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Regulation of Protein A Synthesis by the *sar* and *agr* Loci of *Staphylococcus aureus*

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The synthesis of protein A in *Staphylococcus aureus* is regulated by global regulatory loci such as *sar* and *agr*. Phenotypic data indicate that both *sar* and *agr* suppress protein A synthesis; like *agr*, *sar* also regulates protein A production at the transcriptional level. To determine the genetic requirement of *sar* in protein A suppression, we transformed shuttle plasmids containing various *sar* fragments into a *sar* mutant. Our results indicated that the 560-bp sarA transcript, or, more probably, the SarA protein (13.5 kDa), is sufficient for suppressing protein A gene transcription when introduced on a multicopy plasmid or as a single copy in the chromosome. Immunoblot analysis with a chicken anti-protein A antibody also confirmed the reduction in protein A expression in these *sar* mutant clones. Complementation studies revealed that the transcription of the protein A gene can be suppressed in a *sar* mutant background by a plasmid containing RNAIII. Surprisingly, in *agr* deletion mutant clones and in clones derived from the *agr-sar* double mutant, protein A gene transcription can also be suppressed by plasmids containing the sarA transcript plus additional upstream sequence but not the sarA transcript alone. These data suggest that the *sar* locus can down-modulate protein A gene transcription via both RNAIII-dependent and RNAIII-independent pathways. Consistent with the hypothesis of an RNAIII-independent pathway is an additional genetic requirement for protein A suppression in the *agr* deletion mutant RN6911 as well as the isogenic double *sar-agr* mutant, whereas in the *sar* mutant background, the sarA transcript encoding the SarA protein alone is sufficient. These data suggested that both *sar* and *agr* are coregulators of protein A synthesis in *S. aureus*.

Protein A (SpA) is a major surface protein that is present in over 90% of *Staphylococcus aureus* strains (10, 27). SpA makes up about 7% of the *S. aureus* cell wall (12). Based on its immunoglobulin G (IgG)-binding property, it has been estimated that there are ~80,000 IgG-binding sites per cell in *S. aureus* Cowan 1 (18). Although the molecular mass of Cowan 1 SpA has been estimated to be 42 kDa (3), immunological analysis suggested that size heterogeneity in SpA exists among serologically distinct strains (5). Besides being an integral cell wall protein, SpA is actively secreted into the supernatant of actively growing cultures. SpA is best studied for its interesting immunological properties. For instance, it can activate complement in vitro (11). Additionally, the IgG-binding property of this protein has been exploited extensively in a variety of immunological assays (13).

The biological properties of SpA have been extensively studied (10). In vitro assays demonstrated that *S. aureus* strains with high SpA content are more resistant to phagocytosis than are strains with less SpA (25). Presumably, the antiphagocytic effect may be mediated by the binding of SpA to the Fc portion of IgG, thus competing with phagocytic cells for available IgG-Fc sites and thereby resulting in diminished IgG-mediated opsonization (25). Similarly, Greenberg et al. (14) found that hyperimmune serum to Cowan 1 SpA was associated with highly efficient opsonophagocytosis and killing of the homologous strain. However, the precise biological function of SpA in vivo remains poorly defined.

As a surface protein, SpA has been shown to be regulated by at least two distinct global regulatory loci, *agr* and *sar* (15, 17). The *agr* locus is composed of two divergent transcripts, RNAII and RNAIII, which include at least five genes (*agrA*, *agrB*, *agrC*, *agrD*, and *hld*). Inactivation of the *agr* locus has led to increased synthesis of SpA. As RNAIII is the *agr* regulatory molecule ultimately responsible for the expression of the Agr^+ phenotype during the postexponential growth phase (1, 22), it is likely that RNAIII acts to suppress SpA **gene** transcription during this part of the growth cycle (23, 26).

We recently described another regulatory locus, designated *sar*, which is also involved in the pleiotropic expression of both extracellular and cell wall proteins (8). Transcriptional analysis revealed that the *sar* locus is composed of three overlapping transcripts, designated sarA, sarC, and sarB (sizing at 0.58, 0.84, and 1.15 kb, respectively) (2). These three transcripts terminate in a common 3′ end but originate from three distinct promoters (2). Complementation analysis indicated that one of the genes (*sarA*, loc-on the 0.58-kb transcript) within the *sar* locus appears to be necessary for the optimal expression of RNAIII in the *agr* locus (2, 14, 15). Like *agr*, the *sar* locus also suppresses SpA synthesis (8, 15). However, the pathway by which the *sar* locus down-modulates the expression of SpA has not been previously defined.

We report here that the transcription of the SpA gene is coregulated by *sar* and *agr*. More specifically, the suppression of SpA by the *sar* locus can occur via both *agr* (RNAIII)-dependent and *agr*-independent pathways. Either of these pathways alone, if active, is sufficient for repression. In addition, the genetic requirement for down-modulating SpA gene transcription in a *sar* mutant background differs from that of the *agr* mutant background.

MATERIALS AND METHODS

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 *sar* and *agr* mutants.

Media and antibiotics. CYGP and 0.3GL media (21) were used for the growth of *S. aureus*, while Luria-Bertani broth was used for the growth of *Escherichia*

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TABLE 1. Bacterial strains and plasmids used in this study

Strain or plasmid	Reference or source	Comments
S. aureus		
RN4220	19	A mutant of strain 8325-4 that accepts foreign DNA
RN6112	17	A mutant derived from RN6390 carrying an *agr*::Tn*551* mutation
RN6911	17	An RN6390-derived *agr* mutant in which the *agr* locus has been replaced by the *tetM* gene
RN6390	19	Laboratory strain that maintains its hemolytic pattern when propagated on sheep erythrocytes and has a genetic background similar to that of 8325-4
ALC70	14	ALC136 with pALC70
ALC103	14	ALC136 with pALC103
ALC136	8	Isogenic mutant of RN6390 carrying a *sar*::Tn*917*LTV1 mutation
ALC184	9	ALC136 with pRN6735 and pI524
ALC475	This study	ALC136 with pSPT181
ALC488	This study	Isogenic mutant of RN6390 carrying a *sarA*::*ermC* mutation
ALC519	This study	RN6112 carrying pALC103
ALC520	This study	RN6112 carrying pALC70
ALC521	This study	RN6112 carrying pALC406
ALC556	2	ALC136 with pALC549
ALC557	This study	ALC136 carrying pALC557
ALC599	This study	ALC136 with *geh*::pCL84
ALC600	This study	ALC136 with *geh*::pALC555
ALC626	This study	RN6112 carrying pALC549
ALC624	This study	RN6112 carrying pALC557
ALC842	This study	A double *sar-agr* mutant in which the *agr* mutation in RN6911 has been transduced into ALC488 (with the *sarA*::*ermC* mutation)
ALC863	This study	RN6911 carrying pALC213
ALC864	This study	RN6911 carrying pALC861
ALC865	This study	RN6911 carrying pALC862
ALC868	This study	ALC842 carrying pALC213
ALC869	This study	ALC842 carrying pALC861
ALC870	This study	ALC842 carrying pALC862
ALC890	This study	RN6911 carrying pALC888
ALC891	This study	ALC842 carrying pALC888
E. coli		
XL-1 Blue		A highly transformable strain
InvαF′	Invitrogen	Host strain for the TA cloning vector (pCRII)
RN7037		A clone containing an 800-bp *Hin*dIII-*Pst*I fragment of the *spa* gene in pBluescript
Plasmids		
pCL84	18	*S. aureus* integration vector that inserts into the lipase gene (*geh*)
pCRII	Invitrogen	*E. coli* cloning vector for direct cloning of PCR fragments
pSPT181	15	Shuttle vector
pSK236	20	Shuttle vector comprising pUC19 cloned into the *Hin*dIII site of pC194
pRN6735	17	A derivative of pC194 containing the *bla* promoter and two-thirds of the *blaZ* gene followed by a 1.5-kb RNAIII fragment lacking its promoter
pI524	17	30-kb *S. aureus* plasmid containing the β-lactamase repressor
pALC70	14	pSPT181 encompassing the sarA, sarC, and sarB transcriptional units (Fig. 1)
pALC103	2, 14	pSPT181 containing the sarA transcript (nucleotides 620 to 1349 according to published sequence)
pALC213	This study	pSK236 containing the sarA transcript (nucleotides 620 to 1349)
pALC406	15	pSPT181 containing only the promoter region of *sar* but lacking the *sarA* coding sequence
pALC549	This study	pSPT181 containing the sarA transcript and a 189-bp upstream fragment (nucleotides 531 to 1349)
pALC555	This study	pCL84::sarA
pALC557	This study	pSPT181 containing the sarA and sarC transcripts
pALC861	This study	pSK236 containing the sarA and sarC transcripts
pALC862	This study	pSK236 encompassing the sarA, sarC, and sarB transcriptional units
pALC888	This study	pSK236 with the sarA transcript plus 189 bp upstream but lacking the promoter for sarC (nucleotides 531 to 1349)

coli. Antibiotics were used at the following concentrations: erythromycin, 10 μg/ml; tetracycline, 5 μg/ml; ampicillin, 50 μg/ml.

Cloning and sequencing strategies. DNA fragments encompassing the *sar* locus (Fig. 1) were amplified by PCR with genomic DNA of *S. aureus* RN6390 as the template and cloned into the TA cloning vector pCRII (Invitrogen, San Diego, Calif.). The authenticity of the amplified fragments was confirmed by DNA sequencing. The PCR fragments were cleaved from pCRII, ligated to the shuttle vector pSPT181 or pSK236, and transformed into *E. coli* XL-1 Blue. The presence of the correct inserts in the plasmids was confirmed 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 (8). For transduction, phage ϕ11 was used to produce a phage lysate of strain RN4220 containing recombinant shuttle vectors (pSPT181 or pSK236) with *sar* DNA fragments. The phage lysate was then used to infect either *agr* mutants (transposon mutant RN6112 or deletion mutant RN6911) or *sar* mutants (transposon *sar* mutant ALC136 [previously designated mutant R] or *sar* insertion mutant ALC488) as described previously (8) (Table 1).

The *sar* insertion mutant ALC488 was constructed by allele replacement from the temperature-sensitive shuttle plasmid pSPT181 containing the *sarA* gene into

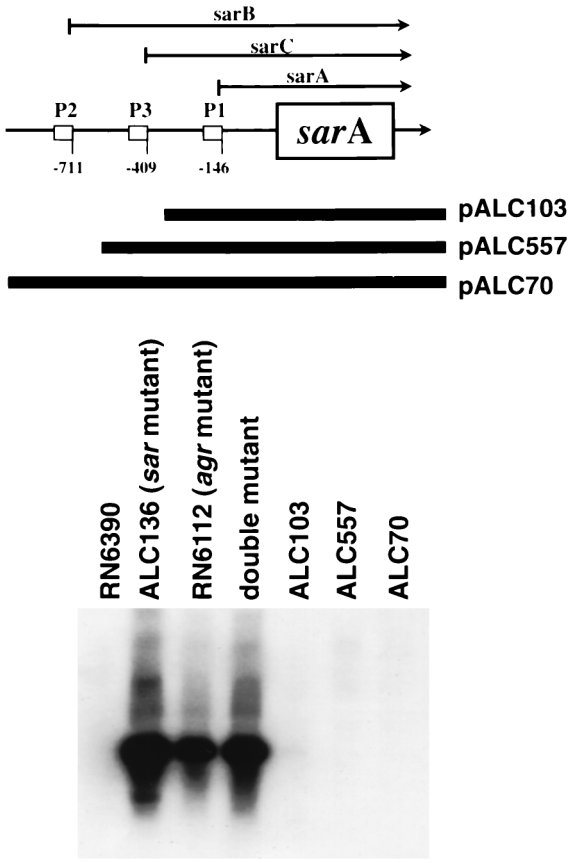

FIG. 1. Northern blot of *sar* mutant clones probed with an 800-bp *spa* fragment. RNA was obtained from cells harvested at the late log phase. *sar* mutant strains have the following designations: ALC136 is the transposon-mediated *sar* mutant; ALC103, ALC557, and ALC70 are *sar* mutant clones derived from ALC136 carrying recombinant shuttle plasmids pALC103, pALC557, and pALC70, respectively. Plasmids pALC103, pALC557, and pALC70 contain the sarA transcript, sarA and sarC transcripts, and sarA, sarC, and sarB transcripts, respectively. The numbers adjacent to the promoter boxes above correspond to the transcription start sites and are expressed as the number of nucleotides upstream of the sarA translation start. The double mutant was constructed by transducing the *sar* mutation into the *agr* mutant RN6911.

which an *ermC* gene has been inserted via the internal *Eco*RI site of the *sarA* coding region. Following transformation into RN4220, the recombinant plasmid was transduced into strain RN6390. The isolate containing the recombinant shuttle plasmid was maintained at 30°C with erythromycin and tetracycline, diluted 1:2,000 in CYGP medium containing erythromycin, and grown at 43°C for 18 h. Growth at the higher temperature was repeated three times to promote loss of plasmid, and the bacteria were plated on blood agar plates to select for nonhemolytic (i.e., *sar* mutant) and tetracycline-sensitive colonies. One isolated clone (ALC488) was confirmed by Southern blot analysis to have the correct chromosomal insertion. Northern blot analysis with a *sarA* probe also verified the disruption of all three *sar* transcripts (data not shown).

To introduce a single copy of the *sar* fragment into the chromosome of *sar* mutant ALC136, we cleaved from pBluescript phagemid a *sar* fragment which was then cloned into the *Pst*I-*Sal*I site of the integration vector pCL84 (19). This vector inserts preferentially into the lipase gene of the staphylococcal chromosome. Following transformation into a derivative of RN4220 which supplies the integrase gene in *trans*, the integrants were selected on DM3 agar (8) with tetracycline at 3 μg/ml. Loss of lipase activity in the transformants was confirmed by unique halo formation on egg yolk agar (19). The integrated fragment was transduced into the *sar* mutant ALC136 with selection for tetracycline and loss of lipase activity. Correct integration was verified by Southern blots with lipase gene- and *sar*-specific probes.

Immunoblot analysis of SpA. To determine levels of SpA production, cell wall-associated proteins were extracted from overnight cultures of *S. aureus* with lysostaphin in a hypertonic medium (30% raffinose) as previously described (7). Equal volumes of extracellular fluid from cells grown to the postexponential phase were harvested and concentrated 50-fold as described previously (8). Equivalent volumes (1 μl each) of either extracellular or cell wall proteins were separated on sodium dodecyl sulfate–10% polyacrylamide gels. Separated proteins were electrophoretically blotted to nitrocellulose and probed with chicken anti-staphylococcal SpA antibody (Accurate Chemicals, Westbury, N.Y.) at a 1:3,000 dilution. Bound antibody was detected with rabbit anti-chicken IgG conjugated to alkaline phosphatase (Jackson Immunoresearch, West Grove, Pa.) (1:5,000 dilution) and visualized as described previously (4).

Isolation of RNA and Northern analysis. Overnight cultures of *S. aureus* were diluted 1:100 in CYPG medium and grown to mid-log, late log, and postexponential phases. The cells were pelleted and processed with the FastRNA isolation kit (Bio101, Vista, Calif.) in combination with 0.1-mm-diameter zirconia/silica beads and a FastPrep reciprocating shaker (Bio101) as described previously (6). A 10-μg portion of RNA from each sample was electrophoresed through a 1.5% agarose–0.66 M formaldehyde gel in MOPS running buffer (20 mM morpholinepropanesulfonic acid [MOPS], 10 mM sodium acetate, 2 mM EDTA [pH 7.0]). Blotting of RNA onto Hybond N membrane (Amersham, Arlington Heights, Ill.) was performed with the Turbo Blotter Alkaline Transfer System (Schleicher & Schuell, Keene, N.H.). For detection of the SpA gene transcript, a gel-purified *Hin*dIII-*Pst*I fragment of the *spa* gene (Table 1) was radiolabeled with [α-^{32}P]dCTP (Amersham) by the random prime method (Ready to Go Labeling Kit; Pharmacia, Piscataway, N.J.) and hybridized under high-stringency conditions. The blots were subsequently autoradiographed.

RESULTS

Regulation of SpA gene transcription by the *sar* locus. In a previous study, we have shown that a shuttle plasmid carrying the *sarA* gene partially restored the defect in hemolysin production to a transposon-mediated *sar* mutant while a larger (1.3-kb) fragment encompassing all three transcripts of the *sar* locus was able to completely reestablish the parental phenotype (2). In addition, the synthesis of SpA as determined by immunoblotting was increased in the *sar* mutant but returned to the parental level upon the introduction of a shuttle plasmid carrying all three *sar* transcripts. To ascertain the mode of SpA control by the *sar* locus, we have chosen to evaluate SpA expression in strain RN6390 because this strain, being a low producer of SpA, show increased sensitivity in the detection of SpA suppression when compared with an isogenic *sar* mutant that overexpresses SpA (15, 21). After transforming various DNA fragments containing either part or all of the *sar* locus into the isogenic *sar* mutant (Fig. 1), we assayed for SpA production as determined by Northern and Western blot analysis.

Northern blot analysis revealed that the transcription of the SpA gene was markedly increased in the *sar* mutant as compared to the parental strain, which exhibited a very low level of the SpA gene transcript (Fig. 1). Upon introduction of a shuttle plasmid comprising the smallest transcript (sarA in ALC103), the transcription of the SpA gene in the mutant decreased to the parental level. Complementation studies with plasmids containing DNA fragments that encompassed two additional *sar* transcriptional units (sarB and sarC) revealed that the SpA gene transcript remained depressed at the parental level. In contrast, a *sar* mutant clone transformed with a plasmid containing only a fragment upstream of *sarA* (pALC406) exhibited an elevated SpA gene transcript level similar to that produced by the *sar* mutant ALC136 alone (data not shown). To assess the role of the *sarA* gene product in SpA suppression, a series of 3′ *sar* deletion clones lacking a part or all of the 339-bp *sarA* open reading frame (ORF) derived from RN6390 were constructed. Remarkably, each of these 3′ *sar* deletion clones displayed a high level of SpA gene transcription analogous to the *sar* mutant (data not shown), thus suggesting that the *sarA* gene product is critical to the control of SpA gene transcription.

We compared the expression of SpA in cell wall extracts of these *sar* mutant clones on immunoblots. As anticipated, the synthesis of cell wall-associated SpA, as detected by using affinity-purified chicken anti-SpA antibody, was markedly enhanced in the *sar* mutant (2) when compared to the parent (Fig. 2). By analogy to the transcriptional data, the expression of SpA in cell

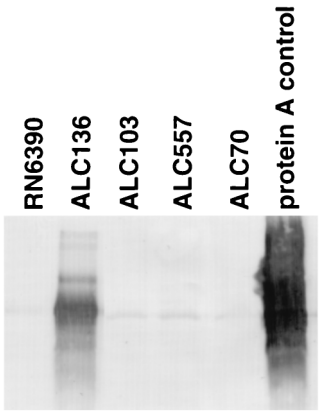

FIG. 2. An immunoblot of cell wall extracts of *sar* mutant clones probed with affinity-purified chicken anti-SpA antibody. The *sar* mutant clones ALC103, ALC557, and ALC70 contain plasmids containing sarA, sarA and sarC, and sarA, sarC, and sarB, respectively.

wall extracts of *sar* mutant clones expressing sarA, sarB, or sarC transcriptional units was also reduced to parental levels. In contrast, clones containing fragments either upstream or downstream of the *sar* locus expressed SpA levels similar to that of the *sar* mutant alone (data not shown). We obtained similar results when we assayed for relative extracellular SpA levels in culture supernatants from these *sar* clones (data not shown).

To avoid the gene dosage problem associated with multicopy plasmids, we introduced a single copy of the fragment encoding the sarA transcript into the *sar* mutant via the integration vector pCL84, which preferentially inserts into the lipase gene of the staphylococcal chromosome. Northern blot analysis revealed that the transcription of the SpA gene in the integrant containing a single copy of *sarA* was reduced to near the parental level (Fig. 3) while the *sar* mutant clone containing only the integration vector pCL84 (ALC599 in Fig. 3) exhibited a high SpA gene transcript level that was slightly lower than that of the *sar* mutant alone. The reason for this slight reduction is not clear, because inactivation of the lipase gene has not been previously shown to affect SpA gene transcription (18a). Immunoblot studies with the cell wall extract of the clone containing a single copy of *sarA* also confirmed the down-regulation in SpA expression to the parental level (data not shown).

Suppression of SpA gene transcription in *sar* and *agr* mutants. As both *sar* and *agr* repress SpA expression (15), we

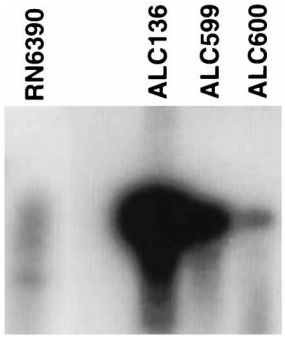

FIG. 3. Northern blot analysis of the SpA gene transcript in a *sar* mutant clone containing a single copy of the sarA transcript. ALC600 is a derivative of *sar* mutant ALC136 with plasmid pALC555 (pCL84::sarA) integrated into the lipase gene of the host chromosome. ALC599 is the *sar* mutant control containing the integrated plasmid pCL84 alone.

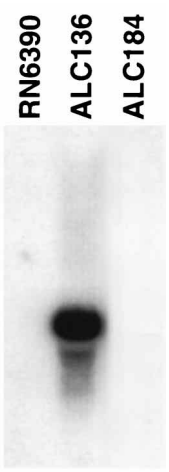

FIG. 4. Northern blot analysis of the SpA gene transcript of a *sar* mutant clone expressing RNAIII. ALC184 carries plasmid pRN6735, which encodes RNAIII and is derived from *sar* mutant ALC136. RNAIII could be expressed in late log phase when the *bla* promoter was induced with a β-lactam compound such as carboxyphenylbenzoyl-aminopenicillanic acid (17).

wanted to determine if the increase in SpA gene transcription in a *sar* mutant was mediated by RNAIII and/or other *agr*-independent but *sar*-specific pathways. We first examined if SpA gene transcription can be suppressed in a *sar* mutant by a plasmid containing the *agr* regulatory molecule RNAIII. Using plasmid pRN6735, which contains a cloned RNAIII fragment under the control of a β-lactamase promoter (22), we were able to show that the transcription of the SpA gene was suppressed in a *sar* mutant clone (ALC184) carrying this plasmid (Fig. 4). As expected, RNAIII was expressed at a higher level in this clone than in the *sar* mutant ALC136 (data not shown). Immunoblot analysis of the cell wall extract also confirmed a reduction in SpA expression in strain ALC184 (data not shown).

To assess the *agr*-independent mechanism in SpA expression, an *agr* mutant, RN6911, with the *agr* fragment, encoding both RNAII and RNAIII replaced by a *tetM* gene (22), was transformed with shuttle plasmid pSK236 containing all three *sar* transcripts. As predicted, the basal SpA gene transcription level was higher in the *agr* mutant than in the parental strain, RN6390 (Fig. 5). Surprisingly, the introduction of a 1.7-kb fragment encompassing all three *sar* transcripts into the *agr* mutant RN6911 yielded a parental level of SpA gene transcription (clone ALC865 in Fig. 5). Parental levels of SpA were also observed in the cell wall and extracellular fractions of the *agr* mutant clone containing this *sar* fragment (data not shown). Similar down-regulation in SpA expression was obtained when the plasmid (pALC862) containing all three *sar* transcripts was transformed into Tn*551*-mediated *agr* mutant RN6112. This mutant, with a transposon insertion in *agrA* (1), also lacked RNAIII transcription as determined by Northern blot analysis (data not shown).

To further assess the role of *sar* in the control of SpA expression, we assayed for SpA gene transcription in the double mutant ALC842 lacking both RNAIII and *sar*-related transcriptions. As expected, SpA gene transcription was not repressed in this genetic background (Fig. 6). However, upon the introduction of a fragment containing all three *sar* transcripts into the double mutant via the shuttle plasmid pSK236, the repression in SpA gene transcription was reestablished (ALC870 in Fig. 6). These data, together with those seen with *agr* mutant RN6911, imply an additional mechanism independent of RNAIII that is responsible for the down-modulation of SpA expression by the *sar* locus.

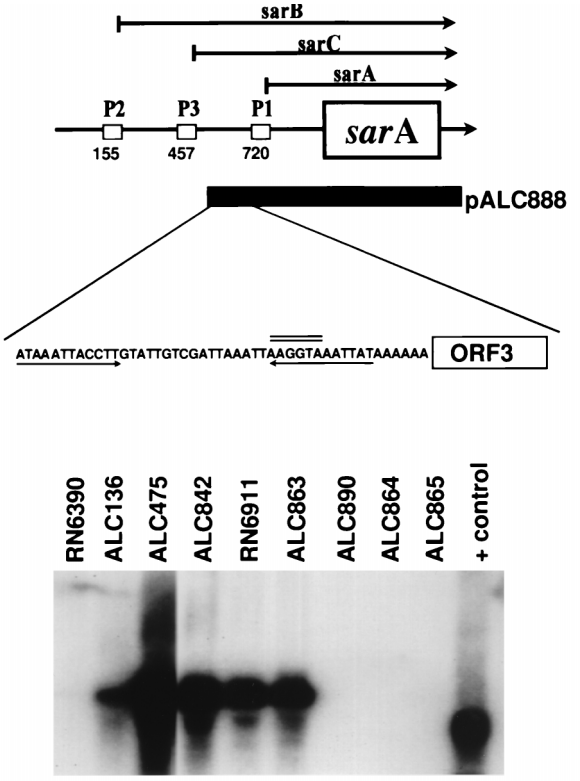

FIG. 5. Northern blot of the SpA gene transcript in *agr* mutant clones expressing various *sar* fragments. RN6911 is the *agr* mutant in which the entire *agr* locus has been replaced by the *tetM* gene (22). ALC863 (sarA), ALC890 (sarA plus 189 bp of upstream sequence), ALC864 (sarA and sarC), and ALC865 (sarA, sarB, and sarC) are derivatives of *agr* mutant RN6911. Positive controls include ALC475 (*sar* mutant ALC136 with pSPT181), the double mutant ALC842, and an 800-bp *spa* gene derived from RN7037 (last lane). Similar results were obtained with the respective RN6112-derived *agr* mutant clones (ALC519 [sarA], ALC626 [sarA plus 189 bp of upstream sequence], ALC624 [sarA and sarC], and ALC520 [sarA, sarB, and sarC]). The 189-bp fragment upstream of *sarA* within pALC888 is highlighted to show the relationship of the inverted repeat (arrows), putative Shine-Dalgarno sequence (double-lined), and the peptide coding region (ORF3). The positions of the three promoters in the 1,349-bp fragment are based on a published sequence (2).

The genetic requirement of the *sar* locus in SpA suppression in an *agr* mutant background. To determine the *sar* genetic requirement in the suppression of SpA gene transcription, we transformed shuttle plasmids (pALC213, pALC888, pALC861, and pALC862) containing progressively longer *sar* fragments into *agr* mutant RN6911 to yield ALC863, ALC890, ALC864, and ALC865, respectively. Plasmids containing similar *sar* fragments were also introduced into Tn*551*-mediated *agr* mutant RN6112 (Table 1). Northern blot analysis revealed that the *sar*-related transcripts were transcribed efficiently in these genetic backgrounds (data not shown). In contrast to the finding of an *agr* mutant clone containing a plasmid with all three *sar* transcripts (ALC865), a clone with a similar genetic background (ALC863) carrying a fragment containing the sarA transcript alone was not successful in suppressing SpA gene transcription (Fig. 5). Surprisingly, an *agr* mutant clone (ALC890) comprising the sarA transcript plus an additional 189-bp upstream sequence (but lacking the sarC-specific P3 promoter) down-modulated SpA gene transcription successfully. As anticipated, *sar* fragments larger than that in pALC888 also repressed SpA gene transcription. Thus, the minimum *sar* genetic requirement for suppressing SpA gene transcription in the RN6911-derived *agr* mutant background

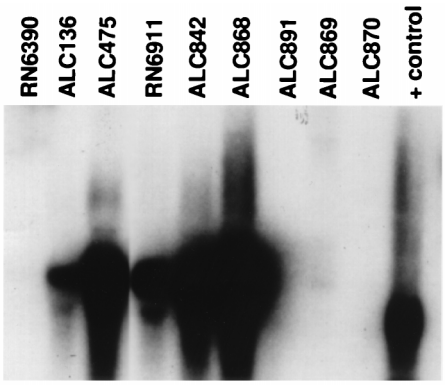

FIG. 6. A Northern blot of the SpA gene transcript in double mutant clones expressing *sar* fragments. The double mutant ALC842 is derived from *sarA* insertion mutant ALC488 (*sarA*::*ermC*), in which the *agr* locus has been replaced with the *tetM* gene. ALC868 (sarA), ALC891 (sarA plus 189 bp of upstream sequence), ALC869 (sarA and sarC), and ALC870 are derivatives of the double mutant ALC842. The positive control is an 800-bp *spa* fragment from RN7037.

appears to require the gene products derived from the sarA transcript plus an additional upstream fragment. A similar genetic requirement was also observed in the transposon *agr* mutant RN6112 (data not shown).

As an additional confirmation of this genetic requirement, we transformed shuttle plasmids encoding various *sar* fragments into the double mutant (ALC842). As seen in Fig. 6, only fragments containing the sarA transcript plus additional upstream sequence was able to repress SpA gene transcription in this mutant (ALC891) while the shuttle plasmid encompassing the *sarA* gene product alone had no effect (ALC868).

Genetic evidence that the *sar* transcripts are not regulated by *agr*. Given the surprising finding that the *sar* gene products were capable of reverting SpA gene transcription to the parental level in an *agr* mutant, we wanted to evaluate if the *agr* locus controls SpA gene transcription via *sar*. For this purpose, we analyzed *sar*-related transcription in an isogenic pair of *agr* strains in a Northern blot with a 732-bp *sarA* probe. As shown in Fig. 7, the *sar* transcript levels were identical between the *agr* mutant and its isogenic parent at all phases of the growth cycle, thus confirming that the *sar* transcripts are not regulated by the *agr* locus.

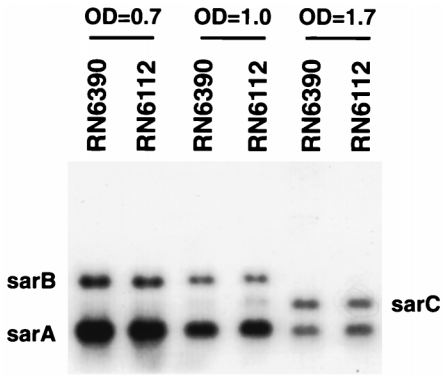

FIG. 7. Northern blot analysis of an isogenic *agr* strain (RN6112) probed with a 732-bp *sarA* fragment contained within plasmid pALC103 (Fig. 1). The optical densities at 650 nm (OD) of 0.7, 1.0, and 1.7 correspond to RNA obtained from cells harvested at the mid-log, late log, and stationary phases, respectively. Similar results were obtained with *agr* deletion mutant RN6911.

DISCUSSION

Expression of SpA, a major cell wall constituent of *S. aureus*, is apparently under the control of global regulatory loci such as *sar* and *agr* (15, 23). Phenotypic analysis suggests that *sar*, like *agr*, acts to repress SpA expression. Consequently, inactivation of the *sar* locus has led to a derepression in SpA synthesis. As in *agr*, repression of SpA synthesis by the *sar* locus occurs primarily at the transcriptional level. Transcriptional analysis revealed that the *sar* locus is composed of three overlapping transcripts that are initiated from a parallel multiple promoter system (2). To determine the genetic requirement of the *sar* locus for SpA suppression, we transformed DNA fragments containing one or more of the *sar* transcriptional units into a *sar* mutant. Our results indicated that a shuttle plasmid carrying a fragment that encoded the 560-bp sarA transcript alone was sufficient to repress SpA gene transcription in the *sar* mutant. More importantly, the introduction of the sarA transcript as a single copy into the lipase gene of the host chromosome via the integration vector pCL84 demonstrated that the effect of SpA suppression by the *sarA* gene product was not attributable to an increased gene dosage as a result of the plasmid multicopy effect. As the sarA transcript in strain RN6390 encodes a 339-bp *sarA* gene product, we speculate that the SarA protein is absolutely necessary for this mode of SpA suppression because 3′ deletion clones lacking the *sarA* ORF were incapable of repressing SpA gene transcription. In addition, the introduction of multiple stop codons into the amino terminus-encoding portion of the *sarA* gene by site-directed mutagenesis abolished the ability of the *sarA* gene to repress SpA gene transcription even when the mutated *sarA* mRNA was appropriately transcribed (unpublished data). This notion contrasts with the *agr* system, in which the regulatory molecule controlling exoprotein synthesis is believed to be the RNAIII transcript itself rather than its translated product (16, 22).

To assess the role of *sar* and *agr* in controlling SpA gene transcription, we first transformed a DNA fragment containing RNAIII into the *sar* mutant. A plasmid (pRN6735) containing the *agr* regulatory molecule RNAIII was able to suppress SpA gene transcription to the parental level in the *sar* mutant under inducible conditions, thus suggesting that the down-regulation in SpA gene transcription is partially mediated by RNAIII of the *agr* system. In studies with *agr* mutants (RN6911 and RN6112), our results showed that with increased *sar* dosage, SpA gene transcription can be suppressed to the parental level even in the absence of RNAIII. Additional studies with the double mutant ALC842 also revealed that SpA gene transcription can be down-modulated by supplying a plasmid carrying *sar* fragments. Taken together, our data support the hypothesis that the *sar* locus down-regulates SpA gene transcription via both *agr*-dependent and *agr*-independent but *sar*-specific pathways.

Interestingly, the *agr* mutant clone (ALC890 as derived from RN6911), as well as the double mutant clone (ALC891) containing an 819-bp DNA fragment (nucleotides 531 to 1349 according to the published sequence) (2) that comprised the sarA transcript together with a 189-bp upstream sequence, was successful in repressing SpA gene transcription to the parental level, while the respective clones expressing the sarA transcript alone did not alter the SpA gene transcript level. This genetic requirement contrasts with that seen in a *sar* mutant background, in which the *sarA* gene product alone was sufficient to down-regulate SpA gene transcription. This disparity in genetic requirements between *agr* and *sar* mutants for SpA suppression also concurs with the premise that there are two different modes of SpA repression by the *sar* locus.

Upon analysis of the sequence that spans the 189-bp region upstream of the sarA transcript, two structural features are apparent. First, this region encompasses a putative but weakly observed Shine-Dalgarno sequence that precedes a 39-amino-acid peptide coding region (ORF3 in Fig. 5). Notably, the *sar* fragment in pALC888, as found in *agr* mutant and double mutant clones (ALC890 and ALC891), did not encompass the putative sarC-specific promoter. Nevertheless, we have not entirely ruled out the possibility that this peptide coding region is transcribed and translated as a result of adjacent active promoters within the shuttle vector. Clearly, additional studies must be done to assess the role of this peptide coding region in modulating SpA gene transcription. Second, the Shine-Dalgarno sequence of ORF3 overlaps with a 12-bp inverted repeat whose axis of symmetry lies 26 bp upstream of a possible (ATG) initiation codon (Fig. 5) (2). We have previously observed that a 5′ deletion clone (pALC103) missing this particular inverted repeat resulted in a significant up-regulation in the sarA transcript, whereas a *sar* mutant clone encompassing this repeat but still lacking the sarC-specific promoter (ALC556) exhibited the parental level of sarA transcription (2). Whether this inverted repeat may exercise a modulatory function in sarA transcription to influence SpA gene transcription indirectly is not certain. However, given that the *sarA* gene is required for SpA suppression, it is unlikely that this inverted repeat plays a dominant role in regulating SpA gene transcription because the sarA transcript containing only the *sarA* gene is overexpressed in the absence of this repeat.

Although a single copy of the *sarA* gene was capable of suppressing SpA gene transcription in a *sar* mutant, this gene dosage was found to be insufficient for repression of SpA gene transcription in *agr* mutant bacteria grown in vitro because we have shown that the *sar* transcripts were expressed at parental levels in these mutants (Fig. 7). For effective attenuation in SpA expression in an *agr* mutant background, our data appear to support the notion that the *sar* gene products may have to be augmented. Whether augmentation in selected *sar* gene products (e.g., sarC) will occur in vivo (e.g., in infected tissues) will not be known until similar studies are done on bacteria harvested directly from infected animals. Interestingly, we have recently observed that the spontaneous *agr* mutation leading to increased SpA production can occur upon routine subculture in blood agar plates (28). Given that SpA is a major *S. aureus* cell wall protein that can make up as much as 7% of the cell wall content (12), one can argue that it is unlikely that the organism will devote significant cellular resources to increasing the production of SpA at a time when SpA no longer confers survival advantages (e.g., antiphagocytic properties of SpA). The availability of *sar* to suppress protein A gene transcription in *agr* mutants may conserve bacterial resources, especially in situations where SpA expression is not needed (e.g., bacteria within abscesses not directly exposed to phagocytes).

Recently, we have reported the existence of a multiple promoter system within the *sar* locus (2). In particular, the middle promoter responsible for *sarC* transcription can also suppress protein A transcription in *agr* mutants (Fig. 5 and 6). This middle promoter has a sigma B-dependent promoter with 100% consensus with that of *Bacillus subtilis* at the −10 position. Sigma B promoters are generally activated in response to stress or in the postexponential phase of the growth cycle. In concordance with the finding in *B. subtilis*, our data revealed that the middle promoter is most active during the stationary phase (Fig. 7). Preliminary reporter fusion studies showed that the middle promoter is active only upon sigma B induction in *B. subtilis* (unpublished data). Thus, it is theoretically plausible that increased sarC transcription in association with stress can occur in vivo to suppress protein A gene transcription in spontaneous *agr* mutants.

In a previous study, Vandenesch et al. showed that the provision of RNAIII in *trans*, preferentially expressed in the late log to stationary phase of the growth cycle, can repress SpA gene transcription in an *agr* mutant (26). Remarkably, the transcription of the SpA gene, as discerned in this study, can also be repressed by RNAIII in the *sar* mutant background. Conversely, we also showed that in the *agr* mutant background, the down-modulation of SpA gene transcription would occur if the SpA gene transcript plus the essential upstream regulatory element are provided in *trans* (Fig. 5 and 6). The ability to curtail SpA gene transcription in either the *sar* or *agr* mutant background suggests that there are coregulatory factors in the capacity of *S. aureus* to down-regulate SpA expression in the stationary phase of the growth cycle. Whether *sar* or *agr* operates in different parts of the stationary growth cycle to regulate SpA gene transcription is not clear. Alternatively, the capacity for coregulation may provide the bacterium with the means to control SpA gene transcription in spontaneous *agr* or *sar* mutants. Notably, spontaneous *agr* mutants have been found on blood agar plates by selecting for α-hemolysin negative colonies (28).

Based on recent gel shift data (15), we have hypothesized that the *sar* transcriptional elements are probably essential to the expression of DNA-binding proteins with binding specificity for the RNAII promoter, thereby activating RNAII and subsequently RNAIII transcription. We further suggested that the *sar* locus may control hemolysin production via the *agr*-mediated pathway. However, the capacity to suppress SpA gene transcription with *sar* fragments in *agr* mutants lacking RNAIII transcription and vice versa implies that the *sar*-mediated pathway of SpA gene transcription has both RNAIII-dependent and RNAIII-independent components and thus differs from the sole RNAIII-dependent mechanism in the *sar*-mediated pathway of hemolysin production. This premise was supported by the finding that *agr* mutant clones containing plasmids including sarC or larger were able to repress SpA gene transcription but did not restore hemolysin production to these mutants (unpublished observation). Additionally, we cannot attribute the *sar*-mediated suppression of SpA gene transcription in *agr* mutants to possible counterregulation of *sar* by *agr*, because the *sar* transcripts were present at parental levels in an *agr* mutant (Fig. 7).

During the growth cycle, SpA is actively synthesized during the log phase. However, the transcription of the SpA gene is repressed as the cells enter the late log to stationary phase of the growth cycle. The suppression of SpA gene transcription is coregulated by two repressor systems, *agr* and *sar*. Notably, the existence of coregulatory factors argues for a mechanism whereby the bacterial host can attenuate SpA gene transcription in different genetic backgrounds.

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REFERENCES

1. **Arvidson, S., L. Janzon, and S. Löfdahl.** 1990. The role of the δ-lysin gene (*hld*) in the *agr* dependent regulation of exoprotein synthesis in *Staphylococcus aureus*, p. 419–431. *In* R. P. Novick (ed.), Molecular biology of the staphylococci. VCH Publishers, New York, N.Y.
2. **Bayer, M. G., J. H. Heinrichs, and A. L. Cheung.** 1996. The molecular architecture of the *sar* locus in *Staphylococcus aureus*. J. Bacteriol. **178:**4563–4570.
3. **Bjork, I., B. A. Petersson, and J. Sjöquist.** 1972. Some physiochemical properties of protein A from Staphylococcus aureus. Eur. J. Biochem. **29:**579–584.
4. **Blake, M. S., K. H. Johnston, G. J. Russell-Jones, and E. C. Gotschlich.** 1984. A rapid sensitive method for detection of alkaline phosphatase-conjugated anti-antibody on Western blots. Anal. Biochem. **136:**175–179.
5. **Cheung, A. L., A. S. Bayer, J. Peters, and J. I. Ward.** 1987. Analysis by gel electrophoresis, Western blot, and peptide mapping of protein A heterogeneity in *Staphylococcus aureus* strains. Infect. Immun. **55:**843–847.
6. **Cheung, A. L., K. Eberhardt, and V. A. Fischetti.** 1994. A method to isolate RNA from gram-positive bacteria and mycobacteria. Anal. Biochem. **222:**511–514.
7. **Cheung, A. L., and V. A. Fischetti.** 1988. Variation in the expression of cell wall proteins of *Staphylococcus aureus* grown on solid and liquid media. Infect. Immun. **56:**1061–1065.
8. **Cheung, A. L., and P. Ying.** 1994. Regulation of α- and β-hemolysins by the *sar* locus of *Staphylococcus aureus*. J. Bacteriol. **176:**580–585.
9. **Cheung, A. L., and S. J. Projan.** 1994. Cloning and sequencing of *sarA* of *Staphylococcus aureus*, a gene required for the expression of *agr*. J. Bacteriol. **176:**4168–4172.
10. **Easmon, C. S. F., and C. Adlam.** 1983. Staphylococci and staphylococcal infections. Academic Press, Inc., New York, N.Y.
11. **Espersen, F.** 1985. Complement activation by clumping factor and protein A from *Staphylococcus aureus* strain E 2371. Acta Pathol. Microbiol. Immunol. Scand. Sect. C **93:**59–64.
12. **Forsgren, A.** 1969. Protein A from *Staphylococcus aureus*. Production of protein A by bacterial and L forms of *S. aureus*. Acta Pathol. Microbiol. Scand. **75:**481–490.
13. **Goding, J. W.** 1978. Use of staphylococcal protein A as an immunological reagent. J. Immunol. Methods **20:**241–253.
14. **Greenberg, D. P., A. S. Bayer, A. L. Cheung, and J. I. Ward.** 1989. Protective efficacy of protein A-specific antibody against bacteremic infection due to *Staphylococcus aureus* in an infant rat model. Infect. Immun. **57:**1113–1118.
15. **Heinrichs, J. H., M. G. Bayer, and A. L. Cheung.** 1996. Characterization of the *sar* locus and its interaction with *agr* in *Staphylococcus aureus*. J. Bacteriol. **178:**418–423.
16. **Janzon, L., and S. Arvidson.** 1990. The role of the δ-hemolysin gene (*hld*) in the regulation of virulence genes by the accessory gene regulator (*agr*) in *Staphylococcus aureus*. EMBO J. **9:**1391–1399.
17. **Kornblum, J., B. Kreiswirth, S. J. Projan, H. Ross, and R. P. Novick.** 1990. *agr*: a polycistronic locus regulating exoprotein synthesis in *Staphylococcus aureus*, p. 373–402. *In* R. P. Novick (ed.), Molecular biology of the staphylococci. VCH Publishers, New York, N.Y.
18. **Kronvall, G., P. G. Quie, and R. C. Williams, Jr.** 1970. Quantitation of staphylococcal protein A: determination of equilibrium constant and number of protein A residues on bacteria. J. Immunol. **104:**273–278.

18a. **Lee, C.** Personal communication.

19. **Lee, C. Y., S. L. Buranen, and Z. H. Ye.** 1991. Construction of single copy integration vectors for *Staphylococcus aureus*. Gene **103:**101–105.
20. **Mahmood, R., and S. A. Khan.** 1990. Role of upstream sequences in the expression of the staphylococcal enterotoxin B gene. J. Biol. Chem. **265:**4652–4656.
21. **Novick, R. P.** 1990. The staphylococcus as a molecular genetic system, p. 1–40. *In* R. P. Novick (ed.), Molecular biology of the staphylococci. VCH Publishers, New York, N.Y.
22. **Novick, R. P., H. F. Ross, S. J. Projan, J. Kornblum, B. Kreiswirth, and S. Moghazeh.** 1993. Synthesis of staphylococcal virulence factors is controlled by a regulatory RNA molecule. EMBO J. **12:**3967–3977.
23. **Patel, A. H., J. Kornblum, B. Kreiswirth, R. Novick, and T. J. Foster.** 1992. Regulation of the protein A-encoding gene in *Staphylococcus aureus*. Gene **114:**25–34.
24. **Patel, A. H., P. Nowlan, E. D. Weavers, and T. Foster.** 1987. Virulence of protein A-deficient and alpha-toxin-deficient mutants of *Staphylococcus aureus* isolated by allele replacement. Infect. Immun. **55:**3103–3110.
25. **Peterson, P. K., J. Verhoef, L. D. Sabath, and P. G. Quie.** 1977. Effect of protein A on staphylococcal opsonization. Infect. Immun. **15:**760–764.
26. **Vandenesch, F., J. Kornblum, and R. P. Novick.** 1991. A temporal signal, independent of *agr*, is required for *hla* but not *spa* transcription in *Staphylococcus aureus*. J. Bacteriol. **173:**6313–6320.
27. **Waldvogel, F. A.** 1985. *Staphylococcus aureus*, p. 1097–1116. *In* G. L. Mandell, R. G. Douglas, Jr., and J. E. Bennett (ed.), Principles and practice of infectious diseases. John Wiley & Sons, Inc., New York, N.Y.
28. **Wolz, C., and A. L. Cheung.** 1996. Characterization of a mutation in *agrC* of *Staphylococcus aureus*, abstr. P119, p. 218. *In* Abstracts of the VIII International Symposium on Staphylococci and Staphylococcal Infections 1996.

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