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 SdB; 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).
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; Haggard 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.

*aer* (accessory gene regulator) was one of the first recognized global regulators of staphylococcal gene expression (Rescei et al., 1986; Janzon et al., 1989). The *agr* system is a quorum sensing one, and consists of an operon of four genes. The first is a membrane-associated protease (*agrB*). The second, *agrD*, produces the pre-pro-peptide that is subsequently modified by AgrB and secreted as a peptide thiolactone consisting of 7–9 amino acid residues, in which the central cysteine residue is covalently linked to the C-terminal amino acid carboxylate forming a cyclic thioester. This functions as the quorum-sensing phenome, which is recognized by a membrane-associated sensor kinase (*agrC*), which in turn activates the cytoplasmic regulator (*agrA*) (Ji et al., 1995; Morfeldt et al., 1996; Lina et al., 1998; Zhang et al., 2002). These genes form the transcript RNAII, and are under the control of the P2 promoter (Novick et al., 1993, 1995; Ji et al., 1995). A second, divergent transcript, RNAIII, is the effector molecule of the *agr* system, and is under the control of the P3 promoter (Janzon & Arvidson, 1990). This molecule is synthesized in response to the environmental concentration of the octapeptide (Ji et al., 1995). Expression of RNAIII is temporal, with maximal expression occurring in the transition from the post-exponential to the stationary phase. It has been postulated that this temporal expression is responsible for the repression of cell surface proteins, and the enhanced expression of exoproteins during the later stages of growth (Kornblum et al., 1990).

The *sar* (staphylococcal accessory 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; Manna 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 (*σ*B) (Bischoff et al., 2004). *σ*B 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 *σ*B activation), *rsbV* (the anti-anti-sigma factor), *rsbW* (the anti-sigma factor which regulates *σ*B 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 *σ*B-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). *σ*B is also involved in the regulation of other regulators. It is well known that the *sarC* transcript is *σ*B dependent (Deora
et al., 1997; Manna et al., 1998; Gertz et al., 2000). However, there are conflicting reports as to whether \( \sigma^B \) influences the levels of SarA (Gertz et al., 2000; Bischoff et al., 2001; Horsburgh et al., 2002). \( \sigma^B \) 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).

\( \text{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 (Giraudou et al., 1994, 1999). The \text{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 } \text{sae mutation has no effect on expression of } \text{agr and sarA (Giraudou et al., 1997). However, } \text{sae is activated by } \text{agr, at least in some strains, by an unknown mechanism (Giraudou et al., 2003; Novick & Jiang, 2003; Goerke et al., 2005). The importance of } \text{sae as a virulence factor } \text{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 } \text{sae is essential for virulence-gene expression } \text{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 \( \text{S. aureus} \), 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 \text{eap and emp} by mapping their transcription start sites, and describe the contribution of the global regulators \text{agr, sarA} and \text{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 μg chloramphenicol ml\(^{-1}\), 10 μg erythromycin ml\(^{-1}\), 5 μg 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 (Infora) at 150 r.p.m. For preparation of RNA for S1 mapping or primer-extension analysis, overnight culture of \( \text{S. aureus} \) 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 and primer-extension analysis, genomic DNA was prepared from \( \text{S. aureus} \) Newman using the Perfect gDNA Blood Mini Kit (Eppendorf). A region encompassing

<table>
<thead>
<tr>
<th>Strain or plasmid</th>
<th>Description</th>
<th>Reference or source</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>\text{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, \text{rsbU}^+</td>
<td>Horsburgh et al. (2002)</td>
</tr>
<tr>
<td>8325-4</td>
<td>Wild-type, \text{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 \text{Δagr::tetM}</td>
<td>Wolz et al. (2000)</td>
</tr>
<tr>
<td>AS3</td>
<td>Newman \text{sar::Tn917}</td>
<td>Steinhuber et al. (2003)</td>
</tr>
<tr>
<td>ALC637</td>
<td>Newman \text{sar::Tn917LT1}</td>
<td>Wolz et al. (2000)</td>
</tr>
<tr>
<td><strong>\text{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>\text{E. coli} cloning vector, Amp(^R)</td>
<td>Stratagene</td>
</tr>
<tr>
<td>pKO10</td>
<td>( \beta )-Galactosidase reporter plasmid</td>
<td>Ohlsen et al. (1997)</td>
</tr>
<tr>
<td>pSB2035</td>
<td>\text{gfp-lux} dual reporter plasmid</td>
<td>Qazi et al. (2001)</td>
</tr>
<tr>
<td>pEAP-gflux</td>
<td>\text{eap} promoter cloned in place of the \text{P3} promoter in pSB2035</td>
<td>This work</td>
</tr>
<tr>
<td>pEMP-KO10</td>
<td>\text{emp} promoter cloned in place of the \text{hla} promoter in pKO10</td>
<td>This work</td>
</tr>
<tr>
<td>pEap1</td>
<td>DNA fragment encompassing the \text{eap} promoter and partial coding sequence cloned in PCRScript</td>
<td>This work</td>
</tr>
<tr>
<td>pEmp1</td>
<td>DNA fragment encompassing the \text{emp} promoter and partial coding sequence cloned in PCRScript</td>
<td>This work</td>
</tr>
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</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>CTGATGCTTTAGCGGTATTTG</td>
<td>S1 mapping and primer-extension analysis</td>
</tr>
<tr>
<td>Universal primer −48</td>
<td>ACGGATAAACATTTGCAACAGGA</td>
<td>S1 mapping and primer-extension analysis</td>
</tr>
<tr>
<td>eap1</td>
<td>GGGTACACTCGCCGCAAGCC-</td>
<td>S1 mapping and primer-extension analysis</td>
</tr>
<tr>
<td>Universal primer −47</td>
<td>CGCCAGGTTTTCATCATCTGGAC</td>
<td>S1 mapping and primer-extension analysis</td>
</tr>
<tr>
<td>eplgF</td>
<td>TTGTAGATCAGCATTATCTCATCC*</td>
<td>Amplification of eap promoter</td>
</tr>
<tr>
<td>eplgR</td>
<td>CATCCGGGAAATTATCTCTCCTTTTGT†</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>capR1</td>
<td>GCCAAGGTATTATGGAATG</td>
<td>Amplification of emp promoter</td>
</tr>
<tr>
<td>empF</td>
<td>CTCAAAATCAACACACTGCATC</td>
<td>Amplification of emp promoter</td>
</tr>
<tr>
<td>empR</td>
<td>GGTGATATCCAGGAACGTGC</td>
<td>Amplification of emp promoter</td>
</tr>
<tr>
<td>empPF1</td>
<td>ACGAATTCATTTATATATTTATGAGCACC*</td>
<td>Amplification of emp promoter</td>
</tr>
<tr>
<td>empPR1</td>
<td>CCTAACCACTTATATAGACTCAATATTACACGC†</td>
<td>Reverse primer for lacZ</td>
</tr>
<tr>
<td>lacR</td>
<td>GGTACGTTTGTGATAGTTGGAAG</td>
<td>Real-time PCR on lacZ</td>
</tr>
<tr>
<td>eapRT_F</td>
<td>AAGCGTTCCTCGCCGCACTA</td>
<td>Real-time PCR on eap</td>
</tr>
<tr>
<td>eapRT_R</td>
<td>TCGATATGCGGACATGCGACTTAGGAA</td>
<td>Real-time PCR on eap</td>
</tr>
<tr>
<td>empRT_F</td>
<td>CAGAATTCGGCTGATATACATCATCCCA</td>
<td>Real-time PCR on emp</td>
</tr>
<tr>
<td>empRT_R</td>
<td>GCTGCCTGGTGTAACAAAAATT</td>
<td>Real-time PCR on emp</td>
</tr>
<tr>
<td>saeRT_F</td>
<td>AAACCTGGTTGATAATGCGCTA</td>
<td>Real-time PCR on sae</td>
</tr>
<tr>
<td>saeRT_R</td>
<td>GTTCTGGTATAATGCGCAATACTTCA</td>
<td>Real-time PCR on sae</td>
</tr>
<tr>
<td>gyrB_F1</td>
<td>GACTGATCGCGAGTTGGA</td>
<td>Real-time PCR on gyrB</td>
</tr>
<tr>
<td>gyrB_R1</td>
<td>ACCGGTGCGTCTGCAATA</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 eapR1, 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 sequencing prior to use in the S1 mapping and primer-extension analysis ducts were subsequently cloned in PCRScript (Stratagene), and then eapR1, and empF and empR, respectively. The amplified PCR products were cloned in PCRScript, and then polymerase (New England Biolabs) and the primers eplgF and eplgR, and empR1, and empF, respectively. The amplified PCR products were cloned in PCRScript, and then digested according to standard protocols (Sambrook et al., 1989), with either EcoRI and Smal (eap promoter), or EcoRI and HindIII (emp promoter), for cloning in their respective vectors. The P3 promoter in pSB2035 (Qazi et al., 2001) was replaced with the eap promoter, and the hla promoter in pKO10 (Ohslen et al., 1997) was replaced with the emp promoter. The resulting constructs were named pEAP-gfpFlux and pEMP-KO10. Following transformation in Escherichia coli, plasmid DNA was prepared using the Qiaprep Spin Miniprep Kit (QiaGen). Ten micrograms of DNA was used to transform S. aureus RN4220 by electroporation. Phage 85 lysate of these strains was prepared, and used to transduce the plasmids to various S. aureus backgrounds. To create a single-copy emp::lacZ fusion, the transformed cells were grown in liquid culture supplemented with 10 μg chloramphenicol ml−1 at 30 °C overnight. Serial dilutions were 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 Ohslen 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 cap 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$_{595}$ 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-32P]ATP (approx. 3 x 10$^6$ 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-32P]ATP (166-5 Tbq mmol$^{-1}$; ICN Biochemicals) and T4 poly-nucleotide 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$^{-1}$), and 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$^{-1}$ DNase-free RNase A, 30 μg ml$^{-1}$ sonicated salmon sperm DNA, 10 μM Tris/HCl, 1 mM EDTA], 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 G+A and T+C sequencing ladders derived from the corresponding end-labelled fragments (EMP, EAP) (Maxam & Gilbert, 1980).

**RNA isolation for real-time PCR.** Cells were grown as described above, and at the pre-determined time points, 0-8 ml culture was removed and centrifuged at 5000 g for 5 min at 4 °C. RNA was prepared with the Nucleospin RNA II kit (Macherey & Nagel), with the following modifications. Following centrifugation, the cells were resuspended in 100 μl TE buffer (pH 8), and then 500 μl of the supplied buffer R1 plus 5 μl β-mercaptoethanol (Roth) was added. This suspension was transferred to a Lysing Matrix B tube (Q-biogene), and spun for 20 s at a speed of 6-5 m s$^{-1}$ in a Fastprep (Thermo Savant). After centrifugation at 14 000 g (Minispin; Eppendorf), the supernatant was collected for further centrifugation. The supernatant was then transferred to the gDNA shredding column supplied with the kit, and the manufacturer’s protocol was then followed. All samples were treated with the supplied DNase I. The absence of contaminating genomic DNA was confirmed by real-time PCR using primers for *gyrB*, as described below.

**Real-time PCR.** cDNA was prepared using the High Capacity cDNA Kit (Applied Biosystems), according to the manufacturer’s instructions. Primers for real-time PCR were designed using Primer Express software (Applied Biosystems). Prior to analysis of the cDNA samples, a number of optimization reactions were performed. For optimizing the primer concentration, real-time PCR with primer concentrations ranging from 50 to 900 nM was performed. The optimal primer concentrations were as follows: *eap* (300 nM, forward and reverse), *emp* (100 nM, forward and reverse), *sae* (50 nM forward, 100 nM reverse) and *gyrB* (300 nM, forward and reverse). To check the efficiency of the PCR, standard curves were made using serial dilutions of cDNA. Real-time PCR was performed on an Applied Biosystems 7000 instrument using the SYBR Green Mastermix (Applied Biosystems), according to the manufacturer’s instructions. *gyrB* was used as an internal control, and the gene-specific transcripts were expressed as the n-fold difference relative to *gyrB*, using the formula 2$^{-ΔCΤ}$, where CT is the threshold cycle value.

**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$_{eap}$ and P$_{emp}$ 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 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 $σ^A$-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*<sup>+</sup>) and 8325-4 (*rsbU<sup>-</sup>). Newman exhibited high levels of expression of both genes, whereas in the 8325

Fig. 1. Mapping the TSP of the *S. aureus* *eap* and *emp* promoters by primer-extension analysis (a) and high-resolution S1-nuclease mapping (b). RNA from *S. aureus* Newman grown to early exponential phase (lane 1) or late exponential phase (lane 2) was used for primer-extension analysis. For S1-nuclease mapping, RNA isolated from Newman was grown to early exponential phase (lane 1), and treated with 100 U S1-nuclease. As a control, 40 μg *E. coli* tRNA was used for hybridization with a particular probe (lane 2). The thin horizontal arrows indicate the positions of the extension products and RNA-protected fragments, and thick bent vertical arrows indicate the nucleotide corresponding to the TSP. (c) The nucleotide sequences of the *S. aureus* *eap* and *emp* promoter regions are shown. The deduced protein products are given in the single-letter amino-acid code in the second position of each codon. The TSPs of the promoters are indicated by bent arrows. The −10 and −35 boxes of promoters are in bold and underlined. The sequences refer to the GenBank/EMBL/DDBJ accession numbers AJ271347 and AJ290973.
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.
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.
DISCUSSION

In this paper, we describe the mapping of the TSPs of the promoters directing expression of \textit{eap} and \textit{emp}, the contribution of a number of important regulators of virulence-gene expression in \textit{S. aureus} to the regulation of the staphylococcal adhesins Eap and Emp, and we identify \textit{sae} as being essential for the regulation of both genes. We also show that both the previously noted differential expression of \textit{eap} and \textit{emp} between strains (Hussain \textit{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 \textit{sae}.

Mapping the TSPs of \textit{eap} and \textit{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 \textit{eap}, there are two genes encoding staphylococcal adhesins immediately upstream of \textit{emp} (Kuroda \textit{et al.}, 2001). The translation start codon of \textit{emp} is only 353 bp downstream of the \textit{vwb} stop codon, raising a possibility of co-transcription. However, previous studies (Bjerketorp \textit{et al.}, 2002) based on sequence analysis of this region have suggested that these genes are not linked, and we can now show that \textit{emp} does indeed have its own promoter.

In order to study the regulation of \textit{eap} and \textit{emp}, we used two different reporter-gene assays. Initial experiments showed that expression of both the \textit{eap} and \textit{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 \textit{et al.}, 2001a). In Newman, enhanced expression of both \textit{eap} and \textit{emp} reporters was obtained. Moreover, using real-time PCR we could confirm that Newman does indeed have much higher levels of expression of \textit{eap} and \textit{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 \textit{eap} and \textit{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 \textit{eap} and \textit{emp}, since SH1000 (8325-4 \textit{rsbU}+) did not show increased expression of either gene compared with 8325-4 (\textit{rsbU}−). These findings are also consistent with the recently published microarray data (Bischoff \textit{et al.}, 2004) showing that \(\sigma^B\) is not essential for expression of either gene. However, since \textit{sarA} is partly under \(\sigma^B\) control (Bischoff \textit{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 \textit{eap} and \textit{emp} in \textit{agr} and \textit{sarA} mutants. In these mutants, expression of both \textit{eap} and \textit{emp} was reduced three- to fivefold (Fig. 3). For \textit{eap}, these findings are in agreement with previous transcriptional profiling of \textit{agr}- and \textit{sarA}-regulated genes in \textit{S. aureus} (Dunman \textit{et al.}, 2001), where it was shown that \textit{eap} is upregulated by both \textit{agr} and \textit{sarA}. However, although \textit{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 \textit{agr} and \textit{sarA}.

Although \textit{agr} and \textit{sarA} were shown to be involved in the regulation of \textit{eap} and \textit{emp}, neither gene was completely repressed in the \(\Delta\text{agr}\) and \(\Delta\text{sarA}\) strains. Our observation that both \textit{eap} and \textit{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 \textit{et al.} (2003) and Novick & Jiang (2003), pointed to \textit{sae} as being a key regulator of \textit{eap} and \textit{emp}. Steinhuber \textit{et al.} (2003) noted that expression of \textit{eap} 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 \textit{et al.} (2004) reported that \textit{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 \textit{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 \textit{eap} and \textit{emp}, that are affected by this change in \(pH\) (Weinrick \textit{et al.}, 2004). This leads to the possibility that changes in \(pH\), such as those that \textit{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 \textit{S. aureus} to changing environmental conditions. It is also possible that \textit{sae} is responding to signals other than \(pH\), as exemplified by our observation that the temporal pattern of \textit{sae} expression was dependent on the growth medium used.

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

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

In conclusion, we have identified \textit{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 \textit{saE} is a crucial regulator in the adaptation of \textit{S. aureus} to changing environments, which consequently may contribute to the intracellular survival of \textit{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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