The numbers are the nucleotide positions from the published sequence (1).

moters in parental strain RN6390. The activities of the P_{21}, P_{22}, and P_{23} promoter fusions were similar, thus suggesting that a small region (i.e., beyond P_{21} from position 49 to −181 [Fig. 1]) upstream of the −35 promoter box is not critical to the activation of the P2 promoter. The P3 promoter was the weakest among these three promoters. Analysis of the expression of P_{11}, P_{12}, P_{15}, P_{13}, and P_{14} promoter fusions in parental strain RN6390 suggested that the activities of P_{11} and P_{12} were two of the strongest among all P1 promoter constructs. With P_{15}, P_{13}, and P_{14}, XylE activities were approximately 2- to 3 1/2-fold lower than the corresponding P_{11} and P_{12} fusions. To assess the discrepancy in the expression of various P1 constructs, we analyzed the upstream region of the P1 promoter and found that P_{11} and P_{12} constructs lacked an inverted repeat region (IR3). Figure 2 shows the proposed secondary structure of the inverted repeat region (repeats at positions 553 to 569 and 578 to 593) among various constructs of the P1 promoter region. With both repeats, the P_{14} promoter fragment is capable of forming a stable stem-loop structure ($\Delta G = -10.4$ kcal), whereas the P_{13} promoter fragment contained a partial stem-loop structure ($\Delta G = -2.4$ kcal) (Fig. 2). The P_{15} construct retained half of the inverted repeats, thus disrupting the stem-loop structure. A lower level of expression for the P_{15} fusion compared with the P_{12} construct indicated that the region comprising approximately one-half of the inverted repeats (nt 567 to 593) likely encompasses the sequence necessary for down-regulating *sarA* transcription at the transcriptional level. Remarkably, the combined P_3-P_1 fusion activity was lower than that of the P_{11} or P_{12} fusion. We speculate that the P3-specific promoter sequence may be a binding site for a repressor protein (see below). Alternatively, the sequence itself may be part of secondary structure which interferes with transcription from the P1 promoter. Interestingly, maximum promoter activity was observed with the combined fusion of the P_2-P_3-P_1 promoters, with the level of activity higher than that of any of the individual *sar* promoters. Using the *bla* promoter as a control, we found that it was stronger than the P2 and P3 promoters but about twofold weaker than the P1 promoter. In contrast, the vector control or the constructs with divergent orientation had little, if any, XylE activity (Table 2).

TABLE 2. Expression of *xylE* fusion from different *sar* promoters in the wild type and an isogenic *sarA* mutant of *S. aureus*

Construct	Orientation with respect to the *xylE* gene	XylE activity (mU/mg of protein)[a]	
		RN6390	ALC488
P_{21}	Convergent	2.9	0.9
P_{22}	Convergent	4.0	1.2
P_{23}	Convergent	5.5	0.8
P_3	Convergent	3.0	0.4
P_{11}	Convergent	313.8	27.6
P_{12}	Convergent	282.3	39.6
P_{13}	Convergent	91.8	13.8
P_{15}	Convergent	132.6	19.0
P_{14}	Convergent	137.2	18.4
P_3-P_1	Convergent	74.6	35.4
P_2-P_3-P_1	Convergent	441.8	29.6
Vector		1.0	1.0
P_{bla}	Convergent	69.8	61.1
P_c[b]	Divergent	1.1	0.8

[a] Activity is defined as milliunits per milligram of total cellular protein. The value for each sample at a particular growth phase (OD_{600} = 1.7) is the mean value assayed at four different time points after the addition of the catechol.

[b] P_c represents the average value of the divergent promoter fusions P_{21-23}, P_3, P_{11-15}, P_3-P_1, P_2-P_3-P_1, P_{bla}, with most values approaching the background level.

Similar expression studies were also performed with the isogenic *sarA* mutant ALC488 (3), in which the *sarA* gene was interrupted by an *ermC* gene inserted at nt 971 (1). Prior transcriptional analysis indicated that the transcription of *sarA*, *-C*, and *-B* was disrupted in this mutant (3). As shown in Table 2, a 2- to 15-fold reduction in promoter activity was observed in assorted transcriptional fusions during the early stationary phase in the mutant background. Similar reductions were also observed in the log and postexponential phases, thus indicating that the *sar* locus is involved in its own expression (data not shown). With the strongest promoter, P1, there was at least a sixfold decrease in promoter activity in all five fusion constructs in the *sar*-negative mutant background. The reduction was about 15-fold in the combined fusion of the P_2-P_3-P_1 promoters. The lower transcriptional activities in the mutant background were not attributable to reduced plasmid copy number, because the plasmid yield was found to be consistent in quantity when isolated from various strains. In addition, we assayed for chloramphenicol acetyltransferase activities for the P_2-P_3-P_1 fusion and found them to be equivalent in strains RN6390 and the *sar* mutant ALC488 harboring the fusion, thus implying similar plasmid copy numbers between the isogenic pair (data not shown). The relative constancy of the plasmid number was also supported by the observation that the control *bla* promoter as well as the divergent fusion constructs had similar promoter activities in the wild type and the isogenic *sarA* mutant. Taken together, these data support the notion that one or more of the *sar* gene products likely activates the expression of its own promoters.

To reconfirm the autoregulatory nature of the *sar* locus, we integrated a single copy of one of these promoter constructs, P_{14}, into the lipase gene on the chromosome of parental strain RN6390 and isogenic *sarA* mutant ALC488 via the recombinant integration vector pCL84 containing the P_{14}-*xylE* fusion in proper orientation. As with the multicopy counterpart, the transcriptional activity was reduced in the *sar* mutant ALC488

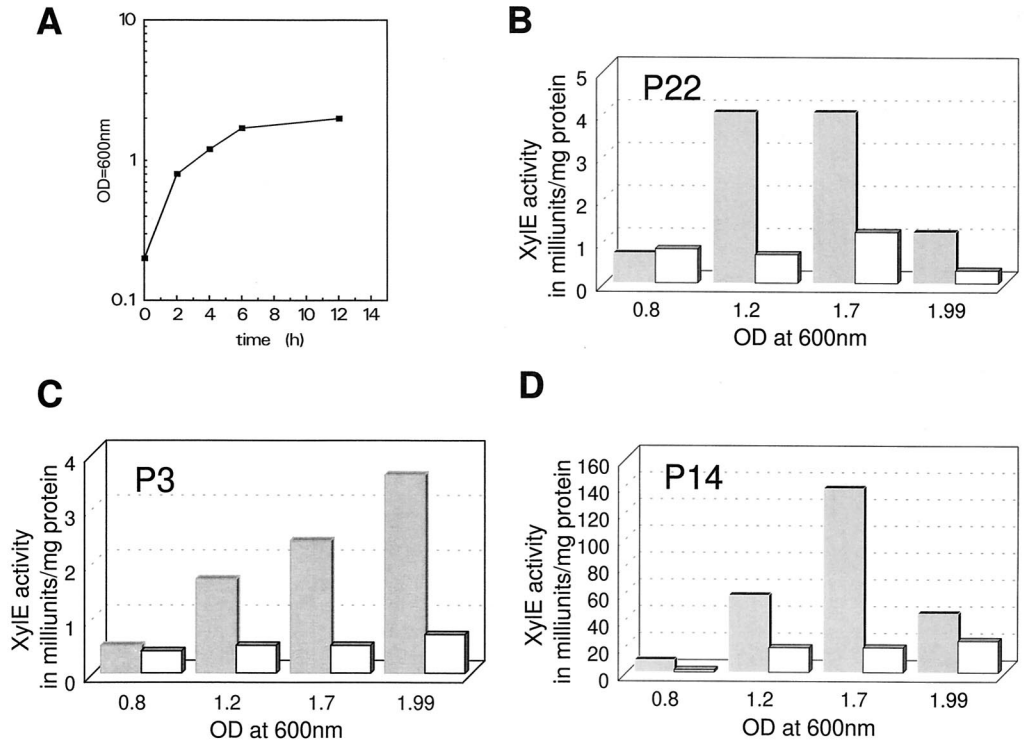

FIG. 3. XylE activity in the P_{22}, P3, and P_{14} constructs (B, C, and D) during the growth cycle. Cells obtained at four sample points during the growth cycle (A [semilog scale]) were lysed with lysostaphin and assayed for catechol 2,3-dioxygenase activity as described previously. The average values for the constructs ($n = 4$) are presented in milliunits per milligram of cellular protein. The open and shaded bars represent data derived from RN6390 and ALC488 (*sar* mutant), respectively.

(2.24 mU/mg of cellular protein) compared with that in the parent (8.12 mU/mg of protein).

Growth-phase-dependent expression of different *sar* promoters. Samples from *S. aureus* strains containing different fusions were assayed for XylE activity during the growth cycle (Fig. 3). With P_{22} and P_{14} promoters, XylE activity generally increased toward the early stationary phase and then tapered off in the late stationary phase (overnight growth). In contrast, the P3 promoter revealed a steady increase in promoter activity, even toward the late stationary phase (3.6 versus 0.5 mU/mg of protein for overnight culture and mid-log phase, respectively). This result is akin to our prior transcriptional data (1), in which we found that the *sarC* transcript that originated from the P3 promoter was maximally transcribed during the postexponential phase. These findings support the notion that the expression of *sar* from different promoters is growth phase dependent.

The P3 promoter is *sigB* dependent. In previous studies, we showed that the P1 promoter shares homology with the σ^{70}-dependent consensus sequences, while the P3 promoter closely resembles the consensus sequence of *sigB*-dependent promoters (1). To investigate whether the P3 promoter is truly *sigB* dependent in vivo, a *sigB* mutant, ALC1001, of *S. aureus* RN6390 was constructed by transducing the mutation from mutant RUSA168 (27). Transcriptional fusions of the *sar* promoters were introduced into the mutant strain ALC1001 and assayed for XylE activity (Table 3). With the exception of the P3 promoter, the pattern of activities for most fusions remained similar to those found in the wild type. Notably, the P3 promoter activity became negligible in this background. As with those found in the parental background, the combined P_3-P_1 fusion activity was also substantially lower than the activity of the P_{11} promoter in the *sigB* mutant background. These results suggest that the central P3 promoter of the *sar* locus likely utilizes a σ^B-dependent form of RNA polymerase to initiate transcription in *S. aureus*. As an additional confirmation of these data, transcriptional analysis of the *sar* locus in the *sigB* mutant ALC1001 revealed that the *sarC* transcript initiated from the P3 promoter was absent (data not shown). A recent in vitro study with purified SigB and RNA polymerase from *S. aureus* has also shown that the P3 promoter of *sar* is dependent on SigB (8).

Transcriptional fusion studies in complemented *sar* mutant strains. In previous studies, we found that the introduction of a *sarC* fragment into a *sar* mutant was sufficient for comple-

TABLE 3. Activities of different *sar* promoters in a *sigB* mutant of *S. aureus*

Construct[a]	XylE activity (mU/mg of protein)[b]	
	Wild-type RN6390	ALC1001
P_{21}	7.4	8.2
P_3	6.1	0.6
P_{11}	302.0	256.0
P_3-P_1	73.1	38.0
P_2-P_3-P_1	486.0	273.0
Vector	0.6	0.8

[a] Note that all constructs (excluding vector) were convergent in terms of their orientation with respect to the *xylE* gene.

[b] Activity is defined as milliunits per milligram of total cellular protein. The value for each sample at a particular growth phase ($OD_{600} \approx 1.9$ from overnight cultures) is the mean value assayed at two different time points after the addition of catechol.

TABLE 4. Expression of *xylE* fusion from different *sar* promoters in *sarA* mutant strain ALC488 complemented with various *sar* fragments

Construct	Orientation with respect to *xylE*	XylE activity (mU/mg of protein)[b]				
		RN6390[a]	ALC488[a]	ALC812 (*sarB*)	ALC996 (*sarA*)	ALC997 (*sarC*)
P_{22}	Convergent	4.00	1.20	4.63	2.94	8.3
P_3	Convergent	3.00	0.4	13.42	4.0	5.0
P_{11}	Convergent	313.8	27.61	407.50	272.4	177.8
Vector		1.02	0.9	0.73	0.55	0.61
P_c[c]	Divergent	1.07	0.83	0.79	0.60	1.08

[a] The data for RN6390 and ALC488 are taken from Table 2.
[b] Activity is defined as milliunits per milligram of total cellular protein. The value for each sample at a particular growth phase (OD_{600} = 1.7) is the mean value assayed at four different time points after the addition of the catechol.
[c] P_c represents the average value of the divergent promoter fusions P_{22}, P_3, and P_{11}.

mentation; however, complete restoration of the *sar*-related phenotypes required the presence of a *sarB* fragment in the mutant (2). In promoter fusion studies involving the parental strain and the *sar* mutant ALC488 (*sarA*::*ermC*), our data clearly indicated that *sar* gene products partially regulate promoter activation from *sar* promoters (Table 2). To determine whether the observed reduction in promoter activity in the *sar* mutant is due to a loss of *sarA* function or to those proteins encoded by the sequence upstream of *sarA* (e.g., ORF3 and ORF4), single copies of *sarA*, *sarC* (*sarA* with ORF3), and *sarB* (*sarA* with ORF3 and ORF4) were introduced into *sar* mutant ALC488 to form ALC996, ALC997, and ALC812 via the integration vector pCL84, which preferentially integrated into the lipase gene (*geh*) on the host chromosome (Tables 1 and 4).

As shown in Table 4, both P2 (P_{22}) and P3 promoter activities in *sarA*-complemented strains (2.94 and 4.0 mU/mg of protein, respectively, for P2 and P3 in ALC996) were generally comparable to those found in parental strain RN6390 (4.0 and 3.0 mU/mg of protein). However, in comparison to the *sar* mutant ALC488, P2 and P3 promoter activities were higher as the size of the complemented *sar* fragments increased from *sarA* to *sarB* (4.63 and 13.42 mU/mg for P2 and P3 activities, respectively [Table 4]). The restoration of P2 and P3 promoter activities in the *sar* mutant ALC488 to parental levels by the *sarA* transcript fragment itself (ALC996) revealed that the SarA protein was probably responsible for modulating these promoter activities in the mutant, since we have previously shown that the effector molecule within *sar* is the protein rather than the *sarA* transcript (2). As with the P2 and P3 promoters, the P1 promoter activity was significantly enhanced in complemented strains (e.g., 272.4 mU/mg for P_{11} in *sarA*-complemented strain ALC996) compared with the mutant ALC488 alone (27.6 mU/mg of protein for P_{11}). The augmentation in XylE activity in P_{11} was higher in the *sarB*-complemented strain (ALC812) than in the *sarA*-complemented strain (ALC996). This finding argues for additional factors other than *sarA* but that are encoded by *sarB* and that may serve to augment transcriptional activity of the P1 promoter of the *sar* locus in *S. aureus*.

Purification of a putative 12-kDa protein. Two experimental observations led us to consider that some factor(s) or protein(s) may bind the upstream region to down-modulate the expression from *sar* promoters. First, transcriptional analysis of the *sar* locus revealed a gradual decrease in *sarA* and *sarB* transcription and an increase in *sarC* transcription as bacterial cells make the transition between the late log and stationary phases of growth (Fig. 3). Second, the differential promoter activity as expressed in the complemented *sar* mutant strain implies that a factor or factors other than SarA protein may bind to the *sar* promoter region (Table 4). A close inspection of the upstream region, including the inverted repeat (IR3) and the minimum 34-bp sequence (nt 567 to 600 in P_{15} [Fig. 2]) required for down-regulating the P1 promoter activity, reveals the presence of a 7- to 8-bp sequence (TAAATTAA) which is repeated 11 times within the P1-P3-P2 promoter region. It seems reasonable to surmise that this sequence may be involved in presenting the binding site for a common regulatory protein. Accordingly, we synthesized a 49-bp DNA sequence of the P2 promoter region (from nt 71 to 119) (1) which encompassed the AT-rich UP box of the P2 promoter region as well as the 8-bp repeat. This DNA fragment was then conjugated to CNBr-activated Sepharose 4B (Pharmacia) to produce an affinity column. Putative DNA protein was purified from lysates of *S. aureus* RN6390 by first using a heparin-Sepharose column followed by the DNA-specific column. The details for purification of the putative protein were described in Materials and Methods. Gel shift analysis of putative fractions obtained during the purification was done with a labeled 51-bp fragment encompassing the 49-bp sequence (data not shown). By this technique, we purified a 12-kDa protein which was analyzed by SDS gels (Fig. 4, lane 6) to be ~90% pure.

In gel shift studies of the labeled 51-bp probe with the purified 12-kDa protein, retardation of the probe was observed with increasing amounts of the purified protein, suggesting binding specificity (Fig. 5, lanes 1 to 5). As expected, unlabeled P2 promoter fragment (P_{21}) competed successfully as an in-

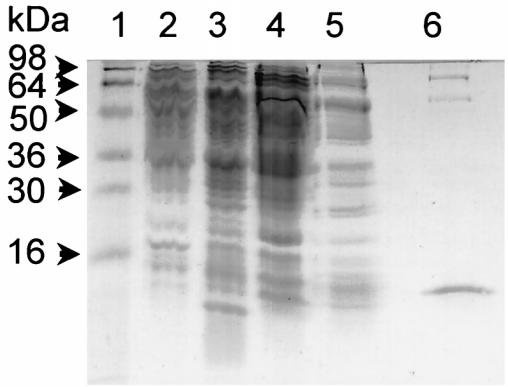

FIG. 4. SDS-polyacrylamide gel showing different stages of purification and the purity of the purified protein. Fractions were subjected to SDS–12% polyacrylamide gel electrophoresis and stained with Coomassie blue dye. Lanes 1, molecular mass marker (Novex, San Diego, Calif.); 2, 10 μg of total cell extract proteins; 3, 10 μg of heparin-Sepharose column-bound fraction; 4, 10 μg of nonbound heparin-Sepharose column fraction; 4, 10 μg of flowthrough fraction of the DNA-specific column; 6, 1 μg of the purified protein fraction from the affinity column.

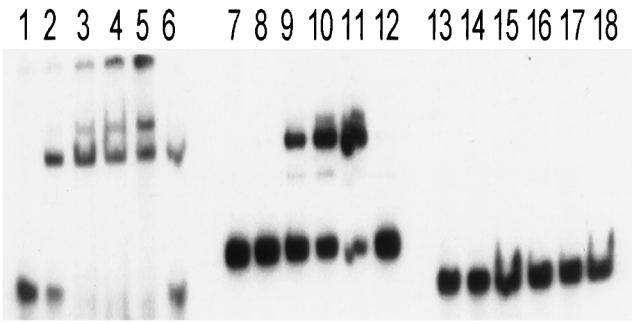

FIG. 5. Autoradiogram of a nondenaturing 10% polyacrylamide gel showing the binding of the 12-kDa purified protein to a 51-bp radiolabeled DNA fragment [GATCC (nt 73) TTAAGACC-TAAATTAA-TGTTATTTTTTAA-TAATTTA-CACC-AAATTAA-(nt 119) G (the nucleotides in the repeated 7- or 8-bp sequence in the proper or complementary orientation are underlined)] (1) of the upstream P2 promoter region, an 80-bp labeled fragment containing the 34-bp sequence with the polylinker region of pUC18, and a 53-bp polylinker fragment from pUC18. Lanes: 1 to 5, mobility of the 51-bp DNA fragment of the upstream P2 promoter in the presence of 0, 30, 100, 200, and 300 ng of the purified protein, respectively; lane 6, mobility of the same fragment in the presence of 300 ng of the purified protein and 40-fold (molar ratio) of unlabeled P_{21} fragment as the specific competitor. Lanes 7 to 11 and 13 to 17 represent mobility shifts of the 80-bp DNA fragment containing the 34-bp binding site for the putative repressor together with flanking polylinker sequence and equivalent amounts of 53-bp polylinker fragment of pUC18, respectively. Lanes 12 and 18 are competition assays with unlabeled P_{21} and 53-bp pUC18 fragments, respectively.

hibitor (lane 6). The retarded bands in the gel shift assay indicate that the 51-bp fragment possibly contains three binding sites for this protein. An alternative explanation will be the binding of 12-kDa multimers to a single binding site. Interestingly, when a labeled 80-bp fragment containing the 34-bp sequence (nt 567 to 600 [Fig. 2]) upstream of P1 and flanking polylinker sequence of pUC18 was used for gel shift assays, a single retarded band was observed (Fig. 5, lanes 7 to 11). In contrast, a labeled 53-bp fragment containing only the polylinker region from pUC18 did not result in any gel shift activity with equivalent amounts of the purified protein (Fig. 5, lanes 13 to 18). Thus, the 34-bp sequence (nt 567 to 600) (Fig. 2) likely constitutes one binding site for this protein. Additional gel shift experiments with overexpressed and highly purified protein suggested that the partially purified protein behaves similarly and thus is specific to its substrates. Because the 34-bp sequence is responsible for down-modulating P1 transcription (see P_{15} versus P_{12}), we speculate that the 12-kDa protein may act as a repressor for *sar* expression. Clearly, more extensive work needs to be done to confirm the functional and physical characterization of this 12-kDa protein. Preliminary analysis suggests that the purified protein is not SarA nor histone (HU)-like protein, but does have limited homology to the SarA protein of *S. aureus*.

DISCUSSION

The *sar* locus is a complex regulatory system with a 372-bp *sarA* gene preceded by an 800-bp extended triple-promoter region. Within the promoter region are multiple repeats and inverted repeats as well as potential peptide coding regions that may form a complex network for *sar* promoter activation (1). To confirm the existence of three distinct promoters and to ascertain the role of these secondary structures and its mode of regulation, we cloned various lengths of putative *sar* promoters, both singly and in combinations, into the transcriptional vector pLC4 by using *xylE* as a reporter gene. The results of our reporter fusion studies (Table 2) clearly confirm our previous transcriptional data (1) that the smaller transcripts do not arise from degradation of the larger *sarB* transcript because these three promoters can exist as distinct entities in transcriptional fusions. Among these, P1 is the strongest individual promoter compared with P2 and P3. Because the region immediately upstream of the P1 promoter including a 16-bp inverted repeat (IR3 in Fig. 2) and a putative ORF (ORF3) may modulate *sarA* transcription from P1 (1), we cloned various lengths of the P1 promoter into the fusion vector and found that shorter promoter fragments (P_{11} and P_{12}) were stronger than their lengthy counterparts (i.e., P_{15}, P_{13}, and P_{14} in Table 2). Recognizing that P_{15}, P_{14}, and P_{13} encompass a promoterless ORF3, it becomes unlikely that ORF3 plays a major role in down-modulating *sarA* transcription from P1. In retaining the entire or partial 16-bp inverted repeats in P_{14} and P_{13} and also preserving only one-half of the repeat in P_{15}, we showed that it is the sequence within the inverted repeat (IR3) rather than the repeat itself (1) that is critical to the down-modulation of *sarA* transcription from the P1 promoter, since comparable promoter activities were found among the P_{13}, P_{14}, and P_{15} constructs (Table 2).

The P2 promoter is at least 20-fold weaker than P1 but comparable to P3 (Table 2). Conceivably, differences in the promoter structure between P1 and P2 may have accounted for the disparate promoter activities. Both the P1 and P2 promoters have features that resemble a σ^{70}-dependent promoter; a comparative analysis revealed that the −10 and −35 consensus sequence of the P1 promoter (TTTACT-N_{18}-TATAAT), like that of the *blaZ* promoter (TTGACA-N_{18}-TATTAT), closely resembles the *E. coli* canonical consensus sequence (TTGACA-N_{14-21}-TATAAT) (22), whereas the P2 promoter (TAGCAAA-N_{17}-TAATAT) is less conserved. However, the direct reliance of the P2 promoter on the σ^A-dependent form of RNA polymerase has not been confirmed in vitro. The effect in varying the extent of the P2 promoter is not significant compared with that of P1, thus implying that the structure 70 bp upstream of P2 (i.e., beyond P_{12}) is not critical to the regulation of P2 transcription. Alternatively, another factor or factors may bind to this region to suppress promoter activity of the transcriptional fusion. Remarkably, when the P2 promoter was fused to P3 and P1, the activity became more potent than that of P_{11}. However, we observed that the P_3-P_1 promoter fusion had lower activity than P1 alone. A plausible explanation for this divergent finding may be that the P3 promoter region may be a binding site for a repressor protein that down-modulates P1 transcription while the region upstream of P2 may be involved in either positive regulation of P1 or negative regulation of P3 promoter. Clearly, more extensive work needs to be done to understand the mode of P2 and P3 promoter activation.

In contrast to P1 and P2, P3 has a putative *sigB*-dependent promoter with a typical −10 promoter box (GGGTAT) (1). Because *sigB* promoters in gram-positive bacteria (e.g., *Bacillus subtilis*) are typically activated during periods of stress, including the postexponential phase (26), our results also showed that P3 was most active in late stationary or overnight cultures (Fig. 3), while the σ^A-dependent promoters of P1 and P2 had lower activities compared to those during the late-log or early stationary growth phases. Wherein P1 and P2 remained active in a *sigB* mutant, the P3 promoter activity became silent in this background (Table 3). These findings coincided with our observation that *sarC* transcription is absent in a *sigB* mutant (unpublished data). In addition, recent in vitro transcription assays with purified SigB protein and core RNA polymerase of *S. aureus* also supported the notion that P3 promoter is *sigB* dependent (8). Taken together, our data con-

firmed that the P3 promoter utilizes the SigB-dependent form of RNA polymerase as found in other stress-response promoters of gram-positive bacteria.

Previously, the transcription of RNAII and RNAIII was found to be dramatically reduced in an *agr* mutant (15), thus revealing the autoregulatory feature of the *agr* system. Similar to *agr*, we discovered that activation of *sar* promoters, either singly or in combination, is dependent on the expression of *sar* gene products (see *sar* mutant ALC488 in Table 2). In contrast, the P_{bla} promoter activity remained unaffected in the *sar* mutant background. To minimize the issue arising from plasmid copy number, we have also introduced single copy of the promoter construct P_{14} to a pair of isogenic *sar* strains. As anticipated, the P_{14}-XylE activity in the *sarA* mutant was reduced approximately sevenfold compared to that of the parental strain when measured with the multicopy fusion and approximately threefold when measured with the single-copy fusion. To further confirm the autoregulatory nature of *sar*, we conducted complementation studies of *sar* mutant strains (ALC488) carrying single copies of *sarA*, *sarC*, or *sarB*. As predicted, promoter activities of P1, P2, and P3 were restored to near-parental levels with the strain containing the largest transcript, *sarB*, while those containing *sarA* and *sarC* were at slightly lower levels. Collectively, our data strongly support the notion that *sar* gene products, consisting of SarA and possibly other encoded elements upstream (2), contribute directly or indirectly to *sar*'s own regulation. However, factors other than *sar* gene products must also play a role in modulating *sar* promoter activity, because significant residual promoter activities (P1, P_3-P_1, and P_2-P_3-P_1) were retained in the *sar* mutant ALC488 (Tables 2 and 4).

In scanning the UP element in P2 and P3 promoters, as well as the 34-bp binding site (Fig. 2) for a putative repressor protein, we found a conserved 7- to 8-bp sequence (TAAAT TAA) which was repeated 11 times in the 800-bp *sar* promoter region, including several repeats in the region upstream of the P1 and P2 −35 promoter boxes. This 7- to 8-bp sequence is also part of the inverted repeat (nt 553 to 593) found within the P_{15}, P_{13}, and P_{14} promoter constructs. Although *S. aureus* chromosomal DNA is 70% AT rich, this 8-bp sequence is rarely found, as demonstrated by the fact that this motif was found only once in a 6-kb *agr* sequence. We thus speculate that this may be a possible binding site for a DNA binding protein involved in regulating *sar* gene expression.

Remarkably, we were able to purify a 12-kDa protein with a DNA-specific column to which a 49-bp sequence (nt 71 to 119) comprising the UP box and this repeat was covalently linked via CNBr-activated Sepharose. Although this sequence was derived from the P2 promoter region, gel shift assays with the purified 12-kDa protein revealed that it also bound to a 34-bp fragment (nt 567 to 600) (Fig. 2) upstream of the P1 promoter in a dose-dependent manner (Fig. 5). Notably, this fragment yielded only one retarded band, whereas the 51-bp fragment resulted in three shifted bands in gel retardation assays (Fig. 5). In comparing these two sequences, we found that the 51-bp fragment contained three 7- to 8-bp repeats (see legend to Fig. 5), while the 34-bp fragment (TGTCGATTAAATTAA-GG-T AAATTA-TAA) encompassed two repeats. We speculate that proper conformation of the conserved 7- to 8-bp sequence, as influenced by the adjoining sequence, may serve as a binding site for the putative 12-kDa protein in *sar* regulation. Whether the formation of three shifted bands with the 51-bp fragment is a result of multiple binding sites or binding of protein multimers with increasing number of repeats is not clear. Because the 34-bp sequence constituting half of the 16-bp inverted repeat plays a role in down-regulating *sarA* expression from the P1 promoter (i.e., P_{15} versus P_{12} activity in Table 2), it is likely that the 12-kDa protein is involved in repressing promoter activity from the P1 promoter by binding to the 34-bp sequence in the wild-type strain. Nevertheless, the functional sequelae as a result of the binding of this protein to the 51-bp sequence upstream of the P2 promoter region are largely undefined but are currently under investigation.

In the *araC* family of regulatory proteins, it is known that environmental parameters may affect DNA topology, which, in turn, alters the transcriptional activity of the promoter. For instance, the invasive gene *virB* of *Shigella flexneri* is activated by an AraC-like regulator called *virF* but negatively regulated by *virR*. Although VirF has a limited sequence similarity to SarA (2, 6), it remains to be determined if the 12-kDa protein is an analogous repressor while SarA serves as an its own activator. Preliminary gel shift assays with purified SarA protein suggest that it also binds to the *sar* promoter fragments in a dose-dependent manner. However, the binding sequence appears to differ from the repressor site (data not shown). Nevertheless, this speculation is preliminary and will require additional confirmatory experiments. With additional functional characterization, including binding studies of P1 and P2 promoter fragments with the 12-kDa protein and SarA and analogous in vitro transcription assays, we will be able to dissect the regulatory mechanism in the expression of *sar* in *S. aureus*.

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