JOURNAL OF BACTERIOLOGY, Feb. 2010, p. 613–623 Vol. 192, No. 3
0021-9193/10/$12.00 doi:10.1128/JB.01242-09

Differential Target Gene Activation by the *Staphylococcus aureus* Two-Component System *saeRS*▽†

Markus Mainiero, Christiane Goerke, Tobias Geiger, Christoph Gonser, Silvia Herbert, and Christiane Wolz*

Interfaculty Institute of Microbiology and Infection Medicine, University of Tübingen, Tübingen, Germany

Received 15 September 2009/Accepted 4 November 2009

The *saePQRS* system of *Staphylococcus aureus* controls the expression of major virulence factors and encodes a histidine kinase (SaeS), a response regulator (SaeR), a membrane protein (SaeQ), and a lipoprotein (SaeP). The widely used strain Newman is characterized by a single amino acid change in the sensory domain of SaeS (Pro18 in strain Newman [SaeS^P], compared with Leu18 in other strains [SaeS^L]). SaeS^P determines activation of the class I *sae* target genes (*coa*, *fnbA*, *eap*, *sib*, *efb*, *fib*, *sae*), which are highly expressed in strain Newman. In contrast, class II target genes (*hla*, *hlb*, *cap*) are not sensitive to the SaeS polymorphism. The SaeS^L allele (*saeS^L*) is dominant over the SaeS^P allele, as shown by single-copy integration of *saePQRS^L* in strain Newman, which results in severe repression of class I target genes. The differential effect on target gene expression is explained by different requirements for SaeR phosphorylation. From an analysis of *saeS* deletion strains and strains with mutated SaeR phosphorylation sites, we concluded that a high level of SaeR phosphorylation is required for activation of class I target genes. However, a low level of SaeR phosphorylation, which can occur independent of SaeS, is sufficient to activate class II target genes. Using inducible *saeRS* constructs, we showed that the expression of both types of target genes is independent of the *saeRS* dosage and that the typical growth phase-dependent gene expression pattern is not driven by SaeRS.

The human pathogen *Staphylococcus aureus* can cause a wide range of diseases. The versatility of this organism is due to its capacity to produce accessory molecules that mediate specific interactions with the host cells. Most of these factors are tightly regulated by global regulatory loci, such as *agr*, *rot*, *sigB*, and *sae*, which act as an interactive regulatory network to ensure that there is coordinated temporal expression of virulence factors. Within the network, the *sae* system appears to be a central downstream regulator. Mutations in *sae* do not affect the transcription of *agr*, *sigB*, *sarA*, or *rot* (18, 22, 35). However, at least in some strains, *agr* activates *sae* transcription, while *sigB* and *rot* repress *sae* transcription (17, 19, 21, 35, 44). The *sae* system controls the expression of major virulence genes, including *hla* (encoding α-hemolysin [Hla]), *hlb* (encoding β-hemolysin [Hlb]), *coa* (encoding coagulase [Coa]), *eap* (encoding extracellular adherence protein [EAP]), and *fnbA* (encoding fibronectin binding protein A [FnBPA]), probably through direct interaction with these target genes (18, 33, 36, 44, 46, 48, 52).

The *sae* locus consists of four open reading frames (ORFs), and the products of two of these ORFs (*saeR* and *saeS*) show strong sequence homology to bacterial two-component regulators composed of a histidine kinase (HK), SaeS, and a response regulator (RR), SaeR. Two additional ORFs, ORF3 (*saeQ*) and ORF4 (*saeP*), which are located upstream of *saeRS*, are likely to be important for the function of the *sae* operon (1, 17, 48). *saeP* encodes a putative lipoprotein, and *saeQ* encodes a membrane protein with four membrane-spanning stretches. Four overlapping *sae*-specific transcripts (T1 to T4) have been detected; the T1 message (3.1 kb) initiates upstream of *saeP*, T2 (2.4 kb) initiates upstream of *saeQ*, and T3 (2.0 kb) initiates upstream of *saeR*. T4 (0.7 kb) represents a monocistronic mRNA encompassing only *saeP* (Fig. 1C). Promoter fusion analysis indicated that the main T2 transcript is generated by endoribonucleolytic processing of the T1 transcript (1, 17). Only two distinct promoter elements (P1 and P3) were detected in the *saeRS* upstream region. The P3 promoter, which is upstream of *saeRS*, generates the T3 transcript and is repressed by *saeRS*. In contrast, the distal P1 promoter is strongly autoregulated (1, 17, 35) and is sensitive to environmental signals, such as pH, H_2O_2, and α-defensins (1, 17).

Two-component systems are the predominant form of signal transduction in bacteria (for a recent review, see reference 15). In response to a specific signal, the HK component uses ATP to autophosphorylate at a conserved histidine residue. The phosphoryl group is then transferred to a conserved aspartate residue in the cognate RR. In most cases, activation of the RR leads to gene activation or repression by interaction with specific binding motifs located upstream of the target genes. In addition, many HKs are predicted to be bifunctional enzymes that also exhibit phosphatase activity towards their cognate RRs (2). Depending on the N-terminal signaling domain of the HK, signaling may occur from the extracytoplasmic space, from the cytoplasm, or from within the membrane. SaeS from *S. aureus* can be classified as a member of the group of intramembrane sensing HKs (38, 39). These small HKs are characterized by a short intramembrane sensing domain consisting of two transmembrane helices with an extracytoplasmic linker consisting of less than 25 amino acids and are thought to sense

* Corresponding author. Mailing address: Institut für Medizinische Mikrobiologie und Hygiene, Universität Tübingen, Elfriede-Aulhorn-straße 6, 72074 Tübingen, Germany. Phone: 49-7071-2980187. Fax: 49-7071-295165. E-mail: christiane.wolz@med.uni-tuebingen.de.

† Supplemental material for this article may be found at http://jb.asm.org/.

▽ Published ahead of print on 20 November 2009.

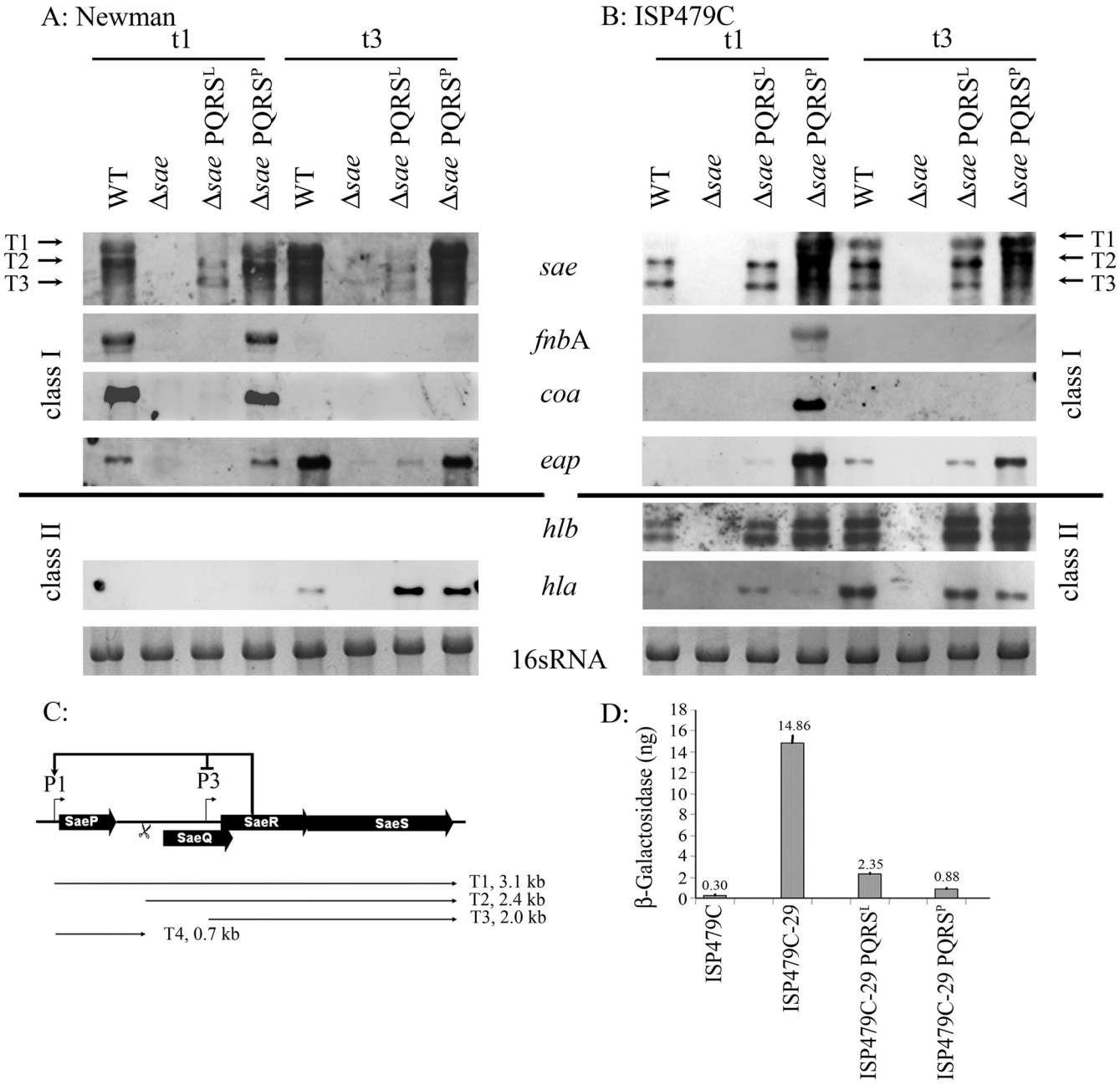

FIG. 1. Influence of N-terminal SaeS polymorphism on *sae* transcription and target gene expression. (A and B) Cross-complementation of *sae* mutants Newman-29 (A) and ISP479C-29 (B). For single-copy complementation the *saePQRS* operon from either strain Newman or strain ISP479C was integrated into the lipase gene of *sae* mutant strains. Bacteria were grown to the exponential (t1) (OD_{600}, 0.5) and postexponential (t3) (OD_{600}, 6) growth phases. RNA was hybridized with digoxigenin-labeled PCR fragments. In strain Newman, the *hlb* gene is inactivated by phage insertion, and therefore no *hlb* was detected. The 16S rRNA detected in the ethidium bromide-stained gels was used as a loading control in the lower lanes. WT, wild type. (C) Organization of the *sae* locus composed of four ORFs. Transcription initiates at the P1 and P3 promoters, generating transcripts T1 and T3, respectively. T2 is generated by processing of T1. P1 is strongly activated and P2 is repressed by SaeR (17). (D) *cap* promoter fusion assays. *cap-lacZ* fusions were integrated into strain ISP479C, the *sae* mutant ISP479C-29, and the complemented *sae* mutants. ISP479C-29 was complemented by integration of pCWSAE50 (ISP479C-29 $PQRS^L$) and pCWSAE51 (ISP479C-29 $PQRS^P$) into *hlb*. Bacteria were grown to postexponential growth phase (t3), equal numbers of bacteria were lysed, and promoter activities were expressed in β-galactosidase equivalents (in nanograms). Standard deviations were derived from data for at least two independent cultures.

their stimulus at or within the membrane. Based on sequence analysis of the conserved domains characterizing HKs (12, 24, 29, 38, 39) or RRs (13, 14, 24), the *saeRS* system can be considered closely related to the EnvZ/OmpR-like two-component systems.

In *S. aureus*, regulatory circuits have been analyzed mainly using derivatives of the prototypic strains 8325-4 and Newman. Strain 8325-4 produces high levels of Hla but little clumping factor A, FnBP, or Coa. This is due in part to a mutation in *rsbU*, which encodes a protein that is important for the activity

of alternative sigma factor B (32). Furthermore, due to a mutation in the *cap5* gene cluster, strain 8325-4 is not encapsulated (53). In contrast, strain Newman contains a functional *rsbU* gene and a *cap5* operon and is known to produce large amounts of Coa, clumping factor A (55), Eap, and Emp (27). FnBPA, although not functional (25), is also transcribed at high levels (56) in strain Newman. Hla expression in strain Newman is low during growth *in vitro*, but during infections it can be activated to the level observed in derivatives of strain 8325-4 (22). Overall, strain Newman seems to be more virulent than strain 8325-4 in animal models of infection (K. Ohlsen, personnel communication; our observations). Sequence analysis revealed a single amino acid variation within the first N-terminal transmembrane loop of SaeS of strain Newman compared to SaeS of strain 8325-4 or of other strains (48). Thus, the *saeS* polymorphism may, at least in part, account for the differences in virulence gene expression between strain Newman and 8325-4 derivatives.

In this study we showed that a single amino acid change in the first transmembrane loop of SaeS with Pro at residue 18 (Sac^P), as found in strain Newman, is indeed responsible for major peculiarities of this strain. The polymorphism probably results in a high kinase/phosphatase ratio for $SaeS^P$, which is necessary for expression of class I target genes (*coa*, *fnbA*, *eap*, *sib*, *efb*) but is not required for expression of class II target genes (*hla*, *hlb*, *cap*). The expression of different target genes is determined by the SaeS polymorphism but is not sensitive to the SaeRS dosage.

MATERIALS AND METHODS

Strains and growth conditions. The strains and plasmids used in this study are listed in Table 1. *In vitro* growth was performed using CYPG (10 g/liter Casamino Acids, 10 g/liter yeast extract, 5 g/liter NaCl, 0.5% glucose, 0.06 M phosphoglycerate) (43) supplemented with the appropriate antibiotics for strains carrying resistance genes (50 μg/ml kanamycin, 10 μg/ml erythromycin, 5 μg/ml tetracycline, 10 μg/ml chloramphenicol). For RNA isolation and promoter fusion assays bacteria from an overnight culture were diluted to obtain an initial optical density at 600 nm (OD_{600}) of 0.05 in fresh medium without antibiotics and grown with shaking at 37°C to the mid-exponential (t1) (OD_{600}, 0.5), late-exponential (t2) (OD_{600}, 3), or postexponential (t3) (OD_{600}, 6) phase. For analysis of extracellular proteins strains were grown for 2.5 h in basic medium (1% tryptone, 0.5% yeast extract, 0.5% NaCl, 0.1% K_2HPO_4, 0.1% glucose).

Replacement of *sae*. The *sae* locus was replaced by a kanamycin resistant cassette. Two fragments flanking the *sae* locus were amplified from strain ISP479C using oligonucleotides A and B and oligonucleotides C and D (see Table S1 in the supplemental material). Oligonucleotides A and D were modified with a KpnI restriction site. The kanamycin resistance cassette (*aphIII*) was amplified from pDG782 using oligonucleotides E and F. Reverse oligonucleotide B for amplification of the *sae* upstream fragment and forward oligonucleotide C for amplification of the *sae* downstream fragment were synthesized with additional nucleotides complementary to the flanking region of *aphIII*. All three fragments were annealed without oligonucleotides and amplified by PCR using oligonucleotides A and D to obtain the complete replacement cassettes. The amplicon was restricted with KpnI and cloned into pBT2 (7). To take advantage of blue-white selection, the fusion fragments were subcloned into the EcoRI and SalI sites of pMAD (3), yielding plasmid pCWSAE29. This temperature-sensitive plasmid was electroporated into *S. aureus* strain RN4220, and *sae* gene replacement mutants were generated as described previously (3). The mutation was transduced into strain Newman and strain ISP479C using phage ϕ11 lysates, resulting in *sae* gene replacement mutants Newman-29 and ISP479C-29. All mutants were verified by Southern hybridization after pulsed-field gel electrophoresis and PCR.

Chromosomal amino acid exchange in SaeS of strain RN1HG and strain Newman. Amino acid exchange in the products of the native *sae* operons of strain RN1 and strain Newman was accomplished by homologous recombination with pMAD vectors in which the *saePQRS* operon was cloned (3). The $pMADsaeS^P$ vector included a 3.5-kb PCR fragment amplified from DNA isolated from *S. aureus* Newman to obtain a chromosomal point mutation in *saeS* (resulting in a change from SaeS with L18 [$SaeS^L$] to SaeS with P18 [$SaeS^P$]) in strain RN1. Similarly, an amino acid change in SaeS from P18 to L18 in strain Newman was obtained by using $pMADsaeS^L$ with a 3.5-kb PCR fragment amplified from strain RN1. Successful mutagenesis of the chromosomal *sae* operons was verified by DNA sequencing. Strain RN1 with the *saeS* allele encoding $SaeS^P$ ($saeS^P$) was designated RN1saeN, and strain Newman with the *saeS* allele encoding $SaeS^L$ ($saeS^L$) was designated NewHG.

Chromosomal integration of different parts of the *sae* operon. Different parts of the *sae* operon were amplified using high-fidelity polymerases (Phusion HF polymerase; Finnzymes, Espoo, Finland) and oligonucleotides (see Table S1 in the supplemental material) modified with restriction sites. The amplicons were either cloned into pCR2.1 and subcloned or directly cloned into the EcoRI site of the respective vectors. Plasmids were verified by restriction digestion and sequencing of the inserts. The 3.5-kb *sae* operons of strain ISP479C(pCWSAE7) (48) and strain Newman(pCWSAE32) were subcloned into the single EcoRI site of pCL84 (34), resulting in pCWSAE28 and pCWSAE33, respectively, and alternatively were subcloned into pCG33, resulting in pCWSAE50 and pCWSAE51, respectively. pCG33 is a derivative of pCG3 with a ϕ13-*attP* integration site. pCG3 was obtained by replacing the *tetK* gene of pCL84 with an *ermB* resistance cassette. The *ermB* was excised from pEC4 (7) with HindIII and SalI and cloned into the corresponding restriction sites of pCL84. For construction of pCG33 the ϕ13-*attP* site was amplified with modified oligonucleotides EcoRIphi13attP-for and Salphi13attP-rev using strain RN1 as the template. The amplicon was cloned into EcoRI-SalI-digested vector pCG3 to replace the L54-specific *attP* site.

saePQR with an intact terminator region was constructed by strand overlap extension PCR in two steps. Two amplicons were generated using oligonucleotide sae-1U with oligonucleotide 2267hybrid (hybrid reverse oligonucleotide) and oligonucleotide 3218hybrid (hybrid forward oligonucleotide) with oligonucleotide sae-3515L. Diluted amplicons were annealed and amplified by PCR using sae-1U and sae-3515L. The fusion product was restricted with EcoRI and cloned in the corresponding restriction site of the pCL84 vector to obtain pCWSAE54.

The phosphorylation site (D51) in *saeR* was mutated by strand overlap extension PCR. Two amplicons were generated with overlapping 3′ and 5′ ends with oligonucleotide pairs sae-1U/1680L29 and 1684U31/sae-3515L. Oligonucleotides 1680L29 and 1684U31 are complementary and have a single nucleotide mismatch that changes codon 51 from an aspartate codon (GAT) to an asparagine codon (AAT). DNA from strains Newman and ISP479C were used as templates. Amplicons were annealed without oligonucleotides and amplified using oligonucleotides sae-1U and sae-3515L. The fusion products were restricted with EcoRI and cloned in the corresponding restriction site of pCL84 to obtain pCWSAE56 [$saePQR(D51N)S^L$] and pCWSAE59 [$saePQR(D51N)S^P$].

The pCL84 derivatives were integrated by electroporation into *geh* of strain CYL316 (34), and the pCG33 derivatives were integrated by electroporation into *hlb* of strain CG18, which contains the cloned integrase gene of ϕ13. The ϕ13 integrase was amplified using modified oligonucleotides listed in Table S1 in the supplemental material, and the amplicons were cloned into the EcoRI and SalI sites of the multicopy vector pSK236, yielding pCG32. RN4220 containing pCG32 was designated strain CG18.

Integrated plasmids were transduced into different strains using ϕ11 lysates. All transductants were analyzed by PCR and/or Southern hybridization of SmaI-digested DNA separated by pulsed-field gel electrophoresis (PFGE) using probes specific for *sae*. Due to the single SmaI site in pCL84 and pCG33, integration of the plasmids resulted in digestion of the *geh*- or *hlb*-containing SmaI fragment into two bands, one of which reacted with the *sae* probe in all cointegrants.

Cloning of different parts of the *sae* operon into pALC2073. To clone different parts of the *sae* operon into the anhydrotetracyline (AHT)-inducible vector pALC2073, PCR fragments were generated using oligonucleotides listed in Table S1 in the supplemental material, or fragments were subcloned from the pCL84 derivatives described above. DNA of strain Newman or strain ISP479C was used as the template. For constructs with a mutated *saeR* gene plasmid pCWSAE56 or pCWSAE59 was used as the template. Amplicons were either cloned into pCR2.1 and subcloned or directly cloned into the EcoRI site of pALC2073. Plasmids and insert orientation were verified by restriction digestion or PCR and sequencing of the inserts. Plasmids were electroporated into strain RN4220 and transduced into different *S. aureus* strains.

Construction of chromosomally integrated promoter-*lacZ* fusions. The *cap8* promoter was amplified with the appropriate oligonucleotides (see Table S1 in the supplemental material) with additional EcoRI restriction sites. Amplicons were ligated into the EcoRI site of pCG6 (17) in front of the promoterless *lacZ*

TABLE 1. Bacterial strains and plasmids

Strain or plasmid	Description[a]	Source or reference
Strains		
One Shot TOP10	Competent *E. coli* strain for plasmid transformation	Invitrogen, Karlsruhe, Germany
CYL316	RN4220(pYL112Δ19), L54 *int* gene, r^-	34
CG18	RN4220(pCG32), ϕ13 *int* gene, r^-	This study
RN4220	Restriction-deficient *S. aureus* strain, r^-	31
RN4220-29	RN4220 Δ*saePQRS*::*kan*	This study
RN1	Strain 8325 lysogenic for three phages	42
RN1saeN	RN1 with $SaeS^P$ from strain Newman	This study
Newman	Wild type	11
NewHG	Newman with $SaeS^L$ from strain RN1	This study
ISP479C	Derivative of 8325-4	45
Newman-29	Newman Δ*saePQRS*::*kan*	This study
ISP479C-29	ISP479C Δ*saePQRS*::*kan*	This study
Plasmids		
pBT2	Low-copy-number shuttle vector with Amp^r in *E. coli* and temperature-sensitive replication with Cm^r in *S. aureus*	7
pCR2.1	Cloning vector	Invitrogen, Karlsruhe, Germany
pMAD	Shuttle vector for gene replacement mutagenesis	3
$pMADsaeS^L$	pMAD with the $saePQRS^L$ operon from strain RN1	This study
$pMADsaeS^P$	pMAD with the $saePQRS^P$ operon from strain Newman	This study
pEC4	pBluescript II KS(+) with *ermB* inserted into ClaI site	7
pCL84	Single-copy integration vector, *att* site for chromosomal integration in *geh*	34
pALC2073	*S. aureus* vector containing a tetracycline-inducible promoter controlling expression of cloned genes, Cm^r	5
pSK236	Shuttle vector containing pUC19 cloned into the HindIII site of pC194	16
pGEM	Cloning vector	Promega, Mannheim, Germany
pCG3	pCL84 with replacement of *tetK* by *ermB*	This study
pCG32	pSK236 with ϕ13 *int* gene	This study
pCG6	pCL84 with integrated *spoVG-lacZ* gene cassette from pKO010	17
pCG18	pCG6 with *cap8* promoter	This study
pCG33	*hlb* integration vector, pCG3 with replacement of L54 *attP* site with *attP* of ϕ13	This study
pCWSAE29	pMAD construct used to generate Δ*saePQRS*::*kan*	This study
pCWSAE7	pGEM with $saePQRS^L$ (nucleotides 1 to 3515),	48
pCWSAE28	pCL84 with $saePQRS^L$ (nucleotides 1 to 3515), subclone from pCWSAE7	This study
pCWSAE50	pCG33 with $saePQRS^L$ (nucleotides 1 to 3515), subclone of pCWSAE7	This study
pCWSAE32	pCR2.1 with $saePQRS^P$ (nucleotides 1 to 3515)	This study
pCWSAE33	pCL84 with $saePQRS^P$ (nucleotides 1 to 3515), subclone from pCWSAE32	This study
pCWSAE51	pCG33 with $saePQRS^P$ (nucleotides 1 to 3515), subclone of pCWSAE32	This study
pCWSAE43	pALC2073 with $saeRS^P$ (nucleotides 1509 to 3449)	This study
pCWSAE45	pALC2073 with $saeRS^L$ (nucleotides 1509 to 3449)	This study
pCWSAE56	pCL84 with $saePQR(D51N)S^L$ (nucleotides 1 to 3515)	This study
pCWSAE54	pCL84 with *saePQR* (nucleotides 1 to 3515, Δ2267–3218)	This study
pCWSAE59	pCL84 with $saePQR(D51N)S^P$ (nucleotides 1 to 3515)	This study
pCWSAE74	pCR2.1 with $saeR(D51N)S^L$ (nucleotides 1509 to 3515), derivative of pCWSAE59	This study
pCWSAE75	PCR2.1 with $saeR(D51N)S^P$ (nucleotides 1509 to 3515), derivative of pCWSAE56	This study
pCWSAE78	pALC2073 with $saeR(D51N)S^L$ (nucleotides 1509 to 3515), subclone from pCWSAE74	This study
pCWSAE79	pALC2073 with $saeR(D51N)S^P$ (nucleotides 1509 to 3515), subclone from pCWSAE75	This study

[a] Nucleotide numbers are based on the *sae* sequence (GenBank accession no. AJ556795).

gene, yielding pCG18. Plasmids were verified by restriction digestion, and the correct orientation of the inserts was verified by PCR. Plasmids were electroporated into *S. aureus* strain CYL316 and transduced into the *sae* mutant of strain ISP479C, which was complemented by integration of $saePQRS^P$ (pCWSAE51) and $saePQRS^L$ (pCWSAE50) into *hlb*. All transductants were verified by PCR.

Promoter activity assays. Promoter activity assays were performed as described previously (17). Briefly, equal numbers of bacteria were harvested at the appropriate growth phase, resuspended in 1 ml of 0.1 M sodium phosphate buffer (pH 7.4), and lysed with 0.5 ml zirconia-silica beads (diameter, 0.1 mm) in a high-speed homogenizer (Savant Instruments, Farmingdale, NY). β-Galactosidase activity was measured using a FluoReporter galactosidase quantitation kit (Invitrogen, Karlsruhe, Germany). Promoter activities were expressed in ng/ml β-galactosidase based on a standard curve generated by using purified β-galactosidase (Sigma-Aldrich, Munich, Germany). All values are means for at least two independently grown bacterial cultures.

Analysis of extracellular proteins. The exoproteins of the supernatant were concentrated with StrataClean resin (Stratagene). Proteins were separated according to their molecular weights by one-dimensional PAGE (12%), and after this the gels were stained using 0.1% Coomassie brilliant blue R250, 10% acetic acid, 50% methanol and destained using 10% acetic acid.

RNA isolation and Northern blot hybridization. RNA isolation and Northern blot analysis were performed as described previously (20). Briefly, approximately 10^9 *S. aureus* cells were lysed in 1 ml Trizol reagent (Invitrogen Life Technologies, Karlsruhe, Germany) with 0.5 ml zirconia-silica beads (diameter, 0.1 mm) in a high-speed homogenizer (Savant Instruments, Farmingdale, NY). RNA was isolated as described in the instructions provided by the manufacturer of Trizol.

Several digoxigenin-labeled probes for detection of specific transcripts were generated using a DIG PCR labeling kit by following the manufacturer's instructions (Roche Biochemicals, Mannheim, Germany). Oligonucleotides used for probe generation are listed in Table S1 in the supplemental material.

RESULTS

Influence of N-terminal SaeS polymorphism on *sae* transcription and target gene expression. The sequences of the products of the *saePQRS* operons of strain Newman (GenBank accession no. AJ556794) and the 8325-4 derivative ISP479C (GenBank accession no. AJ556795) differ at only a single amino acid residue in SaeS (48). All *saeS* sequences derived from genome sequencing projects except those for strain Newman were identical to the *saeS* sequence of strain ISP479C. The mutation in SaeS (Leu18 to Pro18) of strain Newman is located in the first transmembrane loop of the N-terminal signaling domain, indicating that there is a perturbation in the sensing mechanism. Below, $SaeS^P$ indicates SaeS from strain Newman, and $SaeS^L$ indicates SaeS from strain ISP479C. Previously, we and other workers have shown that strain Newman is characterized by an unusually high level of expression of the *sae* operon (1, 17, 48). To evaluate whether the sequence polymorphism in SaeS can account for the differential expression of *sae*, we cloned the $saePQRS^P$ and $saePQRS^L$ operons into the integration vector pCL84. The vector was integrated into the lipase gene of two *sae* gene replacement mutants, Newman-29 and ISP479C-29. In these mutants, the whole *saePQRS* operon was replaced by a *kanA* resistance cassette. The strains were grown to the exponential (t1) and postexponential (t3) growth phases and analyzed by Northern hybridization. The transcriptional patterns of the three *sae*-specific transcripts (T1, T2, and T3) were highly dependent on the *saeS* allele, and the *saeS* polymorphism fully accounted for the strain-specific pattern of *sae* transcription (Fig. 1A and B). Integration of $saePQRS^L$ led to expression of the three *sae* transcripts at levels that are comparable to those in ISP479C. On the other hand, integration of $saePQRS^P$ led to restoration of the *sae* expression pattern typical of strain Newman, namely, elevated expression of T1 and T2. The same results were obtained for derivatives of strain Newman (Fig. 1A) and strain ISP479C (Fig. 1B).

Next, we analyzed the effect of the *saeS* alleles on target gene expression in strain ISP479C and strain Newman. First, prototypic *sae* target genes (*coa*, *fnbA*, and *eap*), which encode cell-associated proteins with specific binding motifs, were analyzed. In the *sae* mutant of strain Newman, integration of $saePQRS^P$ led to full restoration of *coa* and *fnbA* transcription to wild-type levels. In contrast, integration of $saePQRS^L$ did not result in detectable transcription of *coa* and *fnbA*. In strain ISP479C, the levels of transcription of *coa* and *fnbA* were below the detection limit of Northern analysis (Fig. 1B). However, introduction of $saePQRS^P$ into the *sae* mutant of ISP479C resulted in *fnbA* and *coa* expression levels comparable to those obtained for strain Newman. Transcription of *eap* was detected in both wild-type strains, primarily during the postexponential growth phase, and the level of expression in strain Newman was higher than that in strain ISP479C. Again, the higher level of expression of *eap* in strain Newman could be linked to the *sae* allele; complementation of the *sae* mutants with $saePQRS^P$ resulted in stronger activation of *eap* than complementation with $saePQRS^L$. Thus, the high level of transcription of *fnbA*, *coa*, and *eap* in strain Newman compared to the level of transcription in strain ISP479C could clearly be linked to the SaeS polymorphism in the former strain, which seems to be characterized by *sae* hyperactivity. It is noteworthy that strain ISP479C does not express detectable levels of sigma factor B-activated *coa* and *fnbA* due to a mutation in *rsbU* (6). When strain ISP479r, a derivative of ISP479C with a repaired *rsbU* gene (49), was analyzed, a low level of *sae*-dependent expression of *coa* was detected (data not shown). The *sae* mutant of ISP479r was negative for *coa* expression, indicating that $saePQRS^L$ can activate *coa* to some extent, although much less than $saePQRS^P$.

Next, we analyzed genes encoding hemolysins (*hla*, *hlb*) that are also known to be strongly *sae* dependent. In both strains, the *sae* mutation resulted in downregulation of *hla* expression, which could be restored by integration of $saePQRS^P$ or $saePQRS^L$ (Fig. 1A and B). Similarly, *hlb* expression was restored in the *sae* mutant of ISP479C by both *sae* alleles (Fig. 1B). Thus, the regulation of *hla* and *hlb* by SaeRS is largely independent of the SaeS genotype.

The *capAP* operon is one of the few *sae* target operons shown to be upregulated in an *sae* mutant. Previously, this was shown only for strain Newman (48). We examined whether this effect is also linked to the $SaeS^P$ polymorphism by analyzing complemented *sae* mutants of ISP479C. We used promoter fusion assays to analyze the role of *saePQRS* in *capAP* promoter activity. To this end, the *cap* promoter region was cloned in front of *lacZ* and integrated into the chromosomal lipase gene. Cloned *saePQRS* was integrated into the *attB* site of the *hlb*-converting phage ϕ13. It was shown that *sae* also results in severe repression of *cap* promoter activity in strain ISP479C. The repressive effect was independent of the *saeS* allele since integration of $saePQRS^P$ and integration of $saePQRS^L$ similarly complemented the mutant phenotype.

To further show the predominant role of the SaeS polymorphism in target gene repression, we constructed strains in which the native chromosomal *saeS* was mutated; strain RN1saeN (a derivative of 8325) contains an *sae* operon with an $saeS^P$ allele, whereas strain NewHG (a derivative of strain Newman) contains an $saeS^L$ allele. Consistent with the results obtained by cross-complementation (Fig. 1), the gene expression was clearly determined by the SaeS polymorphism (Fig. 2). The extracellular protein profile of strain RN1saeN closely resembles that of strain Newman, namely, high levels of expression of Coa, the IgG binding protein Sbi, and the fibrinogen binding proteins Efb and Fib. This indicates that *sbi*, *efb*, and *fib* also are class I target genes. In contrast, the proteins are clearly downregulated in strain NewHG.

In summary, the *sae*-dependent regulation of *sae*, *fnbA*, *coa*, *eap*, *sib*, *efb*, and *fib* (referred to here as class I target genes) differs markedly from that of *hla*, *hlb*, and *cap* (class II target genes). Class I target genes are highly dependent on $SaeS^P$, whereas class II target genes are independent of the SaeS polymorphism. Below, transcription of *coa* is used mainly as an example of transcription of the class I target genes and *hla* is used as a representative of the class II target genes.

Dose-dependent expression of $saeRS^P$ and $saeRS^L$ using an AHT-inducible promoter system. It was shown previously using

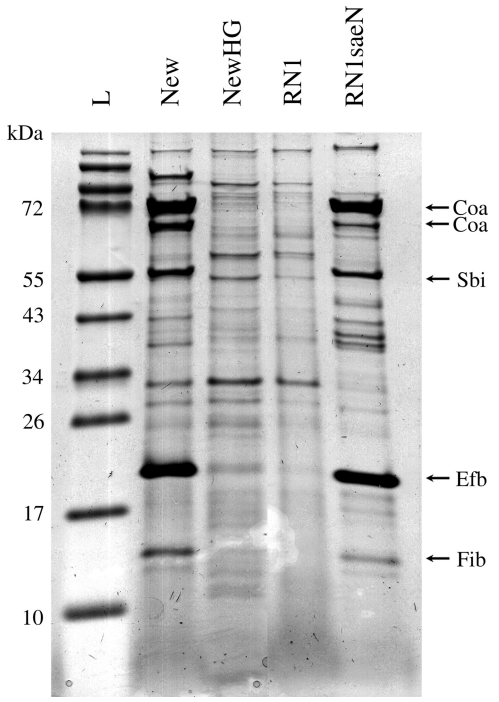

FIG. 2. Exoprotein pattern of *S. aureus* strains with mutagenized *saeS* alleles. Strains were grown for 2.5 h (exponential growth phase), and concentrated proteins from culture supernatants were analyzed by SDS-PAGE. Lane L, ladder; lane New, strain Newman; lane NewHG, strain Newman with $saeS^L$; lane RN1, strain 8325; lane RN1saeN, RN1 with $saeS^P$. The secreted proteins Coa, Sbi, and Efb (MWMN_1069) and the hypothetical fibrinogen-binding MWMN_1066 protein could be identified only in strain Newman and strain RN1 with $saeS^P$.

promoter fusion assays that the level of expression of the autoregulated P1 promoter of the *sae* operon is significantly higher in strain Newman than in other strains (1, 17). As shown in this study, this is solely due to $saeS^P$ in strain Newman (Fig. 1). Thus, the concomitant activation of class I target genes may depend on the higher level of transcription of *saeRS* via $SaeS^P$. To analyze the influence of the *saeRS* transcript level on target genes, $saeRS^P$ and $saeRS^L$ were expressed under control of a tetracycline-inducible promoter and introduced into the *sae* mutants. Low-level *saeRS* expression was observed in both constructs without induction. However, expression could be clearly enhanced by addition of increasing concentrations of AHT (Fig. 3).

hla transcription could be restored in the *saePQRS* mutant by both *saeRS* alleles. A higher induction rate was seen with $saeRS^L$ than with $saeRS^P$ in ISP479C derivatives (Fig. 3A), especially in bacteria from the exponential phase of growth. A similar trend was observed in the single-copy complementation experiments (Fig. 1B). However, different effects of $saeRS^P$ and $saeRS^L$ on *hla* transcription were not observed in the analysis of derivatives of strain Newman (Fig. 3B).

When the transcription of *coa* and *eap* was analyzed, it was evident that transcription of these genes was driven only by the $saeRS^P$ allele. Even a high level of induction of $saeRS^L$ did not result in *coa* expression and yielded only a low level of *eap* transcription. Similar results were obtained when the same constructs in the *saePQRS* mutants of strains ISP479C and Newman were analyzed. These results are in line with the finding obtained using the single-copy strains shown in Fig. 1, which indicated that $saeS^P$ has a direct effect on class I target genes. It can be concluded that this effect is not due to the higher level of expression of *sae* in strains harboring $saePQRS^P$, such as strain Newman.

From these experiments, it also became evident that there is little correlation between the level of *saeRS* and the level of target gene transcription. Increasing the concentration of AHT resulted in a dose-dependent increase in *saeRS* expression without a concomitant increase in target gene expression. Notably, *hla* was already induced to maximum levels of expression in cultures without *saeRS* induction.

We also analyzed whether *saeRS* is involved in the typical

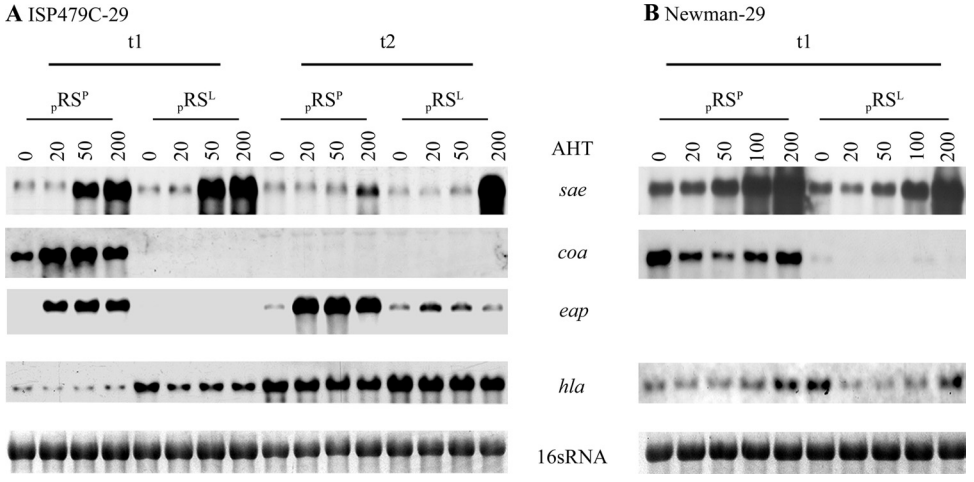

FIG. 3. Dose-dependent *saeRS* expression when an AHT-inducible promoter system was used. *saeRS* from strain Newman ($_pRS^P$) or strain ISP479 ($_pRS^L$) was cloned behind an AHT-inducible promoter and introduced into the *sae* mutant ISP479C-29 (A) or Newman-29 (B). Strains were grown to the exponential (t1) (OD_{600}, 0.5) and postexponential (t3) (OD_{600}, 3) growth phases, which was followed by *saeRS* induction for 1 h with different concentrations of AHT (0, 20, 50, and 200 ng/ml). RNA was hybridized with digoxigenin-labeled PCR fragments. The lane at the bottom contained the 16S rRNA detected in the ethidium bromide-stained gels as a loading control.

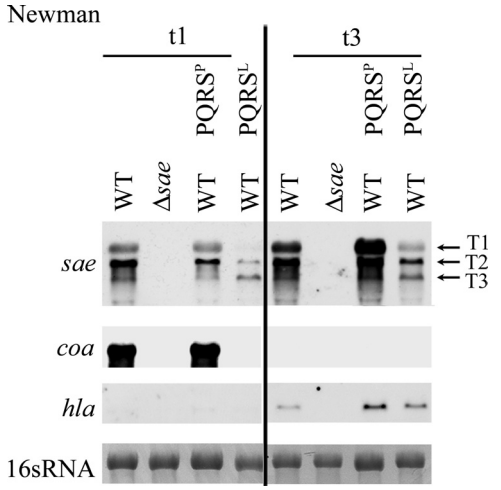

FIG. 4. SaeS^L is dominant over SaeS^P. *saePQRS* from strain Newman (PQRS^P) and *saePQRS* from strain ISP479C (PQRS^L) were integrated into the lipase gene of strain Newman. Bacteria were grown to the exponential (t1) (OD_{600}, 0.5) and late exponential (t2) (OD_{600}, 3) growth phases. RNA was hybridized with digoxigenin-labeled PCR fragments. The lane at the bottom contained the 16S rRNA detected in the ethidium bromide-stained gels as a loading control. WT, wild type.

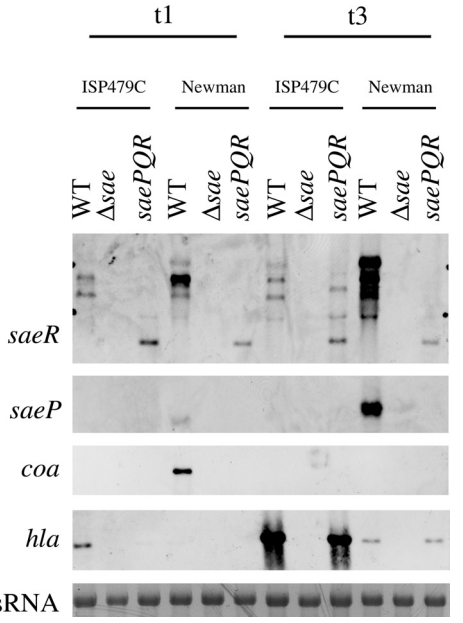

FIG. 5. SaeS-independent expression of class II target genes. The *saePQR* operon (pCWSAE54) was integrated into the lipase gene of the *sae* mutant Newman-29 or ISP479C-29. Bacteria were grown to the exponential (t1) and postexponential (t3) growth phases. RNA was hybridized with digoxigenin-labeled PCR fragments. The lane at the bottom contained the 16S rRNA detected in the ethidium bromide-stained gels as a loading control. WT, wild type.

growth phase-dependent expression of the target genes. Thus, *saeRS* was induced in the exponential and postexponential growth phases by increasing the concentration of AHT for 30 min. Although the AHT induction of *saeRS* was less prominent in the postexponential phase, it could be clearly shown that the growth phase-dependent expression of the target genes *coa* and *hla* is *saeRS* independent. As in wild-type strains, *coa* transcription could be induced only in bacteria from the exponential growth phase. In contrast, *hla* and *eap* are maximally induced during the postexponential phase. Also, the expression of *fnbA*, *coa*, and *hla* is growth phase dependent in *agr* mutants. Thus, other regulatory mechanisms besides *agr* (50, 56) or *sae* are required for growth phase-dependent regulation of virulence factors.

SaeS^L is dominant over SaeS^P. The profound difference in the functionality of the *sae* regulon between *saeS^P*- and *saeS^L*-expressing strains is presumably due to differences in signal transduction from the membrane-bound SaeS to the response regulator, SaeR, which in turn interacts with the target genes. This assumption is based on the general mechanism elucidated for other two-component systems. Using the SWISS-MODEL Model server (http://swissmodel.expasy.org/), it could be predicted that SaeS belongs to the class of bifunctional HKs that exhibit kinase activity as well as phosphatase activity. One can speculate that the overactive SaeS^P protein exhibits constant kinase activity (high levels of phosphorylation of SaeR), whereas in SaeS^L the phosphatase activity is dominant. Different target genes may differ in their requirements for the phosphorylation state of SaeR. If the phosphatase activity of SaeS^L is dominant, one would expect that SaeS^L can titrate the effect of SaeS^P. We therefore introduced a copy of *saePQRS^L* into the *saePQRS^P*-containing wild-type Newman strain (Fig. 4). It was clear that in strain Newman just a single copy of *saePQRS^L* resulted in severe inhibition of T1 and T2, as well as repression of *coa* transcription. As expected, introduction of a second copy of *saePQRS^P* into strain Newman had no effect on either *sae* or *coa* transcription. Thus, the effect of SaeS^L is dominant over the effect of SaeS^P, presumably due to the phosphatase activity of SaeS^L.

SaeS-independent activation of the class II target genes. If SaeS^L exhibits mainly phosphatase activity, then the response regulator would be kept mainly in the unphosphorylated state, which in general is thought to have less activity for transcriptional activation of target genes. However, the activity of response regulators may result in activation of some genes without phosphorylation by the cognate histidine kinase (10, 26, 40). We therefore tested whether SaeR can activate target genes without phosphorylation via SaeS. To this end, *saeS* in the single-copy complementation plasmid was deleted (Fig. 5). The resulting construct contained the native P1 promoter, as well as the native terminator signal downstream of *saeS*. Especially in strain Newman transcription of the shortened *saePQR* operon was strongly decreased compared to transcription of the native *sae* operon in the wild-type strain. The single transcript observed was probably generated by the P3 promoter, which was previously shown not to be autoactivated (17). The main P1 promoter, in contrast, was strongly autoregulated, and the corresponding T1 and T4 (with *saeP*) transcripts were not detectable in the *saeS* deletion construct. This indicates that SaeS is required for P1 activation. Additionally, *coa* expression was undetectable, indicating that SaeS is also needed for the *sae*-dependent activation of class I target genes. In contrast, *hla* could be activated without SaeS. These results suggest either that *hla* can be activated by unphosphorylated

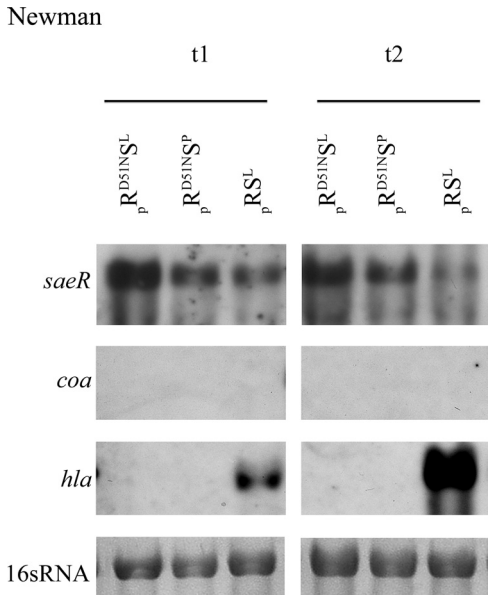

FIG. 6. Role of the SaeR phosphorylation site. To analyze the requirement for SaeR phosphorylation, mutated *saeR*(*D51N*)S^L and *saeR*(*D51N*)S^P were cloned behind an AHT-inducible promoter and introduced into the *sae* mutant ISP479C-29. Strains containing either mutated *saeR*(*D51N*)*S* or wild-type *saeRS* were induced with AHT (0.01 μg/ml) and grown to the exponential (t1) (OD_{600}, 0.5) and late exponential (t2) (OD_{600}, 3) growth phases. RNA was hybridized with digoxigenin-labeled PCR fragments. The lane at the bottom contained the 16S rRNA detected in the ethidium bromide-stained gels as a loading control.

SaeR or that SaeR can be activated by other mechanisms (e.g., by cross-activation via other *S. aureus* HKs).

Role of phosphorylation of the response regulator SaeR. To analyze whether phosphorylation of SaeR is necessary for activation of target genes, the predicted phosphorylation site in SaeR was mutated. Mutation of the conserved Asp was previously shown to lead to complete inactivation of OmpR (28). However, other RRs, such as DegU from *Listeria monocytogenes* (26) and *Bacillus subtilis* (10) and AlgR from *Pseudomonas aeruginosa* (37), were shown to be active in their unphosphorylated forms. Sequence alignment with OmpR class response regulators revealed a conserved Asp residue in SaeR (D51), which is most likely the predicted phosphorylation site. The phosphorylation site was mutated in the AHT-inducible constructs containing *saeRS*^{L} and *saeRS*^{P}, and the plasmids were introduced into the *saePQRS* mutants of strain Newman (Fig. 6) and ISP479C (not shown). The phosphorylation-defective *saeR* gene is indicated by *saeR*(*D51N*). The mutation resulted in complete inactivation of *sae* regardless of which of the *saeS* alleles was present. This suggests that at least a low level of SaeR phosphorylation is required for *sae* activity.

DISCUSSION

Sae hyperactivity in strain Newman. In this study we show that a mutation in the first transmembrane region of SaeS, like that in the widely used strain *S. aureus* Newman, leads to hyperactivation of the *saeRS* system and is the reason for the unusual high levels of transcription of some genes, such as *fnbA*, *coa*, or *eap* (*sae* class I target genes). Additionally, the P1 promoter located upstream of *saeP* also belongs to this class of target genes and results in strong autoactivation of the T1 and T2 *sae* transcripts in strain Newman. It was recently shown that the transcription levels of selected *sae* target genes vary widely between the Newman and Col strains (46). Most of the peculiarities of virulence gene expression in strain Newman are due to the *saeS* polymorphism. The unusual *saeS*^{P} allele probably makes this strain relatively virulent in animal models of infection, which is one reason for its wide application in such studies. However, due to the SaeS polymorphism, as well as the recently described truncation of the fibronectin binding proteins (25), results obtained with this strain may not necessarily be applicable to other *S. aureus* strains. The presence of SaeS in strain Newman results not only in *saePQRS* overexpression but also in a largely altered response to environmental signaling compared to other strains (1, 17); depending on the stimulus analyzed, either $SaeS^P$ is unresponsive (17) or its reaction is opposite that of $SaeS^L$ (47).

In contrast to the transcription of class I *sae* target genes, the transcription of secreted virulence factors, such as the strictly *sae*-dependent *hla* and *hlb* genes, is not elevated in constructs containing $SaeS^P$ compared to constructs containing the $SaeS^L$ allele. Accordingly, strain Newman does not produce large amounts of α-hemolysin. The level of *hla* expression seems to be even lower than that in other virulent *S. aureus* strains, such as the emerging methicillin-resistant strain *S. aureus* USA300 (8). In addition to the effect on *hla* and *hlb*, the repressive effect of *saeRS* on the expression of the capsular polysaccharide was also shown to be not altered by the SaeS polymorphism (Fig. 1D), placing the *capAP* gene cluster in class II of the target genes.

Our results are in perfect agreement with the results obtained by Adhikari and Novick (1), who analyzed an *saeP*::bursa insertion mutant of strain RN6734, a derivative of strain 8325. This mutant curiously resembled strain Newman in the pattern of expression of several virulence factors, such as *coa*, *fnbA*, and *eap*. The original bursa mutation was generated in strain Newman and then transduced into strain RN6734. We assume that upon transduction, the whole *saeQRS*^{P} sequence from strain Newman replaced the wild-type sequence of strain RN6374 due to the homologous recombination necessary for transduction. Therefore, the phenotype described for the *saeP*::bursa mutant of stain RN6734 that resembled the Newman strain can be fully explained by the introduction of the $SaeS^P$ allele, but it is not related to the bursa insertion.

Role of the transmembrane region of SaeS in the differential activation of target genes. In this study we showed that a single amino acid change in the transmembrane region of SaeS results in severe changes in the expression of class I target genes, whereas class II target genes seem to be regulated equally by $SaeS^P$ and $SaeS^L$. This observation can best be explained by differential requirements of the different promoter regions for gene activation via SaeR (Fig. 7). In our working model, the presence of $SaeS^P$ results in a shift toward kinase activity and consequently to a high level of SaeR phosphorylation. For $SaeS^L$, the phosphatase activity is dominant, at least under uninduced conditions, and thus only a few SaeR molecules are phosphorylated. The ratio of SaeR-P to SaeR would then determine which genes are activated. This model is based on

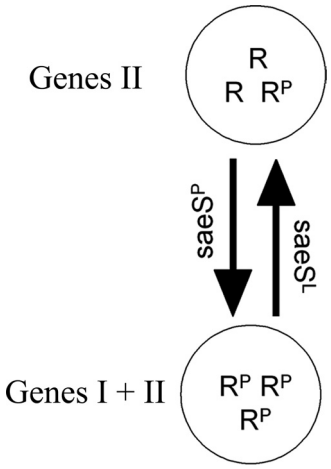

FIG. 7. Model for differential regulation of target genes. R, response regulator SaeR; R^P, phosphorylated SaeR. The class II target genes (II) include *hla*, *hlb*, and *cap*, and the class I target genes (I) include *fnbA*, *coa*, *eap*, and *sae*. Class II target genes require only low levels of phosphorylated SaeR. In contrast, class I target genes require high levels of SaeR phosphorylation, like those obtained with $SaeS^P$ with strong kinase activity. Under conditions in which the *sae* system is not fully activated the phosphatase activity of $SaeS^L$ results in only a low level of phosphorylated SaeR, which is not sufficient to activate class I target genes.

similarities to the EnvZ/OmpR system of *Escherichia coli* and the presumption that, depending on the signal, SaeS can exhibit kinase activity and/or phosphatase activity. It could be predicted that SaeS, like EnvZ, is probably a bifunctional HK. This class of HKs is thought to enhance the suppression of signals from noncognate resources due to the phosphatase activity (2). For the prototypic EnvZ/OmpR system in *E. coli*, it was shown that the ratio of phosphorylated RR to unphosphorylated RR determines which of the target genes are activated. In this model, osmotic signals regulate the level of the phosphorylated RR (OmpR-P) by modulating the ratio of kinase activity to phosphatase activity of EnvZ (57). The target gene *ompC* requires a high proportion of OmpR to be phosphorylated, while *ompF* can be activated with just a few OmpR-P molecules. However, the activation of both target genes, *ompF* and *ompC*, is dependent on at least a low level of OmpR phosphorylation (28), similar to what was found here for SaeR-mediated gene activation. Mutation of the Asp phosphorylation site in the RR eliminated activation of all target genes in both systems (28) (Fig. 6). Interestingly, deletion of SaeS does not result in complete inactivation of target gene expression. Thus, a low level of SaeR phosphorylation which is independent of SaeS can be assumed. This may occur either through unrelated HKs or through small-molecule phospho donors, such as acetyl phosphate (54).

Role of *saeRS* in temporal gene expression. Recently, it was proposed that a different dependence of target genes on the level of RR phosphorylation controls the coordinate multicellular behavior of *B. subtilis* (30, 51). Target genes involved in swarming motility are activated by very low levels of the phosphorylated RR DegU, whereas genes necessary for complex colony architecture require an intermediate level of DegU phosphorylation. Finally, high levels of DegU-P inhibit both swarming motility and complex colony architecture. Whether a temporal program can also be linked to the different *sae* target genes remains to be determined. The class II target genes, which in our model require only low levels of phosphorylation (*hla*, *hlb*, and *cap*), are typically expressed in the late growth phase. For class I target genes, there is no correlation with growth phase; *coa* and *fnbA* are typically expressed only during the exponential phase, whereas *eap* is activated in the postexponential phase. This temporal gene expression pattern is clearly determined by additional factors, as shown by the induction of *saeRS* at different growth phases (Fig. 3). Thus, based on an *in vitro* analysis, a link to the temporal expression cannot be made easily. However, the gene expression patterns during infection or colonization are markedly different from those under *in vitro* growth conditions (21–23), and little is known about the gradual expression of *sae* target genes upon stimulation *in vivo*. Recently, it was proposed that the *sae* system may play an important role in environmental sensing after phagocytosis (17), and it was shown that *saeRS* is important for bacterial escape from the phagocyte through the intracellular activation of toxins (52). One may speculate that the gradual activation of target genes in response to various input signals plays a role after phagocytosis.

Target gene expression is not sensitive to the *saeRS* dosage. Using an inducible expression vector system, we showed that the transcription of both types of target genes is largely not sensitive to the level of *saeRS* transcriptional activation. Induction of a high level of *saeRS* expression did not result in a significant increase in expression of the target genes. Thus, the different target gene activation of $SaeS^P$ and $SaeS^L$ was independent of the *saeRS* dosage. This also suggests that it is the ratio of phosphorylated SaeR to unphosphorylated SaeR mediated by SaeS activity which accounts for the different output, rather than the SaeR concentration. It was also shown that the EnvZ/OmpR system is robust and is only marginally sensitive to the absolute concentrations of EnvZ and/or OmpR (4). However, the robustness seen in the EnvZ/OmpR system is explained by the observation that the translation of OmpR is much more efficient than that of EnvZ, resulting in a >30-fold excess of OmpR over EnvZ (9). Whether there is a similar bias in the translational efficiency of SaeR and SaeS remains to be determined. However, a low ratio of SaeS to SaeR is probable, due to the overlap of the *saeR* stop and *saeS* start codons. The same overlap is present in the genetic organization of *ompR* and *envZ* and was assumed to cause the high OmpR/EnvZ ratio.

Based on our analysis, the autoregulated P1 promoter of the *sae* system can be classified as a promoter of the class I target genes. Thus, the *saePQRS* system, like many other regulatory systems, is positively autoregulated, which seems to contradict the assumption that the system is robust. There would be no point in changing the expression if there were no effect on the output. However, it was recently predicted that positive autoregulation of a two-component system contributes to enhanced output only in the presence of a strong stimulus (41). Since the native signal for SaeS activation is not well defined, we do not know whether our assays were performed under activating conditions. The fact that we could not detect *coa* and *fnbA* in strains harboring *saePQRS* may indicate that the growth conditions used only very weakly stimulated SaeS activation. Thus,

under strong stimulating conditions, the function of $SaeS^L$ may approximate that of the constitutively activated $SaeS^P$ protein. In this way, strain Newman may be a valuable tool for defining the *sae* regulon by mimicking high-stimulation conditions, which are not well defined yet.

ACKNOWLEDGMENTS

We thank Vittoria Bisanzio for excellent technical assistance. We are grateful to A. Cheung for providing the vector pALC2073.

This work was supported by grants to C.W. from the Deutsche Forschungsgemeinschaft (grants Wo578/5, SFB766, and TR34) and from the IZKF of Eberhard Karls University, Tübingen, Germany.

REFERENCES

1. **Adhikari, R. P., and R. P. Novick.** 2008. Regulatory organization of the staphylococcal *sae* locus. Microbiology **154:**949–959.
2. **Alves, R., and M. A. Savageau.** 2003. Comparative analysis of prototype two-component systems with either bifunctional or monofunctional sensors: differences in molecular structure and physiological function. Mol. Microbiol. **48:**25–51.
3. **Arnaud, M., A. Chastanet, and M. Debarbouille.** 2004. New vector for efficient allelic replacement in naturally nontransformable, low-GC-content, gram-positive bacteria. Appl. Environ. Microbiol. **70:**6887–6891.
4. **Batchelor, E., and M. Goulian.** 2003. Robustness and the cycle of phosphorylation and dephosphorylation in a two-component regulatory system. Proc. Natl. Acad. Sci. U. S. A. **100:**691–696.
5. **Bateman, B. T., N. P. Donegan, T. M. Jarry, M. Palma, and A. L. Cheung.** 2001. Evaluation of a tetracycline-inducible promoter in *Staphylococcus aureus* in vitro and in vivo and its application in demonstrating the role of *sigB* in microcolony formation. Infect. Immun. **69:**7851–7857.
6. **Bischoff, M., P. Dunman, J. Kormanec, D. Macapagal, E. Murphy, W. Mounts, B. Berger-Bachi, and S. Projan.** 2004. Microarray-based analysis of the *Staphylococcus aureus* σ^B regulon. J. Bacteriol. **186:**4085–4099.
7. **Bruckner, R.** 1997. Gene replacement in *Staphylococcus carnosus* and *Staphylococcus xylosus*. FEMS Microbiol. Lett. **151:**1–8.
8. **Bubeck Wardenburg, J., T. Bae, M. Otto, F. R. Deleo, and O. Schneewind.** 2007. Poring over pores: alpha-hemolysin and Panton-Valentine leukocidin in *Staphylococcus aureus* pneumonia. Nat. Med. **13:**1405–1406.
9. **Cai, S. J., and M. Inouye.** 2002. EnvZ-OmpR interaction and osmoregulation in *Escherichia coli*. J. Biol. Chem. **277:**24155–24161.
10. **Dahl, M. K., T. Msadek, F. Kunst, and G. Rapoport.** 1992. The phosphorylation state of the DegU response regulator acts as a molecular switch allowing either degradative enzyme synthesis or expression of genetic competence in *Bacillus subtilis*. J. Biol. Chem. **267:**14509–14514.
11. **Duthie, E. S., and L. L. Lorenz.** 1952. Staphylococcal coagulase; mode of action and antigenicity. J. Gen. Microbiol. **6:**95–107.
12. **Fabret, C., V. A. Feher, and J. A. Hoch.** 1999. Two-component signal transduction in *Bacillus subtilis*: how one organism sees its world. J. Bacteriol. **181:**1975–1983.
13. **Galperin, M. Y.** 2006. Structural classification of bacterial response regulators: diversity of output domains and domain combinations. J. Bacteriol. **188:**4169–4182.
14. **Gao, R., T. R. Mack, and A. M. Stock.** 2007. Bacterial response regulators: versatile regulatory strategies from common domains. Trends Biochem. Sci. **32:**225–234.
15. **Gao, R., and A. M. Stock.** 2009. Biological insights from structures of two-component proteins. Annu. Rev. Microbiol. **63:**133–154.
16. **Gaskill, M. E., and S. A. Khan.** 1988. Regulation of the enterotoxin B gene in *Staphylococcus aureus*. J. Biol. Chem. **263:**6276–6280.
17. **Geiger, T., C. Goerke, M. Mainiero, D. Kraus, and C. Wolz.** 2008. The virulence regulator Sae of *Staphylococcus aureus*: promoter activities and response to phagocytosis-related signals. J. Bacteriol. **190:**3419–3428.
18. **Giraudo, A. T., A. L. Cheung, and R. Nagel.** 1997. The *sae* locus of *Staphylococcus aureus* controls exoprotein synthesis at the transcriptional level. Arch. Microbiol. **168:**53–58.
19. **Giraudo, A. T., C. Mansilla, A. Chan, C. Raspanti, and R. Nagel.** 2003. Studies on the expression of regulatory locus *sae* in *Staphylococcus aureus*. Curr. Microbiol. **46:**246–250.
20. **Goerke, C., S. Campana, M. G. Bayer, G. Doring, K. Botzenhart, and C. Wolz.** 2000. Direct quantitative transcript analysis of the *agr* regulon of *Staphylococcus aureus* during human infection in comparison to the expression profile in vitro. Infect. Immun. **68:**1304–1311.
21. **Goerke, C., U. Fluckiger, A. Steinhuber, V. Bisanzio, M. Ulrich, M. Bischoff, J. M. Patti, and C. Wolz.** 2005. Role of *Staphylococcus aureus* global regulators *sae* and sigmaB in virulence gene expression during device-related infection. Infect. Immun. **73:**3415–3421.
22. **Goerke, C., U. Fluckiger, A. Steinhuber, W. Zimmerli, and C. Wolz.** 2001. Impact of the regulatory loci *agr*, *sarA* and *sae* of *Staphylococcus aureus* on the induction of alpha-toxin during device-related infection resolved by direct quantitative transcript analysis. Mol. Microbiol. **40:**1439–1447.
23. **Goerke, C., and C. Wolz.** 2004. Regulatory and genomic plasticity of *Staphylococcus aureus* during persistent colonization and infection. Int. J. Med. Microbiol. **294:**195–202.
24. **Grebe, T. W., and J. B. Stock.** 1999. The histidine protein kinase superfamily. Adv. Microb. Physiol. **41:**139–227.
25. **Grundmeier, M., M. Hussain, P. Becker, C. Heilmann, G. Peters, and B. Sinha.** 2004. Truncation of fibronectin-binding proteins in *Staphylococcus aureus* strain Newman leads to deficient adherence and host cell invasion due to loss of the cell wall anchor function. Infect. Immun. **72:**7155–7163.
26. **Gueriri, I., S. Bay, S. Dubrac, C. Cyncynatus, and T. Msadek.** 2008. The Pta-AckA pathway controlling acetyl phosphate levels and the phosphorylation state of the DegU orphan response regulator both play a role in regulating *Listeria monocytogenes* motility and chemotaxis. Mol. Microbiol. **70:**1342–1357.
27. **Harraghy, N., J. Kormanec, C. Wolz, D. Homerova, C. Goerke, K. Ohlsen, S. Qazi, P. Hill, and M. Herrmann.** 2005. *sae* is essential for expression of the staphylococcal adhesins Eap and Emp. Microbiology **151:**1789–1800.
28. **Kanamaru, K., H. Aiba, and T. Mizuno.** 1990. Transmembrane signal transduction and osmoregulation in *Escherichia coli*. I. Analysis by site-directed mutagenesis of the amino acid residues involved in phosphotransfer between the two regulatory components, EnvZ and OmpR. J. Biochem. **108:**483–487.
29. **Kim, D., and S. Forst.** 2001. Genomic analysis of the histidine kinase family in bacteria and archaea. Microbiology **147:**1197–1212.
30. **Kobayashi, K.** 2007. Gradual activation of the response regulator DegU controls serial expression of genes for flagellum formation and biofilm formation in *Bacillus subtilis*. Mol. Microbiol. **66:**395–409.
31. **Kreiswirth, B. N., S. Lofdahl, M. J. Betley, M. O'Reilly, P. M. Schlievert, M. S. Bergdoll, and R. P. Novick.** 1983. The toxic shock syndrome exotoxin structural gene is not detectably transmitted by a prophage. Nature **305:**709–712.
32. **Kullik, I., P. Giachino, and T. Fuchs.** 1998. Deletion of the alternative sigma factor sigmaB in *Staphylococcus aureus* reveals its function as a global regulator of virulence genes. J. Bacteriol. **180:**4814–4820.
33. **Kuroda, H., M. Kuroda, L. Cui, and K. Hiramatsu.** 2007. Subinhibitory concentrations of beta-lactam induce haemolytic activity in *Staphylococcus aureus* through the SaeRS two-component system. FEMS Microbiol. Lett. **268:**98–105.
34. **Lee, C. Y., S. L. Buranen, and Z. H. Ye.** 1991. Construction of single-copy integration vectors for *Staphylococcus aureus*. Gene **103:**101–105.
35. **Li, D., and A. Cheung.** 2008. Repression of *hla* by *rot* is dependent on *sae* in *Staphylococcus aureus*. Infect. Immun. **76:**1068–1075.
36. **Liang, X., C. Yu, J. Sun, H. Liu, C. Landwehr, D. Holmes, and Y. Ji.** 2006. Inactivation of a two-component signal transduction system, SaeRS, eliminates adherence and attenuates virulence of *Staphylococcus aureus*. Infect. Immun. **74:**4655–4665.
37. **Ma, S., U. Selvaraj, D. E. Ohman, R. Quarless, D. J. Hassett, and D. J. Wozniak.** 1998. Phosphorylation-independent activity of the response regulators AlgB and AlgR in promoting alginate biosynthesis in mucoid *Pseudomonas aeruginosa*. J. Bacteriol. **180:**956–968.
38. **Mascher, T.** 2006. Intramembrane-sensing histidine kinases: a new family of cell envelope stress sensors in Firmicutes bacteria. FEMS Microbiol. Lett. **264:**133–144.
39. **Mascher, T., J. D. Helmann, and G. Unden.** 2006. Stimulus perception in bacterial signal-transducing histidine kinases. Microbiol. Mol. Biol. Rev. **70:**910–938.
40. **Matsubara, M., and T. Mizuno.** 1999. EnvZ-independent phosphotransfer signaling pathway of the OmpR-mediated osmoregulatory expression of OmpC and OmpF in *Escherichia coli*. Biosci. Biotechnol. Biochem. **63:**408–414.
41. **Miyashiro, T., and M. Goulian.** 2008. High stimulus unmasks positive feedback in an autoregulated bacterial signaling circuit. Proc. Natl. Acad. Sci. U. S. A. **105:**17457–17462.
42. **Novick, R.** 1967. Properties of a cryptic high-frequency transducing phage in *Staphylococcus aureus*. Virology **33:**155–166.
43. **Novick, R. P.** 1991. Genetic systems in staphylococci. Methods Enzymol. **204:**587–636.
44. **Novick, R. P., and D. Jiang.** 2003. The staphylococcal *saeRS* system coordinates environmental signals with quorum sensing. Microbiology **149:**2709–2717.
45. **Pattee, P. A.** 1981. Distribution of Tn551 insertion sites responsible for auxotrophy on the *Staphylococcus aureus* chromosome. J. Bacteriol. **145:**479–488.
46. **Rogasch, K., V. Ruhmling, J. Pane-Farre, D. Hoper, C. Weinberg, S. Fuchs, M. Schmudde, B. M. Broker, C. Wolz, M. Hecker, and S. Engelmann.** 2006. Influence of the two-component system SaeRS on global gene expression in two different *Staphylococcus aureus* strains. J. Bacteriol. **188:**7742–7758.
47. **Schafer, D., T. T. Lam, T. Geiger, M. Mainiero, S. Engelmann, M. Hussain, A. Bosserhoff, M. Frosch, M. Bischoff, C. Wolz, J. Reidl, and B. Sinha.** 25 September 2009. A point mutation in the sensor histidine kinase SaeS of

Staphylococcus aureus strain Newman alters response to biocide exposure. J. Bacteriol. doi:10.1128/JB.00630-09.
48. **Steinhuber, A., C. Goerke, M. G. Bayer, G. Doring, and C. Wolz.** 2003. Molecular architecture of the regulatory locus *sae* of *Staphylococcus aureus* and its impact on expression of virulence factors. J. Bacteriol. **185:**6278–6286.
49. **Toledo-Arana, A., N. Merino, M. Vergara-Irigaray, M. Debarbouille, J. R. Penades, and I. Lasa.** 2005. *Staphylococcus aureus* develops an alternative, *ica*-independent biofilm in the absence of the *arlRS* two-component system. J. Bacteriol. **187:**5318–5329.
50. **Vandenesch, F., J. Kornblum, and R. P. Novick.** 1991. A temporal signal, independent of *agr*, is required for *hla* but not *spa* transcription in *Staphylococcus aureus*. J. Bacteriol. **173:**6313–6320.
51. **Verhamme, D. T., T. B. Kiley, and N. R. Stanley-Wall.** 2007. DegU coordinates multicellular behaviour exhibited by *Bacillus subtilis*. Mol. Microbiol. **65:**554–568.
52. **Voyich, J. M., C. Vuong, M. Dewald, T. K. Nygaard, S. Kocianova, S. Griffith, J. Jones, C. Iverson, D. E. Sturdevant, K. R. Braughton, A. R. Whitney, M. Otto, and F. R. Deleo.** 2009. The SaeR/S gene regulatory system is essential for innate immune evasion by *Staphylococcus aureus*. J. Infect. Dis. **199:** 1698–1706.
53. **Wann, E. R., B. Dassy, J. M. Fournier, and T. J. Foster.** 1999. Genetic analysis of the cap5 locus of *Staphylococcus aureus*. FEMS Microbiol. Lett. **170:**97–103.
54. **Wolfe, A. J.** 2005. The acetate switch. Microbiol. Mol. Biol. Rev. **69:**12–50.
55. **Wolz, C., D. McDevitt, T. J. Foster, and A. L. Cheung.** 1996. Influence of *agr* on fibrinogen binding in *Staphylococcus aureus* Newman. Infect. Immun. **64:**3142–3147.
56. **Wolz, C., P. Pohlmann-Dietze, A. Steinhuber, Y. T. Chien, A. Manna, W. van Wamel, and A. Cheung.** 2000. *agr*-independent regulation of fibronectin-binding protein(s) by the regulatory locus *sar* in *Staphylococcus aureus*. Mol. Microbiol. **36:**230–243.
57. **Yoshida, T., S. Phadtare, and M. Inouye.** 2007. Functional and structural characterization of EnvZ, an osmosensing histidine kinase of *E. coli*. Methods Enzymol. **423:**184–202.