

# The Virulence Regulator Sae of *Staphylococcus aureus*: Promoter Activities and Response to Phagocytosis-Related Signals<sup>∇</sup>

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The two-component system SaeRS of *Staphylococcus aureus* is closely involved in the regulation of major virulence factors. However, little is known about the signals leading to *saeRS* activation. A total of four overlapping transcripts (T1 to T4) from three different transcription starting points are expressed in the *sae* operon. We used a  $\beta$ -galactosidase reporter assay to characterize the putative promoter regions within the *saeRS* upstream region. The main transcript T2 is probably generated by endoribonucleolytic processing of the T1 transcript. Only two distinct promoter elements (P1 and P3) could be detected within the *saeRS* upstream region. The P3 promoter, upstream of *saeRS*, generates the T3 transcript, includes a *cis*-acting enhancer element and is repressed by *saeRS*. The most distal P1 promoter is strongly autoregulated, activated by *agr*, and repressed by sigma factor B. In strain Newman a mutation within the histidine kinase SaeS leads to a constitutively activated *sae* system. Evaluation of different external signals revealed that the P1 promoter in strain ISP479R and strain UAMS-1 is inhibited by low pH and high NaCl concentrations but activated by hydrogen peroxide. The most prominent induction of P1 was observed at subinhibitory concentrations of  $\alpha$ -defensins in various *S. aureus* strains, with the exception of strain ISP479R and strain COL. P1 was not activated by the antimicrobial peptides LL37 and daptomycin. In summary, the results indicate that the sensor molecule SaeS is activated by alteration within the membrane allowing the pathogen to react to phagocytosis related effector molecules.

*Staphylococcus aureus* asymptotically colonizes the noses of healthy individuals but also causes a variety of infections in humans. Polymorphonuclear neutrophils (PMNs) provide an effective line of defense against infections. However, *S. aureus* has developed a variety of mechanisms to avoid being killed by PMNs, including the expression of a number of immune modulators and toxins (for a review, see reference 8). The expression of most virulence and adherence factors is directly or indirectly influenced by diverse regulators such as *agr*, the alternative sigma factor B (SigB), *sarA* homologues or *sae* (for reviews, see references 4, 6, 17, and 32). Within this network *sae* appears to be a central downstream regulator that controls the expression of major virulence genes such as *hla* (coding for alpha-hemolysin), *coa* (coding for coagulase), or *fnbA* (coding for fibronectin-binding protein A) (11, 33, 39). Microarray analysis and proteomics have revealed that most of the genes activated by *sae* are involved in bacterial adhesion, immune modulation, or toxicity (25, 29, 38). In addition, the importance of gene regulation by *sae* in vivo was shown in several animal models (3, 15, 16, 29, 43).

The *sae* locus consists of four open reading frames, two of which (*saeR* and *saeS*) show strong sequence homology to response regulators and to histidine kinases (HKs) of bacterial two-component regulators (10). Two additional open reading frames, *saeP* and *saeQ*, both located upstream of *saeRS*, are likely to be important for the functionality of the *sae* operon (33, 39). Four overlapping *sae*-specific transcripts (T1 to T4)

can be detected: the T1 message (3.0 kb) initiates upstream of *saeP*, the T2 message (2.4 kb) initiates upstream of *saeQ*, and T3 (2.0 kb) initiates in front of *saeR*. T4 (0.7 kb) represents a monocistronic mRNA encompassing *saeP* only. Thus far, the signals leading to the activation of the *saeRS* system are only poorly understood. The T1 transcript seems to be activated in the post-exponential-growth phase (33, 39) but downregulated under low pH or osmotic upshift (33). Subinhibitory concentrations of several classes of antibiotics have also been implicated in *sae* activation (glycopeptides,  $\beta$ -lactams) (25, 26) or inhibition (clindamycin) (33). The results on interactions with other virulence regulators such as *agr* and SigB are partially contradictory, which may be due to strain differences (15, 25, 33).

We are able to show here that the *sae* upstream region contains two active promoter elements, P1 and P3. The main strongly autoregulated P1 promoter can be activated by H<sub>2</sub>O<sub>2</sub> and by subinhibitory concentrations of  $\alpha$ -defensins, the key players in the phagocytic killing of bacteria. Thus, *sae* activation may play an important role in the synthesis of virulence factors leading to the destruction of PMNs after phagocytosis.

## MATERIALS AND METHODS

**Strains and growth conditions.** Reference strains COL, MW2, N315, USA300, MRSA252, and MSSA476 were provided by NARSA (i.e., the Network on Antimicrobial Resistance in *Staphylococcus aureus*). Other strains and plasmids are listed in Table 1. *S. aureus* strains were grown in CYPG (Casamino Acids [10 g/liter], yeast extract [10 g/liter], NaCl [5 g/liter], 20% glucose, 1.5 M phosphoglycerate) (31) supplemented with the appropriate antibiotics for strains carrying resistance genes (kanamycin at 50  $\mu$ g/ml, erythromycin at 10  $\mu$ g/ml, and tetracycline at 5  $\mu$ g/ml).

For promoter assays and RNA analysis, bacteria from an overnight culture were diluted to an initial optical density at 600 nm (OD<sub>600</sub>) of 0.05 in fresh

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TABLE 1. Bacterial strains and plasmids

Strain or plasmid	Description	Source or reference
<b>Strains</b>		
One Shot, TOP10	Competent <i>E. coli</i> for plasmid transformation	Invitrogen
CYL316	RN4220, pYL112Δ19, L54 <i>int</i> gene, r <sup>-</sup>	27
RN6390	Derivate of NCTC 8325-4	36
RN6112	RN6390 <i>agr</i> ::Tn551	Richard Novick
8325 ( <i>ΔsigB</i> )	<i>ΔrsbVW-sigB::erm</i>	24
RN4220	Restriction-deficient <i>S. aureus</i> strain	23
RN4220-29	RN4220 <i>Δsae::kan</i>	Unpublished data
Newman	Wild type	7
Newman-29	Newman, <i>Δsae::kan</i>	This study
Newman- <i>agr</i>	Newman, <i>agr</i> ::Tn551	This study
ISP479R	ISP479R, derivative of strain 8325-4, in which the mutation in <i>rsbU</i> was repaired to gain full sigma B activity	40
ISP479R-29	<i>Δsae::kan</i>	This study
ISP479R- <i>agr</i>	<i>agr</i> ::Tn551	This study
ISP479R- <i>sigB</i>	ISP479R, <i>ΔrsbVW-sigB::erm</i>	This study
UAMS-1	Osteomyelitis isolate	9
UAMS-1- <i>agr</i>	<i>agr</i> ::Tn551	This study
UAMS-1- <i>sigB</i>	UAMS-1, <i>ΔrsbVW-sigB::erm</i>	This study
<b>Plasmids</b>		
pDG783	Vector containing kanamycin resistance cassette	18
pKO010	Cloning vector with <i>spoVG-lacZ</i>	34
pCL84	Single-copy integration vector, att-site for chromosomal integration in <i>geh</i>	27
pCG6	pCL84 with integrated <i>spoVG-lacZ</i> gene cassette from pKO010	This study
pCG7	pCG6 with <i>sae</i> P1 (nt 1 to 400)	This study
pCG8	pCG6 with <i>sae</i> P2 + short (nt 496 to 1141)	This study
pCG38	pCG6 with <i>sae</i> P2 + middle (nt 496 to 1258)	This study
pCG39	pCG6 with <i>sae</i> P2 + long (nt 496 to 1269)	This study
pCG9	pCG6 with <i>sae</i> P3 + short (nt 1127 to 1529)	This study
pCG36	pCG6 with <i>sae</i> P3 + middle (nt 974 to 1529)	This study
pCG15	pCG6 with <i>sae</i> P3 + long (nt 496 to 1529)	This study
pCWSAE28	pCL84 with <i>saePQRS</i> operon (long, nt 1 to 3515)	This study
pCWSAE47	pCL84 with partial <i>sae</i> operon including P3 (short, nt 1066 to 3515)	This study
pCWSAE48	pCL84 with partial <i>sae</i> operon including P2 and P3 (middle, nt 724 to 3515)	This study

medium without antibiotics and grown with shaking (222 rpm) at 37°C to the mid-exponential growth phase (OD<sub>600</sub> = 0.5). The influence of *agr* and SigB on P1 activity was determined in bacteria grown to the post-exponential growth phase (OD<sub>600</sub> = 6). The influence of pH, high NaCl concentration, and cell wall antibiotics was evaluated after 1 h of induction. Therefore, bacteria were grown to the mid-exponential growth phase (OD<sub>600</sub> = 0.5) in CYPG and centrifuged (5 min, 5,000 rpm). Pellets were resuspended in CYPG adjusted to pH 7.4 or 5.5 with or without the addition of either 1 M NaCl, H<sub>2</sub>O<sub>2</sub> (0.5 to 20 mM, AppliChem, Darmstadt, Germany), or the cell-wall-active antibiotics vancomycin (5 to 20 μg/ml; Sigma-Aldrich, Munich, Germany) or bacitracin (20 to 40 μg/ml; Sigma-Aldrich). Bacteria were further incubated for 1 h with shaking (222 rpm) at 37°C, the OD<sub>600</sub> was determined, and equal bacterial numbers (corresponding to a 2-ml culture of OD<sub>600</sub> = 1) were harvested.

For induction experiments with antimicrobial peptides, bacteria were grown in a modified Luria-Bertani broth (half yeast extract [2.5g/liter] and half tryptone [5 g/liter], no NaCl) as described elsewhere (37), a protocol that was shown not to inactivate α-defensins. The overnight cultures were diluted to an initial OD<sub>600</sub> of 0.05 in fresh LB medium without antibiotics and grown with shaking (222 rpm) at 37°C to the mid-exponential growth phase (OD<sub>600</sub> = 0.5). Then, 2-ml portions of cultures were supplemented with different concentrations of human neutrophil α-defensins (HNP1 to HNP3; 0.5 to 15 μg/ml), synthetic HNP2 (2 to 15 μg/ml; Peptanova, Sandhausen, Germany), or LL37 (1 to 10 μg/ml) and incubated for 2 h with shaking (222 rpm) at 37°C, and an equal number of bacteria was harvested, corresponding to a 1-ml culture with an OD<sub>600</sub> of 1. For induction experiments using daptomycin (Cubicin Novartis, Nürnberg, Germany), the modified LB medium was supplemented with 50 mg of CaCl<sub>2</sub>/liter. α-Defensins and LL37 were purified from human peripheral blood neutrophils as described previously (37).

**Determination of MICs.** Bacteria from the exponential growth phase (100 μl at an OD<sub>600</sub> of 0.5) were diluted in 1 ml of phosphate-buffered saline. In 96-well microtiter plates 10 μl of bacterial suspension was mixed with 80 μl of medium,

and increasing amounts of H<sub>2</sub>O<sub>2</sub> (0.01 to 20 mM), LL37 (25 to 400 μg/ml), and HNP1 to HNP3 (50 to 400 μg/ml) were added. CYPG medium was used for determination of the H<sub>2</sub>O<sub>2</sub> MICs, and modified LB medium was used for determination of the defensin MICs. Plates were incubated at 37°C, and the turbidity was determined after 24 and 48 h. The bactericidal activity was determined by plating of the dilutions for which no growth was detected.

**Construction of *agr*, *sigB*, and *sae* mutant strains.** The *sae* locus was replaced by a kanamycin-resistant cassette using the temperature-sensitive shuttle vector pMAD (unpublished data). Briefly, two fragments flanking the *sae* locus and the kanamycin-resistant cassette derived from plasmid pCG783 were amplified and annealed by overlapping PCR. The amplicon was cloned into the EcoRI/SalI restriction sites of pMAD to gain pCWSAE29. pCWSAE29 was used to mutagenize strain RN4220 as described previously (2). The obtained *sae* gene replacement mutant strain (RN4220-29) was verified by PCR. In the mutant the whole *sae* operon (nucleotides [nt] 310 to 3279, based on GenBank accession no. AJ556795) was replaced by the kanamycin-resistant cassette.

*sae*, *sigB*, and *agr* mutants of different *S. aureus* strains were obtained by transduction using lysates of strains RN4220-29, 8325 (*ΔsigB*) (24) and RN6112, respectively. Transductants were verified by PCR and pulsed-field gel electrophoresis. All of the *agr* mutants were negative for delta-hemolysin; all of the *sigB* mutants were nonpigmented.

**Chromosomal integration of *sae* into *geh*.** The cloned and sequenced 3.5-kb *sae* operon of strain ISP479C(pCWSAE7) (39) was subcloned into the single EcoRI site of pCL84, resulting in pCWSAE28. Shorter *sae* fragments were generated using oligonucleotides *sae*-724U and *sae*-1066U, together with *sae*-3515L, to generate *sae* fragments encompassing nt 724 to 3515 or nt 1066 to 3515, respectively (Fig. 1A). The fragments were cloned into the integration vector pCL84 (27), resulting in pCWSAE47 and pCWSAE48. Plasmids were integrated into *geh* of strain CYL316 by protoplast transformation and tetracycline selection. All integrants were lipase negative on Baird-Parker plates supplemented with egg

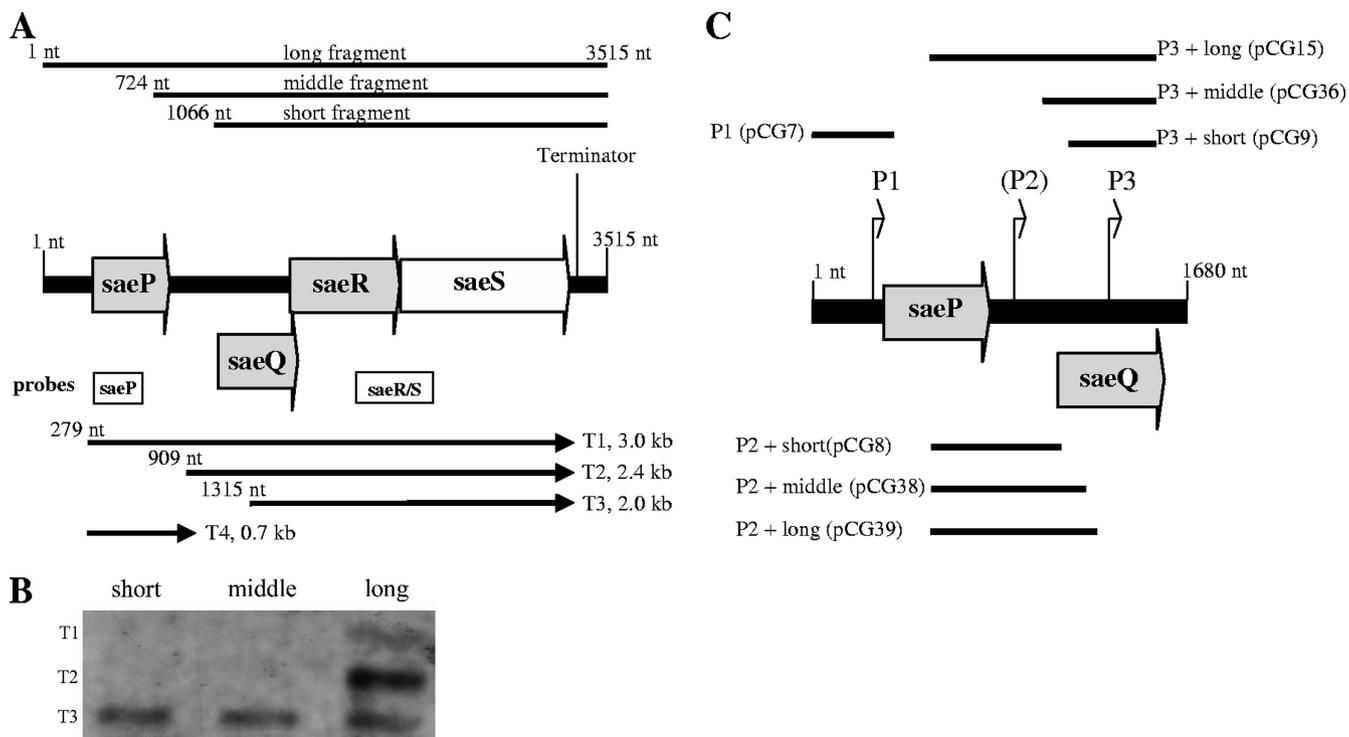


FIG. 1. (A) Genetic organization of the *sae* operon. Indicated are the four transcriptional units (T1 to T4) and the fragments used for complementation of the *sae* mutation. Nucleotide numbers correspond to the *sae* sequence (GenBank accession no. AJ556795). In addition, the probes used for the detection of *saeP* (T1 and T4) and *saeRS* (T1, T2, and T3) in Northern blot analysis are indicated. (B) Northern blot analysis of RNA from the *sae* mutant strain Newman-29 complemented with pCWSAE28 (long), pCWSAE48 (middle), and pCWSAE47 (short) and hybridized with the *saeRS*-specific probe. (C) Indication of fragments upstream of *saeRS* used for promoter fusion assays (for details, see Table 1).

yolk. The integrated plasmids were then transduced into the *sae* mutant Newman-29 using  $\Phi$ 11 lysates.

**Construction of chromosomally integrated promoter-*lacZ* fusions.** The *spoVG/lacZ* gene cassette from the vector pK0010 (34) was purified after

BamHI digestion and cloned into the BamHI restriction site of pCL84 (27), producing the vector pCG6. This vector integrates into the lipase gene (*geh*) of *S. aureus* due to a cloned phage attachment site. Putative *sae* promoter fragments were amplified with the appropriate oligonucleotides with additional EcoRI

TABLE 2. Oligonucleotides

Description	Primer <sup>a</sup>	Sequence
<b>Oligonucleotides used for promoter constructs</b>		
P3 <i>sae</i> promoter with a short upstream region	saeP3U saeP3L	GTCGACGAATTCTCGTGTGGGTTTCAGGTATTGTTATG GTCGACGAATTCACCTCTGTTCTTACGACC
P3 <i>sae</i> promoter with a middle upstream region	sae974U saeP3L	CCGGCGAATTCCTAAGCAACCCATGAGC GTCGACGAATTCACCTCTGTTCTTACGACC
P3 <i>sae</i> promoter with a long upstream region	saeP2U saeP3L	GTCGACGAATTCACCTGTGTAAGGTTAAAGCTG GTCGACGAATTCACCTCTGTTCTTACGACC
P2 <i>sae</i> promoter with a short downstream region	saeP2U saeP2L	GTCGACGAATTCACCTGTGTAAGGTTAAAGCTG GTCGACGAATTCCTGAACCCACACGAATGATAAAATG
P2 <i>sae</i> promoter with a middle downstream region	saeP2U sae1258L	GTCGACGAATTCACCTGTGTAAGGTTAAAGCTG CCGGCGAATTCGCGAAAAACCACTTATAC
P2 <i>sae</i> promoter with a long downstream region	saeP2U sae1269	GTCGACGAATTCACCTGTGTAAGGTTAAAGCTG CCGGCGAATTCCTAATATTTGCGAAAA
P1 <i>sae</i> promoter	saeP1U saeP1L	GTCGACGAATTCCTATTGTGGCAAAAAGGTT GTCGACGAATTCACCTTGATCTTGTGAAT
<i>lacZ</i> control	lacZL	CGGATTGACCGTAATGGGATAGG
<b>Oligonucleotides used for <i>sae</i> cloning in pCL84</b>		
<i>sae</i> complementation with a short fragment	sae-1066U sae-3515L	CAATAAATAGAAAGAATGTGA ATTATTAGCGGCATACAG
<i>sae</i> complementation with a middle fragment	sae-724U sae-3515L	ATCTTCGGCGGCGCTAAA ATTATTAGCGGCATACAG
Full-length <i>sae</i> complementation	sae-1U sae-3515L	TTATTGTGGCAAAAAGGTTT ATTATTAGCGGCATACAG

<sup>a</sup> U, upper primer; L, lower primer.

restriction sites (Table 2) using high-fidelity polymerase (Phusion HF polymerase; Finnzymes, Espoo, Finland). Amplicons were ligated into the EcoRI-digested integration vector pCG6 in front of the promoterless *spoVG/lacZ* gene. Plasmids were verified by restriction digestion, and the correct orientation of the inserts was verified by PCR using the oligonucleotide lacZL together with the respective *sae*-specific upstream oligonucleotides. Plasmids were introduced into *S. aureus* strain CYL316 by protoplast transformation and tetracycline selection. All integrants were lipase negative on Baird-Parker plates. The integrated plasmids were subsequently transduced into different *S. aureus* strains by using  $\Phi$ 11 phage lysates of the CYL316 derivatives. All transductants were verified by PCR as described above, and the *agr* activities were monitored by cross-striking of the strains with RN4220 on sheep blood agar plates as described previously (1).

**RNA isolation, Northern blot hybridization, and real-time RT-PCR.** RNA isolation and Northern blot analysis were performed as described previously (14). Briefly, bacteria were lysed in 1 ml of TRIzol reagent (Invitrogen/Life Technologies, Karlsruhe, Germany) with 0.5-ml zirconia-silica beads (0.1 mm in diameter) in a high-speed homogenizer (Savant Instruments, Farmingdale, NY). RNA was isolated as described in the instructions provided by the manufacturer of TRIzol. Digoxigenin-labeled probes for the detection of specific transcripts were generated by using the digoxigenin labeling PCR kit according to the manufacturer's instructions (Roche Biochemicals, Mannheim, Germany). The oligonucleotides used for probe generation were as described previously (39). Quantification of transcripts coding for *saeP*, *saeR*, *gyr*, *agr*, and *hla* was performed as described by LightCycler reverse transcription-PCR (RT-PCR) (15).

**Promoter activity assay.** For the different promoter activity assays the bacteria were grown in the corresponding media and harvested at the indicated time points with equal numbers of bacteria. Bacteria were centrifuged and resuspended in 1 ml of 0.1 M sodium phosphate buffer (pH 7.4) and lysed with 0.5-ml zirconia-silica beads (0.1 mm in diameter) in a high-speed homogenizer (Savant Instruments) for 10 s at a speed of 6,500 rpm. Aliquots of the supernatants were frozen until use.  $\beta$ -Galactosidase activity was measured by using the FluoReporter galactosidase quantitation kit (Invitrogen). The emerging fluorescence emission was measured in a 96-well microtiter plate (Nunc, Roskilde, Denmark) with a luminescence spectrometer (LS50B; Perkin-Elmer, Waltham, MA). The promoter activities are expressed as ng of  $\beta$ -galactosidase/ml with reference to a standard curve generated by purified  $\beta$ -galactosidase (Sigma-Aldrich) according to the manufacturer's instructions for the FluoReporter galactosidase quantitation kit. All values are means of at least two independently grown bacterial cultures. Significance was calculated by using a Student *t* test.

## RESULTS

**Delineation of P1 and P3 promoter activities of the *sae* upstream region.** We had previously mapped three transcriptional start sites upstream of *saeRS*, indicating the three distinct promoter elements P1, P2, and P3 (39) (Fig. 1). First, we analyzed the activity of the proposed promoters in strain Newman and in strain ISP479R (40), a SigB-proficient derivative of strain 8325-4. Different fragments within the upstream region were amplified and cloned into pCG6, an integration vector that contains *lacZ* with a ribosomal binding site of *B. subtilis* (Fig. 1C). The plasmids were integrated into the chromosomes of different *S. aureus* strains, and promoter activities were assessed by using a  $\beta$ -galactosidase assay.

A fragment containing P1 showed the highest activity in both strains analyzed (Fig. 2). However, the P1 activity in strain Newman was much higher than in strain ISP479R (166 versus 4.7 ng of  $\beta$ -galactosidase) (Fig. 2). This is consistent with previous results from Northern blot analysis showing that the *sae* transcripts T1, T2, and T4 are highly expressed in strain Newman compared to other strains (39). The hyperactivation of *sae* in strain Newman is due to a point mutation within the first membrane-spanning domain of the HK, SaeS. This is supported by our finding that cloning of the *sae* operon from strain Newman and expression in other *sae* mutant strains also leads to constitutive *sae* overexpression (M. Mainiero et al., unpub-

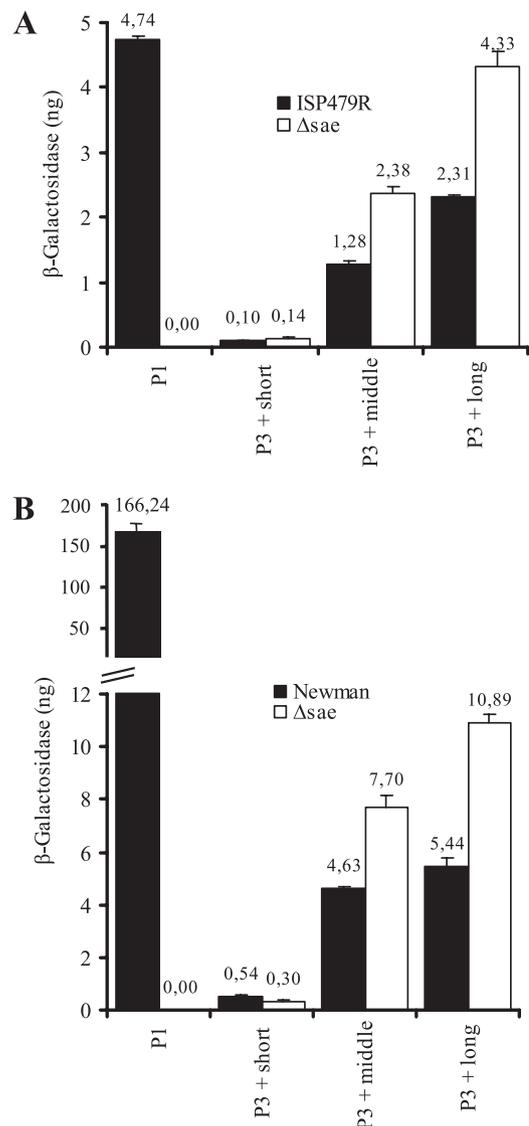


FIG. 2. Promoter fusion assay using strain ISP479R and the *sae* mutant ISP479R-29 (A) or Newman and the *sae* mutant Newman-29 (B). Promoter fragments are indicated as in Fig. 1C. Bacteria were grown to mid-exponential growth phase, equal numbers of bacteria were lysed, and promoter activities are expressed as equivalents of  $\beta$ -galactosidase (in nanograms). Standard deviations are derived from at least two independent cultures.

lished data). This mutation most probably results in constitutive *sae* activation.

Thus far, it is not clear whether the transcriptional start site of T2 is due to a distinct promoter activity or to processing of the T1 transcript. In order to further delineate the activity of a putative P2 promoter, different fragments surrounding the T2 transcriptional start site were analyzed (see Fig. 1C). No  $\beta$ -galactosidase activity was detectable. Also, extending the region surrounding the putative P2 to include downstream sequences but excluding P3 did not result in any activity. For further analysis, we cloned *saeRS* fragments containing different upstream regions into the single-copy integration vector pCL84 and complemented the *sae* mutant strain Newman-29, in which

the whole *sae* locus encompassing *sae*PQRS was replaced by a kanamycin-resistant cassette. Northern blot analysis revealed that full-length complementation resulted in the expected expression of the three distinct *sae* transcripts (T1 to T3) (Fig. 1B). However, complementation with *saeRS* containing the putative P2 promoter (middle fragment, see Fig. 1A) resulted in the expression of only the short T3 transcript, a finding comparable to a construct containing only P3 (short fragment). The results strongly indicate that the T2 transcript is due to RNA processing rather than initiation from an additional internal promoter.

Finally, we analyzed the activity of the putative P3 promoter. A fragment containing a short P3 fragment (pCG9) only was hardly active (Fig. 2). However, P3 activity could clearly be enhanced in constructs with extended upstream regions, indicating the presence of a potential binding site for *cis*-acting enhancer molecules.

**Autoregulation of P1 and P3 promoters.** Bacterial regulatory systems are often characterized by positive feedback activation. Thus, we analyzed the autoregulation of P1 and P3 promoters by the *sae* operon (Fig. 2). Comparison of the *sae*-positive wild-type strains with their isogenic *sae* gene replacement mutants revealed that P1 is strongly autoregulated: no  $\beta$ -galactosidase activity was detectable in the *sae* mutants. In contrast, the P3 activity was increased in the *sae* mutants compared to the wild-type strains. Overall, the results indicate that upon signaling *sae* transcription is mainly driven by the strongly autoactivated P1 promoter.

**Influence of *agr* and *sigB* on *sae* promoter activities.** Previously, we found by Northern blot analysis that in strain Newman neither SigB (15) nor *agr* (data not shown) had a significant impact on the expression of any of the *sae* transcripts. However, results from Novick and Jiang (33) using derivatives of strain 8325-4 indicate that SigB inhibits and that *agr* activates transcription of the *sae* operon. The proposed constitutively active *sae* phenotype of strain Newman may account for its insensitivity toward regulatory signals such as *agr* and SigB. Thus, in further experiments in which we wanted to analyze different signals leading to *saeRS* activation, we focused on strains ISP479R and UAMS-1. The interaction with *agr* and SigB was analyzed by comparison of P1 promoter activities in wild types and their mutants. We analyzed bacteria grown to the postexponential phase (Fig. 3), since *agr* and SigB activity is maximal in the later phases of the growth cycle. Notably, activity of the P1 promoter was >30-fold higher in strain UAMS-1 than in strain ISP479R. Nevertheless, in both strains P1 activity is decreased in the *agr* mutant but increased in the *sigB* mutants compared to the wild-type bacteria. Thus, with the exception of strain Newman, *agr* leads to *sae* activation, and SigB leads to *sae* repression in two unrelated prototypic strains.

**Influence on P1 promoter activity by pH, NaCl concentration, and cell-wall-active antibiotics.** Thus far, the signals leading to *saeRS* activation are not well defined. Since P1 is strongly autoactivated, we assumed that the P1 activity correlates to the signal-specific activation of the HK SaeS. We applied different external signals to exponentially growing bacterial cultures and measured P1 promoter activity after 1 h of induction. First, we analyzed conditions that were already described in the literature as influencing *sae* expression. We were able to confirm that lowering the pH or adding 1 M NaCl leads

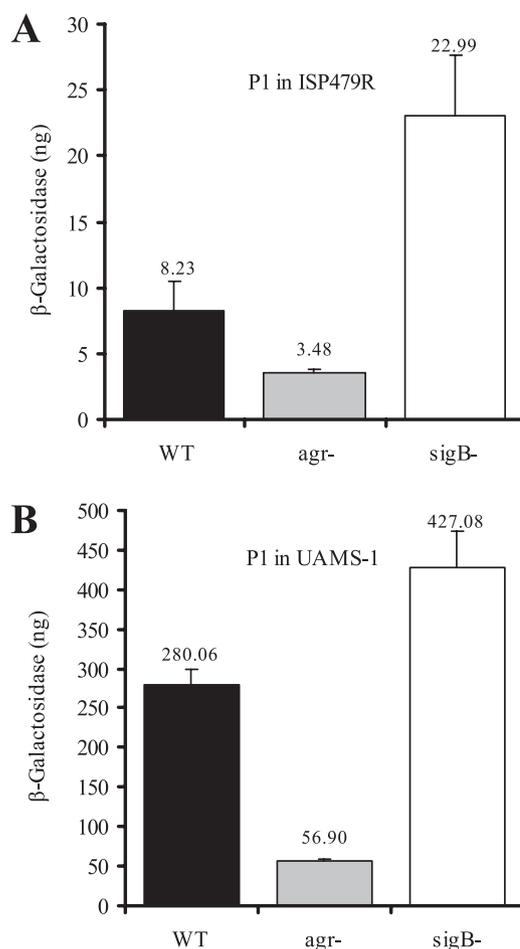


FIG. 3. Promoter fusion assay using strains ISP479R (A) and UAMS-1 (B) and their *agr* and *sigB* mutants with cloned P1 promoter fragments. Bacteria were grown to the post-exponential growth phase, equal numbers of bacteria were lysed, and promoter activities are expressed as equivalents of  $\beta$ -galactosidase (in nanograms). Standard deviations are derived from at least two independent cultures.

to repression of P1 (33) (Fig. 4). Next, we analyzed increasing concentrations of cell-wall-active antibiotics (5 to 20  $\mu$ g of vancomycin/ml and 20 to 40  $\mu$ g of bacitracin/ml) on P1 activity (Fig. 5). Only for vancomycin, at concentrations of >10  $\mu$ g/ml, was a slight but significant ( $P < 0.05$ ) increase in P1 promoter activity observed. However, at subinhibitory concentrations no effect was detectable. In summary, previous results from the literature concerning *sae* signaling could be confirmed with regard to the repression at low pH and high NaCl concentrations (33) and partly with regard to those concerning activation by vancomycin (25).

**Activation of P1 promoter activity by H<sub>2</sub>O<sub>2</sub>.** Recently, it was shown that in community-associated methicillin-resistant *S. aureus* (ca-MRSA) strains phagocytosis leads to the upregulation of several virulence factors (41), many of which are strongly *sae* regulated. We speculated that H<sub>2</sub>O<sub>2</sub> may trigger *sae* activation. We could show that 5 to 20 mM H<sub>2</sub>O<sub>2</sub> resulted in a significant activation of the P1 promoter in strains ISP479R and UAMS-1 (Fig. 6). These concentrations were above the MIC of 1.5 mM. However, under the assay conditions the bacteria were still

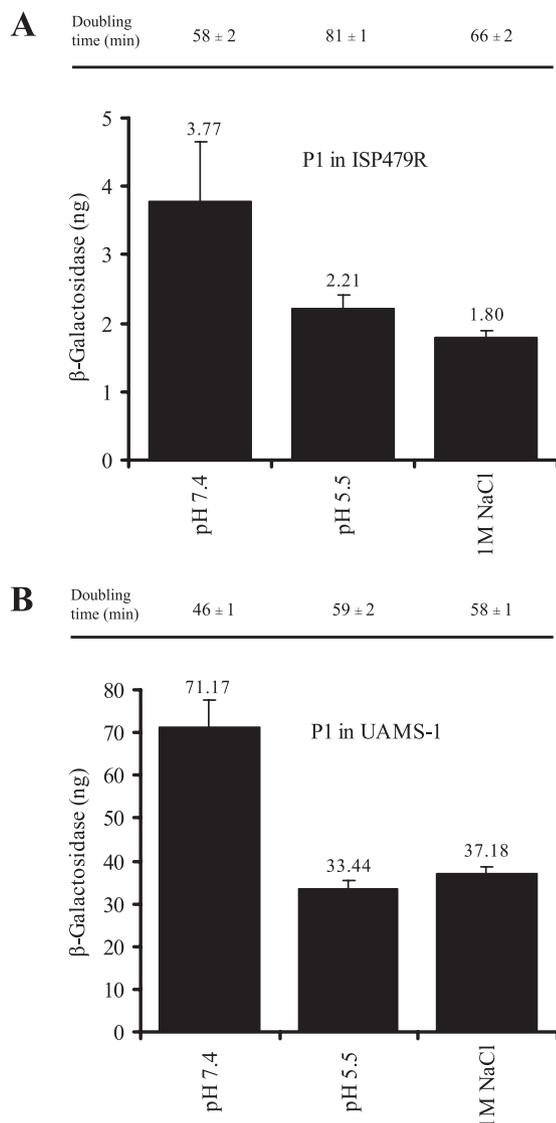


FIG. 4. Promoter fusion assay using strains ISP479R (A) and UAMS-1 (B) with cloned P1 promoter fragment. Bacteria were grown to mid-exponential growth phase, washed, and reincubated for 1 h with medium adjusted to pH 7.4 or 5.5 or in medium supplemented with 1 M NaCl (pH 7.4). Equal numbers of bacteria were lysed, and promoter activities are expressed as equivalents of  $\beta$ -galactosidase (in nanograms). Standard deviations are derived from at least two independent cultures. Bacterial doubling times were assessed during the different incubation conditions based on  $OD_{600}$  measurement and are indicated above the columns.

able to grow at concentrations up to 15 mM, although with slower growth rates than those of the control without  $H_2O_2$ . Lowering the  $H_2O_2$  concentration below 2 mM did not result in a significant activation of P1. We also analyzed whether  $H_2O_2$  can activate P1 under low pH in which P1 is usually repressed. At pH 5.5, a 1.5-fold increase in P1 activation by  $H_2O_2$  could be observed.

**Activation of P1 promoter by human  $\alpha$ -defensin.** We assumed that PMN-derived antimicrobial peptides may also play a role in *sae* signaling. Indeed, the addition of  $\alpha$ -defensins purified from PMN granulae exhibited a strong dose-depen-

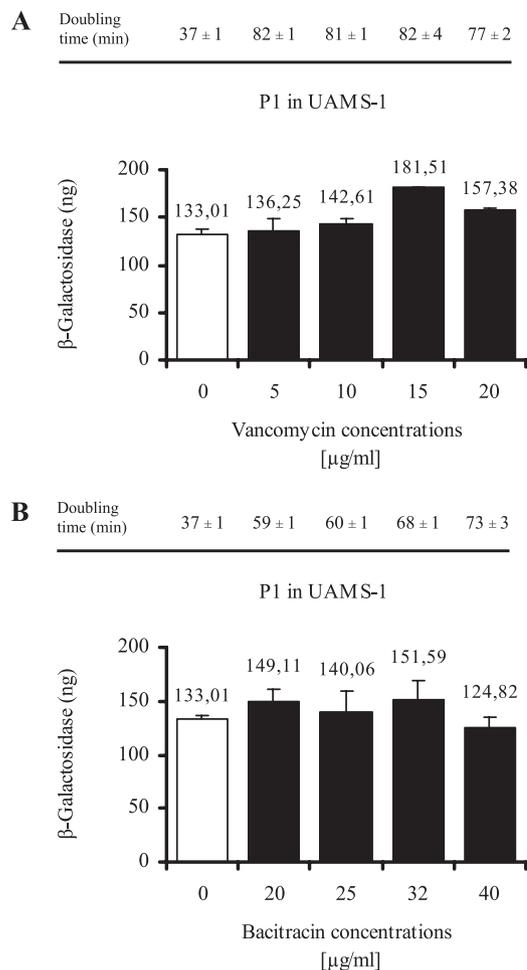


FIG. 5. Promoter fusion assay using strain UAMS-1 with cloned P1 promoter fragment. Bacteria were grown to mid-exponential growth phase, washed, and reincubated for 1 h with (5 to 20  $\mu$ g of vancomycin/ml [A], 20 to 40  $\mu$ g of bacitracin/ml [B]). Equal numbers of bacteria were lysed, and promoter activities are expressed as equivalents of  $\beta$ -galactosidase (in nanograms). Standard deviations are derived from at least two independent cultures. Bacterial doubling times were assessed during the different incubation conditions based on  $OD_{600}$  measurement and are indicated above the columns.

dent activation of the P1 promoter in strain UAMS-1 (Fig. 7). The concentrations used in this assay are subinhibitory for bacterial growth: no inhibitory effect on the growth rate was observed with up to 20  $\mu$ g of  $\alpha$ -defensins/ml. The *sae* sensitivity was also detectable at pH 5.5, where the addition of  $\alpha$ -defensin (5  $\mu$ g/ml) still resulted in a twofold increase in P1 promoter activity. We could confirm that synthetic  $\alpha$ -defensin HNP2 also resulted in a strong P1 activation, indicating that the observed effect is not due to potential impurities in the  $\alpha$ -defensin preparation. We next analyzed whether other antimicrobial peptides can also activate P1. However, neither the antimicrobial peptide LL37 (1 to 10  $\mu$ g/ml) nor the membrane-active antibiotic daptomycin (0.01 to 10  $\mu$ g/ml) activated P1, emphasizing a strong specificity of the *sae* system toward sensing of  $\alpha$ -defensins.

Interestingly, the strong activation of P1 by  $\alpha$ -defensins was not seen in strain ISP479R. To further follow strain-specific

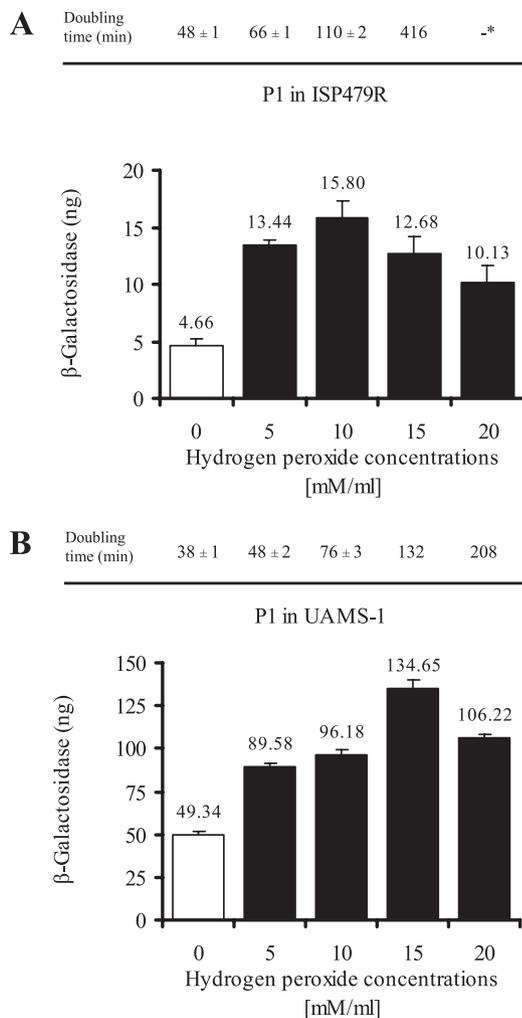


FIG. 6. Promoter fusion assay using strains ISP479R (A) and UAMS-1 (B) with cloned P1 promoter fragment. Bacteria were grown to mid-exponential growth phase ( $OD_{600} = 0.5$ ), followed by incubation with increasing concentrations of  $H_2O_2$  for 1 h. Equal numbers of bacteria were lysed, and promoter activities are expressed as equivalents of  $\beta$ -galactosidase (in nanograms). Standard deviations are derived from at least two independent cultures. Bacterial doubling times were assessed during the different incubation conditions based on  $OD_{600}$  measurement and are indicated above the columns. At 20 mM  $H_2O_2$ , no growth was detectable.

differences, we extended the analysis to a set of genome-sequenced *S. aureus* strains. There was no difference in the MIC or minimal bactericidal activity (both 200  $\mu$ g of HNP1 to HNP3/ml) between the strains analyzed (ISP479R, UAMS-1, and USA300). Strains were incubated with two subinhibitory concentrations (0.5 and 5  $\mu$ g/ml) of  $\alpha$ -defensins for 2 h, and the induction of the *saeP*-specific transcripts (T1 and T4) was analyzed by Northern blot analysis (Fig. 8) and qRT-PCR (Fig. 9). *saeP*-specific transcript levels strongly correlated with the P1 promoter activities determined in strains ISP479R and UAMS-1. Most of the additional strains (MW2, N315, USA300, MRSA252, and MSSA476) analyzed showed a strongly  $\alpha$ -defensin-responsive *sae* phenotype. Other than the constitutively *sae*-activated strain Newman, only strain ISP479R and strain COL turned out to be insensitive with

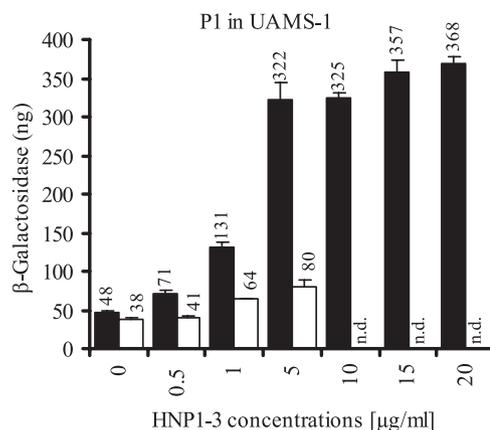


FIG. 7. Promoter fusion assay using strain UAMS-1 with cloned P1 promoter fragment. Bacteria were grown to mid-exponential growth phase in modified LB medium adjusted to pH 7.4 (■) or pH 5.5 (□), followed by incubation with increasing concentrations of  $\alpha$ -defensins (HNP1 to HNP3) for 2 h. Equal numbers of bacteria were lysed, and promoter activities are expressed as equivalents of  $\beta$ -galactosidase (in nanograms). Standard deviations are derived from at least two independent cultures. n.d., not determined.

respect to *sae*-dependent  $\alpha$ -defensin sensing. Of note, strain USA300 showed a strong induction already with 0.5  $\mu$ g of  $\alpha$ -defensins/ml. The degree of induction was also assessed quantitatively by RT-PCR, revealing a 2.5- to 9-fold increase of *saeP*-encoded transcripts after the addition of 0.5  $\mu$ g of  $\alpha$ -defensins/ml and a 9- to 17-fold increase after addition of 5  $\mu$ g of  $\alpha$ -defensins/ml.

To verify that the  $\alpha$ -defensins specifically induce the *sae* system, the expression of RNAIII from the *agr* operon was analyzed in parallel (Fig. 8). Two of the strains (strain COL and strain N315) analyzed did not produce detectable amounts of RNAIII. In none of the strains was RNAIII expression influenced by  $\alpha$ -defensins. Thus, a global effect on gene expression can be excluded.

## DISCUSSION

The central role of the *sae* regulatory system in virulence gene regulation has been demonstrated in several studies (11, 13, 15, 16, 29, 33, 38, 39). However, the mechanisms leading to *sae* activation are only partially understood. Here we could show using promoter fusions that the main P1 promoter of the *sae* locus is repressed by low pH and high NaCl levels, confirming previous results obtained by Northern blot analysis (33). In addition, the P1 promoter could be activated by the addition of  $H_2O_2$  and by  $\alpha$ -defensins. Judging from the genetic structure, the sensing is most probably mediated by the HK SaeS. The N-terminal sensory module of SaeS is composed of only two membrane-spanning stretches separated by a short extracellular loop of 13 amino acids. Signaling toward the cytoplasmic kinase domain is probably mediated by a conserved HAMP domain. This organization indicates that SaeS is a typical member of the intramembrane-sensing HKs recently described in firmicutes (30). Because of the absence of any obvious extracellular or intracellular sensory domains in this class of HKs, it is thought that the sensing occurs via the

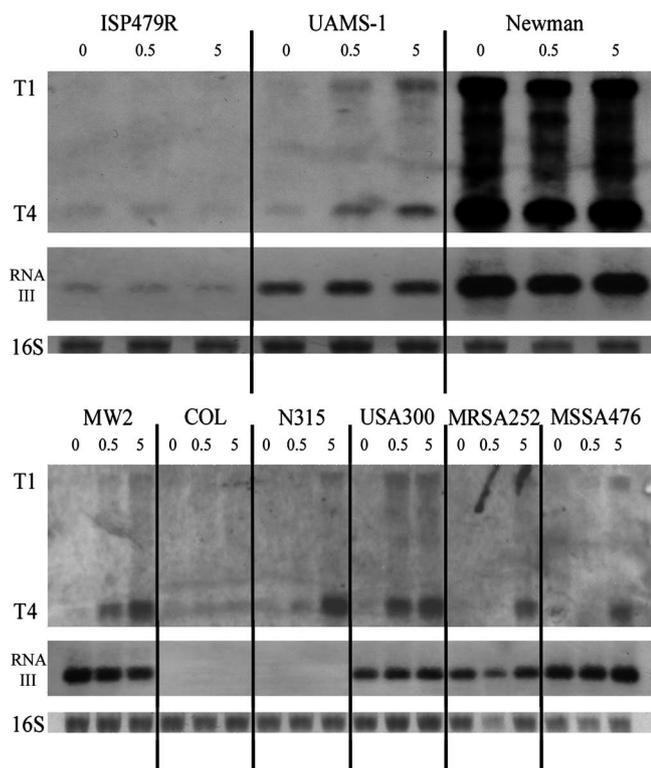


FIG. 8. Northern blot analysis of 2 µg of total RNA from different *S. aureus* strains. Bacteria were grown to mid-exponential growth phase in modified LB medium adjusted to pH 7.4, followed by incubation with 0, 0.5, or 5 µg of α-defensins (HNP1 to HNP3)/ml for 2 h. Blots were hybridized with probes specific for *saeP* and RNAIII (*agr* operon). For descriptions of *sae* transcripts T1 and T4 and probes, see Fig. 1A. In the lower lanes the 16S rRNA detected in the ethidium bromide-stained gels are indicated as loading controls.

transmembrane helices within the membrane. The importance of signal perception by the transmembrane helices is supported by the constitutively active *sae* in strain Newman, which could be traced to a single amino acid exchange in the first of the two transmembrane loops of SaeS (39; unpublished data). According to the constitutively active phenotype, *sae* transcription in strain Newman is independent of *agr* and/or SigB and insensitive toward signals such as H<sub>2</sub>O<sub>2</sub> or α-defensins. It is also possible that SaeS can communicate with other membrane proteins such as SaeQ to become activated. Although there is no direct evidence for this assumption, the similarly organized LiaS-like HK from *B. subtilis* has been shown to interact with the topologically linked membrane protein LiaF (22). LiaS-like HKs were shown to be involved in the sensing of perturbations in the bacterial envelope by cell-wall-active antibiotics, as well as by the antimicrobial peptide LL37 (for a review, see reference 30). However, neither the antimicrobial peptides LL37 nor daptomycin led to *sae* activation, indicating a high specificity of the *sae* system toward α-defensins. The molecular link between the different signals interfering with SaeS may be alterations within the membrane rather than cell wall perturbations.

Four distinct transcripts are expressed within the *sae* operon, of which the T2 transcript initiating between *saeQ* and *saeP* seems to be the most abundant. The results from promoter fusion assays and Northern blot analysis using cloned *sae* fragments void of P1 strongly indicate that the T2 transcript is most probably generated by specific endoribonucleolytic processing of the T1 transcript. Half-life determinations using rifampin indicated that the processed T2 transcript is stabilized compared to T1 (data not shown). Thus, the processing may contribute to a prolonged synthesis of the *sae* gene products once the P1 promoter has been activated. The enzyme responsible

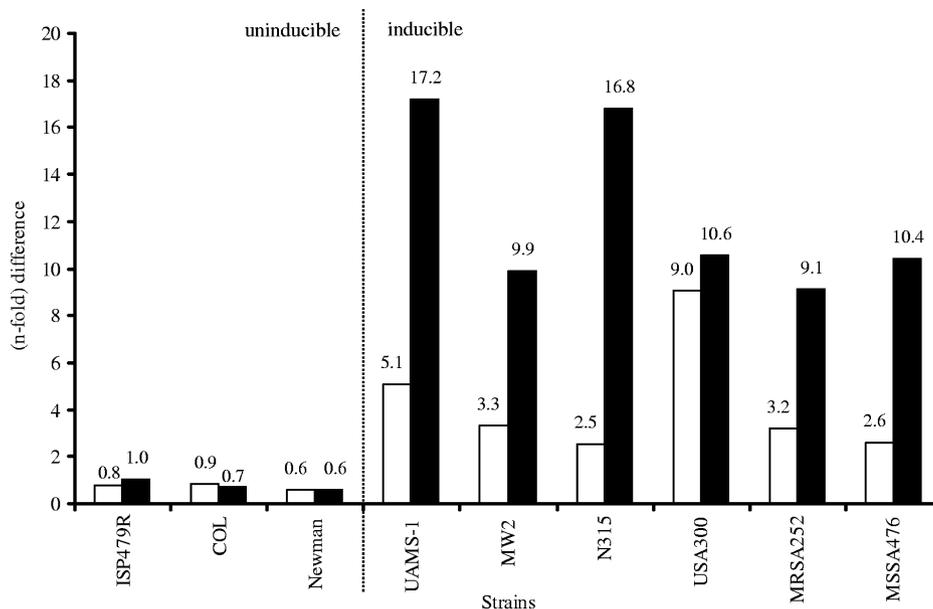


FIG. 9. Quantification of *saeP*-specific mRNA in different *S. aureus* strains by using LightCycler RT-PCR. The same RNA was used as for the Northern blot analysis shown in Fig. 7. Specific *saeP* transcripts were quantified with reference to the constitutively expressed gene *gyr*. Changes in *saeP* expression levels are indicated as factor differences of the expression from a given strain grown with 0.5 µg (□) or 5 µg (■) of HNP1 to HNP3/ml divided by the expression from the strain grown without α-defensins (HNP1 to HNP3).

for this specific RNA cleavage is not known because, in general, information on endoribonucleases in gram-positive bacteria is limited. For *S. aureus* it was recently shown that the double-stranded specific RNase III determines the mRNA stability of the *spa* mRNA (coding for protein A), and the enzyme may also be involved in mRNA processing of the *sae* transcripts (21).

The transcripts T1 and T3 are initiated from the two distinct promoter elements P1 and P3. P3 is autorepressed, and its activity depends on an extensive upstream region, indicating a potential operator domain. SaeRS expressed from P3 may enable the bacteria to sense an appropriate signal, which in turn leads to upregulation on the transcriptional level due to the strongly autoactivated P1 promoter. The P1 activation probably leads to expression of SaeP and SaeQ, which may be linked to the regulatory function of SaeS and SaeR.

For a detailed analysis of the *sae*-specific promoter activities, most of the experiments were performed with strains ISP479R and UAMS-1. ISP479R is a SigB-proficient derivative of the well-characterized 8325-4 (clonal complex 8, multilocus sequence typing database [<http://www.mlst.net/>]). Strain UAMS-1 is a clinical isolate belonging to the widely disseminated strain from clonal complex 30 and was shown to exert a different regulatory pattern with respect to *agr* and *sarA* regulation (5). The low *sae* activity observed in strain ISP479R is partly due to the inhibitory effect of SigB, since the SigB-deficient parental strain ISP479C showed higher levels of *sae* transcription than did strain ISP479R (but still below that of strain UAMS-1 [data not shown]). The high *sae* expression found in strain UAMS-1 is in good agreement with previous results from Cassat et al. (5) and was even further increased in the *sigB* mutant ( $P < 0.05$  [wild type versus *sigB* mutant]). SigB may act indirectly on *sae* expression due to SigB-dependent inhibition of the *agr* system (20). The *agr*-dependent P1 activation is consistent with previous results from others (12, 33) emphasizing that within the regulatory cascade of virulence gene regulation, the *sae* system is placed as a central downstream regulator.

ca-MRSA strains such as MW2 and USA300 are known to account for more severe disease outcomes (44) and to be more resistant toward PMN phagocytosis than hospital-acquired MRSA strains (41). Resistance to phagocytosis was linked to an upregulation of extracellular toxins, most of which are regulated by *sae*. We hypothesized that *sae* may play a major role in virulence gene activation upon PMN interaction. Within the PMNs *sae* can be activated first by the initial rise in the pH due to the generation of reduced oxygen species (45), the production of H<sub>2</sub>O<sub>2</sub>, and most prominently by antimicrobial peptides (HNP1 to HNP3). Sae activation will enable the bacteria to synthesize a plethora of toxic enzymes such as alpha-hemolysin or leukocidins, which may eventually lead to PMN destruction. The *sae* response to  $\alpha$ -defensins was seen in concentrations that are far below the growth inhibitory effect of these peptides and below those found within PMNs. Thus, one may also assume that extracellular  $\alpha$ -defensins released from PMNs result in *sae* activation in the blood or at the side of infection prior to phagocytosis. The plasma levels of  $\alpha$ -defensins detected in infected patients (35) are well within a range in which *sae* activation can occur.

The regulatory properties of  $\alpha$ -defensins seem to be very specific toward *sae* activation. Neither of the two-component

regulatory systems *agr* (Fig. 8) or *graRS* (*aps*) (data not shown) was influenced by  $\alpha$ -defensins (HNP1 to HNP3). Interestingly, other antimicrobial peptides were shown to activate the *graRS* (*aps*) system, leading to a better protection of the bacteria from these peptides (19, 28). In contrast, activation of the *sae* system by  $\alpha$ -defensins probably does not render the bacteria more antimicrobial peptide resistant because there is no indication that *sae* is involved in the regulation of structural compounds of the cell envelope (29, 38). Instead, *sae* specifically activates virulence genes that may be important for the bacteria to evade the hostile environment, e.g., after PMN phagocytosis.

One intriguing finding of the present study was the prominent strain-specific differences in *sae* activation. The unusually high activity seen in strain Newman can be traced to a single amino acid exchange in the first transmembrane loop of the HK SaeS (39). However, sequence analysis of the *sae* operon from other strains did not reveal any link to the different responsiveness of the strains toward  $\alpha$ -defensins. In most strains, including strain USA300, *sae* could be activated by  $\alpha$ -defensins. In the 8325 derivative (ISP479R) and in strain COL the basal low level of P1 activity could not be enhanced by  $\alpha$ -defensins. Strains 8325, COL, and USA300 are evolutionarily related (clonal complex 8) and show 100% sequence identity within the *sae* locus. Thus, the molecular basis for strain-dependent differences must be due to either differences in other regulatory elements interacting with *sae* or in different properties of the cell envelope. Interestingly, the  $\alpha$ -defensin-responsive ca-MRSA strains USA300 and MW2 were shown to be more resistant to phagocytosis than the nonresponsive strain COL (41). More recently, this property could be linked to the expression of phenol-soluble modulins (42). Whether the *sae* responsiveness causes the synthesis of these compounds, the observed phagocytosis resistance, or even differences in strain virulence will be analyzed in future studies.

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