

Repression of *hla* by *rot* Is Dependent on *sae* in *Staphylococcus aureus*[∇]

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The regulatory locus *sae* is a two-component system in *Staphylococcus aureus* that regulates many important virulence factors, including alpha-toxin (encoded by *hla*) at the transcriptional level. The SarA homologs Rot and SarT were previously shown to be repressors of *hla* in selected *S. aureus* backgrounds. To delineate the interaction of *rot* and *sae* and the contribution of *sarT* to *hla* expression, an assortment of *rot* and *sae* isogenic single mutants, a *rot sae* double mutant, and a *rot sae sarT* markerless triple mutant were constructed from wild-type strain COL. Using Northern blot analysis and transcriptional reporter gene green fluorescent protein, fusion, and phenotypic assays, we found that the repression of *hla* by *rot* is dependent on *sae*. A *rot sae sarT* triple mutant was not able to rescue the *hla* defect of the *rot sae* double mutant. Among the three *sae* promoters, the distal *sae* P3 promoter is the strongest *in vitro*. Interestingly, the *sae* P3 promoter activities correlate with *hla* expression in *rot*, *rot sae*, and *rot sae sarT* mutants of COL. Transcriptional study has also shown that *rot* repressed *sae*, especially at the *sae* P3 promoter. Collectively, our data implicated the importance of *sae* in the *rot*-mediated repression of *hla* in *S. aureus*.

Staphylococcus aureus is an important community- and nosocomially acquired pathogen that can cause both local and systemic infections in humans. The pathogenicity of this microorganism depends largely on its successful adaptation to the human host and hence requires the environmentally coordinated expression of virulence factors. The expression of virulence factors in *S. aureus* is regulated by a network of interacting regulators, including two-component regulatory systems and the SarA protein family (2, 19).

The *sae* locus is a two-component regulatory system first described for a Tn551 insertional mutant with an exoprotein-defective phenotype (5). Subsequent works showed that the *sae* locus consists of four open reading frames (ORFs), with two encoding the response regulator (SaeR) and the sensor kinase (SaeS) and the other two encoding the two hypothetical proteins designated ORF3 and ORF4 (6, 20, 28). Transcriptional analysis revealed four overlapping transcripts driven by three promoters (P3, P1, and P2) (20, 28). The *sae* locus was found to be a key element in the regulatory cascade governing the staphylococcal virulon. *In vitro*, it up-regulates many virulence factors, including alpha-hemolysin (encoded by *hla*), beta-hemolysin, DNase, coagulase, the protease SspA, thermolysin, protein A, extracellular adherence protein (Eap), extracellular matrix binding protein (Emp), and FnbpA, and down-regulates capsular polysaccharide at the transcriptional level (4, 5, 8, 10, 24). More importantly, *sae* is an important element for the expression of virulence genes *in vivo* (7, 8, 23).

The *rot* locus was first identified via transposon mutagenesis as a repressor of alpha-hemolysin synthesis in *S. aureus* strain PM614 by partially restoring *hla* expression in an *agr* null mutant, presumably via an *agr*-independent mechanism (17). The *rot* gene product (Rot) is a member of the SarA protein

family that shares homology with the smaller SarA homologs (e.g., SarA, SarR, SarT, SarV, and SarX). Although originally perceived as a 166-residue protein (17), Rot was recently found to be only 133 residues long (16) and is likely a winged-helix protein, as with other members of the SarA protein family (2).

Several studies have shown that *hla* is one of the major virulence factors produced by most *S. aureus* strains (9, 13, 22). Alpha-toxin is a pore-forming toxin that has cytolytic, hemolytic, and dermonecrotic activities. Although alpha-toxin has been shown to be up-regulated by *sae* and down-regulated by *rot* and *sarT*, both of which are repressors of *hla* expression, the exact pathways of *hla* regulation by these three regulators have not been defined. To address this issue, we constructed *sae* and *rot* single- and double-deletion mutants as well as a *sae rot sarT* triple-deletion mutants in the sigma B-positive strain COL to examine *hla* expression at the phenotypic and transcriptional levels. Our results clearly showed that down-modulation of the *sae* P3 promoter, the strongest *sae* promoter among the three promoters *in vitro*, correlated with decreased *hla* expression in *sae*, *sae rot*, and *sae rot sarT* mutants compared to that of the parent. We also demonstrated that *rot* represses *sae* to control *hla* expression. Our studies here thus revealed an intricate network between *sae* and *rot* in the regulation of *hla*.

MATERIALS AND METHODS

Bacterial strains, plasmids, and growth conditions. The bacterial strains and plasmids used in this study are listed in Table 1. *S. aureus* cells were grown at 37°C with aeration in Mueller-Hinton broth (MHB; Difco) supplemented with antibiotics as indicated below. Luria-Bertani (LB) broth was used for cultivating *Escherichia coli*. Antibiotics used for *S. aureus* were erythromycin (5 µg/ml), tetracycline (3 µg/ml), and chloramphenicol (10 µg/ml). For *E. coli*, ampicillin was used at 100 µg/ml.

Construction of single-, double-, and triple-deletion *sae*, *rot*, and *sarT* mutants. To introduce a single deletion of the *rot*, *saeR*, or *saeR* gene, DNA fragments corresponding to the upstream and downstream regions of the gene were amplified by PCR, using chromosomal DNA from strain COL as a template. The PCR products were purified, digested with BamHI and NcoI or EcoRI, and ligated into the temperature-sensitive shuttle plasmid pMAD, containing a temperature-sensitive *S. aureus* origin of replication, an erythromycin resistance cassette, and the β-galactosidase gene (*l*). The resulting plasmids (Table 1)

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TABLE 1. Bacterial strains and plasmids

Strain or plasmid	Comment	Reference
Strains		
<i>E. coli</i> XL-1 Blue	General-purpose host strain for cloning	14
<i>S. aureus</i>		
RN4220	Mutant strain of 8325-4 that accepts foreign DNA	18
COL	Methicillin-resistant laboratory strain	21
ALC4481	COL <i>sae</i> single-deletion mutant	This study
ALC4909	COL <i>rot</i> single-deletion mutant	This study
ALC4988	COL <i>sae rot</i> double-deletion mutant	This study
ALC5245	COL <i>sae rot sarT</i> triple-deletion mutant	This study
ALC3227	COL with pALC1740	This study
ALC5097	ALC4481 with pALC1740	This study
ALC5098	ALC4909 with pALC1740	This study
ALC5099	ALC4988 with pALC1740	This study
ALC5256	ALC5245 with pALC1740	This study
ALC5018	COL with pALC4989	This study
ALC5019	COL with pALC4990	This study
ALC5020	COL with pALC4991	This study
ALC5370	ALC4481 with pALC4991	This study
ALC5371	ALC4909 with pALC4991	This study
ALC5373	ALC4988 with pALC4991	This study
ALC5376	ALC5245 with pALC4991	This study
Plasmids		
pALC1484	Modified pALC236 shuttle vector with a promoterless <i>gfp_{uvr}</i> reporter gene preceded by an <i>S. aureus</i> ribosome binding site	11
pALC1740	pALC1484 with the <i>hla</i> promoter	15
pALC4989	pALC1484 with the <i>sae</i> P1 promoter fused with the <i>gfp_{uvr}</i> reporter gene at the EcoRI and XbaI sites	This study
pALC4990	pALC1484 with the <i>sae</i> P2 promoter fused with the <i>gfp_{uvr}</i> reporter gene at the EcoRI and XbaI sites	This study
pALC4991	pALC1484 with the <i>sae</i> P3 promoter fused with the <i>gfp_{uvr}</i> reporter gene at the EcoRI and XbaI sites	This study
pMAD	Vector for allelic replacement in gram-positive bacteria	1

containing the upstream and downstream fragments in tandem were then amplified in *E. coli* XL1-Blue. The recombinant pMADs were then extracted from *E. coli* and transformed into *S. aureus* RN4220 by electroporation. Plasmids obtained from RN4220 were then transformed into *S. aureus* strain COL. Transformants of *S. aureus* were selected at 30°C on Trypticase soy agar containing 2.5 µg/ml erythromycin and 150 µg/ml 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-Gal). The allelic exchange in the absence of a selection marker was performed as previously described (1). Briefly, recombinant pMAD was integrated into the chromosome through homologous recombination at the non-permissive temperature (42°C). From the 42°C plate containing erythromycin and X-Gal, one light-blue colony was picked into 10 ml of Trypticase soy broth and incubated overnight at 30°C without antibiotic. Tenfold serial dilutions of this culture were plated on Trypticase soy agar plates containing X-Gal. White colonies, which were sensitive to erythromycin and hence no longer contained the pMAD plasmid, were selected, and the gene deletion was confirmed by PCR and DNA sequencing. We used a similar strategy to sequentially construct *sae rot* double mutants and *sae rot sarT* triple mutants, using the pMAD plasmid containing the fragments flanking the deleted genes, and then confirmed the sequences with PCR and DNA sequencing.

Isolation of RNA and Northern blot hybridization. Overnight cultures of *S. aureus* were diluted 1:100 in MHB and grown in 18-mm borosilicate tubes to early log phase (optical density at 650 nm [OD₆₅₀] = 0.7), late exponential phase (OD₆₅₀ = 1.2), and post-exponential phase (OD₆₅₀ = 1.7). The cells were harvested and processed with Trizol (Invitrogen, Gaithersburg, MD) in combination with 0.1-mm sirconia-silica beads in a Biospec reciprocating shaker to yield RNA as described previously (3). RNA concentrations in the extracts were measured by determining absorbance at 260 nm using an Eppendorf Bio-Photometer (Brinkmann, Westbury, NY). Twenty to 40 µg of each sample was electrophoresed in a 1.5% agarose-0.66 M formaldehyde gel in 4-morpholinepropanesulfonic acid (MOPS) running buffer (20 mM MOPS, 10 mM sodium acetate, 2 mM EDTA, pH 7.0) and blotted onto Hybond N+ membranes (Amersham, Arlington Heights, IL), as previously described (3). Prior to being blotted, the gel was viewed under UV light to ensure that equivalent amounts of ethidium bromide-stained rRNA bands were present for each sample. After being blotted,

the gel was again viewed under UV light to confirm complete RNA transfer. For the detection of specific transcripts (*hla*, *saeRS*, and *rot*), digoxigenin (DIG)-labeled probes (the oligonucleotides listed in Table 2) generated by PCR were prepared by using the DIG labeling PCR kit according to the manufacturer's instructions (Roche Biochemicals, Mannheim, Germany). The blotted membrane was prehybridized in 25 ml Dig-Easy-Hyb buffer (Dig High Prime DNA labeling and detection starter kit II; Roche) for 2 h at 50°C with rotation and hybridized in the same Dig-Easy-Hyb buffer containing 25 ng/ml DIG-labeled probe overnight at 50°C. The hybridized membrane was washed first with 2× SSC (1× SSC is 0.15 M NaCl plus 0.015 sodium citrate)–0.1% sodium dodecyl sulfate (SDS) for 30 min at 37°C, second with 0.5× SSC–0.1% SDS for 30 min at 37°C, and finally twice with 0.5× SSC–0.1% SDS for 15 min at 37°C, all with rotation. After being washed with 1× washing buffer (Roche) for 5 min, the membrane was incubated with blocking solution for 60 min and antibody solution (anti-DIG–alkaline phosphatase, 75 mU/ml) for 60 min at 37°C with rotation. The membrane was then washed, covered with 1 ml chemiluminescent substrate for alkaline phosphatase, and incubated for 15 min at 37°C according to the manufacturer's protocol. The membrane was immediately exposed to film (Kodak Biomax MR film) for 3 to 30 min.

Transcriptional fusion studies of different promoters linked to the *gfp_{uvr}* reporter gene. Promoter fragments of *hla*, *sae*, and *rot* were cloned into the shuttle vector pALC1484 upstream of the *gfp_{uvr}* reporter gene to generate transcriptional fusions. Restriction analysis and DNA sequencing confirmed the orientation and authenticity of the promoter fragments. The recombinant plasmids containing these promoters were first amplified in *E. coli* and transformed into *S. aureus* strain RN4220 by electroporation (26). Plasmids purified from RN4220 transformants were then electroporated into COL and their isogenic mutants.

For the assay, overnight cultures of *S. aureus* strains harboring the recombinant plasmids were diluted 1:100 and grown at 37°C with shaking in MHB with chloramphenicol (10 µg/ml). The control strains contained only the vector pALC1484. Aliquots (200 µl) were transferred hourly or every 2 h to microtiter wells to assay for cell density (OD₆₅₀) and fluorescence for 8 h and then overnight in a model FL600 fluorescence spectrophotometer (BioTek Instru-

TABLE 2. Primers used in this study

Use	Primer ^a
<i>sae</i> deletion	5'GTCAGGATTCGTACGGATACCACTATAGAT3' 5'TCCGATTTATTTATAAAAATAAAATGCAAAGACTAAAAAGAAGCTC3' 5'CATTTTATTTTATAAAATAAATCGGACTATTTTTTCACCTCTGTTCTTACGA3' 5'GATCCCATGGTCCAGATTTTATACGTCTACCTAACAA3'
<i>rot</i> deletion	5'GATCGGATCCCACGAGGTTTACAATGAGC3' 5'CCCAACAATCCCAAACTTGTATGTGCT3' 5'ATACAAGTTTTGGGATTGTTGGGGTTAATAGCATAAAAAAGAGGT3' 5'GATCCCATGGTACTCAAGAAGAGTACACAAAC3'
<i>sarT</i> deletion	5'GTCAGGATTCATCCTTTTCATCTGCAAGGGATCGT3' 5'CACCAAGATATTAATACTCGCAAATCATTCATCAAGTCTTC3' 5'GAAGACTTGATGAATGATTTGCGAGATTTAATATCTTGGTC3' 5'GTCAGAATTCATGGTTATTTGCCACTCTAAC3'
<i>sae</i> P1 promoter- <i>gfp</i> fusion	5'GTCAGAATTCGCAATGGTTGACTACGAT3' 5'GTCATCTAGAAATTTATTGTGTGTAATTTATATAAAACA3'
<i>sae</i> P2 promoter- <i>gfp</i> fusion	5'GTCAGAATTCCTTAGTACCAGTCATCGCTAAC3' 5'GTCATCTAGACTTACGACCTCTAAAGTAATTAATGAT3'
<i>sae</i> P3 promoter- <i>gfp</i> fusion	5'GTCAGAATTCCTTATTGTGGCAAAGGTTTATAAAA3' 5'GTCATCTAGACAATTTGATAAGTTAAGTTTAAAAAT3'

^a Restriction sites are in bold and underlined.

ments, Winooski, VT). Promoter activities were plotted as mean fluorescence divided by the OD₆₅₀ from triplicate samples to minimize variations due to cell density.

The production of alpha-hemolysin. After overnight growth in MHB, 2 μl of supernatant from each culture was placed on 2.5% defibrinated rabbit blood agar for 24 h at 37°C. The clear zone around bacterial growth represented alpha-hemolysis. The titer of each culture supernatant was also determined for hemolytic activity against rabbit erythrocytes. The method was modified from the work of Kehoe et al. (12). Briefly, the rabbit erythrocytes were washed and resuspended in phosphate-buffered saline to a final concentration of 1% (vol/vol). Erythrocytes were mixed with culture supernatants at equal proportions and incubated at 37°C for 1 h in microtiter wells. The highest dilution giving rise to visibly detectable lysis was defined as the hemolytic titer (12).

RESULTS

Effects of *sae*, *rot*, *sae rot*, and *sae rot sarT* mutations on *hla* expression in COL. Previous studies have shown *rot* and *sae* to be negative and positive regulators, respectively, of *hla* expression. To determine the relationship between these two regulators, we constructed *sae* and *rot* single-deletion mutants and also *sae rot* double-deletion mutants in the COL background. COL was chosen because methicillin resistance in this strain is clinically relevant. In addition, this strain has an intact *sigB* operon with a functional *rsbU* gene, which has been shown to be important in down-modulating *hla* expression. To minimize the issues of the ectopic promoter and disruption of gene transcription within the same operon, we created in-frame deletions of *saeRS* and *rot* with pMAD, while leaving promoters and the transcription termination sites intact. In concordance with previous observations (8, 20, 23), *hla* transcription was completely absent in the *sae* mutant of strain COL compared with that in the isogenic parent (Fig. 1A). To our surprise, *hla* transcription was significantly up-regulated in the *rot* deletion mutant compared with that in the parent. This contrasts with data from previous studies showing that a *rot* mu-

tation by itself, in the absence of an *agr* mutation, does not have any effect on *hla* expression (17, 25). To determine if *rot* expression is dependent on *sae*, we constructed a *rot sae* double mutant in the COL background. Northern blot analysis revealed that the *sae rot* double mutant displayed significant down-regulation of *hla* transcription in comparison to the *rot* single mutant (Fig. 1A). This indicated that the effect of *rot* on *hla* transcription is likely dependent on *sae* and not vice versa since the up-regulation of *hla* was not observed in the *sae rot* double mutant of COL. Previous studies have shown that *sarT* is a repressor of *hla* expression (27). To determine if *sarT* impacts *hla* expression via the *sae* pathway, we constructed the *sae rot sarT* triple-deletion mutant of strain COL. In comparison to what occurred with the *sae rot* double mutants, introduction of a *sarT* mutation into the *sae rot* double mutant in the COL background did not increase *hla* expression in the double mutants, thus indicating that *sarT* may act upstream of *sae* or *rot* and not downstream of *sae* in *hla* repression.

To confirm the effect of *sae* on *hla* transcription on Northern blots, we performed *hla* promoter fusion assays. For this assay, we transformed strain COL and its isogenic *sae* mutant, *rot* mutant, *sae rot* double mutant, and *sae rot sarT* triple mutant with the shuttle plasmid carrying the *gfp_{uvr}* reporter gene as driven by the *hla* promoter (15). Green fluorescent protein (GFP) levels, expressed as fluorescence units per OD₆₅₀ unit to minimize the effect of bacterial cell densities, also confirmed the effect of *rot* and *rot sae* mutations on *hla* expression in the COL background, thus confirming our data from Northern blots (Fig. 1B). As a positive control, we also included a *sarT* mutant of COL, which displayed elevated *hla* promoter expression, as expected.

The phenotypic expression of alpha-hemolysin in parental strain COL and its isogenic mutants was measured on rabbit blood agar containing 2.5% rabbit erythrocytes as shown in

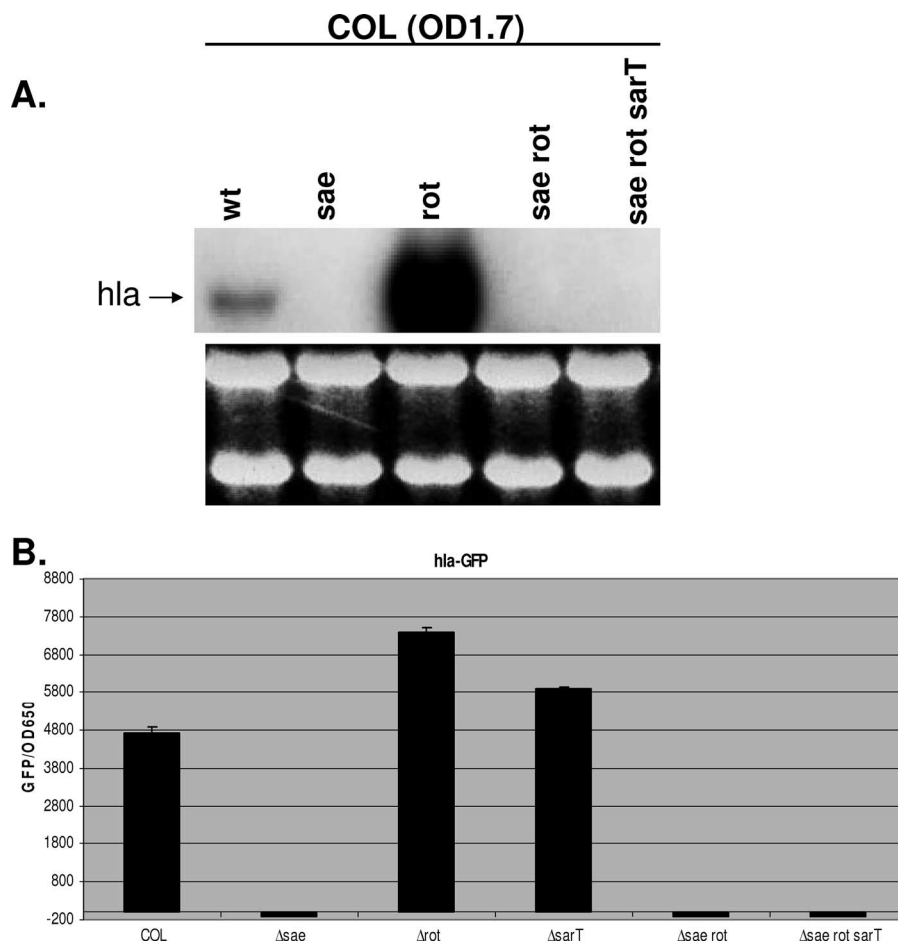


FIG. 1. Transcription of *hla* in COL and its isogenic mutants. (A) Northern blot analysis of *hla*. RNA was harvested from cells grown to an OD₆₅₀ of 1.7 (OD1.7) at a time when *hla* expression was expected to be the strongest. One typical blot with RNA with an OD₆₅₀ of 1.7 is shown. An RNA gel below shows equal sample loadings. wt, wild type. (B) The expression of GFP driven by the *hla* promoter was measured. The fluorescence and cell density (OD₆₅₀) were measured hourly for 10 h and then overnight by transferring aliquots (200 μ l) to microtiter plates in triplicate. The results from one time point (8 h; OD₆₅₀ of 1.7) of promoter activation are shown and are plotted as mean fluorescence divided by the OD₆₅₀ using average values from triplicate readings.

Fig. 2A. The expression of alpha-hemolysin in the parent COL and the *sae* mutant on the rabbit erythrocyte agar plate was low. In agreement with the data on *hla* transcription as determined by Northern blot analysis and GFP reporter fusion assays, the hemolytic zone was noticeably bigger in the *rot* mutant but was reduced in the *sae rot* double mutant to the level in the *sae* single mutant. Likewise, a *sarT* mutation did not alter the hemolytic profile of the *sae rot* double mutant. Quantitation of the titers, defined as the highest dilution from overnight cultures giving rise to erythrocyte lysis, correlated well with those observed on the rabbit erythrocyte agar plates (Fig. 2B).

Differential effects of the three different promoters of the *sae* locus. Previous studies have shown that the *sae* locus is composed of three promoters, resulting in four major transcripts (Fig. 3A) (20, 28). Besides containing the two-component system involving *saeR* and *saeS*, the 3.1-kb transcript bears two ORFs, ORF3 and ORF4, the functions of which are not yet defined. As we have shown above that *rot* may have an impact upon *sae* in the regulation of *hla*, we wanted to determine if

differential levels of expression exist for each of the *sae* promoters. For this assay, we cloned each of the *sae* promoters, based on the transcription start sites derived by Steinhuber et al. (28), upstream of the GFP_{uvr} reporter gene into shuttle plasmid pALC1484. Recombinant plasmids containing the *sae* P1, P2, and P3 promoters (designated pALC4989, pALC4990, and pALC4991, respectively) were then introduced into parental strain COL. Fluorescence assays of bacterial cells every 2 h and overnight disclosed that the *sae* P3 promoter driving both the 0.4- and the 3.1-kb transcript was the strongest promoter in strain COL, and the results of one typical GFP experiment from post-exponential-phase cultures (OD₆₅₀ = 1.7) are shown in Fig. 3B. The *sae* P3 promoter was at least 2 to 5 times stronger than the P2 promoter and more than 10 times stronger than the *sae* P1 promoter in strain COL. Interestingly, the *sae* P1 promoter was silent in both wild-type strains. Thus, it is conceivable that the *sae* P1 promoter may not be a true promoter and hence may represent an mRNA degradation product in the primer extension experiment (communication from Christaine Wolz, Tuebingen, Germany). To confirm that our

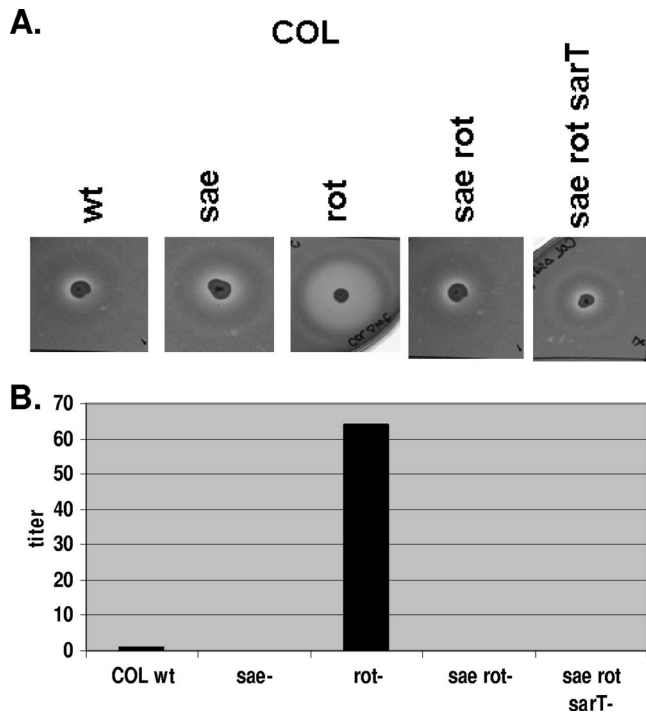


FIG. 2. Production of alpha-hemolysin on 2.5% rabbit blood agar in COL and its isogenic mutants. (A) Two microliters each of supernatant from an overnight culture was placed on rabbit blood agar plates for 24 h at 37°C. wt, wild type. (B) One percent rabbit erythrocytes were mixed with culture supernatants in equal proportions, and the mixture was incubated at 37°C for 1 h in microtiter wells. The highest dilution giving rise to visibly detectable lysis was defined as the hemolytic titer.

results were not unique to strain COL, we also introduced pALC4989, pALC4990, and pALC4991 into strains Newman, SH1000, and clinical isolate MW2, with similar results with the *sae* P3 and P1 promoters (data not shown).

Impact of *rot* and *sae* on the *sae* P3 promoter. Since we have shown that the effect of *rot* on *hla* expression is dependent on *sae* and that the *sae* P3 promoter is the strongest promoter within the *sae* locus, we wanted to examine whether *rot* represses *sae* P3 promoter activation, thereby down-modulating *hla* expression in *rot* and *rot sae* mutants (Fig. 1). For this purpose, we introduced pALC4991 containing the *sae* P3 promoter fused to the *gfp_{uvr}* reporter gene into wild-type strain COL and its isogenic *sae*, *rot*, and *sae rot* deletion mutants as well as the *sae rot sarT* triple-deletion mutant. As shown in Fig. 4, a deletion of *rot* in strain COL resulted in elevated expression of the *sae* P3 promoter compared with that in the respective parents. In *rot sae* double mutants, the *sae* P3 promoter became silent, similarly to what occurred with the vector control in the parental background (data not shown). Deletion of the *sarT* gene in the triple mutants did not alter *sae* P3 promoter activity as assessed by GFP-mediated fluorescence, thus indicating that *sarT* does not play a major regulatory role with the *sae* P3 promoter in the absence of *rot* and *sae*. These data, in conjunction with those on *hla* expression (Fig. 1 and 2), indicated that *rot* may repress the *sae* P3 promoter to down-regulate *hla* expression.

Interestingly, a deletion of *sae* alone also resulted in significant down-modulation of *sae* P3 promoter activity compared with the activity in the parent COL. This finding implied the

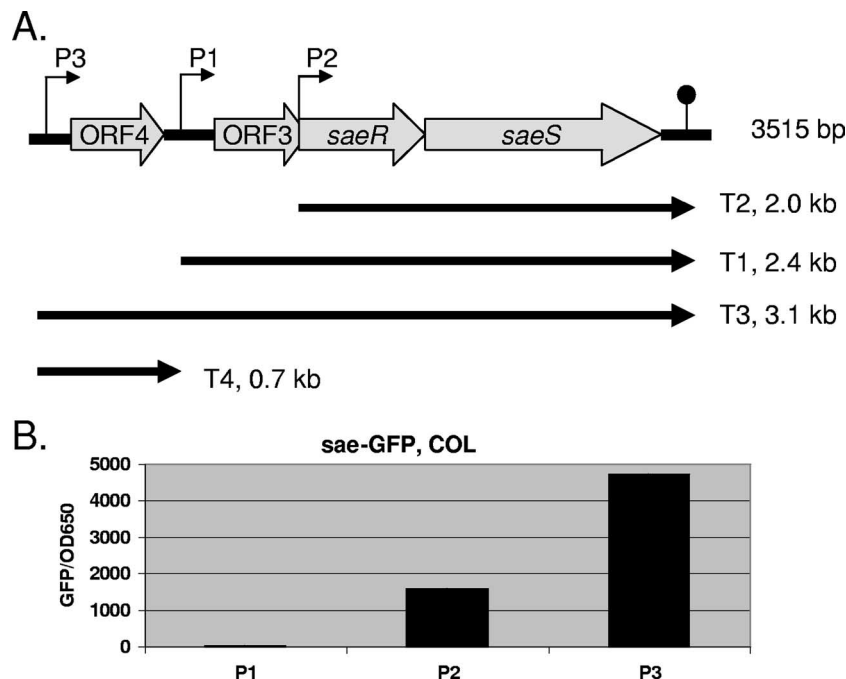


FIG. 3. Transcription of *sae*. (A) Schematic drawing of the *sae* locus modified from the work of Steinhuber et al. (28). Three promoters yielding four overlapping transcripts are indicated. (B) Expression of GFP driven by three different *sae* promoters (P1, P2, and P3) in wild-type strain COL. Fluorescence as the indicator of promoter activity was measured hourly for 10 h and then overnight. The results of one typical assay ($OD_{650} = 1.7$; 8 h) were plotted as mean fluorescence divided by the OD_{650} , using average values of triplicate readings from experiments with three clones from each transformant. These experiments were repeated at least three times with similar results.

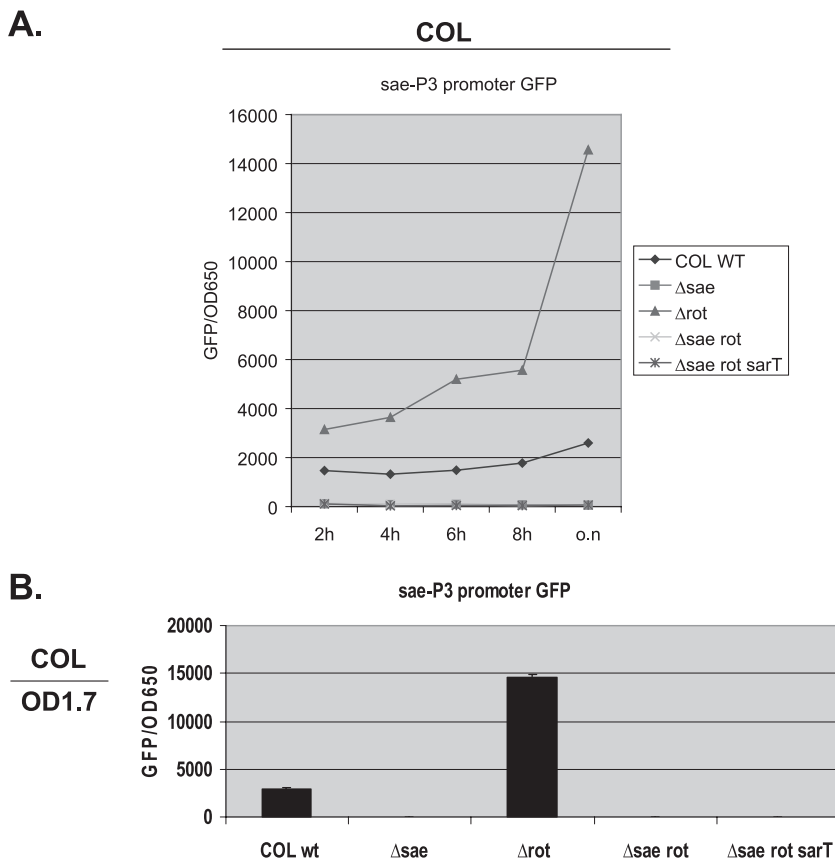


FIG. 4. Expression of GFP driven by the *sae* P3 promoter in COL and its isogenic mutants. (A) Promoter activation was measured every 2 h for 8 h and then overnight (o.n) and plotted as mean fluorescence divided by the OD₆₅₀, using average values of triplicate readings from experiments with three clones of each transformant. wt, wild type. (B) Representative readings at an OD₆₅₀ of 1.7 (OD1.7) for one of the experiments are shown.

existence of an autoregulatory circuit in the *sae* locus, with the major effect being primarily on the strongest *sae* P3 promoter.

rot is a repressor of sae but not vice versa. To further confirm the repressive effect of *rot* on *sae* promoters, we analyzed *sae* transcription in both wild-type COL and its isogenic *sae* and *rot* mutants by Northern blotting. As shown in Fig. 5, expression of the monocistronic T4 transcript (0.7 kb) and, to a much lesser extent, the 3.1-kb *sae* T3 transcripts, both driven by the strongest *sae* P3 promoter, was increased in the *rot* mutant of COL. Therefore, these data indicated that *rot* likely represses the *sae* P3 promoter to down-regulate *hla* expression.

To determine if *sae* regulates *rot* in *hla* expression, we detected *rot* transcripts in *sae* mutants by Northern blot analysis. The *rot*-hybridizing bands were preserved in both the parents and the isogenic *sae* mutant (data not shown). We also transduced pALC5552, a shuttle plasmid containing a 338-bp *rot* promoter fragment fused with the *gfp_{uvr}* reporter, into strain COL and its isogenic *sae* and *rot* mutants. The GFP values of *rot* promoter activity were similar between the parents and the isogenic *sae* mutant, thus confirming that *sae* does not regulate *rot* (data not shown). We also confirmed that *rot* promoter activity was not diminished in the *rot* mutant, thus indicating that the *rot* locus was not autoregulatory.

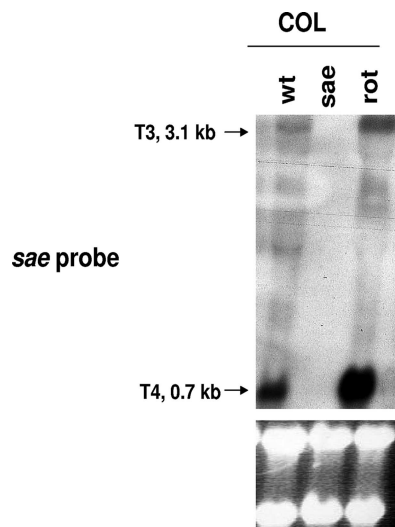


FIG. 5. Northern blot analysis of *sae* transcripts in COL (wild type [wt]) and its isogenic *sae* and *rot* mutants. The probe for *sae* was a 402-bp fragment containing ORF4 of *sae*, which yielded two transcripts (T3 and T4) driven only by the *sae* P3 promoter. RNA was harvested from cells grown to an OD₆₅₀ of 1.7. The RNA gels below show equivalent sample loadings.

DISCUSSION

The *rot* locus was first identified by the restoration of *hla* expression in a transposon mutant with an *agr* null mutation (17). Remarkably, a transposon-mediated *rot* mutation by itself was reported to have no effect on alpha-hemolysin expression in strain RN6390, which is partially SigB deficient by virtue of an *rsbU* nonsense mutation (17). A subsequent microarray study of *rot* transcription confirmed that *rot* is a negative regulator of toxins, but only in an *agr*-negative background (25). In this report, we showed that the *rot* mutant was able to express *hla* at a higher level than the parental strain, COL, and that an *agr* mutation was not necessary to elicit the increased *hla* response, contrary to the data from McNamara et al. (17). In support of our Northern blot data, we discerned corroborative data on *hla* expression with GFP transcriptional fusion and also hemolytic titer assays, indicative of increased alpha-toxin activity in *rot* mutants (Fig. 1 and 2). This discrepancy in *hla* expression in *rot* mutants between our data and those of previous studies may be due to differences in the deletion or in the genetic background since our mutants have an in-frame *rot* deletion in a strain with an intact *sigB* operon, whereas previous studies with *rot* were performed with a *sigB*-deficient strain with a Tn917 insertion in which activation of a cryptic transposon promoter or polar effect on downstream genes may occur.

As the effect of *rot* on *hla* expression is likely indirect (25), we hence ascertained if *sae*, one of the major regulators of *hla* expression in vitro and in vivo (8, 20, 24), contributes to this regulatory effect. Our results showed that the repression of *hla* by *rot* was *sae* dependent since the *rot sae* double mutant expressed alpha-hemolysin at a very low level, similar to that of the *sae* single mutant. This conjecture was supported by the finding that *rot* repressed *sae* transcription (Fig. 5).

The *sae* transcription pattern is complex, with four overlapping transcripts (3.1, 2.4, 2.0, and 0.7 kb) arising from three promoters (20, 28). The *sae* P3 promoter drives two transcripts: the 3.1-kb transcript containing *saeRS* and two additional ORFs and the monocistronic 0.7-kb transcript containing ORF4 only (Fig. 3A). Among the three promoters within the *sae* locus, transcriptional fusion data disclosed that the *sae* P3 promoter was the strongest in the parental strain, COL, followed by the *sae* P2 promoter (Fig. 3B). Similar trends were also discerned in other laboratory strains, including SH1000, Newman, and methicillin-resistant *S. aureus* clinical strain MW2 (data not shown). Importantly, the two *sae* transcripts of 3.1 and 0.7 kb, originating from the P3 promoter and as detected by a probe comprising ORF4, were augmented in the *rot* mutant, compared with their levels in the parent, but not in the *sae* mutant (Fig. 5 and 6). Notably, levels of *sae* P2 expression as detected by a *saeRS* probe on Northern blots did not differ significantly between the *rot* mutant and the parent (data not shown). These results demonstrated that the *rot* gene product specifically represses the *sae* P3 promoter, the strongest promoter in the *sae* locus.

The *rot* gene was recently found to encode a 113-residue protein (16) rather than a 166-residue protein as initially thought (17). Previous Northern blots with *rot* have shown one and possibly two transcripts (25) that were not well delineated. However, recent data by Manna and Ray indicated that the *rot*

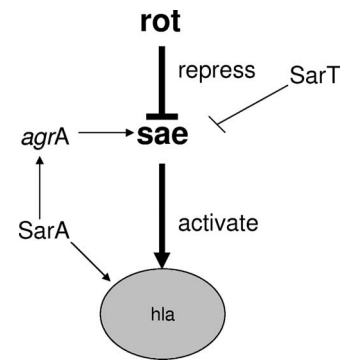


FIG. 6. Proposed regulation scheme of *hla* by *sae*, *rot*, and *sarT*.

gene is driven by three and possibly four different promoters, as deduced by primer extension analysis (16). Our Northern blot analysis with a 338-bp probe containing most of the *rot* ORF clearly revealed three distinct transcripts, with one at 1.8 kb and two at 0.7 and 0.8 kb (data not shown). An examination of the *S. aureus* COL genome reveals that there are two ORFs upstream of the *rot* gene that are transcribed in the same orientation, with one coding for a pseudogene that bears resemblance to a truncated transposase in the 8325-4 genome and the other coding for a 828-bp putative phospholipase gene in the 8325-4 and COL genomes. It is conceivable that the 1.8-kb transcript may comprise both ORFs upstream and *rot* downstream. Thus, these results revealed that *rot* may be transcribed in a more complex pattern than has been revealed until now. The finding that *rot* is flanked by a truncated transposase suggests that *rot* may be horizontally transferred to *S. aureus* from a related species.

Another gene, *sarT*, which is homologous to *sarA*, was initially identified by genomic scanning and was subsequently shown to be a repressor of *hla* (27). To define the contribution of *sarT* to *hla* expression with regard to *sae* and *rot*, we constructed *sarT sae rot* triple-deletion mutants of strain COL. This triple mutant did not rescue the low level of *hla* expression seen in the *sae rot* double mutants (Fig. 1). This finding and the fact that we did not find strong experimental evidence that *sarT* regulates *rot* or vice versa (data not shown) suggest that *sarT* may impact *hla* expression in a *sae*-dependent but *rot*-independent manner (Fig. 1).

It has been suggested that the *sae* locus is autoregulatory (20). In our promoter activation assay, expression from the P3 promoter, the strongest *sae* promoter, was almost completely silent in all *saeRS* deletion mutants of COL (Fig. 4) and SH1000 (data not shown), similar to what occurred in the negative-control vector, thus implying autoregulation of *saeRS* on the *sae* P3 promoter. We also checked the native *sae* P3-P1-P2 promoters in isogenic *saeRS* strains in COL and RN6390 and also found them to be silent in the *sae* mutants (data not shown). Accordingly, we confirmed that *saeRS* likely has a positive feedback on its own promoter.

In this study, we showed that the *sae* locus is autoregulatory by having an impact upon its own promoter. We found that a mutation in *rot* on its own can lead to the up-regulation of *hla* in the absence of any *agr* mutation, which is contrary to previous data. A similar *rot* mutation in strain Newman also led to

elevated *hla* expression compared with that in the parental strain. Accordingly, it will not be feasible to explain our finding based only on differences in RNAPIII expression levels between strains (unpublished data). Based on our study, it is highly likely that *sae* is an important checkpoint for *hla* expression. It has been established previously that both *rot* and *sarT* are repressors of *hla* (17, 27). We have now provided evidence that the repression of *hla* by *rot* and, possibly, by *sarT* is *sae* dependent. More specifically, Rot represses the *sae* P3 promoter, the strongest promoter within the *sae* locus, to control *hla* expression. As the *sae* P3 transcript also encodes ORF4 (Fig. 3), it will be of interest to evaluate the role of ORF4 in regulating SaeRS expression. Additionally, investigations on whether the effect of Rot on *sae* is direct or indirect will be of interest.

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