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Studies on the Expression of Regulatory Locus *sae* in *Staphylococcus aureus*

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Abstract. Global regulatory locus *sae* consists of a two-component signal transduction system coded by *saeR* and *saeS* genes that upregulates the transcription of several exoproteins. Northern analysis carried out in this study reveals the synthesis at late and post-exponential phases of a cotranscript of *saeR* and *saeS* structural genes of about 2.4 kb. This transcript is diminished in the isogenic *agr*::*tetM* mutant. Likewise, transcriptional fusion experiments show that *sae* expression is downregulated in the *agr* null mutant. Complementation analyses with plasmids carrying fragments of about 1.2 or 0.2 kbp upstream of *saeR-saeS* genes, which restore fully or only partially, respectively, the wild-type phenotype to the *sae* mutant, are in agreement with two initiation start points of transcription revealed by primer extension experiments. This work, as well as previous studies, reveals a complex hierarchical regulatory network involving several loci that control the expression of virulence determinants in *S. aureus.*

Staphylococcus aureus is a major pathogen of man and animals; it synthesizes a large number of extracellular and cell wall-associated proteins that contribute to its virulence. Several global regulatory loci, such as *agr*, *sar* and *sae*, have been found to regulate the production of these virulence factors [2, 7, 13].

The *agr* locus consists of two divergent transcripts, RNAIII transcribed from the P3 promoter, which encodes δ-hemolysin and acts in the regulation of secretory and cell wall-associated proteins, and RNAII, transcribed from the P2 promoter, which encodes the products of *agrB, D, C,* and *A.* Genes *agrD* and *agrB* encode and process an autoinducing peptide that acts as a signal that activates the expression of RNAII and RNAIII through a two-component signal system coded by *agrC* and *agrA.* Activation of RNAIII leads to increased production of α- and β-hemolysins, serine protease, DNase, and other exotoxins and to repression of the production of protein A, coagulase, and other cell wall-associated proteins [12, 14, 18].

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The main product of the *sar* locus is a DNA-binding protein, SarA, which is transcribed from three different promoters. SarA acts on the expression of virulent factors indirectly by upregulating RNAIII transcription and also stimulating or repressing the transcription of several virulence genes such as *hla*, *fnbA*, *cna*, *spa*, and *ssp* (which code for α-hemolysin, fibronectin, and collagen-binding proteins, proteinA and serin-protease, respectively) in an *agr*-independent way [1, 4, 15, 25].

The regulatory locus designated *sae* (for *S. aureus* exoprotein expression) encodes a two-component regulatory system involving *saeR*, a response regulator, and *saeS*, a histidine protein kinase, of 687 and 1062 bp, respectively, and upregulates the production of α- and β-hemolysins, DNase, and coagulase at the transcriptional level [9, 10].

More recently, other virulence regulators have been described. The *sarH1* or *sarS* locus, which belongs to a family of *sar* homologs, represses the transcription of α-hemolysin and activates *spa* transcription while its own transcription is repressed by *sarA* and *agr* [3, 23]. Other characterized genes of this family are *sarT*, which like *sarH1* is negatively controlled by *sarA* and *agr* and

represses the expression of *hla* [21], and *sarR*, which represses *sarA* [16]. The two-component system *arlR-arlS*, which is upregulated by *sarA* and *agr*, modulates the synthesis of RNAII, RNAIII, and *sarA* [6].

In this work we study the expression of the *sae* locus during growth and its interaction with *agr*.

Materials and Methods

Strains, plasmids, and media. *Staphylococcus aureus* strains used were: ISP479 (derivative of 8325-4 [22]) and its *sae*::Tn*551* and *agr*::*tetM* derivatives, RC106 and RC185, respectively [8]; RN4220, restriction-deficient, as intermediate host for plasmids isolated from *E. coli*. *Escherichia coli* DH5α was used as recipient for plasmids derived from pGEM-T-Easy (Promega). Phage 80α was used in transduction experiments in *S. aureus*.

Plasmids used were: pGEM-T-Easy (Promega), for cloning into *E. coli;* pMK4, bifunctional vector Amp-R Cm-R; pLC4, transcriptional bifunctional fusion vector Amp-R Cm-R carrying promoterless *xylE* gene [5]; pRC9, pBluescript with a 3.4-kb *Eco*RI-*Cla*I fragment involving the *sae* locus [10]; pRC91, pMK4 derivative carrying the 3.4-kb *Eco*RI-*Cla*I fragment from pRC9 [10].

Brain heart infusion (BHI) was used for growth of *S. aureus*, and LB for *E. coli*. Antibiotics were used at the following concentrations: ampicillin (Amp) at 50 μg ml^{-1}; chloramphenicol (Cm) and erythromycin at 10 μg ml^{-1}, and tetracycline at 5 μg ml^{-1}.

Northern blotting and primer extension analysis. RNA was extracted following the procedure described by Sambrook et al. [20]. RNA from agarose-formaldehyde gels in Mops running buffer was transferred to a nylon membrane and hybridized with probes labeled with digoxigenin-11-dUTP (DIG DNA Labelling Kit; Boehringer-Mannheim) by random primer extension. Labeled hybrids were detected by using anti-digoxigenin F′ (ab)2 antibody fragments conjugated to alkaline phosphatase and a chemiluminiscent substrate according to the manufacturer's instructions (Boehringer Mannheim).

The transcriptional mapping of the *sae* locus was carried out by primer extension with Moloney murine leukemia virus reverse transcriptase (Promega) with the oligonucleotides PE3 5′-$^{-264}$GTCGTATGTGCAACTATATTTGCG^{-287}-3′ and S2 5′-$^{-8}$ TTCACCTCTGTTCTTACGACCTC^{-30}-3′ and 10 μg of total RNA extracted from cultures at the post exponential phase, in the presence of ^{32}P-dATP. DNA sequencing was carried out by the dideoxy chain termination method by using a T7 polymerase sequencing kit (Sequenasa 2.0, United States Biochemical Corp) and ^{32}P-labeled dATP. Single-stranded M13 phage was used as template.

The sequence of the *sae* region involving *saeR-saeS* genes and 227 nt (nucleotides) upstream was based on the data of Giraudo et al. [10] (GenBank accesion number 129010); the sequence upstream of −227 nt was from genome database of Oklahoma University of *S. aureus* strain NCTC8325 (www.genome.ou.edu).

Construction of transcriptional fusions. A 180-bp fragment of the *sae* locus located upstream of the ORF was amplified by the polymerase chain reaction (PCR) on pRC91 as template by using upper primer S1: 5′-$^{-188}$TGCTGCTAGTTTCTTTGGAG^{-169}-3′ and downstream primer S2: 5′-$^{-8}$TTCACCTCTGTTCTTACGACCTC^{-30}-3′. This fragment was cloned into pGEM-T and recloned into bifunctional plasmid pLC4 to obtain vector pFU5 which carries the fusion to *xylE* promoterless gene. From pRC9 an *Eco*RI-*Eco*RV fragment involving 1.15 kb upstream of *saeR* and 138 bp of *saeR* [10] was cloned into pBluescript to generate pRC10. The 1.3-kb *Eco*RI-*Eco*RV fragment of pRC10 was recloned into pLC4 to obtain fusion vector pFU9. Upper primer S1 was also used with downstream primer S4: 5′-^{1862}AATGATGAGAAGGATGCCCA^{1843}-3′ to generate a PCR-amplified fragment of 2050 bp. This fragment, which included *saeR* and *saeS* genes, was cloned into pGEM-T and recloned into pMK4 to generate plasmid pRC31.

The pLC4 and pMK4 derived plasmids were transferred by electroporation to intermediate strain *S. aureus* RN4220 and from it to ISP479 by ϕ80α transduction.

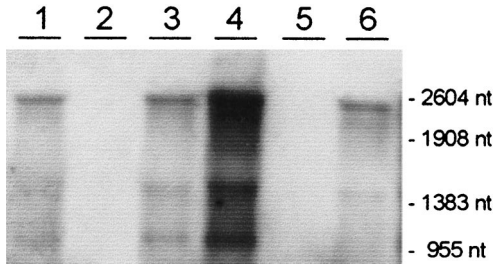

Fig. 1. Northern blot analysis of the *sae* locus in wild type and *sae* and *agr* mutants during growth. Lanes 1 and 4: ISP479 (wt); lanes 2 and 5: RC106 *sae*::Tn*551*; lanes 3 and 6: RC185 *agr*::*tetM*, at 4 and 6 h of growth (OD at A_{600} of about 4 and 6 corresponding to late exponential and post-exponential phases, respectively). A fragment involving 188 bp upstream of *saeR*, *saeR*, and 50 bp at the 5′ end of *saeS* was used as probe. Size markers are indicated on the right.

Catechol 2,3-dioxygenase assays. For enzymatic assays of *xylE* expression, overnight cultures were diluted in BHI with chloramphenicol to an initial OD of about 0.2 at 660 nm and grown with shaking at 37°C. At different time intervals, samples were removed, washed, suspended in 100 mM potassium phosphate buffer (pH 7.5) supplemented with 10% acetone and 25 μg ml^{-1} of lysostaphin, incubated at 37°C for 30 min, and iced for 15 min [5]. The supernatants of the centrifuged extracts were assayed spectrophotometrically at 375 nm in potassium phosphate buffer (pH 8.0) with 0.2 mM catechol, as described [19]. One milliunit corresponds to the formation at 30°C of 1.0 nmol of 2-hydroxymuconic semialdehyde per min. Specific activity is defined as milliunits per milligram of cellular protein.

Exoprotein activities. Supernatants of cultures grown in BHI broth in a rotatory shaker at 37°C for 15 h were assayed for DNase, hemolysins, and coagulase as described previously [7].

Results

Transcription of the *sae* locus. Northern analysis carried out with strain ISP479 revealed at late exponential and post-exponential phases of growth a major transcript specific to the *sae* locus of about 2.4 kb (Fig. 1). Transcription was enhanced at the post-exponential phase, suggesting that activation of the promoter was growth dependent. Using primer extension analysis (Fig. 2), a transcription start point, designated P1, was located 626 nucleotides (nt) upstream of the predicted translation start point of *saeR*. The size of the cotranscript is consistent with the size of *saeR-saeS* genes plus the transcribed upstream fragment (Fig. 3). Inactivation of the *agr* locus markedly decreased the expression of the *sae*

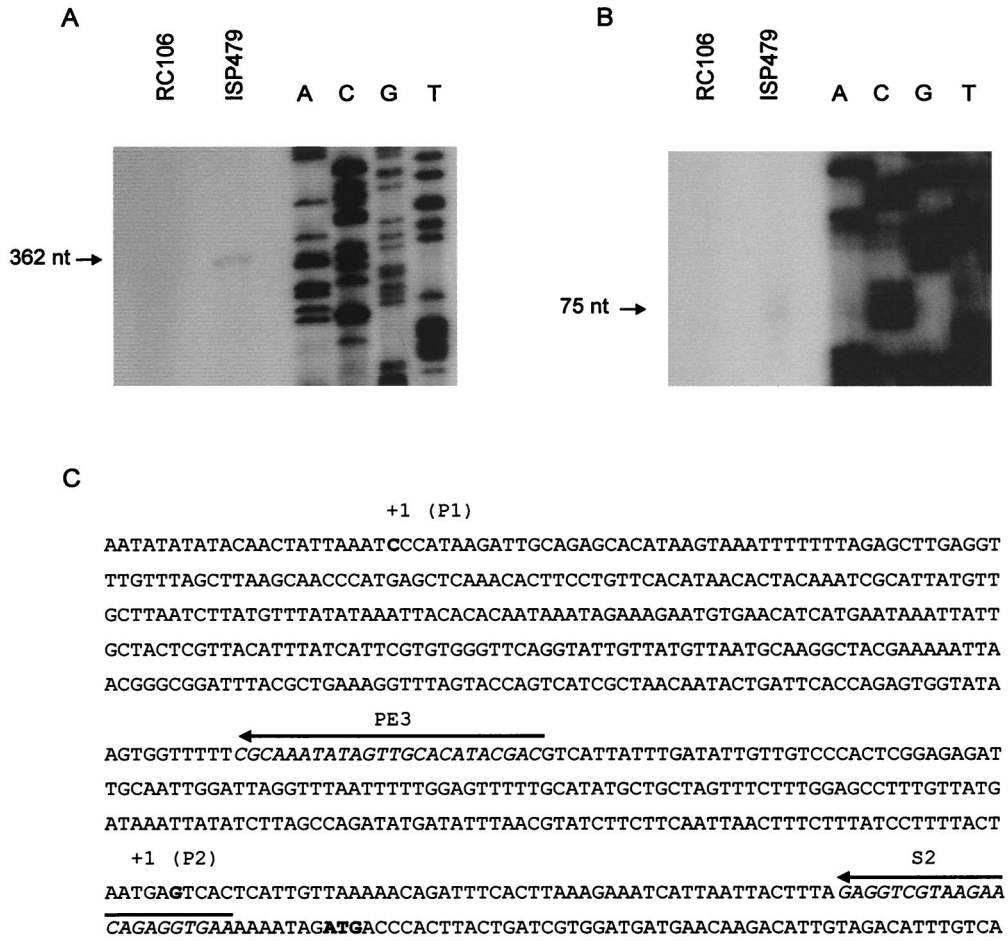

Fig. 2. Primer extension analysis of the transcription start point. The autoradiograms show primer extension experiments performed with RNA from strains RC106 and ISP479 extracted from cultures at the post exponential phase with: A) primer PE3 and B) primer S2. Lanes A, C, G, T show sequencing reactions performed on M13 with the −40 primer. The horizontal arrows indicate the hybridizing extension products. C) Upstream sequence of the *sae* locus. The transcription initiation sites (+1) from promoters P1 and P2 and the ATG of *saeR* ORF are in boldface. Arrows indicate the primers used.

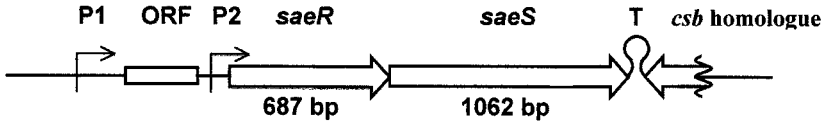

Fig. 3. Organization of the *sae* locus involving *saeR-saeS* genes. P1 and P2 promoters (bent arrows) are located at 626 and 83 nt upstream of *saeR-saeS*, respectively. An ORF of 327 nt located upstream of *saeR-saeS*, which codes for a hypothetical hydrophobic protein, is indicated. T: putative termination loop; *csbB:* homolog of *csbB* gene of *Bacillus subtilis.*

locus at the post-exponential phase (Fig. 1), which indicates that *agr* upregulates *sae* expression.

Two other bands of about 1.5 and 0.9 kb were detected in the wild-type strain but not in the *sae* mutant, which might result from alternative promoter or termination sites or from processing of the major transcript (Fig. 1).

Fusions to promoterless *xylE* gene of two fragments located upstream of the *sae* coding region were assayed for expression during the post-exponential phase. The level of expression of *xylE* fused to an upstream segment of 1150 bp, carried by pFU9, was similar for the wild-type strain and the *sae* mutant (Fig. 4). Expression was diminished in the *agr*::*tetM* mutant, confirming the upregulation effect of *sae* expression by *agr* observed by Northern analysis. Studies with plasmid pFU5, which carries a gene fusion involving an upstream fragment of 180 bp, showed a very low expression of *xylE* in wild

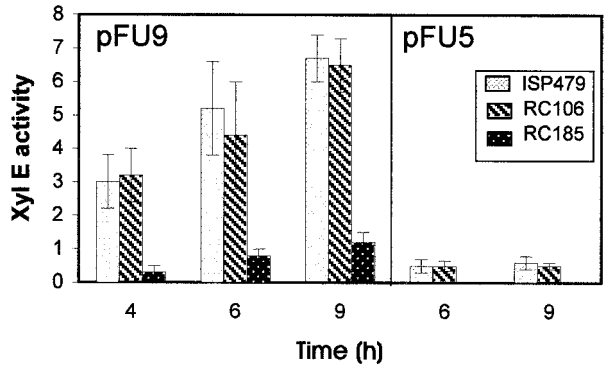

Fig. 4. XylE activity in strains harboring plasmids pFU9 and pFU5 carrying transcriptional fusions of promoterless *xylE* to upstream fragments of the *saeR-saeS* coding region at 4, 6, and 9 h and 6 and 9 h, corresponding to late exponential, post-exponential, and early stationary phases (approximate OD at A_{600} of 4, 6, and 7, respectively). Values, given as milliunits per milligram of cellular protein, are the means of four determinations.

Table 1. Complementation of *sae* mutant RC106 by plasmids carrying two different *saeR-saeS* upstream fragments.

	Production (U ml^{-1}) of			
Strain	α-Hemolysin	β-Hemolysin	Coagulase	DNase ($\times 10^3$)
ISP479 (wt)	1400	15800	8	10
RC106 *sae*::Tn*551*	80	650	<2	0.8
RC106 (pRC31)	500	2900	<2	8
RC106 (pRC91)	2000	22400	32	14

pRC31 and pRC91 carry upstream fragments of 188 bp and 1150 bp, respectively. Data are the mean of three experiments.

type and *sae* mutant backgrounds (Fig. 4). This observation agrees with the results of primer extension experiments, which revealed a second weaker transcription start site, designated P2, located 83 nt upstream of *saeR* (Fig.2).

Complementation experiments. Complementation studies involving *sae*::Tn*551* mutant RC106 and plasmids carrying *saeR* and *saeS* genes with upstream fragments of 1150 and 188 bp were carried out. Plasmid pRC91, which carries the 1.1 kb upstream region, when introduced into the *sae* mutant RC106 restored the wild-type phenotype, as determined for α- and β-hemolysins, DNase and coagulase production (Table 1). On the other hand, plasmid pRC31, carrying the 188-bp upstream fragment, complemented only partially the production of the exoproteins assayed and did not complement for coagulase production; these observations agree with the presence of the second weaker start point of transcription located within this region, as revealed by primer extension experiments (Fig. 2).

Discussion

Northern blot analysis carried out in this work revealed the synthesis during late and post-exponential phases of a cotranscript specific to the *sae* locus, of about 2.4 kb, the expected size for *saeR-saeS* open reading frames plus a transcription start site located 626 nt upstream.

Complementation analysis of a *sae*::Tn*551* mutant with plasmids carrying *saeR-saeS* genes with upstream fragments of 1.1 kbp and 180 bp, which showed complete and partial restoration of the wild-type phenotype, respectively, in the production of several exoproteins, as well as the expression of transcriptional fusions of these fragments with *xylE*, are in agreement with the results of primer extension experiments, which also revealed the presence of a second weaker start point of transcription, located 83 nt upstream of the *saeR* predicted start point.

Previous studies have shown that *sae* does not affect the transcription of *agrA* or RNAIII [9]. In this work, results of Northern analysis, as well as of transcriptional fusion experiments with *xylE* as reporter gene with a 1.1-kb fragment of the region upstream of *saeR-saeS* coding region, revealed that *agr* upregulates *sae* expression. These observations might explain the previously observed interactions between these two loci in the production of several exotoxins, such as those coded by *hla* and *hlb* [8].

This study also showed that *sae* is expressed, though at a lower level, in the absence of the *agr* product. Several experimental evidences have indicated the presence of significant levels of *hla* mRNA during the post-exponential phase of growth in *agr*, *sarA agr*, and *sarA agr sarH1* mutants, suggesting that a distinct phase-dependent signal, independent of *agr*, *sarA*, and *sarH1*, was needed for activation of *hla* [17, 23, 24]. This signal might reflect the upregulation effect of *sae* on *hla* transcription. Interestingly, in vivo assays have shown that *sae*, but not *agr* or *sarA*, is needed for *hla* expression [11].

The observations of this study on *sae* as well as work on *sar* and *agr* regulators reveal that the production of exotoxins and cell wall-associated proteins in *S. aureus* is controlled through a complex interactive regulatory network. The complexity of the regulation of virulence determinants is highlighted by the reported interactions of regulatory loci *sarHI*, *sarT*, and *arlR-arlS* with target genes and with regulatory loci *sarA* and *agr* [6, 21, 23].

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