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Cloning and Sequencing of *sarA* of *Staphylococcus aureus*, a Gene Required for the Expression of *agr*

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To evaluate the effect of a *sar* mutation on the *agr* locus, Northern (RNA) blotting was performed to determine the levels of RNAIII, the *agr* regulatory molecule, in two isogenic pairs of *Staphylococcus aureus* strains. Our results demonstrated that RNAIII was either significantly diminished or absent in both *sar* mutants compared with the parents. The RNAIII level was partially restored in *sar* mutants complemented with an intact *sar* gene (designated *sarA*). Additionally, we were able to complement selected *sar* phenotypes with a plasmid carrying RNAIII (pRN6735). These studies suggest that the *sarA* gene is necessary for the optimal expression of *agr*. The *sarA* gene of strain RN450 was subsequently cloned and sequenced. Sequence analysis revealed an open reading frame of 372 bp with a predicted molecular size of 14,718 Da and a deduced pI of 8.52. The deduced protein sequence has a predominance of charged residues (33%) and shares sequence similarity with the *virF* gene of *Shigella flexneri*.

Staphylococcus aureus is a major human pathogen that has the ability to produce a variety of extracellular and cell wall-associated proteins, many of which are involved in pathogenesis (6). In vitro, most of these exoproteins are usually synthesized and secreted in the postexponential phase (6). However, synthesis of a number of surface-associated proteins (protein A, coagulase, fibrinogen, and fibronectin-binding proteins) that clearly play a role in infection is repressed postexponentially (6, 11).

In *S. aureus*, the postexponential-phase regulation of virulence determinants and other exoprotein genes involves at least three global regulatory systems, *agr*, *xpr*, and *sar* (3, 11, 20). Most of the exoprotein regulated by *agr* are either not synthesized or synthesized at a reduced rate in *agr* mutants, while the synthesis of surface proteins is upregulated (11). The *agr* locus has been cloned (11) and found to consist of at least five genes, *agrA*, *agrB*, *agrC*, *agrD*, and the *hld* (δ-hemolysin) gene. Sequence analysis indicated that it has features suggestive of a two-component regulatory system as described in other procaryotes (11). In particular, the *agrB* is the signaling component, while *agrA* corresponds to the transcription activation element (11, 14). The *agr* locus is composed of two divergent transcription units designated RNAII (*agrA*, *-B*, *-C*, and *-D* genes) and RNAIII (*hld* gene). Mutation in either *agrA* or *agrB* has led to decreased transcription of RNAIII (11, 16). RNAIII, which also encodes the 26-residue δ-hemolysin polypeptide, is essential for the transcriptional control of exoprotein synthesis (e.g., α hemolysin) (11).

A second locus, termed *xpr*, was recently identified by Tn*551* insertion into the staphylococcal chromosome. Northern (RNA) blot studies indicated that the *xpr* locus regulates exoprotein synthesis at the mRNA level (9). Interestingly, both *xpr* and *agr* mutants produced greatly reduced amount of δ-hemolysin. This finding together with the observation that the RNAIII level is decreased in an *xpr* mutant suggest that the *xpr* and *agr* loci may behave as interactive regulatory genes (9, 20).

We recently reported an additional locus in *S. aureus*, designated *sar*, that is involved in the regulation of exoproteins (3). Inactivation of this locus by Tn*917*LTV1 insertion has resulted in decreased expression of several extracellular (e.g., β-hemolysin) and cell wall proteins (3). Phenotypic, Southern blot, and genetic mapping analyses indicated that this locus is distinct from *agr* and *xpr* (3, 4). Using the DNA sequence flanking the Tn*917*LTV1 insertion as a probe, we have subsequently cloned and sequenced the *sar* gene that is involved in the regulation of exoprotein synthesis. Additional transcriptional and phenotypic studies revealed that this *sar* gene is necessary for the optimal expression of *agr*.

Bacterial strains, plasmids, and phage. The bacterial strains and plasmids used in this study are listed in Table 1. Phage ϕ11 was used as a transducing phage for strains RN4220, RN450, and RN6390 (3). The following media were used for bacterial growth: CYGP broth for *S. aureus* (15) and Luria-Bertani broth for *Escherichia coli* (13). Antibiotics were used at the following concentrations: ampicillin at 50 μg/ml for *E. coli*; tetracycline at 5 μg/ml and erythromycin at 10 μg/ml for *S. aureus*. Carboxyphenylbenzoyl-aminopenicillanic acid (CBAP; Sigma), an inducer of the β-lactamase promoter in pRN6735, was used at a concentration of 4 μg/ml.

Cloning and sequencing strategies. The transposon Tn*917* LTV1 inserted into the *sar* locus of the host chromosome contains an *E. coli* replicon carrying ampicillin as a selective marker (2). Taking advantage of the unique restriction sites (*Xho* and *Bal*I) within the transposon, we transformed ligation mixtures of *sar* mutant 11D2 (3) chromosomal DNA digested with one of these enzymes into *E. coli* HB101. Two plasmid clones (pALC1 and pALC2) composed partly of transposon and adjacent staphylococcal chromosomal sequences were generated (Table 1). Plasmid pALC1 was purified (13) and digested with *Xho*I and *Sal*I to release a 4-kb flanking chromosomal fragment, which was then cloned into pUC18 to form the pALC3. The 4-kb insert was subsequently released from pUC18 by digestion with *Sac*I and *Sal*I, gel purified, and labeled with ^{32}P ([α-^{32}P]dCTP; Amersham) (7) to probe a λZap genomic library (Stratagene, La Jolla, Calif.) of *S. aureus*

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TABLE 1. Bacterial strains and plasmids

Strain or plasmid	Reference	Comments
Bacterial strains		
S. aureus		
DB	3	Wild-type blood isolate
RN450	15	Prototypic strain; derivative of NTCC 8325 cured of prophages; secretes β- but not α-hemolysin
RN6390	15	Laboratory strain that maintains its hemolytic pattern when propagated on sheep erythrocytes
RN4220	15	Mutant of 8325-4 that accepts foreign DNA
11D2	3	Mutant derived from DB with a *sar*::Tn*917*LTV1 mutation
A	This study	Isogenic mutant of RN450 with a *sar*::Tn*917*LTV1 mutation
R	This study	Isogenic mutant of RN6390 carrying a *sar*::Tn*917*LTV1 mutation
RN7372	22	Derivative of RN6911 (Tc^r) carrying two plasmids, pRN6735 and pI524
A35	This study	Derivative of mutant A (Tc^s) containing pRN6735 and pI524
C7	This study	Derivative of mutant R (Tc^s) carrying pRN6735 and pI524
E. coli HB101	13	Highly transformable strain
Plasmids		
pALC1	This study	*E. coli* plasmid comprising partly of transposon Tn*917*LTV1 and flanking *sar* sequence (near the *erm*-proximal end) generated by ligating *Xho*I chromosomal digests of *sar* mutant 11D2
pALC2	This study	*E. coli* plasmid generated by ligating *Bal*I digests of 11D2; contains flanking *sar* sequence distal to the *erm*-proximal end of the transposon
pALC3	This study	pUC18 with a 4-kb chromosomal insert from pALC1
pSPT181	10	Shuttle vector
pCRII		Vector for cloning PCR fragment
pALC4	This study	pSPT181 containing a 732-bp PCR fragment of *sarA* gene of RN6390
pI524	22	30-kb *S. aureus* plasmid encoding the β-lactamase repressor
pRN6735	22	Derivative of pC194 (15) 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

DB as instructed by the manufacturer. Two pBluescript phagemids with inserts of 4.7 and 6 kb, respectively, were obtained. Plasmids were purified by Magic Maxiprep (Promega, Madison, Wis.). By using both T3 and T7 primers, bidirectional plasmid sequencing was performed with ^{35}S sequencing mix and Sequenase (U.S. Biochemicals) by the chain termination method of Sanger et al. (13, 19). Additional primers were obtained for sequencing from within the insert. On the basis of the sequence generated, additional primers were also made to amplify the *sar* gene by PCR (designated *sarA* henceforth) from chromosomal DNA of prototypic *S. aureus* RN6390 and RN450 (15). The PCR fragment (732 bp) was cloned into pCRII (Invitrogen, San Diego, Calif.), cleaved with *Xba*I and *Kpn*I, and recloned into shuttle vector pSPT181 (10) in *E. coli* XL-1 blue.

Evidence that *sarA* is necessary for the optimal expression of *agr*. Shuttle plasmid pSPT181 carrying the cloned *sarA* gene (designated pALC4) from strain RN6390 was transformed into RN4220 by protoplast transformation (3) to select for Tc^r colonies at 32°C. A φ11 lysate of an RN4220 Tc^r transformant, which had been verified to carry the reconstructed plasmid pALC4 by restriction analysis (3), was prepared and used to infect *sar* mutants R and A (derived from RN6390 and RN450, respectively) to obtain Tc^r Erm^r transductants. Positive transductants were verified by restriction analysis.

To verify that the cloned *sarA* gene is responsible for the production of selected hemolysins in the complemented *sar* mutants, we assayed for α-, β-, and δ-hemolysin production on plain and cross-streaked sheep and rabbit erythrocyte agar, using specific indicator strains as standards as previously described (17). With this approach, we confirmed the restoration of α- and β-hemolysin production in mutants R and A, respectively, upon the introduction of plasmid pALC4 (Table 2). Using the culture supernatants of RN6390 and mutant R as positive and negative controls, respectively, we also verified the secretion of α-hemolysin in complemented mutant R by probing the cultured supernatant of this strain with affinity-purified anti-α-hemolysin antibody in an immunoblot as previously described (4) (data not shown).

To evaluate the effect of a *sar* mutation on RNAIII (the *agr* regulatory molecule), bacterial RNA was prepared from two pairs of isogenic *S. aureus* strains (RN450 and RN6390 together with their *sar* mutants) and their corresponding complemented mutants, using a method described by Kornblum et al. (12). For Northern blots, equal volumes (≈7.5 μl) of samples extracted from equivalent number of bacterial cells at late log phase were electrophoresed through a 1.5% agarose–0.66 M formaldehyde gel in morpholinepropanesulfonic acid (MOPS) running buffer (20 mM MOPS, 10 mM sodium acetate, 2 mM EDTA [pH 7.0]) (13). RNA was transferred onto a Hybond N membrane (Amersham) as instructed by the manufacturer and allowed to hybridize in 50% formamide at 42°C overnight with a ^{32}P-labeled (random-primed) gel-purified RNAIII probe (1.5-kb *Mbo*I fragment of pRN6735; Table

TABLE 2. Complementation of α- and β-hemolysin expression in *sar* mutants

Strain	Complementation		
	α-Hemolysin	β-Hemolysin	δ-Hemolysin
RN450	−	++	+/−
A	−	−	−
A with pALC4	−	++	+/−
A35 (no CBAP)	−	+	−
A35 (CBAP)	−	++	+/−
RN6390	+	++	+
R	−	−	−
R with pALC4	+	++	++

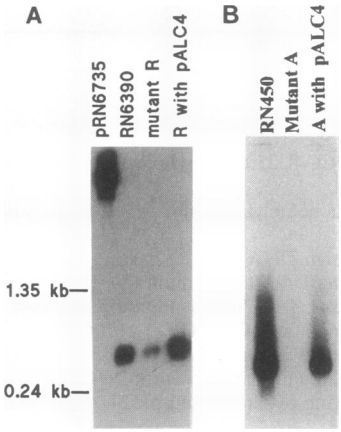

FIG. 1. Northern blots of RNAIII transcript in two sets of *S. aureus* strains. The first set (A) includes RN6390, mutant R, and R complemented with pALC4 (carrying an intact *sarA* gene). The second set (B) comprises of RN450, mutant A, and A complemented with pALC4. Plasmid pRN6735 is a positive control and comprises of RNAIII cloned into pRN6725 (22).

1) (13). Following hybridization, the membrane was washed twice in 2× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate) with 0.1% sodium dodecyl sulfate (SDS) at room temperature for 10 min each time, washed once with 1× SSC with 0.1% SDS at 55°C for 15 min, and finally autoradiographed.

As shown in Fig. 1, the levels of RNAIII in mutants A and R were either absent or significantly diminished compared with levels in the parents. However, mutants A and R transformed with the shuttle plasmid pALC4, which carried an intact *sarA* gene (from RN6390) as confirmed by a Northern blot with a *sarA* probe, were able to partially restore RNAIII levels to that of the parents (Fig. 1).

If *sarA* is assumed to be a positive regulator of *agr*, one would expect complementation of selected *sar* mutant phenotypes with a plasmid carrying RNAIII (i.e., pRN6735; Table 1). As the cloned RNAIII fragment in pRN6735 was under the control of a β-lactamase promoter that was normally repressed in the presence of pI524 (22), the production of RNAIII in mutant A was low at the basal level but was highly inducible by the addition of a β-lactam compound such as CBAP. Using this approach, we found that the production of β-hemolysin in the complemented mutant A35 was reestablished compared with *sar* mutant A (Table 2).

Sequence analysis of the *sarA* gene. With a 732-bp cloned *sarA* gene as a probe, Southern blot hybridization of chromosomal DNA digested with *Eco*RI (internal to the structural gene), *Hin*dIII, and *Eco*RV (external to *sarA*) revealed one copy of this gene in three *S. aureus* strains, DB, RN450, and RN6390 (data not shown). Of these, the complete *sarA* gene of strains DB and RN450 was sequenced. Sequence analysis and comparisons with entries in known data bases were conducted with the sequence analysis software package from the Genetics Computer Group (GCG package; University of Wisconsin, Madison) (5). Sequence data revealed identical *sarA* sequences between the two strains. By sequencing plasmids (pALC2 and pALC3) composed partly of transposon and flanking chromosomal sequences, we were able to locate the insertion site of the transposon Tn*917*LTV1 to 5 bp downstream from the translation start in strain DB (Fig. 2). The *sarA* gene has an open reading frame of 372 bp. The sequence has a GC content (27%) similar that found in the staphylococcal genome (30%) (6). A putative ribosomal binding site (underlined) is indicated in Fig. 2. The mature protein has a predicted molecular size of 14,718 Da and a deduced basic pI of 8.52. The deduced protein sequence has a predominance of charged residues (33%). Four major residues constitute ≈44% of its composition: lysine (12.9%), glutamic acid (11.2%), leucine (10.4%), and isoleucine (9.6%). In addition, there is an absence of glycine and tryptophan residues. Garnier analysis of the deduced amino acid sequence suggested that the molecule is primarily α helical (77%) (5). Additional conformational analysis with the GCG package indicated that a helix-turn-helix motif is not apparent in the deduced sequence. It also does not appear to

FIG. 2. (A) Nucleotide sequence of the *sarA* gene. The ribosomal binding site is underlined. The arrow marks the site of transposon insertion. (B) Protein sequence similarity between *sarA* and *virF*.

have significant similarity with sequence elements of the two-component signal transduction system that are located in the C-terminal domain of the signaling component and in the N-terminal domain of the activator component (14). Comparison of this protein sequence with others in the Gen Bank data base revealed similarity to the *virF* gene of *Shigella flexneri* (18) (Fig. 2).

Implications. Previous studies have shown that inactivation of the *sar* locus has resulted in alterations of expression of exoproteins in three different *S. aureus* isolates (strains DB, RN6390, and RN450) (3, 4). Using both α- and β-hemolysin genes as probes, transcriptional studies of strains with well-defined genetic backgrounds (i.e., RN6390 and RN450) revealed that the *sar* locus probably regulates exoprotein genes positively at the mRNA level (4). The regulation of exoprotein genes (e.g., α- and β-hemolysin genes) by the *sar* locus in vitro was found to begin at mid-log phase and continued into the postexponential phase (4). This mode of regulation is similar to that of *agr* on target exoprotein gene transcription.

To elucidate the interaction between the *sar* and *agr* loci, we assayed for the level of the RNAIII transcript in *sar* mutants as well as mutants complemented with an intact *sarA* gene. Our data suggested that the levels of RNAIII were related to a functional *sarA* gene (Fig. 1). We were also able to overcome the deficiency in β-hemolysin expression in a *sar* mutant by introducing a plasmid carrying RNAIII under the control of a promoter uninfluenced by *sar*. To rule out the possibility of some concerted interaction, we also determined by Northern blot analysis that the levels of *sar* mRNA did not appear to be altered appreciably in an *agr* mutant background (RN6911) (11) compared with the wild-type parent RN6390 (data not shown). Taken together, these data suggest that the *agr* locus may be under the control of *sar*.

Analysis of the *sarA* gene sequence leads to several interesting observations. First, there is no helix-turn-helix motif identifiable in a protein sequence that has a predicted α-helical conformation. Second, glycine residues which are frequently found in a helix-turn-helix motif (1) as well as in two-component signal transduction systems (14) are noticeably absent. Third, a small molecular size together with a high percentage of charged residues (33%) and a basic charge are molecular properties that are consistent with those found in other DNA-binding proteins (21). Fourth, in contrast to the *agr* locus, direct sequence comparison of the *sarA* gene with prototypic sensor and activator genes in *E. coli*, *Salmonella typhimurium*, and *Bacillus subtilis* did not reveal any significant similarity to two-component regulatory systems (14). Finally, sequence similarity with *virF*, which is a positive regulator of invasive genes in a regulon carried on a large plasmid in *Shigella flexneri* (8, 18), is of comparative interest. Like *virF*, which regulates target genes via the control of another positive regulatory gene, *virB*, the *sarA* gene may govern the expression of exoprotein genes (e.g., α- and β-hemolysin genes) by positively controlling the level of RNAIII. However, the exact mechanism by which the *sarA* gene product interacts with the *agr* locus is not apparent from sequence analysis.

The postexponential regulation of exoprotein genes in *S. aureus* involves at least three global regulatory systems (*sar*, *agr*, and *xpr*). Although we provided evidence that the *sarA* gene may control exoprotein synthesis via the control of *agr*, the relationship between *sarA* and *xpr* is not clear. Nevertheless, the observation that both *agr* and *xpr* mutants produce greatly reduced amounts of RNAIII transcript has led to the idea that *agr* and *xpr* loci may behave as interactive regulatory genes. Given our data, it is conceivable that the *sarA* gene may interact with the *xpr* locus as well.

It should be noted, however, that the restoration of RNAIII transcript (Fig. 2), upon the introduction of the *sarA* gene in pALC4, was never complete. This finding raised the possibility that additional signals may be required for a normal pattern of RNAIII transcription, thereby leading to optimal expression of exoproteins at the postexponential phase. On the basis of the pattern of transcription of an exoprotein gene such as the α-hemolysin gene in an *agr*$^+$ parent, Vandenesch et al. suggested that a separate postexponential signal independent of *agr* may be needed for augmented α-hemolysin transcription during the postexponential phase (22).

The exact mechanism by which the *sarA* gene controls *agr* is not well understood. Whether the gene product of *sarA* binds to the promoter region of RNAIII is a hypothesis that needs to be explored. Additional phenotypic analysis on a double *sar agr* mutant of RN6390 that we have recently constructed suggested that *sarA* may also affect exoprotein genes independently of *agr*. This notion is based on the observation that a double *sar agr* mutant produces lesser amounts of hemolysins than either mutant alone (unpublished data). Experiments are under way to elucidate the exact nature of this interaction.

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