The staphylococcal saeRS system coordinates environmental signals with agr quorum sensing

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sae is a two-component signal transduction system in Staphylococcus aureus that regulates the expression of many virulence factors at the transcriptional level and appears to act synergistically with agr in some cases. In this study, the interactions between sae and agr have been characterized in some detail. It was found that the sae locus is larger and more complex than originally envisioned, in that it is expressed from several promoters, giving rise to four or five transcripts, at least three of which are initiated upstream of saeRS and contain two additional reading frames, here designated saeP and saeQ, which are likely to have important roles in sae function. The upstream transcripts are induced during exponential phase concomitantly with the onset of RNAIII synthesis and their induction requires the agr effector, RNAIII, but is blocked by several environmental signals that override the effects of RNAIII. saeR is also required for the induction of these transcripts, so that the sae locus contains an autoinduction circuit. It is suggested that sae is downstream of agr in the exoprotein activation pathway (and also epistatic with agr), that it coordinates the effects of environmental signals with the agr quorum-sensing system, and therefore that it is a key intermediary in the overall regulatory strategy by which S. aureus senses and responds to its environment.

INTRODUCTION

Staphylococcus aureus strains produce a large number of extracellular proteins, including virulence factors, which enable the organisms to adapt to various external exigencies, especially the hostile in vivo environment. These extracellular proteins are accessory proteins – not required for the basic processes of growth and multiplication – and, like other accessory proteins, are produced conditionally. For staphylococci, two types of conditional regulatory inputs combine to determine the overall pattern of extracellular protein production: cell density, which activates the agr two-component signal transduction system, an extensive global regulon, and external stimuli, which affect defined sets of exoproteins, referred to as stimulons. These two types of regulatory inputs act through and are coordinated by intracellular regulatory mediators whose activities combine in a complex manner according to growth and environmental conditions, to generate the observed extracellular protein patterns. Pleiotropic mutations affecting the production of extracellular proteins have resulted in the identification of several other two-component systems, saeRS, scrRS, arlAB, an alternative sigma factor, σB, a family of winged-helix transcription factors, SarA and its homologues, and unrelated transcription factors including SvrA (Garvis et al., 2002). Readers are referred to several recent reviews of the activities and interactions of these various regulators, by Cheung & Zhang (2002), Arvidson & Tegmark (2001) and Novick (2003). It is noted that the synthesis of many cytoplasmic proteins is also controlled by these same regulators and these may also have a role in pathogenesis. For present purposes, however, the focus is on the extracellular accessory proteins.

In our studies of the regulation of extracellular protein production, we have thus far concentrated on the agr system. The agr locus controls genes encoding most extracellular staphylococcal proteins, which constitute the agr regulon, and is conserved throughout the staphylococci. Regulation of the component genes is primarily at the level of transcription, though several of the genes are secondarily regulated at the translational level. Nearly all the currently available data on the regulation of extracellular protein genes are from studies on S. aureus; results with other staphylococci are consistent with these (Vuong et al., 2000). In general, during aerobic planktonic growth in vitro, genes encoding secreted proteins are up-regulated during the post-exponential phase, whereas genes encoding surface proteins are up-regulated very early in growth and down-regulated shortly thereafter. The intracellular effector of both types of agr-determined regulation is a regulatory RNA, RNAIII. However, several of the environmental factors acting as external inputs into this regulatory system inhibit the production of protein A (a surface protein), as well as of
many secreted proteins, and act independently of agr, whereas others may interact with agr (Chan & Foster, 1998a; Lindsay & Foster, 1999). saeR appears to be a key element in the regulatory cascade governing the staphylococcal virulon. saeRS was originally identified as a Tn551 insertion with an exoprotein-defective phenotype (Giraudo et al., 1994) and subsequently shown to be a two-component signal transduction module with the transposon insertion in saeR, the putative response regulator gene (Giraudo et al., 1999). In preliminary studies (Ross & Novick, 2001; Novick, 2003), we have observed that sae is a more complicated locus than originally envisioned (Giraudo et al., 1999) and that it has a complex transcriptional pattern that is profoundly influenced by agr and by certain environmental stimuli. In this report, we present a detailed study of the agr–sae interaction, the results of which suggest that sae may have a major role in the integration of cell density signalling with signalling through environmental stimuli and other regulatory elements.

METHODS

Bacterial strains and growth conditions. Strains and plasmids used in this study are listed in Table 1. Strain RC106 containing a Tn551 insertion was kindly provided by R. Nagel (CEFIBO, Serano 669, Buenos Aires 1414, Argentina). The inserted transposon was transduced to RN6734, giving strain RN9808, and to RN7206, giving RN9809. Throughout the text, ‘Agr’ refers to saeR::Tn551, and ‘Agr’ refers to Δagr::tetM, i.e. agr-null. Media and growth conditions were as described previously (Novick, 1991) except where noted. Chloramphenicol, tetracycline and erythromycin were each added to GL agar (Novick, 1991) at 10 mg l\(^{-1}\) not noted. Chloramphenicol, tetracycline and erythromycin were each

### Table 1. Strains and plasmids used in this study

<table>
<thead>
<tr>
<th>Strain/plasmid</th>
<th>Genotype/description</th>
<th>Reference</th>
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<tr>
<td><strong>Strain</strong></td>
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<tr>
<td>RN6734</td>
<td>φ13 Lysogen of RN6390</td>
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<td>RN7206</td>
<td>φ13 Lysogen of RN6911 (Δagr::tetM)</td>
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<td>RN9808</td>
<td>RN6734 saeR::Tn551; transductant of RC106</td>
<td>This work</td>
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<tr>
<td>RN9809</td>
<td>RN7206 Δagr::tetM saeR::Tn551; transductant of RC106, φ13 lysogen</td>
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<td>RN9360</td>
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<td><strong>Plasmid</strong></td>
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<td>pJ524</td>
<td>Naturally occurring β-lactamase plasmid; source of bla repressor</td>
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<td>pRN5548</td>
<td>PC194-based vector; pRN5543::P(_{\text{bla}})</td>
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<td>pRN7143</td>
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<tr>
<td>PRN7144</td>
<td>pRN6848 with KpnI site replacing RNAIII terminator loop</td>
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RESULTS

Comparison of exoprotein profiles

As the Tn551 insertion in saeR had a profound defect in exoprotein production that was quite different from that seen with Agr\(^{-}\) mutants, we began by analysing exoprotein profiles through time. Fig. 1 shows comparative exoprotein profiles of agr, sae and double mutants in comparison with that of a standard wild-type strain, RN6734, using shake-culture supernatants obtained at several time points during standard growth in CYGP broth without glucose. Each of these samples represents the supernatant from 2.5 × 10\(^{10}\) cells. As can be seen, the two mutations have major, but considerably different, effects on exoprotein production. At T = 3 h, the patterns are fairly similar for all four strains, with the major differences being the presence of several high molecular mass bands in the Agr\(^{-}\) and double mutant lanes.
that are absent from the wild-type and Sae<sup>-</sup> and several bands in the wild-type that are absent or greatly diminished in the mutants. There is a rather dramatic change between $T = 3$ h and $T = 6$ h. Many bands disappear and several new bands appear in the wild-type lane but not in the mutants. It is noted that the agr system is activated shortly before $T = 3$ h in this strain (Vandenesch et al., 1991) so that some of the changes seen in the wild-type pattern are attributable to agr, which down-regulates some proteins and up-regulates others. Additionally, however, many bands present in the mutant supernatants at $T = 3$ h disappear by $T = 6$ h. As this is true for both Agr<sup>-</sup> and Sae<sup>-</sup>, it obviously cannot be attributed to down-regulation by agr, and remains unexplained. By the 9 h sample, representing early-stationary phase, the patterns have become more complex; the agr and sae mutant patterns differ considerably from each other and from the wild-type, whereas the agr and double mutant patterns are very similar, suggesting that sae

Table 2. PCR primers used in this study

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<th>Gene</th>
<th>Primer sequence (5’→3’)*</th>
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<td>saeS</td>
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<td></td>
<td>R: GTATCTGATACGACGCC</td>
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<td>R: CATAACAAAGGCTCAGGAAAGAC</td>
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<tr>
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<td></td>
<td>R: CTAAGTCTAGGAAACTAATG</td>
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<td>R: GCCGAGTCTTACATGACACAG</td>
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<td>saeR</td>
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<td></td>
<td>R: GTCCGGAATTCAGTCTTGGAAAATGATGAGGAGGATGGCC</td>
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*F, forward; R, reverse.

Fig. 1. Exoprotein profiles. CYGP broth cultures, without glucose, were sampled at 3, 6, 9 and 15 h and centrifuged. Supernatants, each representing $2.5 \times 10^{10}$ cells, were TCA-precipitated and analysed by SDS-PAGE according to the method of Laemmli (1970). Lanes 1–4 represent strains RN6734 (wild-type), RN9808 (Sae<sup>-</sup>), RN7206 (Agr<sup>-</sup>) and RN9809 (Agr<sup>-</sup> Sae<sup>-</sup>), respectively.
has little, if any, regulatory activity in the absence of a functional agr. The concurrence of agr and sae effects after T = 3 h could indicate an interaction between these two regulators. At a later time point, T = 15 h, several regulatory classes can be readily discerned. These will be detailed at a later date after the identities of the proteins have been determined. Note that these patterns are considerably different from those reported by Nagel and coworkers, who looked only at an 18 h time point and used a different growth medium (Giraudo et al., 1996).

To carry this analysis one step further, we analysed transcription of some of the exoprotein genes. Results with hla, encoding ζ-haemolysin, sspA, encoding V8 serine protease, and spa, encoding protein A, are shown in Fig. 2. As can be seen in Fig. 2(a), at T = 0 h, there is no detectable signal for either RNAIII or hla, whereas at T = 5 h there is a strong signal for RNAIII in the wild-type and Sae− strains, but not in the Agr− or double mutant. hla, however, is detectably transcribed only in the wild-type strain, suggesting that sae is downstream from agr in the regulatory pathway, or possibly the two are epistatic. This result is consistent with that previously reported by Giraudo et al. (1997). Fig. 2(b) shows the results for Agr+ and Agr− strains with the saeR::Tn551 mutant (Giraudo et al., 1994), complemented with pRN7143, which contains a Pbla::saeRS transcriptional fusion, without induction of the Pbla promoter. As can be seen, spa is more strongly expressed in an sae agr double mutant than in a single sae mutant, and the same effect has been reported for an agr mutant (Giraudo et al., 1997). Furthermore, the saeRS clone can complement the sae mutant but not an agr mutant or a double mutant for spa repression; full down-regulation is seen only when both determinants are present. Similarly, sspA is not detectably activated in either of the single mutants or in the double, and is detectably expressed only in the Agr+ Sae− mutant strain complemented by the saeRS clone. Since both regulatory determinants are individually required for each of these genes, sae is epistatic to agr as well as being downstream; if sae were purely downstream of agr, it would fully complement the effect of any agr mutant. Note that the upstream region of the sae locus is intact in the saeR::Tn551 mutant and, as shown below, is induced by the sae two-component signal transduction system.

**Effect of agr on sae transcription**

An analysis of sae transcription in an Agr+ strain (RN6734) through time is shown in Fig. 3(a). As can be seen, there is clearly a switch in the transcription pattern immediately after the onset of RNAIII synthesis, in that a 2-1 kb transcript (A) disappears and two larger ones (B and C) as well as a smaller one (D, which is not shown in this blot) appear. With the agr-null strain (RN7206), the 2-1 kb transcript is made at a diminishing rate throughout growth but at a much lower level, and the larger transcripts are hardly detectable. Similarly, in a sarA mutant, the larger transcripts are barely detectable and the 2-1 kb species behaves as in the wild-type strain. To determine whether RNAIII is responsible for the difference between the Agr+ and Agr− strains, we analysed an agr-null strain complemented by pRN6848, which contains a transcriptional fusion of RNAIII to the inducible staphylococcal β-lactamase promoter, Pbla. As shown in Fig. 3(b), the upstream sae transcripts, B and C, appear following the induction of RNAIII in this strain. It must be pointed out, however, that variations in the sae transcription pattern occur in different strains and under differing growth conditions, such as under different conditions of aeration and with different batches of medium. For example, transcript C is sometimes seen throughout growth and in some experiments a fifth transcript, X, smaller than A, appears. An example of this is seen with the Dra mutant strain in Fig. 6. Under all conditions, however, the switch
from A to B and the appearance of transcript D are RNAIII-dependent.

Note that sae induction is weaker with an RNAIII mutant in which the terminator loop sequence has been replaced with a KpnI site (m), and is not seen with the vector alone (v) or without induction of the Pbla promoter. In contrast, spa and an unrelated response regulator, srrA (Yarwood et al., 2001), are down-regulated by RNAIII, even without induction (Fig. 3b). It is concluded that RNAIII (and not some other agr component) is required for sae activation but sae is not required for agr activation. Thus, sae is downstream from agr in the exoprotein activation pathway, even though RNAIII also acts independently of sae in the regulation of several target genes.

The sae locus

It is clear from these blots that the sae locus is larger and more complex than originally envisioned by Nagel and coworkers (Giraudo et al., 1999). Analysis of the sae transcription pattern at two different time points, T=1 h (representing early-exponential phase) and T=5 h (representing post-exponential phase), using different probes is shown in Fig. 4(b, c). Henceforth, the 2-1 kb sae transcript is referred to as A, the 2-6 kb sae transcript is referred to as B, the 3-1 kb sae transcript is referred to as C and the 0-5 kb sae transcript is referred to as D. These blots show that sae transcripts A, B and C all terminate at or near the end of saeS, and therefore have different 5’ ends. The fourth transcript, D, is seen only with the upstream probe, P, and is therefore homologous to a region between the 5’ ends of B and C. The directionality of this transcript is not known presently. One possibility is that B and C are transcribed from two different promoters and that D is transcribed independently from a third. Another possibility is that C is processed to give B+D. Transcript C includes two ORFs, of 146 and 157 codons, 5’ to saeR, of which the latter is within B. Since the two upstream ORFs are within the sae operon, they are likely to be important for sae function and are here designated saeP and saeQ, respectively. There is a strong potential translational start within saeQ leading to a possible C-terminal protein, SaeQ’. Additionally, the potential secondary structure of the saeR transcript suggests that the translational start of Saer is occluded, that the C-terminal end of the saeQ reading frame overlaps with the saeR start, and therefore that saeQ would have to be translated in order to permit translation of saeR.

The predicted product of saeP has no significant match in the protein database, whereas that of saeQ appears to be a membrane protein that is closely related to a variety of...
transporters, especially an oligopeptide permease (OppA) of Bacillus subtilis. Experiments are in progress to determine whether these two have any role in sae function.

**sae autoregulation**

Examination of the transcription pattern seen with the saeR::Tn551 mutation (Fig. 5, left-hand side) shows that transcript A is present (indicated as A*), is longer owing to the inserted transposon and its presence is prolonged through the post-exponential phase, suggesting that it may be autorepressed by native SaeR. Furthermore, the upstream transcripts are absent (lower panel), suggesting that these may be autoinduced by SaeR. As expected, saeRS, cloned under Pbla control, restores the upstream transcripts in the saeR mutant (not shown). The right-hand part of Fig. 5, in which RNA prepared from the agr-null, the wild-type and the saeR::Tn551 strains was blot-hybridized with a combination of upstream probes P and Q (Fig. 4a), demonstrates both the agr requirement and the saeR requirement for the expression

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**Fig. 4.** Northern blots of sae transcripts. (a) Map of the sae locus. (b, c) Whole-cell RNA was prepared by the method of Komblum et al. (1988), separated on formamide/agarose and blot-hybridized with the indicated probes. Probes were prepared by PCR using [32P]dATP. The probe ‘S’ samples were duplicated and exchanged to provide size comparisons. B, csbB probe; P, saeP probe; Q, saeQ probe; R, saeR probe; S, saeS probe.

**Fig. 5.** sae autoregulation. Left-hand side, time course of saeR expression in strain RN9808 (saeR::Tn551). RNA samples prepared from hourly time points were blot-hybridized with an saeR probe (see R Fig. 4) or with an RNAIII probe. Note that the saeR transcript is greatly elongated owing to the insertion of the 5-2 kb transposon. Note that the upstream transcripts B and C are not present in the lower half of the left panel. Right-hand side, whole-cell RNA prepared from 2 and 5 h samples of cultures of RN7206 ( Agr-), RN6734 (wild-type) and RN9808 (Sae+) were blot-hybridized with a combined probe specific for saeP and saeQ (see Fig. 4).
of the upstream transcripts B, C and D. This result confirms the autoinduction of these transcripts and the cooperation between sae and agr for their activation.

**Effects of \( \sigma^B \) and rsbU**

The alternative sigma factor \( \sigma^B \) has an important role in exoprotein gene regulation, is generally antagonistic to agr and is essentially inactive in strains of the NCTC 8325 lineage owing to an 11 bp deletion in rsbU (Kullik et al., 1998), which is required for \( \sigma^B \) activation. As shown in Fig. 6, replacement of the defective rsbU with a functional copy of the gene (Nicholas et al., 1999) results in considerable attenuation of the sae transcripts, but does not eliminate the transition. Moreover, inactivation of the \( \sigma^B \) gene itself appears to increase sae expression in comparison to the wild-type (RsbU\(^{\text{+}}\)). Also, Fig. 6 shows a direct comparison of the sizes and levels of the sae transcripts seen with different strains at T = 3 h.

**Effects of environmental stimuli**

It has been observed by several investigators that certain environmental stimuli profoundly affect the expression of certain exoprotein genes through \( \sigma^B \) and SarA independently of agr (Chan & Foster, 1998a, b; Chan et al., 1998; Cheung et al., 1999; Cheung & Zhang, 2002).

Accordingly, we analysed the transcription pattern of sae under the influence of certain environmental stimuli. Fig. 7(a) shows a series of Northern blots of whole-cell RNA samples prepared during growth in CYGP broth with the addition of 28 mM glucose, 1 M NaCl or subinhibitory clindamycin (SBCL). In cultures of this type grown with 28 mM glucose, the glucose is used up by the beginning of the stationary phase, by which time the pH has fallen to 5–5.5 (R. P. Novick & D. Jiang, unpublished data). As can be seen in the glucose panel, in which pH was monitored, the usual switch in transcription pattern occurs at T = 2 h but as soon as the pH drops below 6, at T = 4 h, sae transcription is turned off. We have observed independently that TSST-1 and other agr-regulated exoproteins, previously considered to be catabolite repressed (Hallis et al., 1991; Iandolo & Shafer, 1977; Coleman, 1983), are produced in the presence of glucose but are not produced below pH 6 (B. Weinrick, H. F. Ross & R. P. Novick, unpublished data). This result suggests that sae may mediate the effects of a moderate decline in pH and that a considerable proportion of the apparent catabolite repression of exoprotein synthesis may actually be a pH effect. Similarly, as seen in the NaCl and SBCL blots, both 1 M NaCl and SBCL eliminate the temporal switch in sae transcription, consistent with their
observed effects on overall exoprotein synthesis (Chan & Foster, 1998a; Herbert et al., 2001). Since the experiments were performed with an Agr− strain, and since these stimuli do not affect agr induction of the sae switch. This was confirmed for SBCL in an experiment with the Pbla::RNAIII fusion in an agr-null background. As can be seen in Fig. 7(b), SBCL prevented the switch despite the induction of RNAIII. Note that, even without induction in this strain, transcript A is not detectable and there is a weak band representing transcript B. This seems inconsistent with results shown in Fig. 3 and is probably owing to a difference in growth conditions and the earlier time at which the samples shown in Fig. 3 were collected.

**DISCUSSION**

In this report, we have shown that sae is a key element in the regulatory network that governs the expression of exoprotein (and very likely other) genes. sae affects the production of a variety of exoproteins throughout the growth cycle and it acts at the level of transcription. It does not, however, affect the transcription of any of the other known regulatory genes, including agr, σB and sarA, whereas these genes all affect the level of transcription of sae, placing sae downstream of all three in the regulatory network. However, no regulatory target for σB or sarA has been identified within the sae locus, suggesting that σB and sarA affect sae transcription indirectly (for agr, no regulatory target has yet been identified).

The sae transcription pattern is complex and undergoes a critical change during the growth cycle. This change involves the disappearance of a 2·1 kb transcript (A) that is present from the outset and the appearance of three new ones, of ~0·5 kb (D), 2·6 kb (B) and 3·1 kb (C), coincident with the onset of agr RNAIII synthesis. Partial confirmation of this has recently been reported by Giraudo et al. (2003). The new transcripts contain two additional reading frames, upstream of saeR, here designated saeP and saeQ, which are likely to be translated. The transcriptional switch is effected by sae, indicating that the locus is autoregulated. It is not affected by σB, but is blocked in agr and sarA mutants. Since SarA enhances RNAIII production, the effects of a sarA mutation on sae may be related to this. Whether other regulatory determinants also affect the sae switch is presently under investigation.

The sae transcription pattern suggests that the sae locus is more complex than originally envisioned and suggests that the upstream ORFs P and Q, or the transcripts B, C and D may play a role in the regulation by sae of at least some of the exoprotein genes. Other genes may be regulated by the sae two-component system, consisting of SaeR and S, alone. The potentially different roles of the sae upstream and downstream regions in the regulation of different exoprotein genes may explain the different classes of exoproteins in the exoprotein profiles in Fig. 1, and may also explain the paradox of sae appearing to be both epistatic to and downstream of RNAIII.

Activation of the sae system can be envisioned as starting (in vitro) with the activation of SaeS by an unknown ligand, possibly external, followed by the activation of SaeR, presumably by phosphorylation, though de-phosphorylation is certainly possible. Somewhat later, in mid-exponential phase in vitro, there is a critical regulatory transition in which agr–RNAIII, in conjunction with activated SaeR (or, much less likely, SaeS), and possibly other regulatory elements, induces the three upstream transcripts, leading to production of SaeP and SaeQ. Concomitantly with induction of the upstream promoters, SaeR down-regulates (auto-represses) transcript A. Since transcripts B and C read through saeRS, the continuing transcription of SaeR is ensured. Translation of saeQ, however, is likely to be required for translation of saeR, and would therefore be an important feature of the sae autoregulation mechanism. Thus, the key regulatory transition must be a function of the upstream transcripts, presumably through SaeP and SaeQ, though possibly through an RNA-mediated effect. The alternative possibility that SaeR is the sole effector of sae-mediated regulation and that the upstream transcripts/products affect the production or activity of SaeR has not been ruled out.

Remarkably, the switch in sae transcription is blocked by diverse environmental signals, including 1 M NaCl, pH below 6 and SBCL. These stimuli have been shown to act downstream of agr and not through it; it is not presently known, however, whether they act directly or through other regulatory genes. Thus, sae may be a key intracellular coordinator of the agr quorum-sensing system with a variety of environmental signals that are well known to have profound effects on exoprotein synthesis (Chan & Foster, 1998a; Lindsay & Foster, 1999).

It is concluded that, although the overall regulatory network governing the staphylococcal virulon seems to involve reciprocal interactions among various regulatory determinants (Novick, 2003), a central linear pathway in which saeRS is directly downstream from agr is beginning to take shape. At this stage, critical unknowns are the putative ligand for SaeS, and the mechanisms by which RNAIII induces and environmental stimuli block the mid-exponential regulatory transition responsible for activation of the sae system. Future studies will address these mechanisms and will also investigate the questions of translational and post-translational regulation of sae, the activity of SaeR and the role of its phosphorylation, and the regulatory role(s) of the upstream sae region.

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