sae is essential for expression of the staphylococcal adhesins Eap and Emp

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Eap and Emp are two *Staphylococcus aureus* adhesins initially described as extracellular matrix binding proteins. Eap has since emerged as being important in adherence to and invasion of eukaryotic cells, as well as being described as an immunomodulator and virulence factor in chronic infections. This paper describes the mapping of the transcription start point of the *eap* and *emp* promoters. Moreover, using reporter-gene assays and real-time PCR in defined regulatory mutants, environmental conditions and global regulators affecting expression of *eap* and *emp* were investigated. Marked differences were found in expression of *eap* and *emp* between strain Newman and the 8325 derivatives SH1000 and 8325-4. Moreover, both genes were repressed in the presence of glucose. Analysis of expression of both genes in various regulatory mutants revealed that *sarA* and *agr* were involved in their regulation, but the data suggested that there were additional regulators of both genes. In a *sae* mutant, expression of both genes was severely repressed. *sae* expression was also reduced in the presence of glucose, suggesting that repression of *eap* and *emp* in glucose-containing medium may, in part, be a consequence of a decrease in expression of *sae*.

INTRODUCTION

Adhesion of *Staphylococcus aureus* to eukaryotic cells and implanted devices is an important step in the initiation of staphylococcal infection. Adhesion by *S. aureus* may be mediated by specific cell-surface proteins, or be a result of interactions of cell-surface proteins with host proteins such as von Willebrand factor, fibronectin, fibrinogen and collagen (Höök & Foster, 2000). By these means, *S. aureus* can adhere directly to eukaryotic cell receptors or, alternatively, can bind to plasma-coated inserted devices.

*S. aureus* produces two types of adhesin (reviewed by Navarre & Schneewind, 1999). One set has a characteristic LPXTG motif that anchors the adhesin to the staphylococcal cell surface (Mazmanian et al., 1999). The members of this family of adhesins are called MSCRAMM molecules, and they include protein A, the fibronectin-binding proteins (FnBPs), clumping factors A and B (ClfA and B), and more recently described molecules, such as IsdA, (also known as FrpA and SfbA; Wiltshire & Foster, 2001; Mazmanian et al., 2002; Morrissey et al., 2002; Taylor & Heinrichs, 2002), Bsp (Tung et al., 2000) and HarA (Dryla et al., 2003). Many of these proteins have been implicated as bridging molecules between the bacterium and the host cell (e.g. Sinha et al., 1999; Hartleib et al., 2000; Massey et al., 2001). Members of the second set of adhesins are noncovalently anchored to the cell surface, and include the fibrinogen-binding protein (Efb; Palma et al., 2001), coagulase (Boden & Flock, 1989), and Eap and Emp (discussed below).

Abbreviations: FnBP, fibronectin-binding protein; TSP, transcription start point.
In recent years it has emerged that staphylococcal adhesins may have additional, diverse functions (e.g. Chavakis et al., 2002; Bjerketorp et al., 2004; Heilman et al., 2005), and that bacteria may alter expression of these molecules in response to changing environmental conditions (e.g. Clarke et al., 2004). Moreover, a number of studies have implicated staphylococcal adhesins as being important in the host response to infection (Jahreis et al., 1995, 2000; Miyamoto et al., 2001; Chavakis et al., 2002; Lee et al., 2002). Our work focuses on two of these adhesins, Eap and Emp. These proteins were initially identified by their ability to bind to various extracellular matrix proteins (Boden & Flock, 1992; Jönsson et al., 1995; Palma et al., 1999; Hussain et al., 2001b). Eap has been shown to be involved in the adherence to and invasion of eukaryotic cells by S. aureus (Hussain et al., 2002; Kreikemeyer et al., 2002; Haggar et al., 2003). Moreover, Eap has emerged as being important in modulation of the immune response to infection by interfering with neutrophil recruitment (Chavakis et al., 2002), as well as inhibiting the delayed-type hypersensitivity response, and inducing T-cell death (Lee et al., 2002). It also appears to be important in chronic infections (Lee et al., 2002). Recently, a novel function for Eap as a potent inhibitor of angiogenesis has been described (Sobke et al., 2004). The importance of Emp during infection is not yet known, but, like Eap, it also binds to fibronectin, fibrinogen and vitronectin (Hussain et al., 2001b).

For both Eap and Emp, little is known about the environmental conditions and regulators that affect their expression. The expression of staphylococcal virulence genes is controlled by a complex regulatory network; for a more comprehensive overview of virulence gene regulation in S. aureus, the reader is referred to a number of excellent review articles (Arvidson & Tegmark, 2001; Cheung & Zhang, 2002; Cheung et al., 2002, 2004; Novick, 2003), while a short review of the regulators that are the focus of this study is given below.

The *agr* (accessory gene regulator) locus was identified by Cheung et al. (1992), and encodes a single DNA-binding protein, SarA. However, upstream of *sarA* are three distinct promoters that produce three distinct transcripts (*sarA*, *sarB* and *sarC*), with the *sarA* and *sarB* transcripts preferentially expressed during the exponential phase, and maximal expression of the *sarC* transcript occurring during the late stationary phase (Bayer et al., 1996; Manola et al., 1998; Blevins et al., 2002). All of the transcripts terminate at the same stem–loop structure (Bayer et al., 1996), thereby resulting in constitutive production of SarA (Blevins et al., 1999). In the past few years, analyses of the staphylococcal genome sequence have revealed the presence of a large number of SarA homologues, termed the SarA protein family (reviewed by Cheung et al., 2002, 2004). These homologues appear to be involved in the control of SarA expression.

SarA is believed to regulate RNAIII expression by binding to the P2 (and, to a lesser extent, P3) promoter of the *agr* system, resulting in enhanced transcription of RNAII and correspondingly RNAIII (Heinrichs et al., 1996; Cheung et al., 1997; Chien & Cheung, 1998; Chien et al., 1998). SarA does not mediate its effects only through *agr*. For example, the FnBPs are regulated by *agr*, but also by SarA, in an *agr*-independent manner (Saravia-Otten et al., 1997; Wolz et al., 2000). An *agr* mutation has no obvious effect on *sarA* transcription (Cheung et al., 1997; Horsburgh et al., 2002).

Another staphylococcal regulator recently subjected to microarray analysis is SigmaB (σB) (Bischoff et al., 2004). σB was initially identified in *Bacillus subtilis* as being an important regulator of the general stress and heat-shock response. The homologous operon in *S. aureus* has been mapped, and found to consist of a four-gene operon, consisting of *rsbU* (required for σB activation), *rsbV* (the anti-anti-sigma factor), *rsbW* (the anti-sigma factor which regulates σB activity post-translationally) and *sigB* (Wu et al., 1996; Kullik & Giachino, 1997; Miyazaki et al., 1999, Giachino et al., 2001; Palma & Cheung, 2001). During the course of their study on the *sigB* operon, Kullik & Giachino (1997) noticed that there was an 11 bp deletion in the *rsbU* gene in derivatives of 8325. This effectively rendered these strains σB-negative. Unfortunately, the 8325 lineage had up to this time been used extensively in gene regulation studies and in vivo models of infection. However, since then, several *rsbU* strains in the 8325 background have been constructed (Giachino et al., 2001; Horsburgh et al., 2002). σB is also involved in the regulation of other regulators. It is well known that the *sarC* transcript is σB dependent (Deora...
et al., 1997; Manna et al., 1998; Gertz et al., 2000). However, there are conflicting reports as to whether \( d^R \) influences the levels of SarA (Gertz et al., 2000; Bischoff et al., 2001; Horsburgh et al., 2002). \( d^R \) is also involved in the regulation of agr, but in this case it has been reported to have a negative effect (Bischoff et al., 2001; Horsburgh et al., 2002).

sae (\( S. aureus \) exoprotein expression) has emerged as an important regulator of virulence gene expression. This regulator was initially identified following isolation of a mutant that was defective in the production of several exoproteins (Giraudo et al., 1994, 1999). The sae locus is composed of four ORFs, two of which encode a classical two-component system, which is now known to be more complex (Novick & Jiang, 2003; Steinhuber et al., 2003). A sae mutation has no effect on expression of agr and sarA (Giraudo et al., 1997). However, sae is activated by agr, at least in some strains, by an unknown mechanism (Giraudo et al., 2003; Novick & Jiang, 2003; Goerke et al., 2005). The importance of sae as a virulence factor in vivo has been demonstrated in several animal models (Rampone et al., 1996; Benton et al., 2004; Goerke et al., 2005). Furthermore, it has been shown that sae is essential for virulence-gene expression in vivo (Goerke et al., 2001, 2005).

The interactions of the various regulatory loci are only partially understood, and have been analysed mainly in relation to the coordinate expression of selected extracellular or cell-bound proteins. Little is known about the regulation of the adhesins that are non-covalently attached to the cell wall. Given the importance of Eap and, possibly, Emp as virulence factors of \( S. aureus \), understanding the regulation of these genes may give additional information to the overall picture of staphylococcal virulence, as well as contributing to our understanding of the staphylococcal global regulatory network. In this study we investigate the regulation of eap and emp by mapping their transcription start sites, and describe the contribution of the global regulators agr, sarA and sae to the regulation of eap and emp.

### METHODS

**Bacterial strains, plasmids and growth conditions.** The strains and plasmids used in this study are listed in Table 1. Bacteria were grown in Modified B-Broth (MBB; Ohlsen et al., 1997) supplemented with the appropriate antibiotics (5 or 10 \( \mu \)g chloramphenicol ml\(^{-1}\), 10 \( \mu \)g erythromycin ml\(^{-1}\), 5 \( \mu \)g tetracycline ml\(^{-1}\)). Overnight cultures prepared in this medium were inoculated 1:20 in MBB or MBB containing 0-5% glucose (without antibiotics) in 100 ml Erlenmeyer flasks, giving an OD\(_{600}\) of approximately 0-1 (measured using a GeneQuant Pro photometer; Amersham Pharmacia). They were grown at 37°C on a rotary shaker (Infors) at 150 r.p.m. For preparation of RNA for S1 mapping or primer-extension analysis, an overnight culture of \( S. aureus \) strain Newman was diluted 500-fold in 50 ml portions of fresh MBB medium, and incubated at 37°C on a rotary shaker at 150 r.p.m. to the exponential phase (4 h), or the end of the exponential phase (6 h).

**Cloning.** The primers used in this study are listed in Table 2. For preparation of plasmids for S1 mapping or primer-extension analysis, genomic DNA was prepared from \( S. aureus \) Newman using the Perfect gDNA Blood Mini Kit (Eppendorf). A region encompassing

### Table 1. Bacterial strains and plasmids

<table>
<thead>
<tr>
<th>Strain or plasmid</th>
<th>Description</th>
<th>Reference or source</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>S. aureus</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Newman</td>
<td>Wild-type</td>
<td>T. J. Foster, Trinity College, Dublin</td>
</tr>
<tr>
<td>SH1000</td>
<td>8325-4 derivative, rsbU(^+)</td>
<td>Horsburgh et al. (2002)</td>
</tr>
<tr>
<td>8325-4</td>
<td>Wild-type, rsbU(^-)</td>
<td>Novick (1967)</td>
</tr>
<tr>
<td>RN4220</td>
<td>Restriction-negative strain, 8325 derivative</td>
<td>Kreiswirth et al. (1983)</td>
</tr>
<tr>
<td>ALC355</td>
<td>Newman Δagr::tetM</td>
<td>Wolz et al. (2000)</td>
</tr>
<tr>
<td>AS3</td>
<td>Newman sae::Tn917</td>
<td>Steinhuber et al. (2003)</td>
</tr>
<tr>
<td>ALC637</td>
<td>Newman sae::Tn917LT1V</td>
<td>Wolz et al. (2000)</td>
</tr>
<tr>
<td><strong>E. coli</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>XL-10</td>
<td></td>
<td>Stratagene</td>
</tr>
<tr>
<td><strong>Plasmids</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>pPCRScript</td>
<td>E. coli cloning vector, Amp(^R)</td>
<td>Stratagene</td>
</tr>
<tr>
<td>pKO10</td>
<td>β-Galactosidase reporter plasmid</td>
<td>Ohlsen et al. (1997)</td>
</tr>
<tr>
<td>pSB2035</td>
<td>gfp-lux dual reporter plasmid</td>
<td>Qazi et al. (2001)</td>
</tr>
<tr>
<td>pEAP-gfplux</td>
<td>eap promoter cloned in place of the P3 promoter in pSB2035</td>
<td>This work</td>
</tr>
<tr>
<td>pEMP-KO10</td>
<td>emp promoter cloned in place of the hla promoter in pK010</td>
<td>This work</td>
</tr>
<tr>
<td>pEap1</td>
<td>DNA fragment encompassing the eap promoter and partial coding sequence cloned in PCRScript</td>
<td>This work</td>
</tr>
<tr>
<td>pEmp1</td>
<td>DNA fragment encompassing the emp promoter and partial coding sequence cloned in PCRScript</td>
<td>This work</td>
</tr>
</tbody>
</table>
Table 2. Oligonucleotides used in this study

<table>
<thead>
<tr>
<th>Primer name</th>
<th>Primer sequence</th>
<th>Function</th>
</tr>
</thead>
<tbody>
<tr>
<td>emp1</td>
<td>CTGATGCTTTCAGGTTAGTTG</td>
<td>S1 mapping and primer-extension analysis</td>
</tr>
<tr>
<td>Universal primer – 48</td>
<td>AGCGGATAACAAATTTCCACAGGAC</td>
<td>S1 mapping and primer-extension analysis</td>
</tr>
<tr>
<td>cap1</td>
<td>GGCTTATCTGGCGGACAGCCG</td>
<td>S1 mapping and primer-extension analysis</td>
</tr>
<tr>
<td>Universal primer – 47</td>
<td>CCGCAGGGTTTTCCACACGAC</td>
<td>S1 mapping and primer-extension analysis</td>
</tr>
<tr>
<td>eplgF</td>
<td>TTTGAAATCCAGCCATATTACCTCC</td>
<td>Amplification of eap promoter</td>
</tr>
<tr>
<td>eplgR</td>
<td>CATCCCAGGAAATTACATCCCTCCTTTTTG</td>
<td>Amplification of eap promoter</td>
</tr>
<tr>
<td>empF</td>
<td>CTCAAATACAAAACCTGACATC</td>
<td>Used with eplgF to amplify a section of the eap promoter and coding sequence for S1 mapping and primer-extension analysis</td>
</tr>
<tr>
<td>empR</td>
<td>GCGTGATATTCCAGGAAACTG</td>
<td>Used with empF to amplify a section of the emp promoter and coding sequence for S1 mapping and primer-extension analysis</td>
</tr>
<tr>
<td>empPF1</td>
<td>ACAGAATTCAATTATTTATATATGACCC</td>
<td>Used with empR to amplify a section of the emp promoter and coding sequence for S1 mapping and primer-extension analysis</td>
</tr>
<tr>
<td>empPR1</td>
<td>CCTAAAGCTTTTTATATAAGCTCAATATTATAAC</td>
<td>Amplification of emp promoter</td>
</tr>
<tr>
<td>lacZR</td>
<td>GGTACGTTGTGTTAGATGG</td>
<td>Reverse primer for lacZ</td>
</tr>
<tr>
<td>capRT_F</td>
<td>AAGCGCTTCGCAGCGACTA</td>
<td>Real-time PCR on eap</td>
</tr>
<tr>
<td>capRT_R</td>
<td>TGCATAGGAAACTGGAAGTAGAA</td>
<td>Real-time PCR on cap</td>
</tr>
<tr>
<td>empRT_F</td>
<td>CAGATGCGCTATAGATACACATCCA</td>
<td>Real-time PCR on emp</td>
</tr>
<tr>
<td>empRT_R</td>
<td>GCTGCGCTTGTGTAACAAAAAT</td>
<td>Real-time PCR on emp</td>
</tr>
<tr>
<td>saeRT_F</td>
<td>AAACTTGGCTATATAAGCCTCAA</td>
<td>Real-time PCR on sae</td>
</tr>
<tr>
<td>saeRT_R</td>
<td>GTTCGGTATATCCATGCAATCCTCA</td>
<td>Real-time PCR on sae</td>
</tr>
<tr>
<td>gyrB_F1</td>
<td>GACTGATGCCCCGATGGA</td>
<td>Real-time PCR on gyrB</td>
</tr>
<tr>
<td>gyrB_R1</td>
<td>AACGGTGGGCTGTGCAATA</td>
<td>Real-time PCR on gyrB</td>
</tr>
</tbody>
</table>

*EcoRI site is underlined. †Smal site is underlined. ‡HindIII site is underlined.

at least 400 bp upstream and 200 bp downstream of the translation start codon of eap and emp was amplified by PCR using Vent DNA polymerase (New England Biolabs) and the primers eplgF and capR1, and empF and empR, respectively. The amplified PCR products were subsequently cloned in PCRScript (Stratagene), and sequenced prior to use in the S1 mapping and primer-extension analysis. These plasmids were termed pEap1 and pEmp1. Amplification of the eap and emp promoters for cloning in the reporter gene vectors was performed using Vent Polymerase, genomic DNA from strain 8325-4 as the template, and primers eplgR and eplgF, and empPF1 and empPR1 to amplify the eap and emp promoters, respectively. The amplified products were cloned in PCRScript, and then made and plated on BHI agar containing 10 µg chloramphenicol ml⁻¹, and incubated at 43 °C. Integration of the plasmid was checked by PCR on genomic DNA using primer empPF1 with lacZ.

**β-Galactosidase assay.** Cells were grown as described above, and, at pre-determined time points, 1 ml culture was removed, and centrifuged (Hermle) at 20,000 g for 5 min. The cells were prepared for the assay using the protocol of Ohlsen et al. (1997). Briefly, the pellet was washed in PBS, and then the cells were adjusted to an OD₆₀₀ of 1-0 in a volume of 500 µl. The cells were sedimented by centrifugation at 11,000 g for 5 min. The cell pellet was resuspended in 500 µl lysis buffer (0-01 M potassium phosphate buffer, pH 7-8, 0-015 M EDTA, 1% Triton X-100) containing 20 µl 2 mg lyso- staphin ml⁻¹, and incubated at 37 °C for 30 min, with gentle shaking. The culture supernatants were then stored at −70 °C. β-Galactosidase assays were performed using the Galacto Light Plus chemiluminescent reporter assay system (Applied Biosystems), according to the manufacturer’s instructions. The assays were performed in triplicate in a 96-well white plate (Greiner) using 5 µl culture supernatant. Luminescence was measured in a Victor 2 multilabel reader (Perkin Elmer).

**Luminescence assay.** For assaying eap expression, cells were diluted 1:100 in MBB and MBB plus 0.5% glucose, both supplemented with 5 µg chloramphenicol ml⁻¹, and 200 µl of the cell suspension was transferred to wells of a 96-well clear-bottom white
plate (Greiner). All samples were assayed in triplicate. The plate was then incubated with shaking at 37 °C in a Victor 2 multilabel reader. Optical density and luminescence readings were taken every 30 min. To normalize the data, the luminescence values were divided by the corresponding OD<sub>695</sub> value.

**Isolation of RNA and S1-nuclease mapping.** At pre-determined time points, the cell suspension of S. aureus Newman was immediately poured into a 50 ml Falcon tube containing about 15 ml crushed ice that was prechilled to −80 °C, and total RNA was prepared essentially as recently described in Kormanec (2001). High-resolution S1 nuclease mapping was performed according to Kormanec (2001). Samples (40 μg) of RNA were hybridized to approximately 0-02 pmol of a suitable DNA probe labelled at one 5' end with [γ-<sup>32</sup>P]ATP (approx. 3 x 10<sup>6</sup> c.p.m. per pmol probe). The probes used were prepared by PCR amplification from the corresponding plasmids as follows: probe EMP was a 1100 bp DNA fragment prepared by PCR amplification from the plasmid pEmp1 using the 5' end-labelled reverse primer emp1 from the emp coding region, and the direct universal primer −48; probe EAP was a 600 bp DNA fragment prepared by PCR amplification from the plasmid pEap1 using the 5' end-labelled reverse primer eap1 from the eap coding region, and the direct universal primer −47 (Table 2). Oligonucleotides were labelled at their 5' ends with [γ-<sup>32</sup>P]ATP (160-5 Tbp mmol<sup>−1</sup>; ICN Biochemicals) and T4 polynucleotide kinase (New England Biolabs). The protected DNA fragments were analysed on DNA sequencing gels, together with G + A and T + C sequencing ladders derived from the end-labelled fragments (Maxam & Gilbert, 1980). Before assigning the transcription start point (TSP), 1-5 nt were subtracted from the length of the protected fragment to account for the difference in the 3' ends resulting from the S1-nuclease digestion, and the chemical sequencing reactions. All mapping experiments were done twice with independent sets of RNA with similar results.

**Primer-extension analysis.** A 50 μg quantity of total RNA was dissolved in 60 μl hybridization buffer (40 mM PIPES, pH 6-4, 1 mM EDTA, 0-4 M NaCl, 80 %, v/v, formamide) at 65 °C, denatured together with 0-5 pmol of the 32P-labelled oligonucleotide primer (emp1 or eap1) for 5 min at 95 °C, and annealed for 4 h at 45 °C. DNA samples were ethanol-precipitated, dissolved in 9 μl water, and the following components were added: 0-75 μl RNasin (Promega), 3 μl 5 × AMV-RT buffer (Promega), 0-75 μl 5 mM each of dATP, dGTP, dTTP and dCTP, and 0-75 μl actinomycin (4 mg ml<sup>−1</sup>). The mixture was incubated for 2 min at 42 °C. The primer extension was initiated by adding 1-3 μl (26 U) AMV-RT (Finnzymes), and incubated for 2 h at 42 °C. The reaction was terminated with 25 μl RNase mix [100 μg ml<sup>−1</sup> DNase-free RNase A, 30 μg ml<sup>−1</sup> sonicated salmon sperm DNA, TE buffer (10 mM Tris/HCl, 1 mM EDTA), pH 8.1] and incubated for 30 min at 37 °C. After addition of 20 μl 1 M NaCl, the mixture was extracted with alkaline phenol/chloroform, and DNA was precipitated with ethanol. The pellet was dissolved in 5 μl loading buffer (80 %, v/v, formamide, 10 mM NaOH, 1 mM EDTA, 0-05 % xylene cyanol, 0-05 % bromophenol blue), heated for 2 min at 95 °C, and an aliquot was loaded on a 6 % denaturing gel, and separated together with the corresponding plasmids as follows: probe EMP was a 1100 bp DNA fragment prepared by PCR amplification from the plasmid pEmp1 using the 5' end-labelled reverse primer emp1 from the emp coding region, and the direct universal primer −48; probe EAP was a 600 bp DNA fragment prepared by PCR amplification from the plasmid pEap1 using the 5' end-labelled reverse primer eap1 from the eap coding region, and the direct universal primer −47 (Table 2). Oligonucleotides were labelled at their 5' ends with [γ-<sup>32</sup>P]ATP (160-5 Tbp mmol<sup>−1</sup>; ICN Biochemicals) and T4 polynucleotide kinase (New England Biolabs). The protected DNA fragments were analysed on DNA sequencing gels, together with G + A and T + C sequencing ladders derived from the end-labelled fragments (Maxam & Gilbert, 1980). Before assigning the transcription start point (TSP), 1-5 nt were subtracted from the length of the protected fragment to account for the difference in the 3' ends resulting from the S1-nuclease digestion, and the chemical sequencing reactions. All mapping experiments were done twice with independent sets of RNA with similar results.

**RESULTS**

**Identification of the TSPs of the eap and emp promoters.** In order to identify the TSPs of the promoter(s) directing expression of the S. aureus eap and emp genes, primer-extension analysis was performed using RNA isolated from S. aureus Newman grown to early and late exponential phase, as described in Methods. As shown in Fig. 1(a), a single extension product was identified for each promoter. The identified fragments corresponded to the P<sub>eap</sub> and P<sub>emp</sub> promoters, with a TSP at T and A, 37 and 64 bp upstream from the corresponding translation initiation codon, respectively (Fig. 1c). As primer-extension analysis sometimes produces false signals caused by a premature pausing of reverse transcriptase, positions of the promoters were confirmed by another method, high-resolution S1-nuclease mapping (see Methods). As shown in Fig. 1(b), a single RNA-protected fragment was identified for each promoter with a TSP at the identical position as for primer extension analysis. No RNA-protected fragment was identified with tRNA as a control (Fig. 1b, lane 2). For both promoters, the nucleotide sequence upstream of TSP was identical to the consensus sequence of principal sigma factor σ<sup>A</sup>-dependent promoters in the −10 region (TATAAT); however, only a weak similarity was found in the −35 region (Fig. 1c). In conclusion, these results indicate that S. aureus eap and emp expression is directed by a single promoter.

**Differential expression of eap and emp between strain Newman and the 8325 derivatives SH1000 and 8325-4.** To investigate the regulators and environmental conditions affecting expression of eap and emp, both genes were
analysed in strains Newman, SH1000 and 8325-4 using reporter-gene assays and real-time PCR. Using the reporter-gene assays, it was seen that in strain Newman, expression of both \(eap\) and \(emp\) increased during the exponential growth phase, and declined thereafter (Fig. 2). The peak point of expression for both genes appeared to be 4 h post-inoculation. Although expression of \(emp\) appeared to increase during the later stages of growth (Fig. 2b), we believe this was due to accumulation of the reporter protein (Chan & Foster, 1998), since using real-time PCR we could show that expression of both genes was reduced in the stationary phase (Fig. 2c, d). With the reporter assays, marked differences in expression of \(eap\) and \(emp\) were seen in strain Newman compared with the 8325 derivatives SH1000 (\(rsbU^+\)) and 8325-4 (\(rsbU^-\)). Newman exhibited high levels of expression of both genes, whereas in the 8325
derivatives, expression of both genes was almost undetectable (data not shown). These differences were in spite of the fact that the same construct was used in both strains. Using real-time PCR, we could confirm the differential expression of eap and emp between Newman and the 8325 derivatives (data not shown). This suggests that differential expression of a regulator between Newman and the 8325 derivatives may be responsible for the enhanced expression of eap and emp in Newman.

Glucose represses expression of eap and emp

Since the regulation of some of the important global regulators of S. aureus is affected by glucose (Regassa et al., 1992; Novick & Jiang, 2003), we investigated the effect of glucose on expression of eap and emp. A marked decrease in expression (four- to fivefold at the 4 h time point) of both genes was seen when the cells were grown in MBB containing 0-5% glucose compared with MBB (Fig. 2a, b). Using real-time PCR, we could show that this decrease was due to a reduction in gene expression, rather than degradation of the reporter-gene product (Fig. 2c, d), suggesting that a decrease in the expression of (a) global regulator(s) in S. aureus in the presence of glucose is responsible for the decrease in expression of eap and emp.

Global regulators affecting expression of eap and emp

In order to identify the main regulator of eap and emp, we investigated the contribution of a number of glucose-repressed and important regulators of virulence-gene expression in S. aureus to the regulation of eap and emp. This was performed using real-time PCR in defined regulatory mutants. The initial experiments, showing enhanced expression of eap and emp in Newman compared with the 8325 derivatives SH1000 and 8325-4, suggest that σB is not essential for expression of eap and emp, since neither gene showed enhanced expression in SH1000 (rsbU+) when compared with 8325-4 (rsbU–). This was in spite of the fact that the level of σB activity in SH1000 was similar to Newman, as assayed by real-time PCR, using expression of asp23 as an indicator of σB activity (data not shown). The expression of eap and emp was also examined in agr, sarA and sae mutants (ALC355, ALC637 and AS3, respectively). In both the agr and sarA mutants, expression of eap and emp was reduced three- to fivefold at the 3 h time point (Fig. 3). However, in the sae mutant, expression of both genes was severely repressed, which was particularly evident at the 3 h time point (Fig. 3), thereby demonstrating that sae is essential for expression of eap and emp.

Fig. 2. Expression of eap and emp in Newman grown in MBB (black bars) or MBB containing 0-5% glucose (hatched bars), assayed using reporter-gene assays (a, b) and real-time PCR (c, d). (a, c) eap expression profiles. (b, d) emp expression profiles. Assays were performed as described in Methods. The data shown are the means ± SEM of at least two experiments. RLU, relative light units.
Is differential expression of *eap* and *emp* in Newman and 8325 derivatives, and their repression in the presence of glucose, a result of alterations in expression of *sae*?

Since the analyses in the regulatory mutants showed that *sae* is essential for expression of *eap* and *emp*, we were interested to know if our previous observations, i.e. the enhanced expression of *eap* and *emp* in strain Newman compared with the 8325 derivatives, as well as the repression of *eap* and *emp* in glucose-containing medium, could be attributed to variations in *sae* expression. In Fig. 4(a), it can be seen that expression of *sae* in strain Newman is considerably higher than that of either SH1000 or 8325-4 at the 3 h time point. This suggests that higher levels of *sae* in strain Newman may be responsible for the enhanced expression of *eap* and *emp* in this strain. Moreover, in medium containing 0-5% glucose, it can be seen that *sae* expression is about half that of *sae* at the 3 and 6 h time points (Fig. 4b). Therefore, it is possible that the reduced expression of *eap* and *emp* in glucose-containing medium is a consequence of a reduction in expression of *sae*. It should be noted that although our expression profiles of *sae* (maximal expression during exponential phase) appear to differ from previously published data (maximal expression during the post-exponential phase), this is a result of the different media and time points used in our study. Control experiments using CYPG medium (Novick, 1991) and bacteria harvested at the same time points as Steinhuber et al. (2003) showed that maximal expression of *eap* and *emp* was shifted to the post-exponential phase, corresponding to the time at which maximal expression of *sae* occurred (data not shown). Recently it was reported that repression of *sae* in glucose-containing medium occurs as a result of changes in pH (Novick & Jiang, 2003), and that alterations in the pH rather than glucose per se are responsible for the changes in gene expression (Weinrick et al., 2004). Therefore, we monitored the changes in the pH of the medium over time. We found that in MBB, the pH becomes more basic as time progresses (pH 7-1, 8-3 and 8-7 at 3, 6 and 10 h, respectively), whereas in MBB containing 0-5% glucose, the medium becomes acidic with time (pH 5-0, 4-6 and 4-5 at 3, 6 and 10 h, respectively). These observations provide additional support for the hypothesis that the reduced expression of *eap* and *emp* in glucose-containing medium may be, in part, a result of a pH-mediated decrease in expression of *sae*. 

**Fig. 3.** Analysis of *eap* (a) and *emp* (b) expression in various regulatory mutant backgrounds at different time points using real-time PCR as described in Methods. The data shown are the means ± SEM of two independent experiments.

**Fig. 4.** Differential expression of *sae* in Newman and the 8325 derivatives SH1000 and 8325-4 (a), and repression of *sae* in the presence of 0-5% glucose (b), assayed by real-time PCR as described in Methods. The data shown are the means ± SEM of two independent experiments.
DISCUSSION

In this paper, we describe the mapping of the TSPs of the promoters directing expression of cap and emp, the contribution of a number of important regulators of virulence-gene expression in S. aureus to the regulation of the staphylococcal adhesins Eap and Emp, and we identify sae as being essential for the regulation of both genes. We also show that both the previously noted differential expression of cap and emp between strains (Hussain et al., 2001a), and the repression of both genes in the presence of glucose, occur at the transcriptional level. We believe that these observations are due to variation in the expression of a key regulator, and that this regulator is sae.

Mapping the TSPs of cap and emp revealed that both genes are transcribed from a single promoter. This was an important point to establish, since while there are no genes in the immediate vicinity of cap, there are two genes encoding staphylococcal adhesins immediately upstream of emp (Kuroda et al., 2001). The translation start codon of emp is only 353 bp downstream of the vwb stop codon, raising a possibility of co-transcription. However, previous studies (Bjerketorp et al., 2002) based on sequence analysis of this region have suggested that these genes are not linked, and we can now show that emp does indeed have its own promoter.

In order to study the regulation of cap and emp, we used two different reporter-gene assays. Initial experiments showed that expression of both the cap and emp constructs in the 8325 background was very low. We therefore decided to transfer the constructs to strain Newman, a strain that has previously been shown to produce considerably more Eap than other strains (Hussain et al., 2001a). In Newman, enhanced expression of both cap and emp reporters was obtained. Moreover, using real-time PCR we could confirm that Newman does indeed have much higher levels of expression of cap and emp compared with SH1000 and 8325-4 (data not shown), thereby confirming the reporter-gene assay data. Taken together, these data showed that the differential expression of cap and emp in strains Newman and SH1000 and 8325-4 was not due to differences in the promoter sequences (since the 8325-4 promoter of both genes was used for the reporter assays in all strains), or to the proteins (e.g. as a consequence of enhanced protein stability), and therefore must be due to differential expression of (a) key regulator(s). The difference in $\sigma^B$ activity between strains Newman and 8325-4 is unlikely to be responsible for the observed differences in expression of cap and emp, since SH1000 (8325-4 rsbU$^+$) did not show increased expression of either gene compared with 8325-4 (rsbU$^-$. These findings are also consistent with the recently published microarray data (Bischoff et al., 2004) showing that $\sigma^B$ is not essential for expression of either gene. However, since sarA is partly under $\sigma^B$ control (Bischoff et al., 2001), one should not exclude the possibility that $\sigma^B$ is functioning either directly or indirectly at the post-transcriptional level (N. Harraghy, unpublished observation).

We next examined expression of cap and emp in agr and sarA mutants. In these mutants, expression of both cap and emp was reduced three- to fivefold (Fig. 3). For cap, these findings are in agreement with previous transcriptional profiling of agr- and sarA-regulated genes in S. aureus (Dunman et al., 2001), where it was shown that cap is upregulated by both agr and sarA. However, although emp did not emerge in this study as being under control of either regulator, our data indicate that it is also under control of both agr and sarA.

Although agr and sarA were shown to be involved in the regulation of cap and emp, neither gene was completely repressed in the Δagr and ΔsarA strains. Our observation that both cap and emp were repressed in the presence of glucose, and their enhanced expression in strain Newman compared with the 8325 derivatives, taken together with the recently published data of Steinhuber et al. (2003) and Novick & Jiang (2003), pointed to sae as being a key regulator of cap and emp. Steinhuber et al. (2003) noted that expression of emp was reduced in ISP479C (an 8325 derivative) compared with Newman, an observation that we could confirm in our 8325 derivatives, SH1000 and 8325-4 (Fig. 4). Novick & Jiang (2003) and Weinrick et al. (2004) reported that sae expression is repressed in the presence of glucose as a consequence of changes in the pH that occur when bacteria are grown in such a medium. We could also confirm that there were marked differences in the pH of MBB containing glucose compared with MBB without glucose, and that in the glucose-containing medium, expression of sae was repressed. The recent transcriptome analysis of genes induced under mild acidic conditions, such as those seen when bacteria are grown in the presence of glucose, has revealed a wide range of genes, including cap and emp, that are affected by this change in pH (Weinrick et al., 2004). This leads to the possibility that changes in pH, such as those that S. aureus would encounter following arrival at a new niche, or invasion of eukaryotic cells, may alter expression of key regulators, thereby altering expression of virulence factors, and hence aid the adaptation of S. aureus to changing environmental conditions. It is also possible that sae is responding to signals other than pH, as exemplified by our observation that the temporal pattern of sae expression was dependent on the growth medium used.

From the data presented in this paper, it is clear that sae is essential for transcription of both cap and emp. However, the exact mechanism of sae control is still not known. The interactions between the various global regulators of S. aureus are highly complex, and often paradoxical. For example, Novick & Jiang (2003) have suggested that sae is both dependent and independent of agr, and both agr-dependent and -independent regulation of genes has been described (e.g. Saravia-Otten et al., 1997; Blevins et al., 1999; Wolz et al., 2000). Since repression of cap and emp in the agr mutant is modest in comparison with that seen in the sae mutant, this may be indicative of a decrease in sae expression.
as a result of the agr mutation. Alternatively, it may indicate that RNAIII interacts directly with the eap and emp promoters, and that regulation of eap and emp by sae is independent of agr.

Repression of eap and emp in the sarA mutant may be a direct consequence of a decrease in agr expression, considering that the eap transcript levels are very similar in both the agr and sarA backgrounds. The additional repression of emp seen in the sarA mutant as compared with the agr mutants suggests that, in addition to any effect via agr, SarA may act directly on the sae or emp promoters. However, it is also possible that the decrease in expression of both genes is a result of SarA acting independently of agr. It should also be noted that although in strain Newman eap and emp appear to be under similar regulatory control, the expression of eap is about 10-fold higher than that of emp. This suggests the interaction of additional transcription factors with the eap and emp promoters. Characterization of the eap and emp promoters, and the factors binding to them, is currently under investigation in our laboratories.

In conclusion, we have identified sae as an important regulator of the staphylococcal adhesins Eap and Emp, which is capable of modulating their expression under different environmental conditions. It may well be that sae is a crucial regulator in the adaptation of S. aureus to changing environments, which consequently may contribute to the intracutaneous survival of S. aureus (e.g., altering virulence-gene expression in response to changing pH), or may orchestrate complex interactions with the eukaryotic immune system via expression of these secreted adhesins.

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