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Hyperproduction of Alpha-Hemolysin in a *sigB* Mutant Is Associated with Elevated SarA Expression in *Staphylococcus aureus*

AMBROSE L. CHEUNG,[1]* YUEH-TYNG CHIEN,[1] AND ARNOLD S. BAYER[2,3,4]

Laboratory of Bacterial Pathogenesis and Immunology, The Rockefeller University, New York, New York 10021[1]; St. John's Cardiovascular Research Center[2] and Division of Infectious Diseases,[3] Harbor-UCLA Medical Center, Torrance, California 90509; and UCLA School of Medicine, Los Angeles, California 90024[4]

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To evaluate the role of SigB in modulating the expression of virulence determinants in *Staphylococcus aureus*, we constructed a *sigB* mutant of RN6390, a prototypic *S. aureus* strain. The mutation in the *sigB* gene was confirmed by the absence of the SigB protein in the mutant on an immunoblot as well as the failure of the mutant to activate σB-dependent promoters (e.g., the *sarC* promoter) of *S. aureus*. Phenotypic analysis indicated that both alpha-hemolysin level and fibrinogen-binding capacity were up-regulated in the mutant strain compared with the parental strain. The increase in fibrinogen-binding capacity correlated with enhanced expression of clumping factor and coagulase on immunoblots. The effect of the *sigB* mutation on the enhanced expression of the alpha-hemolysin gene (*hla*) was primarily transcriptional. Upon complementation with a plasmid containing the *sigB* gene, *hla* expression returned to near parental levels in the mutant. Detailed immunoblot analysis as well as a competitive enzyme-linked immunosorbent assay of the cell extract of the *sigB* mutant with anti-SarA monoclonal antibody 1D1 revealed that the expression of SarA was higher in the mutant than in the parental control. Despite an elevated SarA level, the transcription of RNAII and RNAIII of the *agr* locus remained unaltered in the *sigB* mutant. Because of a lack of perturbation in *agr*, we hypothesize that inactivation of *sigB* leads to increased expression of SarA which, in turn, modulates target genes via an *agr*-independent but SarA-dependent pathway.

Staphylococcus aureus is a major cause of human infections, such as superficial abscesses, pneumonia, endocarditis, and sepsis (6). The control of a multitude of extracellular and cell wall virulence determinants in *S. aureus* is growth phase dependent. In particular, cell wall proteins are normally synthesized in the logarithmic phase, while exoproteins are generally produced postexponentially. The growth phase dependence of these virulence factors is mediated in part by global regulatory loci, such as *sar* (12) and *agr* (22). These modulators may either interact with the target gene directly (e.g., RNAIII with *hla* [alpha-hemolysin gene] mRNA) or control another regulatory molecule (e.g., *sar* regulation of the *agr* gene product) which, in turn, alters the transcription of the target gene.

The *sar* locus is composed of three overlapping transcripts, designated *sarA* (0.56 kb), *sarC* (0.8 kb), and *sarB* (1.2 kb), initiated from the P1, P3, and P2 promoters, respectively. Because of this multiplicity of promoters, the activation of *sar* leading to the expression of SarA, the major *sar* regulatory molecule, is complex and may be growth phase dependent. Whereas the *sarB* transcript and the more abundant *sarA* transcripts are maximally expressed during the exponential phase, the transcription of *sarC* from the P3 promoter is most active during the postexponential phase (3). Additional transcriptional analysis indicated that the P3 promoter is σB dependent (17, 20, 25).

In contrast to the primary sigma factor (σA), which is required for the expression of housekeeping genes, SigB (σB) is an alternate transcription factor that has been shown to respond to environmental stresses (e.g., stationary phase of growth) in gram-positive bacteria (20). The core RNA polymerase associated with a particular sigma factor recognizes a specific set of promoters with conserved sequence motifs to initiate the transcription of genes programmed to respond to certain environments (20, 22). For *Bacillus subtilis*, σB activity is regulated posttranslationally by complex networks of protein-protein interactions governed by a variety of environmental stresses (1, 20). Because one of the promoters (P3) within the *sar* locus is σB dependent, it is conceivable that the SigB protein influences *sar* expression. As the *sar* locus activates the synthesis of alpha-hemolysin at the transcriptional level, presumably in part through the interaction of SarA with the *agr* locus (15), we speculate that *sigB* may modulate *sarA* expression and the ensuing *hla* transcription.

In this study, we report the construction and characterization of a *sigB* mutant of RN6390, a prototypic *S. aureus* strain. The specificity of the mutation was confirmed by the absence of the SigB protein on an immunoblot, but the protein was restored in the mutant by a shuttle plasmid carrying the *sigB* gene. Phenotypic analysis revealed that the *sigB* mutant strain secreted more alpha-hemolysin than the parental strain, as determined by immunoblotting and Northern analysis. Complementation of the mutant with the *sigB* gene in *trans* reestablished alpha-hemolysin expression to near parental levels. Interestingly, the hyperproduction of alpha-hemolysin coincided with elevated SarA expression in the *sigB* mutant. Using the rabbit endocarditis model, we found that the *sigB* mutation was stable in vivo. We hypothesize that the hyperproduction of alpha-hemolysin in *S. aureus* as a result of the *sigB* mutation is mediated by an increase in the SarA level which, in turn, enhances the transcription of *hla* via a direct pathway (i.e., *agr* independent).

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

TABLE 1. Strains and plasmids used in this study

Strain or plasmid	Reference(s) or source	Description
Strains		
S. aureus		
RN6390	22, 26	Laboratory strain that maintains its hemolytic pattern when propagated on sheep erythrocytes and has a genetic background similar to that of 8325-4
RN4220	26	Mutant of 8325-4 that accepts foreign DNA
COL	30	Methicillin-resistant *S. aureus* strain
RUSA168	30	*sigB* mutant of COL (*sigB*::Tn*551*)
ALC1001	This study	*sigB* mutant of RN6390
ALC1497	This study	ALC1001 complemented with shuttle plasmid pALC1496 (with the *sigB* gene)
E. coli		
BL21	Novagen	Host strain for pET14b
DU5384	27	Host strain carrying pBR322 with a 3-kb *Eco*RI-*Hin*dIII fragment of the *hla* gene
Plasmids		
pCR2.1	Invitrogen	PCR cloning vector
pET14b	Novagen	*E. coli* expression vector
pALC103	3	pSPT181 with a *sar* fragment from nucleotides 620 to 1349
pALC1270	This study	pET14b with the *sigB* coding region cloned into the *Nde*I-*Bam*HI sites
pDG148	18	*B. subtilis-E. coli* shuttle plasmid (8.2 kb) containing the pSpac promoter (IPTG inducible) followed by a polylinker site and *lacI*
pSK236	19	*S. aureus-E. coli* shuttle vector with pUC19 cloned into the *Hin*dIII site of pC194
pALC1456	This study	pSK236 containing a 1.6-kb fragment derived from pDG148 and comprising, sequentially, the pSpac promoter, a polylinker site, and the *lacI* repressor
pALC1496	This study	pALC1456 with the *sigB* gene (*rbs* + coding region) cloned into the polylinker site (*Sal*I-*Pst*I) derived from pDG148

MATERIALS AND METHODS

Bacterial strains, plasmids, and growth media. The bacterial strains and plasmids used in this study are listed in Table 1. Phage φ11 was used as the transducing phage for *S. aureus* strains. CYGP, 0.3GL medium (26), and tryptic soy broth (TSB) were used for the growth of *S. aureus* strains, while Luria-Bertani medium was used for growing *Escherichia coli*. Antibiotics were used at the following concentrations: erythromycin at 5 μg/ml and ampicillin at 50 μg/ml.

Genetic manipulations in *S. aureus*. A *sigB* mutant of RN6390 was constructed as described previously (12) by transducing the parental strain with a phage lysate of strain RUSA168 carrying the *sigB* mutation (30). Transductants were selected on agar containing erythromycin. Correct insertion of Tn*551* into the *sigB* locus of RN6390 was confirmed by Southern blotting with Tn*917* and *sigB* probes as described previously (12). One transductant, ALC1001, was chosen for further studies.

To complement the *sigB* mutation in ALC1001, we introduced into the shuttle plasmid pSK236 a 1.6-kb fragment (derived from pDG148) containing the pSpac promoter followed by the polylinker site and the *lacI* repressor of *E. coli*, yielding pALC1456. Plasmid pALC1496 was constructed by cloning the *sigB* open reading frame into the multiple cloning site of pALC1456. The shuttle plasmid was electroporated into RN4220 and then transduced into *sigB* mutant ALC1001 with phage φ11 as described previously (12). The presence of the recombinant plasmid was confirmed by restriction mapping.

Production of anti-SigB monoclonal antibodies. Based on the published sequence (30), we amplified by PCR the 768-bp *sigB* gene with the following primers: 5′-GCCAT^{2687}ATGGCGAAAGAGTCGAAATCAGCT^{2710}-3′ (*Nde*I site underlined) and 5′-GCGGATCCCTA^{3454}TTGATGTGCTGCTTCTTG^{3437}-3′ (*Bam*HI site underlined). The correct PCR product, verified by automated sequencing, was cloned into the *Nde*I-*Bam*HI sites of expression vector pET14b (Novagen, Madison, Wis.) and transformed into *E. coli* BL21(DE3).pLys.S. The resulting plasmid, pALC1270, contained an N-terminal His tag and the entire *sigB* coding region.

Recombinant protein expression was induced by adding isopropyl-β-D-thiogalactopyranoside (IPTG) (1 mM) to a growing culture (30°C) at an optical density at 600 nm (OD_{600}) of 0.5. Three hours after induction, the cells were harvested, resuspended in binding buffer (5 mM imidazole, 0.5 M NaCl, 20 mM Tris-HCl [pH 7.9]), and sonicated on ice. After removal of the cellular debris by centrifugation (15,000 × *g* for 15 min), the clarified supernatant was purified on a nickel affinity column in accordance with the manufacturer's instructions. The protein (≈30,000 kDa) eluted from the column with elution buffer (1 M imidazole, 0.5 M NaCl, 20 mM Tris-HCl [pH 7.9]) appeared homogeneous on a sodium dodecyl sulfate gel (data not shown) and was authenticated by sequencing of the N-terminal 15 residues after the His tag had been removed by digestion with thrombin.

Purified SigB was used to immunize two (BALB/c × SJL/J) F_1 mice (100 μg each) to obtain monoclonal antibodies as described by Jones et al. (21). The titers of the immune sera were determined by an enzyme-linked immunosorbent assay (ELISA) in which diluted sera were added to microtiter wells precoated with purified SigB (5 μg/ml). After hybridoma fusion, antibodies from limiting dilutions were screened by an ELISA with immobilized SigB protein. Monoclonal antibody 2D7 was purified from culture supernatants with a protein A affinity column (21) and tested for reactivity with purified SigB on immunoblots.

Phenotypic analysis of the *sigB* mutant and its isogenic parent. Several virulence traits of the *sigB* mutant were evaluated. First, the production of alpha-, beta-, and delta-hemolysins in the *sigB* mutant and its parental counterpart was assessed by cross-streaking the tested strain with indicator strains as described previously (9). To verify the production of alpha-hemolysin on immunoblots, equivalent volumes of extracellular proteins that had been harvested at the stationary phase and concentrated 50-fold by use of a Centriprep concentrator (Amicon Inc., Beverly, Mass.) were blotted onto nitrocellulose and probed with rabbit anti-alpha-hemolysin antibody (a gift from B. Menzies, Nashville, Tenn.) diluted 1:2,000 and then with the $F(ab)_2$ fragment of the goat anti-rabbit antibody–alkaline phosphatase conjugate (Jackson ImmunoResearch, West Grove, Pa.) as described previously (7). Reactive bands were visualized as described by Blake et al. (5).

Taking advantage of the cytolytic effects of alpha-hemolysin upon rabbit erythrocytes and platelets (2, 4), two functional assays for alpha-hemolysin production were performed. First, alpha-hemolysin levels were quantitated by assaying the hemolytic titers of serially diluted stationary-phase culture supernatants for 1% washed rabbit erythrocytes as described previously (2, 4). Purified alpha-hemolysin was used as the positive control. The data were expressed as mean units of hemolytic activity per milliliter of culture supernatant from two separate runs. The hemolytic units were defined as the reciprocal of the highest dilution of the culture supernatant causing 50% erythrocyte lysis. Second, the extent of alpha-hemolysin production was ascertained by measuring platelet lysis spectrophotometrically (at OD_{600}) upon exposure to bacterial supernatants as described previously (2).

In addition to hemolysins, we also measured other putative virulence traits, such as fibronectin- and fibrinogen-binding capacities. The fibronectin-binding capacity of the isogenic pair was compared with an ^{125}I fibronectin-binding assay as described previously (9). The fibrinogen-binding capacity was determined semiquantitatively by immunoblotting. Cell wall proteins from equivalent numbers of bacterial cells grown overnight were extracted as described previously (10). Equivalent volumes of cell wall extracts (10 μl) were resolved on sodium dodecyl sulfate gels and transferred to nitrocellulose. The nitrocellulose membranes were then blocked with blocking buffer (0.01 M Tris, 0.5 M NaCl, 0.5% Tween 20 [pH 8.2]) containing 1% bovine serum albumin for 1 h at 37°C,

incubated at 37°C with fibrinogen (1 mg/ml) in the same buffer, and washed three times with blocking buffer and finally with goat antifibrinogen antibody conjugated to alkaline phosphatase (1:1,000) as described previously (13). As both coagulase and clumping factor bind fibrinogen (14, 29), we assayed for the presence of these proteins in cell wall extracts by immunoblotting. Nitrocellulose membranes containing cell wall extracts were incubated with blocking buffer containing 0.1% human serum (for the blocking protein A-Fc interaction) for 1 h at room temperature. Rabbit anticoagulase (1:1,000) and anti-ClfA (1:1,000) antibodies were then added to separate blots, followed by goat anti-rabbit antibody–alkaline phosphatase conjugate (1:10,000). All reactive bands were visualized as described by Blake et al. (5).

Isolation of RNA and Northern blot hybridization. Overnight cultures of *S. aureus* were diluted 1:50 in CYGP and grown to the mid-log (OD_{650}, 0.7), late log (OD_{650}, 1.1), and postexponential (OD_{650}, 1.7) phases. The cells were pelleted and processed with a FastRNA isolation kit (Bio 101, Inc., Vista, Calif.) in combination with 0.1-mm-diameter sirconia-silica beads in a FastPrep reciprocating shaker (Bio 101) as described previously (8). Ten micrograms of each sample was 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]). Blotting of RNA onto Hybond N^+ membranes (Amersham, Arlington Heights, Ill.) was performed with a Turboblotter alkaline transfer system (Schleicher & Schuell, Inc., Keene, N.H.). For detection of specific transcripts (*agr*, *sar*, and *hla*), gel-purified DNA probes were radiolabeled with [α-^{32}P]dCTP by the random-primer method (Ready-To-Go labeling kit; Pharmacia) and hybridized under high-stringency conditions (7). The blots were subsequently washed and autoradiographed.

Preparation of cell extracts and immunoblot analysis of SigB and SarA in the *sigB* mutant. Cell extracts were prepared for strain RN6390 and the corresponding *sigB* mutant. After being pelleted, the cells were resuspended in 1 ml of TEG buffer (25 mM Tris, 5 mM EGTA [pH 8]), and cell extracts were prepared from lysostaphin-treated cells as described previously (15).

Cell extracts were immunoblotted onto nitrocellulose membranes as described above. For the detection of SigB and SarA, monoclonal antibodies 2D7 (1:1,000 dilution) and 1D1 (1:2,500 dilution), respectively, were added to an immunoblot and allowed to incubate with the membrane for 3 h, followed by another h of incubation with a 1:10,000 dilution of goat anti-mouse antibody–alkaline phosphatase conjugate. The reactive bands were visualized as described previously (5).

Determination of SarA levels by a competitive ELISA. Microtiter wells were coated with purified SarA protein (ca. 3 μg/ml) in 0.1 M Tris–0.3 mM $MgCl_2$ (pH 10) for 3 h at 37°C. After being washed with phosphate-buffered saline (PBS)–Brij (pH 7.4), the wells were blocked with PBS-Brij containing 1% bovine serum albumin overnight at 4°C. The optimal dilution of antibody, defined as the dilution at which 50% of the antibody (i.e., 50% of the maximum amount of bound antibody) bound to the protein-coated wells, was determined to be 1:20,000. Using anti-SarA antibody 1D1 at the optimal dilution (1:20,000), we constructed a standardized curve by adding known quantities of purified SarA (0 to 25 μg/ml) to compete for binding to the anti-SarA antibody, resulting in inhibition of binding to the immobilized SarA protein. For the competitive assay, triplicate cell extracts prepared from individual *sar* mutant clones (see above for preparation methods) were substituted for purified SarA. The protein concentrations in the cell extracts were determined with a protein assay kit from Bio-Rad Laboratories, Hercules, Calif. By comparing the levels of inhibition to the standardized curve, the relative concentration of the SarA protein in each extract was derived.

Rabbit model of endocarditis. To ascertain the stability of the *sigB* mutation in vivo, we chose the rabbit model of endocarditis. Briefly, a bacterial suspension harvested from an overnight culture was diluted in PBS, and bacterial numbers were confirmed by plate counting. Endocarditis on the aortic valves of New Zealand White rabbits (2 kg) was induced by catheterization as previously described (9). At 48 h postcatheterization, groups of animals (three each) were separately challenged intravenously with an inoculum of either 2×10^5 or 2×10^6 CFU. Catheters remained in place until animals were sacrificed by lethal intravenous injection of sodium pentobarbital (100 mg/kg of body weight). At the time of sacrifice (48 h postinfection), aortic valves and left ventricular vegetations from infected animals were removed, pooled, homogenized, and quantitatively cultured. Some colonies were then examined for the retention of the *sigB* mutation after in vivo passage by Southern (with a Tn*551* probe) and Western blotting.

RESULTS

Construction of a *sigB* mutant of RN6390. The mutation in a Tn*551* insertion mutant, RUSA168, was previously mapped to the *sigB* gene of *S. aureus* COL (30). To assess the phenotypic effect of the *sigB* mutation in a genetic background with well-defined virulence determinants (9), we elected to transduce the mutation from RUSA168 to RN6390 to yield mutant ALC1001. Southern blot hybridization with a Tn*917* probe which shows significant homology with Tn*551* revealed that the transposon insertion in ALC1001 was analogous to that found in RUSA168 (data not shown). To further confirm the mutation, we made use of the observation that the P3 promoter of the *sar* locus of *S. aureus* is σB dependent (17, 25). Northern analysis revealed that P3-initiated *sarC* transcription was absent in strain ALC1001, whereas the σA-dependent P1 promoter of *sar* was unaffected (Fig. 1). Complementation studies with plasmid pALC1496, carrying the *sigB* gene, revealed that complemented mutant ALC1497 showed *sarC* transcription. Of note, the pSpac promoter was found to be active in *S. aureus* even in the absence of the inducing agent IPTG, suggesting that, contrary to observations made for *B. subtilis* (18), this promoter is leaky in the staphylococcal background.

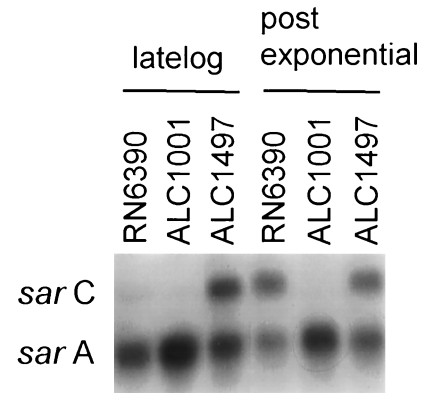

FIG. 1. Northern analysis of *sar* transcripts of *S. aureus* RN6390, its isogenic *sigB* mutant ALC1001, and complemented mutant ALC1497. Northern analysis revealed that the *sigB* gene was transcribed in ALC1497 (data not shown). Ten micrograms of total cellular RNA obtained at the stationary phase (OD_{650}, 1.7, as determined with an 18-mm borosilicate glass tube) was applied to each lane. The probe was a 730-bp *sarA* fragment (nucleotides 620 to 1349, based on the published sequence) (3). Because the transcription of *sarB*, the largest transcript within the *sar* locus, was minimal during the late log and stationary phases, only data for *sarA* and *sarC* transcripts are shown.

To ascertain if the mutation in ALC1001 was indeed due to *sigB*, we probed a cell lysate of the mutant with anti-SigB monoclonal antibody 2D7 in an immunoblot. As shown in Fig. 2, SigB was not detectable in the mutant, while the parental strain (RN6390) as well as the complemented mutant strain (ALC1497) were found to contain SigB, as confirmed by the reactivity of the cell extract with the anti-SigB antibody. Additionally, SigB was not found in the mutant strain carrying the vector alone (data not shown). Consistent with the observation

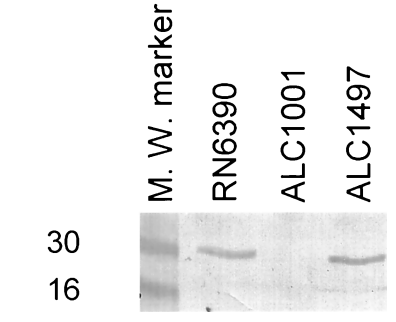

FIG. 2. Immunoblot of cell extracts of the parental strain, *sigB* mutant strain ALC1001, and complemented mutant strain ALC1497 probed with anti-sigB monoclonal antibody 2D7 (1:1,000 dilution). About 50 μg of cellular proteins was applied to each lane. The experiment was repeated two times, with essentially the same results. M. W., molecular weight. Numbers at left are in thousands.

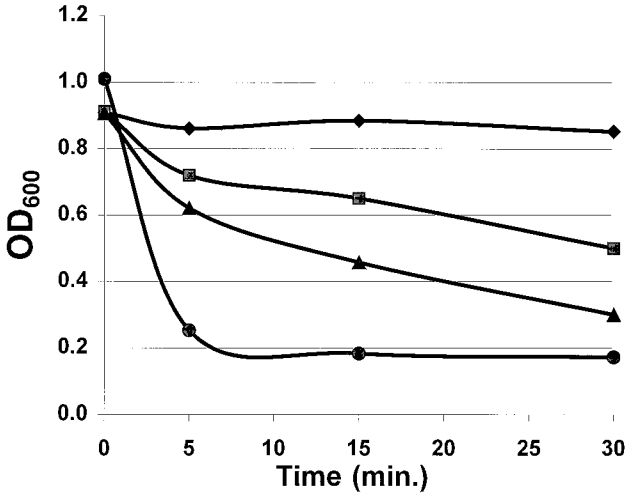

FIG. 3. Lysis of platelets monitored on the basis of OD_{600}. The supernatants of 18-h bacterial cultures in TSB were mixed with platelets (10^9 platelets/ml). Platelet lysis was monitored by measuring the decrease in the OD_{600} over time (2). The medium and purified alpha-toxin served as the negative and positive controls, respectively. Symbols: diamonds, TSB control plus washed platelets; squares, RN6390 (supernatant) plus washed platelets; triangles, SigB (supernatant) plus washed platelets; circles, purified alpha-toxin (1 mg/ml) plus washed platelets.

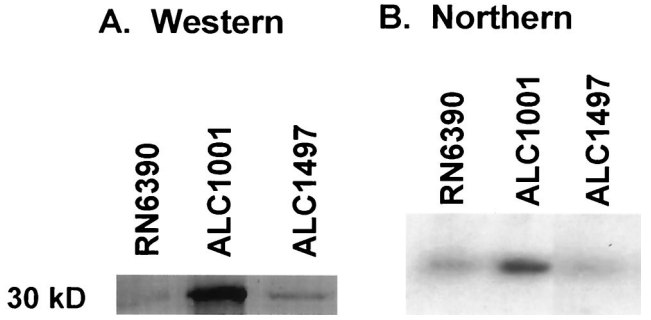

FIG. 4. Western and Northern analyses of the alpha-hemolysin gene product. (A) An immunoblot of extracellular proteins of ALC1001, its isogenic parent RN6390, and complemented strain ALC1497 from the late log phase was probed with rabbit anti–alpha-hemolysin antibody (1:2,500 dilution). (B) Ten micrograms of total cellular RNA obtained from the late log to early stationary phases was applied to each lane. The probe was a 3-kb *Eco*RI-*Hin*dIII fragment of the alpha-hemolysin gene (7). Similar complementation results were obtained with another *sigB* mutant (RUSA168) and plasmid pALC1496. These experiments were repeated three times, with similar results. The results of a representative experiment are shown.

that SigB, being a regulatory molecule, is present in a low quantity in cells, we were able to discern the presence of SigB only upon loading at least 50 μg of cellular proteins from the parental strain.

To determine the stability of the *sigB* mutation in vivo, we infected rabbits that had been previously catheterized to produce thrombotic endocarditis with ALC1001. After harvesting the bacteria obtained from the cardiac vegetations of this animal model, we confirmed by Southern and Western analyses that the *sigB* mutation remained stable after in vivo passage (data not shown).

Phenotypic characterization of *sigB* mutant ALC1001. When ALC1001 and RUSA168 were streaked on blood agar plates, it was observed that the clear zone of hemolysis surrounding 24-h cultures was significantly enhanced in both *sigB* mutants compared with the respective parental strains. As the virulence determinants of RN6390 are well described (9), we chose to focus our analysis on *sigB* mutant ALC1001. In quantitative hemolysis assays for alpha-hemolysin, the culture supernatant of mutant ALC1001 yielded a mean hemolytic titer of 1,560 U/ml, while the corresponding parental strain titer was 490 U/ml. Purified alpha-hemolysin yielded a titer of 22,736 U/mg of protein. As an additional assay for the functional aspects of alpha-hemolysin, we also found that the culture supernatant of ALC1001 had a greater capacity to lyse platelets than the isogenic parental strain, as monitored by a decrease in the OD_{600} in a turbidimetric assay (Fig. 3). An immunoblot of equivalent amounts of extracellular proteins of ALC1001, RN6390, and complemented strain ALC1497 disclosed that alpha-hemolysin was synthesized at a higher level in the *sigB* mutant than in the parental strain but was present at near the parental level in ALC1497 (Fig. 4A). In measuring the transcriptional activity of *hla* by Northern blotting, we also found that the *hla* mRNA level of ALC1001 was higher than that of RN6390 (Fig. 4B). Complementation of *sigB* mutant ALC1001 with a *sigB*-carrying plasmid (pALC1496) expressing the *sigB* transcript (data not shown) restored *hla* transcription to the parental level (Fig. 4B). Similar results on the expression of alpha-hemolysin were observed with RUSA168 complemented with the *sigB*-carrying plasmid pALC1496 (data not shown). Collectively, these data imply that the *sigB* mutation is associated with enhanced *hla* expression initiated at the transcriptional level.

The effect of the *sigB* mutation on beta-hemolysin secretion, as assayed on immunoblots, was more equivocal, with only a marginal increase in ALC1001 and no change in RUSA168 compared with the results for the respective parental strains. In comparison to that in RN6390, the expression of delta-hemolysin (*hld*) in ALC1001 on cross-streaked blood agar plates was not altered (see below for the RNAIII transcript encoding *hld*).

As cell wall-associated proteins such as fibronectin- and fibrinogen-binding proteins likely play a role in mediating the binding of *S. aureus* to catheters and host valvular tissues (9, 11, 24, 28), we also assessed the ability of the isogenic pair to bind fibronectin and fibrinogen in vitro. The binding of mutant strain ALC1001 to radiolabeled fibronectin was similar to that of parental strain RN6390 (11,480 ± 856 [mean ± standard error of the mean] cpm versus 11,455 ± 816 cpm). In contrast, the *sigB* mutant bound more fibrinogen than its parental counterpart (Fig. 5A). Recognizing that multiple fibrinogen-binding proteins may be affected by the *sigB* mutation, we probed immunoblots containing cell wall extracts of the isogenic pair with anti-clumping factor (ClfA) and anticoagulase (Coa) antibodies (Fig. 5B and 3C). In comparison to that in RN6390, the expression of ClfA and Coa in *sigB* mutant ALC1001 was augmented, confirming the observation that the *sigB* mutant strain has a higher fibrinogen-binding capacity than the isogenic parental strain.

Expression of *agr* and *sar* in *sigB* mutant ALC1001. The expression of alpha-hemolysin is influenced by global regulatory loci such as *agr* and *sar*. To assess if the *sigB* mutation in ALC1001 affected the ability of these loci to modulate alpha-hemolysin expression, we performed Northern blotting and showed that the expression of RNAII and RNAIII of the *agr* locus was not altered in the mutant strain relative to the parental strain (data not shown).

We have shown that the *sarC* transcript was absent in *sigB* mutant ALC1001 but was restored upon complementation (Fig. 1). Concomitant with the decrease in *sarC* transcription in *sigB* mutant ALC1001 was the repeated observation of an

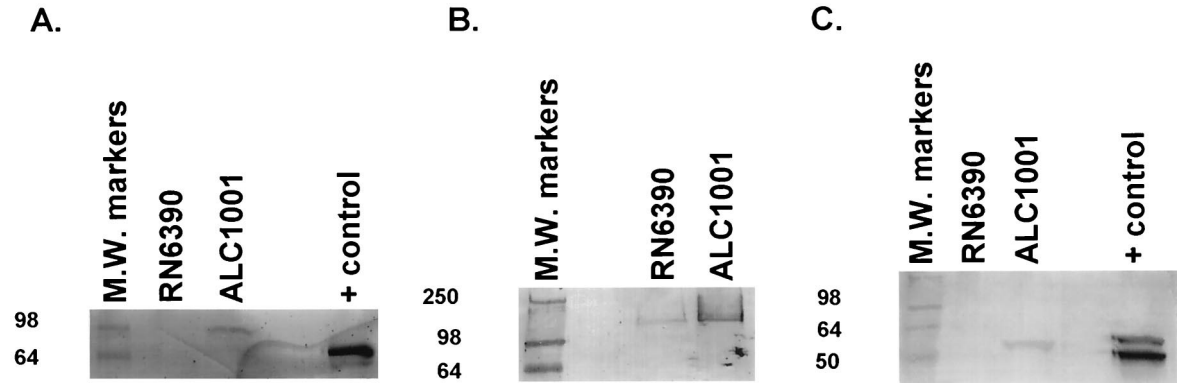

FIG. 5. Immunoblots of cell wall extracts of RN6390 and ALC1001 probed with fibrinogen (A), anti-ClfA antibody (B), and anti-Coa antibody (C). Equivalent amounts of cell extracts were applied to the lanes. The positive controls were fibrinogen (A) and purified coagulase (C). M.W., molecular weight. Numbers at left are in thousands.

increase in *sarA* transcription in that strain (Fig. 1) as well as in RUSA168 (data not shown). Notably, *sarA* transcription was present at near the parental level in complemented mutant ALC1497. In contrast to RNA-mediated control of the *agr* locus, genetic analyses have indicated that the major regulatory molecule of *sar* is the SarA protein (7, 15). To assess SarA expression, we probed an immunoblot of cell extracts of the isogenic pair with anti-SarA monoclonal antibody 1D1. The binding epitope of this antibody was recently mapped to residues 16 to 43 of the N terminus of the SarA molecule (16). Our data indicated that the expression of SarA, as determined by immunoblotting, was higher in ALC1001 than in its isogenic parent RN6390 (Fig. 6). This pattern of SarA expression held true in three repetitions of the experiment. Using a recently described competitive ELISA (16) and the cell extracts of these two strains as competitors of 1D1 binding to immobilized purified SarA, we confirmed that the SarA level was elevated in mutant strain ALC1001 (280 ± 25 ng/mg of extract proteins) compared with the parental strain (170 ± 9 ng/mg). Collectively, these data support the notion that a *sigB* mutation results in enhanced SarA expression, thereby leading to an ensuing increase in alpha-hemolysin expression via an *agr*-independent mechanism.

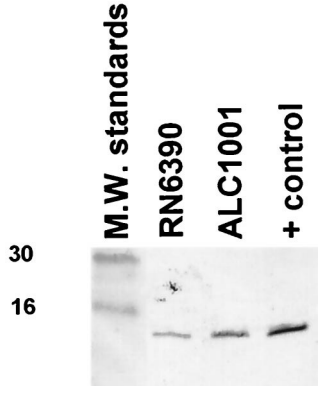

FIG. 6. Immunoblot of cell extracts of RN6390 and ALC1001 probed with anti-SarA monoclonal antibody 1D1 (1:2,500 dilution). About 30 μg of protein was applied to each lane. The positive control was purified SarA protein (14.5 kDa). M.W., molecular weight. Numbers at left are in thousands.

DISCUSSION

The *sigB* locus of *S. aureus*, contrary to the eight-gene *sigB* operon of *B. subtilis* (*rsbR-rsbS-rsbT-rsbU-rsbV-rsbW-sigB-rsbX*), comprises in sequential order four open reading frames which show sequence similarity to the *rsbU-rsbV-rsbW-sigB* gene cluster of *B. subtilis* (Fig. 7) (30). Transcriptional analysis indicated that the *sigB* operon in *B. subtilis* is transcribed from two distinct but convergent promoters, the σA and σB promoters upstream of *rsbR* and *rsbV*, respectively. Although sequence analysis of the *sigB* locus of *S. aureus* disclosed potential σA- and σB-like promoter sequences upstream of *rsbU* and *rsbV*, respectively, the transcription start sites attributed to these putative promoters have not been experimentally confirmed. Irrespective of whether these two putative promoters or possibly another or others are active, it is likely that *sigB* is the last gene encoded with the mRNA message.

Because the Tn*551* insertion site in *sigB* mutant RUSA168 has been mapped to approximately nucleotide position 3254 (30) (or 189 residues from the initiation codon and 67 residues from the termination codon), we wanted to assess if the SigB protein was indeed absent in *sigB* mutant ALC1001. Immunoblot analysis with anti-SigB monoclonal antibody 2D7 revealed the absence of the SigB protein in the mutant strain compared with parental strain RN6390 (Fig. 2). This finding was also confirmed with an additional blot probed with mouse anti-SigB polyclonal antibody (data not shown), indicating that it is unlikely that SigB is synthesized as a truncated form. As the transposon insertion site in the *sigB* mutant is near the 3′ end of the *sigB* coding region and *sigB* may be the last gene transcribed in the polycistronic message, it is unlikely that the Tn*551* insertion in the mutant significantly alters the transcription and translation of genes upstream (i.e., *rsbU*, *rsbV*, and *rsbW*). As an additional confirmation that *sigB* was altered in mutant ALC1001, we took advantage of the fact that the *sarC* promoter within the triple-promoter system of *sar* is σB dependent (17, 25). Predictably, the *sarC* transcript was absent in mutant ALC1001 but was restored upon complementation with a plasmid carrying a functional copy of the *sigB* gene (Fig. 1). Collectively, these data clearly indicated that mutant ALC1001 is defective in the synthesis of SigB.

In analyzing some of the phenotypes related to virulence in *sigB* mutant ALC1001, we found that both extracellular and cell wall proteins were altered. In particular, the capacity to bind fibrinogen as well as the production of alpha-hemolysin

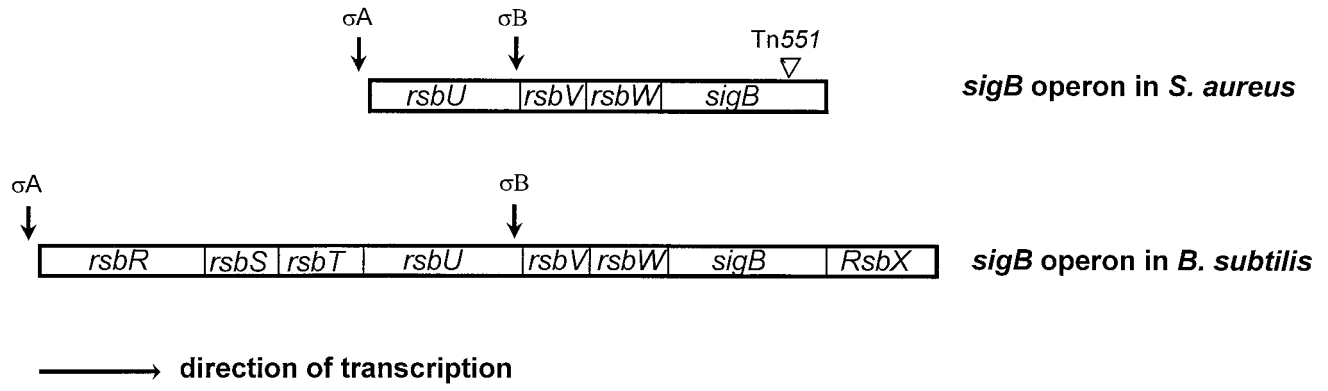

FIG. 7. Organization of the *sigB* operon in *B. subtilis* and *S. aureus*. In contrast to those in *B. subtilis*, the functions of the putative *rsbU*, *rsbV*, and *rsbW* genes in *S. aureus* have not been demonstrated. Similarly, the putative σA and σB promoters in *S. aureus* have not been experimentally confirmed.

were enhanced in the *sigB* mutant compared with the parent. The augmented fibrinogen-binding capacity was probably mediated by upregulation in the expression of both clumping factor and coagulase in *sigB* mutant ALC1001 (Fig. 5). Clearly, the *sigB* mutation was linked to elevated expression of functional alpha-hemolysin at the transcriptional level in two *S. aureus* strains (ALC1001 and RUSA168) that we examined (Fig. 4B). This linkage was confirmed by complementation studies showing that the hyperproduction of alpha-hemolysin in the mutant could be restored to near the parental level when *sigB* was supplied in *trans* on a shuttle plasmid. In addition to these phenotypic alterations, Kullik et al. (23) recently reported that a cytoplasmic protein identified as alkaline shock protein 23 was missing in a *sigB* deletion mutant, while staphylococcal thermonuclease, an extracellular protein, was secreted at a higher level than in the nonmutated controls. However, despite the pleiotropic nature of SigB in modulating a variety of genes, our results demonstrated that the effect of a *sigB* mutation on target gene expression can also be selective. For instance, a mutation in *sigB* in *S. aureus* can upregulate the expression of alpha-hemolysin but leave beta-hemolysin expression relatively unaltered. Similarly, such a mutation can enhance the expression of coagulase and clumping factor but not fibronectin-binding proteins.

It was recently shown that the SigB protein of *S. aureus* can activate σB-dependent promoters in vitro (17). In addition, we have shown (25), as have others (17), that one of the promoters within the *sar* locus (i.e., the *sarC*, or P3, promoter) is σB dependent. Because of the observation that a *sigB* mutant has phenotypes consistent with the upregulation of *sar* (increased alpha-hemolysin production and increased fibrinogen-binding capacity), it seemed reasonable to explore if SigB may directly or indirectly modulate global regulatory loci, such as *sar* and/or *agr*, to effect these phenotypic changes. However, scrutiny of the *agr* promoter region did not reveal any σB-dependent promoter sequences. Additionally, Northern analysis indicated that the transcription of RNAII and RNAIII in the *agr* locus was not altered in ALC1001, implying that SigB does not modulate *agr*-related transcription.

The *sar* locus has a triple-promoter system for which the major regulatory element is the 14.5-kDa SarA protein (124 residues) (3, 7, 15). The *sarA* gene is encoded by each of three transcripts initiated from three distinct promoters, designated P1, P2, and P3 (3). In particular, the σA-dependent proximal P1 and distal P2 promoters of *sar* are maximally expressed during the exponential phase, while σB-dependent P3 promoter expression peaks postexponentially (7, 25). We speculate that differential promoter activation within *sar* may lead to disparate expression of the SarA protein (16, 25), the major regulatory molecule in the control of hemolysin production in *S. aureus* (7). To assess if a *sigB* mutation would lead to an increase in SarA expression and hence overproduction of alpha-hemolysin, we first established by immunoblotting and an ELISA that the SarA level was higher in the *sigB* mutant than in the parental control. Based on the phenotypes related to *sar* (9), a higher SarA protein level will likely enhance the expression of alpha-hemolysin (16). This notion was confirmed by Northern and Western analyses showing that the expression of *hla* was increased in a *sigB* mutant but was restored to normal levels upon complementation with a plasmid carrying *sigB*. Notably, the effect of a higher SarA level on target gene expression could not be mediated by *agr*, as evidenced by the fact that *agr*-related transcription was no different in mutant ALC1001 than in RN6390. In previous studies (9, 14), we inferred from phenotypic analysis that the *sar* locus may have its own direct effect on target gene expression (e.g., beta-hemolysin and fibrinogen-binding proteins). In addition, we demonstrated that the SarA protein may bind to target gene promoters (e.g., the fibronectin-binding protein gene) in gel shift assays (14). Collectively, these data suggested that an increase in alpha-hemolysin expression as well as an upregulation of fibrinogen-binding proteins may occur by an *agr*-independent but SarA-dependent mechanism. As *agr* is not substantially altered in the *sigB* mutant, it is likely that a factor(s) other than SarA also is involved in the expression of genes perturbed by SigB. Because SigB participates in the general stress response of gram-positive bacteria (20), it seems plausible that σB-dependent genes act in concert with SarA to modulate target gene expression.

The mechanism by which inactivation of *sigB* leads to elevated SarA protein levels is not fully understood. Using *sar* promoter-XylE reporter fusions, we recently showed that a combined P3-P1 promoter is less active than a P1 promoter alone, suggesting that the P3 promoter may have a downregulatory effect on transcription from the P1 promoter of *sar*. A careful analysis of the sequence between the P3 and P1 promoters revealed a 16-bp inverted repeat. Gel shift studies revealed that a 12-kDa protein binds to this repeat (25). Additional genetic analysis indicated that the 12-kDa protein may be a repressor (unpublished data). Based on these data, we hypothesize that the P3 promoter (from which the *sarC* transcript is generated), upon activation by SigB, may undergo conformational changes to allow binding by the repressor protein. Presumably, a lack of SigB may interfere with the binding

of this repressor protein, resulting in enhanced transcription from the P1 promoter and the ensuing elevated SarA expression. In concordance with this hypothesis is the observation that the level of *sarA* transcription was found to be consistently higher in *sigB* mutant strain ALC1001 than in the parental strain but returned to near the parental level upon complementation (Fig. 1). Additional experiments to address the interaction between SigB and this repressor protein are currently in progress.

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