INTRODUCTION

_Staphylococcus aureus_ is a major human pathogen, which produces a variety of virulence determinants. To study environmental regulation of virulence-determinant production, several transcriptional reporter gene fusions were constructed. Chromosomal fusions were made with the staphylococcal accessory regulator (sarA), _α_-haemolysin (hla), surface protein A (spa) and toxic-shock syndrome toxin-1 (tst) genes. The effect of many different environmental conditions on the expression of the fusions was examined. Expression of _hla_, _tst_ and _spa_ was strongly repressed in the presence of sodium chloride (1 M) or sucrose (20 mM), but _sarA_ was relatively unaffected. The global regulator of expression of virulence-determinant genes, _agr_ (accessory gene regulator) was not involved in the salt or sucrose repression. Novobiocin, a DNA gyrase inhibitor, did not significantly increase the expression of _tst_ in wild-type or _agr_ backgrounds and failed to relieve the salt suppression. Expression of _tst_ was strongly stimulated in several low-metal environments, independently of _agr_, whilst _spa_ levels were significantly reduced by EGTA. The complex, interactive role of environmental factors in the control of expression of the virulence determinants is discussed.

**Keywords:** _Staphylococcus aureus_, virulence, regulation, toxin, surface protein

*Abbreviations:* MUG, 4-methylumbelliferyl β-D-galactopyranoside; TSS, toxic shock syndrome.
required for maximal expression of \( \text{agr} \) since in a SarA mutant, levels of RNAIII are reduced (Cheung & Projan, 1994; Heinrichs et al., 1996). SarA is a DNA-binding protein which binds to the \( \text{agr} \) promoter region affecting control of target genes (Morfeldt et al., 1996; Cheung et al., 1997a). Furthermore, Sar also is able to modulate expression of certain virulence determinants independently of \( \text{agr} \) (Cheung et al., 1997b). Both sarA- and \( \text{agr} \)-defective mutants exhibit reduced exoprotein biosynthesis and increased levels of cell-wall proteins, and are less virulent in several animal models (Cheung et al., 1992, 1994; Kornblum et al., 1990; Abdelnour et al., 1993). Hence, sar and \( \text{agr} \) are major components in the hierarchical control of virulence factor biosynthesis.

The environmental stimuli which activate the sar and \( \text{agr} \) systems and how these signals are transduced to alter virulence-determinant expression are poorly understood. Limited studies have shown that \( \text{agr} \) is repressed by alkaline pH and glucose (Regassa et al., 1992, 1994; Kornblum et al., 1997). The role of magnesium in the regulation of \( \text{tst} \) expression has been a matter of some controversy (Bergdoll, 1989). Hence, identification of the environmental conditions which control virulence-determinant production and the mechanism by which these signals are transduced to bring about changes in gene expression are crucial in understanding the ability of \( S. \) aureus to cause such a wide range of diseases.

In this work, using single-copy transcriptional reporter gene fusions, we have identified key environmental signals which affect expression of representative virulence-determinant genes (sar, spa, bha and \( \text{tst} \)). Furthermore, epistasis experiments have defined the role of \( \text{agr} \) in the signal transduction pathways responding to major stimuli.

**METHODS**

**Bacterial strains, plasmids and media.** The bacterial strains and plasmids used in this study are described in Table 1. \( S. \) aureus strains were routinely grown in Brain Heart Infusion (BHI) (Oxoid) containing erythromycin (5 \( \mu \)g ml\(^{-1} \)) or tetracycline (5 \( \mu \)g ml\(^{-1} \)) where appropriate. *Escherichia coli* was grown at \( 37 \) °C in Luria-Bertani (LB) medium with ampicillin (50 \( \mu \)g ml\(^{-1} \)), tetracycline (10 \( \mu \)g ml\(^{-1} \)) or spectinomycin (50 \( \mu \)g ml\(^{-1} \)) selection where necessary. Strains were stored in 15% (v/v) glycerol in BHI at \(-70\) °C. Sheep-blood plates were prepared by adding 10% (v/v) defibrinated sheep blood (TCS Biologicals) to molten blood-agar base (Difco). All chemicals were purchased from Sigma unless otherwise stated.

**Recombinant DNA techniques.** All molecular biological methods were carried out as described by Sambrook et al. (1989).

**Enzyme assays.** In both the \( \beta \)-galactosidase and luciferase assays, \( S. \) aureus 8325-4 was used as a control.

**\( \beta \)-Galactosidase.** Cells were assayed for \( \beta \)-galactosidase (LacZ) production using MUG (4-methylumbelliferyl \( \beta \)-d-galacto-pyranoside) as substrate, based on the method of Youngman (1990). Briefly, bacterial cells (0.5 ml culture) were harvested by centrifugation (11,000 \( g \), 3 min, room temperature), the supernatant removed and the cell-pellet fraction snap-frozen at \(-70\) °C. Cells of high-level LacZ-producing strains were typically diluted 1 in 100, compared to original culture density, in AB buffer (100 mM NaCl, 60 mM \( \text{KH}_2\text{PO}_4 \), 40 mM \( \text{K}_2\text{HPO}_4 \) and 50 \( \mu \)l diluted cells lysed for 10 min at \( 37\) °C with lysostaphin (5 \( \mu \)l, 120 \( \mu \)g ml\(^{-1} \)) prior to addition of MUG (5 \( \mu \l, 4 \text{mg ml}^{-1} \)) and incubated for 100 min at 25 °C. \( \beta \)-Galactosidase activity was determined by fluorescence using a Dyna Quant 200 Fluorometer (Hoefer Pharmacia Biotech) using a range of concentrations of 4-methylumbelliferone as standards.

**Luciferase.** Luciferase activity was determined by adding 20 \( \mu \l \) n-decylaldehydine (1%, v/v, in ethanol) to 1 ml bacterial culture and rapidly mixing. Luminescence was measured on an Optocomp I lumimeter (Celsis) (Hill et al., 1993).

**Growth conditions for reporter-gene fusion experiments.** Fusion strains were streaked from glycerol stocks onto BHI plates containing suitable antibiotics, grown overnight at \( 37\) °C and subsequently used to inoculate 100 ml pre-warmed BHI (containing no antibiotics) in 250 ml conical flasks. Pre-cultures were grown to mid-exponential phase at \( 37\) °C in a shaking water bath (Grant OLS 200) at 250 r.p.m. for 3 h (OD\(_{660}\) ~ 1.0; Jenway 6100 spectrophotometer) and used to inoculate 100 ml pre-warmed BHI (same batch as pre-culture, no antibiotics) in test flasks (250 ml) to a starting OD\(_{660}\) of 0.1 and again incubated as above. Samples were taken over a 24 h period. Strains were simultaneously plated out on sheep-blood plates to confirm their expected \( \beta \)-haemolytic properties.

The effect of sub-growth-inhibitory concentrations of various compounds was tested by adding them to BHI prior to inoculation. All aerobic cultures reached a final OD\(_{660}\) of 8–11 after 16–18 h incubation at \( 37\) °C.

For limiting oxygen conditions, cultures were grown in a Variable atmosphere incubator (Don Whitley) either micro-aerobically (8% \( \text{O}_2, 5\% \text{CO}_2, 87\% \text{N}_2 \)) with agitation on a shaking platform for 1 d (final OD\(_{660}\) 3–5) or anaerobically (10% \( \text{H}_2, 10\% \text{CO}_2, 80\% \text{N}_2 \)) without shaking for 2 d (final OD\(_{660}\) 2–3), both at \( 37\) °C. For the pH experiments, the initial pH of BHI was altered (from pH 7.2±0.2) over a pH range 6.5–9.0, in increments of 0.5 pH unit, using HCl or NaOH. All results are representative of at least three individual experiments (unless otherwise stated) which all showed the same trends.

**In vitro construction of lacZ reporter-gene fusions**

**sarA::lacZ.** To obtain a SarA clone, a primer internal to the sarA gene was designed (5'-CGTATGGACATGATGAAA-GAAGCTTATTA-3') from published sequence (Cheung & Projan, 1994) and end-labelled with digoxigenin using terminal transferase (Boehringer Mannheim). This primer was used to probe a previously constructed partial Sau3AI ZAP Express chromosomal library of \( S. \) aureus 8325-4 (Foster, 1995) by plaque lifting (Sambrook et al., 1989). Several \( \lambda \) clones containing sarA inserts were excised as pBKCMV-derived phagemids. A phagemid clone (pSAR100) in \( E. \) coli XLOLR was confirmed to contain the sarA gene by restriction mapping and partial DNA sequencing. The sarA::lacZ reporter was constructed by excising a 1·1 kb EcoRI fragment from pSAR100 that encompassed all three sar promoters (Bayer et al., 1996) and the 5' end of the sarA gene. One EcoRI site was derived from the pBKCMV multiple-cloning site and the other was internal to the sarA coding region. The 1·1 kb EcoRI fragment was ligated into EcoRI-cut and

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Table 1. Bacterial strains and plasmids

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<th>Strain/plasmid</th>
<th>Genotype/phenotype</th>
<th>Source/reference</th>
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<tr>
<td>S. aureus 8325-4</td>
<td>Wild-type strain cured of known prophages</td>
<td>Novick (1967)</td>
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<td>RN4220</td>
<td>t&lt;sup&gt;+&lt;/sup&gt; m&lt;sup&gt;+&lt;/sup&gt;</td>
<td>Kreiswirth et al. (1983)</td>
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<td>PC901</td>
<td>agr&lt;sup&gt;A&lt;/sup&gt;: lacZ&lt;sup&gt;+&lt;/sup&gt; T&lt;sup&gt;c&lt;/sup&gt;</td>
<td>This study; Novick et al. (1993)</td>
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<tr>
<td>WA200</td>
<td>agr&lt;sup&gt;A&lt;/sup&gt;: Tet&lt;sup&gt;+&lt;/sup&gt; E&lt;sup&gt;c&lt;/sup&gt;</td>
<td>Morelli et al. (1988)</td>
</tr>
<tr>
<td>CYL36</td>
<td>RN4220/pTYL112A1 (a plasmid carrying the lysis gene) Cm&lt;sup&gt;+&lt;/sup&gt;</td>
<td>Lee et al. (1991)</td>
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<td>PC016</td>
<td>sar&lt;sup&gt;A&lt;/sup&gt;: sar&lt;sup&gt;A&lt;/sup&gt;: lacZ&lt;sup&gt;+&lt;/sup&gt; E&lt;sup&gt;c&lt;/sup&gt; (in RN4220 background)</td>
<td>This study</td>
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<tr>
<td>PC161</td>
<td>sar&lt;sup&gt;A&lt;/sup&gt;: sar&lt;sup&gt;A&lt;/sup&gt;: lacZ&lt;sup&gt;+&lt;/sup&gt; E&lt;sup&gt;c&lt;/sup&gt;</td>
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<td>PC203</td>
<td>spa&lt;sup&gt;A&lt;/sup&gt;: spa&lt;sup&gt;A&lt;/sup&gt;: lacZ&lt;sup&gt;+&lt;/sup&gt; E&lt;sup&gt;c&lt;/sup&gt;</td>
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<td>sar&lt;sup&gt;A&lt;/sup&gt;: sar&lt;sup&gt;A&lt;/sup&gt;: ag&lt;sup&gt;D&lt;/sup&gt;: tet E&lt;sup&gt;c&lt;/sup&gt;</td>
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<td>PC206</td>
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<td>PC1001</td>
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<td>This study</td>
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**E. coli**

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<th>Source/Genotype/phenotype</th>
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<td>pBR322</td>
<td>Promega</td>
</tr>
<tr>
<td>DH5α</td>
<td>pBK-CMV</td>
<td>Stratagene</td>
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**Plasmids**

- **pAZ106**: E. coli cloning vector, Ap<sup>+</sup>
- **pBR322**: E. coli cloning vector, Km<sup>+</sup>
- **pAZ106**: Promoterless transcriptional lacZ fusion vector, Ap<sup>+</sup> (E. coli), E<sup>c</sup> (S. aureus)
- **pMUTIN4**: Promoterless transcriptional lacZ fusion vector, Ap<sup>+</sup> (E. coli), E<sup>c</sup> (S. aureus)
- **pAC34**: Vector for integration into geh (lipase) gene of S. aureus, Sp<sup>+</sup> (E. coli), T<sup>c</sup> (S. aureus)
- **pSR100**: 2.5 kb Smal fragment carrying sar operon from a S. aureus 8325-4 ligated into BamHI-cut pBK-CMV; Km<sup>+</sup>
- **pSR721**: 4.5 kb EcoRI fragment containing complete spa operon in pACYC184; T<sup>c</sup>
- **pDU1122**: 15 kb HindIII-EcoRI fragment containing complete spa operon in pBR322, linked to a 3.4 kb Cm<sup>+</sup> marker; Ap<sup>+</sup> (E. coli), Cm<sup>+</sup> (S. aureus)
- **pB7001**: 0.4 kb EcoRI fragment containing the tet promoter from a S. aureus FR11187 inserted into EcoRI-cut luxAB transcriptional vector, pB330 (in the same direction as I<sup>tr</sup>AB); Ap<sup>+</sup>, Cm<sup>+</sup>
- **pAZ3**: 1 kb EcoRI fragment from pSR100 containing the 5'S region of the sar<sup>A</sup> gene and upstream sequence in EcoRI-cut pAZ106; Ap<sup>+</sup> (E. coli), E<sup>c</sup> (S. aureus)
- **pUBSPA1**: 12 kb EcoRI-HindIII fragment from pSR721 containing the 5'S region of the spa gene and upstream sequence in EcoRI-HindIII-cut pBS1; Ap<sup>+</sup>
- **pUSPA25**: 1 kb BamHI-HindIII fragment from pUBSPA1 containing the 5'S region of the spa gene and upstream sequence in BamHI-Smal-cut pAZ106; Ap<sup>+</sup> (E. coli), E<sup>c</sup> (S. aureus)
- **pPF4**: 2.2 kb HindIII-ScaI fragment from pDU1122 containing the 5'S region of the hla gene and upstream sequence in HindIII-Smal-cut pBK-CMV; Km<sup>+</sup>
- **pH16**: 2.2 kb EcoRI-NorI fragment from pPF4 containing the 5'S region of the hla gene and upstream sequence in EcoRI-NorI-cut pMUTIN4; Ap<sup>+</sup> (E. coli), E<sup>c</sup> (S. aureus)
- **pPC1**: 30 kb partial EcoRI-BamHI fragment from pB7001 containing the tet::lux fusion in EcoRI/HindIII-cut pCL84; Sp<sup>+</sup> (E. coli), T<sup>c</sup> (S. aureus)

**Integration of the lacZ fusions into the S. aureus chromosome.** LacZ fusion plasmids (5-50 μg DNA) were introduced into the chromosome of S. aureus RN4220 by electroproporation (Schenk & Laddaga, 1992) and recombinants selected on erythromycin (5 μg ml<sup>-1</sup>). Since the lacZ vectors do not contain a replicon active in S. aureus, erythromycin-resistant clones occur as a result of a homologous recombination event between the plasmid inserts and the host chromosome. The fusions were transferred into S. aureus 8325-4 by phage transduction, using Φ11 as the carrier (Novick, 1991), and selected on erythromycin (5 μg ml<sup>-1</sup>). All fusions were blue when streaked on BH plates containing X-Gal (40 μg ml<sup>-1</sup)), indicating a functional lacZ gene, whilst the 8325-4 parental strain was white on X-Gal. The expected integrational events were confirmed by Southern blot analysis using the appropriate DNA inserts as probe (results not shown). All fusions were haemolytic when checked on sheep-blood plates containing erythromycin (5 μg ml<sup>-1</sup>). The agr<sup>D</sup>: tetM genotype of RN6911 (Novick et al., 1993), which has the whole of the agr locus deleted, was introduced into the 8325-4 and fusion backgrounds by phage transduction using Φ11. agrD::tetM transductants were non-haemolytic when...
grown on sheep-blood plates containing erythromycin and tetracycline (both at 5 μg ml⁻¹).

**Construction of tst::lux fusion.** Plasmid pBT001 carrying a tst::lux fusion (Timmins et al., 1995) was digested to completion with BamHI and then partially cut with EcoRI to release a 3·0 kb DNA fragment containing the tst::lux region. This was ligated into EcoRI/BamHI-cut pCL84 (a vector carrying the L54a attachment site for integration into the lipase gene of the S. aureus chromosome; Lee et al., 1991). The resulting construct, pPC1, was electroporated into S. aureus CYL316 (S. aureus RN4240 containing plasmid pYL112Δ19, which carries the integrase gene necessary for recombination into the lipase structural gene). A non-lipase-producing, tetracycline-resistant transformant was selected and the chromosomal construct transferred into S. aureus 8325-4 using 11 transduction to give strain PC1072. The correct insertional event was confirmed by Southern blot and PCR analysis (results not shown). The agrA::Tn551 mutation of WA250 (Morfeldt et al., 1988) was transferred into PC1072 by 11 transduction to give a tetracycline- and erythromycin-resistant strain, PC1093. Transductant PC1072 was haemolytic whilst PC1093 was totally non-haemolytic on sheep-blood plates.

**RESULTS**

**Expression of virulence determinants during growth**

Chromosomal transcriptional-reporter gene fusions of the virulence-determinant genes sarA, spa, bla and tst with the reporter genes lacZ or luxAB were made in S. aureus 8325-4 (Table 1). The lacZ fusions were constructed such that following a single-crossover integration event between the plasmids and the host genome, the fusions contain an intact copy of the virulence gene plus a truncated copy fused to the lacZ gene, stably inserted into the chromosome of S. aureus 8325-4. As S. aureus 8325-4 does not contain the tst gene, the tst::lux fusion was introduced into S. aureus 8325-4 by site-specific integration into the lipase structural gene. All constructs were confirmed by Southern-blot analysis. A representative Southern blot is shown in Fig. 1 for the sarA::lacZ construct. The correct insertion is created on integration of pAZ3 into the chromosome. This results in the expected extra hybridizing band of 1·1 kb when Southern blots are probed with the sarA fragment used for insertion (Fig. 1). The hybridization pattern and the relative intensities of the hybridizing bands show that the integration has led to a single extra copy of the pAZ3 insert.

All our studies were performed in BHI, since, of a wide range of complex and defined media tested, BHI gave the highest toxin expression with high growth yield (results not shown). Preliminary studies showed that slight alterations in growth conditions had a significant effect on expression of virulence determinants. Hence, specific growth conditions were defined as described in Methods and strictly adopted. Furthermore, an exponential-phase pre-inoculum prepared in BHI was used in all our experiments to prevent carry-over from stationary-phase cultures affecting virulence-determinant expression (Saravia-Otten et al., 1997).

The expression of each fusion was followed during growth under standard aerobic growth conditions (Fig.

**Fig. 1.** Construction of a sarA::lacZ fusion. (a) Diagram showing the integration of pAZ3 into the S. aureus RN4220 chromosome to give PC016 (sarA::lacZ) (not to scale). Phage transduction was used to transfer the construct into the 8325-4 background (see Methods) to give strain PC161 (sarA::lacZ). The sizes of important EcoRI restriction fragments are shown. (b) Southern blot of S. aureus 8325-4 (lane 1) and PC161 (lane 2) genomic DNA cut with EcoRI and probed with the digoxigenin-labelled 1·1 kb EcoRI insert from pAZ3. The sizes of the corresponding fragments are highlighted in bold in (a). The sizes of markers run on the same gel are indicated on the left.
All fusion strains showed identical growth kinetics to each other (Fig. 2) and to the parental strain 8325-4 (results not shown). Expression of sarA, blA and spa was determined by measuring the amount of β-galactosidase produced (encoded by lacZ) whilst tst expression was monitored by measurement of luciferase activity (encoded by lux). Using the sarA::lacZ fusion, which measures expression from all three sar promoters (Bayer et al., 1996), sarA expression was found to increase during the growth phase, reaching approximately 5000 MUG units of β-galactosidase activity in stationary phase ($t=18$ h) (Fig. 2a). When spa expression was monitored during growth, levels increased during the exponential phase, achieving a maximum of 120 MUG units in late-exponential phase ($t=3$ h), followed by a decline to 50 MUG units on entry into stationary phase ($t=15$ h) (Fig. 2b). In contrast, maximal expression of α-haemolysin was observed in the transition between post-exponential and stationary phases (Fig. 2c). Early in growth there was a basal level of blA expression (340 MUG units, $t=1-3$ h) followed by a rapid increase in blA transcription during the post-exponential phase ($t=4-8$ h) with levels staying unchanged during the stationary phase (130000 MUG units, $t=12-24$ h). Like blA, tst was preferentially expressed in the transitionary period from exponential to stationary phases of growth. Luciferase activity reached a maximum of 4000 light units at 12 h into growth and remained high during the stationary phase (Fig. 2d).

**Role of agr in expression of virulence-determinant genes**

To examine the role of agr in virulence-determinant gene expression, the agr mutation was introduced into all fusion strains by phage transduction. When sarA

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**Fig. 2.** Expression of virulence-determinant gene fusions during growth of *S. aureus* 8325-4 and the effects of 1 M NaCl. Chromosomal fusions (a) PC161 (sarA::lacZ), (b) PC203 (spa::lacZ), (c) PC322 (bla::lacZ) and (d) PC1072 (tst::lux) were grown in BHI at 37 °C with shaking at 250 r.p.m. as described in Methods. Growth of cultures in BHI (○) or BHI containing 1 M NaCl (■) was measured as OD$_{600}$. Fusion expression was measured by β-galactosidase or luciferase activity in cultures grown in BHI alone (●) or in BHI supplemented with 1 M NaCl (■) as described in Methods. Results are representative of a minimum of three independent experiments.
expression was compared in wild-type and agr mutant backgrounds (PC161 and PC162 respectively), no obvious difference in sarA expression was observed (Fig. 3a). The introduction of the agr mutation into the spa fusion caused a dramatic alteration in spa expression (Fig. 3b). There was a loss of growth-dependent expression of spa in the agr mutant (PC206), with levels of spa increased approximately 100-fold at late-exponential phase ($t=3$ h) and 20-fold at stationary phase ($t=15$ h), as compared to the wild-type background (PC203). Hence, spa is normally strongly repressed by agr. In contrast, bla expression in an agr mutant (PC324) was dramatically reduced post-exponentially ($t=12$ h) to approximately 8% of wild-type levels (PC322), consistent with agr positively regulating bla transcription (Fig. 3c). Interestingly, bla levels were still detectable in the agr mutant (13000 MUG units, $t=18$ h), suggesting there are other components besides agr involved in its regulation. When tst expression was examined in an agr mutant background (PC1093), the fusion showed less than 4% of wild-type activity (PC1072) in 12 h cultures, indicating that tst expression is also upregulated by agr (Fig. 3d).

**Effect of sodium chloride on virulence-determinant gene expression**

*S. aureus* is able to tolerate and grow in high-salt environments (Troller, 1986). An NaCl concentration of 1 M was found to be the maximum which gave a high growth yield and hence this concentration was chosen for further studies. As seen in Fig. 2, the addition of 1 M
NaCl to BHI led to a dramatic decrease in spa, hla and tst transcription during growth, with levels reduced to less than 10, 2 and 1% of expression in BHI alone at \( t = 3 \) h, 12 h and 12 h, respectively. In contrast, sarA expression remained relatively unaffected by 1 M NaCl, although a moderate increase in sarA expression (less than twofold in stationary phase, \( t = 18 \) h) was observed (Fig. 2a). Cultures grown in the presence of 1 M NaCl showed a slight reduction in growth rate and final cell yield compared to control cultures (Fig. 2).

To determine whether the salt effect on virulence-determinant gene expression is mediated via agr, expression of the fusions in strains carrying an agr mutation (Fig. 3) were compared with wild-type expression in Methods. Growth was measured as OD\(_{600}\) as described in Methods. Results are representative of a minimum of three independent cultures.

**Effect of sucrose on expression of virulence-determinant genes**

To ascertain whether the salt effect on virulence-determinant gene expression was due to osmolarity, the effect of sucrose on fusion expression was investigated. The addition of 0.02, 0.5 and 1 M sucrose to BHI typically led to a final growth yield (OD\(_{600}\) at \( t = 16 \) h) of 10, 6.5 and 0, respectively. The effect of adding 20 mM sucrose to BHI, on expression of sarA, hla and tst in stationary-phase (\( t = 16 \) h) and spa in late-exponential phase (\( t = 3 \) h) cultures, was 22, 2, 3 and 37% of the control expression, respectively (results not shown). In the agr mutant background, 20 mM sucrose still reduced expression of sarA, hla and tst in the same relative proportion as in the wild-type, which suggests the sucrose signal is transduced via an agr-independent mechanism.

**Effect of novobiocin on virulence-determinant gene expression**

NaCl has also been shown to inhibit expression of epidermolytic toxin A (eta) and this was suggested to occur by DNA-supercoiling effects (Sheehan et al., 1992). The effects of novobiocin, an inhibitor of DNA gyrase, on tst::lux fusion expression were examined. Novobiocin at 0.5 ng ml\(^{-1}\) did not significantly affect growth or tst expression in the post-exponential phase (\( t = 12 \) h) in the wild-type or agr mutant (results not shown). To determine whether DNA supercoiling plays a role in the salt repression of tst expression, the combined effects of novobiocin (0.5 ng ml\(^{-1}\)) and NaCl (1 M) were determined. Salt repression of tst was not reversed by the addition of novobiocin (results not shown). Novobiocin had no effect on spa, hla or sarA expression.

**Effect of the availability of magnesium, calcium and iron on virulence-determinant gene expression**

The effects of divalent cations on virulence-determinant production were studied using the fusion strains. The addition of MgCl\(_2\) (20 mM) to BHI resulted in an approximately twofold post-exponential decrease in tst expression (\( t = 12 \) h) (Fig. 4). Similarly, CaCl\(_2\) (5 mM) or FeSO\(_4\) (0.05 mM) added to BHI led to an approximately 50% or 70% final activity respectively (\( t = 17 \) h) compared to the control flasks (results not shown). EDTA (0.5 mM), caused a threefold increase in tst expression (\( t = 12 \) h) (Fig. 4). This stimulatory effect of EDTA was confirmed to be due to the chelation of Mg\(^{2+}\) or Ca\(^{2+}\), since the addition of an excess of either of these divalent cations (20 and 5 mM respectively) to cultures already containing EDTA (0.5 mM) resulted in a decrease in tst expression to almost control levels (results not shown). Furthermore, the addition of other metal chelators such as sodium citrate (0.25%) or EGTA (0.5 mM) to BHI led to a five- or threefold increase in tst expression, respectively (\( t = 12 \) h). A low-iron environment (BHI with 0.4 mM 2,2'-dipyridyl) also resulted in an approximately twofold increase in tst transcription in post-exponential phase cultures (\( t = 12 \) h) (results not shown). In an agr mutant background, the addition of EDTA or EGTA to BHI also enhanced tst levels two- to threefold, demonstrating that the induction may occur independently of agr (results not shown).
In contrast, the expression of sarA and hla was not greatly influenced by the addition of MgCl₂, EDTA, EGTA, sodium citrate or 2,2'-dipyridyl at the above concentrations to the growth medium. Interestingly, EGTA (0.5 mM) strongly repressed spa expression during late-exponential phase (t = 4 h, 10-fold) compared to expression in BHI alone (results not shown). Both EDTA (0.5 mM) and sodium citrate (0.25 %) were also inhibitory to spa expression in late-exponential-phase cultures (two- to fourfold), whilst 2,2'-dipyridyl had no effect.

**DISCUSSION**

A range of reporter gene fusions have been created in *S. aureus*, allowing the effect of physiological and environmental factors on expression of virulence determinants to be determined. The fusions are stable and thus do not require maintenance by the addition of drugs, which may affect toxin production (Doss et al., 1993; Vandenesch et al., 1991). Furthermore, they are in single copy so as not to titrate out regulatory elements sometimes associated with high-copy-number plasmid reporters. Insertion of the fusions into the chromosome also leaves an intact copy of the locus (where applicable).

All the reporter fusions show the growth-phase expression kinetics expected from previous studies. Using the transcriptional-reporter gene fusion, we have shown that sarA expression increases during the exponential growth phase, which is consistent with RNA analysis (Bayer et al., 1996). However, the expression of hla and tst, like RNAIII, occurs preferentially in the transition between the post-exponential and stationary phases of growth (Janzon et al., 1989; Kornblum et al., 1990; Vandenesch et al., 1991). In contrast, spa is optimally expressed late in log phase and decreases during stationary phase (Vandenesch et al., 1991). The apparent expression of spa and hla in 24 h cultures may be explained by an accumulation of the β-galactosidase reporter rather than actual continued transcription. In an agr mutant background, expression of both hla and tst was greatly reduced (more than 10-fold) during the post-exponential phase, thus confirming that agr is a positive regulator of hla and tst expression at the transcriptional level (Kornblum et al., 1990). The spa::lacZ fusion created in this study gave a 20- to 100-fold increase in β-galactosidase levels in an agr mutant, consistent with expected results (Recsei et al., 1986, Foster et al., 1990; Cheung et al., 1997b). The agr mutation did not affect sarA expression, which agrees with sarA being at a higher level in the regulatory hierarchy (Heinrichs et al., 1996). The reporter-gene fusions provide a convenient assay for virulence-determinant gene expression.

A wide range of environmental factors have been tested for their effects on virulence-determinant expression in *S. aureus*. The addition of NaCl to the growth medium had a dramatic effect on the production of both toxins and surface proteins. Expression of the virulence genes spa, hla and tst was greatly suppressed. Our studies also show that sarA expression, like RNAIII, is not altered by high NaCl levels (Regassa & Betley, 1993). The NaCl repression of sarA, spa, hla and tst expression is apparently independent of agr. Hence, under certain environmental conditions, transcription of the agr-dependent genes hla, tst and spa may be diverted from sarA and agr regulation, suggesting that additional mechanisms are also involved in their control. During the course of this study, Ohlsen et al. (1997), using a chromosomally encoded bla::lacZ fusion in *S. aureus* Wood 46, similarly observed bla repression at high concentrations of the osmolytes NaCl, KCl and sodium glutamate. Expression of other agr-upregulated exoproteins such as Eta and staphylococcal enterotoxins B and C (SEB and SEC) are also repressed by high osmolyte levels (Sheehan et al., 1992; Genigeorgis & Sadler, 1966; Regassa & Betley, 1993).

The influence of sucrose on virulence-determinant transcription was also examined to determine whether salt repression was a general osmotic-stress effect, or due to sodium or chloride ions specifically. We found that sucrose, at a concentration as low as 0.02 M, was sufficient to suppress gene expression of hla and tst, and to a lesser extent, spa. Interestingly, sarA expression was also sensitive to sucrose repression. This suggests that sucrose regulation of virulence-determinant gene expression is mediated via additional mechanisms independent of osmolarity regulation. Catabolite repression may be involved, since our results show that equivalent glucose levels (10-50 mM) also inhibited sarA, spa, hla and tst at the transcriptional level (results not shown). Sucrose regulation of virulence-gene expression, like that by NaCl, occurs independently of agr. Sheehan et al. (1992), using an eta::lux plasmid fusion, observed an agr-independent NaCl and sucrose repression of eta expression and noted that this repression was five times greater in the presence of 0.44 M sucrose compared with growth in 0.3 M NaCl.

In several pathogens, environmental changes in osmolarity trigger virulence-gene expression by alterations in supercoiling (Dorman, 1991). Whether DNA topology affects virulence-determinant expression in *S. aureus* was examined using novobiocin, a DNA gyrase inhibitor (Surcliffe et al., 1989). Expression of tst, hla and spa, and their repression by NaCl, were not affected by novobiocin. In contrast, studies by Sheehan et al. (1992) linked both the decreased expression of eta and increased negative supercoiling of *S. aureus* plasmid DNA in the presence of high NaCl levels, with the observation that novobiocin (which reduces negative supercoils) increases eta fusion activity. The effects of novobiocin, and thereby DNA supercoiling, may be limited to specific gene promoters in the osmoregulation of virulence expression.

The salt repression of toxin expression may form part of a sophisticated environmental-response mechanism, limiting toxin production where it would be inappropriate. Osmoprotectants were found to relieve the NaCl-induced repression of SEC (Regassa & Betley,
parameters which modulate virulence-determinant production, further enhanced the stimulatory effect of
involving the regulatory mechanisms to rapidly respond and possibly other cations from the growth environment.
S. aureus is a successful colonizer of a diverse range of environments in the human host having evolved sophis-
ticated regulatory mechanisms to rapidly respond and adapt to environmental changes. S. aureus can also cause food poisoning by the ingestion of preformed enterotoxins. This study has revealed key environmental parameters which modulate virulence-determinant production, seemingly independent of the well-charac-
terized regulatory elements, sarA and quorum-sensing involving the agr locus. Additional pleiotropic regulators such as sae (Giraudo et al., 1994), sigB (Wu et al., 1996) and other unidentified components (Vandenesch et al., 1991) may have a role in the environmental regulation of virulence-factor biosynthesis. Identification of novel regulators of virulence-determinant production and elucidation of the mechanism whereby they transduce environmental stimuli to bring about changes in gene expression forms the focus of our continuing research.

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