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The SaeRS Two-Component System Is a Direct and Dominant Transcriptional Activator of Toxic Shock Syndrome Toxin 1 in *Staphylococcus aureus*

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ABSTRACT

Toxic shock syndrome toxin 1 (TSST-1) is a *Staphylococcus aureus* superantigen that has been implicated in both menstrual and nonmenstrual toxic shock syndrome (TSS). Despite the important role of TSST-1 in severe human disease, a comprehensive understanding of staphylococcal regulatory factors that control TSST-1 expression remains incomplete. The *S. aureus* exotoxin expression (Sae) operon contains a well-characterized two-component system that regulates a number of important exotoxins in *S. aureus*, although regulation of TSST-1 by the Sae system has not been investigated. We generated a defined deletion mutant of the Sae histidine kinase sensor (*saeS*) in the prototypic menstrual TSS strain *S. aureus* MN8. Mutation of *saeS* resulted in a complete loss of TSST-1 expression. Using both luciferase reporter experiments and quantitative real-time PCR, we demonstrate that the Sae system is an important transcriptional activator of TSST-1 expression. Recombinant SaeR was able to bind directly to the *tst* promoter to a region containing two SaeR consensus binding sites. Although the stand-alone SarA transcriptional regulator has been shown to be both a positive and a negative regulator of TSST-1, deletion of *sarA* in *S. aureus* MN8 resulted in a dramatic overexpression of TSST-1. As expected, mutation of *agr* also reduced TSST-1 expression, but this phenotype appeared to be independent of Sae. A double mutation of *saeS* and *sarA* resulted in the loss of TSST-1 expression. This work indicates that the Sae system is a dominant and direct transcriptional activator that is required for expression of TSST-1.

IMPORTANCE

The TSST-1 superantigen is an exotoxin, produced by some strains of *S. aureus*, that has a clear role in both menstrual and nonmenstrual TSS. Although the well-characterized *agr* quorum sensing system is a known positive regulator of TSST-1, the molecular mechanisms that directly control TSST-1 expression are only partially understood. Our studies demonstrate that the Sae two-component regulatory system is a positive transcriptional regulator that binds directly to the TSST-1 promoter, and furthermore, our data suggest that Sae is required for expression of TSST-1. This work highlights how major regulatory circuits can converge to fine-tune exotoxin expression and suggests that the Sae regulatory system may be an important target for antivirulence strategies.

Staphylococcus aureus is both a common commensal of humans and a prominent bacterial pathogen that is responsible for an assortment of illnesses ranging from self-limiting superficial infections to life-threatening invasive diseases (1). While most infections caused by *S. aureus* involve the coordinated expression of numerous virulence factors, a few select diseases, including staphylococcal scalded skin syndrome, food poisoning, and toxic shock syndrome (TSS), require the expression of specific staphylococcal exotoxins (2).

Staphylococcal TSS is a rare but devastating disease that is caused by *S. aureus* strains that secrete high levels of superantigens. Superantigen exotoxins function by binding directly to lateral surfaces of both T-cell receptor β-chains and major histocompatibility complex (MHC) class II molecules, which forces the massive activation of numerous T cells and can induce a T-cell-dependent cytokine storm (3). TSS symptoms are characterized by the acute onset of fever, rash formation, hypotension, multisystem involvement, and desquamation in patients that recover (4), although strict use of this definition likely underestimates the actual number of TSS cases (5). There are two major forms of TSS, including menstrual TSS (mTSS) and nonmenstrual TSS (3). Although the latter can occur from virtually any *S. aureus* infection, mTSS typically occurs within ~2 days prior to or ~2 days following menstruation and is often associated with the use of tampons (6, 7). The staphylococcal superantigen toxic shock syndrome toxin 1 (TSST-1) is believed to be responsible for nearly all cases of menstrual TSS (3, 8), as well as many cases of nonmenstrual TSS (5, 9).

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TABLE 1 Bacterial strains used in this study

Strain	Description	Source or reference
E. coli		
XL-1 Blue	Cloning strain	Stratagene
SA30B	DNA methylation strain	20
BL21(DE3)	Protein expression strain	New England Biolabs
S. aureus		
RN4220	DNA methylation strain	19
MN8	Prototypic menstrual TSS strain, tst^+	17
MN8 Δ*agr*	*S. aureus* MN8 with *agr* operon replaced with 3-kb *tetR* marker	18
MN8 Δ*saeS*	*S. aureus* MN8 containing a deletion within *saeS*	This study
MN8 Δ*saeS* (+*sae*)	*S. aureus* MN8 Δ*saeS* containing pALC2073::*sae*	This study
MN8 Δ*sarA*	*S. aureus* MN8 containing a deletion within *sarA*	This study
MN8 Δ*sarA* (+*sarA*)	*S. aureus* MN8 Δ*sarA* containing pALC2073::*sarA*	This study
MN8 Δ*saeS* Δ*sarA*	*S. aureus* MN8 containing deletions in both *saeS* and *sarA*	This study

Despite the well-recognized role of TSST-1 in both forms of TSS, a comprehensive understanding of the genetic elements required for the regulation and expression of TSST-1 remains incomplete. Although the prototypical positive regulator of TSST-1 is the well-characterized accessory gene regulator (*agr*) quorum sensing system (10), a number of additional regulatory factors also impact TSST-1 expression. For example, mutation of the staphylococcal respiratory response (*srrAB*) two-component system resulted in a decrease in TSST-1 production, particularly under conditions of low oxygen (11). However, overexpression of *srrAB* in *trans* massively upregulated RNAIII, the major effector molecule of the *agr* system, with a near complete repression of TSST-1, demonstrating that regulation of TSST-1 by *agr* can be uncoupled (11). This is also consistent with a study that could not correlate *agr* polymorphisms, including *agrC*-inactivating mutations, with varied production of TSST-1 (12). Also, the staphylococcal accessory regulator (SarA) has previously been demonstrated to bind directly to the *tst* promoter region (13), and depending upon the genetic background, has been reported to function as either a positive or negative regulator of TSST-1 expression (13–15). Furthermore, carbon catabolite protein A (CcpA) acts as a repressor of TSST-1 (16), which explains the initial observation that TSST-1 expression is repressed by glucose (17). Although mutation of the repressor of toxins (*rot*) transcriptional regulator was reported to have little effect on TSST-1 expression, overexpression of Rot in *trans* strongly repressed TSST-1 (15). In addition to these experiments, prior work from our laboratory has demonstrated that interspecies communication between *Lactobacillus reuteri* and *S. aureus* can result in a dramatic repression of TSST-1 expression (18). Although this phenotype correlated with repression of *agr*, and deletion of *agr* in *S. aureus* MN8 reduced TSST-1 expression, TSST-1 could be further repressed in the *agr*-null background by the *L. reuteri* signaling molecules. This additional repression of TSST-1 correlated with decreased transcription of the *S. aureus* exotoxin expression (Sae) system and increased transcription of stand-alone transcriptional regulator SarA (18). The aim of the present study was to investigate the role of both Sae and SarA in production of TSST-1 in *S. aureus* MN8, a prototypic menstrual TSS *S. aureus* strain.

MATERIALS AND METHODS

Bacterial strains and growth conditions. The bacterial strains used in this study are listed in Table 1. All cloning was carried out utilizing *Escherichia coli* XL-1 Blue (Stratagene) grown on Luria-Bertani (LB) broth (Difco) or brain heart infusion (BHI) (Becton Dickinson) plates supplemented with 1.5% agar. For *E. coli*, antibiotics (Sigma-Aldrich) were added to growth media at the following concentrations: 200 μg/ml ampicillin (Amp), 150 μg/ml erythromycin (Erm), and 10 μg/ml chloramphenicol (Cm). *S. aureus* MN8 was isolated prior to 1980 and is a prototypic mTSS strain (17). *S. aureus* strains were routinely grown in tryptic soy broth (TSB) (Difco) or BHI broth or on solid agar with antibiotics as appropriate. For *S. aureus*, antibiotics were added to growth media at the following concentrations: 5 μg/ml Erm and 10 μg/ml Cm.

Molecular cloning. The plasmids and primers used in this work are listed in Tables 2 and 3, respectively. Plasmid DNA was isolated from *E.*

TABLE 2 Plasmids used in this study

Plasmid	Description[a]	Source or reference
pMAD	Temp-sensitive integration vector with blue-white selection; Em^r	21
pMAD::Δ*saeS*	*saeS* deletion plasmid; Em^r	This study
pKOR1	Temp-sensitive integration vector with inducible counterselection; Em^r	22
pKOR1::Δ*sarA*	*sarA* deletion plasmid; Em^r	This study
pALC2073	*S. aureus* complementation plasmid; Cm^r	23
pALC2073::*sae*	Complementation plasmid containing *saeQRS* operon; Cm^r	This study
pALC2073::*sarA*	Complementation plasmid containing *sarA*; Cm^r	This study
pAmilux	Luminescence reporter plasmid; Cm^r	24
pAmilux::P_{tst}	TSST-1 promoter reporter plasmid; Cm^r	18
pET28::*saeR*	Protein expression clone containing full-length SaeR; Kan^r	25
pMCSG19::*saeS*[c]	Protein expression clone encoding residues 93–351 of SaeS; Amp^r	25

[a] Em^r, erythromycin resistant; Cm^r, chloramphenicol resistant; Kan^r, kanamycin resistant.

TABLE 3 Primers used in this study

Primer	Sequence (5′→3′)[a]
Primers for *saeS* deletion and complementation plasmid construction	
SaeS up BamHI (forward)	GGGGGATCCAACAACGACAACTAGCGGTAAAGA
SaeS up SalI (reverse)	AGTGTCGACACACCATTATCGGCTCCTTTCA
SaeS down EcoRI (forward)	CCCGAATTCTAGCCCATGATTTAAAAACACCTT
SaeS down BglII (reverse)	CCCAGATCTTTCCTACATCTATATCACTGCTTACACTG
SaeRS Comp II KpnI For	TTTTTGGTACCGGTAAATTAATGCTTACTAACTACAA
SaeRS Comp II EcoRI Rev	CCCCCGAATTCTTATGTCGTAATGTCTAATTTGTG
Primers for *sarA* deletion and complementation plasmid construction	
SarA upstream For attB1	GGGG**ACAAGTTTGTACAAAAAAGCAGGCT**AGGTGCAGCATTAACAACACT
SarA upstream Rev[b]	AATTGCCATGGTTAAAACCTC
SarA downstream For[b]	GAACTATAATTTTGTTTAGCG
SarA downstream rev primer attB2	GGGG**ACCACTTTGTACAAGAAAGCTGGGT**TGAGGGAGGTGTCACAATGA
SarA complement KpnI For	TTTTTGGTACCTTTAACATTTAGCTTATCATTTTAA
SarA complement SacI Rev	CCCCCGAGCTCTTATAGTTCAATTTCGTTGTTTGCT
Primers for sequencing	
SaeRS screen For	CTGGGGGATATGTTTTACC
SaeRS screen Rev	GTCCCTATGCGTATTAAGGA
pMAD seq FP	GGGGAAGGCCATCCAGCCTCGCGTC
pMAD seq RP	AATCTAGCTAATGTTACGTTACACA
SarA flank For	TCTTATCATTAAAACTGCACTGGGA
SarA flank Rev	GCGGTGGCAATTCGTTCATT
pKOR1For	CAGATCCATATCCTTCTTTTCTGA
pKOR1 Rev	GTGTGGAATTGTGAGCGGATA
pALC2073 Seq For	GGTTGCATGCCTGCAGGTCGACGG
pALC2073 Seq Rev	CAGTCACGACGTTGTAAAACG
Primers for qRT-PCR analysis	
tst-For	CTGATGCTGCCATCTGTGTT
tst-Rev	GTAAGCCCTTTGTTGCTTGC
agrA-For (P2)	GTGAAATTCGTAAGCATGACCCAGTTG
agrA-Rev (P2)	TGTAAGCGTGTATGTGCAGTTTCTAAAC
rnaIII-For (P3)	TAGATCACAGAGATGTGA
rnaIII-Rev (P3)	CTGAGTCCAAGGAAACTAACTC
saeR-For	CCAAGGGAACTCGTTTTACG
saeR-Rev	ACGCATAGGGACTTCATGAC
sarA-For	TGTTTGCTTCAGTGATTCGTTT
sarA-Rev	CATCAGCGAAAACAAAGAGAAA
rpoB-For	TCCTGTTGAACGCGCATGTAA
rpoB-Rev	GCTGGTATGGCTCGTGATGGTA
Primers for EMSA experiments	
SaeP1-For-biotin	ATTAGTTAAGCGATATTTAAACGAAGTTAAGAATTAGTTAATGGCA
SaeP1-Rev	TGCCATTAACTAATTCTTAACTTCGTTTAAATATCGCTTAACTAAT
p*tst*-For-biotin	AAAGTGACTTTAAAGAATATAACTA
p*tst*-Rev	TTTTAATTCTCCTTCATTCAAATGT
Probe1-For-IRDye800	GTAACAAACACTTTTTAATTAATATATATTTAAACAATAATTTAGA
Probe1-Rev	TCTAAATTATTGTTTAAATATATATTAATTAAAAAGTGTTTGTTAC
Probe 2-For-IRDye800	TTTTTAATTAATATATATTTAAACAATAATTTAGAGATGGTTAATTGATT
Probe2-Rev	AATCAATTAACCATCTCTAAATTATTGTTTAAATATATATTAATTAAAAA
Probe3-For-IRDye800	AATTTAGAGATGGTTAATTGATTCATTTAAATAATATTTATACATTCT
Probe3-Rev	AGAATGTATAAATATTATTTAAATGAATCAATTAACCATCTCTAAATT

[a] Restriction sites (indicated in the primer name) are underlined in the primer sequence, and *attB* sites are shown in boldface.
[b] Primer contained a phosphate group for blunt-end ligation.

coli using the Qiagen Miniprep kit. Routine PCR amplifications were carried out using an MJ Research PTC-200 instrument with primers obtained from Sigma. Digestions were carried out utilizing restriction enzymes provided by New England BioLabs or Roche. Ligations were performed utilizing T4 DNA ligase (New England BioLabs). *E. coli* cells were made competent using the $RbCl_2$ method (3). Transformation of plasmids into *S. aureus* was carried out as described previously (18), and *S. aureus* RN4220 (19) and *E. coli* SA30B (20) were used as

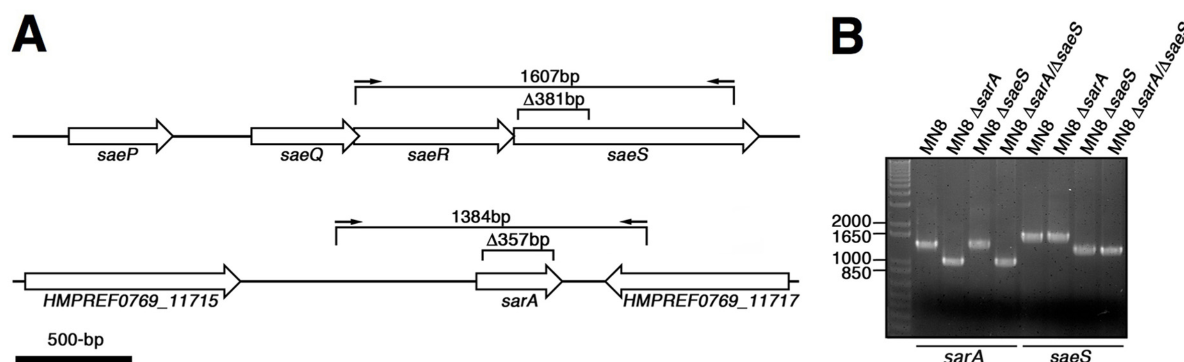

FIG 1 Schematic and PCR analysis of the *saeS* and *sarA* in-frame deletions in *S. aureus* MN8. (A) Scale schematic of the 381-bp deletion in *saeS* (top) and the 358-bp deletion in *sarA* (bottom) and location of the PCR products used for analysis and sequencing of the corresponding deletions. (B) DNA agarose gel analysis using PCR products indicated in panel A for the individual and double MN8 *sarA* and *saeS* deletions as indicated.

cloning intermediates to methylate DNA prior to electroporation in *S. aureus* MN8.

To generate a markerless deletion in the *saeS* gene, 610 bp of DNA (including the first 2 codons of *saeS*) was PCR amplified and cloned into the BamHI and SalI sites of the temperature-sensitive integration plasmid pMAD (21). Next, 616 bp of DNA downstream of and within *saeS* was PCR amplified and cloned into the EcoRI and BglII sites of this plasmid to create pMAD::Δ*saeS* (Table 2). The *saeS* deletion was constructed in the *S. aureus* MN8 chromosome as described previously (21) to create a 381-bp deletion within the 5′ region of the *saeS* gene, which was replaced by a 31-bp "scar" (Fig. 1). The correct deletion was confirmed by PCR and DNA sequencing analysis.

To generate a markerless deletion in the *sarA* gene, 1,122 bp of DNA (including the first 3 codons of *sarA*) and 1,063 bp of DNA downstream of *sarA* (including the last 3 codons) was PCR amplified from the chromosome of *S. aureus* MN8. These products were ligated together and cloned into pKOR1 using the Gateway BP Clonase II system (Life Technologies). The *sarA* deletion was constructed in the *S. aureus* MN8 chromosome as described previously (22) to create a 358-bp, in-frame deletion within the *sarA* coding region (Fig. 1). The correct deletion was confirmed by PCR and DNA sequencing analysis.

To generate a complementation plasmid for the *saeS* mutation, a 2,755-bp fragment containing full-length *saeQ*, *saeR*, and *saeS* genes was PCR amplified and directionally cloned using KpnI and EcoRI into pALC2073. To generate a complementation plasmid for the *sarA* mutation, a 1,321-bp fragment contain full-length *sarA* and upstream promoter region was PCR amplified and directionally cloned using KpnI and SacI into pALC2073. As pALC2073 contains a tetracycline-inducible promoter, the complementation genes were overexpressed in some experiments by the addition of increasing concentrations of anhydrotetracycline (Sigma-Aldrich), as previously described (23).

Analysis of exoprotein profiles and TSST-1 expression. *S. aureus* MN8 and the various mutants were subcultured to a starting optical density at 600 nm (OD_{600}) of 0.05 in BHI medium and grown for indicated time points at 37°C in a shaking incubator. Cells were centrifuged, and cell-free supernatants equivalent to 42 OD_{600} units ($\sim 4 \times 10^{10}$ CFU) of cultures were collected. Proteins were precipitated 30 min on ice using trichloroacetic acid (TCA [Fisher Scientific]) at a final concentration of 6%, washed with ice-cold acetone, and resuspended in 250 μl 8 M urea. Proteins were analyzed by 12% SDS-PAGE, and specific proteins of interest were "picked" from Coomassie brilliant blue R-250 (Bio-Rad)-stained gels using an Ettan Spot Picker (GE Healthcare), in-gel digested using a Waters MASSPrep automated digestor (PerkinElmer), and identified by matrix-assisted laser desorption ionization–time of flight (MALDI-TOF) mass spectrometry at the University of Western Ontario MALDI Mass Spectrometry Facility.

For Western blot analysis of TSST-1 expression, protein samples were transferred to polyvinylidene difluoride (PVDF) membranes (Millipore) at 100 V for 1 h. The membrane was blocked at room temperature for 2 h with phosphate-buffered saline (PBS) containing 5% skimmed milk, 10% normal horse serum (NHS [Life Technologies]), and 10% fetal calf serum (FCS) (Wisent, Inc.). After removal of the blocking solution, the membrane was incubated at 4°C overnight with rabbit polyclonal anti-TSST-1 antisera (kindly provided by Patrick Schlievert, University of Iowa, IA) diluted 1:1,500 in PBS containing 2.5% skimmed milk, 5% NHS, and 5% FCS. Membranes were washed 3 times with PBS containing 0.1% Tween 20 (PBST) (Fisher Scientific) followed by incubation with IRDye 800-conjugated donkey anti-rabbit IgG antibody (Rockland) diluted 1:10,000 in PBS containing 2.5% skimmed milk, 5% NHS, and 5% FCS at room temperature for 1 h in the dark. After the membrane had been washed 3 times with PBST, the membrane was imaged using an Odyssey imager (LI-COR Biosciences, Lincoln, NE).

TSST-1 ELISA. A sandwich enzyme-linked immunosorbent assay (ELISA) procedure was used for quantification of TSST-1 in *S. aureus* culture supernatants. Affinity-purified rabbit IgG anti-TSST-1 antibody (Abcam) diluted to 10 μg/ml in 0.01 M carbonate buffer at pH 9.6 (coating buffer) was adsorbed to high-binding polystyrene 96-well plates (Corning) at 37°C for 18 h. Unbound anti-TSST-1 was removed by being washed 4 times with PBST. A 1% bovine serum albumin (Sigma) blocking solution prepared in coating buffer was added to the wells at 37°C for 1 h, and wells were washed with PBST. Filtered supernatant samples were diluted in 1% normal rabbit serum (Thermo Fisher) prepared in PBST. Each diluted supernatant was incubated at room temperature for 30 min to eliminate possible interference of protein A. Recombinant TSST-1 reference standards (serially diluted in PBST from 10 ng/ml to 0.039 ng/ml) and supernatants were added to the wells, and the mixture was incubated at 37°C for 2 h. The plate wells were washed, and incubated with horseradish peroxidase-conjugated affinity purified rabbit IgG anti-TSST-1 antibody (Abcam) diluted 1:300 in PBS at room temperature for 1 h. After the wells were washed, 3,3′,5,5′-tetramethylbenzidine (TMB) substrate solution (BD) was added, and plates were incubated at 37°C for 20 min. A 2 N H_2SO_4 solution was added to stop the enzymatic reaction. Optical density was read at 450 nm in a microplate reader (Biotek Synergy H4). A standard curve was generated using the data of the reference standards. The model R^2 of linear regression analysis was ≥98%. Total amounts of TSST-1 are reported as nanograms per milliliter.

Luciferase reporter assays. The *tst* gene promoter (P_{tst}) was previously cloned into the luciferase reporter plasmid pAmilux (18, 24). The pAmilux::P_{tst} plasmid was transformed into the different MN8 clones. Cells were grown in BHI medium and subcultured to a starting OD_{600} of 0.01 (200 μl/well) in half-BHI medium (1:2 dilution of BHI medium in Milli-Q water). Each strain was tested for expression of luminescence over time by growth in a Biotek Synergy H4 multimode plate reader at 37°C with shaking in a flat, clear-bottom 96-well Microfluor 2 White plate

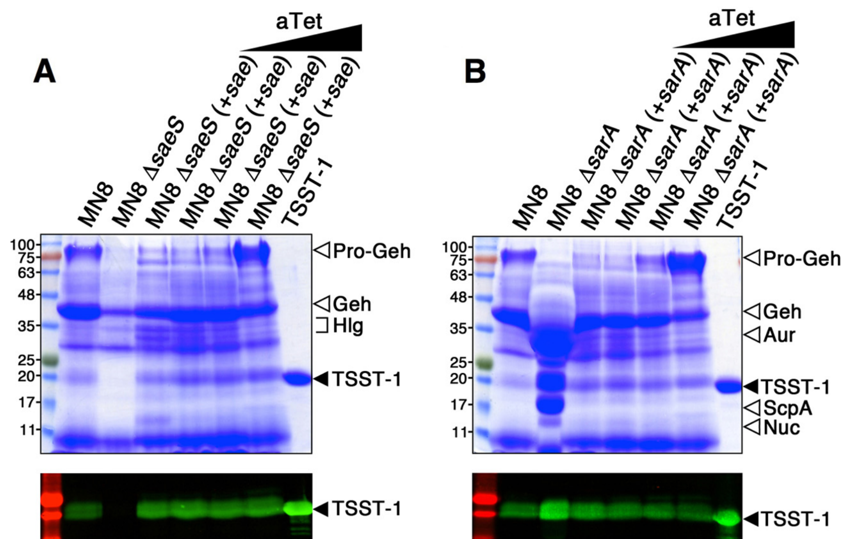

FIG 2 Sae is a positive regulator and SarA is a negative regulator of TSST-1 expression in *S. aureus* MN8. Shown are exoprotein profiles (top panels) and Western blot analysis (bottom panels) of TSST-1 for wild-type *S. aureus* MN8 and the corresponding (A) *saeS* and (B) *sarA* deletion mutant and complemented strains. Concentrated supernatants from the indicated strains grown in BHI medium for 18 h were loaded onto 12% SDS-PAGE gels. Increasing concentrations (0, 5, 50, and 500 ng/ml) of anhydrotetracycline (aTet) were used to induce the promoter in the complemented strains. Molecular mass markers were loaded on the left and labeled in kilodaltons, and purified recombinant TSST-1 was loaded on the right and is indicated by the solid arrowheads.

(Thermo) sealed using Thermo acetate plate sealers. OD_{600} and luminescence readings were taken every hour with individual clones grown separately in quadruplicate.

qRT-PCR. For quantitative real-time PCR (qRT-PCR), *S. aureus* strains were grown in 5 ml of TSB medium for 18 h. Cells were then subcultured at an OD_{600} of 0.05 in 3 ml of medium and grown for 4 and 8 h. RNA extraction was carried out using the RNeasy minikit (Qiagen) with the addition of 250 μg/ml lysostaphin to the lysis solution. First-strand cDNA was synthesized using Super-Script II reverse transcriptase and random primers (Invitrogen) from 500 ng of total RNA. PCR mixtures with the primers listed in Table 3 were prepared using iQ SYBR green supermix (Bio-Rad) and performed with the Rotor-Gene real-time analyzer (Corbett Life Science). All RNA samples were prepared using four biological replicates and analyzed against the expression of the housekeeping *rpoB* gene (18).

Recombinant protein expression and purification. The protein expression plasmids for full-length SaeR and the C-terminal effector domain of SaeS ($SaeS^c$) (25) were kindly provided by Taeok Bae (Indiana University School of Medicine). Protein purification was carried out as previously described, with some modifications (25). Briefly, cells were grown at 37°C in LB medium to an OD_{600} of ~0.6, and 1 mM isopropyl-β-D-thiogalactopyranoside (IPTG) was added. The culture for SaeR expression was further incubated at room temperature overnight, while the culture for $SaeS^C$ expression was induced at 16°C with shaking. Cells were resuspended in 10 mM Tris-HCl–500 mM NaCl (pH 7.5) and lysed at 25,000 lb/in^2 using a cell disruptor (Constants Systems, Ltd.). The His_6-tagged proteins were purified using nickel column chromatography using a previously described protocol (26). The purified SaeR and $SaeS^c$ were dialyzed in buffers consisting of 10 mM Tris-HCl, 138 mM NaCl, and 2.7 mM KCl (pH 7.5) and 50 mM Tris-HCl, 50 mM KCl, and 1 mM $MgCl_2$ (pH 8.0), respectively (27), and concentrated up to 10-fold using Amicon centrifugal filter devices (Millipore). The protein concentration was determined by the bicinchoninic acid assay (Thermo Scientific), and purified proteins were stored in buffers containing 10% glycerol at −80°C until used.

EMSAs. Electrophoretic mobility shift assays (EMSAs) were performed using double-stranded fluorescently labeled DNA probes. The DNA probes for the P_{saeP1} and P_{tst} promoter sequences were PCR amplified with 5′-biotin end-labeled primers (Sigma-Aldrich), and IRDye 800-conjugated streptavidin (Rockland) was used (1:2500 dilution) to detect DNA binding. Custom 5′ IRDye 800 end-labeled oligonucleotides (for probes 1, 2, and 3) were purchased from Integrated DNA Technologies (IDT) (Table 3). Equal amounts of forward and reverse DNA oligonucleotides (20 μM) were heated to 100°C for 3 min and allowed to anneal. Phosphorylation of SaeR and the EMSA were done as previously described (25, 28), with some modifications. Proteins and DNA probes were incubated at room temperature in a 25-μl volume containing phosphorylation buffer (10 mM Tris-HCl [pH 7.4], 50 mM KCl, 5 mM $MgCl_2$, 10% glycerol), 240 μg/ml of bovine serum albumin, and 12 μg/ml poly (dI-dC). First, $SaeS^c$ was preincubated with 1.6 mM ATP (Sigma-Aldrich) for 10 min. Increasing amounts of SaeR were added to the mixture, and the mixture was further incubated for 10 min. DNA probes (40 nM) were mixed with various amounts of test proteins and incubated for 20 min. Samples were separated in 8% nondenaturing polyacrylamide gels in TBE buffer (10 mM Tris, 89 mM Tris-borate, 2 mM EDTA [pH 8.3]) at 85 V for 85 min. Gels were visualized using an Odyssey imager (LI-COR Biosciences).

RESULTS

The Sae two-component system is a positive regulator of TSST-1 expression. To investigate a role for the Sae regulatory system on TSST-1 expression, we generated a markerless deletion within the histidine kinase-encoding *saeS* gene in the prototypical menstrual TSS strain *S. aureus* MN8 (Fig. 1). Analysis of the exoprotein profile of *S. aureus* MN8 Δ*saeS* resulted in a dramatic reduction of multiple proteins, including the effectual elimination of TSST-1, as determined by mass spectrometry and Western blot analysis (Fig. 2A). Also as identified by mass spectrometry, we saw a large reduction in both the pro- and mature forms of the glycerol ester hydrolase (pro-Geh and Geh, respectively) and a minor reduction in components of the gamma hemolysin (Hlg) (Fig. 2A). Using the inducible complementation plasmid pALC2073, in *trans* complementation of the *sae* operon (including *saeQRS*) restored production of TSST-1, although induction with 500 ng/ml an-

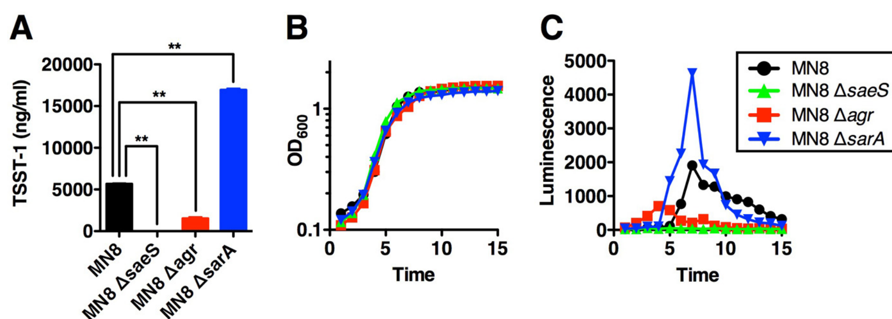

FIG 3 TSST-1 quantitation, growth curve analysis, and TSST-1 promoter activity of wild-type *S. aureus* MN8 and the corresponding *saeS*, *sarA*, and *agr* mutants. (A) *S. aureus* MN8 strains were grown for 18 h in BHI medium, and TSST-1 secretion was quantified by ELISA. The data represent the means ± standard errors of the means (SEM) from three biological replicates (**, $P < 0.05$ by one-way ANOVA with Tukey's multiple comparison test). (B and C) The indicated *S. aureus* strains were grown at 37°C in a Biotek Synergy H4 multimode plate reader, and OD_{600} and luminescence readings were taken every hour for 15 h. Results are expressed as OD_{600} units (B) and luminescence in relative light units (defined as counts per minute) (C). The data represent the means from three independent experiments, each done with quadruplicate technical replicates.

hydrotetracycline was necessary to fully restore pro-Geh expression. These experiments indicate that the Sae two-component system is a positive regulator of TSST-1 expression in *S. aureus* MN8.

The SarA stand-alone transcription regulator is a repressor of TSST-1 expression in *S. aureus* MN8. Previous work has shown that depending upon the genetic background, SarA may function as either a positive (13) or negative (15) regulator of TSST-1 expression. To investigate a role for TSST-1 regulation by SarA in *S. aureus* MN8, we generated a markerless deletion within the *sarA* gene (Fig. 1). Analysis of the exoprotein profile of *S. aureus* MN8 Δ*sarA* resulted in a dramatic increase in TSST-1, the aureolysin protease (Aur), and the staphopain A protease (ScpA), a minor increase in nuclease (Nuc), and a notable reduction in expression of pro-Geh (Fig. 2B). Using the inducible complementation plasmid pALC2073, in *trans* complementation of *sarA* restored production of TSST-1, although similar to the *saeQRS* complementation plasmid, induction with 500 ng/ml anhydrotetracycline was necessary to fully restore pro-Geh to wild-type levels. These experiments indicate that SarA is an important negative regulator of TSST-1 expression in *S. aureus* MN8.

Transcriptional control of TSST-1 by Sae, SarA, and Agr. Given the striking, but qualitative, data in Fig. 2, we quantified TSST-1 production by ELISA after 18 h of growth (Fig. 3A). Compared with wild-type *S. aureus* MN8, TSST-1 production was reduced to barely detectable levels in the MN8 Δ*saeS* mutant. In *S. aureus* MN8 Δ*agr*, TSST-1 protein was significantly reduced but not abolished, whereas MN8 Δ*sarA* produced ~2.5× the amount of TSST-1 relative to wild-type MN8.

To evaluate if the changes in TSST-1 expression were mediated at the transcriptional level, we transformed the luciferase reporter plasmid pAmilux (24) containing the *tst* promoter (P_{tst}) (18) into each of the *S. aureus* strains. Although each strain containing pAmilux::P_{tst} grew equivalently (Fig. 3B), we noted significant differences in luciferase activity for each of the three regulatory mutants (Fig. 3C). Transcription of *tst* in wild-type MN8 peaked at the late exponential phase (~7 h), and activity declined during the stationary phase. Deletion of the *agr* locus resulted in a shift to earlier expression, peaking by 4 h, corresponding to the early exponential phase, and expression was subsequently reduced to background levels when cells entered stationary phase. Consistent with the lack of TSST-1 protein expression (Fig. 2A and 3A), deletion of *saeS* resulted in a complete reduction of *tst* expression over the entire growth curve. However, and also consistent with enhanced TSST-1 protein expression (Fig. 2B and 3A), the *sarA* mutant demonstrated a temporally similar curve to wild-type MN8, although with a marked increase in luminescent activity from the mid- to late-exponential phase.

In order to confirm our findings with the luciferase reporter system, we conducted qRT-PCR experiments at both 4 and 8 h, representing the early and late exponential phases of growth, respectively. In this analysis, we included the *tst* gene, as well as *saeR*, *sarA*, *agrA*, and the RNAIII transcripts. The qRT-PCR experiments with *tst* largely confirmed the results of the luciferase assays (Fig. 4A), although qRT-PCR could detect transcripts prior to the detection of luminescence by ~2 h, and the levels of *tst* transcripts were thus very low at the 8-h time point in wild-type MN8. Both the *agr* and *saeS* mutants demonstrated a clear reduction in *tst* transcript levels for both time points, while the *sarA* mutant demonstrated a clear increase in *tst* transcript levels. Importantly, for each of the regulatory mutants, transcription of the corresponding mutant gene was not detectable above background; however, none of the regulatory mutants had a significant impact on transcription of the other regulatory systems at either time point. Secretion of TSST-1 from the different regulatory mutants at 4 and 8 h (Fig. 4B) was consistent with the transcriptional analysis at these time points. These collective experiments demonstrate that the Sae system is a positive transcriptional regulator, while SarA is a negative transcriptional regulator, of *tst* expression in *S. aureus* MN8. These experiments also further confirm that the Agr system is a positive regulator of *tst* (10, 18).

SaeR binds to the TSST-1 promoter. Although SarA is known to bind to P_{tst} (13), given the complete repression of TSST-1 in the *saeS* deletion background, we hypothesized that SaeR, the response regulatory of the Sae system, would directly regulate TSST-1 transcription. We first assessed the P_{tst} promoter for a potential SaeR binding motif (25, 29). We located two sequences that could theoretically represent SaeR binding motifs, highlighted by the TTAA(N_7)TTAA motif (Fig. 5A). One of the potential SaeR motifs was located immediately upstream of the −35 site (13), which is a common feature of genes directly regulated by SaeR (25, 29). To test binding of SaeR to the P_{tst} promoter, we produced recombinant SaeR, as well as the cytoplasmic C-terminal domain of the Sae histidine kinase $SaeS^c$ (25). $SaeS^c$ was used to

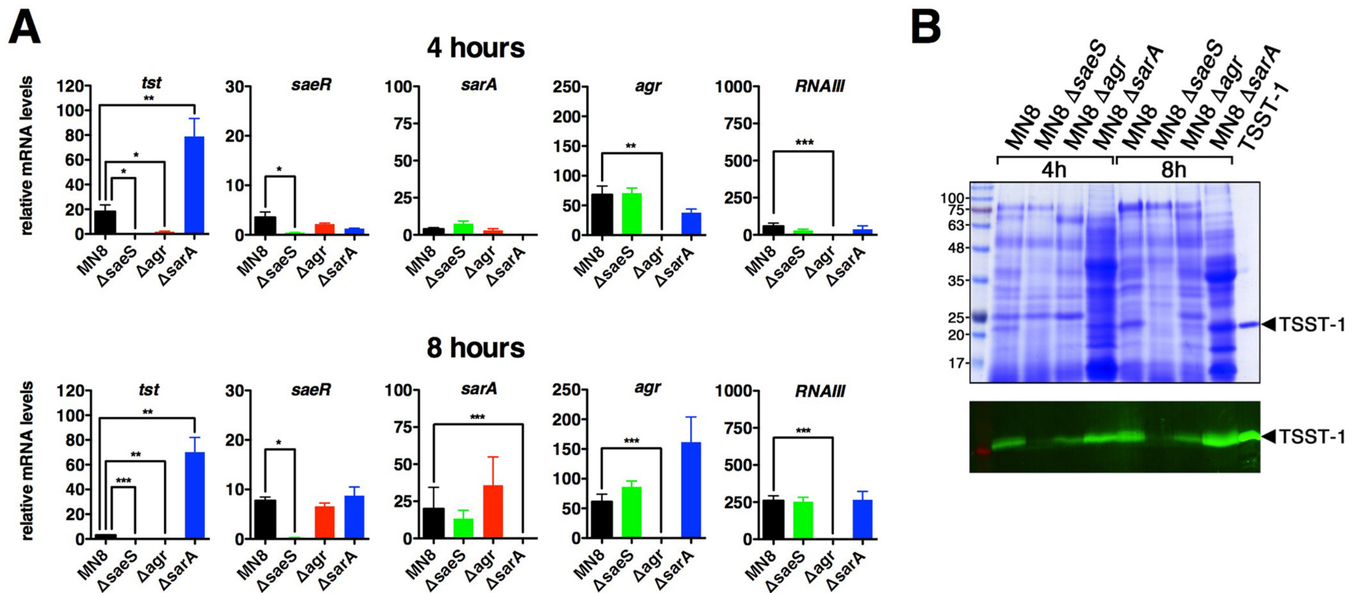

FIG 4 qRT-PCR analysis of *tst* transcripts and relevant regulators in wild-type *S. aureus* MN8 and the *saeS*, *sarA*, and *agr* mutants. (A) cDNA was prepared at the indicated time points, and copies were normalized to the housekeeping *rpoB* gene. Data represent the means ± SEM from four biological replicates (*, $P < 0.05$, **, $P < 0.01$, and ***, $P < 0.001$, by one-way ANOVA with Tukey's multiple comparison test). (B) Exoprotein profiles (top panel) and anti-TSST-1 Western blot (bottom panel) analysis of the indicated *S. aureus* strains at the 4- and 8-h time points. Molecular mass markers were loaded on the left (labeled in kilodaltons), and purified recombinant TSST-1 was loaded on the right (indicated by solid arrowheads).

phosphorylate SaeR *in vitro*, and we first assessed the ability of phosphorylated SaeR to bind to a known target promoter, namely, the P1 promoter of the Sae operon (P_{saeP1}) (25). Phosphorylated SaeR (SaeR~P) bound to P_{saeP1} in a concentration-dependent manner (Fig. 5B). Removal of ATP from the *in vitro* phosphorylation reaction to preclude phosphorylation of SaeR prevented binding to P_{saeP1} (Fig. 5B, lane 6). Next, we tested binding of SaeR~P to an intergenic 271-bp fragment located immediately upstream of *tst* (Fig. 5C). This experiment demonstrated binding of SaeR~P to the P_{tst} promoter region. Again, no binding was detected when ATP was omitted from the phosphorylation reaction. Competition experiments using excess, unlabeled P_{tst} inhibited binding to labeled P_{tst} in a concentration-dependent manner (Fig. 5C, right panel). Next, we generated three sets of smaller probes to contain each potential SaeR binding motif (probes 1 and 3) or one probe to contain both potential SaeR binding motifs (probe 2) (Fig. 5A). A very weak shift was detected with probe 1 (Fig. 5D), and probe 2 demonstrated two apparent shifts (Fig. 5E), while probe 3 demonstrated a single, weak shift similar to probe 1 (Fig. 5F). In each case, binding was dependent upon the phosphorylation status of SaeR. These collective experiments demonstrate that SaeR~P binds directly to P_{tst} and that tandem sequences consistent with the SaeR consensus binding site appear to be important for optimum binding.

Sae is a dominant regulatory system that is essential for TSST-1 expression. Given the data demonstrating that mutation of *sarA* dramatically increased TSST-1 expression (Fig. 2A, 3A and C, and 4), while mutation of *saeS* dramatically reduced TSST-1 expression (Fig. 2B, 3A and C, and 4), to investigate which regulatory system played a more dominant role, we generated a double mutation of both regulatory systems in *S. aureus* MN8 (Fig. 1). Despite the lack of the SarA repressor, the *S. aureus* Δ*saeS* Δ*sarA* double mutant demonstrated a dramatic reduction in TSST-1 expression that essentially phenocopied the single *saeS* mutation (Fig. 6), while Aur and ScpA expression appeared to phenocopy the *sarA* mutation. These findings indicate that although both SarA and Sae are key and opposing regulators of TSST-1, expression of TSST-1 requires functional Sae, even in the absence of repression by SarA.

DISCUSSION

Prior work from our laboratory discovered that particular strains of lactobacilli are able to produce small signaling cyclic dipeptide molecules that repressed the expression of the TSST-1 superantigen in *S. aureus* MN8 (18). In that work, repression of TSST-1 was clearly correlated to repression of *agr*, a well-established positive regulator of TSST-1 expression (10), although the precise details as to how TSST-1 is regulated by *agr* are not known. Nevertheless, mutation of *agr* does not completely abolish TSST-1 expression (10, 13, 15, 18), and quorum sensing inhibitors based on the *agr* autoinducing peptide structure also do not eliminate TSST-1 expression (30). Furthermore, in the *agr* mutant background, TSST-1 could be further repressed by the lactobacillus signals (18), indicating that repression of TSST-1 was not entirely dependent upon *agr*. Herein, this work confirms that although *agr* functions as an important positive regulator of TSST-1, the *sae* regulatory system is the principal regulator that directly controls TSST-1 production and is essential for expression of this important exotoxin.

During the kinetic analysis experiments, transcription of *tst* in wild-type MN8 was predictably initiated at the late exponential phase (Fig. 3) (10). In MN8 Δ*sarA*, *tst* transcription occurred at coinciding time points relative to wild-type MN8, but at much higher levels, which was entirely consistent with the protein expression levels (Fig. 2B, 3A, and 4B) and qRT-PCR analysis (Fig. 4A). Since SarA binds directly to the *tst* promoter (13), this implies

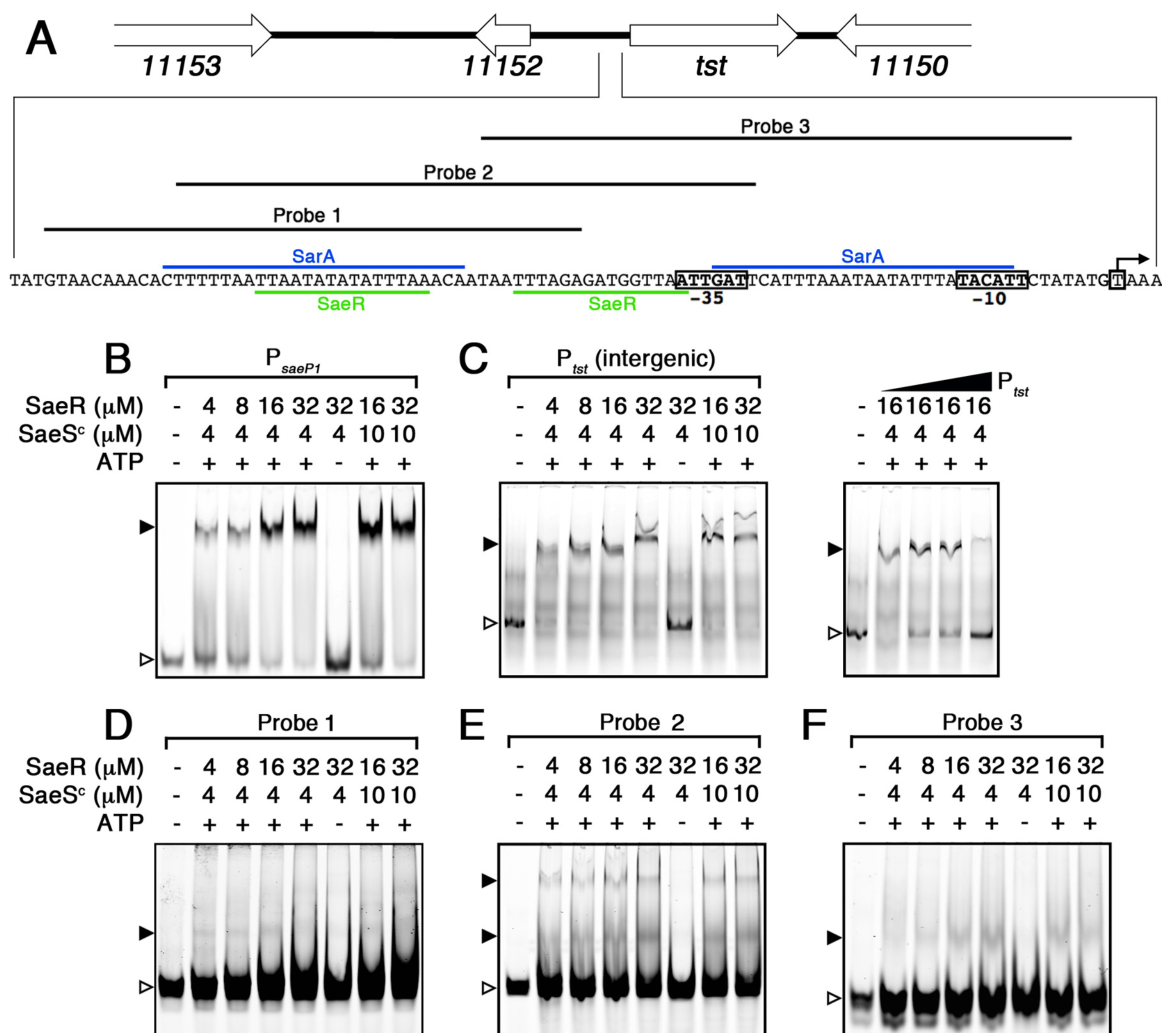

FIG 5 DNA binding analysis of phosphorylated SaeR (P∼SaeR) to the P_{saeP1} and P_{tst} promoters. (A) Schematic map and nucleotide sequence of P_{tst} in *S. aureus* MN8 (locus tag prefix HMPREF0769). The characterized transcriptional start site and −10 and −35 regions are labeled with boxes, and the characterized SarA binding sites is highlighted in blue (13). The proposed SaeR binding sequences are highlighted in green. Probes 1, 2, and 3 correspond to the EMSAs shown in panels D, E, and F, respectively. (C to F) DNA binding analysis of P∼SaeR to P_{saeP1} (B), P_{tst} (C), probe 1 (D), probe 2 (E), and probe 3 (F). Panel C (right panel) also shows a competitive binding analysis using 0-, 5-, 10-, and 50-fold excess unlabeled P_{tst} (gradient denoted by the triangle). Unbound DNA probes are indicated by open arrowheads, and DNA shifts are indicated by solid arrowheads. Protein concentrations, and the presence of ATP in the reaction buffer, are indicated for each lane.

that in the context of TSST-1 regulation in *S. aureus* MN8, the role of SarA is to compete with SaeR-mediated activation of *tst* transcription. This is also consistent with the overlapping binding sites of SarA (13) and the SaeR binding motifs localized by the EMSAs (Fig. 5). SarA has been reported to upregulate RNAIII in *S. aureus*, through enhancing transcription of the *agr* P2 promoter (31). With *S. aureus* MN8, however, there was no apparent cross talk between *agr*, *sae*, and *sarA* during the early (4-h) and late (8-h) exponential phases (Fig. 4A), consistent with other *S. aureus* strains (32). Thus, the reduced levels of TSST-1 in MN8 Δ*agr* were likely, for the most part, independent of *sae* or *sarA*.

Earlier studies using a P_{tst}::*lux* reporter system integrated into the lipase gene of *S. aureus* 8325-4 (14) provided evidence that SarA may function as a transcriptional activator of *tst* expression (13). However, 8325-4 does not carry *tst* but as noted (15) is known to harbor a mutation in *rsbU* resulting in a sigma B defect (33). Deletion of the alternative sigma factor *sigB* gene in strain RN4282, which does carry *tst*, resulted in increased expression of *tst*, which also resulted in decreased levels of *sarA* transcript and increased levels of RNAIII (15). In *S. aureus* RN4282, SarA functioned to repress TSST-1 production (15). As the effects of *sigB* disruption on *sarA* have been previously noted (34), SigB likely acts indirectly, through the derepression of *tst*, due to decreased *sarA*. Combined with our work in *S. aureus* MN8, which is a prototypical menstrual TSS isolate (17), SarA is a TSST repressor in *tst*-containing *S. aureus* strains.

S. aureus MN8, similar to other contemporary clonal complex 30 isolates, contains a G55R mutation within AgrC that is known to result in decreased levels of RNAIII and reduced virulence in a mouse model of bacteremia (35). Consistent with this mutation, levels of RNAIII transcripts are reduced in *S. aureus* MN8 compared with other *S. aureus agr* groups (18). Yet, *S. aureus* MN8

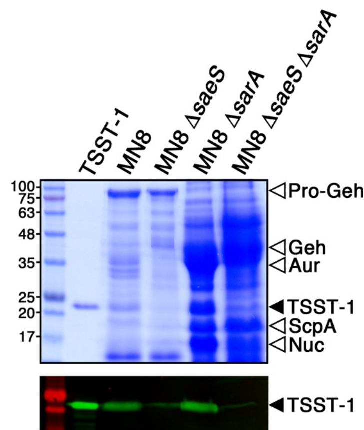

FIG 6 The Sae two-component regulator is necessary for TSST-1 expression in the absence of repression by SarA. Shown are exoprotein profiles (top panel) and Western blot (bottom panel) analysis of TSST-1 for wild-type *S. aureus* MN8 and the corresponding mutants. Concentrated supernatants from the indicated strains grown in BHI medium were loaded onto 12% SDS-PAGE gels. Molecular mass markers in kilodaltons are shown on the left. Purified recombinant TSST-1 is indicated by the solid arrowhead. The locations of pro-Geh, mature Geh, Aur, ScpA, and nuclease (Nuc) are indicated by open arrowheads.

Δ*agr* consistently produced reduced, but detectable, levels of TSST-1. The shift to the early expression profile in MN8 Δ*agr* is entirely consist with the protein analysis, where expression was not abolished but peaked at a relatively low cell density, accounting for the small quantities of TSST-1 produced. Evidence exists that *agr* can antagonize Sae (36), and thus in the absence of *agr*, the *sae* system may potentially function prior to 4 h, which could account for the temporal shift in transcription. In addition, activation of *agr* may function to relieve repression of TSST-1 by the repressor of toxins (Rot), which would no longer be repressed by RNAIII (37, 38).

There are two proposed DNA binding motifs for SaeR (25, 29). Both motifs indicate a highly conserved "primary" TTAAN$_7$ TTAA sequence that overlaps the 5′ end of the −35 promoter region. As noted previously, many Sae-regulated genes contain an additional but less conserved "secondary" TTAA repeat motif (25). Figure 7 shows an alignment of the SaeP1 promoter region, and the Sbi promoter region, both of which are highly regulated by the Sae system in *S. aureus* USA300 (29). In addition, the Geh promoter appears to be Sae regulated in *S. aureus* MN8, as well as the TSST-1 promoter. Mutation analysis of the SaeP1 promoter demonstrated that the presence of a G residue upstream of either primary TTAA sequence facilitates direct binding by SaeR (25). Of note, the P_{tst} "primary" motif sequence does not appear to be optimal, yet *tst* transcript is completely repressed in the absence of a functional Sae system. The evident weak binding of SaeR to P_{tst} may reflect, in part, the *in vitro* nature of the system, which lacks RNA polymerase known to enhance SaeR binding to P_{saeP1} (39). However, based on these findings, we predict that an additional element may function to enhance SaeR binding to P_{tst}. Although Rot is generally considered an exotoxin repressor, Rot and SaeR both cooperate to enhance promoter activity for the staphylococcal superantigen-like genes (40). Since Rot is likely a repressor of *tst* transcription (15), we do not expect that Rot promotes SaeR binding to P_{tst}. The staphylococcal respiratory response (*srr*) two-component system has been shown to influence TSST-1 expression, primarily under microaerophilic conditions. SrrA has also been demonstrated to weakly bind P_{tst}, and thus it is possible that Sae cooperates with other systems, such as Srr, in the regulation of TSST-1. Nevertheless, the apparent weak binding of SaeR to P_{tst} *in vitro* is consistent with other studies in which Sae is a key regulator (e.g., *nuc* and *ssl* promoters) (40, 41).

Apart from regulation of TSST-1 in *S. aureus* MN8, the reduction of pro-Geh expression in MN8 Δ*saeS* (Fig. 2) is consistent with Sae functioning as a positive transcriptional regulator of *geh* in USA300 (29), as well as a potential SaeR binding site (25, 29) located upstream of *geh* (Fig. 7). In MN8 Δ*sarA*, as expected (42, 43), we noted a prominent increase in expression of the aureolysin (Aur) metalloprotease and staphopain A (ScpA) cysteine protease. Aur is responsible for maturation of pro-Geh to mature Geh (44), which explains the near complete lack of pro-Geh in the *sarA* mutant. Thus, although the regulatory role of SarA and Sae for these other important exoproteins was largely predicted, a dominant role for regulation by Sae is selective for particular exotoxins.

Consistent with a prior model as to how phosphorylated SaeR recruits RNA polymerase to Sae-regulated target promoters (25), we herein show that the Sae system is required for expression of TSST-1 and thus functions as a dominant transcriptional activator of TSST-1 expression. Although the regulatory queues that control Sae activation are complex, the specific signals that govern Sae control of TSST-1 expression during the context of menstrual TSS remain uncharacterized. Given that increased expression of *saeRS* from the inducible complementation system did not enhance TSST-1 expression (Fig. 2A), we would predict that host factors that alter SaeR phosphorylation, rather than autoinduction of the *sae* system, would play a more important role for enhanced TSST-1 expression. Apart from the role of TSST-1 in both menstrual and nonmenstrual forms of TSS, another major superantigen with a clear role in nonmenstrual TSS is staphylococcal enterotoxin B (SEB) (9). SEB expression is known to be controlled by Sae (32, 45), while this two-component regulator had no effect on transcription of *sea* or *sel-k* (45). Immediately upstream of the

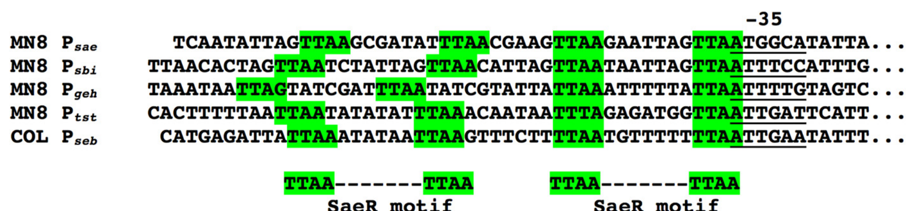

FIG 7 Alignment of promoter element nucleotide sequences of SaeR binding motifs located upstream of P_{saeP1}, P_{sbi}, P_{geh}, P_{tst}, and P_{seb}. The −35 promoter binding regions are underlined, and green highlights outline the primary and secondary SaeR binding motifs.

−35 promoter region of *seb* (46) is a potential SaeR consensus binding sequence (Fig. 7). In an *in vivo* rabbit model of nonmenstrual TSS, *agr* activation was not necessary for the expression of SEB and the development of TSS (47). Thus, potentially, Sae may also be the dominant regulator of SEB expression in *S. aureus*. A picture has therefore emerged whereby the Sae system seems to have evolved to be a critical regulator of *S. aureus* "trademark" superantigen exotoxins involved in TSS, and thus Sae may represent an important target for the rational design of targeted therapeutics with a focus to disarm the exotoxin arsenal of *S. aureus*.

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