Regulation of α- and β-Hemolysins by the sar Locus of
Staphylococcus aureus

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We recently identified a locus on the Staphylococcus aureus chromosome, designated sar, for staphylococcal accessory regulator, that is involved in the global regulation of extracellular and cell wall-associated proteins. Previous phenotypic and Southern blot analyses with Tn917 and agr probes indicated that this locus is distinct from agr, a previously described global regulator of exoproteins in S. aureus. To understand the mode of regulatory control of exoprotein synthesis by the sar locus, the sar genotype was transduced from the original sar mutant 11D2 into two prototypic S. aureus strains, RN6390 and RN450, with well-defined genetic backgrounds. An analysis of extracellular protein profiles by use of silver-stained sodium dodecyl sulfate gels revealed alterations in the pattern of exoprotein production in the late log-early stationary phase in the sar mutants in comparison with the corresponding parents. In addition, most of the phenotypic changes that occurred in the conversion from the sar+ genotype to the sar genotype in mutant 11D2 were also found in these mutants. Northern (RNA) blot analyses of two exoproteins transcripts (α- and β-hemolysins) from strain RN6390 and its corresponding sar mutant revealed downregulation of these transcripts in the mutant. Serial studies of these hemolysin transcripts at various growth intervals demonstrated that the transcriptional regulation of the hemolysin genes by the sar locus began during the log phase and continued into the postexponential phase. These data suggested that the sar locus probably regulates exoprotein genes at the transcriptional level. This mode of regulation is similar to that of exoprotein target gene transcription by agr.

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 postulated to be involved in pathogenesis (1). The global regulation of a number of these proteins, including α-hemolysin, β-hemolysin, coagulase, protein A, and fibronectin binding protein, is mediated by agr, a well-characterized global regulatory locus in S. aureus (8). Most of the exoproteins (including β- and δ-hemolysins) that are regulated by agr either are not synthesized or are synthesized at a reduced rate in agr mutants, while the synthesis of surface proteins is increased (8). Additional analyses of the agr locus indicated that it acts independently at the transcriptional level in regulating α-hemolysin, toxic shock syndrome toxin, and protein A (8, 16). Because of the complexities of this regulation, it has been speculated that other loci may be involved in the regulatory control of exoproteins.

We recently identified by Tn917 insertion mutagenesis an additional regulatory locus, designated sar, for staphylococcal accessory regulator, that is also involved in the global regulation of exoproteins and surface proteins (4). A detailed Southern blot analysis with Tn917 and agr probes revealed that the sar locus is distinct from the agr locus. Inactivation of the sar locus as a result of a single Tn917LTV1 insertion into the chromosome of a wild-type S. aureus isolate (strain DB) resulted in the diminished expression of β- and δ-hemolysins in an isogenic sar mutant (strain 11D2 with a sar::Tn917LTV1 mutation), while the production of serine proteases and lipase was increased (4). In contrast, the synthesis of cell wall-associated proteins (e.g., fibrinogen and fibronectin binding proteins) was downregulated in the sar mutant in comparison with the parental strain. Some of these phenotypic alterations (e.g., diminished expression of selected cell wall-associated proteins) were different from those found in switching from the agr+ phenotype to the agr mutant phenotype.

To determine whether the sar locus regulates exoprotein synthesis at the transcriptional level, we transduced the sar genotype into two S. aureus strains, RN6390 and RN450, with well-described genetic backgrounds. With α- and β-hemolysins as prototypic exoproteins, Northern (RNA) blot and Western blot (immunoblot) analyses with an isogenic strain pair indicated that the sar locus is likely to act in trans at the transcriptional level.

MATERIALS AND METHODS

Bacteria, plasmids, and bacteriophages. The bacterial strains used in this study are listed in Table 1. Transducing phage Φ71 for parental strain DB was kindly provided by P. Pattee (Iowa State University, Ames). Phage Φ11 was used as a transducing phage for strain RN4220.

Media and antibiotics. CYGP and 0.3GL media (4, 14) were used for the growth of S. aureus, while Luria-Bertani broth was used for growing Escherichia coli. Antibiotics were used at the following concentrations: erythromycin, 10 μg/ml; and ampicillin, 50 μg/ml.

Plasmid purification. Plasmid DNA was purified from E. coli strains by a standard alkaline sodium dodecyl sulfate (SDS) miniprep protocol (12). To obtain DNA fragments as probes, plasmids were digested with appropriate restriction enzymes, resolved on a 0.7% Tris-acetate-EDTA (TAE) gel, and purified by use of SpinBind columns (FMC Corp., Rockland, Maine). All restriction enzymes were purchased from New England BioLabs (Beverly, Mass.).

Transduction. Phage Φ71 was used to produce a phage
lysat of sar mutant strain 11D2 as previously described (4). The phage lysisate was then used to infect strain RN4220 at a low multiplicity of infection (phage/recipient ratio, 1:10). Transductants were selected on 0.3% agar with erythromycin. A φ11 phage lysisate of an RN4220 erythromycin-resistant transductant was prepared and used to infect strains RN6390 and RN450 to obtain transductants.

**Phenotypic characterization.** For phenotypic characterization, the measurements included α-, β-, and δ-hemolysin production measured on plain and cross-streaked sheep and rabbit erythrocyte agar (19), serine protease assayed by the fibrin agar method (Boehringer Mannheim Biochemicals, Indianapolis, Ind.), lipase production measured on 1% Tween agar plates, fibronecin binding protein content assayed by 125I-labeled fibronecin binding (6), and fibrinogen binding capacity determined by 125I-labeled fibrinogen binding (11).

**SDS-PAGE and immunoblot analyses of extracellular proteins.** Equal volumes of extracellular fluids from strains RN6390 and RN450 and their corresponding sar mutants at mid-log, late log, and postexponential phases were harvested as previously described (19). In brief, an overnight bacterial culture grown in CYGP medium (or CYGP medium with erythromycin for the sar mutants) was inoculated 1:50 into 10 ml of fresh medium without antibiotics. Prior studies had established the stability of the transposon insertion when grown in this manner (4). At ~2, 2.75, and 6 h after inoculation, corresponding to an optical density at 650 nm (OD650) of 0.7, 1.1, and 1.7, respectively, 10 ml of extracellular fluid was obtained directly from the cell culture without further volume adjustment, filtered through a 0.22-μm-pore-size filter, dialyzed against 1 mM Tris-0.15 M NaCl (pH 8), and concentrated 50-fold with an Amicon (Danvers, Mass.) Centriprep concentrator. To evaluate the extracellular protein profiles of parental strains and their respective sar mutants, 2 μl of concentrated extracellular fluid was resolved on SDS–9% polyacrylamide slab gels (10). After polyacrylamide gel electrophoresis (PAGE), the gels were stained with silver (4). To assay for the amount of α-hemolysin present, 7 μl of concentrated fluid was immunooblotted onto nitrocellulose (22). The immunoblot was incubated with affinity-purified sheep anti-α-hemolysin antibody (Toxin Technology, Sarasota, Fla.) for 2 h at room temperature at a dilution of 1:500. A rabbit anti-sheep antibody–alkaline phosphatase conjugate (Kirkegaard & Perry Laboratories, Inc., Gaithersburg, Md.) was then added as a secondary antibody (1:1,000 dilution) for 1 h at room temperature. The reactive bands were visualized as described by Blake et al. (2).

**Southern blot hybridization.** SarB chromosomal DNA was prepared from lysostaphin (Applied Microbiology, New York, N.Y.)-lysed cells as previously described (4). Southern blot hybridization was performed with randomly primed samples of gel-purified DNA as probes (12). Chromosomal DNA was digested with EcoRI or NcoI, transferred to a Hybond N+ membrane (Amersham, Arlington Heights, Ill.), hybridized with a 32P-labeled 1.5-kb HindIII fragment of Tn917 (4) at 65°C, and washed with SSPE (1 × SSPE is 0.18 M NaCl, 10 mM NaPO4, and 1 mM EDTA [pH 7.7]) according to the membrane manufacturer's instructions (4). The membrane was then autoradiographed with an intensifying screen at ~70°C.

**Extraction of bacterial RNA.** Bacterial RNA was prepared by a modification of the method described by Kornblum et al. (9). In brief, bacteria were grown in CYGP medium (or CYGP medium with erythromycin) overnight, diluted 1:75 in fresh medium, and grown to mid-log, late log, and postexponential phases, corresponding to an OD650 of 0.7, 1.2, and 1.7, respectively. To ensure that equal amounts of cells were available for RNA extractions, cells were adjusted to the lowest OD650 (i.e., 0.7) with CYGP medium at harvest. Two milliliters of these cells was quickly frozen in an ethanol-dry ice bath and then slowly thawed at 4°C. The bacteria were pelleted (8,000 × g for 5 min at 4°C), washed with ice-cold TS buffer (20 mM Tris, 20% sucrose, 10 mM EDTA [pH 7.5]), and resuspended in 100 μl of cold TS buffer. Lysostaphin (15 μg) was added, and the cell mixture was incubated for 20 min at 4°C and then for 3 min at 37°C to generate protoplasts. To lyse the protoplasts, 2% SDS (100 μl) and proteinase K (5 μl of a stock of 10 mg/ml) were added and the mixture was incubated at room temperature for 15 min. To reduce the viscosity of the cell lysate, the sample was frozen (~70°C) and thawed (55°C) twice. The integrity of the RNA was verified by running the sample in a formaldehyde gel as described previously (12).

**Northern blot hybridization.** Equal volumes (7.5 μl) of samples 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]) (12). The intensity of the 23S and 16S rRNA bands stained with ethidium bromide was verified to be equivalent among all the samples before transfer. RNA was transferred to a Hybond N membrane (Amersham) according to the manufacturer’s instructions and allowed to hybridize in 50% formamide at 42°C overnight with 32P-labeled (randomly primed) gel-purified DNA probes (12). Following hybridization, the membrane was washed twice with 2 × SSC (1 × SSC...
is 0.15 M NaCl plus 0.015 M sodium citrate)–0.1% SDS at room temperature for 10 min each time, washed once with 1× SSC–0.1% SDS at 55°C for 15 min, and finally autoradiographed.

RESULTS

Transduction of the sar mutant genotype. Transduction was used to transfer the sar mutant genotype from the original sar mutant 11D2 into two prototypic S. aureus strains, RN6390 and RN450, with well-described genetic backgrounds (13). Southern blots of chromosomal DNAs from transductants digested with either EcoRI or NcoI revealed that a single EcoRI fragment and two NcoI fragments from these transductants hybridized to a 1.5- kb HindIII fragment of Tn917 as a probe (Fig. 1). This hybridization pattern was analogous to that seen with sar mutant 11D2, suggesting that the location of the Tn917LTV1 insert in these transductants was identical to that in mutant 11D2.

Phenotypic characterization of transductants from RN6390 and RN450. As most exoproteins are secreted during the late log and postexponential phases (8), total extracellular proteins at corresponding growth intervals (OD500 = 1.1 and 1.7) from strain RN6390 and its isogenic sar mutant R were evaluated for protein profiles. When equivalent volumes of concentrated extracellular fluid were applied to the gel, the parental strain displayed a protein profile distinct from that of the corresponding isogenic mutant (Fig. 2). Likewise, mutant A also exhibited an extracellular protein profile that differed from that of parental strain RN450 (data not shown). The expression of individual proteins is presented in Table 2. Compared with the situation in parental strain RN6390, α-, β-, and δ-hemolysin levels were reduced in sar mutant R. Similarly, the production of β-hemolysin in sar mutant A was decreased compared with that in parental strain RN450. As α-hemolysin is not produced by strain RN450, the effect of the sar locus on α-hemolysin expression could not be determined in mutant A. In contrast, the secretion of extracellular serine proteases was increased in both mutants. The phenotypic consequence of the sar genotype on lipase production was more variable. More specifically, lipase production was enhanced in mutant R but remained unchanged in mutant A.

Alterations were also observed with cell-bound proteins. For instance, the expression of fibronectin binding protein was diminished in both sar mutants in comparison with their respective parents. Similarly, the expression of fibrinogen binding protein was markedly decreased in mutant A in comparison with parental strain RN450. However, the reduction in fibrinogen binding capacity in mutant R compared with parental strain RN6390 was not statistically significant.

With the exception of α-hemolysin, the pattern in switching from the sar+ phenotype to the sar phenotype is analogous to that found in an isogenic pair previously described (i.e., parent DB and sar mutant 11D2) (4). To further confirm the switching in α-hemolysin production, the extracellular proteins of parental strain RN6390 and isogenic sar mutant R at serial growth intervals were evaluated for α-hemolysin expression by Western blotting. As shown in Fig. 3, α-hemolysin was rendered undetectable in sar mutant R compared with parental strain RN6390 throughout the growth cycle. Of interest is the observation that protein A, which reacted with sheep antibody at a low affinity, was produced by sar mutant R at mid-log phase but not at late log and postexponential phases. In contrast, parental strain RN6390 did not synthesize extracellular protein A at a detectable level throughout the growth cycle.

Evidence that the sar locus regulates α- and β-hemolysin transcripts. As the expression of α- and β-hemolysins was downregulated in sar mutant R (Table 2), Northern blotting was performed to determine whether these hemolysins are regulated at the transcriptional level. As displayed in Fig. 4, the levels of transcripts of both exoproteins were reduced in mutant R in comparison with parent strain RN6390. In distinction from the α-hemolysin transcript, there were two hybridizing bands associated with the β-hemolysin transcript
TABLE 2. Phenotypic characterization of RN6390 and RN450 and their corresponding sar mutants

<table>
<thead>
<tr>
<th>Strain</th>
<th>α-Hemolysina</th>
<th>β-Hemolysinb</th>
<th>δ-Hemolysina</th>
<th>Lipaseb</th>
<th>Proteaseb</th>
<th>Fibrinogen binding proteinb</th>
<th>Fibronectin binding proteinb</th>
</tr>
</thead>
<tbody>
<tr>
<td>RN6390</td>
<td>++</td>
<td>+</td>
<td>++</td>
<td>+</td>
<td>+</td>
<td>5,701 ± 53</td>
<td>3,314 ± 35</td>
</tr>
<tr>
<td>sar mutant R</td>
<td>−</td>
<td>−</td>
<td>−</td>
<td>±</td>
<td>−</td>
<td>5,156 ± 94</td>
<td>2,368 ± 81</td>
</tr>
<tr>
<td>RN450</td>
<td>−</td>
<td>+</td>
<td>±</td>
<td>+</td>
<td>+</td>
<td>6,138 ± 119</td>
<td>23,558 ± 398</td>
</tr>
<tr>
<td>sar mutant A</td>
<td>−</td>
<td>−</td>
<td>±</td>
<td>−</td>
<td>++</td>
<td>3,462 ± 449</td>
<td>2,107 ± 402</td>
</tr>
</tbody>
</table>

a ++, strong producer; +, moderate producer; ±, very weak producer; −, weak producer.
b Data are presented as counts of 125I-fibronectin bound per minute to 10^9 CFU and are reported as the mean ± the standard error of the mean (n = 4). The reduction in fibrinogen binding of sar mutant A in comparison with parental strain RN450 was statistically significant (P < 0.008; t test).
c Data are presented as counts of 125I-fibronectin bound per minute to 10^9 CFU and are reported as the mean ± the standard error of the mean (n = 4). The reduction in fibrinogen binding of sar mutant R in comparison with parental strain RN6390 was significant (P < 0.003; t test). The reduction in fibrinogen binding of sar mutant A in comparison with parental strain RN450 was significant (P < 0.0001; t test).

(1.5 kb [broken arrow] and 2.9 kb [solid arrow]) (Fig. 4B). On the basis of the size of the previously reported β-hemolysin gene (17), it is likely that the 1.5-kb band represents the intact β-hemolysin transcript, while the larger hybridizing fragment may arise as a result of an overlapping transcript or cross-reacting transcript. Notably, other investigators also found that the intact β-hemolysin probe hybridized to at least two bands in Northern blot studies (16b). A serial analysis at different growth phases also revealed that the transcription of both hemolysins was detected at the mid-log phase and was maximal at the postexponential phase. More specifically for parental strain RN6390, there were more transcripts at an OD650 of 0.7 than at an OD650 of 1.1, while the levels of the transcripts were highest at an OD650 of 1.7. Remarkably, the levels of transcripts of both α- and β-hemolysins were decreased for mutant R throughout the growth cycle.

DISCUSSION

It was shown in our previous study that a single insertion of transposon Tn917LTV1 into the sar locus of a wild-type clinical isolate, strain DB, resulted in a pleiotropic effect on the expression of a number of extracellular and cell wall-associated

FIG. 3. Western blotting of the extracellular fluids of strain RN6390 and mutant R probed with anti-α-hemolysin antibody. Equivalent amounts of extracellular fluids obtained at serial growth intervals were applied to the lanes. The blot was developed with affinity-purified sheep anti-α-hemolysin antibody. Purified α-hemolysin (Toxin Technology) was used as a positive control. In some samples, the sheep antibody also reacted weakly with protein A present in the extracellular fluids (arrow). As the volume of extracellular fluid was not adjusted for the amounts of CFU present in the culture, a comparison was only valid with samples obtained at identical optical densities.

FIG. 4. Northern blots of α-hemolysin (A) and β-hemolysin (B) transcripts of parental strain RN6390 and sar mutant R during the growth cycle. (A) A single α-hemolysin transcript of ~1.8 kb was noted. The positive control was the plasmid from E. coli DU5384 digested with EcoR1-HindIII. (B) Two bands (1.5 kb [broken arrow] and 2.9 kb [solid arrow]) were noted to hybridize with the β-hemolysin probe. The positive control was the intact plasmid from E. coli RN6929.
proteins in sar mutant 11D2 (4). The location of the Tn917LTV1 insertion in the staphylococcal chromosome was mapped to the SmaI-D fragment, which is in a region distinct from two regulatory loci (i.e., agr and xpr) previously described for S. aureus (16a, 19, 20). To understand the mode of regulatory control of exoprotein synthesis by the sar locus, we transduced the sar genotype from mutant 11D2 to two S. aureus strains, RN6390 and RN450, with well-defined genetic backgrounds (8, 19). The insertion of Tn917LTV1 into the sar locus in both mutants A and R was supported by Southern blot analysis (Fig. 1). As an additional confirmation that the sar locus was insertionally inactivated in both mutants A and R, we also complemented the extracellular protein profiles as well as hemolysin production in these mutants with a plasmid carrying a cloned sar gene (unpublished data).

With the exception of α-hemolysin, most of the phenotypic changes that occurred with the conversion of the sar+ genotype to the sar genotype in mutant 11D2 (4) were also found in mutants A and R (Table 2). For instance, the expression of a cell wall-associated protein, such as fibronectin binding protein, was reduced for both sar mutants in comparison with the parents. Similarly, both β- and δ-hemolysins were downregulated in sar mutants. Like that in the original sar mutant 11D2, the secretion of extracellular proteases was increased in mutants A and R in comparison with the parents. Likewise, lipase activity was either increased (mutant R) or remained unchanged (mutant A) in sar mutants. Although most of the exoproteins were expressed at lower levels in the mutants, the observation that lipase activity was increased in mutant R suggested that the augmented extracellular protease activity was unlikely to have accounted for all the observed alterations in phenotypes. In addition, the finding that some higher-molecular-weight proteins were detected in the extracellular protein profiles of mutant R but not RN6390 is also inconsistent with such a hypothesis (Fig. 2).

Northern blot analysis of the β-hemolysin gene in sar mutant R confirmed a decrease in the level of this transcript in this sar mutant. The transcriptional activity of the α-hemolysin gene was also reduced in this mutant in comparison with parental strain RN6390. These studies suggest that the sar locus is likely to regulate exoprotein genes at a transcriptional level. However, the possibility that the sar locus may alter transcript stability together with translational control cannot be entirely ruled out.

The data obtained in evaluating the levels of α- and β-transcripts at serial growth intervals revealed that the transcriptional regulation of these genes by the sar locus began at the mid-log phase and continued into the postexponential phase. Of interest is the observation that both α- and β-hemolysin transcript levels were higher at an OD_{650} of 0.7 than at an OD_{650} of 1.1 for parental strain RN6390. Given a 2-h latency period between inoculation and harvest at an OD_{650} of 0.7, it is unlikely that the transcript observed at the mid-log phase was due to a carryover of the inoculum. However, the reduction in transcriptional activity from an OD_{650} of 0.7 to an OD_{650} of 1.1 which occurred within a 45-min interval was consistent with either mRNA decay or a decrease in the rate of transcription. Notably, both α- and β-hemolysin transcripts were at their highest level during the postexponential phase. In a previous study, Vandenesch et al. (23) also reported that the transcription of the α-hemolysin gene in an agr+ parent began at the log phase and peaked during the postexponential phase. This pattern of transcription is consistent with the hypothesis that a separate postexponential signal independent of agr is needed for augmented α-hemolysin transcription during the postexponential phase (23). However, the relationship of this postexponential signal to the sar locus with regard to exoprotein expression remains to be elucidated.

The postexponential regulation of exoprotein genes in S. aureus involves at least three global regulatory systems. In addition to sar, the best-described regulatory element is the agr locus, which is composed of two divergent transcription units. One of these transcription units, RNAIII, which also encodes the 26-residue δ-hemolysin polypeptide, is required for the transcriptional control of exoprotein synthesis (7, 8, 23). Vandenesch et al. (23) had shown that the production of the α-hemolysin transcript had to be preceded by that of RNAIII. In the absence of RNAIII (i.e., agr), the mRNA transcriptional activity of the α-hemolysin gene was significantly diminished during the log and postexponential phases of the growth cycle (23). Thus, there appears to be a similar trend in the temporal and transcriptional control of exoprotein genes by the agr locus and the sar locus.

Another regulatory locus, designated xpr, was recently described by Smeltzer et al. (20). A Tn551 chromosomal insertion into the xpr locus in S. aureus S6C resulted in the reduced expression of exoproteins (lipase, enterotoxin B, α- and δ-hemolysins, protease, and nuclease) (23). In contrast to the agr phenotype, coagulase production was not increased in the xpr mutant (23). Whether the xpr locus regulates exoprotein production at a transcriptional or a translational level is not clear. Of particular interest is the observation that both xpr and agr mutants produce greatly reduced amounts of δ-hemolysin, which is encoded within the RNAIII transcript, the regulatory molecule of agr. Because of this finding and the similarities in exoprotein expression between xpr and agr mutants, it has been speculated that xpr and agr may behave as interactive regulatory genes (20).

As described in this and previous (4) studies, inactivation of the sar locus in three different S. aureus strains (strains DB, RN6390, and RN450) resulted in alterations of the expression of both extracellular and cell wall-associated proteins in the corresponding sar mutants. Although most of the phenotypic changes were similar among the sar mutants, it is also clear that host factors may play a role in gene expression. For instance, α-hemolysin was downregulated in mutant R, while it is upregulated in the original sar mutant 11D2. Thus, the expression of α-hemolysin in two diverse sar genetic backgrounds (i.e., strains DB and RN6390) may differ as a consequence of unknown host factors which can influence α-hemolysin gene expression via transcriptional and/or translational control. In support of this hypothesis is the finding by Compagnone-Post et al. (5) that S. aureus strains carrying single and apparently identical copies of the agr locus can produce different amounts of RNAIII, thereby resulting in transcriptional regulation of agr by a host factor(s).

The control of exoprotein synthesis in S. aureus is a complicated process involving several regulatory genetic elements (5, 7, 8, 18, 20, 23). Two of these determinants, sar and agr, appear to regulate exoprotein expression via transcriptional control, while the control mechanism of the third element (i.e., xpr) is not known. Whether these regulatory elements operate independently or in conjunction with each other (4, 20, 23) at a transcriptional level will be important to our understanding of the regulatory control of virulence determinants in S. aureus. More specifically, it will be of interest to know whether the transcription of sar is affected by agr and/or xpr. Another issue concerning sar regulation is whether this locus controls rates of gene transcription or mRNA decay. Future gene fusion studies would help clarify this aspect.
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REFERENCES


16a.Pattee, P. A. Personal communication.

16b.Projan, S. J. Personal communication.


