

Organizational Requirements of the SaeR Binding Sites for a Functional P1 Promoter of the *sae* Operon in *Staphylococcus aureus*

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In *Staphylococcus aureus*, the SaeRS two-component system controls the expression of multiple virulence factors. Of the two promoters in the *sae* operon, P1 is autoinduced and has two binding sites for the response regulator SaeR. In this study, we examined the organizational requirements of the SaeR binding sites in P1 for transcription activation. Mutational studies showed that both binding sites are essential for binding to phosphorylated SaeR (P-SaeR) and transcription activation. When the 21-bp distance between the centers of the two SaeR binding sites was altered to 26 bp, 31 bp, 36 bp, or 41 bp, only the 31-bp mutant retained approximately 40% of the original promoter activity. When the -1 -bp spacing (i.e., 1-bp overlap) between the primary SaeR binding site and the -35 promoter region was altered, all mutant P1 promoters failed to initiate transcription; however, when the first nucleotide of the -35 region was changed from A to T, the mutants with 0-bp or 22-bp spacing showed detectable promoter activity. Although P-SaeR was essential for the binding of RNA polymerase to P1, it was not essential for the binding of the enzyme to the alpha-hemolysin promoter. When the nonoptimal spacing between promoter elements in P1 or the coagulase promoter was altered to the optimal spacing of 17 bp, both promoters failed to initiate transcription. These results suggest that SaeR binding sites are under rather strict organizational restrictions and provide clues for understanding the molecular mechanism of *sae*-mediated transcription activation.

Staphylococcus aureus is a Gram-positive human pathogen that commonly inhabits the skin, anterior nares, and mucous membranes. It causes a wide spectrum of diseases, ranging from superficial skin infections to life-threatening infections (2, 37). Its pathogenic versatility is due partly to the production of numerous virulence factors, such as surface- and cell-associated proteins, secreted toxins, and enzyme proteins (2, 17). The expression of most virulence factors is coordinated by various regulatory factors, including DNA binding proteins (e.g., MgrA, SarA, and Rot), alternative sigma factors (e.g., σ^B , σ^H , and σ^S), and two-component systems (TCSs; e.g., *agr*, *saeRS*, *srrAB*, and *arlRS*) (9, 13, 18, 30, 34, 38, 41, 42, 46).

In bacteria, TCSs play an important role in the sensing of and response to a wide range of environmental stimuli (48). The key elements of this signaling system are a sensor histidine kinase (HK) and a response regulator (RR). Upon the recognition of cognate stimuli, the HK autophosphorylates the conserved histidine residue and then the phosphate group is transferred to an aspartate residue of its cognate RR. The phosphorylation typically activates the function of the RR, such as protein-protein interaction, enzymatic activity, or DNA binding activity, and brings about the desired response to the environmental stimuli (20, 48).

S. aureus genomes typically contain 16 TCSs; among them, the SaeRS TCS is essential for the production of multiple virulence factors such as alpha-hemolysin (Hla), coagulase (Coa), and fibronectin-binding proteins (24, 25, 42, 47). The *sae* operon consists of two promoters, P1 and P3, and four open reading frames (ORFs), *saeP*, *saeQ*, *saeR*, and *saeS* (Fig. 1) (22, 23). P3, located inside *saeQ*, is a fairly constitutive promoter and transcribes *saeR* and *saeS*, the genes for the RR and HK, respectively. On the other hand, P1, located in front of *saeP*, can transcribe all four ORFs (Fig. 1) (21, 31, 50). P1 is a well-characterized target promoter of *sae*, and its transcription requires phosphorylated SaeR (P-SaeR) (43, 50). P1 contains two SaeR binding sites, the primary binding site with no mismatch and the secondary binding site with one

mismatch (Fig. 1). Previously, we showed that the primary binding site is essential for SaeR binding and P1 promoter activity (50). However, the role of the secondary binding site in SaeR-mediated transcription activation remains to be determined.

Depending on strain backgrounds, the SaeRS TCS has been reported to control from 18 to more than 220 genes (36, 52). In our previous study, when one mismatch was allowed, more than 130 genes were found to contain at least one SaeR binding site, and more than 20 genes carry more than one SaeR binding site (50). In addition, among the *sae* target promoters whose transcription start sites (TSSs) are known, the spacing between the SaeR binding site and the -35 promoter region seems to vary; for example, while the spacing is -1 bp (i.e., 1-bp overlap) in P1 and the promoter for coagulase (*Pcoa*), it is 0 bp or 22 bp in the promoters for *emp* (*Pemp*) and alpha-hemolysin (*Phla*), respectively (10, 11, 47) (Fig. 1). Without further knowledge of the organizational requirements for functional SaeR binding sites, however, it is difficult to differentiate the functional SaeR binding sites from nonfunctional ones in the putative *sae* targets. In addition, it is unclear what roles the spacing between the SaeR binding site and the -35 region plays in SaeR-mediated transcription activation. In this study, therefore, we investigated the organizational requirement for the functional SaeR binding site using the *sae* P1 promoter as a model system. In addition, we purified RNA polymerase (RNAP) from *S. aureus* RN4220 to study the role of SaeR binding in transcription activation of the target promoters.

Received 19 December 2011 Accepted 18 March 2012

Published ahead of print 23 March 2012

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doi:10.1128/JB.06771-11

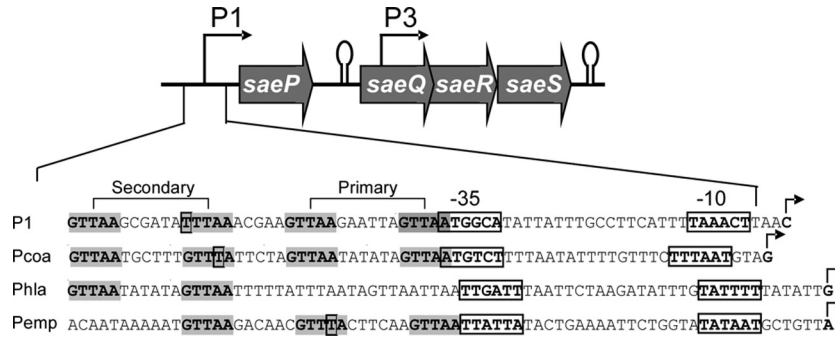


FIG 1 Schematic of the *sae* operon and *sae* target promoters. The *sae* operon consists of four ORFs and two promoters, P1 and P3. The sequence of P1 is illustrated under the map with other *sae* target promoters whose TSSs and promoter elements are reported. The SaeR binding sequence (GTTAAN₆GTAA) is in boldface and shaded in gray. The boxed nucleotides at the binding sites are the nucleotides mismatched with the consensus sequence. The -35 and -10 promoter regions are boxed and indicated. The TSS is in boldface and indicated by a right-angled arrow. Primary, the primary binding site; Secondary, the secondary binding site; Pcoa, promoter for coagulase (*coa*); Phla, promoter for alpha-hemolysin (*hla*); Pemp, promoter for extracellular matrix binding protein (*emp*). Note that Pemp contains three half-binding sites for SaeR.

MATERIALS AND METHODS

Bacterial strains and culture conditions. The strains and plasmids used in this study are listed in Table 1. *Escherichia coli* and *S. aureus* were grown in Luria-Bertani broth and tryptic soy broth (TSB), respectively. When necessary, antibiotics were added to the growth medium at the following

concentrations: ampicillin, 100 µg/ml; erythromycin, 10 µg/ml; chloramphenicol, 5 µg/ml.

DNA manipulation. The restriction enzymes and DNA modification enzymes were purchased from New England BioLabs (NEB). DNA isolation and manipulation in *E. coli* and *S. aureus* were carried out according

TABLE 1 Bacterial strains and plasmids used in this study

Strain or plasmid	Relevant characteristic(s)	Source or reference
<i>E. coli</i> DH5α	Plasmid free, restriction deficient	New England Biolabs
<i>S. aureus</i>		
RN4220	Restriction deficient, prophage free	33
Newman	Clinical isolate, L18P substitution in SaeS	15
USA300-P23	USA300-0114 without plasmids 2 and 3, wild-type SaeS	32
ΦNΞ-01594	Newman with <i>saeR</i> transposon mutation	Phoenix mutant library
RN4220rpoChis	RN4220 with pKOR1-rpoC integrated into chromosome	This study
Plasmids		
pCL-lacZ	pCL55 carrying promoterless <i>lacZ</i>	50
pCL-PgyrB-lacZ	<i>gyrB-lacZ</i> fusion in pCL-lacZ	This study
pCL-P1-lacZ	P1- <i>lacZ</i> fusion in pCL-lacZ	50
pCL-P1-M1-lacZ	P1 mutant with perfect secondary SaeR binding site	This study
pCL-P1-M2-lacZ	P1 mutant with no secondary SaeR binding site	This study
pCL-P1-M3-lacZ	P1 mutant with no primary SaeR binding site	This study
pCL-P1+5-lacZ	10-bp spacing between SaeR binding sites	This study
pCL-P1+10-lacZ	15-bp spacing between SaeR binding sites	This study
pCL-P1+15-lacZ	20-bp spacing between SaeR binding sites	This study
pCL-P1+20-lacZ	25-bp spacing between SaeR binding sites	This study
pCL-P1+1A-lacZ	0-bp spacing between SaeR binding site and promoter	This study
pCL-P1+1T-lacZ	pCL-P1+1A-lacZ with A-T mutation in -35 region	This study
pCL-P1+6A-lacZ	5-bp spacing between SaeR binding site and promoter	This study
pCL-P1+6T-lacZ	pCL-P1+6A-lacZ with A-T mutation in -35 region	This study
pCL-P1+11A-lacZ	10-bp spacing between SaeR binding site and promoter	This study
pCL-P1+11T-lacZ	pCL-P1+11A-lacZ with A-T mutation in -35 region	This study
pCL-P1+23A-lacZ	22-bp spacing between SaeR binding site and promoter	This study
pCL-P1+23T-lacZ	pCL-P1+23A-lacZ with A-T mutation in -35 region	This study
pCL-P1 ₋₁ -lacZ	pCL-P1-lacZ with 17-bp spacing between promoter elements	This study
pCL-Pcoa-lacZ	<i>Pcoa-lacZ</i> fusion in pCL-lacZ	32
pCL-Pcoa ₊₁ -lacZ	pCL-Pcoa-lacZ with 17-bp spacing between promoter elements	This study
pCL-Phla-lacZ	<i>Phla-lacZ</i> fusion in pCL-lacZ	32
pKOR1	Allelic replacement plasmid	3
pKOR1-rpoC	pKOR1 carrying <i>rpoC</i> with His ₁₀ tag at C terminus	This study

to standard procedures (26, 32). Plasmids and genomic DNA were extracted with the Zippy plasmid miniprep kit (Zymo research) and the Wizard genomic DNA purification kit (Promega), respectively. The primers used in this study are listed in Table 2. DNA fragments were PCR amplified with the high-fidelity DNA polymerase Phusion (NEB), and the PCR products were purified with the QuickClean PCR extraction kit (GenScript).

Mutagenesis of P1 and *Pcoa*. To mutate the SaeR binding sites in P1, two DNA fragments were PCR amplified from pCL-P1-lacZ (Table 1) (50) with primer pair P671/P1081, P671/P1083, or P671/P1671 for the first fragment and primer pair P673/P1080, P673/P1082, or P673/P1670 for the second fragment (Table 2). To increase the spacing between the primary and secondary binding sites, the primer pairs used were P671/P1472, P671/P1474, P671/P1508, or P671/P1510 for the first fragment and P673/P1471, P673/P1473, P673/P1507, or P673/P1509 for the second fragment. To insert nucleotides between the primary SaeR binding site and the -35 promoter region, the primer pairs used were P671/P1064, P671/P1580, P671/P1582, P671/P1584, P671/P1606, P671/P1608, P671/P1610, or P671/P1612 for the first fragment and P673/P1063, P673/P1579, P673/P1581, P673/P1583, P673/P1605, P673/P1607, or P673/P1609 for the second fragment. To alter the spacing between the promoter elements, DNA fragments were PCR amplified using primer pairs P671/P1673 (P1) and P1161/P1683 (*Pcoa*) for the first fragment and P673/P1672 (P1) and P1162/P1682 (*Pcoa*) for the second fragment. The first and second fragments were mixed and further subjected to PCR amplification with primer pair P671/P673 (P1) or P1161/P1162 (*Pcoa*). The resulting fragments were digested with EcoRI and KpnI and inserted into plasmid pCL-lacZ (50). The intended mutations were all verified by DNA sequencing analysis. The resulting plasmids were electroporated into *S. aureus* RN4220 and then transduced with ϕ 85 into strain Newman or USA300-P23 (Table 1).

Electrophoretic mobility shift assay. The DNA probes were PCR amplified from plasmid DNA carrying the target promoter sequences (Table 1) with primer pairs P671/P673 (P1), P1492/P1162 (*Pcoa*), and P1306/P1307 (*Phla*) (Table 2), and then the 5' ends of the PCR products were labeled with [γ - 32 P]ATP (Perkin-Elmer) using T4 polynucleotide kinase (NEB). The purified SaeR protein (32 μ M) was phosphorylated with the cytoplasmic domain of SaeS (SaeS^C, 4 μ M) in 10 mM Tris-HCl (pH 7.4)-50 mM KCl-5 mM MgCl₂-10% glycerol as described previously (50). The probe (2 ng) was mixed with various amounts of phosphorylated SaeR (P-SaeR) in a 20- μ l reaction mixture containing 10 mM Tris-HCl (pH 7.4), 50 mM KCl, 5 mM MgCl₂, 10% glycerol, and 3 μ g/ml sheared salmon sperm DNA. In RNAP binding experiments, purified RNAP (0.7 μ g) was mixed with the probes in the presence of either non-phosphorylated SaeR or P-SaeR. Binding reaction mixtures were incubated at room temperature for 15 min, and then samples were analyzed by 5% PAGE. The gels were dried and exposed to X-ray film (Fuji). Images were quantified with the ImageJ software (NIH).

Insertion of His₁₀ tag sequence into C terminus of *rpoC*. A 1,068-bp *rpoC* fragment containing a His₁₀ tag sequence at its C terminus was PCR amplified with primer pair P1038/P1079 and reamplified with primer pair P1038/P1156. The PCR product was inserted into pKOR1 (3) using the BP clonase (Invitrogen). The resulting plasmid, pKOR1-*rpoC*, was electroporated into *S. aureus* RN4220 and incubated at 42°C. A strain with the plasmid integrated into the chromosome was identified by PCR analysis and named RN4220rpoChis.

Purification of *S. aureus* RNAP. *S. aureus* RN4220rpoChis was grown in TSB at 30°C for 16 h and cooled down to 4°C. Cells were harvested by centrifugation, suspended in column buffer (50 mM Tris-HCl, pH 7.8, 300 mM NaCl, 5 mM MgCl₂, 20% glycerol) containing lysostaphin (100 μ g/ml), and incubated on ice for 1 h. After sonication, cell debris was precipitated by centrifugation at 12,000 \times g for 1 h. The supernatant was applied to a Ni-nitrilotriacetic acid (GenScript) affinity column, and then the column was washed with column buffer containing 60 mM imidazole (Sigma). Proteins were eluted with column buffer containing 400

mM imidazole and dialyzed against storage buffer (10 mM Tris-HCl, pH 8.0, 10 mM MgCl₂, 1 mM EDTA, 100 mM KCl, 10% glycerol). Protein concentrations were determined with the bicinchoninic acid protein assay kit (Pierce). Purified RNAP was separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), and the first four protein bands were analyzed by liquid chromatography-tandem mass spectrometry (MS) analysis at the Proteomics Core of the Indiana University School of Medicine.

***In vitro* transcription assays.** *In vitro* transcription assays were performed as described previously (19), with minor modifications. DNA templates were PCR amplified from a plasmid carrying the target promoter sequences with primer pair P850/P641 (Table 2). The purified RNAP (0.3 μ g) and DNA templates (87.5 nM) were mixed with SaeR (4 μ M) or P-SaeR (4 μ M) in transcription buffer (20 mM Tris-HCl, pH 7.9, 20 mM NaCl, 20 mM MgCl₂, 0.1 mM EDTA) containing 10 mM β -mercaptoethanol; 0.25 mM ATP, CTP, and GTP; 0.025 mM UTP; 10 U of RNase inhibitor (Invitrogen); and 5 μ Ci of [α - 32 P]UTP. After incubation at 37°C for 15 min, transcripts were extracted with phenol-chloroform-isoamyl alcohol (25:24:1) and suspended in 3 \times loading buffer (6 M urea, 0.1 M EDTA, 5% glycerol, 0.25% bromophenol blue, 0.25% xylene cyanol). Samples were denatured at 95°C for 2 min, electrophoresed with a 6% acrylamide gel containing 6 M urea, and exposed to X-ray film (Fuji). The gel images were quantified by ImageJ (NIH).

β -Galactosidase assays. β -Galactosidase activity was measured as described previously (50), with minor modifications. Briefly, the test strains were grown in TSB containing appropriate antibiotics at 37°C for 16 h. For human neutrophil peptide 1 (HNP-1) induction assays, the test strains were grown to an optical density at 600 nm (OD₆₀₀) of 0.6 and induced with HNP-1 (5 μ g/ml; Bachem) for 2 h. The cells in 1 ml of culture were collected by centrifugation, washed with AB buffer (100 mM potassium phosphate, 100 mM NaCl, pH 7.0), and suspended in 100 μ l of AB buffer containing lysostaphin (0.1 μ g/ml). After incubation at 37°C for 15 min, 900 μ l of ABT buffer (AB buffer containing 0.1% Triton X-100) was added. Then 50 μ l of cell lysate or its diluent was mixed with 10 μ l of 4-methylumbelliferyl- β -D-galactopyranoside (4 mg/ml; Sigma) and incubated at room temperature for 1 h. A standard curve was obtained by using MU (4-methylumbiliferone; Sigma). The emission of fluorescence was measured with a plate reader (355-nm excitation and 455-nm emission wavelengths; Molecular Devices). β -Galactosidase activity was normalized by cell density (OD₆₀₀), and then the activity was determined in AU (arbitrary units), where 1 AU corresponds to the generation of 1.2×10^{-8} mol of MU h⁻¹ ml⁻¹ OD₆₀₀ unit⁻¹.

RESULTS

The secondary SaeR binding site of P1 is essential for promoter function. Of the two SaeR binding sites in P1, the primary binding site was shown to be essential for SaeR-mediated transcription activation; however, the role of the secondary binding site remains unknown. Therefore, to identify the role of the secondary binding site, we altered the SaeR binding sequence such that mutant M1 has two binding sites with a perfect sequence match, M2 does not have the secondary binding site, and M3 does not have the primary binding site but has the secondary binding site with a perfect sequence match (Fig. 2A). Then we examined the effects of the sequence alterations on the SaeR binding and *in vivo* promoter activities. As shown in Fig. 2B and C, the M1 mutant promoter showed approximately normal SaeR binding and promoter activities. On the other hand, the mutant M2 and M3 promoters showed similarly reduced affinities for P-SaeR (M2 and M3 in Fig. 2B), as well as either greatly reduced (M2) or no (M3) transcription activity (Fig. 2C).

The assays described above were done with strain Newman (15), in which SaeS is in a constitutively active state due to the L18P mutation in the first transmembrane domain (1, 21). To

TABLE 2 Primers used in this study

Purpose and primer	Sequence (5' → 3') ^a
RNAP	
P1038	GGGGACAAGTTTGTACAAAAAAGCAGGCTCACCTGAAATTGCTAAGAAAATTAC
P1079	TTATTAATGATGGTGATGATGATGATGGTGATGATGTTCCGTTACTTCAGTTTGAGATTC
P1156	GGGGACCACCTTTGTACAAGAAAGCTGGGTTTATTAATGATGGTGATGATGATG
P1 amplification	
P671	AACGAATTCCTGGTACTTGTATTTAATCGTCTATC
P673	AAAGGTACCGTTGTGATAACAGCACCAGCT GC
<i>PgyrB</i> amplification	
P918	CTAGAATTCAAAGGTGACGACTCGGTAACG
P919	CTAGGTACCGTGTATTTAACTTCATTGTTCCACC
<i>Phla</i> amplification	
P931	CCCGAATTCGAGTTTATAATATTATCAACTCTGTG
P639	CCCGGTACCGTGTGACTGACTATACGTGTTTTTC
P1306	TTTTCTCTATTTCTATTTATTAATTTACACTA
P1307	CTTTAAACTAATGATTTGTTTGATTAAAAA
<i>Pcoa</i> amplification	
P1161	GGAATTCGAATTGTAAACTTTCTAATC
P1162	GGGGTACGCGCCTAGCGAAATTATTTGC
P1492	GTTGTCATGCTTTGTTACTCCTTTG
<i>In vitro</i> transcription	
P641	TAACGCCAGGGTTTTCCCGGTGCGAC
P850	CCACCTGACGTCTAAGAAACC
Mutagenesis of SaeR binding sites	
P1080	CTTAACTTCGTTTAACTATCGCTTAAC
P1081	GTTAAGCGATAGTTAAACGAAGTTAAG
P1082	CTTAACTTCGTTTATAATATCGCTATACTAAATG
P1083	CAATATTAGTATAGCGATATTATAACGAAGTTAAG
P1157	CCATTAACTAATTCTTGGCTTCGTTTAACTATCGC
P1158	GCGATAGTTAAACGAAGCCAAGAATTAGTTAATGG
Insertion mutagenesis between SaeR binding sites	
P1471	GTTAAGCGATATTAAACGAAACGAAGTTAAGAATTAG
P1472	CTAATTCCTTAACTTCGTTTCGTTTAAATATCGCTTAAC
P1473	GTTAAGCGATATTAAACGAAACGAAACGAAGTTAAGAATTAG
P1474	CTAATTCCTTAACTTCGTTTCGTTTAAATATCGCTTAAC
P1507	CTAATTCCTTAACTTCGTTTCGTTTCGTTTAAATATCGCTTAAC
P1508	GTTAAGCGATATTAAACGAAACGAAACGAAGTTAAGAATTAG
P1509	CTAATTCCTTAACTTCGTTTCGTTTCGTTTAAATATCGCTTAAC
P1510	CTAATTCCTTAACTTCGTTTCGTTTCGTTTAAATATCGCTTAAC
Insertion mutagenesis between SaeR binding site and -35 region	
P1579	GTTAAGAATTAGTTAAGAATTATGGCATATTATTTGC
P1580	GCAAATAATATGCCATAATTCCTTAACTAATTCCTTAAC
P1581	GTTAAGAATTAGTTAAGAATTAGAATATGGCATATTATTTGC
P1582	GCAAATAATATGCCATAATTCCTTAACTAATTCCTTAAC
P1583	GTTAAGAATTAGTTAATTTTATTTAATATTTAATTAATTTGGCATATTATTTGC
P1584	GCAAATAATATGCCAATTAATTAATTTAATTAATTAATTAATTTGGCATATTATTTGC
P1603	GTTAAGAATTAGTTAATTTGGCATATTATTTGC
P1604	GCAAATAATATGCCAATTTAACTAATTCCTTAAC
P1605	GTTAAGAATTAGTTAATTTGGCATATTATTTGC
P1606	GCAAATAATATGCCAATTTAACTAATTCCTTAAC
P1607	GTTAAGAATTAGTTAAGAATTTTGGCATATTATTTGC
P1608	GCAAATAATATGCCAATTTCTTAACTAATTCCTTAAC
P1609	GTTAAGAATTAGTTAAGAATTAGAATTTGGCATATTATTTGC
P1610	GCAAATAATATGCCAATTTCTTAACTAATTCCTTAAC
P1611	GTTAAGAATTAGTTAATTTTATTTAATATTTAATTAATTTGGCATATTATTTGC
P1612	GCAAATAATATGCCAATTTAATTAATTAATTAATTAATTTAACTAATTCCTTAAC

(Continued on following page)

TABLE 2 (Continued)

Purpose and primer	Sequence (5' → 3') ^a
Spacing mutagenesis between promoter elements	
P1672	GAATTAGTTAATGGCATATTATTGCCTTCATTTTAAACTTAACTTATC
P1673	GATAAGTTAAGTTAAAAATGAAGGCAATAATATGCCATTAACCTAATTC
P1682	GTCTTTAATATTTTGTTCCTTAATGTAGATTGGG
P1683	CCCAATCTACATTAAGAAACAAAAATATTAAGAGAC

^a Underlined sequences are restriction enzyme sites.

examine whether the SaeR binding sites play the same role in a strain with wild-type (WT) SaeS activity, we repeated the assays with strain USA300-P23, a strain producing WT SaeS (21, 31). As shown in Fig. 2D, although the promoter activities were much lower, similar results were obtained: without either binding site, the promoter activity was either greatly reduced (M2) or abolished (M3). In addition, neither defective P1 promoter responded to induction by HNP-1, a known inducer of the SaeRS TCS (Fig. 2D) (21). Taken together, these data demonstrate that, at P1, both the primary and secondary sites are essential for SaeR-mediated transcriptional activation and the response to HNP-1.

In vitro transcription assays confirm the essential role of the secondary binding site. To gain further insight into the molecular mechanism of transcription activation by SaeR, we performed *in vitro* transcription assays by using RNAP purified from *S. aureus* RN4220 (see Materials and Methods; Fig. 3A). SDS-PAGE analysis of purified RNAP produced five protein bands, and MS analysis

identified the first four protein bands as β' , β , and α subunits and σ^A , respectively. Although not analyzed by MS, the last protein band is presumed to be the ω subunit (Fig. 3A) (45). When used for an *in vitro* transcription assay for the *gyrB* promoter, a SaeR-independent constitutive promoter, the purified RNAP produced transcripts in a concentration-dependent manner (Fig. 3B), confirming the functionality of the enzyme. When the P1 promoter was subjected to the *in vitro* transcription assay, WT P1 produced transcripts only when both P-SaeR and RNAP were present (Fig. 3C), confirming the essential role of P-SaeR in P1 transcription (compare lanes 3 and 4). Of the mutant P1 promoters, M1 produced transcripts at the WT level (lane 8 in Fig. 3C), recapitulating the *in vivo lacZ* assay result in Fig. 2C. However, no transcripts were produced from either M2 or M3, demonstrating the essential roles of both the primary and secondary binding sites in transcription initiation at P1.

SaeR binding sites should be on the same side of the DNA helix. As the centers of the primary and the secondary binding

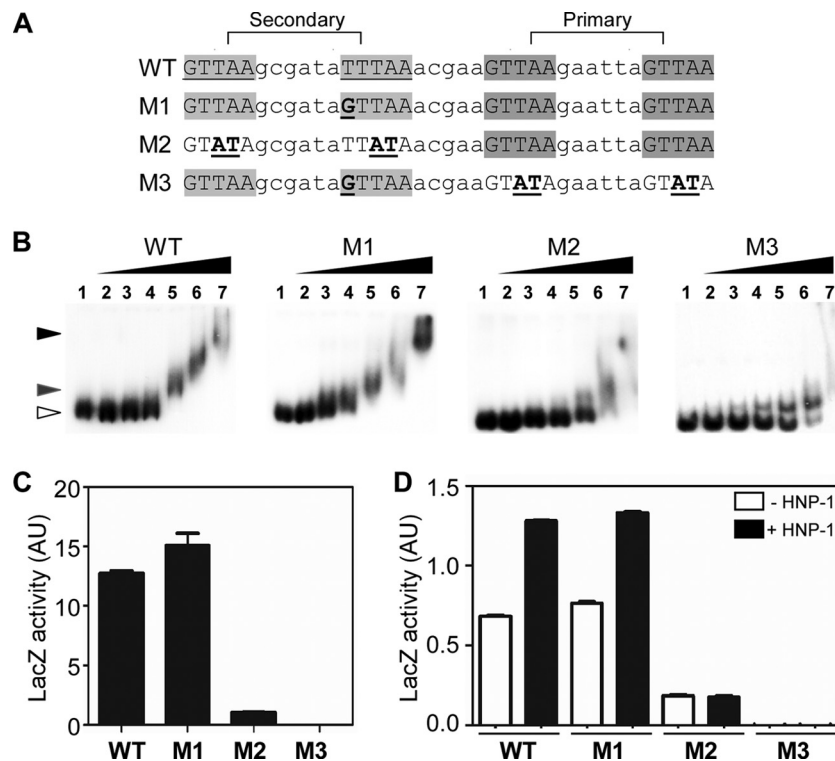


FIG 2 The secondary SaeR binding site in P1 is essential for promoter function. (A) Sequences of WT and mutant P1 promoters. SaeR binding sequences are indicated by gray shading. Mutated nucleotides are in boldface and underlined. (B) Effects of mutations on affinity for phosphorylated SaeR (P-SaeR). The P1 promoter probes were PCR amplified and end labeled with [γ -³²P]ATP. The labeled DNAs (2 ng) were incubated with 3 μ g/ml salmon sperm DNA and increasing amounts of P-SaeR (0 μ M, 0.25 μ M, 0.5 μ M, 1 μ M, 2 μ M, 4 μ M, and 8 μ M in lanes 1 to 7). The protein-DNA complexes were analyzed by 6% PAGE. The white arrowhead indicates free DNA probes. (C and D) Effects of mutations on *in vivo* promoter activity in strains Newman (C) and USA300 (D). The data presented are representative of results obtained from three independent experiments. Error bars indicate standard deviations.

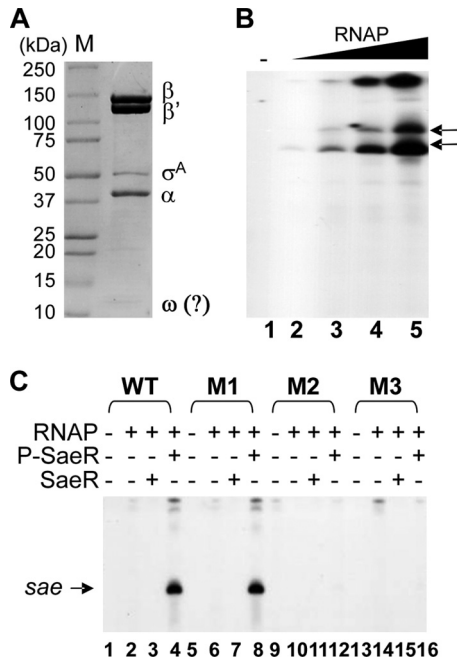


FIG 3 *In vitro* transcription assays confirm the essential role of the secondary SaeR binding site in P1 transcription. (A) SDS-PAGE analysis of purified *S. aureus* RNAP. The subunits of *S. aureus* RNAP (β , β' , σ^A , α , and ω) are indicated. M, molecular size marker proteins. (B) *In vitro* transcription assay for *gyrB* promoter. The PCR-amplified *gyrB* promoter was subjected to *in vitro* transcription assays in the presence of increasing amounts of *S. aureus* RNAP (0 μ g, 0.15 μ g, 0.3 μ g, 0.6 μ g, and 1.2 μ g in lanes 1 to 5). The *gyrB* transcripts are indicated by arrows. -, no RNAP. (C) *In vitro* transcription assays for WT and mutant P1 promoters. DNA templates were PCR amplified and mixed with the RNAP (0.3 μ g), SaeR (4 μ M), and P-SaeR (4 μ M) proteins in various combinations. M1 to M3, mutant P1 promoters shown in Fig. 2A. +, presence; -, absence.

sites are 21 bp apart, a distance allowing two turns of the DNA helix, the P-SaeR molecules bound to DNA are expected to reside on the same side of the DNA helix (WT in Fig. 4A). To further examine the binding requirement of SaeR, we altered the binding phases by inserting 5 bp, 10 bp, 15 bp, or 20 bp of random nucleotides between the binding sites and then measured their SaeR binding and promoter activities. As shown in Fig. 4B to D, the insertion of either 5 bp or 15 bp (+5 and +15), which puts the centers of the binding sites on opposite sides, greatly reduced both SaeR binding and promoter activities. On the other hand, the insertion of 10 bp, which would increase the distance to 31 bp (i.e., three helix turns), did not alter SaeR binding significantly, and the resulting mutant retained 30% to 40% of the WT promoter activity (+10 in Fig. 4B to D). The insertion of 20 bp, although it would be expected to maintain the binding phase, greatly reduced the SaeR binding and promoter activities (+20 in Fig. 4B to D). We also noted that the *in vitro* results did not always agree with the *in vivo* results. For example, although the +5-, +15-, and +20-bp insertion mutants showed similar *in vitro* promoter activities (16% to 18% of the WT level), under *in vivo* conditions, only the +5-bp insertion mutant showed significant promoter activity (approximately 10% of the WT level) and the other mutants completely lost their promoter activities (+5, +15, and +20 in Fig. 4D). These results imply that the requirements for efficient transcription are not identical under those two conditions. When the

measurement of the *in vivo* promoter activity was repeated in the strain USA300-P23 background, we observed similar results. When the SaeR binding sites are expected to be on opposite sides of the DNA helix, only the +5 mutant showed detectable promoter activity while the +15 mutant lost its activity completely (+5 and +15 in Fig. 4E). When SaeR binding sites are expected to reside on the same side of the DNA helix, the promoter activity was inversely correlated with the distance of the binding sites (compare WT, +10, and +20 in Fig. 4D). Intriguingly, HNP-1 treatment induced promoter activity in the WT and the +5, and +20 mutants but not in the +10 mutant. These results suggest that for efficient SaeR-mediated transcription at P1, the SaeR binding sites should be on the same side of the DNA helix and that their optimal spacing is 21 bp.

In P1, the optimal spacing between the SaeR binding site and the -35 region is -1 bp. Next, we investigated the role of the spacing between the SaeR binding site and the -35 region in P1 transcription. Although the spacing is -1 bp in P1 (i.e., there is a 1-bp overlap), in other *sae* target promoters, such as *Pemp* and *Phla*, it is 0 bp and 22 bp, respectively (Fig. 1) (11, 27). To investigate whether that spacing is also optimized for SaeR-mediated transcription activation, we altered the spacing to 0 bp and 22 bp (Fig. 5A). In addition, to examine the effect of the binding phase, we also changed the spacing to 5 bp or 10 bp. When their promoter activity was measured by *in vitro* transcription and the *lacZ* reporter assays, the mutant P1 promoters showed no or very low activity (0, 5, 10, and 22 in Fig. 5B and C), suggesting that in P1, the -1-bp spacing is critical for SaeR-mediated transcriptional activation.

Considering the fact that the 0-bp and 22-bp spacings are found in other known *sae* target promoters, the nearly complete loss of promoter activity was unexpected. Since successful transcription at *sae* target promoters would require intricate interactions between SaeR and RNAP, we suspected that the poor sequence conservation of the P1 promoter might be responsible for the inability of those spacing mutants to initiate transcription. Comparison of the promoter sequences shows that one of the main differences between P1 and *Pemp/Phla* is the first nucleotide of the -35 region: it is nonconsensus nucleotide A in P1, while it is consensus nucleotide T in *Pemp* and *Phla* (Fig. 1). Therefore, we changed the first nucleotide of the -35 region of P1 from A to T (Fig. 5A) and measured the promoter activity in the presence or absence of P-SaeR. As shown in Fig. 5B and C, while the mutant P1 promoters with 5-bp or 10-bp spacing still completely lost their promoter activity, partial restoration of P-SaeR-dependent promoter activity was observed in mutant P1 with 0-bp or 22-bp spacing (5T and 10T versus 0T and 22T), suggesting that, along with -1 bp, the 0-bp and 22-bp spacings allow efficient interaction between P-SaeR and RNAP.

RNAP requires phosphorylated SaeR for binding to P1. The -1-bp spacing between the primary SaeR binding site and the -35 sequence in P1 suggests that SaeR directly interacts with RNAP and, possibly, recruits RNAP to the promoter. To test this possibility, we examined RNAP binding to P1 in the presence of either unphosphorylated SaeR or phosphorylated SaeR (P-SaeR). As shown in Fig. 6, when unphosphorylated SaeR was present, RNAP binding to P1 was almost undetectable (top panel of Fig. 6). Intriguingly, the free probe appears to shift slightly in the presence of RNAP (compare lanes 1 to 5 and 6 to 10). However, since it was not consistently reproducible (data not shown), it seems that the

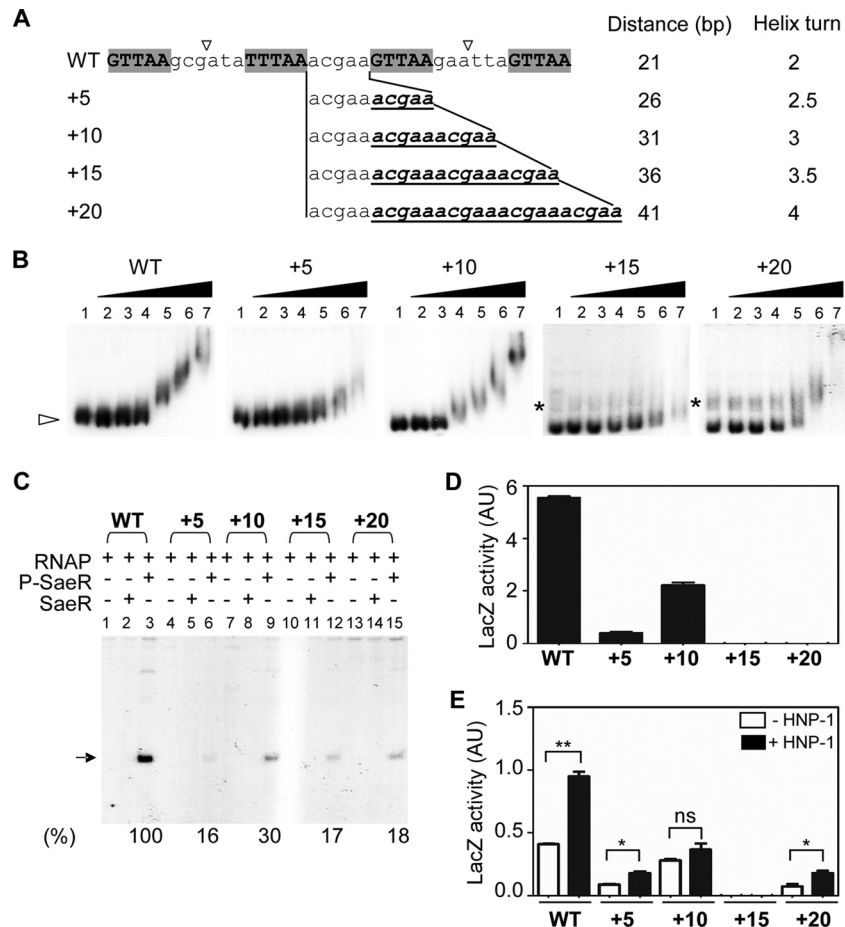


FIG 4 SaeR needs to bind to the same side of the DNA helix. (A) Sequences of WT and insertion mutant P1 promoters. SaeR binding sites are indicated by capital letters and gray shading. Shown to the left are the numbers of nucleotides inserted, which are in boldface italics and underlined. The distance between the centers (∇) of the SaeR binding sites is shown with the corresponding number of DNA helix turns. (B) Effects of nucleotide insertions on SaeR binding affinity. P1 promoters (2 ng) labeled with ^{32}P were incubated with 3 $\mu\text{g}/\text{ml}$ salmon sperm DNA and 0 μM , 0.25 μM , 0.5 μM , 1 μM , 2 μM , 4 μM , or 8 μM P-SaeR (lanes 1 to 7) and then subjected to 6% PAGE and autoradiography. The white arrowhead indicates free DNA probes, and the asterisks denote contaminant DNA unrelated to P1. (C) *In vitro* transcription assays for WT and mutant P1 promoters in various combinations of RNAP (0.3 μg), SaeR (4 μM), and P-SaeR (4 μM). The P1 transcript is indicated by the arrow. The quantification values of the transcripts are shown at the bottom, where the level of transcripts from WT P1 was set to 100%. +, presence; -, absence. (D and E) *In vivo* activity of WT and mutant P1 promoters measured by promoter-*lacZ* fusion assays in the background of strain Newman (D) or USA300 (E). Data are representative of results obtained from three independent experiments. Error bars indicate standard deviations. In panel E, statistical analysis was carried out by two-tailed *t* test. *, $P < 0.05$; **, $P < 0.005$; ns, not significant.

slight shift might be caused by irregularity in the electrophoresis conditions. When P-SaeR was used in the binding assay, RNAP bound to P1 in a P-SaeR concentration-dependent manner (lanes 7 to 10 in the bottom panel of Fig. 6), demonstrating that P-SaeR is essential for efficient RNAP binding to P1. When the same assay was repeated for the mutant P1 with a T substitution, as shown in Fig. 5A, the functional promoters (i.e., WT and 0T and 22T mutant P1) produced a ternary complex of P-SaeR, RNAP, and P1 (WT, 0T, and 22T in Fig. 5D). On the other hand, the nonfunctional promoters, 5T and 10T, only showed a faster-migrating band or smears, implying that the promoters cannot form a stable ternary complex with RNAP and P-SaeR. These results further demonstrate that P-SaeR can efficiently interact with RNAP at a spacing of -1 bp, 0 bp, or 22 bp.

RNAP can bind to the alpha-hemolysin promoter without P-SaeR. Unlike P1 or *Pcoa*, the alpha-hemolysin promoter (*Phla*) has only one binding site and a relatively large spacing, 22 bp, between the SaeR binding site and the -35 region (Fig. 1). In

addition, for transcription initiation, *Phla* requires a lower concentration of P-SaeR than P1 or *Pcoa* does (31, 39). Nevertheless, we observed that all of the promoters bound to P-SaeR with similar affinities under *in vitro* conditions (Fig. 7A) and required SaeR for their activity (Fig. 7B). To examine whether P-SaeR plays a distinct role in *Phla* transcription, we repeated the RNAP binding assay with this promoter. Surprisingly, although RNAP bound to P1 and *Pcoa* only in the presence of P-SaeR, RNAP bound to *Phla* even in the absence of P-SaeR (lane 3 of *Phla* in Fig. 7C). The presence of P-SaeR increased RNAP binding to P1 2-fold (compare lanes 3 and 4 of *Phla* in Fig. 7C). These results suggest that RNAP can bind to *Phla* without P-SaeR and that P-SaeR further accelerates the binding of RNAP to *Phla*.

In P1 and *Pcoa*, alteration of the spacing between promoter elements abolishes promoter activities. The spacing between the -35 and -10 promoter elements in P1 and *Pcoa* is 18 bp and 16 bp, respectively, and deviates from the optimal spacing of 17 bp. Recently, Reynolds and Wigneshweraraj reported that when the

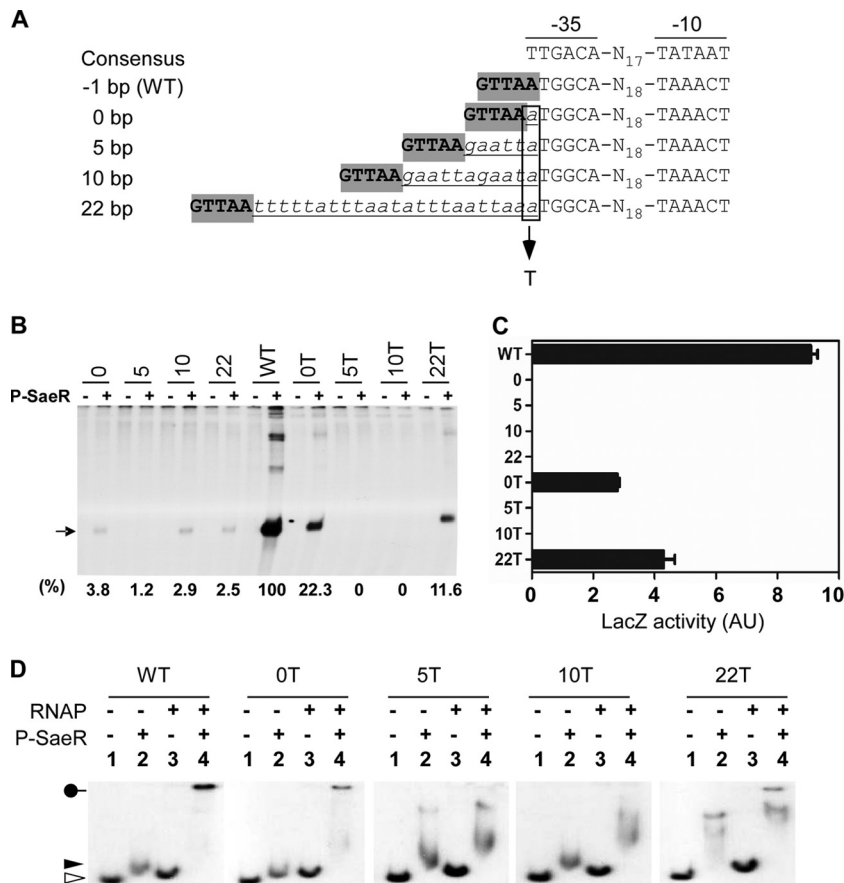


FIG 5 In P1, the optimal spacing between the SaeR binding site and the -35 region is -1 bp. (A) Nucleotide insertion mutations and A-T transversion mutation in P1. The resulting spacing between the primary SaeR binding site and the -35 region is shown to the left. The consensus -35 and -10 promoter sequences are shown at the top. Gray shading indicates the half sequence of the primary SaeR binding site. The inserted nucleotides are italicized and underlined. The position of the A-T transversion mutation is indicated by a vertical rectangle and an arrow. The spacing between the -35 and -10 regions is indicated by the letter N with the nucleotide number. (B) *In vitro* transcription assays for the WT and mutant P1 promoters. Each reaction mixture contained $0.3 \mu\text{g}$ of RNAP and $4 \mu\text{M}$ either SaeR ($-$) or P-SaeR ($+$). Shown above is the spacing between the primary SaeR binding site and the -35 region, where T denotes the A-T transversion mutation in the -35 region. The arrow points to the specific *sae* transcripts from the promoters. At the bottom are the quantified values for the transcripts, where the level of transcripts from the WT P1 was set to 100%. (C) *In vivo* activity of the WT and spacing mutant promoters. Data are representative of results obtained from three independent experiments. Error bars depict standard deviations. (D) Effects of spacing mutations on binding to P-SaeR and RNAP. The WT and mutant promoters were mixed with RNAP ($0.7 \mu\text{g}$) and/or P-SaeR ($0.5 \mu\text{M}$), incubated for 15 min at room temperature, and then loaded onto a 5% polyacrylamide gel. The promoters tested are shown at the top. The white arrowhead indicates the free DNA probe, and the black arrowhead points to the DNA probes bound to P-SaeR. The black pinhead denotes the ternary complex of the DNA probe, P-SaeR, and RNAP. $+$, presence; $-$, absence.

20-bp spacing of the *agr* P3 promoter was reduced to 17 bp, the resulting mutant promoter showed improved promoter activity (45). To examine whether the spacing between promoter elements plays any role in SaeR-mediated transcriptional activation, we altered the spacing of both promoters to 17 bp ($P1_{-1}$ and $Pcoa_{+1}$ in Fig. 8A) and measured the resulting promoter activity by using *lacZ* reporter assays. As shown in Fig. 8B and C, the mutations almost abolished the promoter activity of P1 and *Pcoa* in both the Newman and USA300-P23 backgrounds. Importantly, those mutant promoters did not respond to induction by HNP-1, suggesting that in P1 and *Pcoa*, the spacing of the promoter elements is required for their SaeR-mediated transcriptional activation.

DISCUSSION

The importance of the SaeRS TCS in staphylococcal pathogenesis has been well recognized. It can affect the expression of more than 200 genes and is required for bacterial survival in mouse models

and human blood (52). Not surprisingly, the binding sequence for SaeR has been identified in more than 130 genes (43, 50). However, it has not been clear why only some of the more than 130 genes are regulated by the TCS. In this study, using RNAP purified from *S. aureus* RN4220 and the *sae* P1 promoter, we investigated various organizational requirements of SaeR binding sites, as well as the role of SaeR in transcription activation. Although our study focused mainly on the *sae* P1 promoter, the results of this study can probably be applied to other *sae* targets and assist in identifying genuine targets of the SaeRS TCS.

When two SaeR binding sites are present, both sites appear to be essential for SaeR binding and transcription activation. In P1, when either the primary or the secondary binding site was eliminated by mutation, the resulting promoter failed to effectively initiate transcription under both *in vitro* and *in vivo* conditions (Fig. 2 and 3). SaeR binding to those two binding sites might be cooperative. Mutant P1 with only one binding site did not show

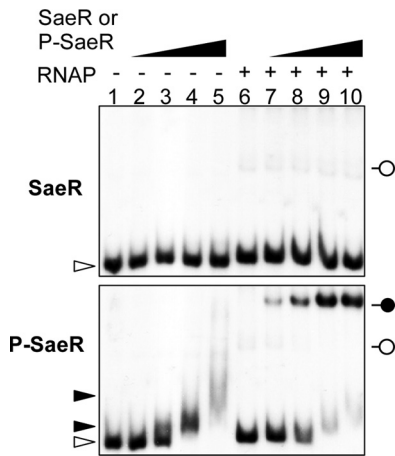


FIG 6 RNAP requires P-SaeR for efficient binding to P1. The P1 probe labeled with ^{32}P was mixed with RNAP (0.7 μg) and increasing amounts of either unphosphorylated SaeR (SaeR) or phosphorylated SaeR (P-SaeR). Lanes 1 and 6, no SaeR proteins; lanes 2 and 7, 62.5 nM; lanes 3 and 8, 125 nM; lanes 4 and 9, 250 nM; lanes 5 and 10, 500 nM. The mixtures were incubated for 15 min at room temperature and then loaded onto a 5% polyacrylamide gel. The white arrowheads indicate free DNA probe, while black arrowheads denote DNA probes bound to P-SaeR. The white pinheads indicate possible P1-RNAP complexes, while the black pinhead depicts the ternary complex of P1, P-SaeR, and RNAP.

the supershifted bands, an indicator of multiple bindings of P-SaeR, and reduced the affinity for P-SaeR (compare lanes 5 of WT and M1 with those of M2 and M3 in Fig. 2B). When we assume that P-SaeR binds to DNA as a dimer, the disappearance of the supershifted band might indicate the loss of dimer-dimer interac-

tions. When the SaeR binding sites were placed on opposite sides of the DNA helix, the resulting mutant P1 promoters showed greatly reduced SaeR binding and transcription activities (+5 and +15 in Fig. 4B to E), suggesting that P-SaeR molecules need to be on the same side of the DNA helix for efficient protein-protein interactions. This notion is further corroborated by the finding that the 10-bp insertion mutant form of P1 showed normal SaeR binding and retained a significant level of *in vivo* promoter activity (40% of the WT level) (+10 in Fig. 4). On the other hand, when a 20-bp sequence was inserted, the resulting mutant P1 lost both the P-SaeR binding and promoter activities in the Newman background (+20 in Fig. 4B to D), implying that the distance imposed by the 20-bp insertion (~ 6.8 nm in the B-form DNA helix) might be prohibitive for P-SaeR protein-protein interactions. Since a single SaeR binding site alone cannot activate transcription at P1 (M2 and M3 in Fig. 2C), the occupation of both binding sites by P-SaeR seems to be essential for the interaction with RNAP and subsequent transcription initiation (Fig. 6 and 7). Certainly, further research is required to examine whether P-SaeR binds to DNA as a dimer and whether the protein-protein interactions of P-SaeR are cooperative.

Depending on the locations of their DNA binding sites in the promoter region, transcription factors activate transcription by different mechanisms. For promoters whose transcription is activated by a single transcription factor, three mechanisms have been suggested, class I and II activation and activation by conformation change (6, 16). In class I activation, the binding site is located upstream of the -35 region and the bound transcription factor recruits RNAP by interaction with αCTD of RNAP (16, 54, 55). In class II activation, the binding site overlaps the -35 region and the

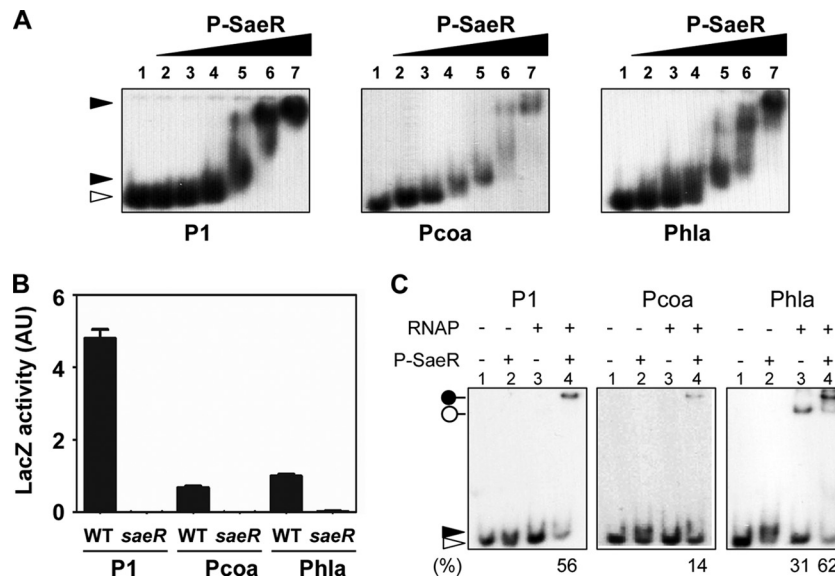


FIG 7 RNAP can bind to *Phla* without P-SaeR. (A) SaeR binding of the three target promoters. Promoters (2 ng) labeled with ^{32}P were mixed with 3 $\mu\text{g}/\text{ml}$ salmon sperm DNA and 0 μM , 0.25 μM , 0.5 μM , 1 μM , 2 μM , 4 μM , or 8 μM P-SaeR (lanes 1 to 7); incubated at room temperature for 15 min; and analyzed by 5% PAGE and autoradiography. The white arrowhead indicates free probes. (B) Dependence of the three *sae* target promoters on the SaeRS TCS. Promoter-*lacZ* fusion plasmids were inserted into WT or *saeR* mutant strain Newman, and then promoter activity was measured by *lacZ* expression. *saeR*, *saeR* mutant. Data are representative of results obtained from three independent experiments. Error bars represent standard deviations. (C) Binding of RNAP to the three target promoters. DNA probes were mixed with RNAP (0.7 μg) and/or P-SaeR (0.5 μM), incubated for 15 min at room temperature, and then analyzed by 5% PAGE and autoradiography. The white and black arrowheads indicate free and P-SaeR-bound probes, respectively. The white pinhead denotes the DNA probe-RNAP complex, and the black pinhead represents the DNA probe-P-SaeR-RNAP ternary complex. The percentage of DNA probe in the protein-DNA complex is shown at the bottom.

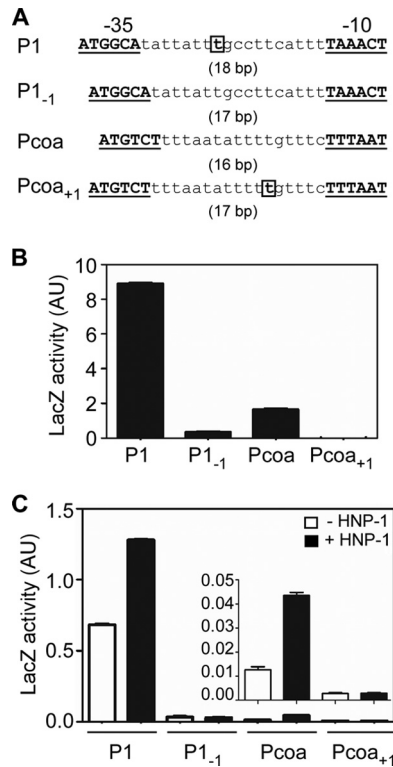


FIG 8 In P1 and *Pcoa*, alteration of the spacing between promoter elements abolishes promoter activity. (A) Promoters with altered spacing between the promoter elements. The nucleotides either deleted (in P1) or inserted (in *Pcoa*) are in boldface and boxed. The spacing of each promoter is shown in parentheses. (B and C) *In vivo* activities of the WT and mutant promoters, measured by promoter-*lacZ* reporter assay, in the background of strains Newman (B) and USA300 (C). Since *Pcoa* activity was very low in the USA300 background, a magnified graph image is shown for *Pcoa* above the original graph. Data are representative of results from three independent experiments. Error bars denote standard deviations.

bound transcription activator can interact with various components of RNAP (i.e., α CTD, α NTD, and sigma factor), recruiting RNAP and activating the conversion from a closed to an open RNAP complex (8, 14, 35, 53). In activation by conformational change, the transcription activator most often binds to near promoter elements and elicits conformational changes in the promoter region, resulting in enhanced interaction of the region with RNAP (5, 28). Since, in *Phla*, the SaeR binding site resides 22 bp upstream of the -35 region, *Phla* seems to be a class I promoter and P-SaeR probably interacts with α CTD and recruits RNAP to the promoter. Indeed, the presence of P-SaeR enhanced the binding of RNAP to *Phla* (*Phla* in Fig. 7C). Interestingly, however, RNAP appears to be able to bind to *Phla* without P-SaeR at least under *in vitro* conditions (*Phla* in Fig. 7C, lane 3). Since the presence of P-SaeR is essential for transcription from *Phla* (39), the results suggest that RNAP bound to *Phla* cannot initiate transcription without P-SaeR. Therefore, in *Phla*, P-SaeR seems to play at least two roles, (i) further recruitment of RNAP to *Phla* and (ii) assistance with transcription initiation, possibly by repositioning of RNAP in *Phla*. On the other hand, because the SaeR binding sites overlap the -35 region by 1 bp (Fig. 1), P1 and *Pcoa* resemble class II promoters. In those promoters, P-SaeR is expected to activate transcription by interacting with sigma factor, α NTD, or α CTD.

Several studies have suggested that certain transcription activators and RNAP need to be on the same side of the DNA helix for their interactions and subsequent transcription activation (40, 49, 51, 55). The cyclic AMP receptor protein (CRP), a DNA binding protein, regulates the transcription of a large number of genes by directly interacting with RNAP (7, 12). With the *lac* promoter, where the CRP binding site is located at -61.5 bp from the TSS, Mandecki and Caruthers showed that while a 5-bp insertion between the CRP binding site and the -35 promoter region abolished transcription activation, an 11-bp insertion partially restored activation (40). Indeed, CRP and FNR, another well-studied transcription factor, can activate transcription when their binding sites are positioned -41.5 bp, -61.5 bp, -72.5 bp, 82.5 bp, or 92.5 bp from the TSS (16, 40, 49, 51, 55). In the P1 promoter, where the center of the SaeR binding site is at -40.5 bp, when the position of the SaeR binding site was altered to -45.5 (5-bp insertion), -50.5 (10-bp insertion), or -62.5 (22-bp insertion), only mutant P1 with the SaeR binding site at -62.5 showed significant SaeR-mediated transcription activation (22T in Fig. 5B), suggesting that, for successful transcription activation, SaeR might also need to be on the same side of the DNA helix as RNAP. Noteworthy is that, like CRP and FNR (55), SaeR failed to activate transcription at the P1 mutant, where the SaeR binding site resides at the -50.5 -bp position, suggesting that positioning P-SaeR and RNAP on the same side of the DNA helix is not sufficient and that a certain minimum distance is also required for efficient interaction between P-SaeR and α CTD.

Recently, staphylococcal RNAP has been purified and used to study the molecular mechanism of transcription regulators (44, 45). Reynolds and Wigneshweraraj purified core RNAP from *S. aureus* NCTC 8325 by affinity chromatography and formed a holoenzyme by adding the vegetative sigma factor σ^A that was expressed and purified from *E. coli* (45). On the other hand, Reyes et al. attached a His₁₀ tag sequence to the C terminus of *rpoC*, the gene encoding the RNAP β' subunit, and purified RNAP from a *sarA agrA sarR* triple mutant of *S. aureus* SH1000, a σ^B -positive strain, by Ni column chromatography (30, 44). As Reyes et al. did, we also attached a His₁₀ tag sequence at the C terminus of the RNAP β' subunit (*rpoC*, NWMN_0505) and purified RNAP by Ni column chromatography from *S. aureus* strain RN4220, a strain derived from the 8325-4 (= RN0450) (29). Since strain RN4220 contains inactive σ^B (4), the majority of the sigma factors in the purified RNAP holoenzymes are expected to be σ^A (*rpoD*, NWMN_1464). In fact, MS analysis confirmed that the 51-kDa protein in purified RNAP is σ^A (Fig. 3A). Interestingly, despite its higher molecular mass, the band intensity of σ^A was lower than that of the RNAP α subunit, implying that a significant portion of the purified RNAPs are core enzymes without a sigma factor. Since *S. aureus* has two additional alternative sigma factors, σ^H (23 kDa) and σ^S (19 kDa) (41, 46), we cannot exclude the possibility that a certain portion of the purified RNAP contains one of the alternative sigma factors. However, the absence of protein bands corresponding to those alternative sigma factors in Fig. 3A suggests that the portion of RNAP with those alternative sigma factors, if there is any, will be minor. Nonetheless, the overall agreement of *in vitro* transcription assays with *lacZ* reporter assays demonstrates that purified RNAP can be utilized for functional analysis of σ^A -dependent promoters.

ACKNOWLEDGMENTS

We thank Gary M. Dunny and Christopher M. Johnson at the University of Minnesota for their technical assistance in generating staphylococcal RNAP and conducting *in vitro* transcription assays. We also thank Olaf Schneewind and Dominique Missiakas at the University of Chicago for providing the *saeR* transposon mutant.

This study was supported by scientist development grant 0835158N from the American Heart Association and by AI077564 from the National Institute of Allergy and Infectious Diseases.

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