Regulatory organization of the staphylococcal sae locus

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This paper describes an investigation of the complex internal regulatory circuitry of the staphylococcal sae locus and the impact of modifying this circuitry on the expression of external genes in the sae regulon. The sae locus contains four genes, the saeR and S two-component signalling module (TCS), and saeP and Q, two upstream genes of hitherto unknown function. It is expressed from two promoters, PAsae, which transcribes only the TCS, and PCsae, which transcribes the entire locus. A bursa aurealis (bursa) transposon insertion in saeP in a derivative of Staphylococcus aureus NCTC 8325 has a profound effect on sae function. It modifies the activity of the TCS, changing the expression of many genes in the sae regulon, even though transcription of the TCS (from Pcsae) is not interrupted. Moreover, these effects are not due to disruption of saeP since an in-frame deletion in saeP has essentially no phenotype. The phenotype of S. aureus strain Newman is remarkably similar to that of the saeP::bursa and this similarity is explained by an amino acid substitution in the Newman saeS gene that is predicted to modify profoundly the signalling function of the protein. This concurrence suggests that the saeP::bursa insertion affects the signalling function of saeS, a suggestion that is supported by the ability of an saeQR clone, but not an saeR clone, to complement the effects of the saeP::bursa insertion.

INTRODUCTION

sae is a key regulatory locus in staphylococci, coordinating environmental signals with the internal regulatory circuitry governing virulence and other adaptive processes (Novick & Jiang, 2003). It was identified on the basis of a Tn551 insertion in saeR (Giraudo et al., 1994a), which profoundly affected the expression of a large set of virulence genes (Giraudo et al., 1997), largely, but not entirely, parallel to the regulatory activities of the agr system (Giraudo et al., 1996). The sae locus contains a classical two-component signalling module (TCS), of which SaeS is the receptor kinase and SaeR the response regulator (Giraudo et al., 1999). It also contains two additional genes, saeP and saeQ (Novick & Jiang, 2003), upstream of the TCS, whose functions are presently unknown. In previous studies (Novick & Jiang, 2003; Steinhuber et al., 2003), we and others have analysed the transcription/expression pattern of the sae locus, identifying four transcripts, A, B, C and D, and two promoters, PAsae and PCsae, as diagrammed in Fig. 1(a). Transcript A, 2.1 kb, initiated from promoter PAsae and covering saeR and saeS, is present at the earliest time point and diminishes in intensity during the mid-exponential phase of growth. PAsae is within the saeQ coding sequence. Transcripts B (2.4 kb), C (3.1 kb) and D (0.5 kb), all initiated from PCsae, appear during the mid-exponential phase. Transcript C covers the entire locus and may be processed to generate B, which covers saeQ, saeR and saeS; D, which covers saeP only, may be a processed product of C or it may be a de novo transcript. PCsae is (auto)induced by saeR and S. Thus, saeR and saeS are transcribed throughout growth, first from promoter PAsae and then from PCsae. The mid-exponential-phase activation of PCsae represents a profound regulatory transition within the sae locus and is critically dependent on various regulatory determinants, including agr, sarA and, as noted, saeR/S, and is affected by various environmental stimuli, including low pH, high NaCl concentration and subinhibitory concentrations of antibiotics (Novick & Jiang, 2003; Steinhuber et al., 2003). The present study was initiated with the intention of determining the roles of saeP and/or saeQ in the regulatory function of the overall sae locus, starting with a set of bursa aurealis (bursa) insertions, isolated and identified by Bae et al. (2004) and kindly provided by D. Missiakas (University of Chicago), and continuing with an in-frame deletion in saeP. We observed that the bursa insertion and in-frame deletions in saeP had different effects that could be explained only partially by the polarity of the bursa insertion. In particular, the in-frame deletion had no detectable effect on sae transcription or regulatory function, whereas the bursa insertion caused
a sharp upregulation of $P_{Csaec}$ probably owing to subtle effects on downstream components of the $sae$ locus.

The upregulation of $P_{Csaec}$ by the $saeP::bursa$ mutation is reminiscent of the $sae$ transcription pattern reported for the wild-type *Staphylococcus aureus* strain Newman (Blickwede et al., 2005). This led to the rather striking finding that strain Newman, unlike any of nine other *S. aureus* strains for which the sequence of $sae$ is known, has an amino acid substitution in the receptor domain of $saeS$, which is partially responsible for its phenotype, including the upregulation of $P_{Csaec}$ and the hyperproduction of coagulase, a phenotype that is rather different from that of NCTC 8325 derivatives and of other *S. aureus* strains.

**METHODS**

**Bacterial strains and plasmids.** These are listed in Table 1. In analysing the functions and regulatory circuitry of the $sae$ locus, we have been fortunate in obtaining *bursa aurealis* insertions (Bae et al., 2004) in all of the genes, kindly provided by Dominique Missiakas (University of Chicago). We outcrossed all the transposon mutations to several different strains, confirming in all cases linkage of the observed phenotype to the mutation, ruling out adventitious mutations in other genes.

**Media and growth conditions.** General methods for *S. aureus* were as described by Novick (1991). Standard CYGP medium without glucose (Novick, 1991) was used for RNA and exoprotein extraction. Culture densities were determined with a Klett–Summerson colorimeter at 540 nm. A Klett reading of 100 corresponds to $-3 \times 10^8$ cells ml$^{-1}$. Bacteria stored at $-80\, ^\circ\text{C}$ were inoculated on GL plates (Novick, 1991) and grown overnight with selective antibiotics as required for plasmid maintenance. For time-course experiments, cultures were resuspended in CYGP to give a Klett reading of 20 ($K=20$), grown to K=50 at 37 $\, ^\circ\text{C}$ with shaking, then diluted to K=20 and regrown to K=50; this point was taken as time zero ($t_0$). Samples taken at 3 and 6 h are listed as $t_3$ and $t_6$ samples, respectively.

**Exoprotein profile and lipase zymogram.** For determination of exoprotein profiles, culture samples (1–10 ml) were centrifuged in an Eppendorf centrifuge. The supernatant was centrifuged to remove any residual organisms, then precipitated with a 10 % volume of 50 % trichloracetic acid, and the pellet analysed by SDS-PAGE according to the method of Laemmli (1970). The lipase zymogram was generated by standard techniques. Briefly, TCA-precipitated exoprotein (native; without reducing agent or boiling) gel was run with TGE buffer. The gel was washed with 20 % 2-propanol and then with water at room temperature. The gel was then transferred onto an agarose plate containing 1 % Tween 20 and incubated overnight at 37 $\, ^\circ\text{C}$. White precipitates represent the lipase activities.

**RNA preparation.** Cell pellets were treated with RNA Protect reagent (Qiagen) and mechanically disrupted by agitation with glass beads using the Bio101 FastPrep apparatus. RNA was purified using the Qiagen RNeasy kit, and its integrity checked by agarose gel electrophoresis (Novick, 1991).

**Northern blot hybridization.** DNA samples corresponding to equal numbers of cells were separated by gel electrophoresis through 1 % denaturing agarose (MOPS/formaldehyde), vacuum-blotted to Hybond-N+ membranes (Amersham), and UV cross-linked. Blots were hybridized overnight to [$\alpha$-$^{32}$P]dATP-labelled, PCR-generated probes. Washed blots were exposed to Phosphorimager screens that were read by a Molecular Dynamics Phosphorimager. Primers (Integrated DNA Technologies) are listed in Supplementary Table S1(a), available with the online version of this paper.

**DNA procedures.** Most clonings were done by ligating restriction-enzyme-digested PCR products obtained with oligonucleotide primers tagged with restriction sites (Integrated DNA Technologies), as listed in Supplementary Table S1(b), to comparably digested vector DNA. Primers containing 5’ *PstI* or *KpnI* sites were used to clone *sae* genes under the *Phead* promoter of pCN51 in pRN9160. Plasmid and chromosomal DNAs were isolated by using a QIAprep Spin Miniprep Kit from Qiagen. PCR products were purified by using a QIAquick PCR Purification kit also from Qiagen. Primers listed in Table S1(b) were used to clone different *sae* genes and promoter fusions. Primers 7000PF and 6300KR, 6100PF and 5702KR, 5700PF and 4900KR, 4900PF and 3900KR were used to clone *saeP*, *saeQ*, *saeR* and *saeS*, respectively. Similarly, primers 7000PF and 3900KR, 6100PF and 3900KR, 5700PF and 3900KR, 6100PF and 4900KR, and 7000PF and 4900KR were used to clone *saePQRS*, *saeQR*, *saeRS*, *saeR* and *saeS*, respectively. For transcriptional *blaZ* fusions, primers 7300PF and 6900KR, 6500PF and 6300KR, 6100PF and 5700KR were used to clone *P_3saec*, *P_blaZ* and *P_sae* promoters, respectively. Promoters were cloned into pRN7240, upstream of the *blaZ* reporter.
Table 1. Strains and plasmids

<table>
<thead>
<tr>
<th>Strain or plasmid</th>
<th>Genotype/description</th>
<th>Reference or source</th>
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<tbody>
<tr>
<td><strong>E. coli</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>DH5α</td>
<td>F' 80d lacZΔM15 (lacZYA-argF) U169 deoR recA1 endA1 hsdR17 (r_{K} m_{K})&lt;sup&gt;+&lt;/sup&gt;</td>
<td>Promega</td>
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<tr>
<td><strong>S. aureus</strong></td>
<td></td>
<td></td>
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<tr>
<td>RN420</td>
<td>Restriction-deficient mutant of strain 8325-4</td>
<td>Kreiswirth et al. (1983)</td>
</tr>
<tr>
<td>RN6734</td>
<td>813 lysogen of RN6390B (group 1 agr)</td>
<td>Novick et al. (1993)</td>
</tr>
<tr>
<td>RN2076</td>
<td>813 lysogen of RN6911</td>
<td>Novick et al. (1993)</td>
</tr>
<tr>
<td>HF6134</td>
<td>RN6734 saeP::bursa*</td>
<td>Bae et al. (2004)</td>
</tr>
<tr>
<td>HF6131</td>
<td>RN6734 saeS::bursa</td>
<td>Bae et al. (2004)</td>
</tr>
<tr>
<td>HF6160</td>
<td>RN10149 saeS::bursa; transductant of HF6131</td>
<td>This work</td>
</tr>
<tr>
<td>HF6162</td>
<td>RN10149 saeP::bursa; transductant of HF6134</td>
<td>This work</td>
</tr>
<tr>
<td>HF6165</td>
<td>RN10149 saeR::Tn551; transductant of HF6135</td>
<td>This work</td>
</tr>
<tr>
<td>RN9360</td>
<td>RN6734 rsbU&lt;sup&gt;+&lt;/sup&gt; (complemented)</td>
<td>Novick &amp; Jiang (2003)</td>
</tr>
<tr>
<td>RN9375</td>
<td>RN6734 sigB::Tn917</td>
<td>Novick &amp; Jiang (2003)</td>
</tr>
<tr>
<td>RN10824</td>
<td>RN10149 sigB::Tn917 transductant of RN9375</td>
<td>This work</td>
</tr>
<tr>
<td>RN10825</td>
<td>RN6734 ΔsaeP (in-frame saeP deletion)</td>
<td>This work</td>
</tr>
<tr>
<td>RN10149</td>
<td>Newman</td>
<td>This work</td>
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<td><strong>Plasmids</strong></td>
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<td>Charpentier et al. (2004); This work</td>
</tr>
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<td>pRN7240</td>
<td>pRN7044, pE194 replicon, Tc&lt;sup&gt;R&lt;/sup&gt;</td>
<td>Novick lab collection; Vojtov et al. (2002)</td>
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<td>pRN9160</td>
<td>pCN51 (Em marker replaced with Cm)</td>
<td>Charpentier et al. (2004); This work</td>
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<td>pRN9180</td>
<td>P&lt;sub&gt;C&lt;/sub&gt;sae::blaZ cloned with KpnI and PstI sites in pRN7240</td>
<td>This work</td>
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<td>pRN9181</td>
<td>saeP cloned with KpnI and PstI sites in pRN9160</td>
<td>This work</td>
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<td>pRN9183</td>
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<td>This work</td>
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<td>pRN9184</td>
<td>saeR cloned with KpnI and PstI sites in pRN9160</td>
<td>This work</td>
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<tr>
<td>pRN9185</td>
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<td>This work</td>
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<tr>
<td>pRN9186</td>
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<td>This work</td>
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<td>saePQRS cloned with KpnI and PstI sites in pRN9160</td>
<td>This work</td>
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<td>saeQRS cloned with KpnI and PstI sites in pRN9160</td>
<td>This work</td>
</tr>
<tr>
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<td>saeRS cloned with KpnI and PstI sites in pRN9160</td>
<td>This work</td>
</tr>
<tr>
<td>pRN9190</td>
<td>saeQR cloned with KpnI and PstI sites in pRN9160</td>
<td>This work</td>
</tr>
<tr>
<td>pRN9191</td>
<td>saePQR cloned with KpnI and PstI sites in pRN9160</td>
<td>This work</td>
</tr>
<tr>
<td>pRN9192</td>
<td>saePR::blaZ, saeP&lt;sub&gt;A&lt;/sub&gt; cloned with KpnI and PstI sites in pRN7240</td>
<td>This work</td>
</tr>
<tr>
<td>pRN9193</td>
<td>saeP&lt;sub&gt;b&lt;/sub&gt;::blaZ, saeP&lt;sub&gt;A&lt;/sub&gt; cloned with KpnI and PstI sites in pRN7240</td>
<td>This work</td>
</tr>
<tr>
<td>pMAD</td>
<td>Plasmid used to construct saeP in-frame deletion mutant</td>
<td>Toledo-Araná et al. (2005); a gift from J. R. Penades</td>
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</tbody>
</table>

*K*sa<sup>+</sup>* and sae<sup>S</sup>* are the sae<sup>S</sup> genes from strains RN6734 and Newman, respectively.

*`bursa' indicates the bursa aurealis transposon.*

In KpnI and PstI restriction sites. pRN7240 was constructed by replacing the pT181 replicon and Em<sup>R</sup> marker of pRN7044 with the pE194 replicon and tet<sup>R</sup> marker. pRN9160 contains the cadmium-inducible P<sub>Cad</sub> promoter, which has a relatively high basal activity and is generally used without cadmium induction (Novick, 1991). All plasmids were first transferred into S. aureus RN4220 then to other S. aureus strains by standard transduction techniques with phage 80α (Novick, 1991).

**Construction of in-frame saeP mutant.** An in-frame saeP deletion was constructed using pMAD (kindly provided by José R. Penades, Centro de Investigación y Tecnología Animal, Instituto Valenciano, Spain). We amplified 1 kb regions at each end of saeP, including four and six amino acid codons at the N- and C-termini, respectively, using primers listed in Table S1(b). These segments were cloned to pMAD and the resulting construct electroproporated into RN4220. Duplications resulting from the Campbell insertion were eliminated by outcrossing and the mutation confirmed by sequencing.

**β-Lactamase assays.** To 50 μl of sample 10 μl 1 μM sodium azide was added. Nitrocefin (50 μl) was added as β-lactamase substrate; activities were recorded using a Thermomax ( Molecular Devices) microtitre plate reader. β-Lactamase units are defined as [V<sub>max</sub>/OD<sub>650</sub>] (Li et al., 1997).

**Coagulase test.** To 100 μl samples, 100 μl rabbit plasma (BBL coagulase plasma, rabbit with EDTA) was added and the mixture incubated at 37 °C for 3 h. Results of twofold dilution series were interpreted as titles, the reciprocal of the highest dilution showing coagulation.
RESULTS

Previous studies had suggested that the upstream ORFs, saeP and Q, had important but undefined roles in sae regulation (Novick & Jiang, 2003). To commence the study of their roles, we analysed the effects of available bursa insertions in these two ORFs. As the saeQ insertion was downstream of P_Asae, its polarity made it uninformative and we have not studied it for this report, concentrating on the saeP::bursa insertion. Although we expected that the saeP transposon insertion would also have polar effects, we found that such effects could not explain all of its properties, and therefore that a comparison of the four congenic strains illustrated in Fig. 1 could provide us with insights into the workings of the locus.

Phenotypic comparison

On sheep blood agar, the wild-type (WT) strain RN6734 and the saeP::bursa derivative produced high and comparable levels of α- and δ-haemolysins, while the saeR::Tn551 and saeS::bursa derivatives produced only δ-haemolysin (Fig. 1a). This shows that the saeP insertion is not significantly polar on saeR and S, confirms that saeR and S are required for α-toxin production as previously reported (Giraudo et al., 1994b), and suggests that saeP is not required. For most of the phenotypic comparisons, we sampled at three time points, starting with a culture density of 50 Klett units, namely t₀, t₃ and t₆. Growth curves of WT and different sae mutants are shown in Fig. 1(b).

To provide a broader comparison of the phenotypes of the four strains, we analysed their exoprotein profiles during the exponential and post-exponential phases of growth (Fig. 2a, b). During the exponential phase, the profiles of WT and saeR and S mutants contained few proteins and looked very similar. A marked increase in the level and number of proteins was observed with the saeP::bursa mutant. One of the proteins induced by the saeP::bursa mutation was identified as coagulase, and a titration assay of coagulase confirmed coagulase production by this mutant but not by the WT or the two other mutants (Table 2). Northern blot hybridization with a coa-specific probe indicated that the observed regulation of coa is at the level of transcription (Fig. 2c). This was surprising for two reasons: (i) coa, the coagulase gene, has a σ⁸⁶ promoter and RN6734, a variant of 8325-4, is σ⁸⁶ defective owing to a deletion in rsbU, whose product is required for σ⁸⁶

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Fig. 2. Effects of transposon insertions in sae genes P, R and S. (a) Exoprotein profile at time t₀ (exponential phase culture at 50 Klett units). (b) Exoprotein profile at time t₆ (6 h incubation at 37 °C with shaking after t₀). (c) Northern blot at time t₀ with probes as indicated at left. (d) Tween zymogram (for lipase) at time t₆. (e) Northern blot at time t₆. Bacteria were grown in CYGP without glucose.
activation (Kullik et al., 1998); (ii) the saeR/S TCS has been reported to upregulate coa (Giraud et al., 1994a) and these data would suggest that saeP or a regulatory gene affected by the insertion in saeP downregulates it. Further, the expression of fnbB, encoding fibronectin-binding protein B, has also been reported to be dependent on σ\(^B\) (Bischoff et al., 2004) and fnbB is also expressed in the saeP::bursa mutant.

The post-exponential-phase exoprotein profiles present a very different picture. The WT and saeP mutant profiles are similar and are characterized by many abundant proteins, whereas the saeR and S mutant profiles are almost identical and are characterized by few, relatively weak bands (Fig. 2b). These results are consistent with upregulation by the TCS of many exoprotein genes (Giraud et al., 1997) and with the lack of any important effect of saeP::bursa on the TCS. A significant difference between the WT and saeP mutant profiles is the pronounced pro-Geh (lipase) band in the latter, suggesting that pro-Geh is not processed. A Tween (lipase) zymogram confirmed this (Fig. 2d). Aureolysin is the major pro-Geh processing protease and Northern blot hybridization with an aur (aureolysin gene)-specific probe has shown that aur is not transcribed in the saeP mutant (Fig. 2e), although it is well-transcribed in the WT and the saeR and S mutants. This result suggests that aur is upregulated by saeP and downregulated by saeR and S. The Northern blotting data for the three mutants reveal the effects of upstream and downstream mutations and indicate that there is differential regulation of several target genes in addition to aur and geh, including hla (the \(\alpha\)-haemolysin gene) and map, encoding a secreted adhesin.

**sae transcription pattern**

The observed effects of the saeP::bursa mutation on the transcription of target genes suggested that saeP was involved in the internal regulatory circuitry within the sae locus. Accordingly, we analysed the effect of this insertion on the sae transcription pattern, in comparison with the bursa insertions in saeR and S. As noted above, we have previously observed that the RN6734 WT sae locus undergoes a dramatic change in which transcription from the downstream promoter, \(P_s\text{sae}\), generating transcript A, declines and that from the upstream promoter(s), \(P_c\text{sae}\), generating transcripts B, C and D, increases sharply in mid-exponential phase (Fig. 3). Transposon insertions had major effects on this pattern. Thus, we observed that a Tn551 insertion in saeR eliminates transcripts B, C and D, and causes constitutive synthesis of an elongated transcript A (A* in Fig. 3) (Novick & Jiang, 2003), suggesting that saeR and S signalling autoinduces \(P_c\text{sae}\) and autorepresses \(P_s\text{sae}\). In the present study, we analysed the effects of bursa insertions in saeR and in saeP. In the saeP::bursa mutant, the upstream transcripts B, C and D were eliminated, as predicted. We were surprised to observe, however, that in the saeP::bursa mutant they were also eliminated. Elimination of C and D was expected since the insertion site is within the region specifying these transcripts (Fig. 3). Elimination of B was unexpected: its 5′ end is a considerable distance downstream of the bursa insertion and it has been reported that B is transcribed from an independent promoter, \(P_b\text{sae}\) (Steinhuber et al., 2003). However, a \(\beta\)-lactamase fusion containing the region expected to include the putative \(P_b\text{sae}\) had no activity in any of the strains tested, including WT RN6734 and Newman. Fusions with larger promoter fragments encompassing both \(P_c\text{sae}\) and \(P_b\text{sae}\) or \(P_c\text{sae}\) and \(P_b\text{sae}\) had the same activity as the \(P_s\text{sae}\) or \(P_c\text{sae}\) fusion (not shown). Therefore, it is suggested that B is a processed form of C rather than a de novo transcript. In the

![Fig. 3. Northern blot analysis of the sae locus in WT (RN6734) and bursa mutants at times \(t_0\) and \(t_6\) with different sae transcripts indicated on the right. A combined probe specific for saeR and saeP was used. A* is the transcript described in our previously published report (Novick & Jiang, 2003). The primers are listed in Supplementary Table S1(a).](image-url)
saeP::bursa mutant, transcript A was synthesized throughout growth, as in the saeR::Tn551 mutant. These results suggest that the insertion in saeP affects either the level or activation of saeR. They do not, however, explain the difference in target gene expression between the saeP and saeR transposon mutants.

**Pc**sae** promoter activity**

Since saeR and S upregulate Pc_sae (Novick & Jiang, 2003; Steinhuber et al., 2003), and since the saeP::bursa insertion has a major effect on the sae transcription pattern, it seemed likely that the regulation of Pc_sae would be affected. Accordingly, we constructed and tested a Pc_sae–blaZ fusion. The plasmid containing this fusion was introduced into the WT and saeP, saeR and saeS mutants and β-lactamase activity was measured as a function of time in a standard growth experiment. As shown in Fig. 4(a), Pc_sae activity in the WT was low during exponential growth and later increased about sevenfold, while in the saeR and saeS mutants, Pc_sae activity was barely detectable. However, in the saeP mutant, Pc_sae activity was surprisingly high. During early exponential phase, it was eightfold higher than the WT and did not greatly increase beyond this point. The low Pc_sae activity in the saeR and S mutants suggests that the TCS upregulates Pc_sae, and the high Pc_sae activity in the saeP::bursa mutant suggests that saeP or some other regulatory gene whose expression is affected by the insertion downregulates Pc_sae. The mutation thus has a complicated polar effect on the downstream elements of the sae locus; it eliminates readthrough from Pc_sae to saeQ, R and S, and is predicted to eliminate the post-exponential phase blockage of expression from P_Asae.

**Fig. 4.** β-Lactamase activities of a Pc_sae–blaZ transcriptional fusion in different strains, over time. WT refers to the wild-type strain (RN6734 in this experiment). P, R and S refer to strains with bursa insertions in saeP, R and S, respectively. (a) Time-course of Pc_sae expression in CYGP at pH 7.5 without salt or glucose added. (b) Effects of 1 M NaCl and pH 5.5 on Pc_sae expression over time as indicated. The effect of NaCl was evaluated by incorporating NaCl at 1 M into CYGP without glucose. The effect of low pH was evaluated by adjusting the pH of the medium to 5.5. (c) Complementation of saeP::bursa by sae subclones. Letters indicate sae gene segments cloned to pCN51 under control of Pcad. Samples were assayed at time t3 under standard growth conditions for the β-lactamase activities of the Pc_sae::blaZ fusion present in these strains. WT, wild-type strain (RN6734); V, strain containing vector (pRN9160).
The opposing effects of the upstream and downstream sae mutations were also observed with sae target genes geh and tst (encoding TSST-1).

What, then, is responsible for the saeP::bursa phenotype? This phenotype is clearly distinct from the WT or the other sae mutants analysed.

Effects of the saeP::bursa mutation on response to environmental factors

The expression of virulence genes, particularly tst, is exquisitely sensitive to a variety of environmental conditions, including subinhibitory concentrations of antibiotics, high concentrations of NaCl and low pH (Chan & Foster, 1998; Weinrick et al., 2004). Several studies have shown that sae plays an important role in mediating this response (Novick & Jiang, 2003; Weinrick et al., 2004) and that the Pc{sae} activity and, consequently, the post-exponential sae transcription pattern are affected, suggesting that sae may sense and respond to these conditions. Previous studies (Novick & Jiang, 2003; Weinrick et al., 2004) have shown that high salt and low pH block the mid-exponential activation of Pc{sae} and the post-exponential activation of P{tst}, raising the possibility that saeP and/or saeQ, which would not be expressed, transduce these responses. If SaeP were the transducer, then Pc{sae} would no longer be responsive to these signals in strains with the saeP::bursa insertion. This was, indeed, the case, as shown in Fig. 4(b). In this study, we questioned whether the high activities of Pc{sae} in the saeP::bursa mutant were still sensitive to these environmental signals. As demonstrated with blaZ fusions, the bursa insertion in saeP eliminated the inhibition by high salt and altered the response to mild acid of the Pc{sae} promoter (Fig. 4b).

From all of the above data, it is concluded that the bursa insertion in saeP affects exoprotein production at the transcriptional level, causes a dramatic increase in Pc{sae} expression, eliminates the inhibitory effects of high salt and alters the response to low pH.

Effects of an in-frame saeP deletion

To determine whether inactivation of saeP is responsible for the observed phenotype of the saeP::bursa insertion, we constructed an in-frame deletion of saeP. Remarkably, this mutation had no detectable effect on the exoprotein profiles (supplementary Fig. S1A), coagulate activity (Table 2) or Northern blotting patterns for coa, fmbB, geh or aur (not shown). There was also no discernible difference between the sae transcription pattern of the in-frame deletion and the WT except that transcripts B, C and D were commensurately shorter (data not shown). The results with the Pc{sae}-blaZ fusion were consistent with the Northern blotting data. The Pc{sae} activity in the mutant and WT were indistinguishable (supplementary Fig. S1B) and the responses to low pH and high salt were the same in the mutant and WT. The effects of the saeP::bursa mutation are thus dramatically different from those of the saeP in-frame deletion and suggest that inactivation of saeP is not responsible for the saeP::bursa phenotype.

Complementation tests

The lack of any effect of the in-frame deletion in saeP suggested that the phenotypic effects of the saeP::bursa mutation would not be complemented by the cloned saeP. As shown in Fig. 4(c), this was confirmed with a plasmid containing saeP under control of the exogenous Pcad. Thus it seemed likely that one or more of the downstream genes are responsible for these effects. Accordingly, we cloned saeQ, R and S individually behind this same promoter on a multicopy vector. These constructs were introduced into the saeP::bursa mutant strain, containing also the Pc{sae}-blaZ fusion, and β-lactamase activity was measured as a function of time. As shown in Fig. 4(c), Pcad::saeS suppressed the elevated Pc{sae} activity of the mutant, restored the response to environmental signals (not shown) and blocked production of coagulate (Table 2). Thus the high level of SaeS fully complemented the saeP::bursa mutation. As expected, SaeS also complemented the saeS::bursa mutation (not shown). In other words, the increased expression of saeS, driven by Pcad and on a multicopy plasmid, downregulated the very high Pc{sae} activity of the saeP::bursa mutant and, separately, upregulated the very low Pc{sae} activity of the saeS::bursa mutant. In both cases, Pc{sae} activity was restored to the intermediate level characteristic of the WT. These results suggest that either expression of the saeRS signalling module from Pc{sae} — such as in the wild-type configuration — or the overexpression of saeS — such as from a high-copy plasmid — may affect sae-responsive genes differently than when the saeRS signalling module is driven by Pc{sae} (as with the saeP::bursa mutant). Alternatively saeS may have different functions depending on its activation state: that is, activated in the WT strain or upon expression from the clone, or not activated in the saeP::bursa mutant. Since many signal transducers at an increased dosage are activated independently of the usual signal, these possibilities cannot be distinguished on the basis of available data. The fact that saeS could not complement the saeR mutant but saeR could (Fig. 3a, d) indicates that saeR, the response regulator, is absolutely required for z-haemolysin production, and, as shown in Fig. 4(a), for Pc{sae} activity. As expected, the vector (V) alone does not affect the haemolytic pattern of the WT or S and R mutants (Fig. 3b). The fact that saeR could not complement either the saeP or saeS::bursa mutations suggests that saeR alone, even at an increased dosage, is not activated independently of an activated cognate signal transducer saeS, or that an additional function is required. The fact that neither saeP nor saeQ could complement any of the mutants suggests that neither has a role independent of the TCS.

To analyse the interactions and dependencies of the four sae genes, we constructed additional Pcad clones with the
sae genes in different combinations. We introduced the plasmids carrying these clones into the different mutant strains harbouring the P_csaeblaZ fusion and assayed β-lactamase activity. As expected, all combinations with saeS, including S alone, RS, QRS and PQRS, complemented the bursa insertions in both saeP (Fig. 4c) and saeS (not shown), and all combinations with R, except for R alone, but including QR, RS, PQR, QRS and PQRS, complemented saeP::bursa (Fig. 4c) and saeR::Tn551 (Fig. 5c). However, surprisingly, QR complemented both saeP and saeS::bursa mutations (Figs 4c and 5a) even though neither gene individually complemented them. A possible explanation for the saeQ result is that saeQ on a high-copy plasmid titrates saeR, since P_Asaes is within the saeQ coding sequence (Fig. 1a) and is repressed by saeR (Novick & Jiang, 2003). A possible explanation for the saeR result is that saeR evidently does not get activated in the absence of saeS or saeQ. Although saeQ and R are translationally coupled, they do not appear to be required in cis because the saeR clone alone complements the R mutant. In other words, SaeQ has a specific role in sae regulation.

The complementation results suggest that saeQ plays a critical role in sae function, that it acts in conjunction with saeR, that saeS is not required when saeQ and R are expressed from the plasmid and therefore that the role of saeS is either to increase the level of saeQ and R or to activate them. One possible explanation of the effect of the saeP::bursa mutation is that saeR and S expressed from P_Asaes are not activated; an alternative possibility is that they are not produced in sufficient amounts to exert their normal regulatory role.

**Strain variation**

Not surprisingly, this remarkable and rather subtle regulatory scheme shows variation among _S. aureus_ strains, as observed by Blickwede et al. (2005) and by ourselves (unpublished data). For example, the activity of P_csaes in strain Newman is similar to that seen with the saeP::bursa insertion in RN6734, namely a high level of activities (Fig. 5a). One possibility was that the difference was a reflection of σ^B^ activity, which in Newman is typical of most _S. aureus_ strains, but in RN6734 (and all other NCTC 8325 derivatives) is greatly attenuated owing to an inactivating deletion in rsbU (Bischoff et al., 2001). Accordingly, we introduced a σ^B^-inactivating Tn551 insertion into Newman and into an rsbU^+ derivative of RN6734 and compared P_csaes activity in the resulting σ^B^ negative strains with that in the respective σ^B^+ strains. Although σ^B^ had a modest stimulatory effect on P_csaes, only at _t_0, it is clearly not responsible for the difference in P_csaes activity between the two strains (data not shown).

**Fig. 5.** Haemolytic activities on sheep blood agar. Strains were streaked against RN4220, which produces only β-haemolysin (turbid zone). (a) Complementation of saeS::bursa and saeR::Tn551 by saeR (left) or saeQR (right), cloned to pCN51 behind Pcad. (b) Haemolytic pattern of control strains: WT (RN6734), saeS::bursa and saeR::Tn551 (left) or V (vector alone) (right). (c) RN6734-saeR::Tn551 containing the indicated sae subclones, except at upper left, RN6734 (WT) with vector, V (pRN9160). (d) Upper left, WT (RN6734); upper right, saeR::Tn551; lower left, saeR::Tn551 with vector (pRN9160); lower right, saeR::Tn551 with plasmid containing Pcad::saeS. Wide clear zones indicate α-haemolysin activity; Narrow clear zones at the intersection with the β-haemolysin zone indicate δ-haemolysin activity.
Fig. 6. Functional comparison of saeS genes of strains Newman (saeSN) and RN6734 (saeSW). (a) Pc-sae::blaZ activities in Newman WT, RN6734 WT, RN6734 with the saeP::bursa mutation (saePN::bursa) and Newman with this mutation (saePN::bursa). Strains were assayed with vector alone (grey bars), with cloned saeSN (black) or with cloned saeSN (white). (b) Downregulation of Pc-sae in Newman by cloned RN6734 saeRS (saeRSN): effects of 1 M NaCl and pH 5.5 are included. (c) Exoprotein profiles of Newman WT and of bursa insertions in Newman saeP or S. (d) Cross-complementation of Newman and RN6734 saeP::bursa by cloned saeSN and cloned saeSN, as revealed by exoprotein profiles at time t0. Lanes: 1, RN6734; 2, RN6734 saeP::bursa (cloned saeSN); 3, RN6734 saeP::bursa (cloned saeSN); 4, Newman; 5, Newman (cloned saeSN); 6, Newman (cloned saeSN).

Additionally, the complex sae transcription pattern that develops during growth in RN6734 is fully developed in Newman at t0 (not shown), the Newman exoprotein profile at t0 is considerably more robust than that of RN6734 (Fig. 6c), and Pc-sae in Newman does not respond to the saeP::bursa insertion, or to either high salt or low pH (see below). We have initiated the following study of this difference.

Missense mutation in saeS

We first considered the possibility that there might be a difference between the two sae loci, and found by sequencing that there was a single base substitution in saeS of Newman compared to saeS of RN6734. This base substitution, T53C, resulting in the substitution of proline for leucine at position 18, has recently been confirmed by whole-genome sequencing (Baba et al., 2007). SaeS is predicted to have a single pair of transmembrane segments, in the N-terminal region of the protein, separated by an extracellular segment of 9 amino acid residues; the L18P substitution is within the first of these and is predicted to disrupt the surrounding α-helical configuration, changing it to a β-sheet. This change, within the putative receptor domain, could have a significant effect on the signalling function of the protein. To determine whether this difference was responsible for the difference in sae function in the two strains, we performed a series of cross-complementation experiments, introducing the cloned saeS from Newman (saeSN) into an saeS-negative RN6734 and the cloned saeS from RN6734 (saeSN) into Newman, testing for saeS function by means of the Pc-sae-blaZ fusion, by exoprotein profiling and by coagulase production. As shown in Fig. 6(a, d), the cloned saeSN converted the functionality of saeS in Newman to that in RN6734, and vice versa, with respect to Pc-sae expression and overall exoprotein profiles. Fig. 6(d) shows that Newman has a fairly robust exoprotein profile at t0 (lane 4), which is unaffected by the cloned saeS gene from Newman (saeSN) (lane 5), but is converted to the very weak profile seen with RN6734 (lane 1) by the cloned saeS gene from RN6734 (saeSN). Additionally, the saeP::bursa insertion enhances the exoprotein profile of RN6734 and this is unaffected by cloned saeSN (lane 2 – compare with Fig. 2a, lanes 1 and 2) but is reversed by saeSN (lane 3). The t0 profiles are shown because these effects are much less apparent at t6.

As shown in Fig. 6(b), Pc-sae in Newman did not respond to high salt and was stimulated by pH 5.5, especially at t0, in contrast to its behaviour in RN6734 (Fig. 2a); inhibition by both high salt and low pH was restored in Newman by
the cloned saeS<sub>W</sub>. saeS<sub>W</sub> also inhibited coagulase production by Newman, whereas saeS<sub>Q</sub> did not, as shown in Table 2.

**DISCUSSION**

Given that sae contains a classical TCS, the normal presumption would be that the response regulator, saeR, is responsible for the regulatory activities of the locus, and this presumption is entirely consistent with the regulatory functions initially described by Giraud et al. (1999). However, transcription data of the sae locus suggested that the two genes upstream of the TCS, saeP and Q, might be involved. In this study, we have begun to examine the functions of these genes. We observed that a bursa transposon insertion in saeP had a striking phenotype, different from the WT or TCS mutants, suggesting that saeP had an independent regulatory role. However, an in-frame deletion of saeP had no detectable phenotype.

Two key observations provided a possible interpretation for these strange and seemingly paradoxical results. First, a comparison of NCTC 8325 strains, such as RN6734, with strain Newman revealed that the latter strain had many of the properties described for the saeP::bursa mutant of RN6734; thus it showed substantial production of coagulase and high activity of P<sub>C</sub>sae, which did not respond to high salt or low pH. These properties of the Newman sae locus were explained by an amino acid substitution in saeS, L18P, in the first transmembrane helix, which is predicted to convert the a-helix in this region to a b-sheet, and which would be expected to have a major effect on the signalling properties of the protein. This substitution, incidentally, is unique to Newman in that it is not present in any of the other genome-sequenced strains. These properties of strain Newman were all converted to the corresponding properties of RN6734 by a plasmid containing the RN6734 saeS gene, whereas a plasmid containing the Newman saeS gene had no effect. Conversely, an RN6734 saeS::bursa mutant containing the cloned Newman saeS had a phenotype similar to that of the native Newman strain, whereas the plasmid containing the RN6734 saeS gene restored the phenotype of RN6734 saeS::bursa to that of RN6734. In other words, this finding suggests that the phenotype of the saeP::bursa insertion in RN6734 might be the result of an effect on saeS signalling. To explain the apparent difference between Newman WT and Newman saeS::bursa, we suggest that SaeS has two different activities depending on its transmembrane signalling function. Our view is that the saeS mutation in Newman does not represent a knockout, but rather locks SaeS in a configuration specific for one of these activities, whereas the bursa insertion simply destroys the gene, eliminating both.

The second clue was provided by a series of complementation experiments (see Figs 4c and 6), in which various sae subclones were tested for their ability to restore the phenotype of the saeP::bursa mutation, as well as that of other sae mutants to WT. Given that SaeP had no detectable activity, it was not surprising that an saeP clone did not restore the phenotype of the saeP::bursa insertion mutant to that of the WT. The phenotype was restored, however, by each of several sae subclones containing saeS, supporting the idea that the phenotype of the saeP::bursa insertion represented a deficiency in saeS signalling. Since saeQ is expressed by transcription from P<sub>C</sub>sae but not by transcription from P<sub>A</sub>sae, we tested the cloned saeQ for complementation of saeP::bursa; although this result was negative, the cloned saeQ and R, but not the cloned saeR, restored the phenotype of the saeP::bursa strain to that of the WT. Although this result suggests that saeQ is necessary, it may not show the expected complementation because it contains P<sub>A</sub>sae, which would be likely to titrate saeR. Further, saeQ does not seem necessary in the presence of high gene dosages of saeR and S, as also shown in Fig. 4(c).

We have formulated a working hypothesis based on these observations, which has several features, as follows. (i) SaeR, like other response regulators, exists in two (phosphorylation?) states which have different activities. (ii) SaeQ is required for the conversion (dephosphorylation?) of saeR from one state to the other. (iii) It can accomplish this in the absence of saeS. We note that saeQ is not expressed in the saeP::bursa mutant, since transcript C is eliminated and saeR, and, in this form, induces the expression of P<sub>C</sub>sae, the expression of coa, fnbB, map, etc. (v) The L18P substitution in Newman also prevents the conversion of saeR, and is responsible for the phenotype of Newman and its resemblance to the saeP::bursa of RN6734.

**ACKNOWLEDGEMENTS**

We are grateful to Dunrong liang for expert technical assistance. Thanks to Brian Weinrick for plasmid pRN7240. This work was supported by a grant-in-aid from the Skirball Institute to R. P. N.

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Edited by: J. A. Lindsay