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In the *Staphylococcus aureus* Two-Component System *sae*, the Response Regulator SaeR Binds to a Direct Repeat Sequence and DNA Binding Requires Phosphorylation by the Sensor Kinase SaeS[▽]

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Staphylococcus aureus **uses the SaeRS two-component system to control the expression of many virulence factors such as alpha-hemolysin and coagulase; however, the molecular mechanism of this signaling has not yet been elucidated. Here, using the P1 promoter of the *sae* operon as a model target DNA, we demonstrated that the unphosphorylated response regulator SaeR does not bind to the P1 promoter DNA, while its C-terminal DNA binding domain alone does. The DNA binding activity of full-length SaeR could be restored by sensor kinase SaeS-induced phosphorylation. Phosphorylated SaeR is more resistant to digestion by trypsin, suggesting conformational changes. DNase I footprinting assays revealed that the SaeR protection region in the P1 promoter contains a direct repeat sequence (GTTAAN$_6$GTTAA [where N is any nucleotide]). This sequence is critical to the binding of phosphorylated SaeR. Mutational changes in the repeat sequence greatly reduced both the *in vitro* binding of SaeR and the *in vivo* function of the P1 promoter. From these results, we concluded that SaeR recognizes the direct repeat sequence as a binding site and that binding requires phosphorylation by SaeS.**

Staphylococcus aureus is a common gram-positive human pathogen that colonizes skin, the anterior nares, and other mucosal surfaces (2). *S. aureus* can cause a wide range of diseases from soft tissue infections to life-threatening infections such as toxic shock syndrome, necrotizing pneumonia, and endocarditis (2, 36). The bacterium is so versatile that it can infect almost all human body parts. Its versatility is, in part, due to the variety of virulence factors that it produces (e.g., surface proteins, toxins, and immune modulators). The expression of these virulence factors is coordinated by a network of multiple DNA binding proteins (e.g., SigB, Rot, MgrA, SarA, and SarA homologues) and two-component systems (e.g., *agr*, *srrAB*, *arlRS*, *vraSR*, and *saeRS*) (8, 10, 16, 31, 38, 42, 51).

The two-component system is a signal transduction mechanism by which bacteria and lower eukaryotes monitor and respond to environmental stress cues such as nutrient concentrations, ionic strength, and membrane disturbances (27, 47). Typically, the two-component system consists of a sensor histidine kinase (HK) and response regulator (RR) proteins (26). The sensor HK is a membrane protein composed of a signal binding domain and an autokinase domain. The RR is a cytoplasmic protein made up of an N-terminal regulatory domain and the C-terminal effector domain. In a typical RR, the regulatory domain inhibits the function of the effector domain, usually DNA binding activity. Upon sensing its cognate environmental cues, the sensor HK autophosphorylates a histidine residue and then transfers the phosphate group to an aspartate residue in the N-terminal regulator domain of the RR. Commonly, it is the phosphorylated RR that mediates the necessary physiological changes, typically by binding to its target promoters and modulating the level of transcription (26, 27, 49). The phosphorylated RR is dephosphorylated by its intrinsic or the cognate HK-induced phosphorylated RR phosphatase activity, which resets the system to the prestimulus state (27, 47).

The *sae* locus is a staphylococcal two-component system critical for the proper expression of exoproteins (18, 19, 21). This locus is composed of four open reading frames (ORFs): *saeP* (ORF4), *saeQ* (ORF3), *saeR*, and *saeS* (Fig. 1). The two genes *saeS* and *saeR* encode the sensor HK and the RR, respectively. SaeS is a 351-amino-acid (aa) polypeptide with two transmembrane segments at the N terminus. The two membrane segments are separated by only nine extracellular amino acid residues (1), which is regarded as too small a sequence to be a signal binding domain. SaeS, therefore, can be classified as an intramembrane sensing HK, which is hypothesized to monitor membrane disturbances (40). The remaining parts of SaeS are in the cytoplasm and contain three subdomains: HAMP (HKs, adenylyl cyclases, methyl binding proteins, and phosphatases; aa 61 to 114), HisKA (His kinase A; aa 122 to 189), and HATPase_c (HK-like ATPases; aa 234 to 348) (SMART analysis [http://smart.embl-heidelberg.de/]). The amino acid residue for autophosphorylation is predicted to be His131 (18). SaeR is a 228-aa polypeptide with an N-terminal regulatory domain and a C-terminal effector domain with potential DNA binding activity. In the regulatory domain, aspartate 51 is predicted to be phosphorylated by SaeS (18). Although phosphorylated SaeR is assumed to be the mediator of signaling, neither DNA binding nor phosphorylation of SaeR has been shown. The functions of SaeP and SaeQ are completely unknown. SaeP is predicted to be a 146-aa membrane protein which is

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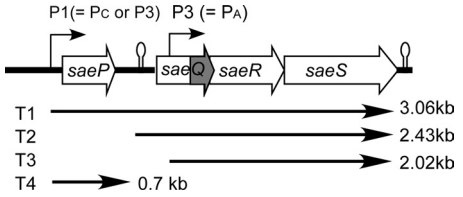

FIG. 1. The *sae* locus of *S. aureus*. Alternative names of the P1 and P3 promoters are in parentheses. The *saeQ* ORF contains a smaller ORF (shown in gray). The transcript species are indicated under the ORF map along with their sizes. This map is adapted from a report by Geiger et al. (17).

not involved in signal transduction (1). Because the *saeQ* ORF contains a smaller ORF, SaeQ can be either a 157-aa protein or a 60-aa polypeptide (Fig. 1). SaeQ is presumed to be involved in signal transduction (1); however, no direct biochemical or genetic evidence has shown the mechanism of its involvement.

The *sae* locus has two promoters, for which various different names have been used in the literature (Fig. 1). The promoter upstream of *saeP* has been called P1, Pc, or P3, while the downstream promoter can be P3 or PA (1, 17, 33). Throughout this study, we will use P1 for the upstream promoter and P3 for the downstream promoter only because those names are found more often in the literature. The P1 promoter is positively autoregulated by the *sae* locus (17, 20, 22), making it a good model system for *sae* regulation studies. The promoter is also positively regulated by *agr* and negatively regulated by *sigB* and *rot* (17, 33). The activity of the promoter is influenced by several environmental stressors. A low pH or a high NaCl concentration represses promoter activity, while stressors such as subinhibitory concentrations of β-lactam antibiotics, H_2O_2, or alpha-defensin activate it (17, 30). However, it is not known how transcription regulators or environmental stressors affect P1 promoter activity.

The SaeRS two-component system plays an important role in staphylococcal gene expression and virulence. Most of all, the two-component system is required for the expression of many virulence factors, including cell wall proteins (Spa, FnbA), cell wall-associated proteins (Map/Eap and Emp), and secreted proteins (SspA, Nuc, Coa, Hla, Hlb, Hlc, SCIN, and CHIPS) (19, 21–23, 34, 43). In particular, the *saeRS* system is an essential positive regulator of alpha-toxin (Hla) production under both *in vitro* and *in vivo* conditions (22, 23, 50). A recent study with clinical isolate MW2 showed that the SaeRS system influences the expression of 212 genes (48), whose functions range from virulence to energy metabolism, ion transport, and DNA repair. Lastly, the *sae* locus is important for the *in vivo* survival of *S. aureus*. Knockout mutations of the locus reduced the survival of the bacterium in animal models including device-related infections (22), murine pyelonephritis (34), intraperitoneal injection (44), and sepsis (48). The *sae* locus also contributes to the apoptosis/death of lung epithelial cells (34), bacterial survival in neutrophils, and killing of neutrophils (48).

Despite its importance in staphylococcal virulence and gene expression, the molecular mechanism of this signaling pathway has not been defined. In this study, we report the identification of the SaeR binding sequence and show that phosphorylation is essential for the DNA binding of SaeR and the signaling process.

MATERIALS AND METHODS

Unless stated otherwise, all of the chemicals used in this study were purchased from Sigma-Aldrich and Fisher and the restriction enzymes were from New England Biolabs.

Bacterial strains, plasmids, and culture conditions. The bacterial strains used in this study are listed in Table 1. Since the genome sequence of strain USA300-0114 is not known, we sequenced the entire *sae* operon of the strain and confirmed that the strain has the same *sae* sequence as strain Newman, except that it does not have the L18P substitution mutation in SaeS. Throughout this study, staphylococci were grown in tryptic soy broth (TSB), except for transduction procedures, for which heart infusion broth supplemented with 5 mM $CaCl_2$ was used. *Escherichia coli* strains were grown in Luria-Bertani broth (LB). When necessary, antibiotics were used at the following concentrations: ampicillin, 100 μg/ml; erythromycin, 10 μg/ml; chloramphenicol, 5 μg/ml.

Recombinant protein expression and purification. (i) Full-length SaeR. The 684-bp ORF of *saeR* was PCR amplified from strain Newman chromosomal DNA with primers SaeRFor_NdeI (5′-GTGTACACATATGACCCACTTACT GATCGTGGATGATGAAC-3′) and SaeRRev_XhoI (5′-GTAGGCACTCGA GTTATCGGCTCCTTTCAAATTTATATCCTAATC-3′). After being digested by NdeI and XhoI, the PCR product was cloned into pET28a (Novagen). The resulting plasmid was transformed first into DH5α and then into BL21 star(DE3). The BL21 star(DE3) strain carrying the plasmid was grown in LB to an optical density at 600 nm (OD_{600}) of 0.6, and then 1 mM isopropyl-β-D-thiogalactopyranoside (IPTG) was added. After overnight induction at room temperature, the cells were harvested and frozen at −80°C. The expressed protein was purified from the frozen cells with a HisTrap column (GE Healthcare, Inc.) by following the column manufacturer's recommendations. The purified protein was supplemented with 20% glycerol and stored at −80°C.

(ii) $SaeR^C$ (SaeRΔN103). For expression of the C-terminal effector domain of SaeR ($SaeR^C$), we used the ligation-independent cloning method (13). The coding regions of $SaeR^C$ (aa 104 to 228) was PCR amplified from strain Newman chromosomal DNA with primers SaeRΔN103_for (5′-TACTTCCAATCCAAT GCCAGTCCAAGGGAACTCGTTTTACGTATTA-3′) and SaeRΔN103_rev (5′-TTATCCACTTCCAATGTTATCGGCTCCTTTCAAATTTATATCCTAA TC-3′). The PCR products were treated with T4 DNA polymerase in the presence of dCTP for 30 min at room temperature. Target vector pMCSG19 (13) was digested with SspI, gel purified, and then treated with T4 DNA polymerase in the presence of dGTP for 15 min at 16°C. The T4 DNA polymerase-treated plasmid vector and PCR product were gel purified, mixed, incubated for 5 min at room temperature, and then transformed into *E. coli* strain DH5α. The resulting plasmid was transformed again into BL21 star(DE3) containing pRK1037(Science Reagents, Inc.), and the transformants were selected on LB agar plates with 150 μg/ml ampicillin and 30 μg/ml kanamycin. The protein was expressed and purified by the same procedures described for full-length SaeR, except that the cells were incubated at 16°C, not at room temperature.

TABLE 1. Bacterial strains used in this study

Species and strain	Relevant characteristics	Source
E. coli		
DH5α		Stratagene
BL21 star(DE3)		Invitrogen
S. aureus		
RN4220	Restriction deficient, prophage cured	29
Newman	Clinical isolate, L18P substitution in SaeS	14
ΦNΞ-9725	Strain Newman with Tn*917* insertion in *saeS*	4
USA300-0114	Clinical isolate, no L18P substitution in SaeS	NARSA[a]
TB3	*S. aureus* Newman with three prophages deleted Δ(φ*NM124*)	3

[a] NARSA, Network on Antimicrobial Resistance in *Staphylococcus aureus*.

TABLE 2. Primers used in EMSA

Primer	Sequence[a]
P1-F	5′AACGAATTCTTGGTACTTGTATTTAATCGTCTATC
P1-R	5′AAAGGTACCGTTGTGATAACAGCACCAGCTGC3′
hla_I_F	5′GTGTACAAACGAAAAAGTATCGTATGTATTTTTAATATAG3′
hla_I_R	5′GTGTACATAAAATAGTTTCATTTTAATCCCCTATC3′
hla_II_F	5′GTGTACAGATTACAATATAAAAATACAAATATCTTAG3′
hla_II_R	5′GTACAATCTATTAGATATTTCTATGTAATGGCAAAATTTATTC3′
empF1	5′ACAGAATTCAATTATTTATAATGCACC3′
empR1	5′CCTAAGCTTTTATATAGACTCAATATTATAAC3′
eapF	5′TTTGAATTCCACCATCATTATCACTCC3′
eapR	5′CATCCCGGGAAATTATCTCTCCTTTTTTG3′
vwbF1	5′TTCGAATTCAGATAGCGATTCGGACTC3′
vwbR1	5′CCTAAGCTTTAATTTTCCCTAATTAAC3′
arlR-F	5′CAATAGTGAAAAGTCAGTATATGAC3′
arlR-R	5′AACGGTACCATTTGCGTCATTTGTACACCTC3′

[a] Restriction enzyme sites are underlined.

(iii) SaeS^C (SaeSΔN92). Except the primers for PCR amplification of the coding region, the production of the cytoplasmic domain of SaeS (SaeS^C aa 93 to 351) was carried out as described for SaeR^C above. The primers used were SaeSΔN92 _for (5′-TACTTCCAATCCAATGCCAAAGAAATTTATGAATTAAATCAATC-3′) and SaeSΔN92 _rev (5′-TTATCCACTTCCAATGTTATCGGCTCCTTTCAAATTTATATCCTAATC-3′).

Electrophoretic mobility shift assays (EMSA). The primers used in EMSA are listed in Table 2. DNA probes were PCR amplified and radiolabeled with T4 polynucleotide kinase (NEB) and [γ-^{32}P]ATP (Perkin-Elmer). The radioactive probe (2 ng) was mixed with various amounts of the test protein in 25 μl of gel shift loading buffer (10 mM Tris-HCl, pH 7.4, 50 mM KCl, 5 mM $MgCl_2$, 10% glycerol, 3 μg/ml sheared salmon sperm DNA). After being incubated at room temperature for 10 min, the samples were analyzed by 8% polyacrylamide gel electrophoresis (100 V for prerun, 85 V for 85 min for sample separation). The gels were dried and subjected to autoradiography on a phosphor screen (BAS-IP; Fuji).

Phosphorylation of SaeR for EMSA. The purified SaeR protein (20 μM) was mixed with purified SaeS^C (1 μM) in phosphorylation buffer (10 mM Tris-HCl, pH 7.4, 50 mM KCl, 5 mM $MgCl_2$, 10% glycerol). One millimolar ATP was then added, and the mixture was incubated at room temperature for 5 min before the addition of ^{32}P-labeled DNA probe. For EMSA or footprinting analyses, the entire reaction mixture was used.

DNase I footprinting assays. The test proteins were mixed with radiolabeled probe in reaction buffer (10 mM Tris-HCl, pH 7.4, 50 ml KCl, 5 mM $MgCl_2$, 1 mM $CaCl_2$, 1 μg/ml yeast tRNA). After the addition of DNase I (0.1 U; New England Biolabs), the samples were incubated at room temperature for 2 min. The reaction was terminated by the addition of 25 μl of stop solution (50 mM Tris-HCl, pH 8.0, 1% [wt/vol] sodium dodecyl sulfate [SDS], 20 mM EDTA) and extracted first with 50 μl of phenol-chloroform (1/1) and then with 50 μl of chloroform. DNA was precipitated with ethanol, washed with 500 μl of 70% ice-cold ethanol, and suspended in 10 μl of loading buffer (98% deionized formamide, 10 mM EDTA, 0.025% [wt/vol] xylene cyanol FF, 0.025% [wt/vol] bromphenol blue). After being denatured at 95°C for 3 min, samples were separated on an 8% urea-polyacrylamide gel. Sequencing ladders consisting of A+G and T+C for the P1 promoter of the *sae* operon were made by the standard Maxam-Gilbert method (41).

Phosphorylation of SaeR by SaeS^C and cell lysates. Cell lysates were prepared from strain Newman, a *saeS* transposon insertion mutant (ΦNΞ-9725), and strain USA300-0114 (= NRS384), which does not contain the L18P substitution mutation in SaeS. The cells were grown in TSB to mid-log phase (OD_{600} = 0.6) and collected by centrifugation. The collected cells were suspended in 1 ml of TSM (50 mM Tris-HCl, pH 7.5, 0.5 M sucrose, 10 mM $MgCl_2$) to which was added 10 μl lysostaphin (2 mg/ml) and incubated at 37°C for 30 min. A 0.6-ml volume of 1.3 M KCl was then added to the cell lysates, and the resulting cell lysates were stored at −80°C. The phosphorylation assays were performed in the presence of 10 μM SaeR and 2 μM SaeS^C or 2-μl cell lysate volumes in phosphorylation buffer (10 mM Tris-HCl, pH 7.4, 50 mM KCl, 5 mM $MgCl_2$, 10% glycerol). The final reaction mixture volume was 20 μl. To initiate the phosphorylation reaction, 0.5 μl [γ-^{32}P]ATP (80 μCi) was added. The reaction mixtures remained at room temperature for 10 min, and then the reactions were stopped by the addition of 10 μl of 2× SDS loading buffer. Samples were analyzed by 13% SDS-polyacrylamide gel electrophoresis (PAGE).

Assay of phosphotransfer between P-SaeS^C and SaeR. SaeS^C (3 μM) was preincubated with 3 μM [γ-^{32}P]ATP in 105 μl of phosphorylation buffer for 1 h at room temperature. As a reference sample, 15 μl was mixed with 15 μl of 2× SDS loading buffer and kept at room temperature. To initiate the phosphotransfer reaction, SaeR (9 μM) was added to phosphorylated SaeS^C. At various time points (0 min, 3 min, 30 min, 2 h, 4 h, and 20 h), 15 μl of the sample was mixed with SDS loading buffer and stored at room temperature until electrophoresis. The samples were analyzed by 13% SDS-PAGE and autoradiography.

Limited trypsin digestion assays. To phosphorylate SaeR, SaeR (50 μM) was mixed with 1 μM SaeS^C in the presence of 1 mM ATP and incubated at room temperature for 5 min. Because the phosphorylated SaeR protein sample contains SaeS^C, to avoid any artifacts from the presence of SaeS^C, an equal amount of SaeS^C was added to the unphosphorylated SaeR sample but without ATP. Trypsin digestion was initiated by the addition of 0.2 μg/μl trypsin in phosphorylation buffer containing 0.1 mM EDTA. The samples were incubated at 37°C. Aliquots of 10 μl were removed from the reaction mixture at defined time intervals and mixed with 10 μl of 2× SDS loading buffer. Samples were separated by 14% SDS-PAGE and stained with Coomassie blue R-250. The digestion patterns were quantified by Quantity One (Bio-Rad).

Random mutagenesis of the P1 promoter. The P1 promoter region was amplified by the error-prone enzymes *Taq* polymerase (NEB) and Mutazyme (Stratagene) with primers P1-F and P1-R (Table 2). After being purified, the PCR product was digested with BamHI/KpnI and then ligated with pYJ-lacZ, a promoterless *lacZ* reporter plasmid derived from pYJ335 (28). The ligated DNA was electroporated into *E. coli* DH5α. Plasmids were purified from the colonies on the transformation plate and electroporated into *S. aureus* strain RN4220, and then the cells were spread on tryptic soy agar (TSA) containing erythromycin and 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-Gal; 80 μg/ml) ($TSA_{erm\ X\text{-}Gal}$). Colonies displaying white or reduced blue color were isolated. In other trials, the RN4220 colonies were pooled and lysed with ϕ85. The phage lysates were used to transduce the plasmids into strain TB3, a strain derived from strain Newman by the deletion of three prophages. This strain has a wild-type *sae* phenotype and, because of the absence of three prophages, is useful for complementation studies. The transduced TB3 cells were spread on $TSA_{erm\ X\text{-}Gal}$. The colonies displaying white or reduced blue color were isolated. To verify that the phenotype was caused by mutations in the P1 promoter, plasmids were purified and the P1 promoter was sequenced. When more than one mutation was found, each mutation was generated by PCR-mediated site-directed mutagenesis; then, the resulting mutation was tested in strain Newman to find the one responsible for the phenotype. During the *lacZ* assay, however, we found that in strain Newman, the plasmid carrying the wild-type P1 promoter was unstable and the majority of the cells displayed a white color on $TSA_{erm\ X\text{-}Gal}$. Characterization of the plasmid purified from the cells revealed that a deletion mutation occurred at the N terminus of LacZ, suggesting that the overexpression of β-galactosidase from the high-copy plasmid is toxic to the cells. Therefore, we moved all of the mutations of the P1 promoter to pCL55-lacZ, a *lacZ* reporter plasmid derived from integration plasmid pCL55 (32). The mutated P1 promoters were PCR amplified by the high-fidelity DNA polymerase Phusion (NEB) with primers P1-F and P1-R (Table 2) and cloned into pCL55-lacZ as described for pYJ-lacZ above. All of the mutant P1 promoter sequences in the pCL-lacZ constructs were verified by DNA sequencing analysis.

LacZ assay. The test strains were grown in TSB containing 5 μg/ml chloramphenicol ($TSB_{chl\text{-}5}$) and incubated at 37°C for 16 h. After being collected by centrifugation, the cells were suspended in AB buffer (100 mM potassium phosphate, 100 mM NaCl, pH 7.0) and treated with lysostaphin (0.1 μg/ml) at 37°C for 15 min. After the addition of 900 μl of ABT buffer (AB buffer containing 0.1% Triton X-100) to the lysostaphin-treated cells, 50 μl of the cell lysate was mixed with 10 μl of MUG (4-methylumbelliferyl-β-D-galactopyranoside, 4 mg/ml; Sigma) and incubated at room temperature for 1 h. Then, 20 μl of the reaction mixture was mixed with 180 μl ABT buffer in a black 96-well plate and the emission of fluorescence was measured by a plate reader (355-nm excitation,

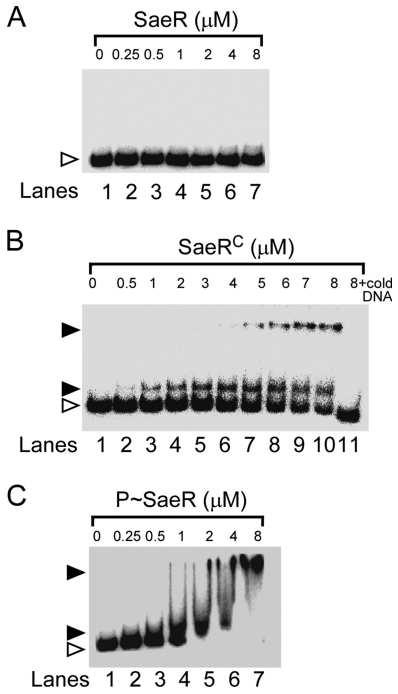

FIG. 2. DNA binding activities of unphosphorylated SaeR (A), the C-terminal effector domain of SaeR (B), and phosphorylated SaeR (C) to the P1 promoter region. In a typical assay, 2 ng of a γ-^{32}P-end-labeled P1 promoter fragment was incubated with the indicated concentration of protein in the presence of 3 μg/ml salmon sperm DNA at room temperature for 15 min. Free DNA is indicated by a white arrowhead, and bound DNA is indicated by a black arrowhead. SaeR, unphosphorylated SaeR; SaeR^C, the C-terminal effector domain of SaeR; P-SaeR, phosphorylated SaeR; + cold DNA, addition of a 10-fold excess of unlabeled DNA.

445-nm emission). The LacZ activity was normalized by cell density at 600 nm, and then the relative activity was calculated by setting the LacZ activity from the wild-type P1 promoter to 100%. The assay was repeated at least twice with similar results.

RESULTS

The N-terminal regulatory domain inhibits the DNA binding activity of the C-terminal effector domain in SaeR. As a first step in identifying the molecular mechanism of *sae*-mediated signal transduction, we examined the DNA binding activity of the RR SaeR. We expressed the full-length SaeR protein in *E. coli* with a His_6 tag at its N terminus and then purified the protein with a Ni column. As a DNA binding substrate, we used the radiolabeled P1 promoter of the *sae* operon because the promoter is highly autoregulated by *sae* (17, 20, 22). Using purified His_6-SaeR and radiolabeled P1 promoter DNA, we performed EMSA. As shown in Fig. 2A, full-length SaeR did not bind to the P1 promoter.

Since the DNA binding activity of the effector domain is commonly inhibited by the N-terminal regulatory domain, we envisioned that if we deleted the N-terminal domain, the C-terminal effector domain alone might be able to bind to its target DNA. To test this hypothesis, we produced the C-terminal effector domain (SaeR^C aa 104 to 228) with a His_6 tag as described in Materials and Methods and performed EMSA

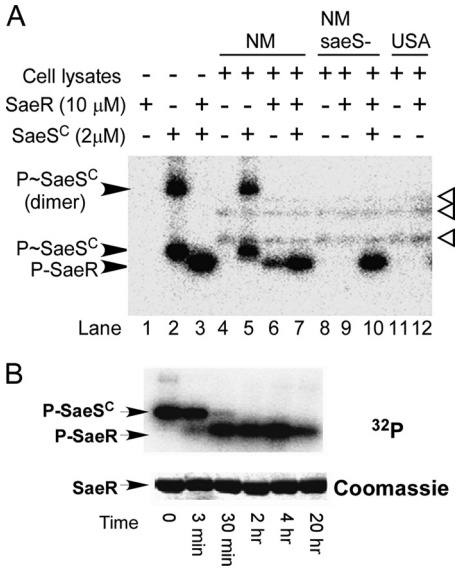

FIG. 3. SaeR phosphorylation by the cytoplasmic domain of SaeS and strain Newman cell lysates. (A) SaeS^C (2 μM) or cell lysates from strains Newman (NM), ΦNΞ-9725 (NMsaeS-), and USA300-0114 (USA) were mixed with SaeR (10 μM) in the presence of [γ-^{32}P]ATP. All of the reactions were performed for 10 min at room temperature. (B) SaeS^C (3 μM) was phosphorylated with [γ-^{32}P]ATP, and then SaeR (9 μM) was added. P-SaeS^C, phosphorylated SaeS^C; P-SaeR, phosphorylated SaeR. The three additional protein bands phosphorylated in the strain Newman cell lysates are indicated by white arrowheads.

with the radiolabeled P1 promoter DNA again. As shown in Fig. 2B, unlike full-length SaeR, SaeR^C bound to the P1 promoter, suggesting that the N-terminal regulatory domain indeed inhibits DNA binding activity. The binding affinity of SaeR^C for the P1 promoter was weak. Even at 8 μM SaeR^C, approximately 30% of the substrate DNA remained unbound. In a densitometry analysis, the dissociation constant of SaeR^C was calculated to be 6 μM. In the EMSA, two shifted bands were observed, suggesting the existence of multiple SaeR binding sites in the P1 promoter. The addition of a 10-fold excess of unlabeled P1 DNA abolished the binding of the radiolabeled DNA, demonstrating the specificity of the protein-DNA interaction (Fig. 2B, lane 11). Based on these results, we concluded that the C-terminal effector domain of SaeR has target DNA binding activity and that in full-length SaeR, this activity is inhibited by the N-terminal regulatory domain.

Phosphorylation is essential for the DNA binding activity of SaeR. Typically, phosphorylation of the N-terminal regulatory domain abolishes its inhibitory effect on the C-terminal effector domain. To examine whether phosphorylation of SaeR can restore the DNA binding activity of SaeR, we decided to phosphorylate SaeR with the sensor kinase SaeS. Since SaeS has its kinase activity in the cytoplasmic domain, we expressed the cytoplasmic autokinase domain of SaeS (SaeS^C aa 93 to 351) with an N-terminal His_6 tag. We purified His_6-SaeS^C and tested the autophosphorylation function of SaeS^C by adding [γ-^{32}P]ATP and Mg^{2+} to the protein. As shown in Fig. 3A, autophosphorylation of SaeS^C was clearly observed (lanes 2), proving that the SaeS^C protein used in the test does contain autokinase activity. When SaeR was added to SaeS^C (SaeR/SaeS^C ratio, 5:1), phosphorylation of SaeR was observed (Fig.

3A, lane 3), suggesting that the purified $SaeS^C$ protein contains phosphotransferase activity and can be used to phosphorylate SaeR. Interestingly, under these experimental conditions, no phosphorylated $SaeS^C$ was detected, implying a very fast phosphotransfer reaction.

In strain Newman, SaeS is constitutively active due to an amino acid substitution mutation (L18P) within the first transmembrane segment (1). Therefore, we anticipated that cell lysates from strain Newman would be able to phosphorylate SaeR. Indeed, when added to SaeR, strain Newman cell lysates clearly phosphorylated the RR within 10 min (Fig. 3A, lane 6). Further addition of purified $SaeS^C$ to the reaction mixture did not change the overall outcome (Fig. 3, lane 7). The fact that the cell lysate from a *saeS* transposon mutant of strain Newman failed to phosphorylate SaeR (Fig. 3, lane 9) strongly suggests that SaeS is responsible for SaeR phosphorylation. Strain USA300-0114 does not contain the L18P substitution mutation in SaeS. When the cell lysate from strain USA300-0114 was used, no significant SaeR phosphorylation was observed, confirming the hyperactivity of SaeS kinase in strain Newman. Noticeably, in the reaction mixtures with cell lysates, three more radiolabeled protein bands appeared (white arrowheads in Fig. 3A), revealing the occurrence of other phosphorylation events in the cell lysate. The identities of these protein bands are unknown.

Although the above result clearly shows that $SaeS^C$ can phosphorylate SaeR, it does not show the phosphotransfer reaction directly due to the fast kinetics of this process. In order to detect that phosphotransfer reaction directly, we first phosphorylated $SaeS^C$ with [γ-^{32}P]ATP and then added SaeR at a lower ratio (SaeR/$SaeS^C$ ratio, 3:1) than in the previous phosphorylation reaction mixture, where the ratio was 5:1. The results are shown in Fig. 3B. Under this condition, a gradual transfer of the phosphate group was observed. After 3 min, only a fraction of SaeR was phosphorylated and the reaction was not complete until 2 h. Interestingly, no significant change in P-SaeR was observed until 4 h after the reaction. Even after 20 h, a significant portion of SaeR remained phosphorylated, indicating that the phosphorylated form of SaeR is stable under the conditions employed.

To examine whether phosphorylation can restore the binding activity of SaeR, we phosphorylated SaeR by adding purified $SaeS^C$ and ATP and used phosphorylated SaeR (P-SaeR) in EMSA. As shown in Fig. 2C, P-SaeR indeed bound to P1 promoter DNA. In a densitometry analysis, the dissociation constant of P-SaeR was calculated to be 0.75 μM, suggesting that the P1 promoter binding affinity of P-SaeR is eight times higher than that of $SaeR^C$ (K_D = 6 μM). To eliminate the possibility that $SaeS^C$ bound to the DNA in the EMSA, we performed EMSA with the mixture of $SaeS^C$ and SaeR in the absence of ATP; no binding was observed (data not shown), suggesting that it is P-SaeR, not $SaeS^C$, that is responsible for the DNA binding observed. Taken together, these results demonstrate that phosphorylation of SaeR is essential for efficient binding to target DNA.

Phosphorylation elicits conformational changes in SaeR. If phosphorylation restores the DNA binding activity of SaeR, we postulated that it would accompany significant conformational changes in SaeR. To examine this postulation, we subjected unphosphorylated SaeR and P-SaeR to a limited trypsin digestion assay where the alteration in protease susceptibility is interpreted as a conformational change in the protein. We digested the two forms of SaeR at a low concentration of trypsin and, by PAGE and Coomassie staining, analyzed the digestion pattern after 2, 5, 15, and 30 min. The digestion results obtained are shown in Fig. 4A. Although almost all of the unphosphorylated SaeR protein was digested by trypsin within 30 min, the majority of the P-SaeR protein remained intact, even after 30 min of digestion (lane 10). This rather dramatic alteration in trypsin susceptibility strongly suggests that phosphorylation elicits significant conformational changes in SaeR which, in turn, lead to restoration of the DNA binding activity of the protein.

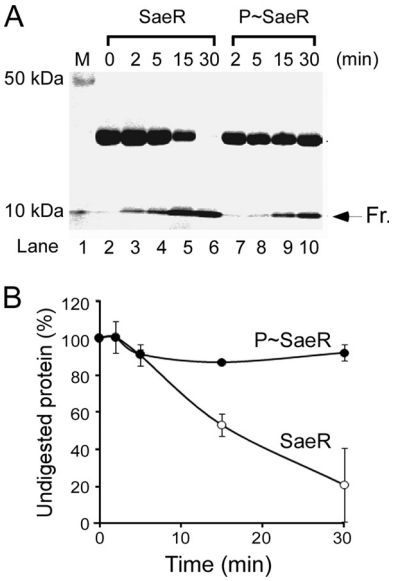

FIG. 4. Limited trypsin digestion analysis of SaeR and P-SaeR. (A) SaeR (50 μM) was phosphorylated by $SaeS^C$ (1 μM) in the presence of 1 mM ATP. An equal amount of $SaeS^C$ (1 μM) was added to unphosphorylated SaeR (50 μM) but without ATP. The proteins were mixed with 0.2 μg/μl trypsin and incubated at 37°C. Aliquots of 10 μl were removed from the reaction mixtures at different time intervals and quenched by the addition of 10 μl 2× SDS loading buffer, followed by heating at 90°C for 5 min. Samples were analyzed by 14% SDS-PAGE and Coomassie straining. Fr, a 10-kDa SaeR fragment resistant to trypsin digestion. (B) The staining results were quantified by Quantity One (Bio-Rad). The error bars represent the standard deviations calculated from two independent experiments.

DNase I footprinting assays identify the SaeR binding sequence. Since both $SaeR^C$ and P-SaeR bound to the P1 promoter, to identify the SaeR binding sequence, we decided to perform a DNase I footprinting assay with the proteins. The radiolabeled P1 promoter was incubated with increasing amounts of either $SaeR^C$ or P-SaeR, and then DNase I was added. After a 2-min incubation, the reaction was terminated and the product was analyzed by PAGE and autoradiography. As shown in Fig. 5A, $SaeR^C$ bound to the P1 promoter in the region from −20 to −54 (bottom strand) or from −35 to −63 (top strand). On the other hand, P-SaeR bound to wider regions of the P1 promoter from −20 to −90 (bottom strand) or from −24 to −101 (top strand).

Because most RRs bind to their target as a dimer, we envisioned that the SaeR binding sequence would be a repeat

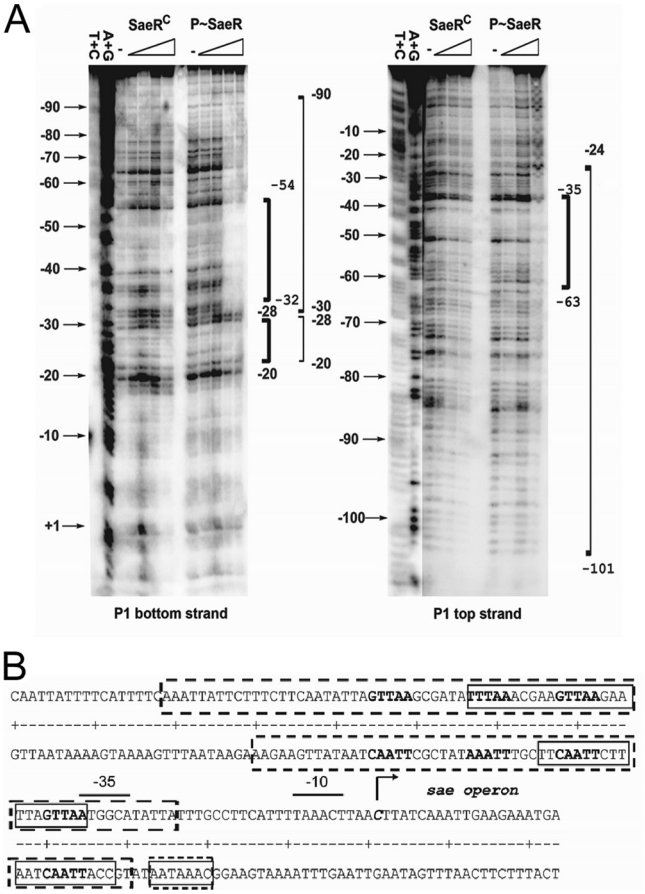

FIG. 5. Identification of SaeR binding sequences. (A) DNase I footprinting analysis of the P1 promoter with $SaeR^C$ and P-SaeR. Sequencing of the DNA probe was carried out by the Maxam-Gilbert method. The nucleotide positions are indicated to the left of the footprinting image. The regions protected by $SaeR^C$ are in bold brackets, and the regions protected by P-SaeR are in plain brackets. Values represent distances from the transcription start site, which was set to +1. (B) P1 promoter sequence with a summary of the DNase I footprinting assay results. The −10 and −35 promoter regions are indicated by solid lines above the sequence. $SaeR^C$-protected regions are in solid boxes, and the P-SaeR-protected regions are in dotted-line boxes. The direct repeat sequences are in boldface. The transcription start site is indicated by a right-angled arrow; and the corresponding nucleotide is in boldface italics.

sequence. Indeed, close inspection of the protected region (−20 to −101 position) revealed a direct repeat sequence ($GTTAAN_6GTTAA$) between positions −35 and −50 and an imperfect repeat sequence ($GTTAAN_6TTTAA$) between positions −56 and −71 (Fig. 5B). To examine whether the repeat sequences are SaeR binding sites, we searched the strain Newman genome, by using Regulatory Sequence Analysis Tools (http://rsat.ulb.ac.be/rsat/), for the genes containing the perfect direct repeat sequence ($GTTAAN_6GTTAA$) in the promoter region. The search results are shown in Table 3. The search identified 21 genes carrying the direct repeat sequence. Of the 21 genes, two pairs of genes (NWMN_0165/0166 and NWMN_1706/1707) shared the same sequence. More importantly, 13 (57%) were genes of the *sae* regulon (30, 34, 45, 46, 48), raising the possibility that the direct repeat sequence is the SaeR binding site.

Interestingly, Table 3 does not contain some of the well-known members of the *sae* regulon such as *map* (also called *eap*), *hlb*, and *scn*. In addition, a recent microarray analysis of strain MW2 showed that the SaeRS system affected the transcription of 212 genes (48), suggesting that the 21 genes identified may not represent the entire *sae* regulon. Therefore, we repeated the sequence search by allowing one mismatch in the sequence; the results are shown in Table 4. The search identified a total of 155 genes, including *map*/*eap*, *hlb*, and *scn*. Of these 155 genes, 36 (23%) were reported to be regulated by *sae*, further supporting the idea that the repeat sequence is the SaeR binding site.

The direct repeat sequence is necessary for binding to SaeR. The gene *hla* encodes alpha-hemolysin and is highly regulated by the *sae* locus (19, 33, 44, 50). The promoter region of *hla* contains two direct repeat sequences: an imperfect repeat sequence ($TTTAAN_6GTTAA$, from −190 to −175) and a perfect repeat sequence (GTTAAN6GTTAA, from −405 to −390) (Fig. 6A). It should be noted that the position is the distance from the ATG start codon, not from the transcription start site (11). The transcription start site corresponds to position −332. As the imperfect repeat sequence is located downstream of the transcription start site and is probably not involved in transcriptional regulation, we focused on the perfect repeat sequence at position −405. We PCR amplified two DNA fragments from the *hla* promoter region: a 180-nucleotide (nt) DNA fragment without the repeat sequence (fragment I) and a 200-nt DNA fragment with the perfect repeat sequence (fragment II). We hypothesized that if the direct repeat sequence is necessary for binding to SaeR, the 180-nt DNA fragment should not bind to P-SaeR while the 200-nt DNA fragment should. After labeling these DNA fragments with [γ-^{32}P]ATP, we mixed each DNA fragment with P-SaeR and performed an EMSA. As shown in Fig. 6B, only DNA fragment II, the DNA with the perfect repeat sequence, bound to P-SaeR, strongly supporting the idea that the direct repeat sequence is the SaeR binding site.

We decided to further examine the role of the direct repeat sequence in SaeR binding with four more genes that contain various combinations of the SaeR binding sequence: *emp*, *map*/*eap*, *vwb*, and *arlR*. The genes *emp*, *map*/*eap*, and *vwb* encode surface proteins important for adhesion and immune evasion (9, 24, 25), while *arlR* encodes the RR for the *arlRS* system that regulates the expression of virulence genes such as *spa* and *cap5* (15, 16, 37). The genes *emp* and *map*/*eap* have been reported to be regulated by the *sae* locus, but *vwb* and *arlR* have not. In addition, only *emp* contains a perfect repeat sequence, while the others have imperfect repeats. It should also be noted that the promoter of *arlR* has two binding sites but those of the others have 1.5 binding sites (Fig. 6C). After radiolabeling these DNA fragments, we performed an EMSA with P-SaeR, and the results are shown in Fig. 6D. All of the promoters bound to P-SaeR. The binding affinity of *emp* containing the perfect repeat sequence appeared stronger than those of the others. These results further support the idea that the direct repeat sequence mediates DNA binding to SaeR.

Mutational changes in the direct repeat sequence in the P1 promoter abolish not only SaeR binding but also promoter function. If the direct repeat sequence is the SaeR binding site,

TABLE 3. Genes containing the perfect direct repeat sequence GTTAAN$_6$GTTAA in the promoter region

Locus ID[b]	Gene	Matching sequence[c]	Product
NWMN_0165		TCTAGTTAATATATAGTTAATGTC	Hypothetical protein
NWMN_0166[a]	*coa*	TCTAGTTAATATATAGTTAATGTC	Coagulase precursor
NWMN_0362[a]		TAAAGTTAATCAAGAGTTAAGATG	Hypothetical protein
NWMN_0369	*set2nmg*	AATAGTTAAAAACAGGTTAATGT	Enterotoxin-like toxin
NWMN_0393	*set6nm*	AATAGTTAAAAAGAGGTTAATTCA	Enterotoxin-like toxin
NWMN_0394[a]	*set7nm*	AATAGTTAAAAAGAGGTTAATTCA	Enterotoxin-like toxin
NWMN_0677[a]	*saeP*	CGAAGTTAAGAATTAGTTAATGGC	Hypothetical protein
NWMN_1066[a]		TTTAGTTAATAAATAGTTAATGTA	Hypothetical protein
NWMN_1073[a]	*hla*	ACTAGTTAATATATAGTTAATTTT	Alpha-hemolysin precursor
NWMN_1074		ACTAGTTAATATATAGTTAATTTT	Hypothetical protein
NWMN_1533	*hisS*	AGCCGTTAAACGTACGTTAAACGT	Histidyl-tRNA synthetase
NWMN_1706[a]	*splA*	AAACGTTAATAAGTGGTTAATTAA	Serine protease
NWMN_1707		AAACGTTAATAAGTGGTTAATTAA	Hypothetical protein
NWMN_1708		TTTAGTTAATAGATAGTTAATACA	Homologue to Ear
NWMN_1719[a]	*lukE*	AATAGTTAATAATCAGTTAATTTT	Leukocidin LukE precursor
NWMN_1880[a]	*sak*	AAATGTTAAATATTTGTTAATTAT	Staphylokinase precursor
NWMN_2317[a]	*sbi*	ATTAGTTAATAATTAGTTAATTTC	IgG binding protein Sbi
NWMN_2319[a]	*hlgC*	AATTGTTAATGAACAGTTAATTAT	Gamma-hemolysin component C
NWMN_2397[a]	*fnbB*	GCGAGTTAATAAAAAGTTAAGATT	Fibrinogen binding protein B
NWMN_2399[a]	*fnbA*	GCGAGTTAATGAAAAGTTAAGATT	Fibrinogen binding protein A
NWMN_2592		AACCGTTAACAACACGTTAACGGG	2-Oxoglutarate/malate translocator

[a] Known gene of the *sae* regulon.
[b] Genome of strain Newman.
[c] Four flanking nucleotides are also shown.

mutational changes in the sequence should abolish SaeR binding and promoter activity. To eliminate bias, instead of site-directed mutagenesis, we used a random mutagenesis strategy. We mutated the P1 promoter by amplifying the DNA with error-prone DNA polymerases and then inserted the PCR products into *lacZ* reporter plasmid pYJ-lacZ, a multicopy plasmid derived from pYJ335 (28). As described in Materials and Methods, the resulting plasmids were inserted into strain RN4220 or TB3, a *sae*$^+$ strain derived from strain Newman, and the cells were spread on TSA plates containing X-Gal. White colonies were selected and subjected to DNA sequencing analysis. Through this process, we identified eight mutations in the SaeR binding site (W4, 62, W6B, W5, DB6, W17A, W104, and W3) (Fig. 7A), confirming that mutational changes in the binding sequence abolish promoter activity (i.e., white color). Among these mutations, DB6 altered the distance between the repeat sequences from 6 nt to 5 nt, suggesting that not only the nucleotide sequence but also the spacing is important for the binding of SaeR. We also identified five mutations in the promoter sequence in the −35 (DW1B, W110) and −10 (DW4, 65A, 32A) regions. As a control, we included a nucleic acid substitution mutant showing blue color (DB5) on further analysis (Fig. 7A). The mutation is located between the −35 and −10 regions. Since no mutations were obtained for three nucleotides in the SaeR binding site, we mutated these three nucleotides by PCR-mediated site-directed mutagenesis. The resulting mutants are M15, M16, and M17 (Fig. 7A). Strain Newman carrying the mutant plasmids also showed reduced blue color, suggesting that these nucleotides are also important for efficient binding to SaeR.

To examine the correlation between the reduced promoter activity of the mutants and the SaeR binding of the promoters, we performed an EMSA for all of the mutants using P-SaeR. As shown in Fig. 7B, all of the mutations in the SaeR binding site, including spacing mutation DB6, reduced DNA binding to SaeR, confirming that the direct repeat sequence is the SaeR binding site. Some residual SaeR binding was observed with M17, W6B, DB6, M16, and M15, suggesting that the contributions of these positions to SaeR binding are different. As expected, none of the other mutations significantly changed DNA binding to SaeR.

To quantitatively measure the effects of the mutations on the function of the P1 promoter, we measured the LacZ activity of the mutants from 16-h overnight cultures. As shown in Fig. 7C, all of the 11 mutations in the SaeR binding sites greatly reduced LacZ activity, confirming the correlation between SaeR binding and the transcriptional activity of the promoter. Of the mutations, M15, M16, and M17 did not completely abolish promoter activity; compared with the wild-type P1 promoter, the promoters with the mutations still retained 10% to 30% activity. These results confirm that for *sae*-mediated transcriptional activation, the contribution of each nucleotide position might not be equal. In EMSA, indeed, the promoters with these mutations showed some residual SaeR binding. As expected, all of the mutations in the promoter regions also abolished or greatly reduced LacZ activity while the DB5 mutant showed nearly wild-type promoter activity (80%), showing that not all of the mutations in the P1 promoter region can abolish LacZ activity and the greatly reduced promoter activities in other mutants are due to the loss of specific functions of the DNA sequence (i.e., SaeR binding or RNA polymerase binding). In summary, through a random mutagenesis strategy, we confirmed that (i) the direct repeat sequence GTTA AN$_6$GTTAA is the SaeR binding site and (ii) the binding site is critical for *sae*-mediated transcriptional activation of the P1 promoter.

DISCUSSION

The SaeRS two-component system is a staphylococcal signaling system that plays a critical role in activating the tran-

TABLE 4. Genes containing the direct repeat sequence with no more than one mismatch

Gene function and ID[a]	Name	Start	Sequence	End	Product
Virulence/defense					
NWMNN_0166[b]	*coa*	−103	GTTAATGCTTTGTTTA	−88	Coagulase precursor
		−82	GTTAATATATAGTTAA	−67	
NWMNN_0388	*set1nm*	−109	GTTAAATGAGGTTTAA	−94	Staphylococcal enterotoxin-like toxin
NWMNN_0389	*set2nm*	−109	GTTAAAAACAGGTTAA	−94	Staphylococcal enterotoxin-like toxin
NWMNN_0390	*set3nm*	−110	GTTAAAAGGGGTTTAA	−95	Staphylococcal enterotoxin-like toxin
		−223	ATTAAAAAGAAGTTAA	−208	
NWMNN_0391	*set4nm*	−109	GTTAAACAAGGTTTAA	−94	Staphylococcal enterotoxin-like toxin
NWMNN_0393	*set6nm*	−145	GTTCAAAAATAGTTAA	−130	Staphylococcal enterotoxin-like toxin
NWMNN_0393	*set6nm*	−134	GTTAAAAAGAGGTTAA	−119	Staphylococcal enterotoxin-like toxin
NWMNN_0394	*set7nm*	−144	GTTCAAAAATAGTTAA	−129	Staphylococcal enterotoxin-like toxin
		−133	GTTAAAAAGAGGTTAA	−118	
NWMNN_0395	*set8nm*	−110	ATTAAACGAGTGTTAA	−95	Staphylococcal enterotoxin-like toxin
		−141	GTTAATGAAGAGCTAA	−126	
		−130	CTTAAATCATTGTTAA	−115	
NWMNN_0396	*set9nm*	−175	ATTAAAAATCAGTTAA	−160	Staphylococcal enterotoxin-like toxin
		−110	TTTAAATGAGCGTTAA	−95	
NWMNN_0397	*set10nm*	−110	TTTAAATCGAGGTTAA	−95	Staphylococcal enterotoxin-like toxin
NWMNN_0400	*set11nm*	−231	GTTAAATAAAGATTAA	−216	Staphylococcal enterotoxin-like toxin
		−153	GTTAACTATTTATTAA	−138	
		−123	ATTAATTTTTAGTTAA	−108	
		−112	GTTAAAGTAAGTTTAA	−97	
		−152	TTTAATAAATAGTTAA	−137	
NWMNN_2619		−190	GTTTAATAGAGGTTAA	−175	Phenol-soluble modulin alpha 1
NWMNN_0758	*ssp/emp*	−126	GTTAAGACAACGTTTA	−111	Extracellular matrix and plasma binding protein
		−115	GTTTACTTCAAGTTAA	−100	
NWMNN_1066		−99	ATTAATGTTTAGTTAA	−84	Similar to fibrinogen binding protein
		−88	GTTAATAAATAGTTAA	−73	
NWMNN_1069	*efb*	−81	ATTAATAATTAGTTAA	−66	Similar to fibrinogen binding protein
NWMNN_1070		−76	TTTAATGACAGGTTAA	−61	Similar to fibrinogen binding protein
NWMNN_1073	*hla*	−405	GTTAATATATAGTTAA	−390	Alpha-hemolysin precursor
		−190	TTTAAATAAAAGTTAA	−175	
NWMNN_1084		−171	TTTAAAATACAGTTAA	−156	Phenol-soluble modulin beta 1
NWMNN_1716	*bsaA2*	−115	GTTAATTTTTTGTAAA	−100	Lantibiotic precursor
NWMNN_1719	*lukE*	−334	GTTAATAATCAGTTAA	−319	Leukocidin LukE precursor
NWMNN_1600		−178	GTTATAATGTAGTTAA	−163	Universal stress protein family protein
		−86	TTTAATGAACAGTTAA	−71	
NWMNN_1664		−357	GTTAAAATATTTTTAA	−342	Arsenical resistance operon repressor
		−356	GTTAAAAATATTTTAA	−341	
NWMNN_1872[b]	*map/eap*	−88	ATTAATATTCAGTTAA	−73	MHC[c] class II analog protein
NWMNN_1873[b]	*hlb*	−307	ATTAATATTCAGTTAA	−292	Truncated beta-hemolysin

Continued on following page

TABLE 4—*Continued*

Gene function and ID[a]	Name	Start	Sequence	End	Product
NWMNN_1876	*scn*	−77	GTTAATGAATAATTAA	−62	Staphylococcal complement inhibitor SCIN
NWMNN_1877	*chp*	−144	TTTAATTTTTAGTTAA	−129	Chemotaxis-inhibiting protein CHIPS
		−112	ATTAATTTCAAGTTAA	−97	
NWMNN_1880	*sak*	−175	GTTAAATATTTGTTAA	−160	Staphylokinase precursor
		−164	GTTAATTATTTTTTAA	−149	
NWMNN_1928[b]	*lukS*	−99	TTTAATAAATAGTTAA	−84	Leukocidin/hemolysin toxin family S subunit
NWMNN_2061	*fmtB /mrp/sasB*	−61	CTTAATCAACTGTTAA	−46	Methicillin resistance determinant FmtB protein,
NWMNN_2317	*sbi*	−92	GTTAATAATTAGTTAA	−77	IgG binding protein Sbi
NWMNN_2318	*hlgA*	−346	GTTAACGAATAGTTCA	−331	Gamma-hemolysin component A
		−85	TTTAACGAACAGTTAA	−70	
		−84	ATTAACTGTTCGTTAA	−69	
NWMNN_2319	*hlgC*	−351	GTTAATAAACAATTAA	−336	Gamma-hemolysin component C
		−86	GTTAATGAACAGTTAA	−71	
NWMNN_2397	*fnbB*	−114	GTTAATAAAAAGTTAA	−99	Fibronectin binding protein B precursor
NWMNN_2399	*fnbA*	−137	ATTAATTTTATGTTAA	−122	Fibronectin binding protein A precursor
		−114	GTTAATGAAAAGTTAA	−99	
NWMNN_2586[b]	*drp35*	−219	GTTATAAGCATGTTAA	−204	Drp35
Cell envelope					
NWMNN_0606	*tagA*	−141	GTTAAACTAATTTTAA	−126	Teichoic acid biosynthesis protein A
NWMNN_1882		−183	GTTAAATGGTTATTAA	−168	Phage holin
NWMNN_2051		−163	GTTGAAAATTTGTTAA	−148	Lytic regulatory protein
NWMNN_2395	*gtaB*	−55	GTTAAAATGACGTTGA	−40	UTP-glucose-1-phosphateuridyl transferase
Transcription					
NWMNN_0038		−110	GTTATCATAGAGTTAA	−95	Similar to LysR family regulatory protein
NWMNN_0636		−82	GTTAAATTTACGTTTA	−67	AraC family regulatory protein
NWMNN_0677	*saeP*	−99	GTTAAGCGATATTTAA	−84	Conserved hypothetical protein
		−78	GTTAAGAATTAGTTAA	−63	
NWMNN_0925		−31	ATTAAATTTTTGTTAA	−16	Similar to cell envelope-related transcriptional attenuator
NWMNN_1328	*arlR*	−169	GTTAATAAATAATTAA	−154	Two-component RR
Translation					
NWMNN_0490	*gltX*	−321	GTTAATTTGAAGTTTA	−306	Glutamyl-tRNA synthetase
		−139	TTTAAGGTCATGTTAA	−124	
NWMNN_0723	*prfB*	−336	GTGAAGAAGTTGTTAA	−321	Peptide chain release factor 2
NWMNN_1478	*rpsU*	−243	GTTAAAACAACGTTAC	−228	30S ribosomal protein S21
NWMNN_1488[b]	*rpsT*	−81	GTTAGACTTTTGTTAA	−66	30S ribosomal protein S20

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TABLE 4—*Continued*

Gene function and ID[a]	Name	Start	Sequence	End	Product
NWMNN_1519	*alaS*	−290	GTTAATGATTTGTTTA	−275	Alanyl-tRNA synthetase
NWMNN_1533	*hisS*	−260	GTTAAACGTACGTTAA	−245	Histidyl-tRNA synthetase
Protein metabolism					
NWMNN_0824	*ppi*	−50	CTTAAAAGTATGTTAA	−35	Cyclophilin type peptidyl-prolyl cis-trans isomerase
NWMNN_0957	*pdf1*	−59	GTAAAAAGGTTGTTAA	−44	Polypeptide deformylase 2
NWMNN_1057	*trxA*	−143	GTTAAAATAATGTAAA	−128	Thioredoxin
NWMNN_1539	*secDF*	−157	GTTAAATTTAAATTAA	−142	Preprotein translocase component SecDF
NWMNN_1706[b]	*splA*	−121	TTTAATAAAACGTTAA	−106	Serine protease SplA
		−110	GTTAATAAGTGGTTAA	−95	
		−99	GTTAATTAATATTTAA	−84	
NWMNN_2203	*ssaA*	−212	ATTAATTATCTGTTAA	−197	Secretory antigen precursor SsaA
NWMNN_2354		−331	TTTAATGATAAGTTAA	−316	Glutamyl-aminopeptidase
		−276	GTTAATGCGTTGTTAT	−261	
Ion transport and metabolism					
NWMNN_0049		−73	GTTAAGATTAGGTAAA	−58	Similar to Na^+ Pi-cotransporter
		−62	GTAAATTTAATGTTAA	−47	
NWMNN_0071		−115	TTTAAGGTATAGTTAA	−100	Glucose/ribitol dehydrogenase
NWMNN_0123		−371	ATTAAGAATTTGTTAA	−356	Similar to surfactin synthetase
NWMNN_0130		−172	GTTAAATCGTTCTTAA	−157	Branched-chain amino acid transport system II carrier protein
		−171	GTTAAGAACGATTTAA	−156	
NWMNN_0158	*uhpT*	−130	ATTAATAAATAGTTAA	−115	Sugar phosphate transport protein
NWMNN_0418	*ndhF*	−345	GTTTAATAGAGGTTAA	−330	NADH dehydrogenase subunit 5
NWMNN_0476	*folP*	−17	ATTAAAGGGTGGTTAA	−2	Dihydropteroate synthase chain A
		−16	GTTAACCACCCTTTAA	−1	
NWMNN_0577	*adh1*	−370	ATTAATCTGTAGTTAA	−355	Alcohol dehydrogenase
NWMNN_0623		−283	GTTTAAACCTTGTTAA	−268	Similar to branched-chain amino acid transportsystem II carrier protein
NWMNN_0630	*vraF*	−80	GTTAGTCATATGTTAA	−65	ABC transporter ATP binding protein VraF
NWMNN_0690		−149	GGTAATCTCCAGTTAA	−134	Osmoprotectant ABC transporter, ATP binding protein
NWMNN_0705		−84	ATTAAAGAAGGGTTAA	−69	Ferrichrome ABC transporter lipoprotein
NWMNN_0853		−110	GTTAATAAAATTTTAA	−95	3-Oxoacyl-(acyl-carrier-protein) synthase III
NWMNN_1040	*isdB*	−165	GTTAAATAAAATTTAA	−150	Iron-regulated heme-iron binding protein IsdB
NWMNN_1060	*sdhC*	−65	GTTAAGCGTACGTTTA	−50	Succinate dehydrogenase cytochrome b558 subunit
NWMNN_1078[b]	*argF*	−359	GCTAAAACTATGTTAA	−344	Ornithine carbamoyltransferase

Continued on following page

TABLE 4—*Continued*

Gene function and ID[a]	Name	Start	Sequence	End	Product
NWMNN_1300	*pstS*	−129	GTTAAGAAAATGTAAA	−114	Phosphate ABC transporter, phosphate binding protein PstS
NWMNN_1305	*asd*	−61	GTAAAATGATTGTTAA	−46	Aspartate-semialdehyde dehydrogenase
NWMNN_1331		−80	GTTTAAAGCATGTTAA	−65	Acetyltransferase, GNAT family protein
		−168	ATTAATTGATTGTTAA	−153	
NWMNN_1337	*dfrA*	−58	GTTAAATTAAAGATAA	−43	Dihydrofolate reductase
NWMNN_1349	*ald*	−122	GTTAATAAATATTTAA	−107	Alanine dehydrogenase 2
NWMNN_1412	*zwf*	−194	GTTATGTCTACGTTAA	−179	Glucose-6-phosphate 1-dehydrogenase
NWMNN_1432	*accB*	−190	TTTAATCAAGAGTTAA	−175	Acetyl coenzyme A carboxylase, biotin carboxyl carrier protein
NWMNN_1453		−145	GTTAATTTAAATTTAA	−130	5-Formyltetrahydrofolate cyclo-ligase
NWMNN_1500		−16	GGTAAAGGTGAGTTAA	−1	Hydrolase, HAD-superfamily, subfamily IIIA
NWMNN_1593	*pfk*	−47	GGTAATTTAGAGTTAA	−32	6-Phosphofructokinase
NWMNN_1595	*accD*	−188	TTTAATTAAACGTTAA	−173	Acetyl coenzyme A carboxylase beta subunit
		−76	GTTAAAAACGGGTTTA	−61	
NWMNN_1871		−22	TTTAAAGAGAGGTTAA	−7	Aspartate transaminase
NWMNN_1929[b]		−288	TTTAATAAATAGTTAA	−273	Similar to succinyl-diaminopimelate desuccinylase
NWMNN_2047	*manA1*	−194	GTTAAAGTACTGTAAA	−179	Mannose-6-phosphate isomerase, class I
NWMNN_2201		−122	GTTAATTTAAGGTTAT	−107	Dehydrogenase family protein
NWMNN_2206		−240	GTTAAATGGATTTTAA	−225	Monooxygenase family protein
		−239	GTTAAAATCCATTTAA	−224	
NWMNN_2271		−82	GTTAATGTATTTTTAA	−67	Acetyltransferase, GNAT family protein
NWMNN_2272		−124	GTTAATGTATTTTTAA	−109	Zinc-containing alcohol dehydrogenase
NWMNN_2592		−220	GTTAACAACACGTTAA	−205	2-Oxoglutarate/malate translocator
		−219	GTTAACGTGTTGTTAA	−204	
NWMNN_2467		−107	CTTAATTAAGGGTTAA	−92	*O*-Acetyltransferase OatA
NWMNN_2526	*phoB*	−87	GTTAAAAATATGTAAA	−72	Alkaline phosphatase III precursor
DNA replication/ recombination/repair					
NWMNN_0090		−27	GGTAATAGCCGGTTAA	−12	Similar to replication initiation protein
NWMNN_0448	*holB*	−170	GTTAATAACTTATTAA	−155	DNA polymerase III delta subunit
NWMNN_1364	*dnaD*	−36	ATTAAGTAATGGTTAA	−21	DNA replication protein DnaD
Nucleotide metabolism					
NWMNN_0249		−37	TTTAAAATACAGTTAA	−22	5′ nucleotidase, lipoprotein e(P4) family protein
NWMNN_0699	*nrdI*	−310	GTTAAATTACTTTTAA	−295	Ribonucleotide reduction-related NrdI protein

Continued on following page

TABLE 4—*Continued*

Gene function and ID[a]	Name	Start	Sequence	End	Product
NWMNN_0809		−86	GTTAATGAACAGTTAT	−71	Similar to pyridine nucleotide-disulfide oxidoreductase
NWMNN_1118		−345	GTTAATTATCCGCTAA	−330	Pseudouridylate synthase
		−249	ATTAAAATTAAGTTAA	−234	
NWMNN_1119[b]	*pryR*	−54	GTTACAATCATGTTAA	−39	Pyrimidine operon regulatory protein
NWMNN_1120[b]	*gmk*	−236	GTTACAATCATGTTAA	−221	Guanylate kinase
NWMNN_1143	*rnc*	−52	TTTAATTTTACGTTAA	−37	RNase III
NWMNN_1185	*ftsK*	−42	GTTAAAACGACGTTAT	−27	DNA translocase FtsK/SpoIIIE family protein
NWMNN_1411		−134	GTTATGTCTACGTTAA	−119	Similar to RNase Z
NWMNN_2091		−55	GTTACTTAAGCGTTAA	−40	Similar to quinone oxidoreductase
Unknown function					
NWMNN_0039		−49	GTTAAAATAGCATTAA	−34	Conserved hypothetical protein
NWMNN_0087		−86	GTAAATTTTTCGTTAA	−71	Conserved hypothetical protein
NWMNN_0157		−22	ATTAATAAATAGTTAA	−7	Conserved hypothetical protein
NWMNN_0165[b]		−123	GTTAATATATAGTTAA	−108	Conserved hypothetical protein
		−102	GTTAATGCTTTGTTTA	−87	
NWMNN_0295		−55	GTTACAAAGAGGTTAA	−40	Similar to ORF041 of Bacteriophage 96
NWMNN_0362		−95	GTTAATCAAGAGTTAA	−80	Conserved hypothetical protein
		−84	GTTAAGATGAATTTAA	−69	
NWMNN_0370		−43	GTTAAGATGCTGTTAT	−28	Conserved hypothetical protein
NWMNN_0387		−378	GTTAAATGAGGTTTAA	−363	Conserved hypothetical protein
NWMNN_0402[b]		−90	TTTAATAAATAGTTAA	−75	Conserved hypothetical protein
NWMNN_0403[b]	*lpl1nm*	−254	TTTAATAAATAGTTAA	−239	Staphylococcal tandem lipoprotein
NWMNN_0503		−103	GTTAAAACCCCCGTTAT	−88	Similar to small methyltransferase
NWMNN_0546		−109	CTTAATAGCTTGTTAA	−94	Conserved hypothetical protein
NWMNN_0605		−98	GTTAAACTAATTTTAA	−83	Conserved hypothetical protein
NWMNN_0632		−239	GTTAAAAATTTGTAAA	−224	Conserved hypothetical protein
NWMNN_0687		−250	GTTAATTGAATGATAA	−235	Conserved hypothetical protein
		−181	GTTAAATCAAAGTTGA	−166	
NWMNN_0746		−147	GTTAAATATGTGTTAT	−132	Conserved hypothetical protein
NWMNN_0759		−126	GTTAATAGTTTGTTCA	−111	Conserved hypothetical protein
		−115	GTTCATCGCAAGTTAA	−100	
NWMNN_0810		−246	GTTAATGAACAGTTAT	−231	Conserved hypothetical protein
NWMNN_0823		−29	CTTAAAAGTATGTTAA	−14	Conserved hypothetical protein
NWMNN_0834		−164	GTTAAGAGAAAATTAA	−149	Conserved hypothetical protein
NWMNN_0956		−262	GTTAACAATAAATTAA	−247	Similar to Zn-dependent hydrolase
		−261	TTTAATTTATTGTTAA	−246	

Continued on following page

TABLE 4—*Continued*

Gene function and ID[a]	Name	Start	Sequence	End	Product
NWMNN_0958		−321	GTAAAAAGGTTGTTAA	−306	Conserved hypothetical protein
NWMNN_1059		−56	GTTACAATATGGTTAA	−41	hypothetical protein
NWMNN_1067		−99	ATTAATGAAGTGTTAA	−84	Conserved hypothetical protein
		−98	GTTAACACTTCATTAA	−83	
NWMNN_1072		−46	GTTAAACTTAAGTTGA	−31	Conserved hypothetical protein
NWMNN_1077[b]		−85	GCTAAAACTATGTTAA	−70	Conserved hypothetical protein
NWMNN_1152[b]		−221	GTTAAATCTAAGTTAC	−206	Conserved hypothetical protein
NWMNN_1153[b]		−195	GTTAAATCTAAGTTAC	−180	Similar to GTP binding protein
NWMNN_1487[b]		−280	GTTAGACTTTTGTTAA	−265	GTP binding protein LepA
NWMNN_1599		−42	GTTATAATGTAGTTAA	−27	CBS domain DNA binding protein
NWMNN_1663		−182	GTTAAAAATATTTTAA	−167	Conserved hypothetical protein
		−181	GTTAAAATATTTTTAA	−166	
NWMNN_1669		−31	TTTAATAAGTTGTTAA	−16	Conserved hypothetical protein
NWMNN_1707[b]		−376	GTTAATTAATATTTAA	−361	Hypothetical protein
		−365	GTTAATAAGTGGTTAA	−350	
		−354	TTTAATAAAACGTTAA	−339	
NWMNN_1708		−109	GTTAATAGATAGTTAA	−94	Conserved hypothetical protein, homologous to ear (MW0758)
NWMNN_1859		−71	GATAAATGATTGTTAA	−56	Conserved hypothetical protein
NWMNN_1860		−214	GATAAATGATTGTTAA	−199	Conserved hypothetical protein
NWMNN_2075		−219	GTTTAAAAAAGGTTAA	−204	Conserved hypothetical protein
NWMNN_2088		−23	GTTAAAGTTAATTTAA	−8	Conserved hypothetical protein
NWMNN_2202		−141	GTTAATTTAAGGTTAT	−126	Conserved hypothetical protein
NWMNN_2228		−109	GTTAAGATGATGTAAA	−94	Conserved hypothetical protein
NWMNN_2408		−184	GTTAAGCGGAATTTAA	−169	Conserved hypothetical protein
NWMNN_2409		−378	GTTAAGCGGAATTTAA	−363	DedA family protein
NWMNN_2415		−183	GTTATAATACAGTTAA	−168	Conserved hypothetical protein
NWMNN_2432		−74	GTTAAGTTGATGTAAA	−59	Conserved hypothetical protein
NWMNN_2436		−46	TTTAAAATCATGTTAA	−31	Conserved hypothetical protein
NWMNN_2508		−202	GTTAATGTGTTGTTCA	−187	Conserved hypothetical protein
NWMNN_2525		−126	GTTAAAAATATGTAAA	−111	Hypothetical protein
NWMNN_2587[b]		−40	GTTATAAGCATGTTAA	−25	Similar to rhodanese family protein

[a] The start point and end point are the distance from the translation start codon. Conserved repeat sequences are underllined.
[b] This gene shares the same repeat sequence with an adjacent gene.
[c] MHC, major histocompatibility complex.

scription of many important virulence genes. However, the molecular mechanism of this activation has not yet been elucidated. Here, we report that SaeR binds to the direct repeat sequence $GTTAAN_6GTTAA$ and phosphorylation is essential for DNA binding.

Several lines of data support our claim that $GTTAAN_6GTTAA$ is the SaeR binding site. First, in DNase footprinting assays, the sequence was found in the P1 promoter regions protected by either $SaeR^C$ or P-SaeR (Fig. 5). Second, the sequence was identified in the promoter region of 36 known

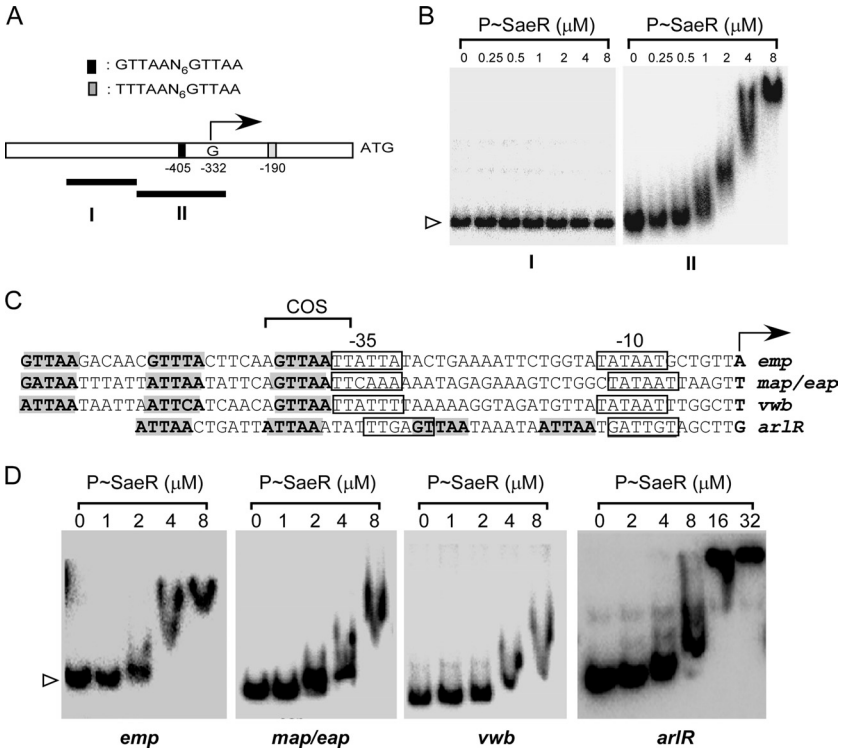

FIG. 6. DNA binding of P-SaeR to various staphylococcal promoters. (A) Schematic map of the *hla* promoter. The perfect repeat sequence is represented by a black box, and the imperfect repeat sequence is represented by a gray box. The sequences of the repeats are also shown above the map, where N represents any nucleotide. Values represent distances from the ATG start codon, which is shown to the right. The transcription start site (G) is shown by a right-angled arrow. The two DNA probes used for EMSA are shown as solid lines with the names under them. (B) DNA binding of P-SaeR to two different regions of the *hla* promoter. The concentration of P-SaeR is indicated at the top. Unbound free DNA probe is indicated by a white arrowhead. I and II represent the *hla* promoter regions shown in panel A. (C) The direct repeat sequences in the promoters of *emp*, *map*/*eap*, *vwb*, and *arlR*. The transcription sites are indicated by a boldfaced letter and a right-angled arrow. The −10 and −35 regions of the promoters are indicated by solid boxes. The direct repeat sequences are in boldface and shaded gray. (D) DNA binding of P-SaeR to the *emp*, *map*/*eap*, *vwb*, and *arlR* promoters. Protein concentrations are indicated at the top. For clarity, only unbound free DNA is indicated.

members of the *sae* regulon (Table 4). Third, although all of the DNA fragments containing the repeat sequence bound to SaeR, a DNA fragment without the repeat sequence did not (Fig. 6). Lastly, mutations of the repeat sequence severely reduced DNA binding to SaeR (Fig. 7).

Interestingly, all of the genes containing the SaeR binding site in their promoter regions have not been reported to be regulated by the SaeRS system. Of the 155 genes containing the SaeR binding site, only 36 (23%) have been reported to be regulated by SaeRS. One possible explanation for the discrepancy is that some of the genes may be genuinely regulated by the SaeRS system but the regulation has not been detected yet. So far, the *sae* regulon has been identified mainly by microarray assay or proteomic analysis. These methods might not be sensitive enough to detect all transcriptional changes. Therefore, in the future, more-sensitive techniques such as high-throughput real-time quantitative reverse transcriptase-mediated PCR might identify new genes of the *sae* regulon. The other possibility is that the binding affinity of some of the SaeR binding sites is too low for them to be genuine targets of *sae*-mediated regulation under physiologically relevant conditions. In our P1 promoter mutagenesis study, single nucleotide changes in the binding site could abolish binding to SaeR and promoter activity. Therefore, many of the imperfect SaeR binding sites may not bind to SaeR *in vivo*. For example, the *arlR* promoter clearly can bind to SaeR at a high concentration of purified P-SaeR *in vitro* (Fig. 6D); but the *arlRS* operon is not regulated by the *sae* two-component system (45). Therefore, the *arlR* binding shown in Fig. 6D might not be physiologically relevant *in vivo*. However, some of the well-known *sae* regulon genes in Table 4, such as *efb*, *hlb*, and *lukS*, have only one imperfect SaeR binding site, suggesting that not only the motif sequence itself but also the context of the motif (e.g., distance from the promoter element, etc.) might play a role in selecting the targets of SaeR binding and *sae*-mediated regulation.

Conversely, the SaeRS system seems to affect the transcription of the genes that do not contain the SaeR binding site. For example, among the 212 genes of the *sae* regulon identified by microarray analysis of strain MW2, 176 do not contain the SaeR binding site. How, then, could the SaeRS system have altered the transcription of these genes? One possibility is that the SaeRS system might affect the expression of some of the 176 genes indirectly. For example, as shown in Table 4, four transcriptional regulators (NWMN_0038, NWMN_0636, NWMN_0925, and *arlR*) have the SaeR binding site in their promoter regions. Although *arlR* is not involved in downstream regulation by *sae*, the other three regulators might play

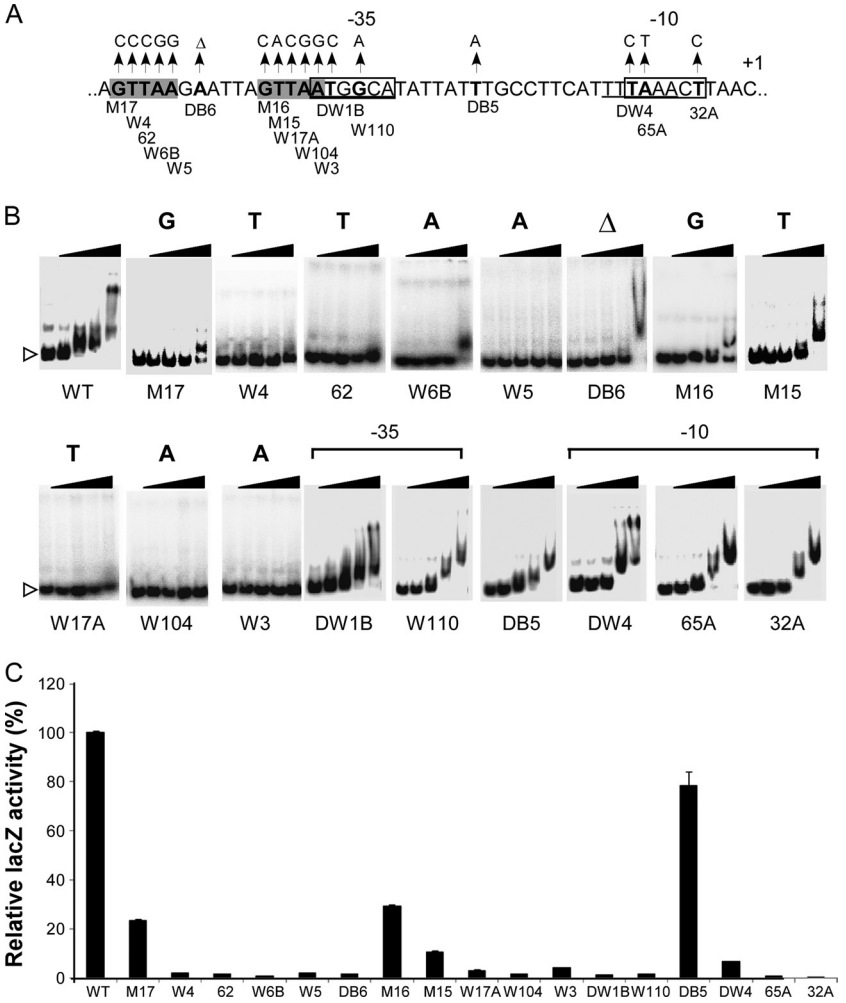

FIG. 7. Effects of P1 promoter mutations on binding to P-SaeR and promoter function. (A) Summary of the mutations in the P1 promoter. Each mutation is indicated by an arrow with the name under the sequence. The −10 and −35 regions are in a solid box. The direct repeat sequence is in boldface and shaded gray. +1, transcription start site; Δ, a deletion mutation. (B) Binding of the mutant promoters to P-SaeR. Increasing amounts of P-SaeR (0, 1, 2, 4, and 8 μM) were used for the binding assay. The mutated nucleotides of the direct repeat and mutated promoter regions are shown at the top. Unbound free DNA probe is indicated by a white arrowhead. Δ, a deletion mutation. (C) Activities of the mutated P1 promoters represented by LacZ expression. The values presented are relative LacZ expression, where the LacZ expression from the wild-type promoter was set to 100%. The *lacZ* assays were repeated two or three times with similar results. Error bars represent standard deviations. WT, wild type.

a role in it. Since the transcriptional regulatory systems in *S. aureus* are interconnected and form a very complex network, transcriptional changes in a regulator protein can be transmitted or amplified via the network. In addition, since the *sae* regulon includes not only virulence genes but also genes involved in various cellular processes such as protein synthesis, ion transport, and energy metabolism (48), we can envision that alterations in those processes might indirectly cause transcriptional changes in other genes. Clearly, more studies are required to identify direct targets and the mechanism of indirect regulation.

In a previous study, Harraghy et al., identified a COS (conserved octanucleotide sequence, AGTTAATT) in 11 genes, of which 6 are genes of the *sae* regulon such as *eap*, *emp*, and *efb* (24). In *emp*, a mutational change of three nucleotides (**AGT** TAATT to **TCA**TAATT, where boldface and underlining indicate the mutated nucleotides and SaeR binding sequences, respectively) decreased *emp* expression by more than half (24). As shown in Fig. 6C, the COS overlaps the SaeR binding site, and the mutational change in the COS in this study also changed two nucleotides in the SaeR binding site from **GT**-TAA to **CA**TAA. Since we observed in our P1 mutagenesis study that even a single nucleotide change could abolish DNA binding to P-SaeR, we suspect that the two nucleotide changes in this study also abolished DNA binding to SaeR. Therefore, we think the effect of the COS mutation on *emp* expression is due to the mutated promoter region's inability to bind to SaeR. In fact, in our analysis of the 11 COS-containing genes, 9 genes (*eap*, *emp*, *vwb*, *efb*, *hlgA*, *hlgC*, *sbi*, *lukE*, and *lukD*) have one or more SaeR binding sites (either perfect or imperfect). Therefore, we propose that COS is a part of the SaeR binding site. In their study, the authors reported that neither unphosphorylated SaeR nor the DNA binding domain of SaeR bound to the three promoters (i.e., *emp*, *map*/*eap*, and *vwb*). In our

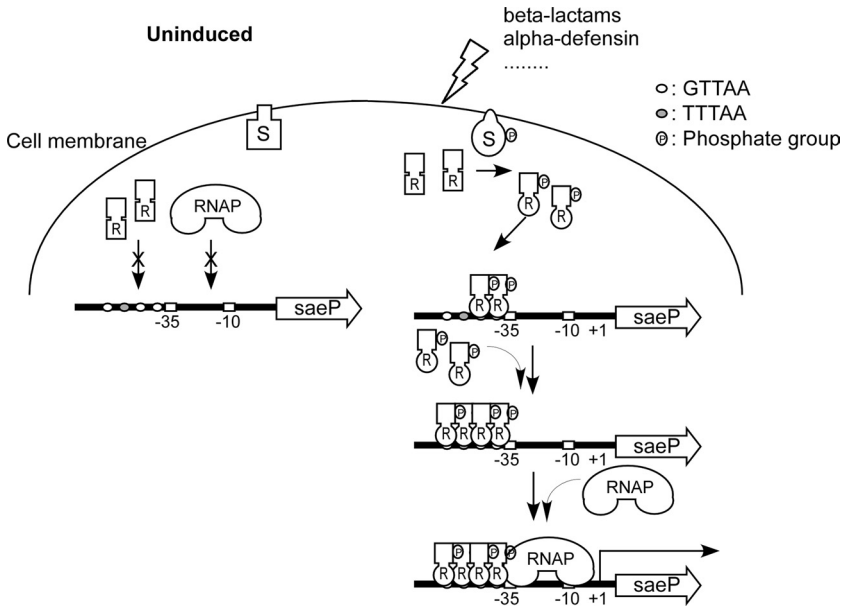

FIG. 8. Model of P1 promoter activation by the SaeRS system. RNAP, RNA polymerase.

study, however, although the binding affinity was very low (K_D = 6 μM), the DNA binding domain (i.e., $SaeR^C$) clearly bound to its target, the P1 promoter (Fig. 2B). It is possible that, compared with the P1 promoter, the three promoters tested previously have lower binding affinities for $SaeR^C$, which, in turn, made it more difficult to detect protein-DNA binding.

In this study, we demonstrated that phosphorylation is essential for SaeR binding to its target DNA (Fig. 2). Recently, Mainiero et al. also showed that SaeR with a mutation at the phosphorylation site (D51N) could not activate transcription from the *hla* promoter (39), further confirming the essential role of SaeR phosphorylation in transcriptional regulation. This essentiality of phosphorylation differentiates the SaeRS two-component system from the VraSR (vancomycin resistance-associated sensor/regulator) system, a well-studied staphylococcal two-component system that coordinates cellular responses to antibiotics inhibiting cell wall synthesis (6, 12, 31). VraR, the RR of the VraSR system, recognizes the DNA motif $ACT(X)_nAGT$ (where X is any nucleic acid and n = 1 to 3); however, phosphorylation is not required for VraR to bind to the most conserved binding site (7). On the other hand, VraR binding to the less conserved secondary binding site does require phosphorylation. Interestingly, as with the primary SaeR binding site, the secondary binding site of VraR in the *vraRS* operon overlaps the −35 region and direct interaction between VraR and sigma factor was postulated (7). The different roles of phosphorylation in the SaeRS and VraSR systems suggest that the signaling mechanism of staphylococcal two-component systems might be diverse and cannot be generalized to one model.

The P1 promoter contains a secondary SaeR binding site with an imperfect repeat sequence ($GTTAAN_6TTTAA$). This secondary site is separated from the primary SaeR binding site ($GTTAAN_6GTTAA$) by 5 nt (Fig. 5B). The EMSA and DNase footprinting analysis results suggest that P-SaeR binds to the secondary binding site as well. In EMSA, two shifted bands were observed, suggesting the existence of two different species of protein-DNA complex (Fig. 2C). In addition, in DNase I footprinting assays, P-SaeR protected not only the primary binding sites but also the secondary binding site (Fig. 5). We envision that P-SaeR binds to the primary binding site first, generating the lower shift band in Fig. 2C, and recruits P-SaeR to the secondary binding site, generating the higher band in EMSA (Fig. 2C). The occupation of the secondary binding site might stabilize P-SaeR binding to the promoter such that P-SaeR can recruit RNA polymerase to the promoter through protein-protein interactions. The close proximity of the primary SaeR binding site and the −35 region resembles the class II promoters in *E. coli*, where the transcription regulator interacts with sigma factor 70 (5, 35). Therefore, we postulate that the interaction between P-SaeR and the sigma factor recruits RNA polymerase to the P1 promoter. Combining all of the data, we propose, for the first time, a model for the SaeRS signal transduction mechanism in the P1 promoter (Fig. 8). Under uninduced conditions, SaeS is not active and does not phosphorylate SaeR. However, when external environmental stressors (e.g., β-lactam antibiotics or alpha-defensin) are present, SaeS experiences conformational changes and autophosphorylation and then transfers the phosphate group to D51 of SaeR. Phosphorylated SaeR binds to the primary SaeR binding domains, forming a dimer, and recruits more P-SaeR to the secondary binding site. As a result, the SaeR-P1 promoter complex becomes stable and recruits RNA polymerase to the promoter by interactions between SaeR and sigma factor σ^A, resulting in transcription initiation at the promoter.

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REFERENCES

1. **Adhikari, R. P., and R. P. Novick.** 2008. Regulatory organization of the staphylococcal *sae* locus. Microbiology **154:**949–959.
2. **Archer, G. L.** 1998. *Staphylococcus aureus*: a well-armed pathogen. Clin. Infect. Dis. **26:**1179–1181.
3. **Bae, T., T. Baba, K. Hiramatsu, and O. Schneewind.** 2006. Prophages of *Staphylococcus aureus* Newman and their contribution to virulence. Mol. Microbiol. **62:**1035–1047.
4. **Bae, T., A. K. Banger, A. Wallace, E. M. Glass, F. Aslund, O. Schneewind, and D. M. Missiakas.** 2004. *Staphylococcus aureus* virulence genes identified by bursa aurealis mutagenesis and nematode killing. Proc. Natl. Acad. Sci. U. S. A. **101:**12312–12317.
5. **Barnard, A., A. Wolfe, and S. Busby.** 2004. Regulation at complex bacterial promoters: how bacteria use different promoter organizations to produce different regulatory outcomes. Curr. Opin. Microbiol. **7:**102–108.
6. **Belcheva, A., and D. Golemi-Kotra.** 2008. A close-up view of the VraSR two-component system. A mediator of *Staphylococcus aureus* response to cell wall damage. J. Biol. Chem. **283:**12354–12364.
7. **Belcheva, A., V. Verma, and D. Golemi-Kotra.** 2009. DNA-binding activity of the vancomycin resistance associated regulator protein VraR and the role of phosphorylation in transcriptional regulation of the vraSR operon. Biochemistry **48:**5592–5601.
8. **Bischoff, M., P. Dunman, J. Kormanec, D. Macapagal, E. Murphy, W. Mounts, B. Berger-Bachi, and S. Projan.** 2004. Microarray-based analysis of the *Staphylococcus aureus* sigmaB regulon. J. Bacteriol. **186:**4085–4099.
9. **Bjerketorp, J., M. Nilsson, A. Ljungh, J. I. Flock, K. Jacobsson, and L. Frykberg.** 2002. A novel von Willebrand factor binding protein expressed by *Staphylococcus aureus*. Microbiology **148:**2037–2044.
10. **Cheung, A. L., K. A. Nishina, M. P. Trotonda, and S. Tamber.** 2008. The SarA protein family of *Staphylococcus aureus*. Int. J. Biochem. Cell Biol. **40:**355–361.
11. **Chien, Y., A. C. Manna, S. J. Projan, and A. L. Cheung.** 1999. SarA, a global regulator of virulence determinants in *Staphylococcus aureus*, binds to a conserved motif essential for sar-dependent gene regulation. J. Biol. Chem. **274:**37169–37176.
12. **Cui, L., H. M. Neoh, M. Shoji, and K. Hiramatsu.** 2009. Contribution of *vraSR* and *graSR* point mutations to vancomycin resistance in vancomycin-intermediate *Staphylococcus aureus*. Antimicrob. Agents Chemother. **53:**1231–1234.
13. **Donnelly, M. I., M. Zhou, C. S. Millard, S. Clancy, L. Stols, W. H. Eschenfeldt, F. R. Collart, and A. Joachimiak.** 2006. An expression vector tailored for large-scale, high-throughput purification of recombinant proteins. Protein Expr. Purif. **47:**446–454.
14. **Duthie, E. S., and L. L. Lorenz.** 1952. Staphylococcal coagulase; mode of action and antigenicity. J. Gen. Microbiol. **6:**95–107.
15. **Fournier, B., and A. Klier.** 2004. Protein A gene expression is regulated by DNA supercoiling which is modified by the ArlS-ArlR two-component system of *Staphylococcus aureus*. Microbiology **150:**3807–3819.
16. **Fournier, B., A. Klier, and G. Rapoport.** 2001. The two-component system ArlS-ArlR is a regulator of virulence gene expression in *Staphylococcus aureus*. Mol. Microbiol. **41:**247–261.
17. **Geiger, T., C. Goerke, M. Mainiero, D. Kraus, and C. Wolz.** 2008. The virulence regulator Sae of *Staphylococcus aureus*: promoter activities and response to phagocytosis-related signals. J. Bacteriol. **190:**3419–3428.
18. **Giraudo, A. T., A. Calzolari, A. A. Cataldi, C. Bogni, and R. Nagel.** 1999. The sae locus of *Staphylococcus aureus* encodes a two-component regulatory system. FEMS Microbiol. Lett. **177:**15–22.
19. **Giraudo, A. T., A. L. Cheung, and R. Nagel.** 1997. The sae locus of *Staphylococcus aureus* controls exoprotein synthesis at the transcriptional level. Arch. Microbiol. **168:**53–58.
20. **Giraudo, A. T., C. Mansilla, A. Chan, C. Raspanti, and R. Nagel.** 2003. Studies on the expression of regulatory locus sae in *Staphylococcus aureus*. Curr. Microbiol. **46:**246–250.
21. **Giraudo, A. T., C. G. Raspanti, A. Calzolari, and R. Nagel.** 1994. Characterization of a Tn551-mutant of *Staphylococcus aureus* defective in the production of several exoproteins. Can. J. Microbiol. **40:**677–681.
22. **Goerke, C., U. Fluckiger, A. Steinhuber, V. Bisanzio, M. Ulrich, M. Bischoff, J. M. Patti, and C. Wolz.** 2005. Role of *Staphylococcus aureus* global regulators *sae* and sigmaB in virulence gene expression during device-related infection. Infect. Immun. **73:**3415–3421.
23. **Goerke, C., U. Fluckiger, A. Steinhuber, W. Zimmerli, and C. Wolz.** 2001. Impact of the regulatory loci agr, sarA and sae of *Staphylococcus aureus* on the induction of alpha-toxin during device-related infection resolved by direct quantitative transcript analysis. Mol. Microbiol. **40:**1439–1447.
24. **Harraghy, N., D. Homerova, M. Herrmann, and J. Kormanec.** 2008. Mapping the transcription start points of the *Staphylococcus aureus eap*, *emp*, and *vwb* promoters reveals a conserved octanucleotide sequence that is essential for expression of these genes. J. Bacteriol. **190:**447–451.
25. **Harraghy, N., J. Kormanec, C. Wolz, D. Homerova, C. Goerke, K. Ohlsen, S. Qazi, P. Hill, and M. Herrmann.** 2005. sae is essential for expression of the staphylococcal adhesins Eap and Emp. Microbiology **151:**1789–1800.
26. **Hoch, J. A.** 2000. Two-component and phosphorelay signal transduction. Curr. Opin. Microbiol. **3:**165–170.
27. **Hoch, J. A., and T. J. Silhavy.** 1995. Two-component signal transduction. ASM Press, Washington, DC.
28. **Ji, Y., A. Marra, M. Rosenberg, and G. Woodnutt.** 1999. Regulated antisense RNA eliminates alpha-toxin virulence in *Staphylococcus aureus* infection. J. Bacteriol. **181:**6585–6590.
29. **Kreiswirth, B. N., S. Lofdahl, M. J. Betley, M. O'Reilly, P. M. Schlievert, M. S. Bergdoll, and R. P. Novick.** 1983. The toxic shock syndrome exotoxin structural gene is not detectably transmitted by a prophage. Nature **305:**709–712.
30. **Kuroda, H., M. Kuroda, L. Cui, and K. Hiramatsu.** 2007. Subinhibitory concentrations of beta-lactam induce haemolytic activity in *Staphylococcus aureus* through the SaeRS two-component system. FEMS Microbiol. Lett. **268:**98–105.
31. **Kuroda, M., H. Kuroda, T. Oshima, F. Takeuchi, H. Mori, and K. Hiramatsu.** 2003. Two-component system VraSR positively modulates the regulation of cell-wall biosynthesis pathway in *Staphylococcus aureus*. Mol. Microbiol. **49:**807–821.
32. **Lee, C. Y., S. L. Buranen, and Z. H. Ye.** 1991. Construction of single-copy integration vectors for *Staphylococcus aureus*. Gene **103:**101–105.
33. **Li, D., and A. Cheung.** 2008. The repression of *hla* by *rot* is dependent on *sae* in *Staphylococcus aureus*. Infect. Immun. **76:**1068–1075.
34. **Liang, X., C. Yu, J. Sun, H. Liu, C. Landwehr, D. Holmes, and Y. Ji.** 2006. Inactivation of a two-component signal transduction system, SaeRS, eliminates adherence and attenuates virulence of *Staphylococcus aureus*. Infect. Immun. **74:**4655–4665.
35. **Lloyd, G. S., W. Niu, J. Tebbutt, R. H. Ebright, and S. J. Busby.** 2002. Requirement for two copies of RNA polymerase alpha subunit C-terminal domain for synergistic transcription activation at complex bacterial promoters. Genes Dev. **16:**2557–2565.
36. **Lowy, F. D.** 1998. *Staphylococcus aureus* infections. N. Engl. J. Med. **339:**520–532.
37. **Luong, T. T., and C. Y. Lee.** 2006. The arl locus positively regulates *Staphylococcus aureus* type 5 capsule via an mgrA-dependent pathway. Microbiology **152:**3123–3131.
38. **Luong, T. T., S. W. Newell, and C. Y. Lee.** 2003. Mgr, a novel global regulator in *Staphylococcus aureus*. J. Bacteriol. **185:**3703–3710.
39. **Mainiero, M., C. Goerke, T. Geiger, C. Gonser, S. Herbert, and C. Wolz.** 2010. Differential target gene activation by the *Staphylococcus aureus* two-component system *saeRS*. J. Bacteriol. **192:**613–623.
40. **Mascher, T.** 2006. Intramembrane-sensing histidine kinases: a new family of cell envelope stress sensors in Firmicutes bacteria. FEMS Microbiol. Lett. **264:**133–144.
41. **Maxam, A. M., and W. Gilbert.** 1977. A new method for sequencing DNA. Proc. Natl. Acad. Sci. U. S. A. **74:**560–564.
42. **Novick, R. P.** 2003. Autoinduction and signal transduction in the regulation of staphylococcal virulence. Mol. Microbiol. **48:**1429–1449.
43. **Novick, R. P., and D. Jiang.** 2003. The staphylococcal saeRS system coordinates environmental signals with agr quorum sensing. Microbiology **149:**2709–2717.
44. **Rampone, H., G. L. Martinez, A. T. Giraudo, A. Calzolari, and R. Nagel.** 1996. In vivo expression of exoprotein synthesis with a Sae mutant of *Staphylococcus aureus*. Can. J. Vet. Res. **60:**237–240.
45. **Rogasch, K., V. Ruhmling, J. Pane-Farre, D. Hoper, C. Weinberg, S. Fuchs, M. Schmudde, B. M. Broker, C. Wolz, M. Hecker, and S. Engelmann.** 2006. Influence of the two-component system SaeRS on global gene expression in two different *Staphylococcus aureus* strains. J. Bacteriol. **188:**7742–7758.
46. **Schäfer, D., T. T. Lam, T. Geiger, M. Mainiero, S. Engelmann, M. Hussain, A. Bosserhoff, M. Frosch, M. Bischoff, C. Wolz, J. Reidl, and B. Sinha.** 2009. A point mutation in the sensor histidine kinase SaeS of *Staphylococcus aureus* strain Newman alters response to biocide exposure. J. Bacteriol. **191:**7306–7314.
47. **Stock, A. M., V. L. Robinson, and P. N. Goudreau.** 2000. Two-component signal transduction. Annu. Rev. Biochem. **69:**183–215.
48. **Voyich, J. M., C. Vuong, M. Dewald, T. K. Nygaard, S. Kocianova, S. Griffith, J. Jones, C. Iverson, D. E. Sturdevant, K. R. Braughton, A. R. Whitney, M. Otto, and F. R. Deleo.** 2009. The SaeR/S gene regulatory system is essential for innate immune evasion by *Staphylococcus aureus*. J. Infect. Dis. **199:**1698–1706.
49. **West, A. H., and A. M. Stock.** 2001. Histidine kinases and response regulator proteins in two-component signaling systems. Trends Biochem. Sci. **26:**369–376.
50. **Xiong, Y. Q., J. Willard, M. R. Yeaman, A. L. Cheung, and A. S. Bayer.** 2006. Regulation of *Staphylococcus aureus* alpha-toxin gene (hla) expression by agr, sarA, and sae in vitro and in experimental infective endocarditis. J. Infect. Dis. **194:**1267–1275.
51. **Yarwood, J. M., J. K. McCormick, and P. M. Schlievert.** 2001. Identification of a novel two-component regulatory system that acts in global regulation of virulence factors of *Staphylococcus aureus*. J. Bacteriol. **183:**1113–1123.