Mol Gen Genet (1999) 262: 323–331

ORIGINAL PAPER

J. A. Lindsay · S. J. Foster

Interactive regulatory pathways control virulence determinant production and stability in response to environmental conditions in *Staphylococcus aureus*

Received: 18 February 1999 / Accepted: 21 May 1999

Abstract The accessory gene regulator (*agr*) and staphylococcal accessory regulator (*sar*) loci are important regulators of toxin production in *Staphylococcus aureus*. In this study we examined how environmental conditions – degree of aeration and salt concentration – affect the transcription and translation of mRNAs for α-haemolysin (Hla) and serine protease (Ssp) via these pathways and influence the stability of these proteins. Using Northern analysis, we have confirmed earlier observations that *sarA* is involved in the upregulation of RNAIII, the effector molecule encoded by the *agr* locus. However, this effect was abolished in highly aerated cultures. While *sarA* does appear to have an up-regulatory effect on *hla* transcription that is independent of *agr*, we propose that the PC1839 (*sarA*) mutant produces less α-haemolysin activity mainly as a result of post-translational inactivation by proteases. The most obvious phenotypic feature of PC1839 (*sarA*) is the upregulation of proteases. In this study we show that *ssp* is repressed by SarA at the transcriptional level. Western analysis using an anti-α-haemolysin antibody identified a major breakdown product that is only present in the supernatant of strains that are overexpressing serine protease. We have also confirmed that *agr* exerts a significant regulatory influence on *hla* at the level of translation, as well as transcription. Finally, the addition of salt upregulates *ssp* transcription and dramatically downregulates transcription of *hla*, and is an example of an environmental parameter that affects toxin production independently of *agr* and *sarA*. How environmental signals are transduced to control α-haemolysin and serine protease production, activity and stability at multiple levels are discussed.

Key words *Staphylococcus aureus* · Regulation · Toxin · Virulence

Communicated by W. Goebel

J. A. Lindsay[1] · S. J. Foster (✉)
Department of Molecular Biology and Biotechnology,
University of Sheffield, Firth Court,
Western Bank, Sheffield S10 2TN, UK
e-mail: s.foster@sheffield.ac.uk
Tel.: +44-114-2224411; Fax: +44-114-2728697

Present address:
[1]Division of Infectious Disease,
St George's Hospital Medical School,
Cranmer Terrace, London SW17 0RE, UK

Introduction

Staphylococcus aureus is a frequent cause of nosocomial and community-acquired bacterial infections, which can range from the trivial to the life-threatening. A worldwide epidemic of methicillin-resistant *S. aureus* (MRSA) strains in hospitals has had a severe impact on infection control policy and antibiotic usage, occasionally resulting in the temporary closure of entire wards. Some of these strains are resistant to all useful antibiotics except vancomycin. In 1997, *S. aureus* strains resistant to intermediate levels of vancomycin were reported in Japanese hospitals and have since been identified in the USA and France (Centers for Disease Control 1997; Hiramatsu et al. 1997; Ploy et al. 1998).

In order to develop new methods for controlling *S. aureus* infection, we need to understand the infection process. *S. aureus* strains are capable of producing dozens of toxins and potential virulence factors, although no single toxin appears essential for all infections (Projan and Novick 1997; Coulter et al. 1998). Recent research has concentrated on the role of the global regulators encoded by *agr* and *sarA*, which control the expression of multiple virulence determinants.

The phenotype of *agr* mutants suggests that the locus acts to repress expression of surface proteins, such as protein A, coagulase and fibronectin-binding proteins, and activate production of extracellular proteins and toxins, such as α- and β-haemolysin, proteases, most enterotoxins and toxic shock syndrome toxin-1 (Bjorkland and Arvidson 1980; Recsei et al. 1986). *agr* mutant strains show partially attenuated virulence in

animal models (Abdelnour et al. 1993; Booth et al. 1995; Gillaspy et al. 1995). The *agr* locus consists of two major divergent operons. The first (P2) comprises four genes, *agrBDCA*. *agrA* and *agrC* encode a two-component sensor regulator, while *agrD* encodes a small peptide that is apparently cleaved by the product of *agrB* to produce an octapeptide (Novick et al. 1995). The octapeptide is secreted and accumulates in the environment to a threshold level, and then activates *agrA* via *agrC* to upregulate both the P2 and P3 operons (Balaban and Novick 1995; Ji et al. 1995). The product of the P3 operon is an mRNA, RNAIII, which is the regulatory effector molecule (Janzon et al. 1989; Novick et al. 1993).

Northern analysis of wild-type strains have shown that transcription of P2 and P3 is switched on in mid- to late-log phase. At this point, expression of surface proteins is switched off and exoprotein production is switched on (Vandenesch et al. 1991; Ji et al. 1995). This is closely analogous to the process thought to be involved in infection: surface proteins are expressed by single cells and used for attachment to tissues, where they begin to multiply. Once the cell number reaches a threshold level – as indicated by the ambient concentration of octapeptide – *agrA* is activated, and extracellular toxins – often degradative enzymes – are secreted, which generate a further source of nutrients.

The RNAIII molecule itself, rather than the protein translated from this mRNA (*hld*), is responsible for the regulatory effects of the *agr* locus on transcription (Janzon et al. 1989; Janzon and Arvidson 1990; Novick et al. 1993). How the RNAIII molecule might achieve this is not known. Although the phenotype of the *agr* mutant suggests that RNAIII is essential for *hla* expression, Morfeldt et al. (1995) isolated a spontaneous mutant of strain V8 that is deficient in RNAIII yet still produces the *hla* transcript. The mutation responsible has not been characterized. This group showed the RNAIII molecule is capable of binding to *hla* mRNA, and proposed that this complex is required for efficient translation of α-haemolysin. At present, *hla* is the only mRNA that is reported to be regulated by RNAIII at the translational level.

The *sar* locus encodes another global regulator first described by Cheung and colleagues. The *sarA* mutant shows altered production of exoproteins (Cheung et al. 1992; Cheung and Ying 1994). The mutant strain shows attenuated virulence in some animal models (Nilsson et al. 1996). There are three promoters upstream of the *sarA* gene. The distal promoters have been predicted to drive transcription of small ORFs encoded between them (Bayer et al. 1996; Manna et al. 1998). Extensive work has shown that SarA is a DNA-binding protein which upregulates transcription of the P2 and P3 promoter regions (Cheung and Projan 1994; Heinrichs et al. 1996; Morfeldt et al. 1996; Cheung et al. 1997; Chien and Cheung 1998). Thus it may exert many of its effects on exoprotein production via *agr*. However, we have previously shown that SarA has a major role as a repressor of protease production (Chan and Foster 1998a).

Earlier studies in our laboratory suggested SarA might be part of the mechanism that controls the response to aeration (Chan and Foster 1998a). In this study we have investigated how α-haemolysin levels are regulated in response to environmental conditions and how *agr* and *sarA* are involved in this process. While SarA may affect the *hla* transcript independently of *agr*, we present evidence to show that the overexpression of proteases in the *sarA* mutant strain PC1839 leads to the cleavage and inactivation of α-haemolysin, thus accounting for the major decrease in haemolytic activity observed in this strain. This suggests that a regulatory mechanism that controls protease activities may modulate virulence factor stability and activity.

Materials and methods

Bacterial strains and growth conditions

The strains used in this study were constructed in the isogenic parental background *S. aureus* 8325-4 (Novick 1967). The mutant strains PC6911 (*agr*), PC1839 (*sarA*) and PC18391 (*agr sarA*) have been described previously (Chan and Foster 1998a).

Growth conditions during experiments were exactly as described previously (Chan and Foster 1998b). Briefly, glycerol stocks were streaked onto BHI agar plates containing appropriate antibiotics, then inoculated into 100 ml of BHI broth in a 250-ml flask at 37° C shaking at 250 rpm. After 3 h, the culture was diluted to an OD_{600} of 0.01 with fresh growth medium, to a final volume of either 10 ml or 100 ml in a 250-ml flask. In some experiments BHI broth was supplemented with 1 M NaCl. Cultures were incubated at 37° C with shaking at 250 rpm.

Northern analysis

Culture samples for Northern analysis were cooled in liquid nitrogen; cells were immediately recovered by centrifugation (13,000 ×*g*, 4° C, 5 min) and RNA was extracted from the pellet. The FastRNA kit and a FastPrep Instrument (Bio 101) were used for RNA extraction according to the manufacturer's instructions: RNA was then stored at −20° C. Aliquots (8 μg) of the nucleic acid (measured using A_{260}) were digested with 5 U of RQ1 RNase-free DNase (Promega) in the presence of 10 mM $CaCl_2$ and 10 mM $MgSO_4$ for 1 h at 37° C to remove residual DNA contamination. Samples were then denatured and fractionated on a 1.1% w/v agarose-formaldehyde gel, transferred onto Hybond N+ nylon membrane (Amersham), fixed using UV irradiation, and detected using the Enhanced Chemiluminesence kit (Amersham). This kit allows a single membrane to be probed several times without stripping. The probes used were PCR products isolated from an agarose gel and purified with the Qiagen gel extraction kit, then labelled with horse-radish peroxidase according to the ECL protocol. Primers used to generate the probes were: for RNAIII, 5′-CACAGAGATGTGATGGAAAATAGTTG-3′ and 5′-TAAATGAAGTAGAACAGCAACGCG-3′, generating a product of 829 bp which is estimated to cover the entire RNAIII region (Genbank Accession No. X52543; Novick et al. 1995); for *hla*, 5′-CACGTATAGTCAGCTCAG-3′ and 5′-CTGAAGAACGATCTGTCC-3′, generating a product of 909 bp covering almost the entire ORF (Genbank Accession No. X01645; Gray and Kehoe 1984); and for *ssp*, 5′-GGAGGTTTTTAGATGAAAGG-3′ and 5′-CGCCATTGTCTGGATTATCAGG-3′, generating a product of 987 bp covering almost the entire ORF (Genbank Accession No. Y00356; Carmona and Gray 1987).

Protein analysis

Extracellular protein samples from 12-h cultures (stationary phase) were prepared as previously described (Foster 1992), boiled and loaded onto 15% w/v SDS-PAGE gels. Gels were stained with Coomassie blue or transferred to Hybond N+ nylon membranes (Amersham) for Western analysis. Polyclonal anti-sera raised in rabbits against α-haemolysin or V8 serine protease were kindly donated by SmithKline Beecham. Labelled bands were visualized using the ECL system (Amersham).

Precast casein protease gels (12% w/v acrylamide; Biorad) were used to detect protease activity. Extracellular proteins in sample buffer (containing β-mercaptoethanol as above) were diluted 1:10 in water and Zymogram sample buffer (Biorad) and boiled again prior to loading on the gel. Protease activity was detected according to the gel manufacturer's instructions.

α-Haemolysin activity was measured using rabbit blood as previously described (Chan and Foster 1998b) but without the addition of PMSF.

Results

The *agr* locus activates, but is not essential, for transcription of *hla*

The growth curve in highly aerated (10-ml) cultures shows a sharp break between exponential and stationary phase, whereas less aerated cultures (100 ml) show a more gradual decrease in growth rate (Fig. 1A and B). We consistently find that both the PC6911 (*agr*) and PC18391 (*agr sarA*) strains reach slightly higher final OD levels than 8325-4. The points in the growth curves at which samples were taken for RNA preparation are indicated in Fig. 1A and B. They are roughly equivalent to log, late-log, early stationary and stationary phase.

As expected, Northern blot experiments showed that the *agr* locus is required for *hla* expression in 10-ml cultures (Fig. 1C). However, in 100-ml cultures, the *hla* transcript is detected in the *agr* mutant (Fig. 1D). This is confirmed by the results with PC18391 (*agr sarA*), as the *hla* transcript is seen under both conditions in the absence of *agr* (Fig. 1C and D). In both cases there is more *hla* transcript in the wild-type than in PC6911 (*agr*), and in PC1839 (*sarA*) compared to PC18391 (*agr sar*). Therefore, *agr* positively regulates *hla* transcription but is not essential for expression.

Transcriptional regulation by *sarA* depends on aeration

In 100-ml cultures, the amount of RNAIII transcript detected on Northern blots was decreased in PC1839 (*sarA*) compared to 8325-4 (Fig. 1D), which supports published data (Cheung and Projan 1994; Heinrichs et al. 1996). A proportionate decrease in the *hla* transcript was seen, suggesting that *sarA* acts on *hla* transcription via *agr*.

However, in 10-ml cultures, the amount of RNAIII transcript was not affected by the *sarA* mutation. This suggests that the upregulatory effect of *sarA* on RNAIII can be abolished by increased aeration. This is not due to an alteration in the amount of *sarA* mRNA produced, as experiments with the *sarA*::*lacZ* chromosomal fusion strain PC161 (Chan and Foster 1998a) show that β-galactosidase production is not affected by aeration (data not shown). Although RNAIII was not affected in the *sarA* mutant, the amount of *hla* transcript nevertheless decreased compared to the wild-type strain (Fig. 1C). This suggests that *sarA* still acts as a positive activator of *hla* but works independently of the amount of RNAIII. Unexpectedly, in the absence of *agr*, *sarA* does not upregulate the *hla* transcript, it represses expression [compare PC6911 (*agr*) to PC18391 (*agr sarA*) in Fig. 1C]. Therefore *sarA* is a repressor of *hla* transcription in the absence of *agr*, and an activator of *hla* transcription in the presence of *agr*. This activation is independent of the amount of RNAIII transcript produced.

Chan and Foster (1998a) noted that β-galactosidase activity expressed from a chromosomal *hla*::*lacZ* fusion construct was delayed in the PC1839 (*sarA*) background. It was proposed that *sarA* played a role in the temporal regulation of *hla*. This effect was confirmed by Northern analysis (Fig. 1D), although it was not seen in highly aerated cultures (Fig. 1C).

RNAIII enhances *hla* translation

In 100-ml cultures, the *hla* transcript is produced, albeit at reduced levels, in the presence of *agr* and *sarA* mutations, while in aerated conditions (10 ml) the *hla* transcript is not seen in the PC6911 (*agr*) strain (Fig. 1C, D). However, the amount of transcript does not correspond to the amount of protein observed. On SDS-PAGE gels (Fig. 2) and Western blots (Fig. 3A) very little α-haemolysin protein (32 kDa) is detectable in PC6911 (*agr*) and PC18391 (*agr sarA*) backgrounds compared to cells in which the *agr* locus is intact, irrespective of the amount of *hla* transcript synthesized (10 ml vs 100 ml cultures). This supports the observations of Morfeldt et al. (1995), who suggested RNAIII was required to activate translation of *hla* mRNA.

Expression of the gene for serine protease (*ssp*) is repressed by SarA at the transcriptional level

The level of *ssp* transcript in 8325-4 is low relative to the *hla* transcript, but its expression is activated by *agr* in 10-ml cultures (Fig. 1C). The product of the *sarA* locus, on the other hand, is a major repressor of *ssp*. Repression occurs in both 10-ml and 100-ml cultures, and it appears that *agr* and *sar* antagonize each other. When both genes are absent, *ssp* transcription returns to levels similar to those seen in the wild type.

In SDS-PAGE gels stained with Coomassie blue, serine protease is the predominant protein (33 kDa) produced by PC1839 (*sarA*) (Fig. 2; Chan and Foster 1998a). The amount of protein seen in all cases corresponds to the amount of *ssp* transcript produced, and

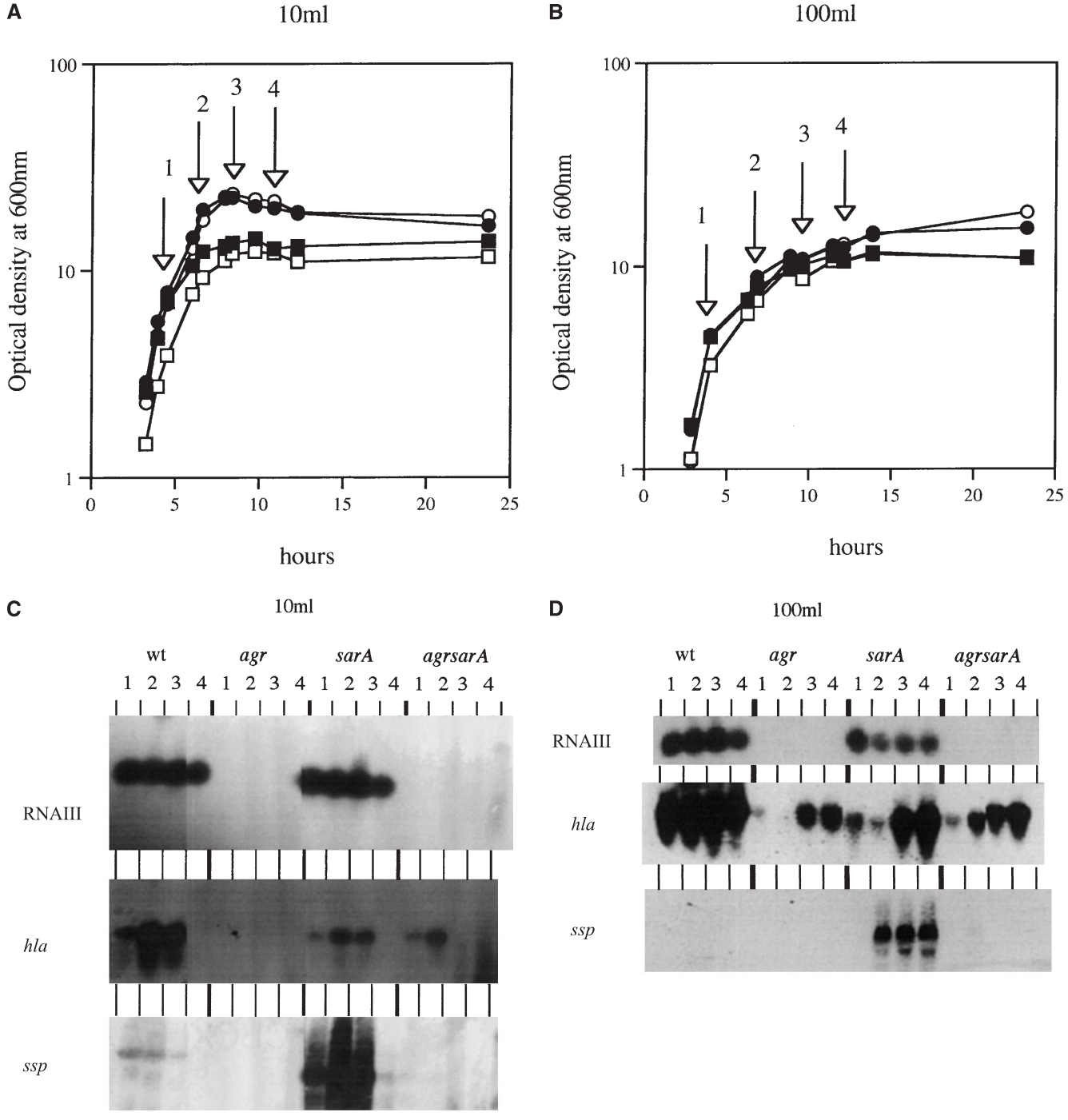


Fig. 1A–D Growth curves and Northern analysis of *S. aureus* in 10-ml and 100-ml cultures. Growth curves (OD at 600 nm; log scale) are shown for the strains 8325-4 (wt, *filled squares*), PC6911 (*agr*, *filled circles*), PC1839 (*sarA*, *empty squares*) and PC18391 (*agr sarA*, *empty circles*) in 10-ml (**A**) and 100-ml (**B**) cultures. The *numbered arrows* refer to points at which samples were taken for RNA preparation. Northern blots from 10-ml cultures (**C**) and 100-ml cultures (**D**) were hybridized consecutively with RNAIII, *hla* and *ssp* probes, which detect bands of approximately 0.5 kb, 1.5 kb and 3 kb, respectively. Results are representative of three experiments and, with one exception, are all from a single membrane. The sample for time point 3 from PC18391 (*agr sarA*) in 100-ml cultures originated from a separate experiment

there appears to be very little regulation of *ssp* at the translational level.

Serine protease is activated in aerated cultures

Serine protease is reported (Janzon and Arvidson 1990) to appear as a double band in Western blots of supernatants grown in baffled flasks (highly aerated). We have confirmed this observation in aerated conditions (Fig. 3B). Protease gels show that the wild-type

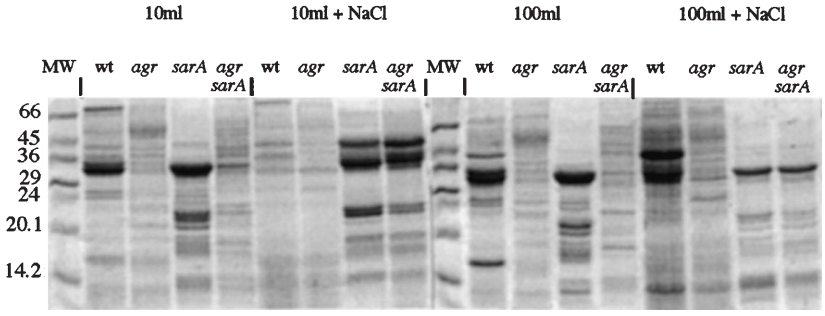


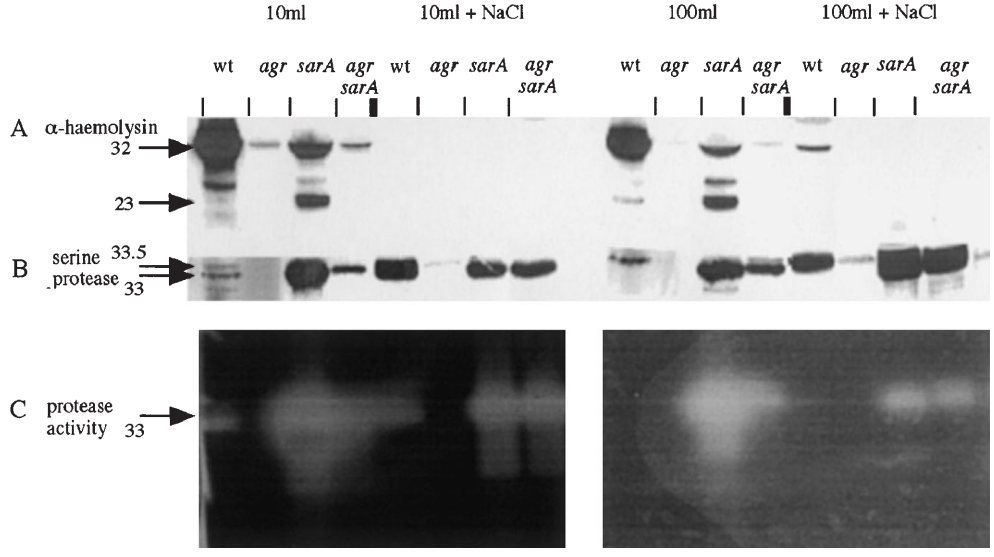
Fig. 2 Analysis of exoproteins on 15% w/v SDS-PA gels. Exoproteins were isolated from stationary-phase cultures of 8325-4 (wt), PC6911 (*agr*), PC1839 (*sarA*) and PC18391 (*agr sarA*) grown in either 10-ml or 100-ml batches with or without supplementation with 1 M NaCl. The sizes of molecular weight markers (MW) are indicated in kDa on the left. α-Haemolysin and serine protease have apparent molecular weights of approximately 32 kDa and 33 kDa, respectively. N-terminal sequencing identified the major band in the 32-kDa region in the wild-type 8325-4 strain as α-haemolysin, while the major band in the 33-kDa region in PC1839 (*sarA*) is serine protease. This was confirmed by Western analysis

strain produces active protease under these conditions (Fig. 3C). The majority of this activity appears as a single band that corresponds in size (33 kDa) to V8 serine protease supplied by Sigma (data not shown). Interestingly, in less aerated conditions (100 ml), serine protease appears as only the larger band in Western blots of supernatants from cultures of the wild-type strain 8325-4 (Fig. 3B), and shows no activity on protease gels (Fig. 3C). This suggests that serine protease is processed from the inactive larger form to the smaller, active form, and this processing is enhanced in aerated conditions. Interestingly, under both conditions, serine protease was activated in PC1839 (*sarA*) and PC18391 (*agr sarA*), suggesting that SarA represses the "activator", which is itself probably another protease.

α-Haemolysin activity is lowered in the PC1839 strain (*sarA*) owing to proteolytic cleavage

α-Haemolysin activity, measured in the rabbit blood cell assay, is greatly reduced in PC1839 (*sarA*) compared to 8325-4 (Fig. 4, 87 ± 14 and 1501 ± 688 activity units, respectively, in 10-ml cultures). However, *hla* is clearly transcribed in PC1839 (*sarA*) cells (Fig. 1C and D) and translated, resulting in significant levels of α-haemolysin that can be detected on Western blots (Fig. 3A). PC1839 (*sarA*) produces large amounts of active serine protease (Figs. 2, 3B, C; Chan and Foster 1998a), and we have noticed a general reduction in the amounts of other higher molecular weight proteins in the supernatant of this strain (Fig. 2). Western analysis of exoproteins with anti-α-haemolysin antiserum revealed a prominent 23-

Fig. 3 **A** Western analysis with antiserum raised against α-haemolysin. The samples shown in Fig. 2 were used. The *arrows* point to α-haemolysin (32 kDa) and a breakdown product seen in PC1839 (*sarA*) of approximately 23 kDa. **B** Western analysis of the samples shown in Fig. 2 with antiserum raised against serine protease. The *arrows* point to the serine protease double band at around 33 kDa. **C** Protease activity of culture supernatants assayed on casein PAGE gels. Supernatant samples are the same as those for Fig. 2, diluted 1:10. The band of activity appears at approximately 33 kDa, the same size as the commercially available serine protease (Sigma, data not shown). All data are representative of at least two experiments



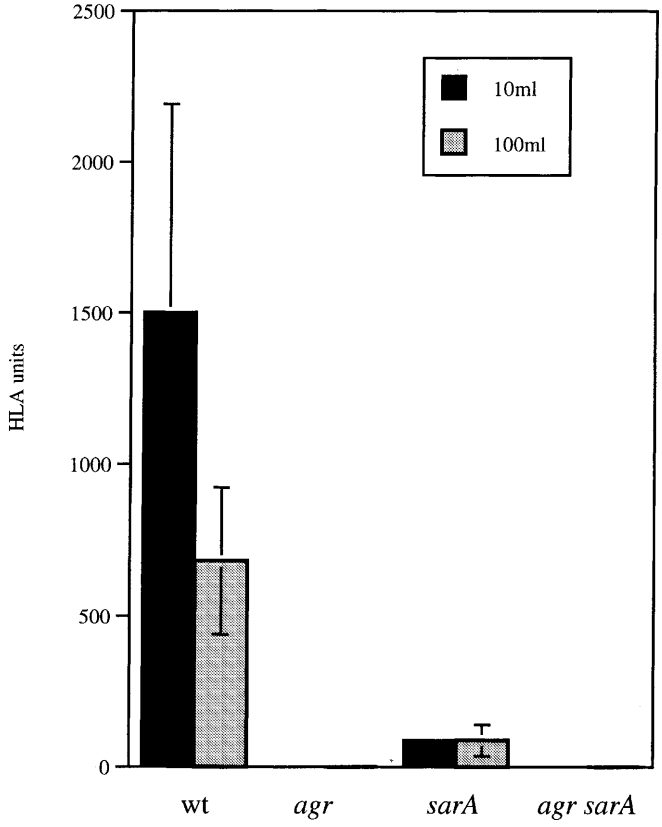


Fig. 4 Haemolytic activity in culture supernatants of *S. aureus*. Supernatant samples were taken at the same time as those for Fig. 2. Results are averages of between three and six experiments and the error bars show the standard deviation

kDa protein in PC1839 (*sarA*). We suggest that this may be a breakdown product of α-haemolysin, which is cleaved by serine protease in the culture supernatant. This could account for the lower level of α-haemolysin activity on rabbit blood cells detected in cultures of PC1839 (*sarA*) (Fig. 4).

NaCl downregulates α-haemolysin and upregulates serine protease at the transcriptional level, and this effect is independent of *agr* and *sarA*

High concentrations of NaCl (1 M) dramatically reduced the amount of *hla* transcript in all the strains. While NaCl had a minor downregulatory effect on RNAIII, the effect of NaCl appeared to be independent of both *agr* and *sarA*. Virtually no α-haemolysin activity was detected in cultures supplemented with NaCl (Fig. 3A), which is consistent with the lack of *hla* transcript. All cultures grown in 1 M NaCl failed to produce detectable haemolytic activity, as measured on rabbit blood cells (data not shown).

On the other hand, the presence of 1 M NaCl enhanced production of the *ssp* transcript (Fig. 1C, D; Fig. 5C, D) and of serine protease (Fig. 3B, C). This enhancement was seen in both the 8325-4 and PC18391 (*agr sarA*) strains. Thus the effects of NaCl on production of serine protease do not appear to be mediated by *agr* or *sarA*.

Discussion

Extensive studies have shown that *agr* positively regulates the production of α-haemolysin and other toxins at the level of transcription (Bjorkland and Arvidson 1980; Recsei et al. 1986; Vandenesch et al. 1991; Abdelnour et al. 1993; Novick et al. 1993). Similarly, studies have shown that SarA is a positive regulator of *agr*, and therefore indirectly regulates *hla* transcription (Cheung and Projan 1994; Heinrichs et al. 1996; Morfeldt et al. 1996; Cheung et al. 1997; Chien and Cheung 1998). The results of our Northern analyses have confirmed both of these observations.

We have now identified an environmental condition (high aeration) where *sarA* appears to have no significant effect on the amount of RNAIII transcript produced. However, under these conditions, the amount of *hla* transcript is partially reduced in a *sarA* mutant strain. This reduction appears to be independent of the level of RNAIII transcript. Other groups have also suggested that *sarA* can act independently of *agr* to regulate virulence factors. Thus, Booth et al. (1997) have shown that a *sarA* mutant strain was not attenuated in virulence in an endophthalmitis model, but the double *agr sarA* mutant strain was less virulent than the *agr* mutant. They concluded that *sarA* continues to influence virulence traits in the absence of *agr*. Gillaspy et al. (1998) have reported that collagen adhesin is primarily regulated by *sarA*, and not by *agr*.

To further investigate this observation, the same experiment was performed in an *agr* mutant background. If *sarA* can act as a positive regulator of *hla* independently of *agr*, we would expect to see a small amount of *hla* transcript in an *agr* mutant and even less transcript in a double mutant. Instead the level of *hla* RNA increased in the double mutant. Therefore, in highly aerated conditions the presence or absence of the *agr* locus determines the function of *sarA* as a regulator of *hla*, although the amount of RNAIII transcript is not affected. Furthermore, in the absence of *agr*, it could be argued that *sarA* acted as a repressor of *hla* transcription.

The role of *sarA* in the regulation of *hla* transcription in an *agr* mutant is academic however. In no case did we find significant α-haemolysin activity in an *agr* mutant. This fully confirms the results of Morfeldt et al. (1995) who suggested RNAIII is required for the translation of *hla*.

While SarA clearly has effects on toxin production both via *agr* and independently of *agr*, the major effect of a *sarA* mutation is the de-repression of proteases (Fig. 3; Chan and Foster 1998a). Our Northern blots demonstrate that *sarA* is a potent repressor of *ssp* production at the level of transcription. While *agr* is re-

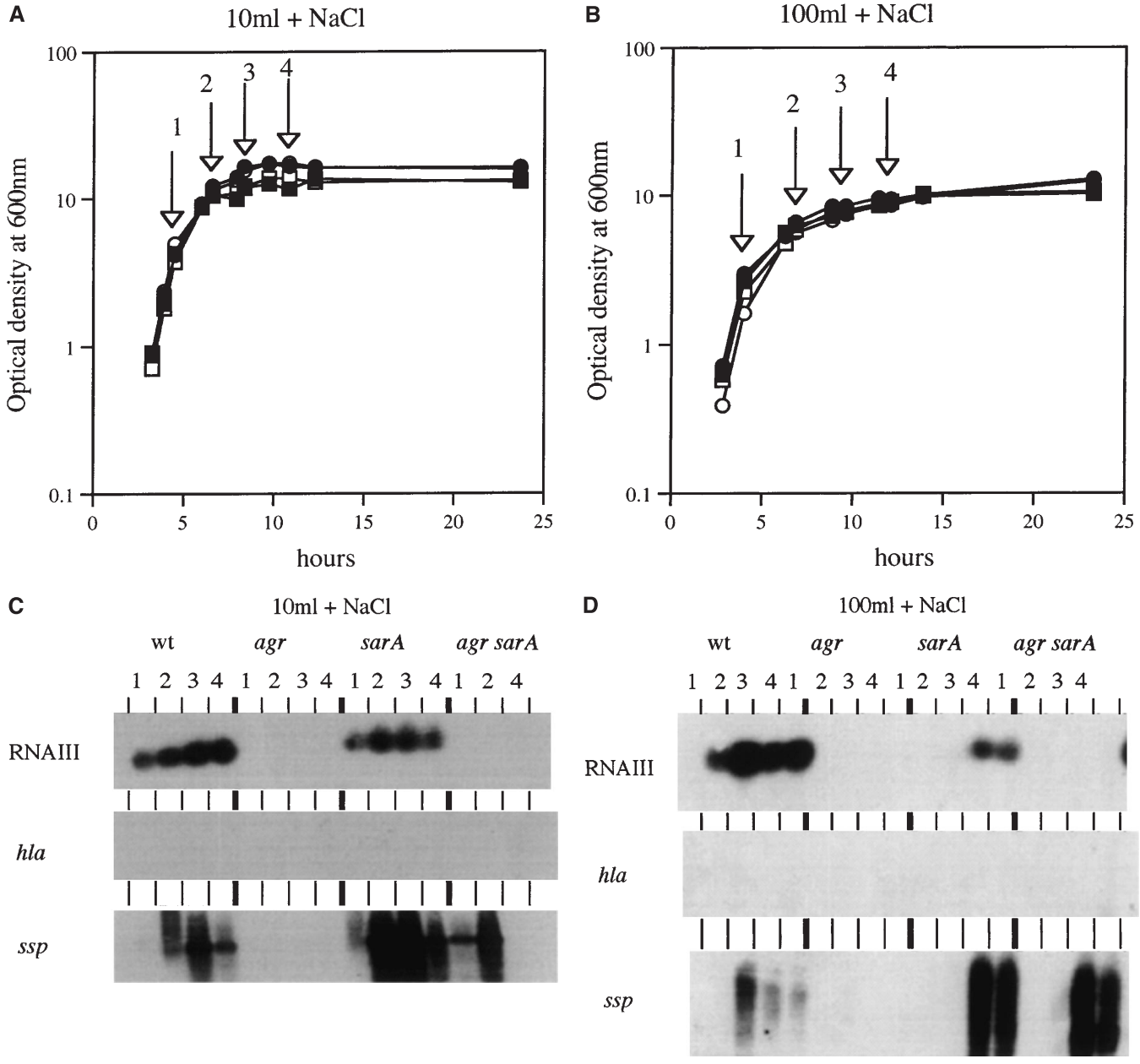


Fig. 5A–D The effect of 1 M NaCl on growth of *S. aureus* and transcription of selected genes in 10-ml and 100-ml cultures. Growth curves were constructed and Northern analysis was performed as in Fig. 1, except that all cultures were grown in BHIB supplemented with 1 M NaCl. The results are presented exactly as in Fig. 1

quired for *ssp* transcription (Janzon and Arvidson 1990), the effect of *sarA* on *ssp* is independent of *agr*. Furthermore, repression of *ssp* by *sarA* is independent of aeration.

In summary, our results suggest that SarA is a partial up-regulator of RNAIII at the transcriptional level, but this effect is blocked by aeration. It is a minor up-regulator of *hla* in the presence of *agr*, but this effect is independent of the amount of RNAIII produced in an *agr*-positive strain. A major regulatory role of SarA in *S. aureus* is the repression of Ssp and possibly other proteases (Chan and Foster 1998a). The effect on *ssp* regulation is independent of *agr* and of aeration.

Both *agr* and *sar* were found to have important, but quite different, post-transcriptional effects on α-haemolysin. As previously mentioned, an intact *agr* locus is required for translation of the *hla* transcript. Morfeldt et al. (1995) demonstrated that an RNAIII/*hla* mRNA complex forms in the wild-type strain 8325-4, and, based on studies with an uncharacterized spontaneous mutant, they proposed that the complex was required for *hla* translation. Here we show that the *hla* transcript is produced in an *agr* or double mutant background under less aerated conditions, but is not translated into protein. This supports their model and provides an explanation for our observations. Thus, a significant

regulatory effect of *agr* on α-haemolysin is exerted at the level of translation.

SarA, on the other hand, appears to have little effect on the translation of *hla*. However, the *sarA* mutant produces large amounts of active serine protease (Chan and Foster 1998a). Here we provide evidence that proteases in the supernatant may cleave α-haemolysin post-translationally, thus reducing its activity. We propose that much of the decrease in α-haemolysin activity seen in PC1839 (*sarA*) is due to this phenomena. Froman et al. (1987) and McGavin et al. (1997) reported a significant role for staphylococcal proteases in the cleavage and inactivation of fibronectin binding protein in wild-type strains. Proteases may therefore have an important role in regulating the stability of virulence factors, an effect that is enhanced in PC1839 (*sarA*).

Post-translational processing of serine protease is required for activation (Drapeau 1978). Since the active form appeared as a slightly smaller band on Western blots, we presume this activation is due to the cleavage of a pro-sequence by a protease, such as the metalloprotease described by Drapeau (1978). In our experiments, the protease is activated under aerated conditions, particularly in the wild-type strain. It also appears that this protease is down-regulated by *sarA*, but does not absolutely require *agr*, as the active serine protease form is present in both *sarA* and *agr sarA* mutant strains.

Therefore, protease production in itself may be an important modulator of the activity of multiple virulence factors. Serine protease is just one of these. Serine protease activity is dependent on regulation by *sarA*, on post-translational activation by another protease which is controlled by *sarA* and *agr*, and on environmental conditions such as aeration.

Finally, while *agr* and *sar* are important regulators of toxin and virulence factor production, other regulators may be just as important. Here we show that 1 M NaCl has a dramatic effect on *hla* transcription and this is independent of both *agr* and *sar*. Regassa and Betley (1993), have demonstrated that enterotoxin C levels are also dramatically reduced under conditions of high osmotic strength, and this effect is independent of *agr*. Sheehan et al. (1992) suggested that the decrease in transcription of the gene for epidermolytic toxin A in high salt was due to an increase in negative supercoiling of DNA – which was *agr* dependent. However, our preliminary experiments suggests that the decrease in *hla* transcription is not due to changes in supercoiling, and cannot be reversed by novobiocin (data not shown; Chan and Foster 1998b). There was no evidence for a role for supercoiling in the regulation of *hla*::*lacZ*, *spa*::*lacZ*, *sar*::*lacZ* or *tst*::*luxAB* expression by NaCl.

NaCl also enhanced the transcription of *ssp* and, as in the control of *hla* transcription, the effect on *ssp* transcription was independent of *agr* and *sar*. NaCl does not affect *sarA* expression, as determined using PC161 (*sarA*::*lacZ*) (data not shown; Chan and Foster 1998b).

S. aureus is a particularly salt-tolerant microbe. This is reflected in its ability to colonize the skin and nares. In this environment, *S. aureus* survives as a commensal. Perhaps salt concentration contributes to lack of pathogenicity by switching off α-haemolysin, enterotoxin C (Regassa and Betley 1993), epidermolytic toxin A (Sheehan et al. 1992) and protein A (Chan and Foster 1998b), and enhancing the production of serine protease – which may act in degrading other toxins. Salt is also reported to alter cell wall morphology in *S. aureus* (Vijaranakul et al. 1995). Therefore, salt and other environmental conditions may trigger important regulatory pathways, resulting in decreased production of toxins.

The importance of complex regulatory control of toxin production by *S. aureus* has been highlighted by studies with α-haemolysin. While α-haemolysin mutants show attenuated virulence in animal models (Bramley et al. 1989), Bayer et al. (1997) demonstrated that strains that produce too much α-haemolysin were also attenuated in a model of experimental endocarditis, possibly due to increased release of microbicidal proteins from platelets. Therefore, there may be sound reasons for the exceedingly complicated regulatory control over the level and timing of production of α-haemolysin and other virulence factors by *S. aureus*.

This study has emphasized the need for careful control of environmental conditions when evaluating toxin production in *S. aureus*. An important change in regulatory activity of *sarA* was seen after simply altering the amount of growth medium in the flask. Seemingly minor differences in growth conditions and media may account for differences in the results reported by different laboratories. Therefore it is imperative that all growth conditions be documented in full when studying regulatory pathways in *S. aureus*. Elucidation of the complex, interwoven, regulatory circuits controlling the pathogenicity of such a successful and versatile pathogen as *S. aureus* is the subject of our continuing research.

Acknowledgements This research programme was supported with funds from SmithKline Beecham (J.A.L.) and the Royal Society (S.J.F.). We wish to acknowledge Martin Burnham at SmithKline Beecham for the kind gift of antisera, and Pan Chan, Mark Clements and Malcolm Horsburgh for assistance with techniques.

References

Abdelnour A, Arvidson S, Bremell T, Rydén C, Tarkowski A (1993) The accessory regulator (*agr*) controls *Staphylococcus aureus* virulence in a murine arthritis model. Infect Immun 61:3879–3885

Balaban N, Novick RP (1995) Autocrine regulation of toxin synthesis by *Staphylococcus aureus*. Proc Natl Acad Sci USA 92:1619–1623

Bayer MG, Heinrichs JH, Cheung AL (1996) The molecular architecture of the *sar* locus in *Staphylococcus aureus*. J Bacteriol 178:4563–4570

Bayer AS, Ramos MD, Menzies BE, Yeaman MR, Shen AJ, Cheung AL (1997) Hyperproduction of alpha-toxin by *Staph-*

ylococcus aureus results in paradoxically reduced virulence in experimental endocarditis: a host defense role for platelet microbicidal proteins. Infect Immun 65:4652–4660

Bjorkland A, Arvidson S (1980) Mutants of *Staphylococcus aureus* affected in the regulation of exoprotein synthesis. FEMS Microbiol Lett 7:203–206

Booth MC, Atkuri RV, Nanda SK, Iandolo JJ, Gilmore MS (1995) Accessory gene regulator controls *Staphylococcus aureus* virulence in endophthalmitis. Invest Ophthalmol Vis Sci 36:1828–1836

Booth MC, Cheung AL, Hatter KL, Jett BD, Callegan MC, Gilmore MS (1997) Staphylococcal accessory regulator (*sar*) in conjunction with *agr* contributes to *Staphylococcus aureus* virulence in endophthalmitis. Infect Immun 65:1550–1556

Bramley AJ, Patel AH, O'Reilly M, Foster R, Foster TJ (1989) Roles of alpha-toxin and beta-toxin in virulence of *Staphylococcus aureus* for the mouse mammary gland. Infect Immun 57:2489–2494

Carmona C, Gray GL (1987) Nucleotide sequence of the serine protease gene of *Staphylococcus aureus*, strain V8. Nucleic Acids Res 15:6757

Centers for Disease Control and Prevention (1997) *Staphylococcus aureus* with reduced susceptibility to vancomycin-United States, 1997. Morbid Mortal Weekly Rep 46:765–766

Chan PF, Foster SJ (1998a) Analysis of the role of SarA in virulence determinant production and environmental signal transduction in *Staphylococcus aureus*. J Bacteriol 180:6232–6241

Chan PF, Foster SJ (1998b) The role of environmental factors in the regulation of virulence-determinant expression in *Staphylococcus aureus* 8325–4. Microbiology 144:2469–2479

Cheung AL, Projan SJ (1994) Cloning and sequencing of *sarA* of *Staphylococcus aureus*, a gene required for the expression of *agr*. J Bacteriol 176:4168–4172

Cheung AL, Ying P (1994) Regulation of α- and β-hemolysins by the *sar* locus of *Staphylococcus aureus*. J Bacteriol 176:580–585

Cheung AL, Koomey JM, Butler CA, Projan SJ, Fischetti VA (1992) Regulation of exoprotein expression in *Staphylococcus aureus* by a locus (*sar*) distinct from *agr*. Proc Natl Acad Sci USA 89:6462–6466

Cheung AL, Bayer MG, Heinrichs JH (1997) *sar* genetic determinants necessary for transcription of RNAII and RNAIII in the *agr* locus of *Staphylococcus aureus*. J Bacteriol 179:3963–3971

Chien Y, Cheung AL (1998) Molecular interactions between two global regulators, *sar* and *agr*, in *Staphylococcus aureus*. J Biol Chem 273:2645–2652

Coulter SN, Schwan WR, Ng EY, Langhorne MH, Ritchie HD, Westbrock-Wadman S, Hufnagle WO, Folger KR, Bayer AS, Stover CK (1998) *Staphylococcus aureus* genetic loci impacting growth and survival in multiple infection environments. Mol Microbiol 30:393–404

Drapeau GR (1978) Role of metalloprotease in activation of the precursor of staphylococcal protease. J Bacteriol 136:607–13

Foster SJ (1992) Analysis of the autolysins of *Bacillus subtilis* 168 during vegetative growth and differentiation by using renaturing polyacrylamide gel electrophoresis. J Bacteriol 174: 464–470

Froman G, Switalski LM, Speziale P, Hook M (1987) Isolation and characterization of a fibronectin receptor from *Staphylococcus aureus*. J Biol Chem 262:6564–71

Gillaspy AF, Hickmon SG, Skinner RA, Thomas JR, Nelson CL, Smeltzer MS (1995) Role of the accessory gene regulator (*agr*) in pathogenesis of staphylococcal osteomyelitis. Infect Immun 63:3373–3380

Gillaspy AF, Lee CY, Sau S, Cheung AL, Smeltzer MS (1998) Factors affecting the collagen binding capacity of *Staphylococcus aureus*. Infect Immun 66:3170–3178

Grey GS, Kehoe M (1984) Primary sequence of the alpha-toxin gene from *Staphylococcus aureus* Wood 46. Infect Immun 46:615–618

Heinrichs JH, Bayer MG, Cheung AL (1996) Characterization of the *sar* locus and its interaction with *agr* in *Staphylococcus aureus*. J Bacteriol 178:418–423

Hiramatsu K, Hanaki H, Ino T, Yabuta K, Oguri T, Tenover FC (1997) Methicillin-resistant *Staphylococcus aureus* clinical strain with reduced vancomycin susceptibility. J Antimicrob Chemother 40:135–136

Janzon L, Arvidson S (1990) The role of the delta-lysin gene (*hld*) in regulation of virulence genes by the accessory gene regulator (*agr*) in *Staphylococcus aureus*. EMBO J 9:1391–1399

Janzon L, Lofdahl S, Arvidson S (1989) Identification and nucleotide sequence of the delta-lysin gene, *hld*, adjacent to the accessory gene regulatory (*agr*) of *Staphylococcus aureus*. Mol Gen Genet 219:480–485

Ji G, Beavis R, Novick RP (1995) Cell density control of staphylococcal virulence mediated by an octapeptide pheromone. Proc Natl Acad Sci USA 92:12055–12059

Manna AC, Bayer MG, Cheung AL (1998) Transcriptional analysis of different promotors in the *sar* locus in *Staphylococcus aureus*. J Bacteriol 180:3828–3836

McGavin MJ, Zahradka C, Rice K, Scott JE (1997) Modification of the *Staphylococcus aureus* fibronectin binding phenotype by V8 protease. Infect Immun 65:2621–2628

Morfeldt E, Taylor D, von Gabain A, Arvidson S (1995) Activation of alpha-toxin translation in *Staphylococcus aureus* by the trans-encoded antisense RNA, RNAIII. EMBO J 14:101–108

Morfeldt E, Tegmark K, Arvidson S (1996) Transcriptional control of the *agr*-dependent virulence gene regulator, RNAIII, in *Staphylococcus aureus*. Molec Microbiol 21:1227–1237

Nilsson I-M, Bremell T, Rydén C, Cheung AL, Tarkowski A (1996) Role of the staphyloccal accessory gene regulator (*sar*) in septic arthritis. Infect Immun 64:4438–4443

Novick R (1967) Properties of a cryptic high-frequency transducing phage in *Staphylococcus aureus*. Virology 33:155–166

Novick RP, Ross HF, Projan SJ, Kornblum J, Kreiswirth B, Moghazeh S (1993) Synthesis of staphylococcal virulence factors is controlled by a regulatory RNA molecule. EMBO J 12:3967–3975

Novick RP, Projan SJ, Kornblum J, Ross HF, Ji G, Kreiswirth B, Vandenesh F, Moghazeh S (1995) The *agr* P2 operon: an autocatalytic sensory transduction system in *Staphylococcus aureus*. Mol Gen Genet 248:446–458

Ploy MC, Grelaud C, Martin C, de Lumley L, Denis F (1998) First clinical isolate of vancomycin-intermediate *Staphylococcus aureus* in a French hospital. Lancet 351:1212

Projan SJ, Novick RP (1997) The molecular basis of pathogenicity. In: Crossley KB, Archer GL (eds) The staphylococci in human disease. Churchill Livingstone, New York, pp 55–81

Recsei P, Kreiswirth B, O'Reilly M, Schlievert P, Gruss A, Novick RP (1986) Regulation of exoprotein gene expression by *agr*. Mol Gen Genet 202:58–61

Regassa LB, Betley MJ (1993) High sodium chloride concentrations inhibit staphylococcal enterotoxin C gene (*sec*) expression at the level of *sec* mRNA. Infect Immun 61:1581–1585

Sheehan BJ, Foster TJ, Dorman CJ, Park S, Stewart GSAB (1992) Osmotic and growth-phase dependent regulation of the *eta* gene of *Staphylococcus aureus*: a role for DNA supercoiling. Mol Gen Genet 232:49–57

Vandenesch F, Kornblum J, Novick RP (1991) A temporal signal, independent of *agr*, is required for *hla* but not *spa* transcription in *Staphylococcus aureus*. J Bacteriol 173:6313–6320.

Vijaranakul U, Nadakavukaren MJ, de Jonge BLM, Wilkinson BJ, Jayaswal RK (1995) Increased cell size and shortened peptidoglycan interpeptide bridge of NaCl-stressed *Staphylococcus aureus* and their reversal by glycine betaine. J Bacteriol 177:5116–5121