The role of environmental factors in the regulation of virulence-determinant expression in *Staphylococcus aureus* 8325-4

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***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

INTRODUCTION

Staphylococcus aureus is an important human pathogen implicated in a wide range of diseases including septicaemia, meningitis, endocarditis, osteomyelitis, septic arthritis, toxic-shock syndrome (TSS) and food poisoning (Waldvogel, 1995). The pathogenic diversity of the bacterium reflects its ability to successfully colonize, adapt and survive in many different host tissues during infection. The production of a repertoire of virulence determinants such as extracellular proteins [including α -haemolysin (encoded by the *hla* gene) and toxic-shock-syndrome toxin-1 (*tst* gene)] and cell-surface-associated proteins [such as protein A (*spa* gene) and fibronectin-binding protein] contributes to the virulence of the bacterium (Iandolo, 1990).

The exo- and surface proteins are expressed coordinately in a growth-phase-dependent manner and are controlled by at least two well-characterized global regulatory loci, *sar* and *agr* (Cheung *et al.*, 1992; Recsei

et al., 1986; Morfeldt *et al.*, 1988; Peng *et al.*, 1988). Other putative pleiotropic regulators such as *xpr* and *sae* have also been described (Hart *et al.*, 1993; Giraudo *et al.*, 1994). The *agr* locus controls virulence-determinant gene expression by way of a regulatory RNA molecule, RNAIII, as an effector (Janzon & Arvidson, 1990; Novick *et al.*, 1993). This both upregulates production of extracellular toxins and represses expression of surface proteins primarily at the transcriptional level (Kornblum *et al.*, 1990; Vandenesch *et al.*, 1991; Novick *et al.*, 1993; Morfeldt *et al.*, 1995). The *agr* locus encodes a two-component sensor-regulator pair, *agrC* and *agrA*, respectively (Novick *et al.*, 1995; Parkinson & Kofoed, 1992). The sensor protein (AgrC) has been proposed to sense the extracellular concentration of a modified octapeptide which is the processed product of the *agrD* gene. This quorum-sensing mechanism leads to expression of RNAIII, which in turn elicits virulence-determinant production in *S. aureus* (Balaban & Novick, 1995; Ji *et al.*, 1995, 1997).

The *sar* locus is expressed as three overlapping transcripts, differentially produced during the growth cycle, all of which encode SarA (Bayer *et al.*, 1996). SarA is

Abbreviations: MUG, 4-methylumbelliferyl β -D-galactopyranoside; TSS, toxic shock syndrome.

required for maximal expression of *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 *agr* promoter region affecting control of target genes (Morfeldt *et al.*, 1996; Cheung *et al.*, 1997a). Furthermore, *sar* is also able to modulate expression of certain virulence determinants independently of *agr* (Cheung *et al.*, 1997b). Both *sarA*- and *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 *agr* are major components in the hierarchical control of virulence factor biosynthesis.

The environmental stimuli which activate the *sar* and *agr* systems and how these signals are transduced to alter virulence-determinant expression are poorly understood. Limited studies have shown that *agr* is repressed by alkaline pH and glucose (Regassa & Betley, 1992; Regassa *et al.*, 1992) but the environmental triggers for *sar* expression are currently unknown. The production of α -haemolysin is affected by glucose, temperature and NaCl (Duncan & Cho, 1972; Wiseman, 1975; Ohlsen *et al.*, 1997). The role of magnesium in the regulation of *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*, *hla* and *tst*). Furthermore, epistasis experiments have defined the role of *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 medium (BHI) (Oxoid) containing erythromycin ($5 \mu\text{g ml}^{-1}$) or tetracycline ($5 \mu\text{g ml}^{-1}$) where appropriate. *Escherichia coli* was grown at 37°C in Luria-Bertani (LB) medium with ampicillin ($50 \mu\text{g ml}^{-1}$), tetracycline ($10 \mu\text{g ml}^{-1}$) or spectinomycin ($50 \mu\text{g ml}^{-1}$) 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 β -galactosidase and luciferase assays, *S. aureus* 8325-4 was used as a control.

β -Galactosidase. Cells were assayed for β -galactosidase (LacZ) production using MUG (4-methylumbelliferyl β -D-galacto-

pyranoside) as substrate, based on the method of Youngman (1990). Briefly, bacterial cells (0.5 ml culture) were harvested by centrifugation (11000 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 K_2HPO_4 , 40 mM KH_2PO_4) and 50 μl diluted cells lysed for 10 min at 37°C with lysostaphin ($5 \mu\text{l}$, $120 \mu\text{g ml}^{-1}$) prior to addition of MUG ($5 \mu\text{l}$, 4 mg ml^{-1}) and incubated for 100 min at 25°C . β -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 μl n-decylaldehyde (1%, v/v, in ethanol) to 1 ml bacterial culture and rapidly mixing. Luminescence was measured on an Optocomp1 luminometer (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 ($\text{OD}_{600} \sim 1.0$; 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_{600} of 0.01 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 β -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_{600} 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% O_2 , 5% CO_2 , 87% N_2) with agitation on a shaking platform for 1 d (final OD_{600} 3–5) or anaerobically (10% H_2 , 10% CO_2 , 80% N_2) without shaking for 2 d (final OD_{600} 2–3), both at 37°C . For the pH experiments, the initial pH of BHI was altered (from $\text{pH } 7.2 \pm 0.2$) 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'-CGTAATGAGCATGATGAAA-GAAGTGTATTA-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* λ ZAP Express chromosomal library of *S. aureus* 8325-4 (Foster, 1995) by plaque lifting (Sambrook *et al.*, 1989). Several λ clones containing *sarA* inserts were excised as pBKCMV-derived phagemids. A phagemid clone (pSAR100) in *E. coli* XL0LR 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

Table 1. Bacterial strains and plasmids

Strain/plasmid	Genotype/phenotype	Source/reference
Strains		
<i>S. aureus</i>		
8325-4	Wild-type strain cured of known prophages	Novick (1967)
RN4220	$r_k^- m_k^+$	Kreiswirth <i>et al.</i> (1983)
PC6911	<i>agrΔ::tetM</i> Tc ^r	This study; Novick <i>et al.</i> (1993)
WA250	<i>agrA::Tn551</i> Ey ^r	Morfeldt <i>et al.</i> (1988)
CYL316	RN4220/pYL112Δ19 (a plasmid carrying the L45a <i>int</i> gene) Cm ^r	Lee <i>et al.</i> (1991)
PC016	<i>sarA⁺ sarA::lacZ</i> Ey ^r (in RN4220 background)	This study
PC161	<i>sarA⁺ sarA::lacZ</i> Ey ^r	This study
PC203	<i>spa⁺ spa::lacZ</i> Ey ^r	This study
PC322	<i>bla⁺ bla::lacZ</i> Ey ^r	This study
PC1072	<i>tst::lux</i> Tc ^r	This study
PC162	<i>sarA⁺ sarA::lacZ agrΔ::tetM</i> Ey ^r Tc ^r	This study
PC206	<i>spa⁺ spa::lacZ agrΔ::tetM</i> Ey ^r Tc ^r	This study
PC324	<i>bla⁺ bla::lacZ agrΔ::tetM</i> Ey ^r Tc ^r	This study
PC1093	<i>tst::lux agrA::Tn551</i> Tc ^r Ey ^r	This study
<i>E. coli</i>		
XL0LR	Δ(<i>mcrA</i>)183 Δ(<i>mcrCB-hsdSMR-mrr</i>)173 <i>endA1 thi-1 recA1 gyrA96 relA1 lac</i> [F' <i>proAB lac</i> ⁺ Δ <i>M15 Tn10</i> (Tc ^r)] Su ⁻ λ ^r	Stratagene
DH5α	φ80 <i>dlc</i> Δ <i>M15 recA1 endA1 gyrA96 thi-1 hsdR17</i> (r _k ⁻ , m _k ⁻) <i>supE44 relA1 deoR</i> Δ(<i>lacZYA-argF</i>)U169	Promega
Plasmids		
pUBS1	<i>E. coli</i> cloning vector; Ap ^r	G. Murphy*
pBK-CMV	<i>E. coli</i> cloning vector; Km ^r	Stratagene
pAZ106	Promoterless transcriptional <i>lacZ</i> fusion vector; Ap ^r (<i>E. coli</i>), Ey ^r (<i>S. aureus</i>)	Kemp <i>et al.</i> (1991)
pMUTIN4	Promoterless transcriptional <i>lacZ</i> fusion vector; Ap ^r (<i>E. coli</i>), Ey ^r (<i>S. aureus</i>)	V. Vagner†
pCL84	Vector for integration into <i>geh</i> (lipase) gene of <i>S. aureus</i> , attP; Sp ^r (<i>E. coli</i>), Tc ^r (<i>S. aureus</i>)	Lee <i>et al.</i> (1991)
pSAR100	2.3 kb <i>Sau3A</i> I fragment spanning <i>sar</i> operon from a λ library of <i>S. aureus</i> 8325-4 ligated into <i>Bam</i> HI-cut pBK-CMV; Km ^r	This study
pSPA721	4.3 kb <i>Eco</i> RI fragment containing complete <i>spa</i> operon in pACYC184; Tc ^r	Patel <i>et al.</i> (1987)
pDU1212	3.0 kb <i>Hind</i> III- <i>Eco</i> RI fragment containing complete <i>bla</i> operon in pBR322, linked to a 3.0 kb Cm ^r marker; Ap ^r (<i>E. coli</i>), Cm ^r (<i>S. aureus</i>)	Fairweather <i>et al.</i> (1983)
pBT001	0.4 kb <i>Eco</i> RI fragment containing the <i>tst</i> promoter from a λ library of <i>S. aureus</i> FR1187 inserted into <i>Eco</i> RI-cut <i>luxAB</i> transcriptional vector, pSB330 (in the same direction as <i>luxAB</i>); Ap ^r , Cm ^r	Timmins <i>et al.</i> (1995)
pAZ3	1.1 kb <i>Eco</i> RI fragment from pSAR100 containing the 5' region of the <i>sarA</i> gene and upstream sequence in <i>Eco</i> RI-cut pAZ106; Ap ^r (<i>E. coli</i>), Ey ^r (<i>S. aureus</i>)	This study
pUBSPA1	1.2 kb <i>Eco</i> RI- <i>Hind</i> III fragment from pSPA721 containing the 5' region of the <i>spa</i> gene and upstream sequence in <i>Eco</i> RI/ <i>Hind</i> III-cut pUBS1; Ap ^r	This study
pSUSPA25	1.2 kb <i>Bam</i> HI- <i>Hinc</i> II fragment from pUBSPA1 containing the 5' region of the <i>spa</i> gene and upstream sequence in <i>Bam</i> HI/ <i>Sma</i> I-cut pAZ106; Ap ^r (<i>E. coli</i>), Ey ^r (<i>S. aureus</i>)	This study
pPF4	2.2 kb <i>Hind</i> III- <i>Sca</i> I fragment from pDU1212 containing the 5' region of the <i>bla</i> gene and upstream sequence in <i>Hind</i> III/ <i>Sca</i> I-cut pBK-CMV; Km ^r	This study
pH16	2.2 kb <i>Eco</i> RI- <i>Not</i> I fragment from pPF4 containing the 5' region of the <i>bla</i> gene and upstream sequence in <i>Eco</i> RI/ <i>Not</i> I-cut pMUTIN4; Ap ^r (<i>E. coli</i>), Ey ^r (<i>S. aureus</i>)	This study
pPC1	3.0 kb partial <i>Eco</i> RI- <i>Bam</i> HI fragment from pBT001 containing the <i>tst::lux</i> fusion in <i>Eco</i> RI/ <i>Bam</i> HI-cut pCL84; Sp ^r (<i>E. coli</i>), Tc ^r (<i>S. aureus</i>)	This study

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dephosphorylated pAZ106 (Kemp *et al.*, 1991) and transformed into *E. coli* XL0LR. The resulting plasmid, pAZ3, was confirmed to contain the truncated *sarA* gene inserted in the correct orientation with *lacZ* to generate a transcriptional fusion by restriction digestion.

***spa::lacZ*.** The 1.2 kb *Eco*RI-*Hind*III fragment containing the control region and 5' end of the *spa* gene from pSPA721 (Patel *et al.*, 1987, 1992) was cloned into *Eco*RI/*Hind*III-cut vector pUBS1 (Foster, 1991), to give pUBSPA1 in *E. coli* DH5α. A *Bam*HI-*Hinc*II fragment was then excised and ligated into *Bam*HI/*Sma*I-cut pAZ106, resulting in the transcriptional-fusion plasmid pSUSPA25.

***hla::lacZ*.** A 2.2 kb *Hind*III-*Sca*I fragment containing the upstream region and 5' portion of the *hla* gene was excised from pDU1212 (Fairweather *et al.*, 1983) and subcloned into *Hind*III/*Sca*I-cut pBKCMV to give plasmid pPF4 in *E. coli* DH5α. The 2.2 kb insert was then excised with *Eco*RI/*Not*I and cloned into *Eco*RI/*Not*I-cut pMUTIN4 (Edwards & Errington, 1997) to create the transcriptional-reporter fusion plasmid pH16.

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 electroporation (Schenk & Laddaga, 1992) and recombinants selected on erythromycin (5 µg ml⁻¹). 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⁻¹). All fusions were blue when streaked on BHI plates containing X-Gal (40 µg ml⁻¹), 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⁻¹). The *agrΔ::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. *agrΔ::tetM* transductants were non-haemolytic when

grown on sheep-blood plates containing erythromycin and tetracycline (both at $5 \mu\text{g ml}^{-1}$).

Construction of *tst::lux* fusion. Plasmid pBT001 carrying a *tst::lux* fusion (Timmins *et al.*, 1995) was digested to completion with *Bam*HI and then partially cut with *Eco*RI to release a 3.0 kb DNA fragment containing the *tst::lux* region. This was ligated into *Eco*RI/*Bam*HI-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* RN4220 containing plasmid pYL112 Δ 19, 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 ϕ 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 ϕ 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*, *hla* 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 integrational 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 *Eco*RI restriction fragments are shown. (b) Southern blot of *S. aureus* 8325-4 (lane 1) and PC161 (lane 2) genomic DNA cut with *Eco*RI and probed with the digoxigenin-labelled 1.1 kb *Eco*RI 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.

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 (*hla::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 OD₆₀₀. 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.

2). 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*, *hla* 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 *hla* expression (340 MUG units, $t=1-3$ h) followed by a rapid increase in *hla* transcription during the post-exponential phase ($t=4-8$ h) with levels staying unchanged during the stationary phase (130 000 MUG units, $t=12-24$ h). Like *hla*, *tst* was preferentially expressed in the transitional 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*

Fig. 3. Role of *agr* in virulence-determinant gene expression during growth of *S. aureus* 8325-4 and the effects of 1 M NaCl. Chromosomal fusions in wild-type background [(○, ●), (a) PC161 (*sarA::lacZ*), (b) PC203 (*spa::lacZ*), (c) PC322 (*hla::lacZ*) and (d) PC1072 (*tst::lux*)] or *agr* backgrounds [(□, ■, △, ▲), (a) PC162 (*agrΔ::tetM*, *sarA::lacZ*), (b) PC206 (*agrΔ::tetM*, *spa::lacZ*), (c) PC324 (*agrΔ::tetM*, *hla::lacZ*) and (d) PC1093 (*agrA::Tn551*, *tst::lux*)] were grown at 37 °C with shaking at 250 r.p.m. as described in Methods. Growth was measured as OD₆₀₀ (○, □, △) and fusion expression 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, *hla* 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 *hla* transcription (Fig. 3c). Interestingly, *hla* 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

Fig. 4. Effect of EDTA and magnesium on *tst::lux* expression during growth. *S. aureus* PC1072 (*tst::lux*) was grown in BHI (○, ●), BHI with 0.5 mM EDTA (□, ■) or BHI with 20 mM MgCl₂ (△, ▲) at 37 °C with shaking at 250 r.p.m. as described in Methods. Growth was measured as OD₆₀₀ (○, □, △) and *tst::lux* expression by luciferase activity (●, ■, ▲) as described in Methods. Results are representative of a minimum of three independent cultures.

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 (Fig. 2) in the presence and absence of 1 M NaCl. In the *agr* mutant, as in the wild-type strain, there was little difference in *sarA* expression in BHI supplemented with salt (Fig. 3a). In contrast, the expression of *spa*, *hla* and *tst*, in *agr* strains, was reduced in media containing 1 M NaCl to 10% at late-exponential (*t*=3 h) and <1% and 5% at post-exponential phases (*t*=12 h), respectively (Fig. 3b, c, d), compared to *agr* with no added NaCl. This suggests that the salt effect is transduced independently of *agr*.

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 (OD₆₀₀ 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 MgCl₂ (20 mM) to BHI resulted in an approximately twofold post-exponential decrease in *tst* expression (*t*=12 h) (Fig. 4). Similarly, CaCl₂ (5 mM) or FeSO₄ (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 Mg²⁺ or Ca²⁺, 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 MgCl_2 , 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 RNIII, 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 RNIII, is not altered by high NaCl levels (Regassa & Betley, 1993). The NaCl repression of *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 divorced 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 *hla::lacZ* fusion in *S. aureus* Wood 46, similarly observed *hla* 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 (Sutcliffe *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,

1993), suggesting that osmoregulation of toxin production may be closely linked to other components involved in the osmotic-stress response. Identification of specific regulators responsible for osmolarity-induced changes in morphology (Vijaranakul *et al.*, 1995) and virulence-determinant expression in *S. aureus* will allow the signal-transduction mechanism to be elucidated.

The availability of metal ions is a key signal in the environmental regulation of virulence-gene expression in many pathogenic bacteria (Mekalanos, 1992). In *S. aureus* pathogenesis, the association between the incidence of menstrual TSS and the use of high-absorbency tampons is unequivocal (Kreiswirth, 1989). Staphylococci have a requirement for Mg^{2+} for growth but the reported effects of magnesium on TSST-1 (TSS toxin-1) biosynthesis are conflicting and very dependent on the growth conditions used. In chemically defined media, several studies have found that aerobic production of TSST-1 is increased when *S. aureus* is limited for Mg^{2+} (Mills *et al.*, 1986; Kass *et al.*, 1987; Sarafian & Morse, 1987). In contrast, TSST-1 biosynthesis decreased under Mg^{2+} -limiting conditions in chemostat culture (Taylor & Holland, 1988). Other metal ions such as Ca^{2+} and Mn^{2+} could partially reduce the effect of limiting Mg^{2+} on TSST-1 levels (Mills *et al.*, 1986). Combining Mg^{2+} , Fe^{2+} and Zn^{2+} in a metal-deficient defined medium, further enhanced the stimulatory effect of Mg^{2+} (Reeves, 1989), suggesting that these divalent ions may have a common role in *tst* regulation. In this study, using a shaker flask system, the addition of EDTA, a chelator of divalent cations, to complex media significantly increased *tst* expression; however, this effect was due to the removal of calcium as well as magnesium, 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 sophisticated 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-characterized regulatory elements, *sarA* and quorum-sensing involving the *agr* locus. Additional pleiotropic regulators such as *sae* (Giraud *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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