

Molecular Architecture of the Regulatory Locus *sae* of *Staphylococcus aureus* and Its Impact on Expression of Virulence Factors

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We characterized the *sae* operon, a global regulator for virulence gene expression in *Staphylococcus aureus*. A Tn917 *sae* mutant was obtained by screening a Tn917 library of the *agr* mutant ISP479Mu for clones with altered hemolytic activity. Sequence analysis of the *sae* operon revealed two additional open reading frames (ORFs) (ORF3 and ORF4) upstream of the two-component regulatory genes *saeR* and *saeS*. Four overlapping *sae*-specific transcripts (T1 to T4) were detected by Northern blot analysis, and the transcriptional initiation points were mapped by primer extension analysis. The T1, T2, and T3 mRNAs are probably terminated at the same stem-loop sequence downstream of *saeS*. The T1 message (3.1 kb) initiates upstream of ORF4, T2 (2.4 kb) initiates upstream of ORF3, and T3 (2.0 kb) initiates in front of *saeR*. T4 (0.7 kb) represents a monocistronic mRNA encompassing ORF4 only. *sae*-specific transcripts were detectable in all of the 40 different clinical *S. aureus* isolates investigated. Transcript levels were at maximum during the post-exponential growth phase. The *sae* mutant showed a significantly reduced rate of invasion of human endothelial cells, consistent with diminished transcription and expression of *fnbA*. The expression of type 5 capsular polysaccharide is activated in the *sae* mutant of strain Newman, as shown by immunofluorescence and promoter-reporter fusion experiments. In summary, the *sae* operon constitutes a four-component regulator system which acts on virulence gene expression in *S. aureus*.

The human pathogen *Staphylococcus aureus* is the causative agent of a wide spectrum of diseases. This organism's capacity to adapt to different environments in vitro and in vivo is due to a global regulatory network comprising several loci such as *agr*, *sar*, *sigB*, *rot*, *arlRS*, *svrA*, and *saeRS* (4, 27). Each of these regulators is involved in the control of the expression of virulence factors such as hemolysins, protein A, fibronectin-binding proteins (FnBPA and FnBPB, encoded by *fnbA* and *fnbB*, respectively), or capsular polysaccharide (CP, encoded by the *cap* operon). *agr*, *sar*, *sigB*, *svrA*, *rot*, and *arlRS* constitute a complex interactive regulatory network (3–5, 8, 9, 25). For instance, SarA has been shown to be necessary for full *agr* activation (5), and SigB (3) and ArlR (8) modulate the transcription of *agr* and *sarA*. Mutation of the proposed regulatory locus *sae* did not affect the transcription of *sarA* and *agr*, indicating an independent regulatory circuit (12). A *sae* transposon mutant was shown to express reduced levels of alpha-hemolysin (Hla, encoded by *hla*), beta-hemolysin, DNase, coagulase (Coa, encoded by *coa*), and protein A compared with those expressed by the parental strain, whereas the expression of delta-hemolysin, proteases, and lipase was not altered (15). *sae* affects target genes on the transcriptional level (12).

The contribution of *sae* to virulence has been shown after intraperitoneal injection of bacteria into mice (14). In addition, it has been shown that *sae*, but not *agr* or *sarA*, is essential for the transcription of *hla* during device-related infection in

guinea pigs (17). In a different tissue-cage model, it was also shown by microarray analysis that in contrast to that of *agr*, transcription of *sae* was not repressed in vivo (42). Sequencing of the transposon insertion site of the *sae* mutant originally described revealed two open reading frames (ORFs), *saeR* and *saeS*, with strong sequence homology to response regulators and histidine kinases of bacterial two-component regulators (11) (GenBank accession number AF129010). According to the general mechanism of these systems (38), SaeS probably functions as a membrane-spanning sensor histidine kinase which, upon sensing the appropriate signal, autophosphorylates and in turn activates the cognate cytosolic response regulator SaeR. The activated response regulator may function as a transcriptional regulator via a specific DNA-binding domain, recognizing motifs near the promoter sequences of target genes.

Since *sae* seems to be an important modulator of virulence, we decided to further characterize this locus at the molecular level. We show that *sae* activates the transcription of *fnbA*, *coa*, and *hla*. In contrast, expression of CP5 is inhibited by *sae*. Transcriptional analysis revealed three overlapping transcripts and two additional putative protein-encoding regions (ORF3 and ORF4) which are cotranscribed with *saeS* and *saeR*. *sae* was found to be differentially expressed depending on the growth phase and the genetic background of the strain studied.

MATERIALS AND METHODS

Bacterial strains and growth conditions. The strains and plasmids used in this study are listed in Table 1. In vitro growth was performed in CYPG (26) supplemented with the appropriate antibiotics for strains carrying resistance genes (erythromycin at 10 µg/ml; tetracycline at 5 µg/ml). For RNA isolation, cells of an overnight culture were diluted to an initial optical density at 600 nm

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TABLE 1. Bacterial strains and plasmids

Strain or plasmid	Description	Reference or source
Strains		
Newman	Wild type	7
ALC355	Newman $\Delta agr::tetM$	40
ISP479C	Derivative of 8325-4	36
ISP479Mu	Spontaneous <i>agrC</i> mutant of ISP479C	Unpublished data
AS1	ISP479Mu <i>sae::Tn917</i>	This work
AS2	ISP479C <i>sae::Tn917</i>	This work
AS3	Newman <i>sae::Tn917</i>	17
AS4	Newman $\Delta agr::tetM$ <i>sae::Tn917</i>	This work
RN6390	Derivative of NCTC8325	31
AS5	RN6390 <i>sae::Tn917</i>	This work
DU5886	Newman <i>fnbA::Tc^r fnbB::Em^r</i>	T. J. Foster Dublin
Plasmids		
pLT4 ^{ts}	Tn917 delivery vector	43
pSN8388	<i>cap5</i> promoter fused to the reporter gene <i>xylE</i> in pCL4	18
pCWSAE7	pGEMTEasy with 3.5-kb fragment encompassing ORF4, ORF3, <i>saeS</i> , and <i>saeR</i> of strain ISP479C	This work
pCWSAE8	pCRII with 3.5-kb fragment encompassing ORF4, ORF3, <i>saeS</i> , and <i>saeR</i> of strain Newman	This work

(OD₆₀₀) of 0.05 and grown with shaking at 37°C to the mid-exponential (OD₆₀₀ = 0.8) or post-exponential (OD₆₀₀ = 8) phase or for 16 h to stationary phase. Clinical isolates were obtained from nose swabs of healthy individuals, from the sputa of patients with cystic fibrosis, and from wound swabs. All isolates were typed by pulsed-field gel electrophoresis (PFGE) as described elsewhere (34).

Tn917 insertional mutagenesis and transduction. The identification of a *sae::Tn917* mutant was described previously (17). Briefly, a Tn917 library of the spontaneous *agrC* mutant strain ISP479Mu (ISP479Mu pTV1^{ts}) was screened for differences in the hemolytic pattern observed after growth in air alone versus air supplemented with 5% CO₂. One colony which remained nonhemolytic under elevated CO₂ was chosen for further investigation. In order to determine the Tn917 insertion site, we digested chromosomal DNA with *EcoRI*, separated it by electrophoresis, blotted it onto a nylon membrane, and hybridized it with a Tn917-specific probe. The reactive fragment was gel eluted, cloned into pUC18, and sequenced. The mutation was transduced into strains ISP479Mu, ISP479C, RN6390, and Newman and the *agr* mutant of strain Newman (ALC355 [40]) by using a ϕ 11 lysate of the original transposon mutant.

RNA isolation and Northern blot hybridization. RNA isolation and Northern blot analysis were performed as described previously (16). Briefly, approximately 10⁹ *S. aureus* cells were lysed in 1 ml of Trizol reagent (Invitrogen Life Technologies, Karlsruhe, Germany) with 0.5 ml of zirconia-silica beads (diameter, 0.1 mm) in a high-speed homogenizer (Savant Instruments, Farmingdale, N.Y.). RNA was isolated as described in the instructions provided by the manufacturer of Trizol.

Several digoxigenin (DIG)-labeled probes for the detection of specific transcripts were generated by using the DIG-labeling PCR kit according to the manufacturer's instructions (Roche Biochemicals, Mannheim, Germany). Oligonucleotides are listed in Table 2.

Cloning and sequencing. A 3.5-kb fragment comprising the *sae* operon was amplified from strain ISP479C or strain Newman by using a high-fidelity polymerase (HF; Roche Diagnostics) and cloned into the PCR cloning vector pGEM-TEasy (Promega, Mannheim, Germany) or pCR2.1 (Invitrogen), respectively. The inserts were sequenced by ABI377 instrumentation using the DY-EnamicET Terminator sequencing kit (Amersham Biosciences, Freiburg, Germany). Sequence analysis was performed with the HUSAR software package (Dkfz, Heidelberg, Germany).

Primer extension analysis. In order to facilitate the identification of the appropriate promoters from which transcription is initiated, we mapped the 5' ends of the different *sae* transcripts by primer extension as previously described (2) with some modifications. Briefly, we adapted the [γ -³²P]ATP-based methodology to a fluorescence ALExpressII sequencer (Amersham Biosciences), utilizing Cy5-fluorescein end-labeled oligonucleotides (Table 2) for reverse transcription and dye-primer sequencing. Sequencing reaction mixtures primed by oligonucleotides identical to those used for primer extension were applied on the gel in parallel lanes.

Promoter-fusion assay and measurement of promoter activity using *xylE* as a reporter gene. The reporter plasmid pSN8388 (carrying the *cap5* promoter fused to the promoterless reporter gene *xylE* in pCL4 [18]) was transduced from strain

Newman into the isogenic *sae* mutant of strain Newman by using ϕ 11. The presence of correct constructs was confirmed by restriction mapping of the isolated plasmid. After *in vitro* growth of the strains under different conditions, the bacterial cells were harvested and lysed, and XylE activity was determined as described previously (24). The promoter activity was expressed as XylE activity per milligram of protein.

Ligand affinity analysis for detection of FnBP. Fibronectin-binding proteins were detected with biotinylated fibronectin as described previously (41). Briefly, cell wall extracts were prepared from an equivalent number of washed cells (10¹¹ CFU/ml). After protoplast formation, cell wall proteins were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to nitrocellulose membranes. Membranes were blocked in 5% dehydrated skim milk in phosphate-buffered saline and incubated first with biotinylated fibronectin and then with streptavidin-peroxidase complex (Pierce, Rockford, Ill.). Bound fibronectin was visualized with the ECL PlusTM Western blotting detection system according to the manufacturer's instructions (Amersham Biosciences). A high-molecular-weight prestained marker (Invitrogen) was used for determination of molecular size (in kilodaltons).

Coagulase assay. Coagulase activity was measured by addition of 100 μ l of serial twofold dilutions of culture supernatants to 200 μ l of rabbit plasma. The titer was the reciprocal of the highest dilution of samples that caused clotting after a 4-h incubation at 37°C.

Endothelial cell binding and invasion assay. Primary human umbilical vein endothelial cells (Labor Glatthaar, Reutlingen, Germany) were seeded in 0.2% gelatin-coated 24-well plates (BD Biosciences, Heidelberg, Germany) and grown in endothelial growth medium (Cell Systems, Katharinen, Germany) without antibiotics at 37°C in an atmosphere containing 5% CO₂. Confluent monolayers of endothelial cells (~10⁵ cells per well) were washed three times with M199 (Sigma Chemical Co., Deisenhofen, Germany) and incubated for 20 min at 37°C with 3% bovine serum albumin (Sigma) in M199 to minimize background adherence. Endothelial cells were then incubated with bacteria from the mid-exponential-growth phase (5 \times 10⁶ CFU of *S. aureus*/well) at 37°C for 2 h. The supernatants containing the nonadherent bacteria were removed, and CFU were determined by plating aliquots on sheep blood agar plates. Cells were subsequently washed three times with M199 and treated with trypsin-EDTA and 0.25% Triton X-100 to lyse endothelial cells, after which the CFU of adherent and intracellular bacteria were counted. In order to determine the number of intracellular bacteria, extracellular bacteria were killed by adding lysostaphin (10 μ g/ml) prior to cell lysis. Each assay was repeated at least once, and within each experiment all samples were processed at least in quadruplicate.

Detection of CP5. CP5 was detected by indirect immunofluorescence as described previously (32). Briefly, protein A was blocked by incubation with human immunoglobulin G, and CP5 antigen was detected by using monoclonal antibodies raised against CP5 (20) and secondary Cy3- or fluorescein isothiocyanate-labeled anti-mouse antibodies.

Nucleotide sequence accession numbers. The sequence data obtained in this study have been submitted to the DDBJ/EMBL/GenBank databases under accession numbers AJ556794 and AJ556795.

TABLE 2. Oligonucleotides used for PCR and primer extension analysis

Description	Basis ^a	Name ^b	Primer sequence
Probe 1	AJ556795	sae285U sae564L	CAAATTGAAGAAATGAGGAGTTA ACCTTTTGATGATTGTAGTTAG
Probe 2	AJ556795	sae727U sae981L	TTCGGCGGCGCTAAATTA TGCTTAAGCTAAACAAACCTC
Probe 3	AJ556795	sae992U sae1283L	TCAAACACTTCCTGTTCACA TGACGTCGTATGTGCAACTA
Probe 4	AJ556795	sae1980U sae2458L	TGGTCACGAAGTCCCTATGC TGCTTGCGTAATTTCCGTTAG
Probe 5	AJ556795	sae3364U csbL	AAACTTTATGGGTATCCTTCTCA TTGGCTTGTATTCTTTTCAATA
<i>fmbA</i> probe	J04151	fmbAU fmbAL	TGCAAATACGACAGATACTT TTGGCCACCTCATAACCTA
<i>coa</i> probe	X16457	coaU coaL	CGAGACCAAGATTCAACAAG AAAGAAAACCACTCACATCA
<i>agr</i> probe	X52543	RNIIIU RNIIIL	TATATTTTAAACGGCGGGTCTCA TTAATTAAGGAAGGAGTGATTT
<i>hla</i> probe	X01645	hlaU hlaL	AGAAAATGGCATGCACAAAAA TGTAGCGAAGTCTGGTGAAAA
Tn917 probe	M11180	Tn917U Tn917L	TAAGAGTGTGTTGATAG ACGATAAGTTGAATAGATAG
<i>sae</i> cloning	AJ556795	sae1U sae3530L	TTATTGTGGCAAAGGTTTAT ATTATTAGGCGGCATACAG
Primer extension 1 (P1)	AJ556795	sae337, CY5	ACCTAAAGCTAATGTTGTGATAACAGCACC
Primer extension 2 (P1, P2)	AJ556795	sae1017, CY5	TAAGATTAAGCAACATAATGCGATTTGTAG
Primer extension 3 (P2)	AJ556795	sae1290, CY5	CAATCTCTCCGAGTGGGACAACAATATC
Primer extension 5 (P2, P3)	AJ556795	sae1772, CY5	GTATCATGTTCTTGTGTTTTGGCAGTTA
Primer extension 6 (P3)	AJ556795	sae1973, CY5	TACGCATAGGGACTTCGTGACCATTACAG

^a GenBank accession number.

^b Numbers indicate nucleotide position in the corresponding reference sequence. U, upper primer; L, lower primer.

RESULTS

Identification of a *sae* Tn917 mutant. In order to identify regulatory loci which act independently of *agr*, we screened a Tn917 library of the spontaneous *agrC* mutant ISP479Mu (ISP479Mu pLT4^{ts}) for clones with differences in the hemolytic patterns observed on sheep blood agar plates after incubation in air alone versus air supplemented with 5% CO₂. The parental strain ISP479Mu was nonhemolytic under regular air conditions but developed visible hemolytic activity after incubation in elevated CO₂. One colony which remained nonhemolytic under elevated CO₂ was selected for further characterization. Sequencing of the transposon insertion site revealed that Tn917 was inserted into *saeS*, encoding a previously described histidine kinase (11). In order to rule out the possibility that additional mutations contributed to the observed alterations of the phenotype, the mutation was transduced back into ISP479Mu as well as into other *agr*-negative (ALC355) and *agr*-positive (ISP479C, RN6390, Newman) strains. As in the previous characterization of a *sae* mutant strain (15), all the transductants showed a severe reduction in hemolysis on sheep blood agar plates (for *agr*-positive strains) or were nonhemo-

lytic (in the *agr*-deficient background). None of the transductants led to visible clotting of rabbit plasma. Northern blot analysis also confirmed (12) that neither *hla* nor *coa* transcripts are detectable in *sae* mutant strains (data not shown). A single chromosomal integration of Tn917 could be verified for all derivatives of strain 8325-4 (AS1, AS2, and AS5) as shown by Southern hybridization of the genomic DNA digested with *Sma*I and separated by PFGE using a Tn917-specific probe (data not shown). According to the physical *Sma*I map designation of strain 8325 (29), the labeled DNA-band corresponded to fragment D. Derivatives of strain Newman showed a single insertion in a DNA fragment of a similar size.

Sequence analysis of the *sae* operon. A 3.5-kb DNA fragment encompassing *sae* was amplified from strains ISP479C and Newman by using primers based on the published genomic sequence of strain N315 (22). The fragments were cloned and sequenced. The sequence derived from strain ISP479C (GenBank accession number AJ556795) was found to be identical to a homologous region of strain COL (available from The Institute for Genomic Research [TIGR] at <http://www.tigr.org>) but showed minor differences from those of N315, Mu50 (22), and

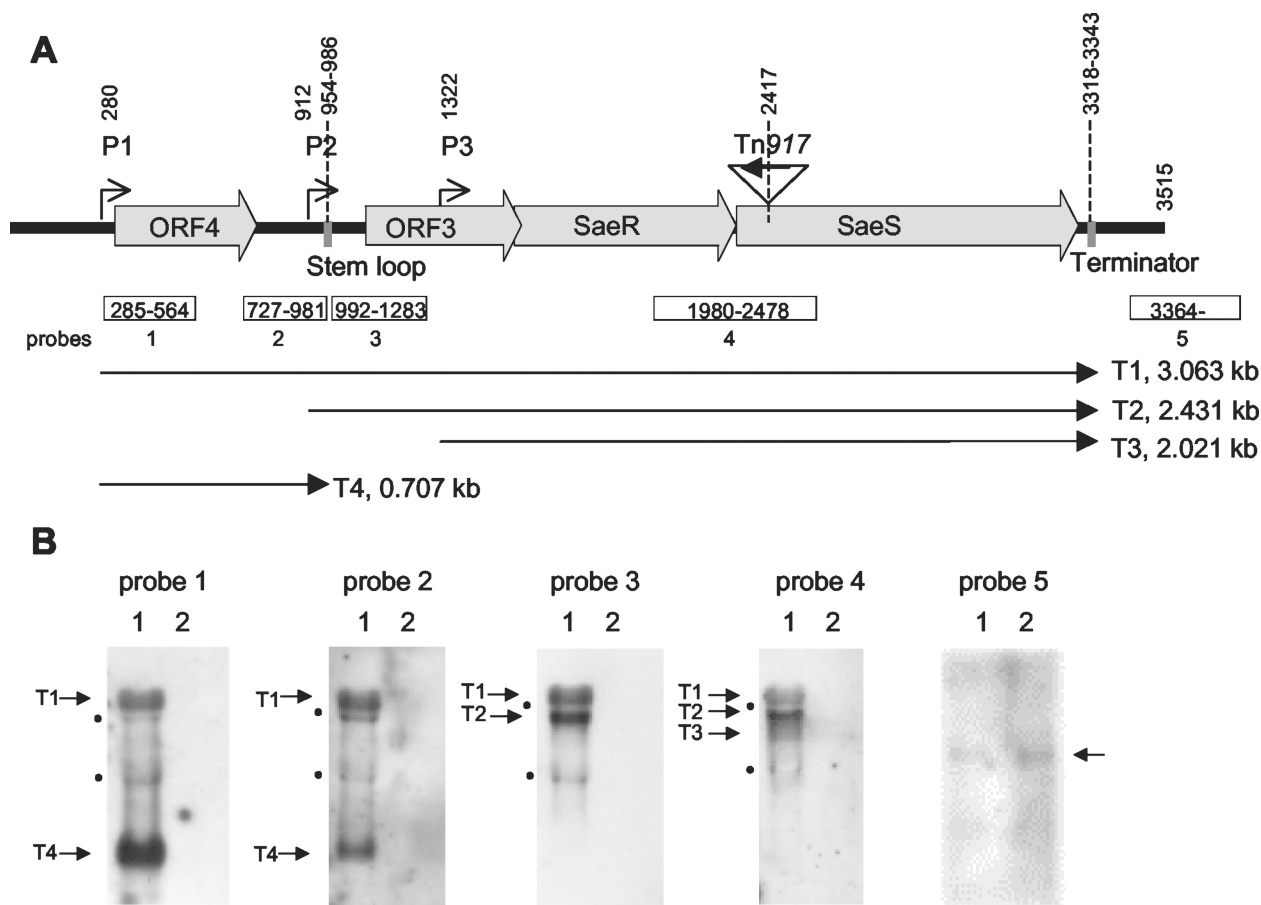


FIG. 1. (A) Scheme of the *sae* operon based on the sequence of pCWSAE7 (GenBank accession number AJ556795). Transcriptional start points (P1, P2, and P3) were determined by primer extension assays. (B) Northern blot analysis of RNA of strain Newman (lanes 1) and its isogenic *sae* mutant (lanes 2) grown to the post-exponential-growth phase. The blots were hybridized using different DIG-labeled PCR fragments within the *sae* operon (probes 1 to 4 [Table 2]) and downstream of the *sae* operon (probe 5 [Table 2]). The sizes of the labeled transcripts were determined by comparison with a molecular weight marker run in parallel. The positions of the rRNA are marked by black dots.

MW2 (1). Sequence comparison between the amplified fragments of strains Newman (GenBank accession number AJ556794) and ISP479 (GenBank accession no. AJ556795) revealed two single-base-pair exchanges, one within *saeR* and the other within *saeS*. However, there were substantial differences from the already published *saeRS* sequence derived from strain Newman (GenBank accession number AF129010) (11). Reamplification and resequencing of all the regions in question yielded results identical to our initial sequencing of the *sae* fragment.

Sequence analysis revealed two additional ORFs (designated ORF3 and ORF4) upstream of *saeR* (Fig. 1A). ORF3 encodes a putative 157-amino-acid (157-aa) polypeptide with a molecular mass of 17.68 kDa and an isoelectric point of 9.76. Due to the presence of an N-terminal signal peptide sequence and four membrane-spanning stretches, the putative gene product of ORF3 is probably membrane associated. The sequence revealed no obvious homology to known proteins. A homologous ORF upstream of *saeR*, encoding only 60 aa, is annotated in the genomes of *S. aureus* strains N315 (NTSA0730), Mu50 (SAV0693) (22), and MW2 (MW0668) (1); its product is identical to the C terminus of the ORF3

product. The different annotations are due not to sequence discrepancies but to different interpretations of the sequence data. In each of the published genomes, the same internal ATG motif within ORF3 was interpreted as the translational start site. In contrast, sequence annotation by TIGR (<http://www.tigr.org>) supports our hypothesis that the putative start site is located further upstream, which is based on the results obtained with a trained GLIMMER program. ORF4 codes for a second putative protein, of 146 aa, with a molecular mass of 16.05 kDa and an isoelectric point of 9.87. There are strain-specific variations in the N-terminal part of the ORF4-deduced polypeptide from strains COL, ISP479C, and Newman versus those from strains N315 and MW2. Sequence analysis of the translated ORF4 sequence revealed neither a signal peptide nor hydrophobic stretches. There is no obvious homology to known proteins in the database.

Transcriptional analysis of the *sae* operon. Northern blot analysis with different probes up- and downstream of *saeRS* showed the presence of three transcripts (T1, T2, and T3) probably initiating at different promoters but all terminating at the same stem-loop sequence downstream of *saeS* (Fig. 1). Interestingly, by using a probe specific for ORF4 (probes 1 and

2), one additional transcript (T4; 0.7 kb) could be detected (Fig. 1B). This transcript probably terminates at a stem-loop sequence upstream of ORF3, since probes downstream of the predicted stem-loop sequence (probes 3 and 4) did not detect T4. Bands of the size of the rRNA molecules are detectable by using different probes. We assume that this is an artifact rather than being due to additional specific transcripts, since these rRNA signals are usually seen when larger transcripts (e.g., *fnbA* [see Fig. 4A]) are analyzed using nonradioactive probes.

When RNA from the *sae* mutant strain was analyzed (Fig. 1B, lanes 2) no *sae*-specific mRNA was detectable with probes 1 to 4. The *sae*-downstream probe 5 reacted with a transcript present both in the wild type and in the *sae* mutant strain, indicating that the marked transcript does not belong to the *sae* operon.

To determine the transcriptional start points more precisely, primer extension experiments were performed using RNA from strain Newman grown to the post-exponential-growth phase. Each start point (P1, P2, and P3) was verified with at least two different primers. The P1 and P2 transcriptional start points could be clearly mapped to positions 280 and 912, respectively. The P3 start point was mapped to position 1322 (Fig. 2). However, the results for the starting point of T3 were more ambiguous, because the primers used to map P3 generated additional signals (data not shown). This is consistent with the results of *sae*-specific Northern blot analysis of strain Newman, in which T3 appeared to be less distinct than T1 and T2 (see Fig. 1B, 3A, and 3B). However, in other *S. aureus* strains, a clearer and stronger signal corresponding to T3 is detectable (see Fig. 3B).

Differential transcription of *sae* during the growth phase and in clinical isolates. Northern blot analysis with a *saeRS*-specific probe revealed that the three transcripts T1 to T3 were differentially expressed during the growth phase, with maximal expression in the post-exponential-growth phase (Fig. 3A). The overall expression of the *sae* transcripts was higher in strain Newman than in strain ISP479C. In order to examine whether *sae* is equally expressed in different clinical isolates, we analyzed *sae* transcripts in 40 epidemiologically independent clinical isolates after subculturing the strains to the post-exponential-growth phase. The three *sae* mRNAs were detectable in all the isolates. However, the total amounts of the transcripts were different for different strains. Furthermore, there was a strain-specific pattern with respect to the relative intensity of the three transcripts. The *sae*-related mRNA patterns of 10 clinical isolates and strain Newman are shown in Fig. 3B. Interestingly, in one isolate (Fig. 3B, lane 2) the size of the T2 transcript was obviously diminished.

Phenotypic characterization of *sae* mutants with respect to FnBPA and cell invasion. In order to further characterize the *sae* regulon, we analyzed the impact of *sae* on the transcription of *fnbA*. No *fnbA*-specific transcript was detectable in strains ISP479C and RN6390 by Northern blot analysis. When RNA from strain Newman was used, maximum transcription of *fnbA* was seen in bacteria from the exponential-growth phase. *fnbA* transcription is inhibited by *agr*, as shown by increased *fnbA* mRNA levels in the *agr* mutant strain (Fig. 4A) (33, 41). In contrast, *fnbA* mRNA was not detectable in the *sae* mutant or the *agr sae* double mutant of strain Newman (Fig. 4A). Thus, *sae* seems to be necessary for *fnbA* activation independently of

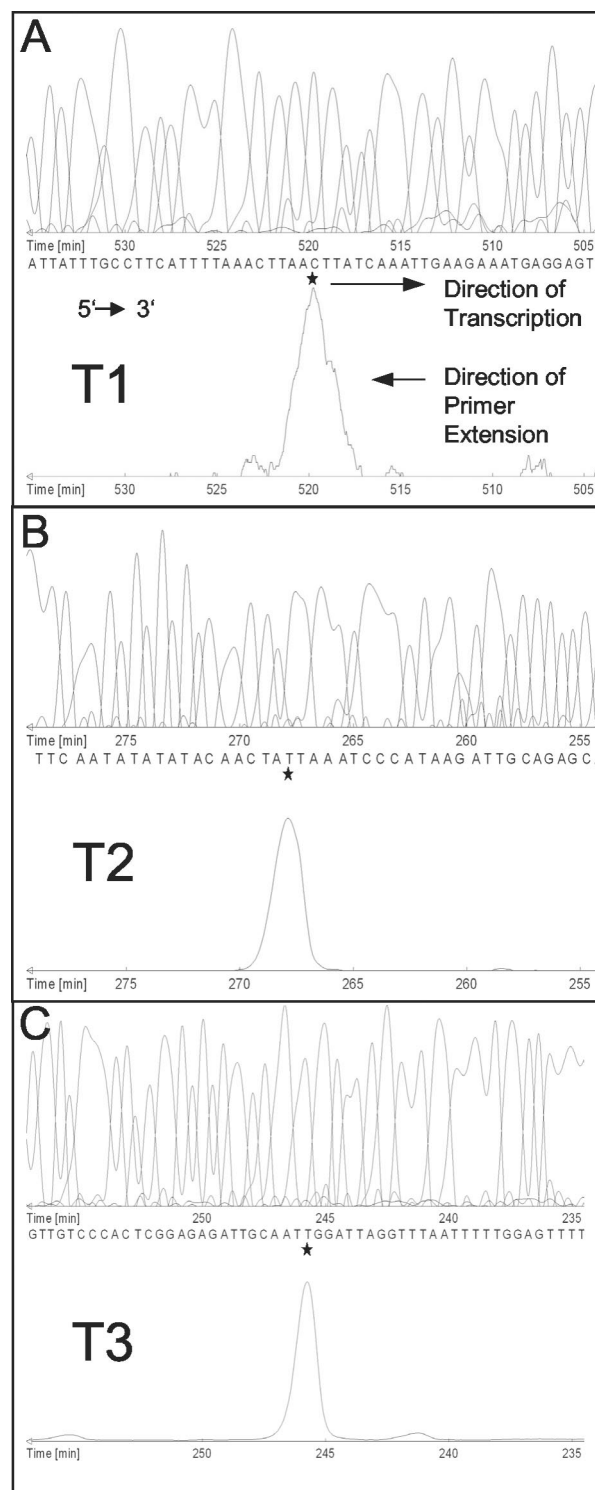


FIG. 2. Mapping of the 5' ends of three *sae* transcripts by fluorescence-based primer extension analysis. The precise base mapping was done by comparing the migration of the extended product with a parallel sequencing reaction primed by an identical Cy5-labeled oligonucleotide. The sequence and product traces are given in complement reverse order, thus corresponding to the 5'-to-3' orientation. The initiation start nucleotide of the mRNA is marked by a star. The *sae* mRNAs of 3 (A), 2.4 (B), and 2 (C) kb are labeled T1, T2, and T3, respectively.

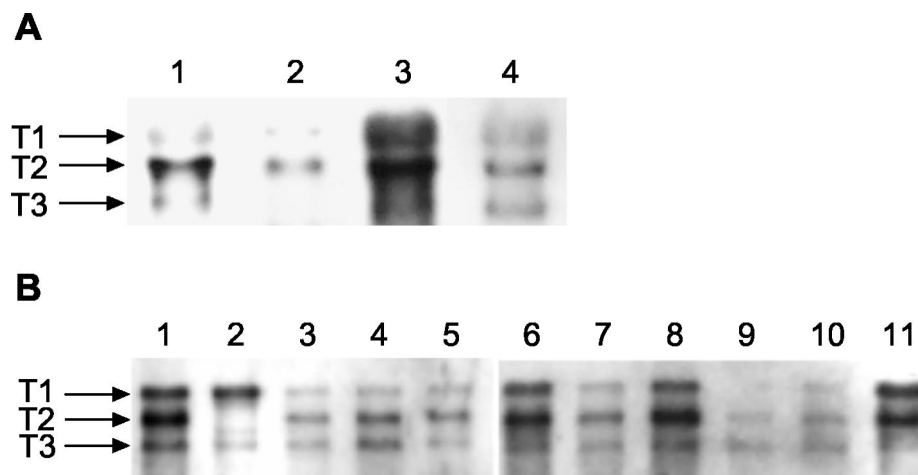


FIG. 3. Northern blot analysis using a DIG-labeled PCR fragment specific for *saeRS* (probe 4 [Table 2]). (A) RNA isolated from strains Newman (lanes 1 and 3) and ISP479C (lanes 2 and 4) grown to the mid-exponential-growth phase (lanes 1 and 2) or the post-exponential growth phase (lanes 3 and 4). (B) RNAs of strain Newman (lane 11) and different clinical isolates (lane 1, GN81; lane 2, GN435I; lane 3, W674; lane 4, W678; lane 5, W681; lane 6, W713; lane 7, CFN724; lane 8, CFN726; lane 9, W684; lane 10, W710) grown to the post-exponential-growth phase. Lanes 1 and 2, nasal isolates from healthy controls; lanes 3 to 6, 9, and 10, wound isolates; lanes 7 and 8, isolates from patients with cystic fibrosis.

agr. In the *sae* mutant strain, no FnBPA was detectable by ligand affinity blotting using cell wall extracts from bacteria in the exponential-growth phase (Fig. 4B). Besides FnBPA, one additional faint band with an apparent molecular mass of 200 kDa was visible in all preparations. This additional putative FnBP is also present in the *fnbAB* mutant and is not regulated by *sae*.

Since FnBPs were shown to be essential for the uptake of *S. aureus* by endothelial cells (30), we examined the interaction of the *sae* mutant strains with endothelial cells. There was no significant difference between the adherence of *sae* mutants to endothelial cells and that of their respective parental strains (Fig. 5A). However, the *sae* mutant of strain ISP479C was significantly less invasive than the parent strain (Fig. 5B) ($P < 0.0001$ by a two-tailed *t* test). Only 0.025% of strain Newman bacteria and 0.007% of bacteria of the *sae* mutant of strain Newman were incorporated by endothelial cells after 2 h of infection.

Phenotypic characterization of *sae* mutants with respect to CP5. Since strain 8325 and its derivatives are CP5 negative (39), the impact of *sae* on the expression of CP5 was evaluated in the CP5-positive strain Newman and its isogenic mutants. The *sae* mutant expressed more capsular antigen than strain Newman, as shown by indirect immunofluorescence (Fig. 6A). Following standard liquid culture, 20% of strain Newman bacteria were shown to be CP5 positive, whereas in the *sae* mutant 78% of bacteria showed detectable CP5 expression (Fig. 6B) ($P < 0.001$ by a two-tailed *t* test). Examination of the *agr* single mutant and the *agr sae* double mutant showed that significantly fewer bacteria were encapsulated in the latter than in the parental strain or the *sae* single mutant. In order to analyze whether *sae* inhibits CP5 expression on the transcriptional level, *cap5*-promoter activity was determined by using a *xylE*-reporter fusion that was introduced into strain Newman and its *sae* mutant. *cap5* promoter activity was significantly higher in the *sae*-negative background than in the *sae*-positive background of the parental strain (Fig. 7A).

As CP5 has been shown to be down-regulated by elevated CO₂ concentrations (19), we tested the impact of CO₂ on the *sae*-dependent up-regulation of CP5 by introducing a *cap5* promoter-reporter gene fusion plasmid. Elevated CO₂ concentrations resulted in down-regulation of the *cap5* promoter in strain Newman as well as in the *sae* mutant (Fig. 7A). Accordingly, when the expression of CP5 was investigated by indirect immunofluorescence, an inhibitory effect of CO₂ could be observed in both strains (Fig. 7B).

DISCUSSION

Here we characterized the *sae* operon, an independent global regulator for virulence gene expression in *S. aureus*. Sequence analysis revealed two additional ORFs upstream of the two-component system *saeRS*. Both ORFs are predicted to code for putative proteins with yet unknown functions. The predicted protein encoded by ORF3 is probably membrane associated, and that encoded by ORF4 is probably cytosolic. Transcriptional analysis leads to the assumption that both ORFs are functionally linked to the *saeRS* two-component regulatory system. ORF3 is cotranscribed with *saeRS* in the major transcripts T1 and T2. ORF4 is cotranscribed with ORF3 and *saeRS* (T1) but is also contained in the monocistronic T4 transcript. All transcripts, including the monocistronic message T4, are absent in the *sae* mutant strain, although the transposon insertion site was shown to be localized further downstream within *saeS*. Thus, SaeRS is probably necessary for transcriptional initiation from P1. Transcriptional initiation from multiple promoters of regulatory genes has also been described for *sarA* (2) and *sigB* (10). This indicates that multiple control elements are essential for the fine tuning of regulatory pathways. Recently, transcriptional analysis of the *sae* operon has been published (13) Two promoters upstream of *saeRS* were mapped, one of which corresponds to the P2 promoter described here. In contrast, the second promoter is not identical to any of the promoters characterized by us. The

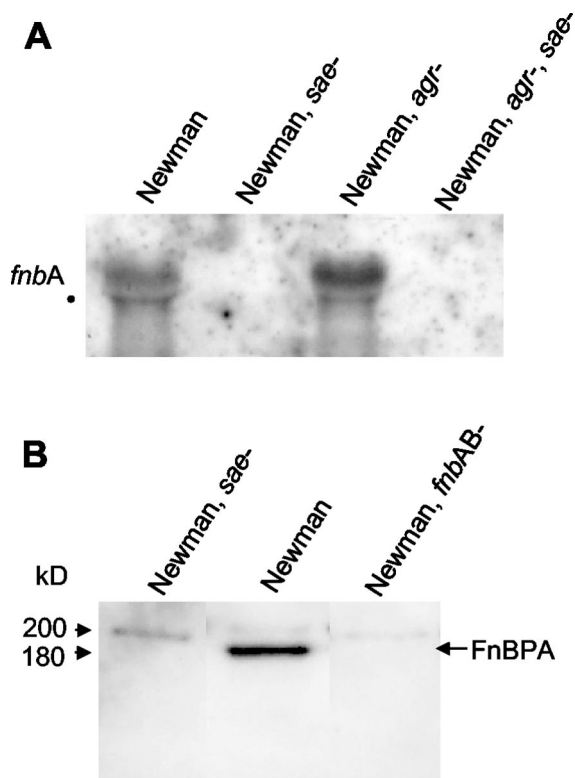


FIG. 4. (A) Northern blot analysis of equal amounts (2 μ g) of total RNA isolated from strains grown to the mid-exponential phase. Hybridization was performed using a DIG-labeled PCR fragment specific for *fnbA*. The position of the 23S rRNA is marked by a black dot. (B) Detection of FnBP by ligand blotting using biotinylated fibronection. Cell wall proteins were isolated from bacteria grown to mid-exponential phase and separated on an SDS-5% polyacrylamide gel.

discrepancy may be due to strain variation. The promoter activity of P2 was confirmed by promoter fusion experiments using a 1.15-kb upstream fragment excluding P1 (13). Thus, the possibility that the proposed T2 transcript is a degradation product derived from T1 can be ruled out.

Although *saeRS* clearly constitutes a classic two-component regulatory system, two additional ORFs are probably involved in its function, resulting in a four-component system. Two-component regulators as part of an operon with additional genes can be seen in quorum-sensing systems, such as *agr* (21). Recently, the regulatory operon *rgf* was shown to modulate genes involved in adhesion and virulence in *Streptococcus agalactiae* (37). *rgf* shows the same gene organization as the *sae* operon: four ORFs which are cotranscribed and predicted to encode one unknown protein, and one putative peptide with a signal sequence followed by a typical response regulator and a histidine kinase. The authors speculate that *rgf* may function as a quorum-sensing system in *S. agalactiae*. However, so far there is no evidence that *S. aureus* is equipped with additional quorum-sensing systems besides *agr*.

We further characterized the effects of *sae* on target genes and show that in addition to *hla* and *coa* (12), *sae* also activates *fnbA*. Previously it had been shown that *sarA* exerts a very similar effect on *fnbA* (41) and *coa* expression (unpublished data). Both *sae* and *sarA* are required for *fnbA* and *coa* tran-

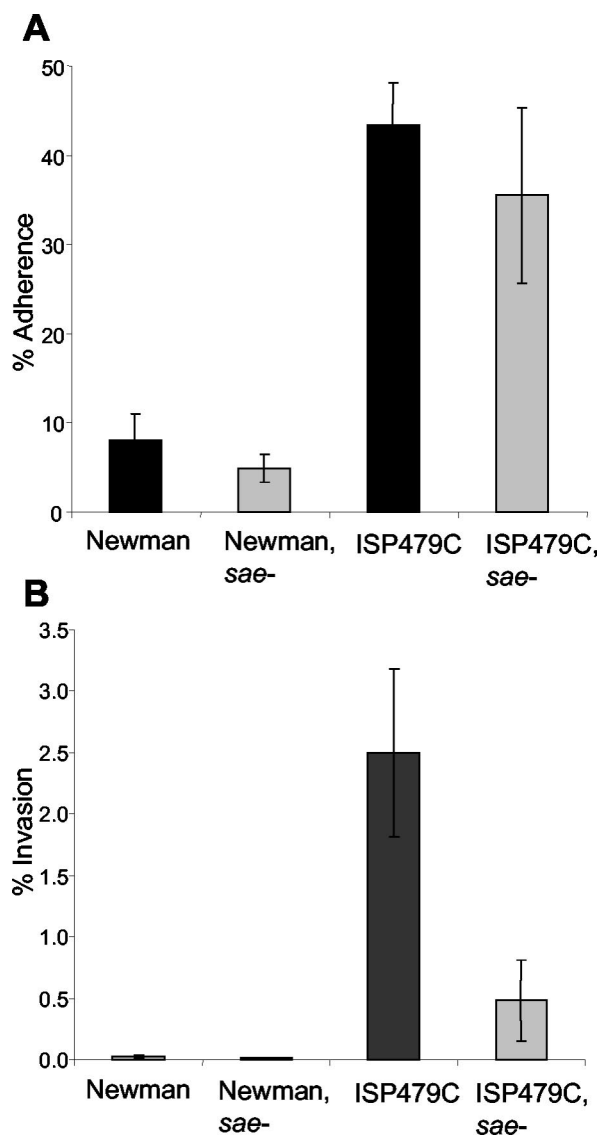


FIG. 5. (A) Adherence of *S. aureus* to primary human endothelial cells. Strains were grown to the mid-exponential-growth phase. Cell monolayers ($\sim 10^5$ cells/well) were incubated with 5×10^6 bacteria/well for 2 h at 37°C. The percentage of adherence was expressed as $100 \times$ (CFU of adherent bacteria/number of inoculated bacteria). (B) Internalization of *S. aureus* by endothelial cells. After 2 h of incubation, extracellular bacteria were removed by lysostaphin treatment. The percentage of invasion was expressed as $100 \times$ (CFU of internalized bacteria/number of inoculated bacteria). Each data point represents the mean of four determinations. Error bars, standard deviations.

scription independently of *agr*, whereas *agr* leads to the inhibition of both genes (33, 40). Since no direct interaction between *sae* and *sarA* could be detected (data not shown), one may assume that *sae* interacts directly with the target genes. However, a comparison of the upstream sequences of *fnbA* and *coa* revealed no obvious common motifs. Additional factors may be needed to explain the observed regulatory pattern of *fnbA* and *coa* expression. This is further emphasized by the observation that both genes are strictly repressed during the late growth cycle, which cannot be explained by any of the regulatory loci studied so far.

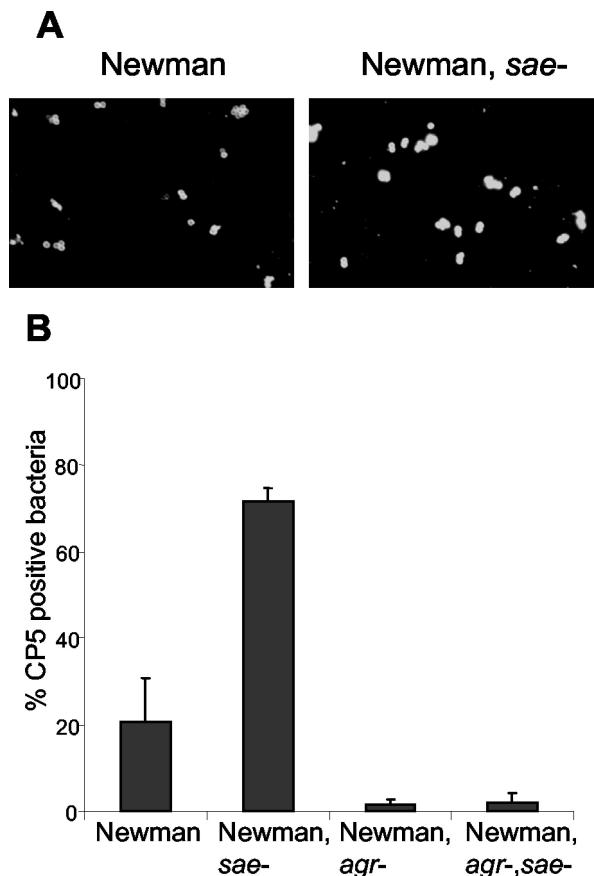


FIG. 6. (A) CP5 expression determined by indirect immunofluorescence of strain Newman and its isogenic *sae* mutant grown to stationary phase at 37°C in CYPG. Bacteria were marked with CP5-specific monoclonal antibodies and stained with Cy3-conjugated anti-mouse antibodies. (B) Percentages of CP5-positive bacteria. Three microscopic fields with about 50 bacteria each were evaluated, and the percentage of CP5-positive bacteria was determined by comparing the number of fluorescent bacteria with the total number of bacteria stained with DAPI (4',6'-diamidino-2-phenylindole).

We could demonstrate that *sae* is necessary for *fnbA* transcription and that accordingly the *sae* mutant did not express FnBPA and was internalized significantly less by endothelial cells. It has been shown convincingly by others that FnBPs are essential and sufficient for bacterial uptake by endothelial cells (30, 35). However, there are striking differences between strains. Whereas in strain Newman more *fnbA*-specific transcripts are detectable than in strain ISP479C, only the latter is effectively taken up by endothelial cells. It remains to be determined why strain Newman is not internalized by these cells, even though it expresses FnBPA. One may speculate that strain-specific differences in the sequence, the surface localization of the protein, or proteolytic processing account for these discrepancies. It is also evident that the adhesion of *S. aureus* to endothelial cells is mediated by additional factors not affected by *sae*, since there was no difference in adhesion between *sae* mutant strains and the parental strains.

Since bacterial adhesion to endothelial cells is inhibited by CP5 (32), we also examined the effect of *sae* on CP5 expression in strain Newman. *sae* leads to the repression of *cap* transcrip-

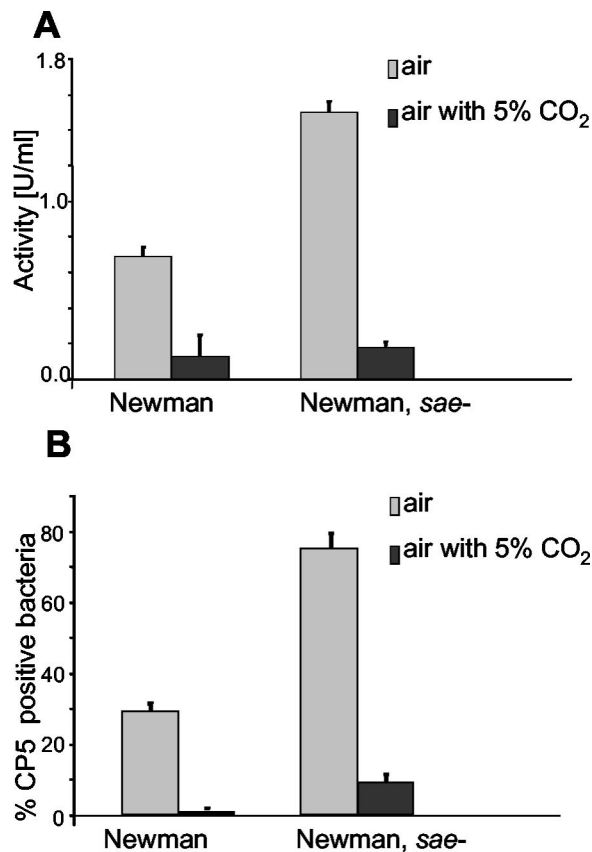


FIG. 7. (A) *cap5* promoter activity in strain Newman and its *sae* mutant containing the *xylE* fusion construct after growth in air alone and in air with 5% CO₂. Promoter activity was expressed as XylE activity per milligram of total protein. (B) Percentages of CP5-positive bacteria of strain Newman and its isogenic *sae* mutant after growth in air alone and in air with 5% CO₂. Three microscopic fields with 50 bacteria each were evaluated, and the percentage of CP5-positive bacteria was determined by comparing the number of fluorescent bacteria with the total number of bacteria stained with DAPI (4',6'-diamidino-2-phenylindole).

tion, as shown by promoter fusion assays. Accordingly, *sae* mutant bacteria express significantly more CP5. In contrast, *agr* was shown to activate the *cap* operon (6, 23, 32). Thus, *agr* and *sae* counteract the target gene expression not only of *coa* and *fnbA* but of *cap* as well. However, this is most probably not due to cross-inhibition of *sae* and *agr*. This is based on the observation that in *sae* mutant bacteria, the expression of the effector molecule RNAIII of the *agr* locus is not altered from that in the parental strain (12) (data not shown). Additionally, *sae* and *agr* exert similar and additive effects on *hla* transcription, which is activated by both regulators.

Since Hla (28) and CP5 (19) are affected in opposite manners by *sae* as well as by elevated CO₂, we analyzed whether *sae* might function as a CO₂ sensor. However, the CO₂ responses with respect to CP5 expression were similar in the *sae* mutant strain and the parental strain. Thus, the appropriate signal for *sae* activation still remains to be determined. Since *hla* activation during device-related infection was shown to be dependent on *sae* (17), we assume that *sae* contributes to the regulatory adaptation of *S. aureus* during infection.

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