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Rot and SaeRS Cooperate To Activate Expression of the Staphylococcal Superantigen-Like Exoproteins

Meredith A. Benson,[a] Sarit Lilo,[a] Tyler Nygaard,[b] Jovanka M. Voyich,[b] and Victor J. Torres[a]

Department of Microbiology, New York University School of Medicine, New York, New York, USA,[a] and Department of Immunology and Infectious Diseases, Montana State University, Bozeman, Montana, USA[b]

Staphylococcus aureus **is a significant human pathogen that is capable of infecting a wide range of host tissues. This bacterium is able to evade the host immune response by utilizing a repertoire of virulence factors. These factors are tightly regulated by various two-component systems (TCS) and transcription factors. Previous studies have suggested that transcriptional regulation of a subset of immunomodulators, known as the staphylococcal superantigen-like proteins (Ssls), is mediated by the master regulators accessory gene regulator (Agr) TCS, *S. aureus* exoprotein expression (Sae) TCS, and Rot. Here we demonstrate that Rot and SaeR, the response regulator of the Sae TCS, synergize to coordinate the activation of the *ssl* promoters. We have determined that both transcription factors are required, but that neither is sufficient, for promoter activation. This regulatory scheme is mediated by direct binding of both transcription factors to the *ssl* promoters. We also demonstrate that clinically relevant methicillin-resistant *S. aureus* (MRSA) strains respond to neutrophils via the Sae TCS to upregulate the expression of *ssl*s. Until now, Rot and the Sae TCS have been proposed to work in opposition of one another on their target genes. This is the first example of these two regulators working in concert to activate promoters.**

Staphylococcus aureus is a major human pathogen responsible for a range of diseases from soft tissue infections to life-threatening infections such as toxic shock syndrome, pneumonia, sepsis, endocarditis, and osteomyelitis. The versatility of this bacterium is largely due to the regulated expression of an arsenal of virulence factors, including cell wall-associated factors, cytotoxins, and immunomodulators (16, 39). Among the immunomodulators, the staphylococcal superantigen-like proteins (Ssls) represent a family of 14 secreted proteins, each with a molecular mass of around 25 kDa, that have a variety of immunomodulatory properties, including inhibition of complement activation and neutrophil recruitment, as well as blocking of opsonization by IgG and IgA (17). Importantly, Ssl overproduction is associated with *S. aureus* pathogenesis in animal models of systemic infection (3, 7, 52).

The expression of virulence factors in *S. aureus* is tightly regulated by two-component systems (TCS) (i.e., accessory gene regulator [Agr] and *S. aureus* exoprotein expression [Sae] TCS), global regulators (i.e., Rot, SigB, SarA, and SarA homologues), and regulatory RNA molecules that interact to ensure the temporal expression of these factors (11, 15, 40). The best-characterized TCS in *S. aureus* is the Agr quorum-sensing system, which is composed of the *agrBCDA* structural genes and a 514-nucleotide regulatory RNA molecule, RNAIII, which is the main effector molecule of the Agr system (41). Upon reaching quorum, the Agr TCS is activated and the autoinducing peptide (AIP), encoded by *agrD*, is produced. AIP is processed and released by AgrB, upon which it is recognized by AgrC, the sensor kinase (41, 50). Subsequently, AgrC activates the response regulator AgrA, resulting in the expression of RNAIII (27, 38). RNAIII directly and indirectly controls the majority of Agr target genes by its antisense function (6, 37). The indirect regulation of virulence genes by RNAIII is mediated principally by posttranscriptional control of the transcription factor repressor of toxins (Rot), by which RNAIII prevents the translation of *rot* (8, 19). Rot is a member of the SarA family of DNA binding proteins involved in the repression of genes that encode various secreted proteins and activation of genes that encode cell-associated proteins (11, 30, 35, 47, 53, 54).

An additional critical TCS involved in the regulation of the staphylococcal virulon is the *S. aureus* exoprotein expression TCS, which is encoded by the *sae* locus (24). The *sae* locus consists of four open reading frames (*saePQRS*), of which *saeR* and *saeS* encode the response regulator and sensor histidine kinase, respectively (21). *saeP* encodes a poorly characterized putative lipoprotein, while *saeQ* encodes a membrane protein involved in the stabilization of SaeS (1, 26). The *sae* locus has two promoters, designated P1 and P3, of which P1 is positively autoregulated and negatively regulated by SigB and Rot (1, 18, 23, 25, 26, 30). Activation of P1 can occur through exposure to neutrophils and phagocyte products, such as low pH, H_2O_2, and the antimicrobial peptide α-defensin (18, 43, 55). Activation of the P3 promoter results in a transcript carrying *saeR*, *saeS*, and a truncated *saeQ*. This promoter demonstrates low, constitutive activity (18). The Sae TCS is primarily known for its requirement for the expression of cytotoxin-encoding genes (22, 24, 42, 46, 56). Consistent with this, the Sae TCS plays a critical role in *S. aureus* pathogenesis in several animal models of infection (25, 36, 42, 56, 57). In addition, the Sae TCS plays an essential role in *S. aureus* survival in human whole blood and in the avoidance of human neutrophil-mediated killing (56). Interestingly, the *S. aureus* strain Newman, a highly virulent and commonly used methicillin-sensitive strain, exhibits high *sae* expression levels, due to a single amino acid substitution in the first N-terminal transmembrane domain of SaeS that results in the change of a leucine at position 18 to proline (1, 4, 18, 48).

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Address correspondence to Victor J. Torres, victor.torres@nyumc.org.

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TABLE 1 *Staphylococcus aureus* strains

Strain	Background	Description	Genotype	Reference or source
VJT 1.01	Newman	Wild type	Wild type	14
VJT 2.84	USA100	USA100	Wild type	NARSA[a]
VJT 2.59	USA200	UAMS-1	Wild type	20
VJT 12.61	USA300	LAC	Wild type	13
VJT 4.79	USA400	MW2	Wild type	2
VJT 2.86	USA500	USA500	Wild type	45
VJT 15.49	USA600	USA600	Wild type	NARSA
VJT 21.05	USA700	USA700	Wild type	NARSA
VJT 21.06	USA800	USA800	Wild type	NARSA
VJT 21.08	USA1100	USA1100	Wild type	NARSA
VJT 7.17	VJT 1.01	Transduction of *agr*::*tet* from RN7206 into VJT 1.01	*agr*::*tet*	7
VJT 9.98	VJT 1.01	Transduction of *rot*::Tn*917* from RN10623 into VJT 1.01	*rot*::Tn*917*	7
VJT 16.99	VJT 1.01	Replacement of *saeQRS* with *aad* in VJT 1.01	*saeQRS*::*spec*	This study
VJT 10.03	VJT 1.01	Transduction of *rot*::*Tn917* from RN10623 into VJT 7.17	*agr*::*tet rot*::Tn*917*	7
VJT 17.27	VJT 1.01	Transduction of *agr*::*tet* from RN7206 into VJT 16.99	*agr*::*tet saeQRS*::*spec*	This study
VJT 19.04	VJT 1.01	Transduction of *rot*::Tn*917* from RN10623 into VJT 17.27	*agr*::*tet saeQRS*::*spec rot*::Tn*917*	This study
AH1263	USA300 LAC	Erythromycin-sensitive clone	Wild type	9
AH1292	USA300 LAC	Transduction of *agr*::*tet* from RN7206 into AH 1263	*agr*::*tet*	7
VJT 28.25	USA300 LAC	Transduction of *saeS*::*bursa* from HF6131 into AH1263	*saeS*::*bursa*	This study
VJT 31.49	USA300 LAC	Transduction of *saeS*::*bursa* from HF6131 into VJT 28.25	*agr*::*tet saeS*::*bursa*	This study
HF6131	RN6734	Transduction of *saeS*::*bursa*	*saeS*::*bursa*	1
RN4220	8325-4	Restriction-deficient cloning host		28

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

This L18P amino acid substitution results in constitutive activation of the Sae TCS, which in turn is responsible for the altered phenotypes exhibited by strain Newman (31, 58), including the increased production of exoproteins observed in this strain (1, 18, 31, 32, 48).

We recently demonstrated that inactivation of *agr* in strain Newman resulted in the production of higher levels of Ssls than in the wild-type (WT) strain and that the ability of Agr to repress *ssl*s was dependent on the RNAIII-mediated regulation of Rot (7). Surprisingly, we found that Rot regulates *ssl* expression by directly binding to and activating the *ssl* promoters. Recently, the Sae TCS has also been implicated in the regulation of *ssl*s (44). In this study, we investigated the molecular mechanism by which Rot and the Sae TCS regulate *ssl* expression. Our genetic and biochemical data demonstrate that Rot and SaeR synergize to bind to the *ssl* promoters and activate the expression of these genes in *S. aureus*, a regulatory scheme that has not been previously reported.

MATERIALS AND METHODS

Bacterial strains and culture conditions. The *S. aureus* strains used in this study are described in Table 1. *S. aureus* were grown in Roswell Park Memorial Institute medium (RPMI) supplemented with 1% Casamino Acids (CAS) (RPMI-CAS) with shaking at 180 rpm, unless otherwise indicated. When appropriate, tryptic soy broth (TSB) and RPMI-CAS were supplemented with chloramphenicol (CM) at a final concentration of 10 μg/ml.

Escherichia coli DH5α was used to propagate plasmids, and T7 *lysY lacQ* (New England BioLabs) was used as an expression strain for recombinant protein. *E. coli* was grown in Luria broth (LB), and when needed, the medium was supplemented with ampicillin at a final concentration of 100 μg/ml or kanamycin at a final concentration of 50 μg/ml.

Construction of mutants. Strains used in this study are described in Table 1. *S. aureus* mutants lacking the *agr* locus, *saeS*, and *rot* were generated by transduction using phage 80α.

The *S. aureus saeQRS*::*spec* mutant strain was generated using the allelic replacement strategy previously described (5). Plasmids for allelic replacement of *saeQRS* were constructed using pGEM-T (Promega) and pKOR-1. Briefly, sequences flanking *saeQRS* were PCR amplified with primers VJT125 and VJT126 for the upstream fragment and primers VJT127 and VJT128 for a downstream fragment. The PCR amplicons were digested with XmaI and PstI and assembled into pGEMT-T (Promega). To generate the plasmid containing the *saeQRS*::*spec* construct, a spectinomycin resistance cassette (*aad9*) was amplified from plasmid pBT-S using primers VJT391 and VJT392 and subsequently digested with XmaI and subcloned into the pGEM-T *saeQRS* plasmid (an internal XmaI site was previously generated between both flanking sequences to facilitate the insertion of antibiotic resistance markers). A PCR amplicon of the resultant *saeQRS*::*spec* construct was then recombined into pKOR-1, resulting in the pKOR-1Δ*saeQRS*::*spec* plasmid. Further allelic replacement was carried out in strain Newman according to previously described methods (5).

Plasmids. Primers and plasmids used in this study are listed in Tables 2 and 3, respectively. Plasmids were isolated from *E. coli* and then transformed into the restriction-deficient *S. aureus* RN4220, followed by electroporation into the respective *S. aureus* strains.

(i) Complementation plasmids. For the construction of a plasmid expressing *rot* under the control of the hemin-inducible *hrtAB* promoter, a PCR amplicon containing the *rot* open reading frame (ORF) (NWMN_1655) into which codons for six histidine residues were inserted at the 3′ end just prior to the stop codon was made using primers 420 and 403. The PCR product was digested with EcoRI and XhoI and ligated into the *E. coli*/*S. aureus* shuttle vector pOS1-P_{hrtAB}, which had been digested with the same restriction enzymes. The ligation product was then transformed into *E. coli* DH5α, and the resulting plasmid was designated pOS1-P_{hrtAB}-*rot*6×His.

For the construction of a plasmid expressing *saeRS* under the control of the hemin-inducible *hrtAB* promoter, a PCR amplicon containing the *saeRS* ORF (NWMN_0675 and NWMN_0674, respectively) was made using primers 481 and 484. Further construction of the vector was carried out as done with the pOS1-P_{hrtAB}-*rot*6×His vector. The resulting plasmid was designated pOS1-P_{hrtAB}-*saeRS*.

For the construction of a plasmid constitutively expressing *saeS*, a PCR amplicon containing the *saeS* ORF (NWMN_0674) was made using prim-

TABLE 2 Primers

No.	Name	Sequence
403	rot-6XHis-3′-EcoRI	5′-CCCCGAATTCTTAGTGATGGTGATGGTGATGCACAGCAATAATTGCGTTTAAAC
420	rot5′-XhoI	5′-GGGGCTCGAGATGAAAAAAGTAAATAACGACACTG
481	saeR-XhoI-R	5′-CCCCCTCGAGATGACCCACTTACTGATCG
484	saeS-EcoRI-F	5′-CCCCGAATTCTTATGACGTAATGTCTAATTTGTG
761	saeS-F-XhoI	5′-CCCCCTCGAGATGGTGTTATCAATTAGAAGTCA
762	saeS-R-BamHI	5′-CCCCGGATCCTTATGACGTAATGTCTAATTTGTG
745	R-XhoI-3 × FLAG-rot	5′-CCCCTCGAGCTTGTCATCGTCATCCTTGTAATCGATATCATGATCTTTATAATCA CCGTCATGGTCTTTGTAGTC-CACAGCAATAATTGCGTTTAAACTA
313	rot-5′-F-NdeI	5′-CCCCCATATGAAAAAAGTAAATAACGACACTG
311	rot-6 × His-3′-R-XhoI	5′-CCCCTCGAGTTAGTGATGGTGATGGTGATGCACAGCAATAATTGCGTTTAAAC
706	saeR-R-BamHI	5′-CCCGGATCCTTATCGGCTCCTTTCAAATTTATATC
438	saeR-5′-NdeI	5′-CCCCCATATGACCCACTTACTGATCGTGGATG
771	saeS-dN92-R-XhoI	5′-CCCCCTCGAGAAAGAAATTTATGAATTAAATCAATC
762	saeS-R-BamHI	5′-CCCCGGATCCTTATGACGTAATGTCTAATTTGTG
305	p-ssl7-R-BIO	5′-BIO-CCCCAGTACTATTCTCCCAATCTATTT
374	pSSL7-R-NdeI	5′-CCCCATATGAGTACTATTCTCCCAATCTATTTA
304	p-ssl9-R-Bio	5′-BIO-CCCCATTTTTTTGCTCCAATCTTAATG
375	pSSL9-R-NdeI	5′-CCCCATATG-ATTTTTTTGCTCCAATCTTAATGTA
82	LukAB intra 3′-BIO	5′-AGTATCACCATCAAGATTCTTC
87	LukAB intra 5′	5′-CCCCGAATTCAAAGAAGGATAATATTGAAAGG
372	LukAB intra 3′	5′-AGTATCACCATCAAGATTCTTC
308	p-ssl11-R-BIO	5′-BIO-CCCC-AATTCTATGCTCCCAATTTTTAG
345	pssl11-F-PstI	5′-CCCCCTGCAGTTAGGCACTGTGAAAGCGC
323	pssl11-R-no RBS-KpnI	5′-CCCCGGTACCTTTTAGTCTATTTGATTTATTCTATTA
341	pssl7-F-PstI	5′-CCCCCTGCAGGCAGACTAGTAATTGTAGGG
319	pssl7-R-no RBS-KpnI	5′-CCCCGGTACCCTATTTATAAATTTTGTCTTAATATATT
757	pSSL7-F-saeR site	5′-AAAAATAGTTAGAAAGAGGTTAATTCATA
758	pSSL7-R-saeR site	5′-TATGAATTAACCTCTTTCTAACTATTTTT
343	pssl9-F-PstI	5′-CCCCCTGCAGGAATGAAAGCTTAAGAAGCGG
321	pssl9-R-no RBS-KpnI	5′-CCCCGGTACCCTTAATGTATTGGATTGTTATTATTA
759	pSSL9-F-saeR site	5′-TTTATTTAGATGAGCGTTAATGTCAG
760	pSSL9-R-saeR site	5′-CTGACATTAACGCTCATCTAAATAAA
552	P1-sae-R-PstI	5′-CCCCTGCAGTTATTGTGGCAAAAGGTTTATAAA
555	P1-sae(full)-F-NdeI	5′-CCCCATATGGCTAACTCCTCATTTCTTCAAT
348	sGFP-R-EcoRI	5′-CCCGAATTCTTAGTGGTGGTGGTGG
347	psarA-F-PstI	5′-CCCCTGCAGCTGATATTTTTGACTAAACCAAATG
125	NWMN_0674-0676 5′-1 AttB1	5′-GGGGACAAGTTTGTACAAAAAAGCAGGCTAATAAAAAGGATGGAC ATAGATG
126	NWMN_0674-0676 3′-1-Xma	5′-TCCCCCCGGGAAAATGCAAAGACTAAAAAGAAG
127	NWMN_0674-0676 5′-2-Xma	5′-TCCCCCCGGGACATTCTTTCTATTTATTGTGTG
128	NWMN_0674-0676 3′-2 AttB2	5′-GGGGACCACTTTGTACAAGAAAGCTGGGTTAGTTCAGATAAATATTTCCTTAC
671	prot-F-PstI	5′-CCCCTGCAGACAGTAGATGCTCATCTTTTTTTAG
517	prot-R-no RBS-KpnI	5′-CCCGGTACCAAATAAACTTGCTTTCTATTCAATTG
391	pSpec.B-Xma	5′-TCCCCCCGGGAAAATTGAAAAAAGTGTTTCCACC
392	pSpec.A-Xma	5′-TCCCCCCGGGCAGGTTATGACCATCTGTGCC

ers 761 and 762 with either Newman genomic DNA (gDNA) or USA300 LAC gDNA as the template. The PCR products were digested with BamHI and XhoI and ligated into the *E. coli*/*S. aureus* shuttle vector pOS1-P_{lgt}, which had been digested with the same restriction enzymes. The ligation products were then transformed into *E. coli* DH5α, and the resulting plasmids were designated pOS1-P_{lgt}-*saeS* (Newman) and pOS1-P_{lgt}-*saeS* (LAC).

For the construction of a plasmid constitutively expressing *rot* with a C-terminal FLAG tag, a PCR amplicon containing the *rot* ORF (NWMN_1655) was made using primers 745 and 313. The PCR product was digested with NdeI and XhoI and ligated into the *E. coli*/*S. aureus* shuttle vector pOS1-P_{lgt}, which had been digested with the same restriction enzymes. The ligation product was then transformed into *E. coli* DH5α, and the resulting plasmid was designated pOS1-P_{lgt}-*rot*-FLAG.

(ii) Generation of reporter plasmids. The construction of *ssl7*, *ssl9*, and *ssl11* reporter plasmids was described previously (7). For construction of a plasmid expressing the green fluorescent protein (GFP) gene under the control of the *sarA* promoter, a PCR amplicon was made using pCM11 as the template to amplify the *sarA* promoter and GFP gene using primers 347 and 348. The PCR product was digested with PstI and EcoRI and ligated into the *E. coli*/*S. aureus* shuttle vector pOS1sGFP, which had been digested with the same restriction enzymes. The ligation product was then transformed into *E. coli* DH5α, and the resulting plasmid was designated pOS1sGFP-P_{sarA}-*sod* RBS.

For the construction of a plasmid expressing the GFP gene under the control of the *sae* P1 promoter, a PCR amplicon containing the P1 promoter region was made using primers 552 and 555. The PCR product was digested with PstI and NdeI and ligated into the *E. coli*/*S. aureus* shuttle vector pOS1sGFP, which had been digested with the same restriction enzymes. The ligation product was then transformed into *E. coli* DH5α, and the resulting plasmid was designated pOS1sGFP-$P_{sae\ P1}$.

For construction of a plasmid expressing the GFP gene under the

TABLE 3 Plasmids

Name	Description	Resistance	Reference or source
pOS1-P_{hrtAB}	Empty vector control containing the *hrtAB* promoter	Cm	51
pOS1-P_{hrtAB}-*rot*6 × His	*hrtAB* promoter controlling expression of *rot* with a C-terminal His tag	Cm	This study
pOS1-P_{hrtAB}-*saeRS*	*hrtAB* promoter controlling expression of *saeRS*	Cm	This study
pOS1-P_{lgt}	*lgt* promoter in an empty vector	Cm	10
pOS1-P_{lgt}-*saeS* (Newman)	*lgt* promoter controlling expression of *saeS* amplified from strain Newman	Cm	This study
pOS1-P_{lgt}-*saeS* (LAC)	*lgt* promoter controlling expression of *saeS* amplified from strain LAC	Cm	This study
pOS1-P_{lgt}-*rot*3 × FLAG	*lgt* promoter controlling expression of *rot* containing a C-terminal FLAG tag	Cm	This study
pCM11	*sarA* promoter and *sod* RBS controlling expression of s*gfp*[a]	Erm	29
pOS1sGFP promoterless	pOS1 containing the *sgfp* with no promoter	Cm	7
pOS1sGFP-P_{ssl7}-*sod* RBS	*ssl7* promoter controlling expression of s*gfp*	Cm	7
pOS1sGFP-P_{ssl9}-*sod* RBS	*ssl9* promoter controlling expression of s*gfp*	Cm	7
pOS1sGFP-P_{ssl11}-*sod* RBS	*ssl11* promoter controlling expression of s*gfp*	Cm	7
pOS1sGFP-P_{sarA}-*sod* RBS	*sarA* promoter controlling expression of s*gfp*	Cm	This study
pOS1sGFP-P_{rot}-*sod* RBS	*rot* promoter controlling expression of s*gfp*	Cm	This study
pOS1sGFP-P_{sae}P1	*sae* P1 promoter controlling expression of s*gfp*	Cm	This study
pOS1sGFP-P_{ssl7}-*saeR*-*sod* RBS	*ssl7* promoter with mutated SaeR binding site controlling expression of s*gfp*	Cm	This study
pOS1sGFP-P_{ssl9}-*saeR*-*sod* RBS	*ssl9* promoter with mutated SaeR binding site controlling expression of s*gfp*	Cm	This study
pET41b	Empty expression vector with IPTG-inducible promoter	Amp	Novagen
pET41b *saeS*ΔN92	*saeS* deltaN92 expression vector	Amp	This study
pET41b *saeR*	*saeR* expression vector	Amp	This study
pET14b	Empty expression vector with IPTG-inducible promoter	Kan	Novagen
pET14b *rot*	*rot* expression vector	Kan	This study
pKOR1	Empty vector used for allelic replacement	Cm	5
pKOR1 Δ*saeQRS*::*spec*	pKOR1 vector containing *saeQRS*-homologous regions with *aad* insertion	Cm	This study

[a] s*gfp*, superfolder green fluorescent protein gene.

control of the *rot* promoter, a PCR amplicon containing the *rot* promoter region was made using primers 671 and 517. The PCR product was digested with PstI and KpnI and ligated into the *E. coli*/*S. aureus* shuttle vector pOS1sGFP, which had been digested with the same restriction enzymes. The ligation product was then transformed into *E. coli* DH5α, and the resulting plasmid was designated pOS1sGFP-P_{rot}-*sod* RBS.

For construction of a plasmid expressing the GFP gene under the control of the *ssl7* or *ssl9* promoter in which the SaeR binding site has been mutated, a PCR splicing by overlap extension (SOE) approach was used, in which the 5′ ends were generated using primers 341 and 758 for the *ssl7* promoter and primers 343 and 760 for the *ssl9* promoter. The 3′ ends were generated with primers 319 and 757 for the *ssl7* promoter and primers 321 and 759 for the *ssl9* promoter. The 5′ and 3′ ends were mixed together as the template for a PCR using primers 341 and 319 for the *ssl7* promoter and primers 343 and 321 for the *ssl9* promoter. PCR products were digested with PstI and KpnI and then ligated into the *E. coli*/*S. aureus* shuttle vector pOS1sGFP, which had been digested with the same restriction enzymes. The ligation products were then transformed into *E. coli* DH5α, and the resulting plasmids were designated pOS1sGFP-$P_{ssl7\text{-SaeR}}$-*sod* RBS and pOS1sGFP-$P_{ssl9\text{-SaeR}}$-*sod* RBS.

GFP reporter assays. GFP reporter assays were performed as described previously (7). Briefly, overnight cultures of *S. aureus* grown in RPMI-CAS plus CM were diluted 1:100 into 5 ml of RPMI-CAS plus CM. The optical density at 600 nm (OD_{600}) and GFP fluorescence were measured at 0, 3, 5, 7, 9, and 24 h postsubculture using a Perkin-Elmer Envison 2103 multilabel reader.

Exoprotein profiles. Exoproteins were produced and processed as described previously (3, 51). Briefly, *S. aureus* strains were grown in 5 ml RPMI-CAS overnight at 37°C with shaking at 180 rpm. The overnight cultures were then diluted 1:100 into 5 ml fresh RPMI-CAS in a 15-ml conical tube. Cultures were grown to stationary phase and then normalized to the same OD_{600}. Bacterial cells were sedimented by centrifugation at 400 × *g* for 15 min, and proteins in the culture supernatant were precipitated using 10% (vol/vol) trichloroacetic acid (TCA) at 4°C overnight. The precipitated proteins were sedimented by centrifugation, washed, dried, resuspended in 1× SDS loading buffer, and boiled for 10 min. Proteins were separated using 15% SDS-PAGE and stained with Coomassie blue.

Immunoblots. *S. aureus* cultures were grown in RPMI-CAS as indicated and normalized to the same OD_{600}. Cells were then pelleted by centrifugation. Exoproteins were prepared from supernatants as described above. For cytoplasmic extracts, cell pellets were resuspended in PBS containing 0.1-mm glass beads. Cells were then disrupted using a FastPrep-24 tissue and cell homogenizer (MP Biomedicals, Solon, OH). The protein concentration was normalized for each sample using a bicinchoninic acid (BCA) protein assay kit (Thermo Scientific). Proteins were resolved by 10, 12, or 15% SDS-PAGE, transferred to nitrocellulose membranes, and probed with the indicated primary antibodies. An Alexa Fluor 680-conjugated anti-rabbit antibody was used as a secondary antibody. Membranes were then scanned using an Odyssey infrared imaging system (Li-Cor Biosciences, Lincoln, NE).

Cloning, expression, and purification of recombinant proteins. (i) SaeR. The *saeR* ORF (NWMN_0675) was PCR amplified from strain Newman genomic DNA with primers 706 and 438. After being digested by NdeI and XhoI, the PCR product was cloned into pET41b (EMD Biosciences, Darmstadt, Germany). The resulting plasmid was transformed first into DH5α and then into T7 *lysY lacQ*. The T7 *lysY lacQ* strain carrying the plasmid was grown in LB at 37°C with shaking at 180 rpm to an optical density at 600 nm of 0.6, followed by addition of IPTG (isopropyl-β-D-thiogalactopyranoside) to a final concentration of 0.1 mM. After overnight induction at 16°C, the cells were harvested and frozen at −80°C. SaeR was purified from the frozen cells using Ni-nitrilotriacetic acid (Ni-NTA) Superflow resin (Qiagen). The purified protein was concentrated using Centriprep spin columns (Millipore) and frozen at −80°C.

(ii) SaeSΔN92. Except for the primers used for PCR amplification of the coding region, the cloning, expressing, and purification of the cytoplasmic domain of SaeS were carried out as described for SaeR above. The primers used were 771 and 762.

(iii) Rot. The *rot* ORF (NWMN_1655) was PCR amplified from strain Newman genomic DNA with primers 311 and 313. After being digested

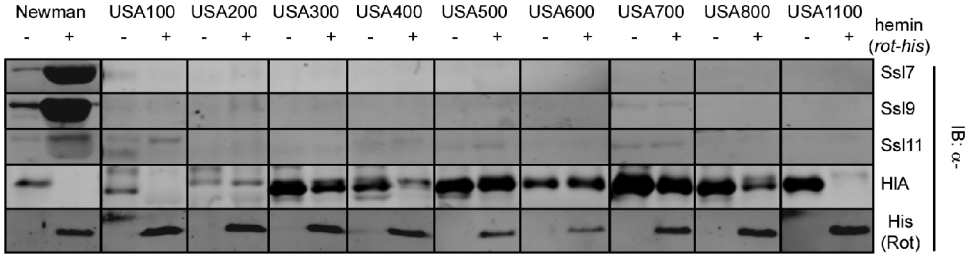

FIG 1 Effect of overproduction of Rot in *S. aureus* clinical isolates. Immunoblot analysis of early-stationary-phase supernatants from various *S. aureus* clinical isolates containing a hemin-inducible Rot vector, grown in inducing (+ hemin) or noninducing (− hemin) conditions, is shown. Exoproteins were collected, precipitated, separated using SDS-PAGE, and transferred to nitrocellulose, and the indicated Ssls and Hla were detected by immunoblotting (IB). Corresponding whole-cell lysates were immunoblotted with an anti-6×His antibody to detect Rot-His.

with XhoI and NdeI, the PCR product was cloned into pET14b (EMD Biosciences, Darmstadt, Germany). The resulting plasmid was transformed first into DH5α and then into T7 *lysY lacQ*. The T7 *lysY lacQ* strain carrying the plasmid was grown in LB to an optical density at 600 nm of 0.6, and then 0.1 mM IPTG was added. After overnight induction at 16°C, the cells were harvested and frozen at −80°C. Rot was purified from the frozen cells using Ni-NTA Superflow resin (Qiagen).

Phosphorylation of SaeR for electrophoretic mobility shift assays (EMSA). Purified SaeR was phosphorylated as described previously (49). Briefly, SaeR was mixed with purified SaeSΔN92 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 15 min before the protein was added to biotinylated DNA probes.

Generation of anti-Rot polyclonal antibody. The *rot* gene was amplified, cloned, expressed, and purified as described above. The Rot polyclonal antibody was generated by Pacific Immunology by immunizing rabbits with the purified protein.

EMSA. The primers used to generate EMSA probes are listed in Table 2. DNA probes of about 300 to 400 bp were PCR amplified using biotinylated primers, and 40 fmol of the biotinylated probes was mixed with various amounts of the desired purified recombinant protein in a final reaction volume of 20 μl in EMSA buffer (10 mM Tris-HCl [pH 7.4], 50 mM KCl, 5 mM $MgCl_2$, 10% glycerol, 5 μg/ml salmon sperm DNA). After being incubated at room temperature for 15 min, samples were analyzed by 6% native polyacrylamide gel electrophoresis (10 mA/gel prerun for 30 min in Tris-borate-EDTA [TBE]). The gels were incubated in streptavidin DyLight (Thermo Scientific, Rockford, IL) diluted 1:1,000 in phosphate-buffered saline (PBS) plus 0.1% Tween and 5% bovine serum albumin (BSA) for 1 h and then visualized using the Odyssey infrared imaging system (Li-Cor Biosciences, Lincoln, NE).

Immunoprecipitation. The indicated *S. aureus* strain Newman mutants were grown to early stationary phase in 15 ml of RPMI-CAS and normalized to the same OD_{600}. A 378-μl portion of 37% formaldehyde was added to each culture, and the tubes were rocked at room temperature for 20 min. Three milliliters of 2.5 M glycine was added to each culture, and the tubes were rocked at room temperature for an additional 5 min. Bacterial cells were sedimented by centrifugation at 400 × *g* for 15 min, and pellets were washed and frozen at −80°C until ready to use.

Pellets were thawed on ice and treated with lysostaphin to remove the cell wall. Protoplasts were then sonicated, and M2 anti-FLAG magnetic beads (Sigma-Aldrich, St. Louis, MO) were added to the lysates. Incubation of beads and lysates was carried out with end-over-end rotation at room temperature for 30 min. Beads were washed extensively, and bound proteins were eluted. An aliquot of this elution was mixed with SDS buffer for immunoblot analysis. The remaining elution product was treated with RNase A and proteinase K and then subjected to PCR analysis of the *ssl7* and *ssl9* promoters using primers 374/241 and 375/343, respectively.

Isolation of PMNs. Blood samples were obtained from anonymous healthy donors as buffy coats from the New York City Blood Center. The New York City Blood Center obtained written informed consent from all participants involved in the study. Primary human neutrophils (PMNs) were isolated under endotoxin-free conditions as described previously (55).

Neutrophil reporter assay and CFU counts. Purified PMNs were diluted to 1 × 10^6 cells/ml in RPMI-CAS. *S. aureus* reporter strains grown overnight in RPMI-CAS plus CM were subcultured 1:100 into 5 ml RPMI-CAS and grown to mid-log phase. Cultures were sedimented by centrifugation at 400 × *g* for 15 min and washed twice with sterile PBS. Cultures were subsequently normalized to an OD_{600} of 1.0. Bacterial cultures were incubated with neutrophils at a multiplicity of infection (MOI) of 20. GFP fluorescence was measured at 0, 3, 5, 7, and 18 h postsubculture using a Perkin-Elmer Envision 2013 multilabel reader. At 5 h postinoculation, cultures were serially diluted and plated on tryptic soy agar (TSA) for CFU enumeration.

RESULTS

Overproduction of Rot in clinical isolates is not sufficient for Ssl overproduction. In *S. aureus* strain Newman, increased production of Rot resulted in the overproduction of Ssls (Fig. 1). However, when Rot was overproduced in several wild-type clinical isolates, we were unable to recapitulate the Ssl overproduction phenotype (Fig. 1). We observed that alpha-toxin levels were reduced in the majority of strains overproducing Rot, due to Rot-dependent repression of *hla* expression (34, 47), indicating that the overproduced Rot was functional in these strains. The inability of Rot to increase Ssl production in strains other than Newman indicated that while Rot is required for Ssl production (7), it alone is not sufficient to induce *ssl* expression in clinical isolates.

A functional Sae TCS is necessary for the Ssl overproduction phenotype exhibited by *agr*-defective strain Newman. Previous studies have revealed that a main difference between strain Newman and other *S. aureus* strains is the hyperactivity of the Sae TCS due to the L18P amino acid substitution in SaeS This substitution leads to the constitutive phosphorylation and activation of SaeR (1, 4, 18) and a series of Newman-specific phenotypes (31, 32, 48, 58). To determine if the Sae TCS is involved in the overproduction of Ssls exhibited by strain Newman, we generated an isogenic *saeQRS* mutant and examined the abundance of Ssls produced by this strain.

Exoprotein profile analysis revealed that deletion of *saeQRS* markedly affected exoprotein production compared to that by the wild-type Newman strain (Fig. 2A), a phenotype complemented by expressing *saeRS* in *trans* (Fig. 2A). In contrast, the exoprotein profile of an isogenic strain lacking *rot* was similar to that of wild-type Newman (Fig. 2A). Complementation studies revealed that overproduction of Rot into the *rot* mutant strain resulted in the

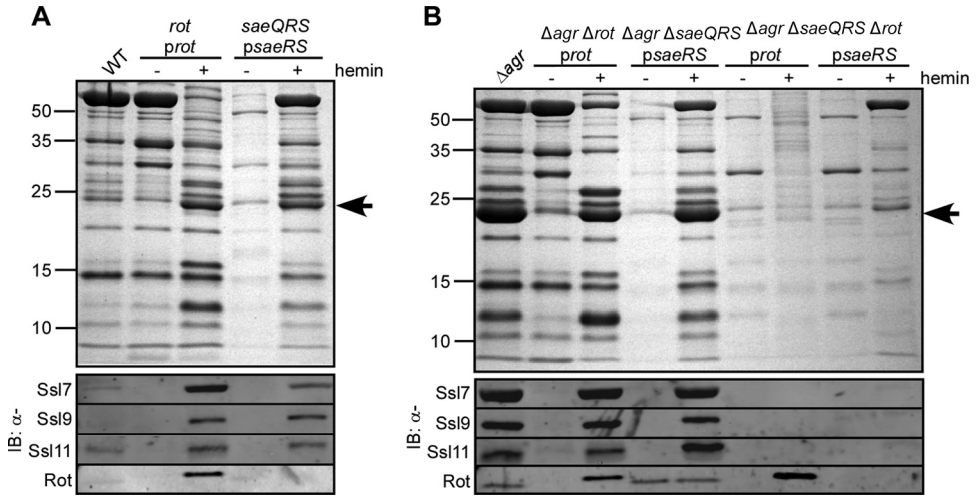

FIG 2 Contribution of Rot and the Sae TCS to Ssl production in strain Newman. The indicated Newman strains were grown to early stationary phase under either inducing (+ hemin) or noninducing (− hemin) conditions. Exoproteins were collected, precipitated, separated using SDS-PAGE, either stained with Coomassie blue (top panels) or transferred to nitrocellulose, and immunoblotted for the indicated Ssls (bottom panel). Corresponding whole-cell lysates were also immunoblotted for Rot. The arrow indicates Ssls.

enhanced appearance of a protein band at ~25 kDa (Fig. 2A), which has been shown to correspond to the Ssls (3, 7, 52). Interestingly, Ssls were also overproduced, albeit to a lower level, when *saeRS* was overexpressed by the complementation plasmid (Fig. 2A). Immunoblot analysis demonstrated that as observed with Rot, the Sae TCS is also required for Ssl production, as the *saeQRS* mutant no longer produced Ssls (Fig. 2A), a phenotype complemented by expressing *saeRS* in *trans*.

To further examine the contribution of the Sae TCS in the regulation of the Ssls and whether this regulation is in conjunction with or independent of Rot, we generated mutants of strain Newman lacking both *saeQRS* and the *agr* loci. In this strain, an *agr* mutation causes overproduction of Ssls, a finding attributed to an increased abundance of Rot (7). Exoprotein profile analysis confirmed this, as the *agr rot* mutant no longer produced the Ssls and their production was restored by expressing *rot* in *trans* (Fig. 2B). As with the *saeQRS* mutant, virtually no exoproteins were produced by the *agr saeQRS* double mutant strain, a phenotype complemented by expressing *saeRS* in *trans* (Fig. 2B). This observation suggests that the overabundance of Rot in the *agr* mutant strain is not sufficient to support production of Ssls.

Immunoblot analysis of *agr rot* and *agr saeQRS* double mutant strains revealed that both Rot and the Sae TCS are required for the production of the Ssls (Fig. 2B). Consistent with this, the lack of Ssls in the *agr rot saeQRS* triple mutant was not rescued by producing either Rot or SaeRS (Fig. 2B). Taken together, these data suggest that Sae and Rot work synergistically to promote the production of Ssls.

The Sae TCS is required for the activation of the *ssl* promoters. We have previously demonstrated that the regulation of *ssl* expression by Rot is due to the promoter activation by this transcription factor (7). To test whether Sae directly regulates the activation of the *ssl* promoters, the *ssl7* and *ssl9* promoters were fused to the GFP gene and the resulting transcriptional reporters were transformed into WT *S. aureus* strain Newman or the *rot* or *saeQRS* isogenic mutants. The strain lacking *saeQRS* was used to examine the effect of knocking out both the SaeS kinase and the SaeR response regulator. We observed that both Rot and the Sae TCS were required for the activation of the *ssl* promoters (Fig. 3A). Interestingly, the *rot* mutant was still able to activate the *ssl9* promoter at low levels (Fig. 3A). This residual activation could be attributed to Sae TCS activity, as the *rot saeQRS* double mutant strain displayed no detectable activation (Fig. 3A).

In an effort to further elucidate the contribution of the Sae TCS to the activation of the *ssl* promoters, we transformed the reporter plasmids into an *agr*, *agr rot*, or *agr saeQRS* mutant strain. The *agr* mutant had higher levels of *ssl* promoter activation (Fig. 3B). This was attributed to increased levels of Rot present in an *agr* mutant, as an *agr rot* double mutant strain exhibited reduced promoter activation (Fig. 3B). In contrast, no promoter activation was observed in the *agr saeQRS* double mutant strain (Fig. 3B). Taken together, these data suggest that both Rot and the Sae TCS are necessary to attain full activation of the *ssl* promoters; however, in the absence of Rot, the Sae TCS can still elicit partial activation.

The requirement of the Sae TCS for the activation of the *ssl* promoters is consistent with the finding that these promoters harbor an SaeR binding site (7, 42, 49). To further examine the influence of SaeR on the activation of the *ssl* promoters, we generated transcriptional reporters in which the SaeR binding sites on the *ssl7* and *ssl9* promoters were disrupted. The *ssl7* promoter contains a perfect SaeR binding site (GTTAA-N_6-GTTAA), while the *ssl9* promoter contains an imperfect SaeR binding site (TTTAA-N_6-GTTAA). Mutation of the last adenine of the SaeR binding palindromic sequence (GTTAA) has been previously shown to disrupt binding of the regulator (49). Thus, we mutated the *ssl7* and *ssl9* promoters to generate a GTTAG-N_6-GTTAA and a TTTAG-N_6-GTTAA SaeR binding site, respectively. The transcriptional reporters harboring these mutated promoters were transformed into wild-type *S. aureus* or an *agr* mutant strain and promoter activation monitored as described above. We observed that mutation of the SaeR binding site resulted in a significant reduction in promoter activation (Fig. 3C, open symbols). These data strongly suggest that SaeR influences the activation of the *ssl*

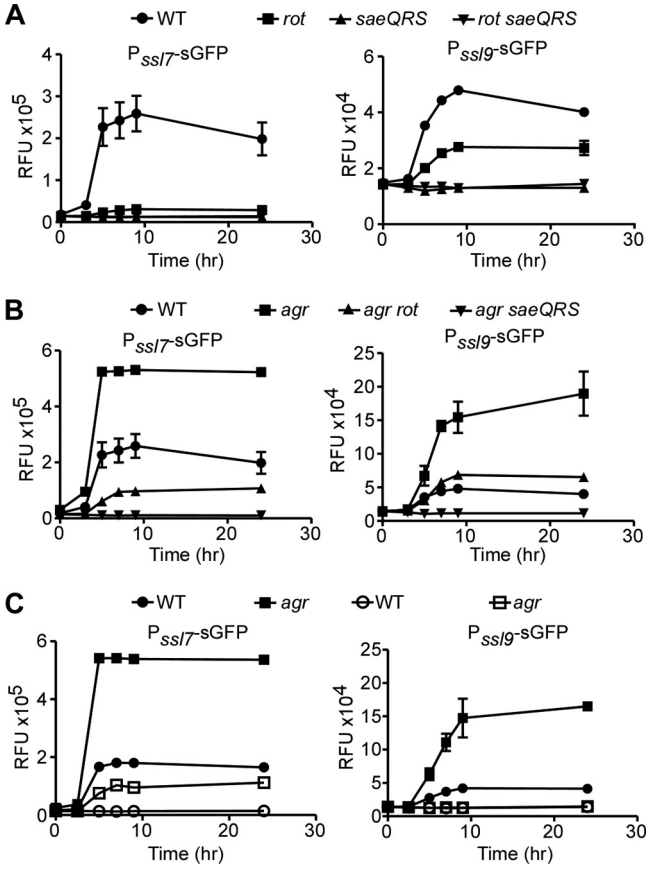

FIG 3 Role of the Sae TCS in the activation of the *ssl* promoters. (A and B) The indicated Newman strains were transformed with plasmids containing the *ssl7* or *ssl9* promoter controlling GFP gene expression, and GFP fluorescence was monitored at the indicated time points. Values represent averages from three independent experiments ± standard deviations (SD). (C) Reporter assay as done for panel A. Closed symbols indicate wild-type promoters, and open symbols indicate promoters in which the SaeR binding site has been mutated to impair SaeR binding.

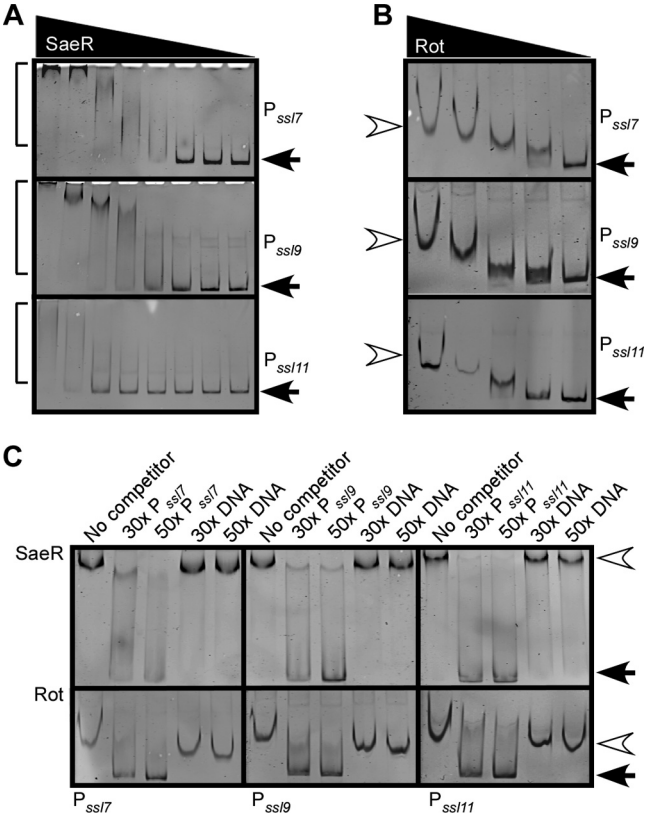

FIG 4 Binding of SaeR or Rot to the *ssl7*, *ssl9*, and *ssl11* promoters. (A) EMSA of purified phosphorylated SaeR incubated with either the *ssl7*, *ssl9*, or *ssl11* promoter containing a biotin tag. Two-fold serial dilutions of SaeR, starting with 150 pmol, were incubated with 40 fmol DNA. Protein-DNA complexes were separated by PAGE, and the DNA probe was visualized using streptavidin DyLight. Arrows indicate free probe, and brackets indicate shifted probe. (B) EMSA of purified Rot 2-fold serial dilutions, starting with 4 pmol, incubated with either the *ssl7*, *ssl9*, or *ssl11* promoter as for panel A. Closed arrows indicate free probe, and open arrowheads indicate shifted probe. (C) EMSA in which 75 pmol of SaeR or 2 pmol of Rot was incubated with 40 fmol of the indicated biotinylated promoter DNA with a 30- or 50-fold molar excess of nonbiotinylated promoter DNA or nonbiotinylated control DNA. The EMSA reaction was performed and visualized as for panel A. Closed arrows indicate free probe, and open arrowheads indicate shifted probe.

promoters, most likely due to direct binding of SaeR to these promoters.

SaeR and Rot directly and specifically interact with the *ssl* promoters. To conclusively demonstrate that SaeR directly binds to the *ssl* promoters, electrophoretic motility shift assays (EMSA) were performed. Recombinant SaeR was purified from *E. coli* and phosphorylated with SaeS as described previously (49). We observed that SaeR interacted with *ssl7*, *ssl9*, and *ssl11* promoter DNAs (Fig. 4A). In contrast, when EMSA where performed with SaeS alone, no shift was observed (data not shown). We also observed that Rot directly interacted with the *ssl* promoters (Fig. 4B). These interactions were specific, as SaeR and Rot were efficiently competed off when nonlabeled *ssl* promoter DNA was added but not when a nonpromoter control DNA was included (Fig. 4C).

Rot and SaeR form a complex with *ssl* promoters *in vitro* and *in vivo*. Based on the genetic and biochemical data presented above, we hypothesized that Rot and SaeR are both able to occupy the *ssl* promoters concurrently. To test this hypothesis, *ssl7*, *ssl9*, or *ssl11* promoter DNA was incubated with either Rot, phosphorylated SaeR, or both together and the samples analyzed by EMSA. While each protein alone was able to cause either a full shift (Rot) or a slight shift (SaeR) in the DNA migration, when present together, a supershift was observed (Fig. 5A). This supershift is indicative of both factors binding to the DNA concurrently, consistent with the finding that both Rot and SaeR are required for *ssl* promoter activation (Fig. 3).

We next examined whether Rot and SaeR bind to *ssl* promoters *in vivo*. To this end, we performed immunoprecipitation studies using an *agr rot* double mutant strain containing a plasmid that produces a FLAG-tagged Rot. Whole-cell lysates were generated from cells previously cross-linked with formaldehyde to preserve protein-protein and protein-DNA complexes. Lysates were subsequently immunoprecipitated using an anti-FLAG antibody and the pulldown samples immunoblotted for SaeR. We found that immunoprecipitation of Rot resulted in the coisolation of SaeR (Fig. 5B). In addition, analysis of the immunoprecipitated material by PCR revealed that Rot could be found associated *in vivo* with the *ssl7* and *ssl9* promoters (Fig. 5B). Collectively, these data suggest that Rot and SaeR interact *in vivo* and form complexes with the *ssl* promoters.

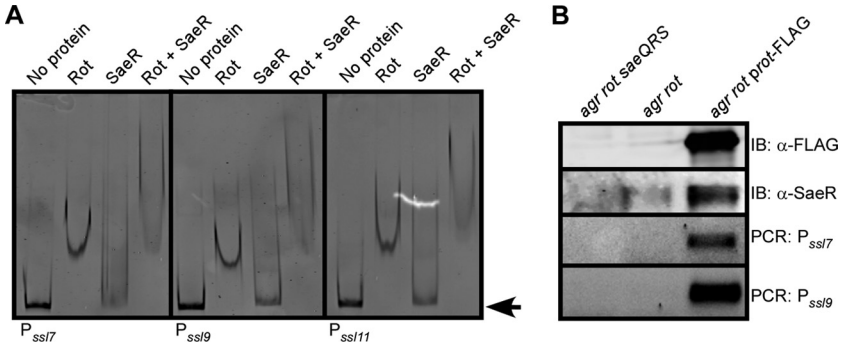

FIG 5 DNA binding properties of Rot, SaeR, and Rot/SaeR complexes. (A) EMSA of 2 pmol of purified Rot, 37.5 pmol of purified phospho-SaeR, or a mixture of Rot and phospho-SaeR incubated with 40 fmol of either the *ssl7*, *ssl9*, or *ssl11* promoter containing a biotin tag. DNA was visualized using streptavidin DyLight. The arrow indicates free probe. (B) Immunoprecipitation of FLAG-tagged Rot from the indicated *S. aureus* Newman strains. The samples were separated using SDS-PAGE, transferred to nitrocellulose, and immunoblotted (IB) for FLAG to detect Rot or for SaeR (top) or used as temple for PCR amplification of the *ssl7* and *ssl9* promoters (bottom).

Hyperactivation of SaeS results in increased production of Ssls. We next investigated whether the *saeS*^{L18P} allele in other *S. aureus* clinical strains would result in overproduction of the Ssls. To test this, we introduced a vector that constitutively expresses Newman *saeS*^{L18P} into an isogenic mutant of USA300 strain LAC with *agr* and *saeS* mutated. As observed previously, inactivation of *agr* in strain LAC resulted in an increase in Ssl7 abundance (7), a phenotype dependent on a functional Sae TCS (Fig. 6, *agr* versus *agr/saeS*). Complementation studies demonstrated that expression of the *saeS*^{L18P} allele induced the overproduction of Ssls, while expression of the *saeS*^{L18} allele rescued Ssl levels only to that of wild-type LAC (Fig. 6). These results demonstrate that constitutive activation of SaeS results in the overproduction of Ssls and that other *S. aureus* strains are capable of producing Ssls, provided that the Sae TCS is activated.

***S. aureus* induces the activation of the *ssl* promoters in response to human neutrophils.** The data presented above demonstrate that the Sae TCS and Rot synergize to activate the expression of *ssl*s. However, production of these proteins *in vitro* is minimal in *S. aureus* strains harboring the *saeS*^{L18} allele (Fig. 1 and 6). Previous studies have demonstrated that *sae* is upregulated in response to neutrophils and neutrophil products (18, 33, 43, 55). Specifically, the P1 promoter has been shown to be activated in the presence of α-defensins, key components in bacterial killing by neutrophils (18). Therefore, we sought to determine if this enhanced promoter activation, and thus expression of the Sae TCS, could induce *ssl* expression in clinical methicillin-resistant *S. aureus* (MRSA) strains following interaction with human neutrophils.

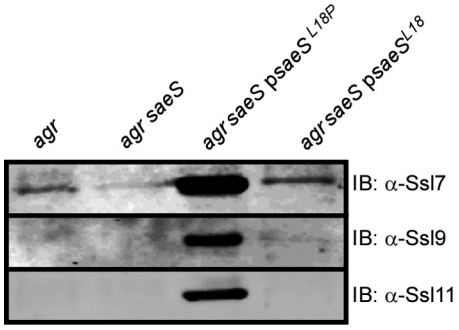

FIG 6 Effect of the SaeS-L18P mutant on the production of Ssls by USA300. The indicated USA300 LAC strains were grown to early stationary phase, and exoproteins were collected, precipitated, separated using SDS-PAGE, and transferred to nitrocellulose. The indicated Ssls were detected by immunoblotting.

To test this hypothesis, we generated an *sae* P1 transcriptional reporter vector and transformed it into the MRSA pulsed-field gel electrophoresis type USA300. When this reporter strain was grown in culture in the presence or absence of primary human neutrophils, we observed an induction in the activation of the *sae* P1 promoter that occurred in a time- and neutrophil-dependent manner (Fig. 7A, *sae* P1 promoter). In addition to *sae* P1 activation, we also observed an increase in the activation of the *ssl* promoters in the presence of neutrophils in USA300 (Fig. 7A, P_{ssl7} and P_{ssl11}). Importantly, neutrophil-mediated activation of *sae* P1 and *ssl* promoters was also observed in other MRSA clinical isolates (USA100, USA500, and USA800) (Fig. 7B). The USA300 *saeQRS* mutant strain was no longer able to activate the *ssl11* promoter, regardless of whether neutrophils were present or not (Fig. 7C), indicating that the enhanced activation of the *ssl* promoters was attributable to the increased activation of *sae*. Rot was also found to be required for this neutrophil-mediated activation, as a *rot* mutant was unable to significantly enhance the activation of the *ssl* promoters, even in the presence of neutrophils (Fig. 7C). Importantly, the neutrophil-mediated activation of the *sae* P1 and *ssl* promoters was specific, since neutrophils did not influence the activation of the *rot* and *sarA* promoters (Fig. 7D). This neutrophil-mediated induction of the *ssl* promoters was not due to altered growth of the tested strains, since enumeration of CFU of USA300 and the isogenic *saeQRS* mutant strain grown for 5 h in the presence or absence of neutrophils demonstrated no difference in bacterial numbers (Fig. 7E). Taken together, these data demonstrate that clinically relevant MRSA strains sense neutrophils to activate the *ssl* promoters, a phenotype dependent on the Sae TCS and Rot.

DISCUSSION

S. aureus is a significant human pathogen capable of infecting most host tissues, a trait dependent on the evasion of the host immune response. Among the staphylococcal virulence factors, the Ssls are believed to be an important component of this host immune evasion strategy (3, 7, 17, 52). Our previous studies have

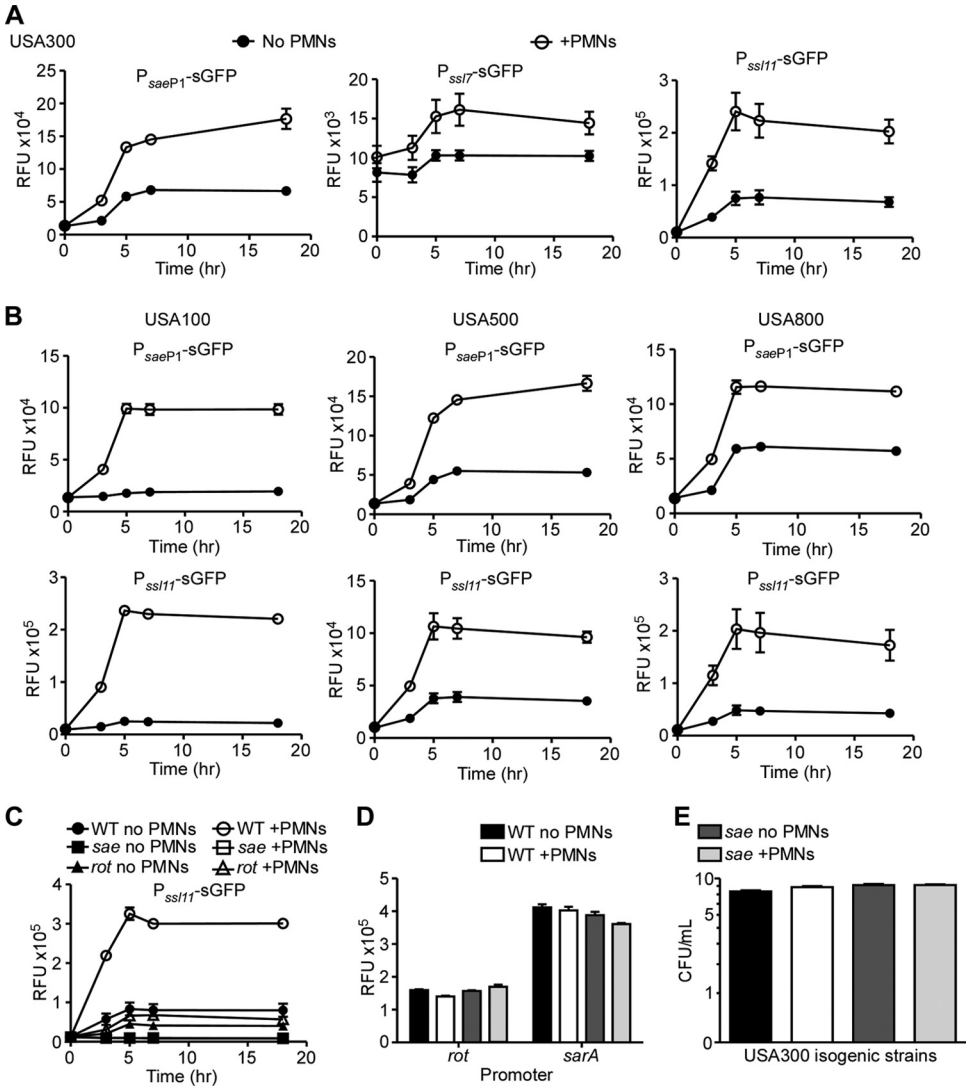

FIG 7 Human neutrophils and activation of the *ssl* promoters. (A) Reporter assay of *S. aureus* USA300 containing either the *sae* P1, *ssl7*, or *ssl11* transcriptional reporter controlling GFP gene expression grown in the absence (closed circles) or presence (open circles) of primary human neutrophils (PMNs). (B) Reporter assay of MRSA USA100, USA500, or USA800 containing the *sae* P1 or *ssl11* transcriptional reporter as for panel A. (C) Reporter assay of wild-type USA300 (WT) (circles), the *saeQRS* mutant (squares), or the *rot* mutant (triangles) containing the *ssl11* transcriptional reporter grown as for panel A. (D) Reporter assay of WT USA300 containing a *rot* or *sarA* transcriptional reporter controlling GFP gene expression grown in the absence (no PMNs) or presence (+PMNs) of primary human neutrophils. (E) CFU counts of WT USA300 or *saeQRS* mutant growth with or without neutrophils for 5 h. Values represent averages from six (A to C) or three (D and E) independent donors ± SD.

demonstrated that the repression of *ssl* expression is due to RNAIII-mediated inhibition of Rot (7). However, while the overproduction of Rot in strain Newman leads to a robust increase in the production of the Ssls, the same phenotype is not observed when Rot is overproduced in clinically relevant *S. aureus* strains, indicating that Rot alone is not sufficient to enhance Ssl production in these strains (Fig. 1). We demonstrate in this study that both the Sae TCS and Rot are required to regulate *ssl* expression by directly binding and activating the *ssl* promoters.

The SaeR binding site has been recently identified (42, 49). Interestingly, each of the *ssl* promoters contains an SaeR binding site that overlaps with the −35 site (7). While some of the promoters contain an imperfect SaeR binding site, none of the mutations found are expected to abolish SaeR binding (49). The *ssl7* promoter contains a perfect SaeR binding site (GTTAA-N_6-GTTAA), while the *ssl9* and *ssl11* promoters contain imperfect sites (TTTAA-N_6-GTTAA, and GTTAA-N_6-TTTAA, respectively). We hypothesize that these differences in SaeR binding site could contribute to the observation that the *ssl7* promoter exhibits more robust activation than the *ssl9* promoter. This hypothesis is also supported by the finding that less SaeR is required to shift the *ssl7* promoter than to shift the *ssl9* or *ssl11* promoter (Fig. 4A). Additionally, the introduction of a single nucleotide change in the SaeR binding site in the *ssl9* promoter completely ablates activation (Fig. 3C), while in the *ssl7* promoter, this single mutation greatly reduces but does not eliminate activation (Fig. 3C).

To our knowledge, the finding that SaeR and Rot work together to directly activate the expression of target genes has not

been described previously. Interestingly, Rot has been found to repress the *sae* P1 promoter *in vitro* (30). We speculate that while Rot could inhibit P1 activation, the low, constitutive activity of the *sae* P3 promoter, which encodes a transcript containing both *saeR* and *saeS*, allows for enough phosphorylated SaeR to be available in the cell to activate the *ssl* promoters. Until now, these transcription factors have been shown to work in opposition to one another to regulate the expression of secreted and cell surface factors. However, with the *ssl*s, these factors work together to provide a dual layer of positive regulation. This complex regulatory scheme may serve as insurance that the Ssls are produced both at the correct time during infection and in the appropriate context. For example, production of these factors could be critical early during infection, when Rot levels would be maximal and when *S. aureus* first encounters neutrophils. Ssls could protect *S. aureus* from the innate immune system until the bacterial density increases and the population reaches quorum, which is required for the activation of the Agr TCS and the subsequent expression of the *S. aureus* virulon.

While we demonstrate that both Rot and SaeR are required to activate the *ssl* promoters and that these factors both bind to the promoter DNA, the exact mechanism by which these two regulators work together is not known. We speculate that Rot may recruit SaeR to the *ssl* promoters, which in turn will recruit the RNA polymerase and activate gene expression (12). The notion that Rot facilitates the recruitment of SaeR to the *ssl* promoters is supported by the fact that less SaeR is required to create a supershift of the *ssl7*, *ssl9*, and *ssl11* promoter DNAs in the presence of Rot than when Rot is absent (Fig. 4A and 5A). The Rot-mediated recruitment of SaeR could be due to direct interaction between Rot and SaeR. Alternatively, Rot could bend the DNA to better expose the SaeR binding site at these promoters. These possibilities are currently being explored.

Taken together, the data presented in this study reveal a new regulatory scheme by which *S. aureus* is able to activate the expression of a subset of genes through the coordinated efforts of two transcription factors previously demonstrated to work in opposition. The finding that neutrophils induce the expression of the *ssl*s in clinically relevant strains of *S. aureus* further supports the role of these molecules in avoidance of the immune response and pathogenesis.

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