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σ^B Modulates Virulence Determinant Expression and Stress Resistance: Characterization of a Functional *rsbU* Strain Derived from *Staphylococcus aureus* 8325-4

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The accessory sigma factor σ^B controls a general stress response that is thought to be important for *Staphylococcus aureus* survival and may contribute to virulence. The strain of choice for genetic studies, 8325-4, carries a small deletion in *rsbU*, which encodes a positive regulator of σ^B activity. Consequently, to enable the role of σ^B in virulence to be addressed, we constructed an *rsbU*$^+$ derivative, SH1000, using a method that does not leave behind an antibiotic resistance marker. The phenotypic properties of SH1000 (8325-4 *rsbU*$^+$) were characterized and compared to those of 8325-4, the *rsbU* mutant, parent strain. A recognition site for σ^B was located in the promoter region of *katA*, the gene encoding the sole catalase of *S. aureus*, by primer extension analysis. However, catalase expression and activity were similar in SH1000 (8325-4 *rsbU*$^+$), suggesting that this promoter may have a minor role in catalase expression under normal conditions. Restoration of σ^B activity in SH1000 (8325-4 *rsbU*$^+$) resulted in a marked decrease in the levels of the exoproteins SspA and Hla, and this is likely to be mediated by reduced expression of *agr* in this strain. By using Western blotting and a *sarA-lacZ* reporter assay, the levels of SarA were found to be similar in strains 8325-4 and SH1000 (8325-4 *rsbU*$^+$) and *sigB* mutant derivatives of these strains. This finding contrasts with previous reports that suggested that SarA expression levels are altered when they are measured transcriptionally. Inactivation of *sarA* in each of these strains resulted in an expected decrease in *agr* expression; however, the relative level of *agr* in SH1000 (8325-4 *rsbU*$^+$) remained less than the relative levels in 8325-4 and the *sigB* mutant derivatives. We suggest that SarA is not likely to be the effector in the overall σ^B-mediated effect on *agr* expression.

The pathogenic bacterium *Staphylococcus aureus* has the ability to cause a wide variety of human diseases ranging from superficial abscesses and wound infections to deep and systemic infections, such as osteomyelitis, endocarditis, and septicemia. This ability has been attributed to the large repertoire of toxins, exoenzymes, adhesins, and immune-modulating proteins that it produces (37, 42). These proposed virulence determinants are believed to be temporally and environmentally regulated in response to the requirements of the organism during growth in vivo (42). Environmental regulation of virulence determinant expression is pertinent to the biology of *S. aureus* since this organism is commonly isolated from the anterior nares, where it lives as a harmless commensal (37).

Two major regulatory genetic determinants, *agr* (accessory gene regulator) (1, 32, 42, 43) and *sar* (staphylococcal accessory regulator) (11, 13, 16, 17, 47), mediate control of virulence determinant expression. Completion of the *S. aureus* genome has revealed a multitude of potential *sarA* product homologues, and some of these, including SarH1 (16, 51), SarT (50), and Rot (39), have been shown to have an impact on the expression of determinants previously found to be Agr and/or SarA regulated. The accessory sigma factor σ^B has been the subject of much interest in *S. aureus* (4, 10, 24, 25, 26, 34, 35). A number of virulence-associated loci, including *coa*, *sarA*, *sarH1*, and *clfA* (2, 21, 51), are transcriptionally regulated by σ^B. In addition, the *S. aureus* σ^B regulon, like that of *Bacillus subtilis* (22, 44, 45), contains many components that are perceived to be important for protecting the cell from various environmental stresses (24). The production of biofilms by *S. aureus* is controlled, possibly indirectly, by σ^B (46), and the ability to form an adherent biofilm has been implicated in the virulence of *Staphylococcus epidermidis* (33). Determining the exact role of σ^B in *S. aureus* has been impeded, however, by the presence of an *rsbU* mutation in the genetic lineage used most frequently for molecular and physiological analyses, 8325-4 (RN6390). This mutation, in a positive regulator of σ^B function, produces a strong defect in σ^B activity (26). The contribution of σ^B to virulence, when σ^B was inactivated in an alternative genetic background with an intact *rsbU* locus, was tested in a variety of animal models in which the *sarA* and *agr* loci are required for virulence (40). This study convincingly demonstrated that inactivation of *sigB* resulted in no significant reduction in virulence. Despite the failure to demonstrate attenuation of a *sigB* mutant in any animal model tested to date, the presence of σ^B promoters in the upstream regulatory regions of many virulence-associated loci demands that the role of this sigma factor be investigated further.

In this paper, we describe the construction and phenotype of an 8325-4-derived, functional *rsbU* strain, designated SH1000 (8325-4 *rsbU*$^+$). This strain is an important prerequisite for effective study of the role of *S. aureus* σ^B in a well-character-

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TABLE 1. Strains, plasmids, and primers used

Strain, plasmid, or primer	Genotype or description	Reference(s) or source
E. coli DH5α	ϕ80 Δ*(lacZ)M15* Δ*(argF-lac)U169 endA1 recA1 hsdR17* (r_K^- m_K^+) *deoR thi-1 supE44 gyrA96 relA1*	48
S. aureus strains		
8325-4	Wild-type strain cured of prophages	Lab stock
RN4220	Restriction-deficient transformation recipient	Lab stock
MJH499	*rsbU rsbV rsbW sigB*::pMAL30 integrant in RN4220	This study
MJH500	*rsbU rsbV rsbW sigB*::pMAL30 integrant in 8325-4	This study
MJH501	Functional *rsbU* derivative of RN4220 *rsbU*$^+$	This study
MJH502	SH1000 *rsbU*$^+$ *sigB*::*tet*	
SH1000	Functional *rsbU* derivative of 8325-4 *rsbU*$^+$	This study
SH1001	SH1000 *agr*::*tet*	This study
SH1002	SH1000 *sarA*::*kan*	This study
PC6911	*agr*::*tet*	9
PC1839	*sarA*::*kan*	9
PC400	*sigB*::*tet*	10
MJH006	8325-4 *katA*::pAZ106 *katA*$^+$	30, 31
MJH506	SH1000 *katA*::pAZ106 *katA*$^+$	This study
MJH606	SH1000 *katA*::pAZ106 *katA*$^+$ *sigB*::*tet*	This study
LES07	8325-4 *sspA*::pAZ106 *sspA*$^+$	This study
LES08	SH1000 *sspA*::pAZ106 *sspA*$^+$	This study
PC161	8325-4 *sarA*::pAZ106 *sarA*$^+$	9
JLA311	SH1000 *sarA*::pAZ106 *sarA*$^+$	This study
PC4030	8325-4 *sarA*::pAZ106 *sarA*$^+$ *sigB*::*tet*	10
JLA313	SH1000 *sarA*::pAZ106 *sarA*$^+$ *sigB*::*tet*	This study
SH101F7	8325-4 *agr* (RNA III)::pAZ106 *agr*$^+$	23
JLA341	SH1000 *agr* (RNA III)::pAZ106 *agr*$^+$	This study
PC604	8325-4 *agr* (RNA III)::pAZ106 *agr*$^+$ *sigB*::*tet*	This study
JLA343	SH1000 *agr* (RNA III)::pAZ106 *agr*$^+$ *sigB*::*tet*	This study
PC600	8325-4 *agr* (RNA III)::pAZ106 *agr*$^+$ *sarA*::*kan*	This study
JLA345	SH1000 *agr* (RNA III)::pAZ106 *agr*$^+$ *sarA*::*kan*	This study
PC602	8325-4 *agr* (RNA III)::pAZ106 *agr*$^+$ *sigB*::*tet sarA*::*kan*	This study
JLA347	SH1000 *agr* (RNA III)::pAZ106 *agr*$^+$ *sigB*::*tet sarA*::*kan*	This study
PC322	8325-4 *hla*::pAZ106 *hla*$^+$	9
JLA371	SH1000 *hla*::pAZ106 *hla*$^+$	This study
PC4044	8325-4 *hla*::pAZ106 *hla*$^+$ *sigB*::*tet*	This study
JLA373	SH1000 *hla*::pAZ106 *hla*$^+$ *sigB*::*tet*	This study
PC3221	8325-4 *hla*::pAZ106 *hla*$^+$ *sarA*::*kan*	This study
JLA375	SH1000 *hla*::pAZ106 *hla*$^+$ *sarA*::*kan*	This study
JLA376	8325-4 *hla*::pAZ106 *hla*$^+$ *sigB*::*tet sarA*::*kan*	This study
JLA377	SH1000 *hla*::pAZ106 *hla*$^+$ *sigB*::*tet sarA*::*kan*	This study
Plasmids		
pAZ106	Promoterless *lacZ erm* insertion vector	30
pMAL30	3-kb OL-80–OL-81 *rsbU rsbV rsbW sigB* PCR fragment in pAZ106	This study
pLES2	1.15-kb OG-15–OG-16 *sspA* PCR fragment in pAZ106	This study
Primers[a]		
OL-15	AATTGGATCCGACCACAATGCCCAATACAACC	
OL-78	TATCTACCAATCTTTGATAATCTCGATAAC	
OL-79	GCTCTAGAGTTCAAGACATTAGATG	
OL-80	GTGAGGATCCGAAGCTTTTCCGATAGAGTGTGAAG	
OL-81	GCTTGAATTCATACGTCTCGGAACATGTACACTCC	
OL-177	ACACTGCAGGAATGGTAACATGGTAATAAT	
OG-15	CCGTCTAGAGTGCCAATGTTCCAGCTCAAATAGC	
OG-16	CCGGGATCCGAATCTTAGGTGTTTGCTGTTTGC	

[a] Restriction sites are underlined.

ized genetic background. We also compare our data with the data obtained for a functional *rsbU* strain described recently.

MATERIALS AND METHODS

Bacterial strains, plasmids, and growth conditions. *S. aureus* and *Escherichia coli* strains and plasmids are listed in Table 1. *E. coli* was grown in Luria-Bertani medium at 37°C. *S. aureus* was grown at 37°C with shaking at 250 rpm in 100 ml of brain heart infusion (BHI) broth (culture/flask volume ratio, 1:2.5; Oxoid) by using defined conditions described previously (8), unless indicated otherwise. When included, antibiotics were added at the following concentrations: ampicillin, 100 mg liter^{-1}; kanamycin, 50 mg liter^{-1}; neomycin, 50 mg liter^{-1}; tetracycline, 5 mg liter^{-1}; and erythromycin and lincomycin, 5 and 25 mg liter^{-1}, respectively.

Construction of functional *rsbU* strain SH1000 (8325-4 *rsbU*$^+$). A 3-kb fragment encompassing the complete *rsbU-rsbV-rsbW* region and part of *sigB* from *S. aureus* strain Newman was amplified by using *Pwo* DNA polymerase (Roche) with primers OL-80 and OL-81 (Table 1). Following purification, the PCR product was digested with *Bam*HI and *Eco*RI and cloned into pAZ106 (30) by using standard cloning techniques (48). The resulting plasmid, pMAL30 (Table 1), was used to transform electrocompetent *S. aureus* RN4220 by the method of Schenk and Ladagga (49). The plasmid was integrated into the chromosome through homology with the parental copy by a Campbell type of event to produce a *sigB-lacZ* fusion. The unresolved locus was transferred into recipient 8325-4

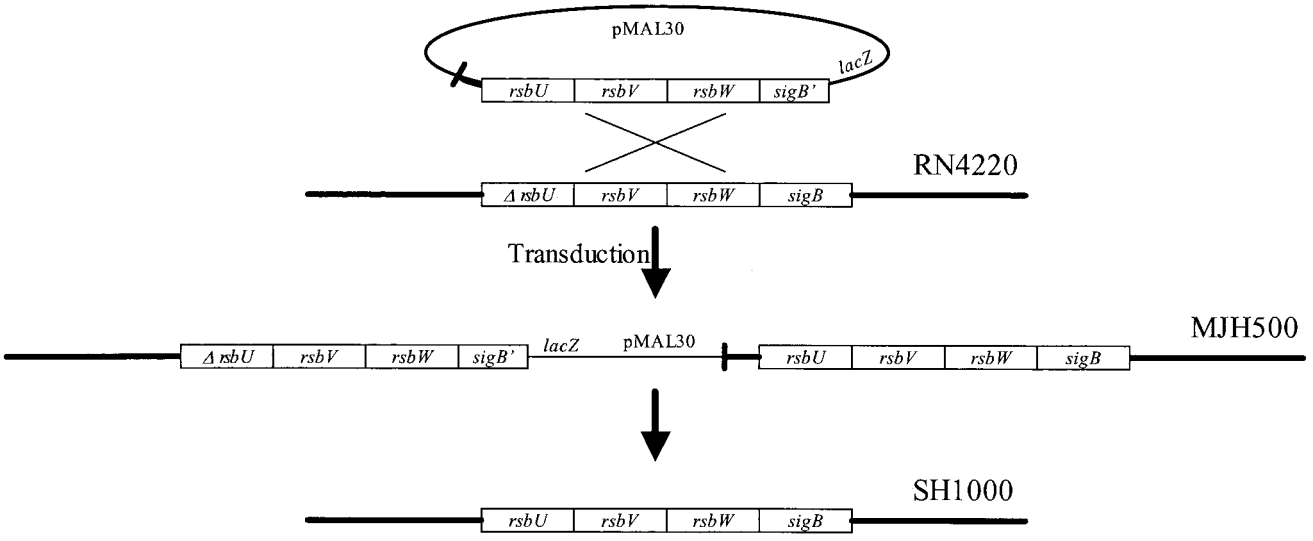

FIG. 1. Schematic diagram illustrating the method used for construction of SH1000 (8325-4 *rsbU*$^+$). A single crossover of pMAL30 into the chromosome of RN4220 produced a *sigB-lacZ* fusion, which was transferred to 8325-4 by transduction. The *rsb-sigB* locus was resolved by overnight growth in antibiotic-free medium followed by selection for white clones (loss of plasmid) and pigment formation (functional *rsbU*). Δ*rsbU* is the defective gene containing an 11-bp deletion.

cells by phage transduction (41) by using ϕ11 as the transducing phage. The *rsb-sigB* locus, which contained a duplication of the *rsb* genes, was resolved by overnight growth in BHI medium with no antibiotic to enable loss of the integrated plasmid, pMAL30, by homologous recombination between the duplicated sets of *rsb* genes (Fig. 1). Plating cells on plates containing X-Gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside) (50 mg liter^{-1}) allowed clones to be isolated that were not blue and thus no longer contained the pMAL30-generated *sigB-lacZ* fusion; these clones were tested for erythromycin sensitivity. The following two methods were used to confirm replacement of the 8325-4 *rsbU* gene with the gene from strain Newman: a PCR performed with primers OL-78 and OL-79 (34) and genomic DNA sequencing (30) performed with the same primers. In addition, the integrity of the *rsb-sigB* locus and its immediate vicinity was verified by Southern blotting (data not shown).

***sigB*::*tet* transductions.** The *sigB*::*tet* mutation was present in 8325-4, which has an *rsbU* deletion mutation. To introduce the *sigB*::*tet* mutation into derivatives of SH1000 without cotransducing the *rsbU* mutation, we screened transductants using the PCR method of Kullik and Giachino (34). Despite the fact that the *rsbU* and *sigB* mutations in these genes are separated by less than 3 kb, cotransduction was found to be only 80%, which facilitated isolation *of sigB*::*tet rsbU*$^+$ derivatives.

Construction of an *sspA-lacZ* reporter. The promoter region of *sspA* was amplified as a 1.15-kb, PCR-generated DNA fragment (position −1,000 to position 150 bp relative to the translational start site) by using OG-15 and OG-16 (Table 1). The purified DNA was digested with *Xba*I and *Bam*HI and cloned into similarly digested pAZ106. *S. aureus* RN4220 was transformed with the resulting plasmid, pLES2, and an integrant confirmed by Southern blotting was transduced into appropriate backgrounds by using ϕ11.

Enzyme assays. Levels of β-galactosidase activity were measured as described previously (30). Fluorescence was measured by using a Victor plate reader (Wallac) with a 0.1-s count time and was calibrated with standard concentrations of 4-methyl-umbelliferone. One unit of β-galactosidase activity was defined as the amount of enzyme that catalyzed the production of 1 pmol of 4-methyl-umbelliferone per min per unit of optical density at 600 nm (OD_{600}). Assays were performed with duplicate samples, and the values were averaged. The results presented here are representative of the results of three independent experiments that showed less than 10% variability.

The levels of alpha toxin (Hla) in culture supernatants were determined as described previously (9). One hemolytic unit was defined as the reciprocal value of the dilution that resulted in 50% lysis of rabbit erythrocytes per OD_{600} unit.

Protein samples, prepared as described previously (9), were resolved on a precast 12% (wt/vol) acrylamide gel containing Zymogram Ready Gel containing gelatin (Bio-Rad). Renaturation and visualization were performed according to the manufacturer's instructions.

Northern hybridization. *S. aureus* strains were grown in 25 ml of BHI medium (culture/flask volume ratio, 1:10) at 37°C with shaking at 250 rpm. RNA was extracted from harvested cells as described previously (30). Ten micrograms of total RNA, which had been separated on a 1% (wt/vol) agarose gel and vacuum blotted onto a nylon membrane (Roche), was probed and washed under high-stringency conditions (0.1× SSC–1% sodium dodecyl sulfate [1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate], 68°C) by using standard conditions (48). The concentration of RNA was measured at 260 nm, and equivalent loading on agarose gels was confirmed by ethidium bromide staining and UV visualization. The *katA* probe was a ^{32}P-labeled *Bam*HI-*Hin*dIII fragment (position −159 to position 943 relative to the translational start point of *katA*) from a digest of PCR DNA amplified by using primers OL-15 and OL-177. Densitometric analysis of autoradiographs with different exposure times was performed by using ImageMaster 3.01 software (Amersham-Pharmacia).

Catalase assays, H_2O_2 challenge, and starvation survival. Catalase activity was assayed spectrophotometrically at 240 nm (ε = 43.6 M liter^{-1} cm^{-1}) as described by Beers and Sizer (3) by using 50 mM potassium phosphate buffer (pH 7.0) with 19.6 mM hydrogen peroxide. Hydrogen peroxide resistance assays were carried out as described previously (30, 52). Comparative starvation survival experiments were performed in amino acid-limiting CDM medium incubated statically at 25°C as described previously (52).

Western blotting. Proteins were blotted onto a polyvinylidene difluoride membrane (Bio-Rad) and were detected by using antisera raised against Hla (36), staphylococcal serine protease (SspA) (36), and SarA (5) (1:6,000, 1:2,500, and 1:400 dilutions of the antibodies, respectively) and standard methods (48). Alkaline phosphatase-conjugated goat anti-rabbit secondary antibody (diluted 1:30,000) was used to detect Hla and SspA colorimetrically, and horseradish peroxidase-conjugated antibody was used to detect SarA with the enhanced chemiluminescence system (Amersham-Pharmacia). Pig serum (20%, vol/vol; Sigma) was included during blocking to improve detection of SarA. Densitometric analysis of autoradiographs with different exposure times was performed by using ImageMaster 3.01 software (Amersham-Pharmacia).

Virulence testing of strains in a murine skin abscess model. *S. aureus* strains were grown to the stationary phase in BHI medium (15 h) and then harvested by centrifugation and washed twice in phosphate-buffered saline (PBS). The cell concentrations were adjusted to 5×10^8 CFU ml^{-1}, and then 200-μl portions of a cell suspension were injected subcutaneously into female 6- to 8-week-old BALB/c mice. After 7 days the mice were euthanatized with CO_2, and skin lesions were aseptically removed and stored frozen in liquid nitrogen. The lesions were weighed, chopped, and homogenized in a mini-blender in 2.5 ml of ice-cold PBS. After 1 h of incubation on ice, the lesions were homogenized again before serial dilution of the suspension, and the total number of bacteria was counted

by growth on BHI agar. The statistical significance of the recovery of strains was evaluated by using the Student *t* test with a 5% confidence limit.

RESULTS

Construction of SH1000 (8325-4 *rsbU*$^+$). The defective copy of *rsbU* in *S. aureus* 8325-4 was replaced with a copy of the intact gene from *S. aureus* Newman, without leaving behind an antibiotic resistance marker. To achieve this, a multiple-step approach was used (Fig. 1). First, the complete *rsbU*, *rsbV*, and *rsbW* genes together with part of the *sigB* gene were amplified as a 3-kb DNA fragment by PCR and cloned into pAZ106. The resulting plasmid, pMAL30, was used to transform *S. aureus* RN4220, and clones were obtained with duplicate copies of the *rsbU*, *rsbV*, and *rsbW* genes. Integration produced a *sigB-lacZ* fusion, and a selected clone, MJH499, was blue on X-Gal plates. The duplicated *rsb-sigB* locus was transferred to *S. aureus* 8325-4 by using φ11-mediated transduction to generate strain MJH500. Finally, to restore the *rsb-sigB* locus, MJH500 was grown overnight in the absence of antibiotics, and the culture was diluted and plated on X-Gal plates. Clones were then picked that were white, indicating loss of the plasmid, and they were screened for the formation of yellow pigment, indicating that a functional *rsbU* gene was present, by overnight growth on BHI agar. The frequency of loss of the plasmid was between 10^{-4} and 10^{-5}.

A number of pigmented clones were isolated and screened for the presence of a functional *rsbU* locus by using the PCR method described by Kullik and Giachino (34). In addition, the integrity of *rsbU* at the site of the previous deletion was verified by genomic DNA sequencing (30). One clone, SH1000 (8325-4 *rsbU*$^+$), was selected for use.

SH1000 (8325-4 *rsbU*$^+$) was characterized phenotypically to compare it with a previously described functional *rsbU* strain of *S. aureus* BB255, which contained a tetracycline resistance marker (26). Expression of the cytosolic, σ^B-regulated protein Asp23 (N-terminal sequence, VDNNXAXQAYDXQ), production of the orange-yellow pigment staphyloxanthin, and a fourfold-greater minimum bactericidal concentration but not MIC of hydrogen peroxide were observed (data not shown), demonstrating that the phenotypic properties of SH1000 were those of a functional *rsbU* strain.

A number of additional phenotypic differences between SH1000 (8325-4 *rsbU*$^+$) and 8325-4 were seen. SH1000 (8325-4 *rsbU*$^+$) exhibited a decreased lag phase (15 to 20 min) before exponential growth began after dilution of an overnight culture into fresh medium (data not shown). Similar to the findings of Giachino et al. (26), we consistently observed a slightly increased growth yield for SH1000 (8325-4 *rsbU*$^+$). The starvation survival capability of SH1000 (8325-4 *rsbU*$^+$) in amino acid-limiting CDM medium measured for 21 days was greater than that of 8325-4 (Fig. 2). Starvation in this CDM medium results in a requirement for a number of oxidative stress resistance components (52).

Catalase expression in SH1000. The increased resistance of SH1000 (8325-4 *rsbU*$^+$) to hydrogen peroxide was hypothesized to be catalase mediated, by virtue of a putative consensus σ^B promoter element in the *katA* promoter region. We have previously shown that the *katA* promoter in 8325-4 contains the −35 and −10 elements of a σ^A promoter-binding site and a binding site for PerR, the peroxide regulon repressor (30, 31). Primer extension of RNA isolated from post-exponential-phase cultures (OD_{600}, 8) of 8325-4, SH1000 (8325-4 *rsbU*$^+$), and PC400 (8325-4 *sigB*) revealed a σ^B promoter, P_B, upstream of *katA* in SH1000 (8325-4 *rsbU*$^+$) (Fig. 3A). This σ^B-regulated transcript was absent in 8325-4 and PC400 (8325-4 *sigB*).

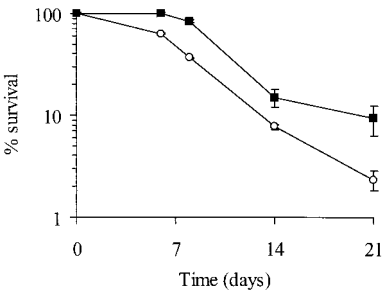

FIG. 2. Starvation survival kinetics of SH1000 (8325-4 *rsbU*$^+$) (■) and 8325-4 (○) during prolonged incubation at 25°C in amino acid-limiting CDM medium. The values are representative of the results of three separate experiments, and the error bars indicate the mean errors.

To assess the contribution of the P_B promoter to the regulation of *katA* expression, we probed RNA isolated from SH1000 (8325-4 *rsbU*$^+$) and MJH502 (SH1000 *rsbU*$^+$ *sigB*) throughout growth (Fig. 3B) by Northern blotting. As measured by densitometry, the levels of *katA* transcript on the blot were between 1.2 and 1.5 times greater in SH1000 (8325-4 *rsbU*$^+$) than in MJH502 (SH1000 *rsbU*$^+$ *sigB*), suggesting that P_B has only a minor role under these conditions. When catalase was assayed during the post exponential and stationary phases of growth (after 5 and 8 h), similar levels of activity were observed (Fig. 3D). In contrast, when expression was measured by using a *katA-lacZ* transcriptional fusion described previously (30, 31), *katA* transcription was four- to sixfold lower during post-exponential-phase growth of MJH506 (SH1000 *katA-lacZ*) than during post-exponential-phase growth of MJH006 (8325-4 *katA-lacZ*) (data not shown). The disparity between the *lacZ* data and the data from the other assays may reflect increased turnover of the *lacZ* transcript via an unknown mechanism. We hypothesize that σ^B has little or no significance in the overall control of catalase expression under the conditions tested.

Expression of Hla and SspA. The exoproteins of *S. aureus* SH1000 (8325-4 *rsbU*$^+$) and 8325-4 were precipitated from culture supernatants and visualized. The level of stationary-phase exoproteins was found to be much lower in SH1000 (8325-4 *rsbU*$^+$) than in 8325-4, and the profiles revealed large reductions for several proteins, including Hla and SspA (Fig. 4A). The expression of Hla is known to be modulated indirectly by σ^B, since inactivation of *sigB* leads to hyperproduction of Hla (14). To quantify the observed difference in Hla expression, cultures were assayed for activity at 10 h, and this analysis showed that 8325-4 had 166.5 alpha toxin units (hemolytic units) and that SH1000 had 77.7 hemolytic units. Furthermore, to show that the reduction was controlled transcriptionally and was not due to alterations in protease activity, we assayed β-galactosidase expression using an *hla-lacZ* transcriptional reporter fusion. Expression of the *hla-lacZ* fusion was much

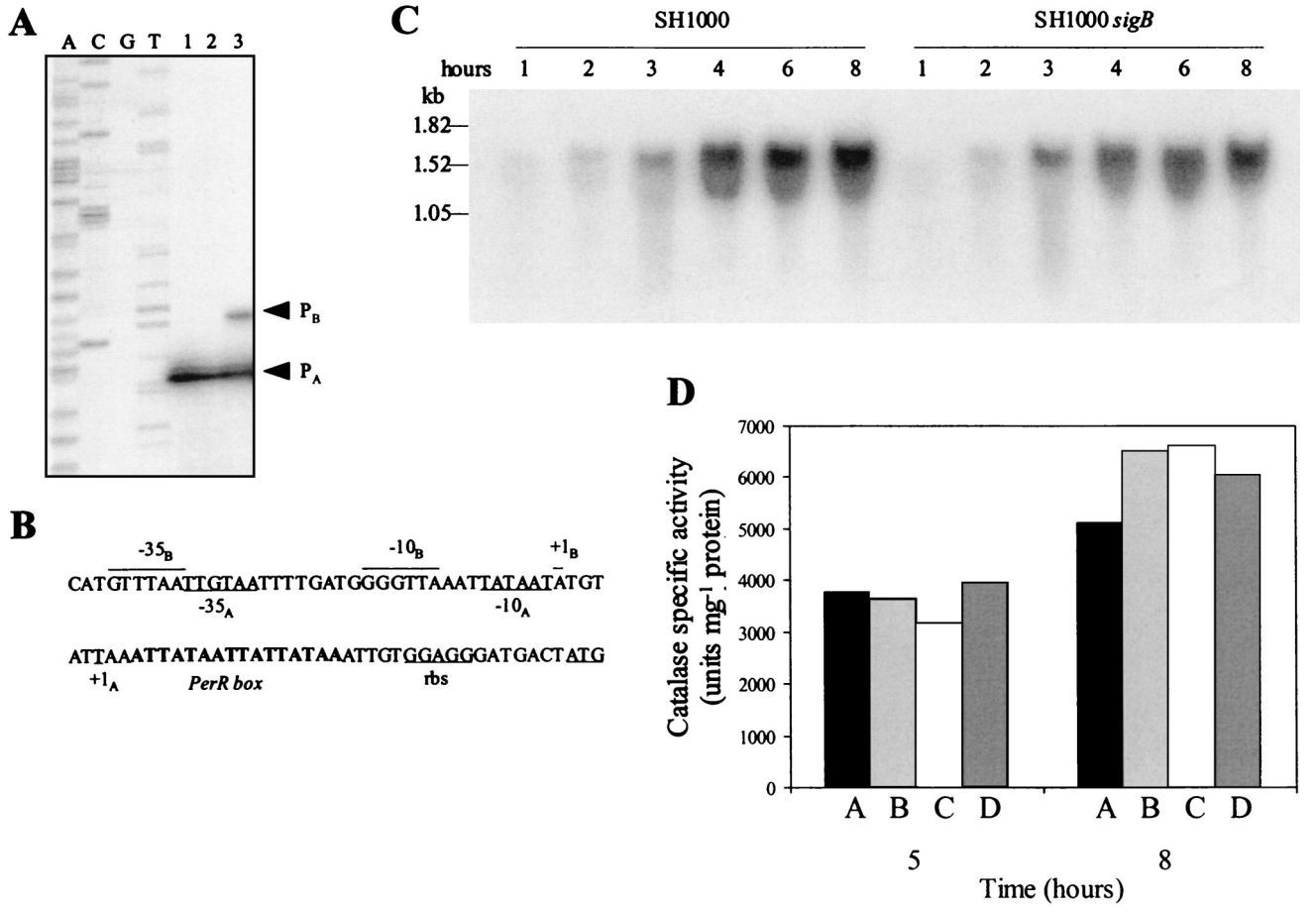

FIG. 3. (A) Mapping of the 5′ ends of *katA* transcripts by primer extension analysis. One-hundred-microgram portions of RNA from post-exponential-phase cultures was used in reactions for 8325-4 (lane 1), PC400 (8325-4 *sigB*) (lane 2), and SH1000 (8325-4 *rsbU*$^+$) (lane 3). Lanes A, C, G, and T show the dideoxy sequencing ladder obtained by using the same primer that was used for primer extension. (B) Potential −35 and −10 regions and transcriptional start sites (+1) for σ^A and σ^B are indicated by the subscripts A and B, respectively. The PerR box is indicated by boldface type, and rbs indicates the translational recognition sequence upstream of the translational start (underlined). (C) Transcript levels of *katA* during growth in 25 ml of BHI medium (medium/flask volume ratio, 1:10) at 37°C with shaking at 250 rpm. Samples were removed at the times indicated at the top, and the extracted RNA was probed by using a radiolabeled *katA* fragment. (D) Assay of catalase activity during growth in 25 ml of BHI medium (medium/flask volume ratio, 1:10) at 37°C with shaking at 250 rpm. Samples removed at different times were washed in PBS and then assayed. Bars A, SH1000 (8325-4 *rsbU*$^+$); bars B, 8325-4; bars C, PC400 (8325-4 *sigB*); bars D, MJH502 (SH1000 *rsbU*$^+$ *sigB*). Assays were performed in triplicate, and the means are shown. The results are representative of the results of two independent experiments.

reduced in SH1000 (8325-4 *rsbU*$^+$), suggesting that the reduction in *hla* expression was primarily controlled at the transcriptional level (Fig. 4B).

The observation of reduced SspA in SH1000 (8325-4 *rsbU*$^+$) (Fig. 4A) led us to examine the activities and amounts of other secreted proteases by using zymograms (Fig. 4C). The activity levels of SspA and a second unidentified protease were much reduced compared to the levels in 8325-4. As with *hla*, this reduction was controlled transcriptionally, since an assay of an *sspA-lacZ* transcriptional fusion determined that there was reduced β-galactosidase activity in SH1000 (8325-4 *rsbU*$^+$) compared to the activity in 8325-4 (Fig. 3D). Inactivation of the *agr* and *sarA* genes in *S. aureus* is known to affect exoprotein production (9, 13, 32, 42), and consequently we examined the exoprotein profiles of SH1000 strains containing *agr* and *sarA* mutations. The *agr* mutant, SH1001, exhibited much reduced levels of exoproteins, such as Hla and SspA, as expected (data not shown). In contrast, SH1002 (SH1000 *sarA*) had increased exoprotein synthesis (Fig. 4A), and notably, the level of SspA was higher, which led to increased proteolysis of the other exoproteins, including Hla; this was confirmed by Western blotting by using antisera raised against Hla and SspA (data not shown).

Effect of σ^B on *sarA* and *agr* expression. Expression of the genes encoding the virulence regulators SarA and Agr was measured in 8325-4 and SH1000 (8325-4 *rsbU*$^+$) by using *lacZ* fusions constructed previously (9). A significant reduction in *agr* (RNA III) expression was observed during growth of SH1000 (8325-4 *rsbU*$^+$) compared to the expression in 8325-4 (Fig. 5B). This finding matched the previous description of reduced *agr* (RNA III) expression by Giachino et al. (26). Since SarA can act as an activator of Agr expression (18), we examined whether the diminished level of *agr* (RNA III) was a consequence of reduced SarA expression. An assay of a *sarA-lacZ* reporter demonstrated that the overall levels of transcription of *sarA* in strains 8325-4 and SH1000 (8325-4 *rsbU*$^+$) and

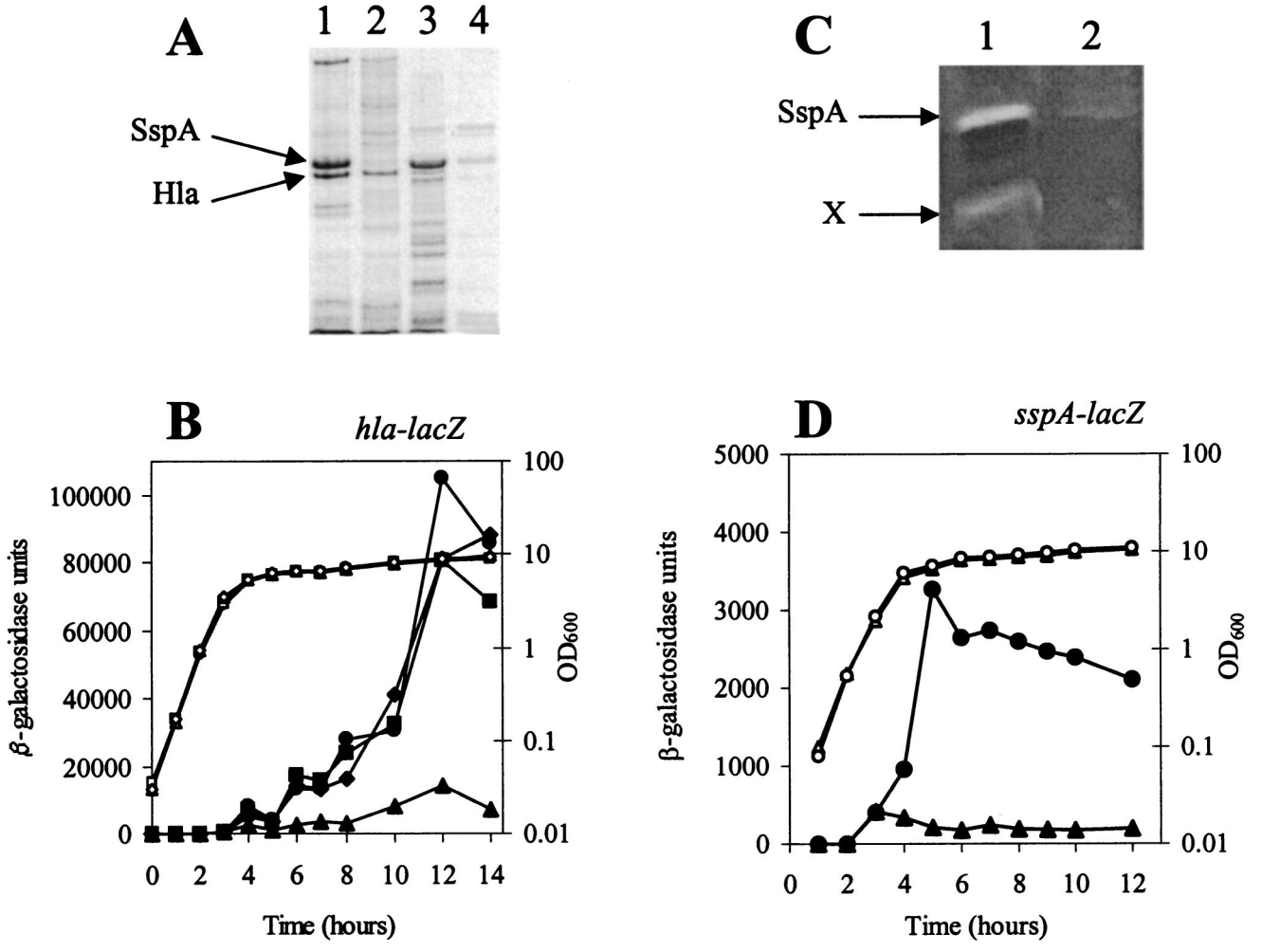

FIG. 4. (A) Exoproteins of 8325-4 (lane 1), SH1000 (8325-4 *rsbU*$^+$) (lane 2), PC1839 (8325-4 *sarA*) (lane 3), and SH1002 (SH1000 *sarA*) (lane 4) purified from culture supernatants after 15 h of growth. The arrows indicate the positions of serine protease (SspA) and alpha toxin (Hla), as verified by Western blotting (data not shown). In PC1839 (8325-4 *sarA*) there was increased proteolysis of Hla, as shown by the number of smaller fragments. (B) Assay of transcription from an *hla-lacZ* fusion during growth. Expression of the reporter fusion in PC322 (8325-4 *hla-lacZ*) (● and ○), JLA371 (SH1000 *hla-lacZ*) (▲ and △), PC4044 (8325-4 *sigB hla-lacZ*) (■ and □), and JLA373 (SH1000 *sigB hla-lacZ*) (♦ and ◇) was measured at different times. ●, ▲, ■, and ♦, β-galactosidase activity; ○, △, □, and ◇, bacterial growth (OD_{600}). The SH1000 *sigB* mutant derivatives were confirmed to be *rsbU*$^+$ by using the PCR method of Kullik and Giachino (34). (C) Protease activities of 8325-4 (lane 1) and SH1000 (8325-4 *rsbU*$^+$) (lane 2) visualized by using a gelatin-containing zymogram. The arrow labeled X indicates the position of unknown protease activity; the position of SspA was verified by comparison with the position of purified protein (V8 protease; Sigma) (data not shown). (D) Assay of transcription from an *sspA-lacZ* fusion during growth. Expression of the reporter fusion in LES07 (8325-4 *sspA-lacZ*) (● and ○) and LES08 (SH1000 *sspA-lacZ*) (▲ and △) was measured at different times. ● and ▲, β-galactosidase activity; ○ and △, bacterial growth (OD_{600}).

sigB mutant derivatives of these strains were very similar (Fig. 5A). The σ^B-mediated reductions in expression of *agr* (RNA III), *hla*, and *sspA* are thus likely to be SarA independent.

The findings described above contrast with the report by Bischoff et al. (4) that *sarA* transcription is increased in a *sigB*$^+$ strain compared to the *sarA* transcription in the *rsbU* deletion strain from which it was generated. Consequently, the SarA protein levels during growth of strains SH1000 (8325-4 *rsbU*$^+$) and 8325-4 and their *sigB* mutant derivatives were determined by using cells grown under our standard defined growth conditions by Western blotting.

Samples removed at 3, 5, and 7 h were lysed, and total cellular proteins were probed by using anti-SarA antibodies (Fig. 6). At these times the levels of SarA were remarkably similar for each of the strains. Thus, when the *lacZ* reporter assay is used or when protein levels are detected by Western blotting, the levels of SarA and temporal regulation of SarA are very similar for strains 8325-4 and SH1000 (8325-4 *rsbU*$^+$) and their *sigB* mutant derivatives.

To confirm the apparent lack of a role for SarA in the overall negative effect of σ^B on the expression of *agr* (RNA III) and *hla,* we introduced a *sarA* mutation into *agr* (RNA III)-*lacZ* and *hla-lacZ* reporter strains by transduction. These reporters in strains 8325-4 and SH1000 (8325-4 *rsbU*$^+$) and their *sigB* mutant derivatives were assayed throughout growth (Fig. 7). The *sarA* mutation decreased expression of each of the reporters, as expected (compare Fig. 7 with Fig. 4B and 5B). However, transcription of both *agr* (RNA III) and *hla* remained

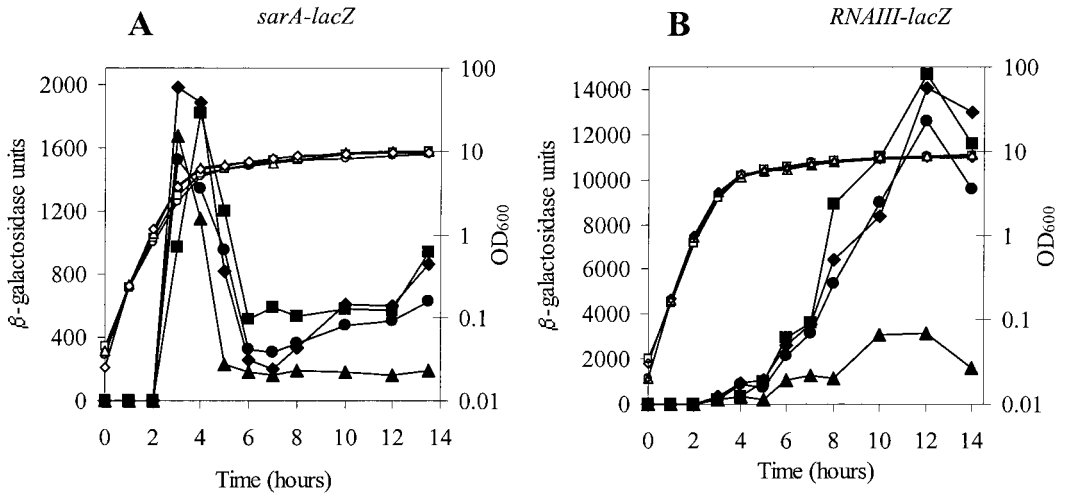

FIG. 5. Assay of transcription from a *sarA-lacZ* fusion and an *agr* (RNA III)-*lacZ* fusion during growth. (A) Expression of the reporter fusion in PC161 (8325-4 *sarA-lacZ*) (● and ○), JLA311 (SH1000 *sarA-lacZ*) (▲ and △), PC4030 (8325-4 *sigB sarA-lacZ*) (■ and □), and JLA313 (SH1000 *sigB sarA-lacZ*) (♦ and ◇) at different times. (B) Expression of the reporter fusion in SH101F7 (8325-4 *agr* [RNA III]-*lacZ*) (● and ○), JLA341 (SH1000 *agr* [RNA III]-*lacZ*) (▲ and △), PC604 (8325-4 *sigB agr* [RNA III]-*lacZ*) (■ and □), and JLA343 (SH1000 *sigB agr* [RNA III]-*lacZ*) (♦ and ◇) at different times. ●, ▲, and ■, β-galactosidase activity; ○, △, and □, bacterial growth (OD_{600}).

lower in SH1000 *sarA* than in 8325-4 *sarA* and the *sigB* derivatives (Fig. 7). The decrease in *agr* and *hla* expression was, therefore, due to a functional *rsbU* gene in SH1000 and not due to the activity of SarA.

Comparison of virulence of 8325-4 and SH1000. The virulence of SH1000 (8325-4 *rsbU*$^+$) was determined by using an established murine subcutaneous skin abscess model of infection (9, 10, 20, 30, 31) and was compared to the virulence of 8325-4 (Fig. 8). When an inoculum of 10^8 CFU was used, the levels of recovery of SH1000 (8325-4 *rsbU*$^+$) and 8325-4 were not significantly different ($P = 0.157$) (Fig. 8). The lesions produced were similar in size and appearance (data not shown).

DISCUSSION

In this study we constructed SH1000 (8325-4 *rsbU*$^+$), a functional *rsbU* derivative of 8325-4. This is an important requirement for studying the control of virulence in *S. aureus*, since in most genetic studies the workers have used the 8325-4/RN6390

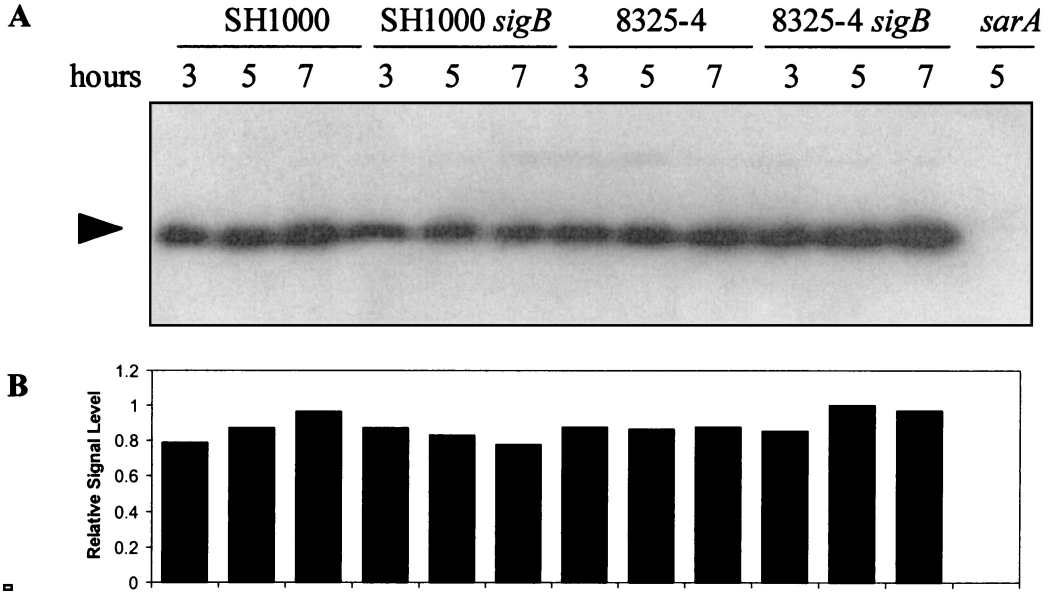

FIG. 6. (A) Equivalent amounts of total cellular proteins (OD_{600} of culture, 0.1), isolated after lysostaphin digestion from cultures grown for 3, 5, and 7 h, were blotted and probed with purified immunoglobulin G antibodies raised against SarA (5). (B) SarA signals quantified on the blot by densitometry. The relative signal level for each lane was compared to the maximum signal level obtained. The results are representative of the results of three independent experiments.

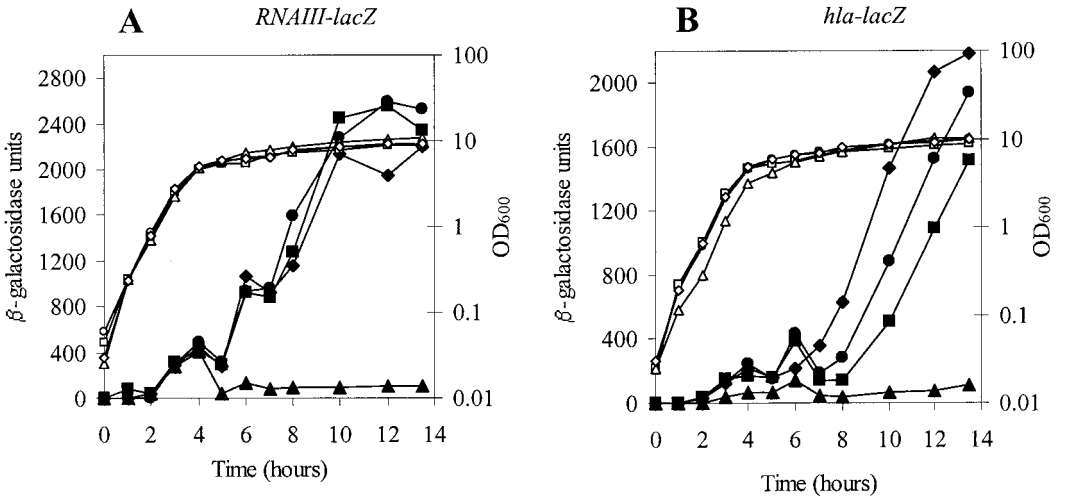

FIG. 7. Assay of an *agr* (RNA III)-*lacZ* fusion and an *hla-lacZ* fusion in a *sarA* mutant background during growth. (A) Expression of the reporter fusion in PC600 (8325-4 *sarA agr* [RNA III]-*lacZ*) (● and ○), JLA345 (SH1000 *sarA agr* [RNA III]-*lacZ*) (▲ and △), PC602 (8325-4 *sarA sigB agr* [RNA III]-*lacZ*) (■ and □), and JLA347 (SH1000 *sarA sigB agr* [RNA III]-*lacZ*) (◆ and ◇) at different times. (B) Expression of the reporter fusion in PC3221 (8325-4 *sarA hla-lacZ*) (● and ○), JLA375 (SH1000 *sarA hla-lacZ*) (▲ and △), JLA376 (8325-4 *sarA sigB hla-lacZ*) (■ and □), and JLA377 (SH1000 *sarA sigB hla-lacZ*) (◆ and ◇) at different times. ●, ▲, and ■, β-galactosidase activity; ○, △, and □, bacterial growth (OD_{600}).

genetic lineage and the absence of antibiotic markers should facilitate future studies. The *rsbU* mutation that is present in the 8325-4 lineage is known to dramatically reduce σ^B activity and consequently affect expression of many virulence-associated loci. The phenotype of SH1000 (8325-4 *rsbU*$^+$) was characterized, and differences between this strain and 8325-4 were observed. These differences included production of the orange pigment staphyloxanthin, decreased levels of alpha toxin, expression of *asp23*, and increased resistance to H_2O_2. While this work was being done, a tetracycline-resistant, functional *rsbU* derivative strain of BB255 was described (4, 26) which effectively has the same lineage, since it was derived from the same parent strain as 8325-4 and RN6390. In the study reported here, we found that in many cases the phenotype of SH1000 (8325-4 *rsbU*$^+$) and the reported phenotype of the BB255 strain were similar; however, there were important differences that are described below.

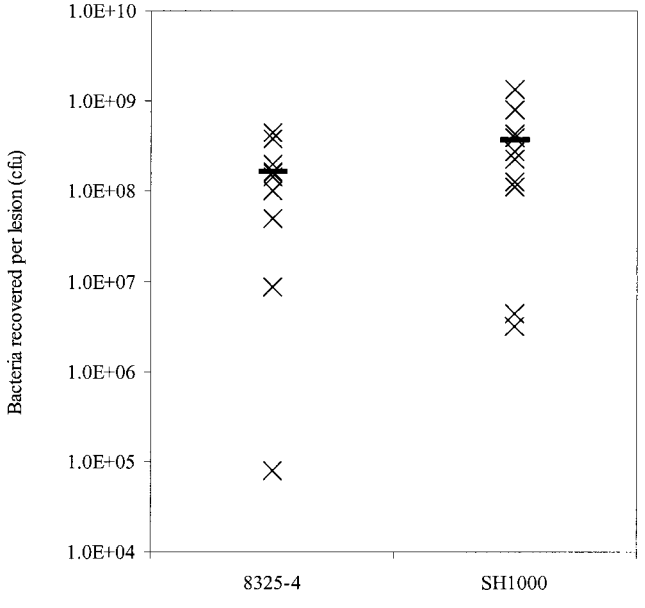

FIG. 8. Virulence of *S. aureus* strains in a murine skin abscess model of infection. Approximately 10^8 CFU of each strain was inoculated subcutaneously into 6- to 8-week-old BALB/c mice. Seven days after infection mice were euthanized, lesions were removed and homogenized, and viable bacteria were counted after dilution and growth on BHI agar plates. The bar indicates the mean recovery for each strain.

The increase in hydrogen peroxide resistance was of interest as one of the major determinants of this characteristic in *S. aureus* is the very active sole catalase, KatA. Previous studies characterized the *katA* locus in 8325-4 and demonstrated the presence of a σ^A promoter, a PerR-binding site, and described positive regulation mediated via Fur (30, 31). Inspection of the identified promoter region of *katA* revealed a putative σ^B motif (Fig. 2A) with strong identity to the consensus sequence described by Gertz et al. (24). Primer extension of *katA* mRNA from SH1000 (8325-4 *rsbU*$^+$), 8325-4, and PC400 (8325-4 *sigB*) revealed that the σ^B-dependent promoter, P_B, was active in SH1000 (8325-4 *rsbU*$^+$) but not in the other two strains. To assess the contribution of P_B to the overall levels of the *katA* transcript, RNA from each of the strains was isolated throughout growth and probed for *katA* by Northern blotting. The amount of the *katA* transcript was found to be between 1.2 and 1.5 times greater in SH1000 (8325-4 *rsbU*$^+$) than in MJH502 (SH1000 *rsbU*$^+$ *sigB*) (Fig. 2B). Assays of catalase activity during growth of SH1000 (8325-4 *rsbU*$^+$), MJH502 (SH1000 *rsbU*$^+$ *sigB*), 8325-4, and PC400 (8325-4 *sigB*) revealed little difference among the strains (Fig. 2C), which is consistent with the similar levels of transcript observed in Northern blot and primer extension analyses. In contrast to these results, assays of a *katA-lacZ* reporter in SH1000 (8325-4 *rsbU*$^+$) and 8325-4 revealed levels of β-galactosidase that were four- to sixfold lower in SH1000 (8325-4 *rsbU*$^+$) than in 8325-4. The reason for

this discrepancy is unclear, but this may reflect differences in turnover of the *katA-lacZ* message in these strains.

The similar transcription of *katA* and the similar expression of catalase activity in SH1000 (8325-4 *rsbU*$^+$) and 8325-4 despite the presence of an active P_B promoter in the former strain suggest that the overall contribution of this promoter is minor, at least under the conditions studied here. Previous work has demonstrated that *katA* is regulated by the PerR and Fur proteins, which control *katA* levels in response to various signals, including peroxide stress and the levels of manganese and iron in the cell (30, 31). The P_B promoter may be important under some environmental conditions, such as alkaline and heat stress, under which expression of a number of σ^B-regulated genes is induced (24, 25, 34). Bischoff et al. (4) remarked that the levels of *katA,* when they were measured by Northern blotting, were equivalent in their marked functional *rsbU*$^+$ strain and its *rsbU* mutant parent. We confirmed the similar transcript levels in SH1000 and 8325-4 and extended this observation to show similar levels of activity; however, importantly, there is potential for σ^B transcriptional control. The lack of an overall effect of σ^B on catalase levels may reflect an ability by the cell to maintain homeostasis via alternative regulatory mechanisms. H_2O_2 resistance is multifactorial, and the observed increase in the minimum bactericidal concentration of H_2O_2 may be due to other σ^B-regulated components that are expressed in SH1000 (8325-4 *rsbU*$^+$) but not in 8325-4. A large regulon of σ^B-regulated genes has already been identified (25).

The most striking phenotype observed for SH1000 (8325-4 *rsbU*$^+$) was the low level of exoprotein expression compared to the level of expression in 8325-4. This phenotype is similar to that observed for clinical strains of *S. aureus* described recently by Blevins et al. (6) and contrasts with that of 8325-4. A major reduction in protease expression, including *sspA* expression, was observed by using protease zymograms, Western blotting, and reporter assays performed with an *sspA-lacZ* fusion. The reduced level of Hla has been reported previously (26), and we have determined that this effect is transcriptional and not due to the changes in protease activity. The overall effect of an intact σ^B locus, therefore, is to reduce the level of expression of the *sspA* and *hla* genes. The mechanism for this is likely to involve the lower levels of *agr* in the cell, since we observed a large reduction in expression of an *agr* (RNA III)-*lacZ* fusion in SH1000 (8325-4 *rsbU*$^+$). Bischoff et al. (4) similarly observed that the level of *agr* (RNA III) was reduced in their functional *rsbU*$^+$ strain of BB255.

The most obvious candidate for mediating the reduction in *agr* levels was SarA, which is a known activator of *agr* expression (18, 19, 29). However, when a *sarA-lacZ* reporter was assayed or when the amount of SarA protein was determined by Western blotting in strains SH1000 (8325-4 *rsbU*$^+$) and 8325-4 and their σ^B mutant derivatives, the levels were found to be very similar. Importantly, inactivation of *sigB* in each of the strains did not alter the level of SarA, suggesting that σ^B had little or no role under the conditions studied here. This is in accordance with the results of Blevins et al. (5), who reported that SarA is expressed constitutively. In their quantitative analysis of SarA during different stages of growth of RN6390 and clinical isolate UAMS-1, these authors observed no variation between the strains (6). We extended this observation to strains SH1000 (8325-4 *rsbU*$^+$) and 8325-4 and their *sigB* mutant derivatives. However, this finding contrasts with the findings of Bischoff et al. (4). In the studies of these authors, expression of a *sarA-lux* fusion reporter strain was found to be greater during the stationary phase of growth in a marked *rsbU*$^+$ strain than in its *rsbU* mutant parent. The reason for the difference in *sarA* expression between the two studies is unclear. However, since different strains, media, and growth conditions were used, we cannot exclude the possibility that genetic or environmental differences had an effect. Gertz et al. (25) showed that there was increased SarA expression in a *sigB* mutant of *S. aureus* COL by using two-dimensional electrophoresis; in this study minimal medium in which constitutive σ^B expression was observed was used. Thus, it is important to quantitatively study a number of environmental conditions to monitor the effect of σ^B on SarA expression. The σ^B-dependent promoter, *sarP*3 (2, 38), is most active during the exponential growth phase in strains with a wild-type *rsbU* locus (4). SarA represses its own transcription via the proximal *sarP*1 promoter but not via the more distal *sarP*3 promoter (7). Therefore, the failure to observe increased expression of SarA despite the presence of a σ^B promoter may be due to the autoregulatory capacity of SarA (5, 7) maintaining constant protein levels during growth. We hypothesize that in certain environmental conditions under which σ^B activity increases, which results in a concomitant reduction in SarA repression, SarA autoregulation increases and maintains the level of SarA. However, since we have found that in the conditions studied here the SarA protein level is relatively constant during growth irrespective of the *rsbU* or *sigB* genotype, the SarA level is unlikely to be the mediator for the reduction in expression of the virulence-associated loci seen in SH1000 (8325-4 *rsbU*$^+$).

The properties of SH1000 (8325-4 *rsbU*$^+$) mean that the 8325-4 lineage now behaves like clinical isolates which are typically low protease producers, have lower Hla levels of expression, and during growth in standard laboratory conditions have constant levels of SarA (6). The effects of σ^B on virulence determinant expression, although multiple and dramatic, did not alter the virulence of *S. aureus* in the mouse abscess model of infection. This result supports previous studies that showed that a *sigB* mutation in an *rsbU*$^+$ parent strain did not produce attenuation in three separate animal models (40).

From the experiments reported here we conclude that mediation of the reduction in *agr* (RNA III) levels in SH1000 is independent of the level of SarA. Consequently, since the SarA level appears not to be the effector of the overall negative σ^B-mediated effect on virulence-associated loci, what then is the effector? The sequence of the *S. aureus* genome has revealed an impressive array of SarA homologues, including SarH1 (51), Rot (39), and SarT (50). These homologues have all been shown to repress exoprotein expression. A number of other candidate loci have an impact on exoprotein synthesis. These loci include *1E3* (12) and *sae* (27, 28), and when inactivated, the latter locus dramatically reduces expression of many exoproteins. To date, we have found that neither SarH1 nor Rot is the missing effector protein (J. L. Aish and S. J. Foster, unpublished data); we are currently attempting to identify this missing effector by epistasis. The σ^B-mediated modulation of expression of virulence-associated loci of *S. aureus* is intriguing, particularly since *S. aureus* colonizes the anterior

nares, where it resides as a harmless commensal. It will be interesting to determine whether σ^B functions to discriminate between environments and to regulate exoprotein synthesis. SH1000 (8325-4 *rsbU*$^+$) provides a useful genetic background to characterize the exact physiological role of σ^B in *S. aureus* and should facilitate comparisons with previous studies for this genetic lineage.

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M.J.H. and J.L.A. contributed equally to this work.

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