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, P_{Asae} , which transcribes only the TCS, and P_{Csae} , 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 P_{Asae}) 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.

Received 2 August 2007
Revised 6 December 2007
Accepted 7 December 2007

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* (Giraud *et al.*, 1994a), which profoundly affected the expression of a large set of virulence genes (Giraud *et al.*, 1997), largely, but not entirely, parallel to the regulatory activities of the *agr* system (Giraud *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 (Giraud *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, P_{Asae} and P_{Csae} , as diagrammed in Fig. 1(a). Transcript A, 2.1 kb, initiated from promoter P_{Asae} and covering *saeR* and *saeS*, is present at the earliest time point and diminishes in intensity during the mid-exponential phase of growth. P_{Asae} is within the *saeQ*

coding sequence. Transcripts B (2.4 kb), C (3.1 kb) and D (0.5 kb), all initiated from P_{Csae} , 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. P_{Csae} is (auto)induced by *saeR* and *S*. Thus, *saeR* and *saeS* are transcribed throughout growth, first from promoter P_{Asae} and then from P_{Csae} . The mid-exponential-phase activation of P_{Csae} 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

Abbreviation: TCS, two-component signalling module.

A supplementary figure, and tables of primers, are available with the online version of this paper.

Fig. 1. The *sae* locus. (a) The *sae* locus consists of four genes, and is transcribed from two promoters, $P_{A_{sae}}$ and $P_{C_{sae}}$, generating four transcripts (A, B, C and D) as shown. The two *bursa aurealis* insertions and the single Tn551 insertion used in this study are indicated by triangles and their haemolytic phenotypes on sheep blood agar are depicted above (see text). (b) Growth curves of *saeP* (●), *S* (◆) and *R* (▲) mutants and the WT strain RN6734 (■). Cultures were grown in side-arm flasks in CYGP without glucose. t_0 was set at 50 Klett units. The arrows indicate the sampling points t_0 , t_3 and t_6 .

a sharp upregulation of $P_{C_{sae}}$, probably owing to subtle effects on downstream components of the *sae* locus.

The upregulation of $P_{C_{sae}}$ 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 $P_{C_{sae}}$ 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 $\sim 3 \times 10^8$ cells ml^{-1} . Bacteria stored at -80°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°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 recentrifuged to remove any residual organisms, then precipitated with a 10% volume of 50% trichloroacetic 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°C . White precipitation represents 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 [α - ^{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' *Pst*I or *Kpn*I sites were used to clone *sae* genes under the *Pcad* 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*, *saeQRS*, *saeRS*, *saeQR* and *saePQR*, respectively. For transcriptional *blaZ* fusions, primers 7300PF and 6900KR, 6500PF and 6300KR, 6100PF and 5700KR were used to clone $P_{C_{sae}}$, $P_{B_{sae}}$ and $P_{A_{sae}}$ promoters, respectively. Promoters were cloned into pRN7240, upstream of the *blaZ* reporter

Table 1. Strains and plasmids

Strain or plasmid	Genotype/description	Reference or source
<i>E. coli</i>		
DH5 α	F ⁻ ϕ 80d <i>lac</i> ZM15 (<i>lac</i> ZYA- <i>arg</i> F)U169 <i>deoR recA1 endA1 hsdR17</i> (r _K ⁻ m _K ⁺)	Promega
<i>S. aureus</i>		
RN4220	Restriction-deficient mutant of strain 8325-4	Kreiswirth <i>et al.</i> (1983)
RN6734	ϕ 13 lysogen of RN6390B (group I <i>agr</i>)	Novick <i>et al.</i> (1993)
RN7206	ϕ 13 lysogen of RN6911	Novick <i>et al.</i> (1993)
HF6134	RN6734 <i>saeP</i> :: <i>bursa</i> *	Bae <i>et al.</i> (2004)
HF6135	RN6734 <i>saeR</i> ::Tn551	Novick & Jiang (2003)
HF6131	RN6734 <i>saeS</i> :: <i>bursa</i>	Bae <i>et al.</i> (2004)
HF6160	RN10149 <i>saeS</i> :: <i>bursa</i> ; transductant of HF6131	This work
HF6162	RN10149 <i>saeP</i> :: <i>bursa</i> ; transductant of HF6134	This work
HF6165	RN10149 <i>saeR</i> ::Tn551; transductant of HF6135	This work
RN9360	RN6734 <i>rsbU</i> ⁺ (complemented)	Novick & Jiang (2003)
RN9375	RN6734 <i>sigB</i> ::Tn917	Novick & Jiang (2003)
RN10824	RN10149 <i>sigB</i> ::Tn917 transductant of RN9375	This work
RN10825	RN6734 Δ <i>saeP</i> (in-frame <i>saeP</i> deletion)	This work
RN10149	Newman	Bae <i>et al.</i> (2004)
Plasmids		
pRN7240	pRN7044, pE194 replicon, Tc ^R	Novick lab collection; Vojtov <i>et al.</i> (2002)
pRN9160	pCN51 (Em marker replaced with Cm)	Charpentier <i>et al.</i> (2004); This work
pRN9180	P _{Csae} :: <i>bla</i> Z cloned with <i>Kpn</i> I and <i>Pst</i> I sites in pRN7240	This work
pRN9181	<i>saeP</i> cloned with <i>Kpn</i> I and <i>Pst</i> I sites in pRN9160	This work
pRN9182	<i>saePQ</i> cloned with <i>Kpn</i> I and <i>Pst</i> I sites in pRN9160	This work
pRN9183	<i>saeQ</i> cloned with <i>Kpn</i> I and <i>Pst</i> I sites in pRN9160	This work
pRN9184	<i>saeR</i> cloned with <i>Kpn</i> I and <i>Pst</i> I sites in pRN9160	This work
pRN9185	<i>saeS_W</i> [†] cloned with <i>Kpn</i> I and <i>Pst</i> I sites in pRN9160	This work
pRN9186	<i>saeS_N</i> [†] cloned with <i>Kpn</i> I and <i>Pst</i> I sites in pRN9160 (<i>saeS</i> from Newman)	This work
pRN9187	<i>saePQRS</i> cloned with <i>Kpn</i> I and <i>Pst</i> I sites in pRN9160	This work
pRN9188	<i>saeQRS</i> cloned with <i>Kpn</i> I and <i>Pst</i> I sites in pRN9160	This work
pRN9189	<i>saeRS</i> cloned with <i>Kpn</i> I and <i>Pst</i> I sites in pRN9160	This work
pRN9190	<i>saeQR</i> cloned with <i>Kpn</i> I and <i>Pst</i> I sites in pRN9160	This work
pRN9191	<i>saePQR</i> cloned with <i>Kpn</i> I and <i>Pst</i> I sites in pRN9160	This work
pRN9192	<i>saePB</i> :: <i>bla</i> Z, <i>saeP_B</i> cloned with <i>Kpn</i> I and <i>Pst</i> I sites in pRN7240	This work
pRN9193	<i>saeP_A</i> :: <i>bla</i> Z, <i>saeP_A</i> cloned with <i>Kpn</i> I and <i>Pst</i> I sites in pRN7240	This work
pMAD	Plasmid used to construct <i>saeP</i> in-frame deletion mutant	Toledo-Arana <i>et al.</i> (2005); a gift from J. R. Penadés

*‘*bursa*’ indicates the *bursa aurealis* transposon.

[†]*saeS_W* and *saeS_N* are the *saeS* genes from strains RN6734 and Newman, respectively.

in *Kpn*I and *Pst*I restriction sites. pRN7240 was constructed by replacing the pT181 replicon and Em^R marker of pRN7044 with the pE194 replicon and *tetM* marker. pRN9160 contains the cadmium-inducible *Pcad* promoter, which has a relatively high basal activity and is generally used without cadmium induction (Charpentier *et al.*, 2004; unpublished data). Plasmids were transformed into *Escherichia coli* DH5 α and then moved into *S. aureus* RN4220 by electroporation (Novick, 1991). All plasmids were first transferred into *S. aureus* RN4220 then to other *S. aureus* strains by standard transduction techniques with phage 80 α (Novick, 1991).

Construction of in-frame *saeP* mutant. An in-frame *saeP* deletion was constructed using pMAD (kindly provided by José R. Penadés, 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 electroporated into RN4220. Duplications resulting from the Campbell insertion were eliminated by outcrossing and the mutation confirmed by sequencing.

β -Lactamase assays. To 50 μ l of sample 10 μ l 1 μ M sodium azide was added. Nitrocefin (50 μ l) was added as β -lactamase substrate; activities were recorded using a Thermomax (Molecular Devices) microtitre plate reader. β -Lactamase units are defined as [V_{max}]/OD₆₅₀ (Ji *et al.*, 1997).

Coagulase test. To 100 μ l samples, 100 μ l 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 titres, 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 $P_{A_{sae}}$, 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 (Giraud *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 t_0 , t_3 and t_6 . 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 σ^B promoter and RN6734, a variant of 8325-4, is σ^B defective owing to a deletion in *rsbU*, whose product is required for σ^B

Fig. 2. Effects of transposon insertions in *sae* genes *P*, *R* and *S*. (a) Exoprotein profile at time t_0 (exponential phase culture at 50 Klett units). (b) Exoprotein profile at time t_6 (6 h incubation at 37 °C with shaking after t_0). (c) Northern blot at time t_0 with probes as indicated at left. (d) Tween zymogram (for lipase) at time t_6 . (e) Northern blot at time t_6 . Bacteria were grown in CYGP without glucose.

Table 2. Coagulase titres

<i>Pcad</i> plasmid	RN6734					Newman		
	WT	Δ <i>saeP</i>	<i>saeP</i> ::bursa	<i>saeR</i> ::Tn551	<i>saeS</i> ::bursa	WT	<i>saeP</i> ::bursa	<i>saeS</i> ::bursa
None	<1	<1	128	<1	<1	256	256	4–8
pRN9160 (vector)	<1	<1	128	<1	<1	256	256	4
pRN9185 (<i>saeS_{WT}</i>)	<1	<1	<1	<1	<1	8	4–8	4–8
pRN9186 (<i>saeS_N</i>)			128		128	256	256	256

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 *fmbB*, encoding fibronectin-binding protein B, has also been reported to be dependent on σ^B (Bischoff *et al.*, 2004) and *fmbB* 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 α -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, $P_{A\text{sae}}$, 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 $P_{A\text{sae}}$. In the present study, we analysed the effects of bursa insertions in *saeS* and in *saeP*. In the *saeS*::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 β -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 $P_{A\text{sae}}$ and $P_{B\text{sae}}$ or $P_{B\text{sae}}$ and $P_{C\text{sae}}$ had the same activity as the $P_{A\text{sae}}$ 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).

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.

P_{Csa} promoter activity

Since *saeR* and *S* upregulate P_{Csa} (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 P_{Csa} would be affected. Accordingly, we constructed and tested a P_{Csa} -*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), P_{Csa} activity in the WT was low during exponential growth and later increased about sevenfold, while in the *saeR* and *saeS* mutants, P_{Csa} activity was barely detectable. However, in the *saeP* mutant, P_{Csa} 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 P_{Csa} activity in the *saeR* and *S* mutants suggests that the TCS upregulates P_{Csa} , and the high P_{Csa} activity in the *saeP*::bursa mutant suggests that *saeP* or some other regulatory gene whose expression is affected by the insertion downregulates P_{Csa} . The mutation thus has a complicated polar effect on the downstream elements of the *sae* locus; it eliminates readthrough from P_{Csa} to *saeQ*, *R* and *S*, and is predicted to eliminate the post-exponential phase blockage of expression from $P_{A_{sae}}$.

Fig. 4. β -Lactamase activities of a P_{Csa} -*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 P_{Csa} expression in CYGP at pH 7.5 without salt or glucose added. (b) Effects of 1 M NaCl and pH 5.5 on P_{Csa} 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 P_{cad} . Samples were assayed at time t_3 under standard growth conditions for the β -lactamase activities of the P_{Csa} ::*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).

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 P_{Csa} 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 P_{Csa} and the post-exponential activation of *Ptst*, raising the possibility that *saeP* and/or *saeQ*, which would not be expressed, transduce these responses. If SaeP were the transducer, then P_{Csa} 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 P_{Csa} 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 P_{Csa} 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 P_{Csa} 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), coagulase 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 P_{Csa} -*blaZ* fusion were consistent with the Northern blotting data. The P_{Csa} 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.

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

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 P_{Csa} -*blaZ* fusion, and β -lactamase activity was measured as a function of time. As shown in Fig. 4(c), $P_{cad}::saeS$ suppressed the elevated P_{Csa} activity of the mutant, restored the response to environmental signals (not shown) and blocked production of coagulase (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 P_{Csa} activity of the *saeP::bursa* mutant and, separately, upregulated the very low P_{Csa} activity of the *saeS::bursa* mutant. In both cases, P_{Csa} activity was restored to the intermediate level characteristic of the WT. These results suggest that either expression of the *saeRS* signalling module from P_{Csa} – 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 $P_{A_{sae}}$ (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. 5a, d) indicates that *saeR*, the response regulator, is absolutely required for α -haemolysin production, and, as shown in Fig. 4(a), for P_{Csa} activity. As expected, the vector (V) alone does not affect the haemolytic pattern of the WT or *S* and *R* mutants (Fig. 5b). 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

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.

sae genes in different combinations. We introduced the plasmids carrying these clones into the different mutant strains harbouring the P_{Csa} -*blaZ* 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_{Asae} 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_{Asae} 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_{Csa} in strain Newman is similar to that seen with the *saeP::bursa* insertion in RN6734, namely a high level of activities (Fig. 6a). 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_{Csa} 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_{Csa} , only at t_0 , it is clearly not responsible for the difference in P_{Csa} activity between the two strains (data not shown).

Fig. 6. Functional comparison of *saeS* genes of strains Newman (*saeS_N*) and RN6734 (*saeS_W*). (a) P_{Csa} activities in Newman WT, RN6734 WT, RN6734 with the *saeP::bursa* mutation (*saeP_W::bursa*) and Newman with this mutation (*saeP_N::bursa*). Strains were assayed with vector alone (grey bars), with cloned *saeS_W* (black) or with cloned *saeS_N* (white). (b) Downregulation of P_{Csa} in Newman by cloned RN6734 *saeRS* (*saeRS_W*): 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 *saeS_N* and cloned *saeS_W*, as revealed by exoprotein profiles at time *t*₀. Lanes: 1, RN6734; 2, RN6734 *saeP::bursa* (cloned *saeS_N*); 3, RN6734 *saeP::bursa* (cloned *saeS_W*); 4, Newman; 5, Newman (cloned *saeS_N*); 6, Newman (cloned *saeS_W*).

Additionally, the complex *sae* transcription pattern that develops during growth in RN6734 is fully developed in Newman at *t*₀ (not shown), the Newman exoprotein profile at *t*₀ is considerably more robust than that of RN6734 (Fig. 6c), and P_{Csa} 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 (*saeS_N*) into an *saeS*-negative RN6734 and the cloned *saeS* from RN6734 (*saeS_W*) into Newman, testing for *saeS* function by means of the P_{Csa} -*blaZ* fusion, by exoprotein profiling and by coagulase production. As shown in Fig. 6(a, d), the cloned *saeS_W* converted the functionality of *saeS* in Newman to that in RN6734, and vice versa, with respect to P_{Csa} expression and overall exoprotein profiles. Fig. 6(d) shows that Newman has a fairly robust exoprotein profile at *t*₀ (lane 4), which is unaffected by the cloned *saeS* gene from Newman (*saeS_N*) (lane 5), but is converted to the very weak profile seen with RN6734 (lane 1) by the cloned *saeS* gene from RN6734 (*saeS_W*). Additionally, the *saeP::bursa* insertion enhances the exoprotein profile of RN6734 and this is unaffected by cloned *saeS_N* (lane 2 – compare with Fig. 2a, lanes 1 and 2) but is reversed by *saeS_W* (lane 3). The *t*₀ profiles are shown because these effects are much less apparent at *t*₆.

As shown in Fig. 6(b), P_{Csa} in Newman did not respond to high salt and was stimulated by pH 5.5, especially at *t*₀, 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_W*. *saeS_W* also inhibited coagulase production by Newman, whereas *saeS_N* 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 Giraudo *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_{Csae}*, 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 α -helix in this region to a β -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_{Csae}* but not by transcription from *P_{Asae}*, 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_{Asae}*, 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 since transcript A is initiated within the *saeQ* coding sequence. (iv) *saeR* expressed in the absence of *saeQ* in the *saeP::bursa* mutant is not converted despite the presence of *saeS*, and, in this form, induces the expression of *P_{Csae}*, the expression of *coa*, *fmbB*, *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 Jiang 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