JOURNAL OF BACTERIOLOGY, June 1997, p. 3963–3971 Vol. 179, No. 12
0021-9193/97/$04.00+0

sar Genetic Determinants Necessary for Transcription of RNAII and RNAIII in the *agr* Locus of *Staphylococcus aureus*

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Received 7 February 1997/Accepted 15 April 1997

The temporal expression of most virulence factors in *Staphylococcus aureus* is regulated by pleiotropic loci such as *agr* and *sar*. We have previously shown that the *sar* locus affects hemolysin production because it is required for *agr* transcription. To delineate the *sar* genetic determinant required for *agr* transcription, single copies of fragments from the *sar* locus, encompassing the individual *sar* transcripts (*sarA*, *sarC*, and *sarB*), were introduced into a *sar* mutant via the integration vector pCL84. Although a DNA fragment encompassing the *sarA* transcript plus a 189-bp upstream region was sufficient for *agr* expression, complementation analysis revealed that the *sarB* transcript was the most effective in augmenting *agr* transcription as determined by RNAII and RNAIII transcription and gel retardation assays with the P2 and P3 promoters of *agr*. As the region upstream of the *sarA* transcript encodes a 39-amino-acid open reading frame, ORF3, it is possible that posttranslational cooperation between the *sarA* gene product and ORF3 may be necessary for optimal *agr* expression. Deletion studies demonstrated that an intact *sarA* gene is essential for *agr* transcription. However, mutagenesis and in vitro translation studies revealed that unlike the *agr* locus, the required element is the SarA protein and not the RNA molecule. Taken together, these results indicate that the *sarA*-encoded protein, possibly in conjunction with peptides encoded in the upstream region, regulates hemolysin production by controlling *agr* P2 and P3 transcription.

The control of extracellular protein production in bacteria is dependent upon global regulatory systems in which a single regulatory locus controls the expression of several unlinked target genes, often in a temporal manner. Regulation of virulence determinants in *Staphylococcus aureus* has been shown to be under the control of at least two genetic loci, *agr* and *sar* (6, 15). The *agr* locus encodes two divergent transcripts (RNAII and RNAIII). The promoter P2 initiates the formation of a 3-kb transcript designated RNAII that encodes four genes (*agrA* through *agrD*), while the promoter P3 initiates the formation of a 500-bp transcript called RNAIII that codes for the δ-hemolysin (15). The *agr* operon mediates control of extracellular and surface proteins via the transcript RNAIII (26). However, in-frame deletions of any of the four genes in the RNAII coding region resulted in diminished transcription of RNAIII, thus indicating that RNAIII transcription is likely dependent on the activation of RNAII (23). We recently reported that the transcription of RNAII and RNAIII was partially dependent on the *sar* locus, which is encoded within a 1.2-kb DNA fragment that encompasses three overlapping transcripts (1, 13) (Fig. 1). These transcripts, designated *sarA* (0.58 kb), *sarC* (0.84 kb), and *sarB* (1.15 kb), have common 3′ ends but three distinct promoters. A major 339-bp open reading frame (ORF), *sarA*, together with widely spaced upstream promoter sequences, is present in this locus (7, 13).

Phenotypic analysis revealed that the *sar* locus is necessary for hemolysin production, probably mediated by interaction of the *sar* gene product(s) with the P2 promoter region of *agr*, thus leading to transcription of RNAII and, subsequently, RNAIII (6, 8, 13). To ascertain the exact *sar* genetic requirement for *agr* expression, *sar* fragments encompassing individual genes were introduced into a *sar* mutant on multicopy plasmids or as single copies integrated into the host chromosome. Analysis of *agr* transcripts as well as gel shift studies with *agr* promoters in this study demonstrated that maximal *agr* expression is dependent on an intact *sarB* transcriptional unit which encodes the *sarA* ORF and potentially two smaller putative peptides (designated ORF3 and ORF4 and consisting of 39 and 18 amino acids, respectively) (1).

In contrast to the RNA-mediated control (via RNAIII) of exoprotein synthesis by *agr* (14, 15), we also reported here that the SarA protein is responsible for modulating α-hemolysin production in *S. aureus*. Serial-deletion analysis of the *sarA* gene product in the carboxyl terminus demonstrated that this region is also necessary for *agr* transcription and the ensuing hemolysin production. Based on the sequence similarity between the *sarA* gene and a DNA-binding domain of the *virF* gene (residues 177 to 196) in *Shigella flexneri* (7, 12), an in-frame deletion of the homologous region (residues 53 to 72) was constructed and introduced into the *sar* mutant via a shuttle plasmid. Phenotypic and molecular analyses indicated that the partially deleted *sarA* gene product was transcribed and translated but was not able to reestablish *agr*-related transcription or restore hemolysin production. Taken together, our data indicated that the SarA protein, rather than the *sarA* transcript, together with the contribution of the upstream sequences from the *sarB* transcript, is required for full expression of transcription of RNAII and RNAIII as well as hemolysin production in *S. aureus.*

MATERIALS AND METHODS

Media and antibiotics. CYGP and 0.3GL media (25) were used for the growth of *S. aureus*, while Luria-Bertani broth or 2X YT (20) was used for growing *Escherichia coli*. Antibiotics were used at the following concentrations: erythromycin at 10 μg/ml, tetracycline at 5 μg/ml, chloramphenicol at 30 μg/ml, and ampicillin at 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 and sequencing strategies. DNA fragments encompassing the *sar* locus (Fig. 1 and 2) were amplified by PCR with genomic DNA of *S. aureus* strain

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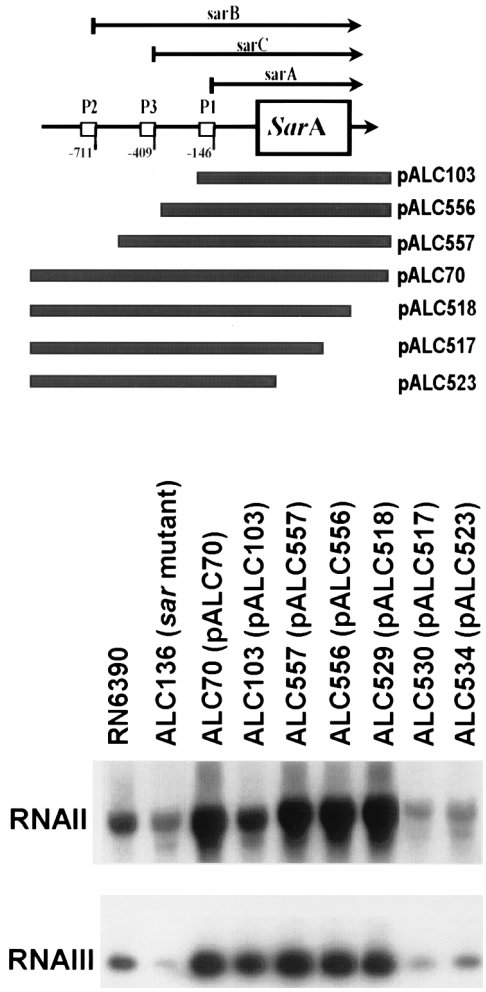

FIG. 1. Northern blots of *agr* transcripts in *sar* mutant clones carrying shuttle plasmids with *sar* fragments. RNA was obtained from cells harvested at late log phase. Ten micrograms of RNA was applied to each lane. The probes for RNAII and RNAIII were ^{32}P-labeled fragments of *agrA* (nt 3830 to 4342) and *hld* (nt 999 to 1510) (15), respectively. The parental strain RN6390 served as a positive control.

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 DNA sequencing. The PCR fragments were then cloned into pBluescript or shuttle vector pSPT181. Following transformation into *E. coli* XL1-Blue, colonies were screened for the presence of plasmids by restriction mapping and sequencing.

Genetic manipulations in *S. aureus*. Protoplast transformation of *S. aureus* RN4220 with shuttle plasmids containing various *sar* fragments was performed as previously described (6). For transduction, phage ϕ11 was used to produce phage lysates of strain RN4220 containing the modified pSPT181 shuttle vector with various *sar* fragments. The phage lysate was then used to infect the *sar* transposon mutant ALC136 as described previously (13). The presence of the correct plasmids was confirmed by restriction mapping.

To introduce a single copy of a specific *sar* fragment into the chromosome of *sar* mutant ALC136, we cleaved the *sar* fragment from a pBluescript phagemid and then cloned it into the *Pst*I-*Sal*I site of the integration vector pCL84 (18). This vector inserts preferentially into the lipase gene of the staphylococcal chromosome. Following transformation into a derivative of RN4220 (CYL316) which supplies the integrase gene in *trans*, the integrants were selected on DM3 agar (3) with tetracycline at 3 μg/ml. Loss of lipase activity in the transformants was confirmed on egg yolk agar (18). The integrated fragment was transduced into the *sar* mutant, with selection for tetracycline resistance and loss of lipase activity. Correct integration was verified by Southern blotting with lipase- and *sar*-specific probes.

Isolation of RNA and Northern analysis. To isolate RNA from *S. aureus* cells, an overnight culture of the *S. aureus* cells was diluted 1:100 in 10 ml of fresh CYGP broth containing the appropriate antibiotics and grown at 37°C. In some experiments, the cultures were grown at 33.5°C to accommodate the temperature-sensitive replicon of the shuttle plasmid. Previous studies have demonstrated that the production of hemolysins and *agr*-related transcripts at this temperature did not differ significantly from that at 37°C (13). Bacterial cells were pelleted (2,500 × *g* for 10 min at 4°C), and total cellular RNA was isolated with the FastPrep system (Bio 101, Inc., Vista, Calif.) as previously described (4). Ten micrograms of RNA was electrophoresed through a 1.5% (wt/vol) 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). 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 0.5 h), hybridized under aqueous conditions at 65°C with gel-purified DNA fragments that had been labeled with [α-^{32}P]dCTP (20), washed, and autoradiographed as described previously (8). Band intensities on Northern blots were quantitated by densitometric scanning with SigmaGel software (Jandel Scientific, San Rafael, Calif.). The values are presented as integrated area units.

Production of cell extracts and gel shift analysis. Cell extracts were prepared for strain RN6390 and the isogenic *sar* transposon mutant (ALC136) as well as for mutant clones complemented with single copies of *sar* fragments (13). In some experiments, *sar* mutant clones carrying shuttle plasmid pSPT181 with various PCR-generated *sar* fragments were also evaluated. 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 by Mahmood and Khan (19).

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 ^{32}P-end-labeled P2 (nucleotides [nt] 1621 to 1773) or P3 (nt 1539 to 1621) promoter probe was then added (15). In some assays, unlabeled P2 promoter DNA was used as a specific competitor while a 163-bp PCR fragment of the α-hemolysin gene was used as a nonspecific competitor. Following incubation at room temperature for 5 min and then on ice for an additional 5 min, the samples were electrophoresed on a 6% polyacrylamide gel in 0.25× Tris-borate-EDTA (TBE) for 2 h at 200 V. Following electrophoresis, the gels were dried and exposed to film.

Phenotypic analysis of hemolysin production by *sar* mutant clones. *Sar* mutant clones containing *sar* fragments in single copies as well as those in multicopy shuttle plasmids were tested in duplicate for hemolysin production on rabbit erythrocytes agar plates with a standard indicator strain as previously described (6). In some experiments, mutant clones carrying shuttle plasmids with the mutated *sarA* gene were also tested (see below). For immunoblots, equivalent amounts of extracellular proteins that had been concentrated 50-fold by use of Centriprep concentrators (Amicon, Beverly, Mass.) were separated on 10% sodium dodecyl sulfate (SDS)-polyacrylamide gels (17), electroblotted onto nitrocellulose (MSI, Westborough, Mass.), and probed with rabbit anti-α-hemolysin antibody (1:2,500) (a gift of B. Menzies) followed by the F(ab′)$_2$ fragment of goat anti-rabbit immunoglobulin G conjugated to alkaline phosphatase (Jackson Immunoresearch, West Grove, Pa.) (1:10,000 dilution) (5, 6). Reactive bands were visualized as described by Blake et al. (2).

Site-directed mutagenesis of the *sarA* gene. The 339-bp *sarA* gene of strain RN6390 contained within a 732-bp fragment was cloned into pBluescript to obtain single-stranded (ss) DNA from the noncoding strand to serve as a template for mutagenesis. Experimentally, the modified pBluescript phagemid was transformed into *E. coli* CJ236, and ssDNA containing uracyl instead of thymidine was produced from this strain as described by Kunkel et al. (16).

Oligonucleotides were designed to incorporate stop codons in the *sarA* ORF (oligonucleotides 293, 365, and 370) or to delete a selected amino acid region (oligonucleotide 378) as shown in Table 2.

Site-directed mutagenesis was performed (16) with the Muta-Gene in vitro mutagenesis kit (Bio-Rad, Richmond, Calif.). After construction of the mutations, the DNA was transformed into XL1-Blue for selection of newly synthesized DNA which did not contain uracil and hence contained the desired mutation. Initially, an *E. coli* mutant (*sar-a1*) which contained a nonsense mutation at codon 2 was constructed by using oligonucleotide 293. To ensure that the SarA protein was not translated, the plasmid was transduced from strain XL1-Blue back into CJ236 to construct a second-round nonsense mutation. Using uracil-containing ssDNA from the *sar-a1* mutation as a template, two additional stop mutations, *sar-a2* (oligonucleotide 365) and *sar-a3* (oligonucleotide 370), were constructed (see Fig. 6). The *E. coli* deletion mutant *sar-a4*Δ53–72 was constructed by using oligonucleotide 378 and the *sarA* gene as the template for ssDNA synthesis by a modified Kunkel method as described by Novick et al. (23).

In vitro transcription-translation. In vitro transcription-translation was performed with plasmid DNA and an *E. coli* S30 extract (Promega, Madison, Wis.) in the presence of [^{35}S]methionine (New England Nuclear, Boston, Mass.) as described by the manufacturer. Two micrograms of plasmid DNA was used for most reactions. For some reactions, an additional 1 μl of T3 RNA polymerase (New England Biolabs, Beverly, Mass.) was added to the reaction mixture to promote in vitro transcription. Reaction mixtures were incubated at 37°C for 1 h. An aliquot was removed, and proteins were precipitated with acetone. The

TABLE 1. Bacterial strains and plasmids used in this study

Strain or plasmid	Reference(s) or source	Description
S. aureus		
RN4220	24	Mutant of strain 8325-4 that accepts foreign DNA
RN6390	24	Laboratory strain that maintains its hemolytic pattern when propagated on sheep erythrocytes
CYL316	18	A derivative of RN4220 supplying the integrase gene in *trans*; the recipient strain for integration vector pCL84
ALC70	13	ALC136 carrying a 1.7-kb fragment encompassing the entire *sar* locus, including *sarA*, *sarC*, and *sarB* (pALC70)
ALC103	13	ALC136 carrying a 730-bp DNA fragment encompassing the *sarA* gene (nt 620 to 1349) (pALC103)
ALC136	7	Isogenic mutant of RN6390 carrying a *sarA*::Tn*917*LTVI mutation
ALC436	This work	ALC136 containing pSPT181 carrying the *sar-a1* mutation
ALC458	This work	ALC136 containing pSPT181 carrying the *sar-a2* mutation
ALC459	This work	ALC136 containing pSPT181 carrying the *sar-a3* mutation
ALC481	This work	ALC136 containing pSPT181 carrying the *sar-a4*Δ53–72 mutation
ALC529	This work	ALC136 with pALC518 (*sarB* lacking the transcriptional termination signal)
ALC530	This work	ALC136 with pALC517 (*sarB* lacking the C-terminal 72 bp in the *sarA* coding region)
ALC532	This work	ALC136 with pALC531 (*sarB* lacking the C-terminal 286 bp in the *sarA* coding region)
ALC534	This work	ALC136 with pALC523 (*sarB* lacking the C-terminal 139 bp in the *sarA* coding region)
ALC556	1	ALC136 with pALC556 carrying *sarA* plus a 189-bp upstream fragment
ALC557	1	ALC136 with pALC557 carrying *sarC* and *sarA*
ALC599	This work	ALC136 with *geh*::pCL84
ALC600	This work	ALC136 with *geh*::pALC555 (carrying a single copy of *sarA*)
ALC601	This work	ALC136 with *geh*::pALC564 (carrying a single copy of *sarACB*)
ALC834	This work	ALC136 with *geh*::pALC814 (carrying a single copy each of *sarA* and *sarC*)
ALC835	This work	ALC136 with *geh*::pALC813 (carrying a single copy of *sarA* plus a 189-bp upstream fragment)
E. coli		
CJ236	16	*dut ung* strain for production of uracil-containing DNA
XL1-Blue	20	Highly transformable strain
Plasmids		
pCRII	Invitrogen	*E. coli* vector for direct cloning of PCR fragments
pSPT181	14	Shuttle vector
pCL84	18	*S. aureus* integration vector that inserts into the lipase gene (*geh*)
pBluescript	Stratagene	*E. coli* cloning vector
sar-a1	This study	pBluescript containing the *sar-a1* mutation
sar-a2	This study	pBluescript containing the *sar-a2* mutation
sar-a3	This study	pBluescript containing the *sar-a3* mutation
pALC70	1, 13	pSPT181 carrying the complete *sar* locus (*sarA*, *sarC*, and *sarB*)
pALC517	This work	pSPT181 carrying a *sarB* fragment as in pALC70 but lacking the transcriptional terminal signal as well as the C-terminal 72 bp in the *sarA* coding region
pALC518	This work	pSPT181 carrying a *sarB* fragment as in pALC70 but lacking the transcription termination signal
pALC523	This work	pSPT181 carrying a *sarB* fragment with a 139-bp deletion in the carboxyl terminus of the *sarA* coding region
pALC531	This work	pSPT181 carrying a *sarB* fragment with a 286-bp deletion in the carboxyl terminus of the *sarA* coding region
pALC555	This work, 1	Integration vector pCL84 containing only the *sarA* coding region (nt 620 to 1349)
pALC556	1	pSPT181 carrying *sarA* plus a 189-bp upstream sequence but lacking the *sarC* promoter
pALC557	1	pSPT181 carrying *sarC* and *sarA*
pALC564	This work	Integration vector pCL84 containing *sarA*, *sarC*, and *sarB*
pALC813	This work, 1	Integration vector containing *sarA* plus a 189-bp upstream sequence (nt 531 to 1349)
pALC814	This work	Integration vector containing *sarA* and *sarC* transcriptional units

samples were then resolved by SDS–12% polyacrylamide gel electrophoresis (PAGE) (17). The gels were dried and exposed to XAR film (Kodak, Rochester, N.Y.) for 24 to 48 h.

RESULTS

Determination of the *sar* genetic requirement for *agr* expression. In previous studies, we determined that the control of hemolysin production by the *sar* locus is partially mediated via the *agr* locus (13). In particular, the production of *agr* transcripts RNAII and RNAIII within *agr*, while diminished in *sar* mutants, was enhanced by the introduction of a 1.7-kb fragment which encodes the *sarA* gene plus adjacent upstream sequences (13). Transcriptional analysis revealed that the 1.7-kb DNA fragment is composed of three overlapping *sar* transcripts (*sarA*, *sarC*, and *sarB*, with sizes of 0.56, 0.8, and 1.2 kb, respectively) originating from three distinct promoters (1). To delineate the *sar* genetic requirement for *agr* expression, *sar* fragments were introduced via shuttle plasmids into *sar* mutant ALC136, and this was followed by Northern analysis for *agr*-related transcription. As expected, the levels of RNAII and RNAIII were diminished in the *sar* mutant compared to the parent (Fig. 1). With the introduction of DNA fragments encoding only the *sarA* gene on shuttle plasmid pSPT181 (pALC103), the transcription of RNAII (Fig. 1) and RNAIII was increased to near parental levels compared to the *sar* mutant (ALC136). In clones with larger fragments encoding *sarC* (pALC557), *sarB* (pALC70), or *sarA* plus a 189-bp upstream sequence but lacking the *sarC* promoter (pALC556), additional enhancement of RNAII and RNAIII levels was observed.

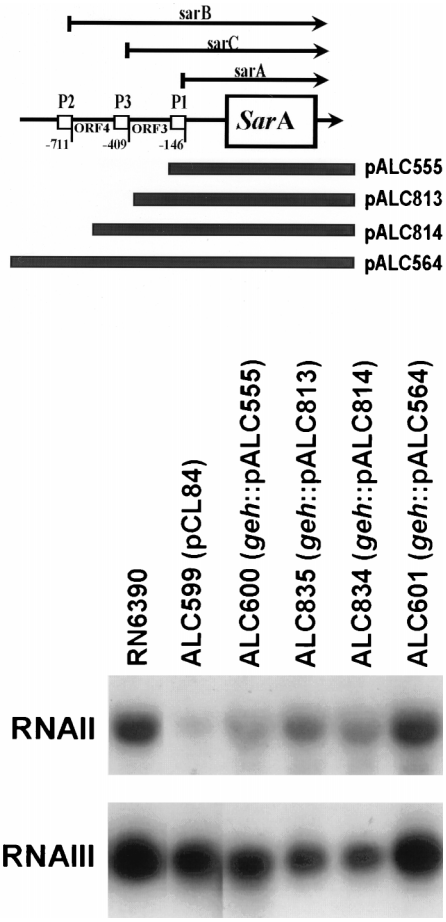

FIG. 2. Northern blots of *agr* transcripts in *sar* mutant clones with chromosomal integration of pCL84 carrying single copies of *sar* fragments in the lipase (*geh*) gene. RNA was obtained from cells harvested at late log to early stationary phase. The bands were quantitated by densitometric scanning, with the data being presented as integrated area units as follows: RNAII values of 7,920, 1,001, 3,154, 5,359, 4,538, and 11,370 and RNAIII values of 11,898, 9,989, 9,256, 6,694, 6,212, and 11,567 for strains RN6390, ALC599, ALC600, ALC835, ALC834, and ALC601, respectively.

To avoid the problem of increased gene dosage associated with a multicopy shuttle plasmid in *agr* expression, we introduced single copies of various *sar* fragments into the *sar* mutant strain ALC136 via the integration vector pCL84, which preferentially inserts into the lipase gene of the staphylococcal chromosome. Northern blot analyses confirmed that these *sar* mutant clones (ALC835, ALC601, and ALC834) expressed the

TABLE 2. Sequences of oligonucleotides used for incorporation of stop codons into the *sarA* ORF

Oligonucleotide	Sequence
293	5′ TGA TTT TTG TAA TTC ACA TGT TTA AA 3′ 19 1
365	5′ ATC ATT GAT TTA TTA AAT TCA CAT GT 3′ 24 1
370	5′ CTC TTT GTT TTA GCT GAT GTA TGT 3′ 144 121
378	5′ TTG AGA TAA AAT ATA GTA TTC TTT C 3′ 226 217 154 144

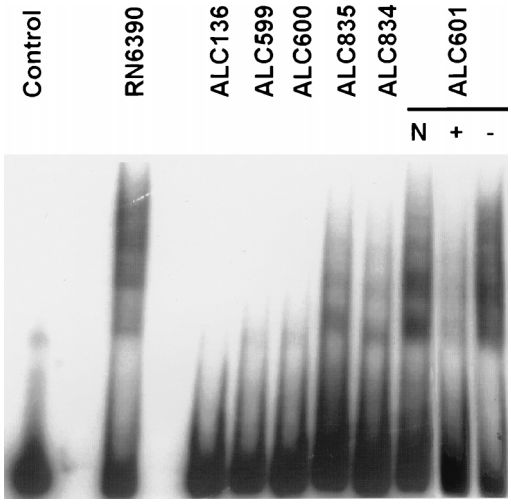

FIG. 3. Effect of cell extracts of *sar* mutant clones (with chromosomally integrated *sar* fragments) on the mobility of a radiolabeled P2 promoter fragment. The P2 promoter fragment (nt 1621 to 1773) (15) was amplified by PCR and cloned into pCRII. The fragment was gel purified and end labeled with ^{32}P. Four micrograms of cell extract was applied to each lane. The designated strains are as follows: RN6390, parent; ALC136, *sar* mutant; ALC599, *sar* mutant with a single copy of integration vector pCL84; ALC600, *sar* mutant with pCL84::*sarA*; ALC835, *sar* mutant with pCL84::*sarA* plus a 189-bp upstream region; ALC834, *sar* mutant with pCL84::*sarC*; ALC601, *sar* mutant with pCL84::*sarB*. Control, P2 fragment without cell extract; N, no competitor; +, 100 ng of cold P2 fragment; −, 100 ng of a 163-bp PCR fragment of the *S. aureus* α-hemolysin structural gene.

sarA, *sarB*, and *sarC* transcripts, respectively (data not shown). However, in contrast to the multicopy counterparts, only the clone (ALC601) containing a single copy of the largest *sar* transcript (*sarB*) produced parental levels of RNAII and RNAIII (Fig. 2). Although clones (ALC834 and ALC835) carrying a single copy of *sarC* or *sarA* plus a 189-bp upstream fragment exhibited higher levels of RNAII than the mutant control (ALC599), their levels did not approach that of the parent (Fig. 2). In addition, these two clones did not appear to augment RNAIII transcription in the *sar* mutant. However, unlike its multicopy counterpart (ALC103 [Fig. 1]), the mutant clone containing only a single copy of the *sarA* gene (ALC600) did not show a significant increase in RNAII transcription compared to the mutant control (ALC599). Taken together, these data demonstrate that the *sar* gene dosage has an appreciable effect on transcription of RNAII and, to some degree, RNAIII. They also imply that sequences upstream from the *sarA* transcript probably play a role in *agr* expression.

As the P2 promoter in the *agr* operon initiates the transcription of RNAII (23), which in turn is necessary for the transcription of RNAIII, the modulation of RNAII and RNAIII transcription by *sar* fragments at physiological levels suggested that the *sar* gene products might play an analogous role, either directly or indirectly, in *agr* promoter activation. In gel shift studies using equivalent amounts of cell extracts (4 μg each) and a radiolabeled P2 promoter (nt 1621 to 1773), retardation of P2 probe mobility occurred most readily with extracts derived from clones containing *sarB* (ALC601) and *sarA* plus a 189-bp upstream fragment but lacking the *sarC* promoter (ALC835) (Fig. 3). Interestingly, extracts from the clone carrying *sarC* (ALC834) have less of a gel retardation effect than clones containing *sarA* plus the 189-bp fragment. With extracts from three clones (ALC835, ALC834, and ALC601) at increasing protein concentrations (2 to 20 μg), it could be demonstrated that the differential effect on gel shift ability among

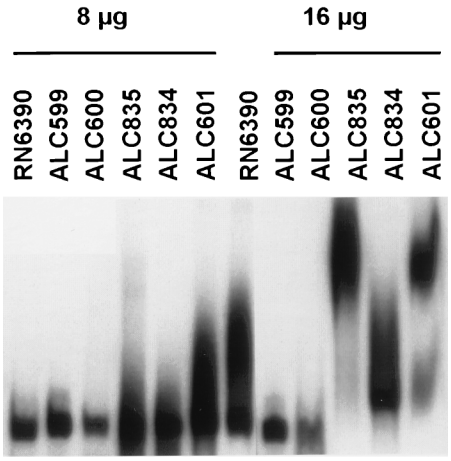

FIG. 4. Effect of extracts of *sar* mutant clone cells on the mobility of a radiolabeled P3 promoter fragment. The P3 fragment (nt 1539 to 1621) (15) was amplified by PCR and cloned into pCRII. The gel-purified fragment was end labeled with ^{32}P. More cell extract was required to retard the mobility of the P3 promoter fragment. Strain designations are listed in the legend to Fig. 3.

these clones persisted at higher extract concentrations, with the clone encompassing all three transcripts (ALC601) having the greatest effect on P2 mobility (data not shown). Remarkably, the extract from clone ALC600, which encompassed only the *sarA* gene, did not retard the mobility of the P2 promoter even at high protein concentrations (up to 20 μg). Since P2 lies adjacent to the P3 promoter in the *agr* operon (15), we also explored the effect of these cell extracts on a labeled P3 promoter probe (Fig. 4). Utilizing a shorter P3 promoter fragment (nt 1539 to 1621) than the one we previously reported (13), we found results analogous to those obtained with the *agr* P2 promoter, albeit at a higher extract concentration (Fig. 4). More specifically, extracts derived from *sar* mutant clones containing *sarB* (ALC601), *sarC* (ALC834), or *sarA* plus the 189-bp upstream fragment (ALC835) retarded the mobility of a P3 promoter probe. As was the case with the P2 promoter, the extract derived from the clone containing only a single copy of the *sarA* gene without any additional upstream sequence (ALC600) had no effect. Taken together, these data, in conjunction with those from Northern analysis, may indicate that the gene products encoded by *sarB* are required for optimal *agr* expression at normal gene dosage.

In previous studies, we postulated that the *sar* gene products likely augment α-hemolysin (*hla*) production by increasing *agr* expression. It has been shown that transcription of RNAIII promotes α-hemolysin production at both the transcriptional (29) and translational (21) levels. To assess the role of these *sar* fragments at single-copy gene dosages in the regulation of *hla* transcription, we performed Northern analysis on several of the strains noted above with an *hla* probe. As predicted, the level of *hla* transcription in the *sar* mutant ALC136 was lower than that in the parent (Fig. 5). However, only the clone encompassing the largest gene (*sarB*) was capable of restoring *hla* transcription in the mutant to the parental level, while those carrying smaller *sar* fragments (ALC835 and ALC834) had a modest effect. Surprisingly, the clone encoding *sarA* alone without the adjacent upstream sequences (ALC600) exhibited an increase in *hla* transcription compared to the mutant (ALC136). The data from immunoblot studies with an anti-α-hemolysin antibody were also consistent with these results (data not shown).

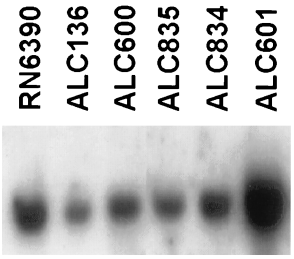

FIG. 5. Northern blot of α-hemolysin transcripts of *sar* mutant clones with chromosomal integration of pCL84 carrying single copies of *sar* fragments. The probe was a 3-kb *Eco*RI-*Hin*dIII fragment of the α-hemolysin gene (8).

Evaluation of *sarA* mutants with nonsense mutations. Sequence analysis revealed that the *sarA* gene is the largest ORF within the *sarB* transcriptional unit (1). In previous studies, we have shown that the *sarA* gene encoded within a multicopy plasmid can promote hemolysin production in *S. aureus* by increasing RNAII transcription (7, 13). However, the exact mode of control by the *sarA* gene product is not clearly defined. Morfeldt et al. (21) and Novick and colleagues (26) independently reported that the *agr* locus, unlike other regulatory paradigms in most prokaryotes, exerts its regulatory effect via an RNA molecule (i.e., RNAIII) (21), presumably by directly interacting with the α-hemolysin mRNA to activate translation (21). To ascertain whether a similar regulatory mechanism is employed by the *sarA* gene product, we created in *E. coli* a pair of mutants (*sar-a2* and *sar-a3*) with at least two nonsense mutations near the amino terminus of the *sarA* gene (Fig. 6). After transforming the recombinant shuttle plasmids into *S. aureus* RN4220, the plasmids were transduced into the *sar* mutant strain ALC136. Northern analysis with a 732-bp *sarA* probe demonstrated that both mutants (ALC458 and ALC459) were able to synthesize transcripts similar in size to the native *sarA* transcript (560 bp) as found in the parental strain RN6390 or the *sar* mutant complemented with the wild-type *sarA* gene (ALC103). However, the *sarA* transcript level in strain ALC458 (carrying the *sar-a2* mutation) was significantly lower than that of strain ALC459 (with the *sar-a3* mutation) or strain ALC103 containing an intact *sarA* gene in *trans* (Fig. 6a). Notably, the amount produced in mutant ALC458 approximated the wild-type (RN6390) level.

To determine whether these transcripts were able to direct the synthesis of the SarA protein, we looked for [^{35}S]methionine-labeled translation products from *E. coli* plasmid templates while using an *E. coli* S-30 extract system for in vitro coupled transcription-translation. Autoradiographs of SDS-PAGE gels containing the resolved proteins revealed an intense band corresponding to the mobility of the predicted 13.5-kDa SarA protein (Fig. 7), as deduced from the construct carrying the wild-type *sarA* gene. Neither of the two plasmids containing nonsense mutations yielded any SarA protein. Notably, a slightly larger protein band (~15 kDa) of unknown origin was present in all protein preparations, including those of the plasmid vector alone.

As the *sarA* gene enhanced α-hemolysin production at the transcriptional level (13), we examined the transcription of this gene in the *sar* mutant complemented with a plasmid carrying the *sarA* gene with the nonsense mutations. By using densitometric scanning of individual bands on Northern blots, it could be shown that the production of α-hemolysin was not detectable in these two clones (ALC458 and ALC459) whereas the parental strain or the *sar* mutant complemented with the native *sarA* gene was able to secrete α-hemolysin (Table 3). As the

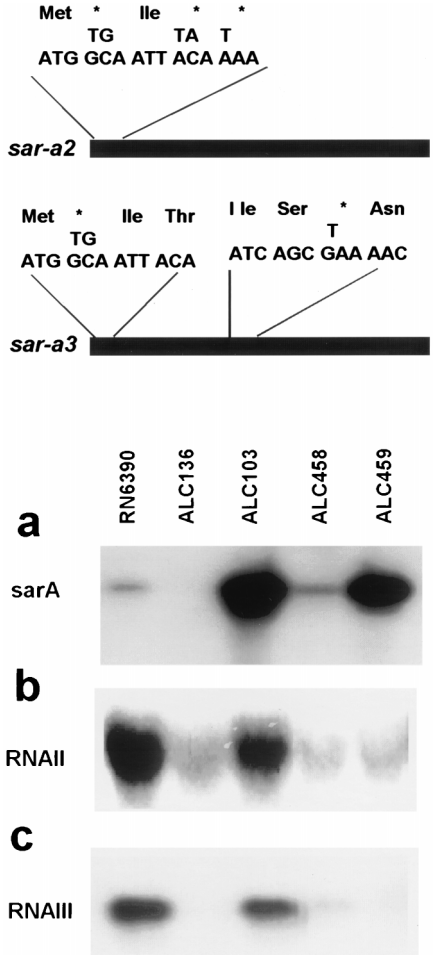

FIG. 6. Northern blots of *sar* mutant clones carrying plasmids with nonsense mutations in the *sarA* coding region. Ten micrograms of RNA was applied to each lane, and the membrane was probed with a ^{32}P-labeled 732-bp fragment encompassing the *sarA* gene (a), a 513-bp *agrA* probe (b), or a 512-bp fragment encompassing the *hld* gene (c). The *agr* probes were the same as those used in the experiment shown in Fig. 1. Strains ALC458 and ALC459 are derivatives of *sar* mutant ALC136 carrying shuttle plasmids with *sar-a2* and *sar-a3* mutations, respectively.

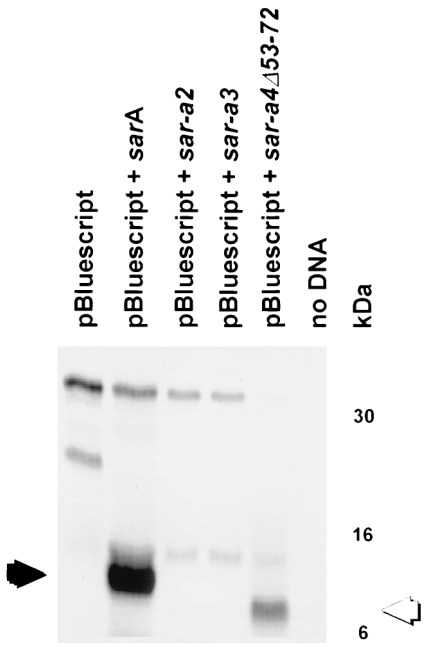

FIG. 7. SDS-PAGE of proteins produced by an in vitro transcription-translation assay. Proteins in 5-μl reaction mixtures were precipitated with acetone, resuspended in denaturing sample buffer, boiled, and loaded on a 12.5% acrylamide gel. After electrophoresis, the gel was dried and exposed to XAR film. The closed arrow indicates the band corresponding to the SarA protein; the open arrow indicates the position of the truncated SarA protein translated from the plasmid with the deletion from *sar-a4*Δ53–72. The mobilities of the molecular mass markers are shown on the right.

synthesis of α-hemolysin in *S. aureus* is regulated by the *agr* system (15, 29), we also assayed for transcription of RNAII and RNAIII in these clones. In Northern analyses, the level of transcription of RNAII and RNAIII was distinctly lower in *sar* mutants carrying plasmids with the altered *sarA* genes than in either the wild type or the *sar* mutant complemented with an intact *sarA* gene (Fig. 6 and Table 3).

Deletion analysis of the *sarA* gene. In a recent study, we have shown that the SarA protein from parental strain RN6390, despite lacking the 11 C-terminal amino acids of the strain DB protein (10), can still reestablish hemolysin production in a *sar* mutant (1). We thus reasoned that the 11 C-terminal amino acids of the SarA protein in strain DB are probably not required for *sar*-related functions. As an extension of this observation, we introduced into the mutant *sar* fragments encoding *sarB* but with progressive deletion of the *sarA* gene in the carboxyl terminus (ALC529, ALC530, ALC534, and ALC532). With a deletion as small as 72 bp in the carboxyl terminus of the *sarA* gene of strain RN6390 (ALC530), the recombinant shuttle plasmid carrying this mutation conferred a *sar* null phenotype and hence failed to activate *agr*-related transcription in a *sar* mutant (Fig. 1). In contrast, a clone (ALC529) that encompassed a *sarB* fragment with an intact *sarA* coding region but lacking the transcription termination signal was able to reestablish *hla* and *agr* transcription in the *sar* mutant.

The amino acid sequences of SarA and the DNA-binding protein VirF of *Shigella flexneri* were compared, and a region of sequence similarity involving the putative DNA-binding domain of VirF was found (9, 10).

```
SarA (RN6390)  53 FKDIINHLNYKQPQVVKAVK  72
                    :: :.::  .  : : ..
VirF          177 LSDISNNLNLSEIAVRKRLE 196
```

To assess the biological significance of this domain in SarA, we constructed an in-frame deletion of this region in *sarA*. North-

TABLE 3. Northern analysis of *sar* nonsense and deletion mutants[a]

Strain (relevant genotype)	Transcript level of:		
	α-Hemolysin[b]	RNAII	RNAIII
RN6390	6,140	11,217	7,368
ALC136 (*sar* mutant)	263	4,477	ND[c]
ALC103 (ALC136 + *sarA*)	1,097	7,806	6,213
ALC458 (*sar-a2*)	ND	1,387	717
ALC459 (*sar-a3*)	ND	1,761	ND
ALC481 (*sar-a4*Δ53–72)	630	3,176	2,181

[a] RNA was obtained from cells harvested at late log phase. Band intensities on Northern blots were quantitated by densitometric scanning with SigmaGel software (Jandel Scientific, San Rafael, Calif.). The values are presented as integrated area units.

[b] α-Hemolysin production was confirmed by streaking colonies on rabbit erythrocyte agar plates, with a specific indicator strain as the standard (7). Additionally, immunoblots of extracellular protein probed with rabbit anti-α-hemolysin antibody also verified these results.

[c] ND, not detectable.

ern blot analysis revealed that the partially deleted *sarA* gene, when inserted into the *sar* mutant, was actively transcribed, yielding a transcript of the appropriate size (data now shown). In vitro linked transcription-translation using an *E. coli* S30 extract and an *E. coli* plasmid template carrying the altered *sarA* gene revealed a translated protein product with the predicted molecular mass (~11 kDa) (Fig. 7). The partially deleted *sarA* gene was ligated to the shuttle vector pSPT181, transformed into *S. aureus* RN4220, and then transduced into the *sar* mutant ALC136. Northern blot analysis revealed that the *hla* mRNAs were transcribed at a significantly lower level in this clone (ALC481) than in either the parental strain or the *sar* mutant carrying an intact *sarA* gene (Table 3). Likewise, the synthesis of two *agr* transcripts, RNAII and RNAIII, was decreased in this mutant compared to the parent, RN6390 (Table 3).

DISCUSSION

Expression of virulence determinants in *S. aureus* is controlled by at least two pleiotropic loci, i.e., *sar* and *agr*. In an earlier study, we established that the *sar* locus encodes a *trans*-acting molecule(s) which is required for the expression of RNAII from the P2 promoter of the *agr* operon (13), thereby stimulating α-hemolysin production. Mapping studies have demonstrated that the *sar* locus is composed of three overlapping transcripts within a 1.2-kb DNA fragment which encodes the 339-bp *sarA* ORF plus an extensive 800-bp upstream promoter region containing three widely spaced but tandemly arranged promoters (1). To delineate the *sar* genetic requirement for *agr* transcription, we assessed transcription of RNAII and RNAIII in strains carrying various segments of the *sar* locus in single copies. In contrast to the multicopy-plasmid experiment, in which the *sarC* gene products could efficiently restore transcription of RNAII and RNAIII (Fig. 1), only the clone containing a single copy of the *sarB* transcriptional unit, which also encompassed both *sarA* and *sarC* transcripts was able to reestablish transcription of RNAII and RNAIII to near parental levels in the *sar* mutant (Fig. 2).

The modulation of RNAII and RNAIII by the *sarB* gene products at the normal gene dosage suggested that these gene products, when expressed at physiological levels, may interact, either directly or indirectly, with the *agr* promoter to regulate the *agr* response. This premise was confirmed by gel shift studies of labeled *agr* P2 promoter fragments with cell extracts of *sar* mutant clones containing single copies of *sar* fragments. At equivalent protein-extract concentrations, the gel retardation effect with the *agr* P2 promoter was most pronounced with the cell extract derived from the clone containing *sarB*, the largest *sar* transcript (Fig. 3). This cell extract also retarded the mobility of a labeled *agr* P3 promoter in a similar fashion. These data contrast with those of our earlier study, in which the cell extract of a *sar* mutant clone carrying a 1.7-kb fragment encoding the entire *sar* locus was able to alter the mobility of the P2 promoter but not that of the P3 promoter (13). The discrepancy may be attributable to the observation that the P3 promoter (nt 1539 to 1621) utilized in this study was shorter than the one (nt 1539 to 1660) previously employed. Conceivably, the presence of secondary structure within a larger DNA fragment may have interfered with the interaction of *sar* gene products with the P3 promoter fragment. The interaction of *sar* gene products to the P3 promoter is consistent with data of Morfeldt et al. (22) in which a DNA fragment encompassing both P2 and P3 promoters (nt 1605 to 1721) was found to bind the SarA protein. However, in comparing equivalent protein extracts from these clones (ALC835, ALC601, and ALC834), the gel retardation effect on the P2 promoter was more pronounced than that on the P3 promoter (2 versus 8 μg for protein required for gel shifts from clone ALC601 for the P2 and P3 promoters, respectively). These data, in conjunction with those from Northern analyses for RNAII and RNAIII in respective clones (Fig. 2), implied that the *sarB* gene products probably bind to the P2 promoter with a higher affinity than they do to the P3 promoter. The observation that the reduction in RNAII transcription in a *sar* mutant was more striking than that of RNAIII (Fig. 2) may imply that there is an additional regulatory factor(s) controlling the transcription of RNAIII as suggested by Vandenesch et al. (29).

The *sarB* transcription unit encompasses the *sarA* ORF together with an extensive 800-bp upstream region (Fig. 1) (1). A scrutinization of the 800-bp upstream sequence revealed two regions between the P1 and P2 promoters that may code for small peptides of 39 (ORF3) and 18 (ORF4) amino acids, respectively. Due to the overlapping nature of a parallel multipromoter system, ORF3 is contained within the *sarC* and *sarB* transcripts while ORF4 is encoded only by the *sarB* transcriptional unit (Fig. 2). Since the extracts yielding *sarB* gene products (as in clone ALC601) were better able to bind to the *agr* promoter than those encoding *sarC* (ALC834) and *sarA* plus the 189-bp upstream fragment (ALC834) (based on the gel shift data shown in Fig. 3 and 4), it is conceivable that post-translational cooperation among the SarA protein and the ORF3 and ORF4 peptides may occur at physiological levels to influence *agr*-related transcription.

As reported previously, the expression of individual transcripts within the *sar* locus is growth cycle dependent (1). In particular, the *sarB* (encoding SarA, ORF3, and ORF4) and the σ^A-dependent *sarA* transcripts are more abundant during the early and mid-log phases and taper off toward stationary phase (1). In contrast, the *sarC* transcriptional unit (encoding SarA and ORF3) carries a σ^B-dependent promoter which, like other σ^B promoters, is most active during stationary phase (1, 11). The growth phase dependency of *sar* suggests the occurrence of a complex interaction between specific *sar* products and the *agr* promoter during the growth cycle in the regulation of the *agr* response. Predicated upon a pattern of *sar* activation in which the expression of *sarC* coincides with the tapering off of *sarB* transcription (1), we speculate that the *sarB* gene products (e.g., ORF4) may serve to modulate the transcription of *sarC*. This premise is supported in part by the observed increase in *sarC* transcription in a *sar* mutant clone carrying *sarB* with a deletion in ORF4 (unpublished data). As *agr*-related transcription is maximal during the postexponential phase (15), we further hypothesize that gene products of *sarC* (i.e., SarA and ORF3), possibly regulated by *sarB*, may be expressed at optimal levels during stationary phase to modulate RNAII transcription. Preliminary gel shift studies revealed that *sar* mutant clones in which ORF3 was deleted from the *sarB* transcriptional unit exhibited lower levels of gel shift activity with a labeled P2 promoter of *agr* (unpublished observations). Additionally, *sar* mutant clone ALC835, which exhibited gel shift activity with the *agr* P2 and P3 promoters, also encompassed ORF3 as well as the *sarA* coding region. Although the *sar* fragment encoded by the recombinant integration vector pALC813 (in ALC835) did not include the putative *sarC*-specific promoter, we have not ruled out the possibility that this putative peptide coding region may be transcribed and translated from adjacent active promoters within the integration vector. Nevertheless, the likelihood that differences in the RNA structures of individual *sar* transcripts may influence the transcription of *agr*-related and other target genes, as has been reported for RNAIII on the *hla* mRNA, cannot be entirely

eliminated (21). Alternatively, it is also conceivable that due to the overlapping nature of the *sar* transcriptional system, the level of *sarA* expression controlled by three upstream promoters that are active during different parts of the growth cycle may play an important role in modulating RNAII and RNAIII transcription.

Based on the data from this and prior studies (7), it is evident that the major ORF, *sarA*, within the *sarB* transcriptional unit is essential for *agr*-dependent regulation of hemolysin production. While the *sarA* gene with increased dosage (ALC103) promoted RNAII transcription in a *sar* mutant, a single copy of the *sarA* gene (ALC600) did not, nor did it manifest gel shift activity with the *agr* P2 promoter. This discrepancy may be related to the gene dosage effect. It is also plausible that the *sarA* gene product, which is present at a low concentration in the cell, interacts with the *agr* promoter with a low affinity. Alternatively, it is conceivable that the peptide encoded within the upstream sequence of *sarB* (i.e., ORF3) positively modulates the interaction of the *sarA* gene product with the *agr* promoter to increase expression of RNAII and RNAIII.

To ascertain whether the *sar* locus, like the *agr* locus, controls α-hemolysin production via an RNA regulatory molecule, two *sar* mutant clones with in-frame stop codons within the *sarA* gene carried on a multicopy plasmid were evaluated for transcription of *hla* and *agr* P2 and P3. These mutations contained either two (*sar-a3*) or three (*sar-a2*) stop codons because previous results with strain ALC436 containing a single nonsense mutation (TGA) at codon 2 did not show complete disruption of the translation of the SarA protein (unpublished data). Northern analysis revealed that the mutant clones containing the *sar-a2* mutation, with three stop codons in close proximity near the amino terminus, had a lower *sarA* transcript level than those containing the mutation (*sar-a3*) with stop codons spaced widely apart. Conceivably, the closely spaced nonsense mutations in *sar-a2* may have contributed to a mild degree of transcript instability. Nevertheless, it should be stressed that the mutated *sarA* genes were transcribed at or above parental levels in their respective clones (ALC458 and ALC459 in Fig. 6), thus implying that the altered transcript was relatively stable. As the putative −10 (TATAAT) and −35 (TTTACT) core promoter boxes of the *sarA* transcript correspond to the canonical hexamers for σ^{70}-dependent promoters derived from *E. coli* (1, 30), we utilized an in vitro coupled transcription-translation system from *E. coli* (S30 extracts) to show that the clone with the wild-type *sarA* gene in pBluescript was capable of synthesizing a SarA protein of the correct molecular size while a clone with a similar plasmid carrying either an *sar-a2* or an *sar-a3* mutation was not (Fig. 7). Concomitant with a lack of translation, the incorporation of multiple stop codons also abolished the capacity of the *sarA* gene to reestablish parental levels of *agr*-related as well as *hla* transcription in the *sar* mutant when these mutant alleles were reintroduced into the mutant strain ALC136. In contrast to the results with *agr*, this finding strongly demonstrates that the *sarA*-encoded protein, but not the corresponding RNA molecule, is essential for controlling hemolysin production. This result concurs with that of Morfeldt et al., who demonstrated that the SarA protein was able to bind magnetic beads to which the *agr* promoter had been coupled (22).

In comparing the *sarA* genes of strains DB and RN6390, it was evident that the 11 C-terminal amino acids in the strain DB gene are absent from the gene of strain RN6390 as a result of a stop codon (TGA) at residue 114 in RN6390 (1). Complementation analysis indicated that the absence of the 11 C-terminal residues in the SarA protein of RN6390 does not affect the functioning of this protein in *agr* activation. With the exception of residue 53 (a leucine in strain DB and a phenylalanine in RN6390), the remainder of the SarA protein sequence is highly conserved (1). By means of serial-deletion studies involving the carboxyl terminus of the *sarA* gene of strain RN6390, we have shown that this region is critical for the functional integrity of the *sarA* gene, because a 72-bp deletion in the carboxyl terminus led to a null *sar* phenotype with regard to *agr* and *hla* activation.

Previous analyses of the *sarA* gene sequence did not reveal an identifiable helix-turn-helix motif, nor is there homology with the two-component regulatory systems (7). Comparison of the sequences of SarA and the VirF protein of *Shigella flexneri* suggested that residues 53 to 72 of SarA have sequence similarity to the DNA-binding domain (residues 177 to 196) of the VirF protein (27). Possibly, this region of the SarA protein somehow participates in the DNA-binding activity to the P2 promoter region of *agr*, thereby increasing RNAII transcription (13). In support of this hypothesis is the finding that an in-frame deletion of this region, while not preventing successful transcription and translation (Fig. 7), diminished the ability of the SarA protein to partially restore RNAII and RNAIII transcription to the *sar* mutant (strain ALC481 in Table 3) compared to the intact *sarA* gene on a shuttle plasmid. Interestingly, the region of SarA (residues 53 to 72) that exhibits sequence similarity to the VirF DNA-binding domain contains a higher percentage of positively charged residues than the remainder of the protein (25% versus 18%). Whether the preponderance of positively charged residues in this region implies that it is a binding domain for the negatively charged DNA molecules remains to be determined.

ACKNOWLEDGMENTS

We thank Chia Lee for providing the plasmid pCL84, Barbara Menzies for supplying anti-α-hemolysin antibody, Steve Projan for helpful suggestions for the manuscript, and Kelly Eberhardt for expert technical assistance.

This work was supported in part by grants-in-aid from the American Heart Association and the New York Heart Association and by NIH grants AI30061 and AI37142. J.H.H. is supported in part by a Participatory Laboratory Award from the New York Heart Association, and M.G.B. is supported in part by a Norman and Rosita Winston Fellowship. A.L.C. is a recipient of the Irma T. Hirshl Career Scientist Award as well as the AHA-Genentech Established Investigator Award from the American Heart Association.

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