Identification and characterization of *msa* (SA1233), a gene involved in expression of SarA and several virulence factors in *Staphylococcus aureus*

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The staphylococcal accessory regulator (*sarA*) plays a central role in the regulation of virulence in *Staphylococcus aureus*. To date, studies involving *sarA* have focused on its activity as a global regulator that modulates transcription of a wide variety of genes (> 100) and its role in virulence. However, there is also evidence to suggest the existence of accessory elements that modulate SarA production and/or function. A reporter system was developed to identify such elements, and a new gene, *msa* (SA1233), mutation of which results in reduced expression of SarA, was identified and characterized. Additionally, it was shown that mutation of *msa* resulted in altered transcription of the accessory gene regulator (*agr*) and the genes encoding several virulence factors including alpha toxin (*hla*) and protein A (*spa*). However, the impact of mutating *msa* was different in the laboratory strain RN6390 and the clinical isolate UAMS-1. For instance, mutation of *msa* caused a decrease in *spa* and *hla* transcription in RN6390 but had a different effect in UAMS-1. The strain-dependent effects of the *msa* mutation were similar to those observed previously, which suggests that *msa* may modulate the production of specific virulence factors through its impact on *sarA*. Interestingly, sequence analysis of *Msa* suggests that it is a putative membrane protein with three membrane-spanning regions, indicating that *Msa* might interact with the environment. The findings show that *msa* is involved in the expression of SarA and several virulence factors.

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INTRODUCTION

Staphylococcus aureus is a prominent member of the normal skin and nasal flora in a significant proportion of healthy individuals, but it is also capable of causing a variety of infections ranging from superficial skin lesions to serious infections including osteomyelitis and endocarditis. The pathogenicity of *S. aureus* arises from its production of an impressive repertoire of virulence factors that includes surface-associated proteins, extracellular toxins and degradative enzymes. Surface-associated proteins are expressed primarily during the exponential growth phase while the exoproteins are produced after the transition into post-exponential growth. This switch is influenced by a growing number of regulatory loci, including the accessory gene regulator (*agr*) and the staphylococcal accessory regulator (*sarA*).

Expression of *agr* is activated in a cell-density-dependent manner as cultures enter the post-exponential growth phase.

Induction of *agr* expression leads to RNAPIII production, which results in decreased production of many surface proteins and increased production of many exoproteins (Janzon & Arvidson, 1990; Novick *et al.*, 1993). In addition to cell density, *agr* expression is also activated by *sarA*. The *sarA* locus encodes three transcripts (*sarP1*, 0.58 kb; *sarP3*, 0.84 kb; and *sarP2*, 1.15 kb), all of which have an identical 3' end and include the ORF (375 bp) encoding the 14.5 kDa DNA-binding protein SarA (Bayer *et al.*, 1996). Transcripts *sarP1* and *sarP2* use σ^A -specific promoters and are preferentially expressed from mid- to late-exponential growth phases. Transcript *sarP3* uses a σ^B -specific promoter and is expressed primarily during the post-exponential growth phase (Manna *et al.*, 1998). There are conflicting reports regarding the impact of transcription from different *sarA* promoters on the production of SarA, but our studies suggest that SarA is produced in similar amounts during all growth phases (Blevins *et al.*, 2002). SarA regulates the expression of *S. aureus* virulence factors via both *agr*-dependent (Cheung *et al.*, 1997; Dunman *et al.*, 2001; Heinrichs *et al.*, 1996) and *agr*-independent pathways

Abbreviation: qPCR, quantitative PCR.

(Bayer *et al.*, 1996; Chan & Foster, 1998a; Blevins *et al.*, 1999). In the *agr*-dependent pathway, *sarA* activates transcription of the *agr* locus at the transition between the exponential and post-exponential growth phase *in vitro* (Chien *et al.*, 1998; Morfeldt *et al.*, 1996) by binding to the intergenic region between the P2 and P3 promoters (Rechlin *et al.*, 1999). In the *agr*-independent pathway, *sarA*-mediated regulation involves a direct interaction between SarA and *cis* elements associated with the target genes (Blevins *et al.*, 1999; Wolz *et al.*, 2000; Chan & Foster, 1998a; Dunman *et al.*, 2001; Sterba *et al.*, 2003).

Expression of *sarA* is regulated by several factors. For instance, some studies suggested that σ^B activates *sarA* expression (Manna *et al.*, 1998; Bischoff *et al.*, 2001); however, Horsburgh *et al.* (2002) showed no difference in the level of *sarA* transcript or protein between wild-type and a σ^B mutant. The reasons for this discrepancy are not clear but it might be due to differences in the strains or growth conditions used in these studies. Another study showed that expression of *sarA* is repressed by SarR, a member of the SarA protein family (Manna *et al.*, 1998). More recently, Rossi *et al.* (2003) identified a membrane-associated protein, MrsR, which also affects expression of *sarA*. Specifically, mutation of *mrsA* resulted in increased expression of SarA. Since MrsR does not have any DNA-binding domain, the authors suggested that its effect on *sarA* is mediated by other factors.

The present study was prompted by several findings that indicated the presence of accessory elements that modulate the production and/or function of *sarA*. For instance, while SarA activates *agr* in growing cells, it inhibited transcription from *agr* promoters in an *in vitro* transcription system (Chakrabarti & Misra, 2000). In addition, our studies indicate that SarA is present in equivalent amounts throughout the growth cycle despite the fact that it modulates transcription of the genes encoding *S. aureus* virulence factors (e.g. *cna*) in a temporal fashion (Blevins *et al.*, 1999). In this report, we identify and characterize a new gene that encodes a putative membrane protein that we designate *msa* (modulator of *sarA*). Mutation of *msa* resulted in reduced transcription of *sarA* and modulated the production of several virulence factors.

METHODS

Bacterial strains and growth conditions. The bacterial strains and plasmids used in this study are listed in Table 1. *S. aureus* cells were grown at 37 °C with constant aeration (200 r.p.m. at a medium/flask volume ratio of 0.5) in tryptic soy broth (TSB) or on tryptic soy agar (TSA) supplemented with antibiotics when appropriate. *Escherichia coli* was also grown in TSB or on TSA. *S. aureus* RN4220 was used as an initial recipient for the transformation of recombinant plasmids. Phage ϕ 11 was used as a generalized transducing phage to move plasmids from RN4220 into other *S. aureus* strains. Antibiotics were used in the following concentrations: for *S. aureus*, 10 μ g erythromycin ml⁻¹, 50 μ g kanamycin ml⁻¹, 3 μ g tetracycline ml⁻¹, 10 μ g chloramphenicol ml⁻¹; for *E. coli*, 100 μ g ampicillin ml⁻¹.

Construction of a *cna-luxABCDE* reporter system. To monitor SarA activity, we constructed a reporter plasmid by fusing the promoter for the collagen-binding adhesion gene (*cna*) with the bioluminescence operon *luxABCDE*. The *luxABCDE* operon is a modified version of the *luxCDABE* from *Photobacterium luminescens* in which Gram-positive ribosome-binding sites were introduced upstream of each ORF in the operon (Francis *et al.*, 2000). The *cna* promoter in UAMS-1 was amplified by PCR using primers B1 and B3 respectively, located at positions -475 and 1596 relative to the ATG start codon (Table 2; Snodgrass *et al.*, 1999). The PCR product was treated with Klenow and polynucleotide kinase to produce phosphorylated blunt ends. The resulting product was then cloned into the *Sma*I site upstream of the *lux* operon carried in the shuttle vector pMK4 *luxABCDE*. Cloning was done in *E. coli* strain TOP10. The resulting clones were sequenced to confirm the correct orientation of the *cna* promoter relative to the *lux* operon. Fusion of the *cna* promoter to the *lux* operon (*cna-luxABCDE*) resulted in the reporter plasmid pMOE59, which was introduced into strain RN4220 by electroporation. The plasmid was then introduced into RN6390 by generalized transduction using phage ϕ 11 as described previously (Blevins *et al.*, 2002). The resulting strain was designated MOE72 and used to generate a transposon-insertion library as described below.

Transposon mutagenesis. Transposon-insertion libraries were constructed using plasmid p1256repA36 to deliver the transposon Tn551. p1256repA36 was introduced into MOE72 by transduction. Transductants were grown at 30 °C in the presence of 10 μ g erythromycin ml⁻¹ and 10 μ g chloramphenicol ml⁻¹ to select for the presence of p1256repA36 and pMOE59. Cultures were diluted in TSB containing 10 μ g erythromycin ml⁻¹ and incubated overnight at 43 °C. Several independent cultures were then diluted and plated on TSA to obtain isolated colonies. Colonies were then streaked on erythromycin and chloramphenicol plates at 37 °C to confirm their identity and screened for aberrant light production using a charge-coupled device (CCD) camera included in the UVP gel documentation system (UVP). Colonies were imaged for 15 min. Colonies exhibiting increased light production relative to the corresponding parent strain were selected for further study. After screening colonies to ensure that the Tn551 insertion did not occur in the *sarA*, *agr* or *cna* loci, a single colony (designated MOE41) was chosen for further study. In order to rule out the possibility that the phenotype observed was due to a secondary mutation, the Tn551 insertion was transduced into a fresh RN6390 background containing the reporter plasmid and the same effect was observed (Fig. 1). The Tn551 insertion in MOE41 was then introduced into strains UAMS-1 and UAMS-174 via generalized transduction as described above. Mutation of *msa* in the recipient strains was confirmed by PCR and verified by Southern blot analysis as previously described (Gillaspay *et al.*, 1995).

Identification of transposon insertion. To identify the Tn551 insertion site in MOE41, we digested chromosomal DNA with *Hind*III and ligated the resulting fragments to *Hind*III-cut pUC18. The resulting plasmids were then introduced into *E. coli* with selection for ampicillin. Ampicillin-resistant colonies were then screened for resistance to erythromycin to identify clones containing Tn551. Sequencing of the cloned DNA and comparison to the *S. aureus* N315 genome sequence revealed that Tn551 insertion occurred inside ORF SA1233 (GenBank accession no. 1124072).

Complementation of *msa* mutant. To complement the mutation, a 750 bp fragment encompassing the Tn551 insertion site was amplified by PCR from *S. aureus* RN6390 chromosomal DNA (Table 2). Plasmid pMOE78 was constructed by cloning the PCR products into the multiple cloning site of vector pCR2.1 TOPO (Invitrogen). Cloning of *msa* was verified by PCR and restriction enzyme analysis. pMOE78 was ligated to the Gram-positive shuttle vector pSK265, resulting in shuttle plasmid construct pMOE83. pMOE83

Table 1. Strains and plasmids

Strain/plasmid	Relevant genotype	Comment and/or parent strain	Source
<i>S. aureus</i>			
RN4220		Restriction-deficient mutant of 8325-4	Novick (1990)
RN6390	<i>cna</i> , <i>rsbU</i>	8325-4	Novick <i>et al.</i> (1993)
UAMS-174	<i>cna</i> , <i>rsbU</i> , <i>geh</i>	RN6390 with <i>cna</i> incorporated in chromosomal lipase gene (<i>geh</i>)	Gillaspy <i>et al.</i> (1998)
UAMS-982	<i>cna</i> , <i>rsbU</i> , <i>agr</i>	RN6390 <i>agr</i> -null transduction donor	Blevins <i>et al.</i> (2002)
UAMS-957	<i>cna</i> , <i>rsbU</i> , <i>sarA</i>	RN6390 <i>sarA</i> ::kan transduction donor	Blevins <i>et al.</i> (2002)
UAMS-981	<i>cna</i> , <i>rsbU</i> , <i>sarA</i> , <i>agr</i>	RN6390 <i>sarA</i> ::kan/ <i>agr</i> -null transduction donor	Blevins <i>et al.</i> (2002)
UAMS-1	Wild-type	Osteomyelitis isolate	Gillaspy <i>et al.</i> (1995)
UAMS-155	<i>agr</i>	UAMS-1	Blevins <i>et al.</i> (2002)
UAMS-929	<i>sarA</i>	UAMS-1	Blevins <i>et al.</i> (2002)
UAMS-930	<i>sarA</i> , <i>agr</i>	UAMS-1	Blevins <i>et al.</i> (2002)
MOE72	<i>cna</i> , <i>rsbU</i>	pMOE59 in RN6390	This study
MOE73	<i>cna</i> , <i>rsbU</i> , <i>sarA</i>	pMOE59 in UAMS-957	This study
MOE41	<i>msa</i> , <i>cna</i> , <i>rsbU</i>	RN6390:: <i>msa</i> from Tn551 mutagenesis	This study
MOE43	<i>msa</i> , <i>cna</i> , <i>rsbU</i>	RN6390:: <i>msa</i> complemented with pMOE83	This study
MOE54	<i>msa</i>	UAMS-1:: <i>msa</i>	This study
MOE163		UAMS-1:: <i>msa</i> complemented with pMOE83	This study
MOE161	<i>msa</i>	UAMS-174:: <i>msa</i>	This study
MOE162		UAMS-174:: <i>msa</i> complemented with pMOE83	This study
Plasmids			
pUC18		High-copy-number <i>E. coli</i> vector Amp ^r	Yanisch-Perron <i>et al.</i> (1985)
pCR2.1		<i>E. coli</i> cloning vector for direct cloning of PCR fragments	Invitrogen
pMK4 <i>luxABCDE</i>		Shuttle plasmid that carries the <i>lux</i> operon	Francis <i>et al.</i> (2000)
pMOE59		<i>cna</i> promoter fused to <i>luxABCDE</i>	This study
pI256repA36		Temperature-sensitive shuttle vector (Tn551)	Iandolo JJ
pSK265		Gram-positive cloning vector	Jones & Khan (1986)
pMOE78		<i>msa</i> gene cloned into pCR2.1	This study
pMOE83		<i>msa</i> in a shuttle vector comprising pCR2.1 and pSK265	This study

was introduced into strain RN4220 by electroporation. Transformants were selected for on TSA plates containing 10 µg chloramphenicol ml⁻¹. The plasmid was subsequently introduced into the RN6390 and UAMS-1 *msa* mutants via generalized transduction (Gillaspy *et al.*, 1998).

Isolation of RNA. For the isolation of RNA, *S. aureus* strains were grown without antibiotic selection and under low-aeration conditions (120 r.p.m. at a medium/flask volume ratio of 0.5) as described by Lindsay & Foster (1999). Cells were harvested at optical densities (OD₅₆₀) of 0.3, 1.5 and 4.0, which correspond to the mid-exponential, late-exponential, and post-exponential growth phases respectively. Total cellular RNA was isolated from the wild-type, the *msa* mutants, and the complemented mutants of both RN6390 and UAMS-1. Briefly, cells were harvested by centrifugation (5000 g for 5 min at 4 °C) and resuspended in TES buffer containing 100 µg lysostaphin ml⁻¹ (AMBI). The samples were incubated at room temperature for 10 min and were then applied to a Qiagen RNeasy Maxi column to isolate total bacterial RNA according to the manufacturer's directions. The optional on-column RNase-free DNase I (Qiagen) was used to remove contaminating DNA. After isolation of RNA, traces of contaminating DNA were further eliminated by treating RNA samples with RNase-free DNase I (DNA-free kit, Ambion) and incubating at 37 °C for 20 min. Samples were used immediately or stored at -80 °C. The quality, integrity and concentration of the RNA were determined by using an Agilent 2100 Bioanalyzer (Agilent Technologies) as described by the manufacturer. RNA preparations were tested for contaminating DNA by no-reverse-transcriptase PCR reactions.

Real-time quantitative PCR. The primers used for real-time quantitative PCR (qPCR) were designed with Primer 3 software (Massachusetts Institute of Technology) to amplify gene fragments with an optimal size of 75–100 bp. The fragment of the gene of interest was cloned into plasmid pCR2.1 TOPO to determine PCR efficiency. Specifically, the plasmid was isolated and a series of 10-fold dilutions was prepared corresponding to 500 000 to 5 plasmid copies. For each primer set, a standard curve was generated to determine the correlation coefficient as an indicator of PCR efficiency. All qPCR reactions were run in triplicate. Melting curve analysis and agarose gel electrophoresis were done to verify primer set specificity. Sequences, correlation coefficient values and PCR efficiencies of primer sets used are listed in Table 2.

Measurements of relative levels of gene expression were done by qPCR. RNA was reverse transcribed into cDNA using iScript cDNA synthesis kit (Bio-Rad). The reverse transcriptase reactions were done at 25 °C for 5 min, 42 °C for 30 min and 85 °C for 5 min. cDNA was stored at -20 °C until needed. PCR reactions were done in 25 µl reactions by using iQ SYBR Green Supermix (Bio-Rad) as recommended by the manufacturer (Bio-Rad). The reaction mixtures contained: 5 µl cDNA; 12.5 µl iQ SYBR Green Supermix; 0.5 µl forward primer (1.5 pmol µl⁻¹); 0.5 µl reverse primer (1.5 pmol µl⁻¹); and 6.5 µl de-ionized H₂O. PCR amplification was done using an iCycler (Bio-Rad) and the amplification parameters were as follows: 94 °C for 3 min, 1 cycle at 95 °C for 3 min, 40 cycles at 95 °C for 30 s, 50 °C for 30 s and 72 °C for 1 min. The final 80 cycles began at 55 °C and increased by 0.5 °C every 10 s. All qPCR reactions were done in

Table 2. Primer sets

Gene	Primer sequence (5'–3')	Correlation coefficient	PCR efficiency
<i>msa</i>	F: GGA TCC ACT AAC ACC TTA CCA AGA AG R: GAA TTC GAT GAC TGT AGA AAA CAT GC	0.983	98.6
<i>sarA</i>	F: TCT TGT TAA TGC ACA ACA ACG TAA R: TGT TTG CTT CAG TGA TTC GTT T	0.991	93.5
<i>agr</i>	F: GGA GTG ATT TCA ATG GCA CA R: ATC CAT TTT ACT AAG TCA CCG ATT	0.999	75.7
<i>agrB</i>	F: CCA GTT TGC CAC GTA TCT TC R: TGC ATC CCT AAA CGT ACT TGC	0.997	82.8
<i>hla</i>	F: GAA AGG TAC CAT TGC TGG TCA R: AAG GCC AGG CTA AAC CAC TT	0.985	99.9
<i>aur</i>	F: GCA CTT TAT CAC CAG CAG CA R: TTG ACC GCA TCA CTC TTT TG	0.995	99.3
<i>sspA</i>	F: TCA AGC AAA CAG CAA ACA CC R: TTT GCG TCA CGT TGT TC	0.995	83.9
<i>lip</i>	F: TAC CGA ATA AGG GGA CAA AGG R: GCC CTT GTT TGG CAA CTT TA	0.989	98.8
<i>nuc</i>	F: ATG GAC GTG GCT TAG CGT AT R: TAG CCA AGC CTT GAC GAA CT	0.997	72.4
<i>clfA</i>	F: CCT GAT GAG CCT GGT GAA AT R: ATC GCT GCC AGA ATC TGA AC	0.983	93.1
<i>fnbA</i>	F: AAC TGC ACA ACC AGC AAA TG R: TTG AGG TTG TGT CGT TTC CTT	0.979	90.0
<i>coa</i>	F: CTC GCC CGA CAT ACA AGA AG R: GCG AGC GCC ATA TGA TGA TAC TT	0.990	96.7
<i>spa</i>	F: AGA TGA CCC AAG CCA AAG TG R: GCT TTC GGT GCT TGA GAT TC	0.996	71.8
<i>geh</i>	F: GCG GCA CAA AAA GAA ACA CT R: TGC TTT CCT GAC TGC ATT TG	0.992	99.3
<i>cna</i>	F: GGA GAG ACA TCG GCA ACA GT R: CAG TTG GTC GTT TTC CGT CT	0.999	93.0
<i>gyr*</i>	F: CAT TGC CAG ATG TTC GTG AC R: CCG GTG TCA TAC CTT GTT CA	0.989	94.1
<i>cna</i> promoter	B1: GGATCCACAGCTTCCGGTTTAATAGGTGTA B3: TCGGTTTTTTTGATTGGTTTTTCAGTATTAG	NA	NA
<i>msa</i> ORF	F: GGATTCACTAACACCTTACCAAGAAAG R: GAATTGATGACTGTAGAAAACATGC	NA	NA

*Gyrase was used as endogenous control.

triplicate and the mean C_T was used for analysis of results. To verify the absence of contaminating DNA, each qPCR experiment included controls that lacked template cDNA or reverse transcriptase. The constitutively expressed gene for gyrase (*gyr*) was used as an endogenous control as described previously (Goerke *et al.*, 2000). Analysis of expression of each gene was done based on at least two independent experiments. Twofold or higher changes in gene expression were considered significant.

Protease assay. Assays for soluble proteases were done as previously described (Smeltzer *et al.*, 1993). In brief, 300 μ l of the culture supernatant was mixed with 800 μ l of 3 mg azocasein ml^{-1} in Tris-buffered saline (pH 7.5) and incubated overnight at 37 °C. To precipitate the undegraded azocasein, 400 μ l of 50% (w/v) trichloroacetic acid was added. The precipitate was then removed by centrifugation and the amount of acid-soluble azocasein was determined by measuring A_{340} .

Haemolysin assay. Haemolytic activity was measured as described previously (Blevins *et al.*, 2002). In brief, supernatants from overnight cultures were harvested and filter-sterilized with a 0.45 μ m (pore-size) acetate syringe filter (CAMEO 25AS, Osmonics). After standardization, 10 μ l culture supernatant was combined with 1% rabbit blood in 10 mM Tris/HCl (pH 7.5)/0.9% NaCl. After incubating 15 min at 37 °C, unlysed blood cells were pelleted by centrifugation. The haemolytic activity was determined by measuring the A_{405} of the cell-free supernatant. SDS (1%) and TSB were used as positive and negative controls respectively.

Binding assays. Binding of *S. aureus* to host proteins (fibronectin, fibrinogen or collagen) was assayed in microtitre plates coated with human fibronectin (BD Biosciences), type 1 fibrinogen from human plasma (Sigma-Aldrich) or type 1 collagen (Sigma). Fibronectin was suspended in sterile distilled water to a starting concentration of 1 mg ml^{-1} . Fibrinogen was suspended in phosphate-buffered saline

Fig. 1. Images of streaks of the *msa* and *sarA* mutants and wild-type RN6390 carrying the *cna-luxABCDE* reporter plasmid. The strains were streaked on TSA with antibiotics when appropriate and grown overnight at 37 °C. The image on the left shows streaks photographed under normal lighting conditions. The image on the right was obtained in complete darkness with a charge-coupled device (CCD) camera included in a gel documentation system. The image on the right was collected for 15 min.

(PBS) to a starting concentration of 15 µg ml⁻¹. Collagen was suspended in PBS to a starting concentration of 1 mg ml⁻¹. The host protein solutions were diluted 1:10 in bicarbonate buffer and 200 µl was used to coat the microtitre plate wells. A solution of BSA (2%) was used as a negative control. After incubating overnight at 4 °C, wells were washed with PBS, and 200 µl of the test culture containing 0.5 optical density units (OD₅₆₀) was added to the wells and incubated at 37 °C for 1 h. The plates were then spun at 200 g in a Jouan T20 rotor for 5 min at room temperature. The absence of cell pellets indicated binding to the host molecule bound to the walls of the wells. The presence of the cell pellet indicated lack of binding. Assays were done in triplicate and were repeated at least three times.

Western blot analysis. Whole-cell lysates used for SarA Western blots were prepared and analysed as previously described (Blevins *et al.*, 1999). Proteins (2 µg) for Western blot analysis were electrophoresed under denaturing conditions on precast 4–20% gradient gels. Polyclonal anti-SarA from rabbits was used as described by Blevins *et al.* (1999).

RESULTS

Identification and characterization of *msa* (SA1233)

To identify accessory elements that modulate SarA production or function, we constructed a reporter plasmid by fusing the *cna* promoter to the bioluminescence operon *luxABCDE*. Because SarA represses transcription of *cna* (Blevins *et al.*, 1999), light emission was increased relative to the wild-type parent strain when the *cna-luxABCDE* reporter was present in a *sarA* mutant (Fig. 1). RN6390 carrying the *cna-luxABCDE* reporter was randomly mutagenized using Tn551. Mutant libraries were screened for enhanced light production as an indication of reduced SarA activity. Once clones with enhanced bioluminescence were identified, we

used PCR analysis to ensure that the insertion did not occur in *sarA* or *agr*. We also did Southern hybridization using an internal fragment of Tn551 as a probe to verify that the mutant contained a single insertion. Characterization of one such clone and comparison to the N315 genome sequence confirmed that the Tn551 insertion occurred at position 197 inside an ORF (SA1233) with no known function. SA1233 is a 402 bp ORF that encodes a protein of 133 amino acids with a predicted molecular mass of 15.6 kDa (Fig. 2a). Since this ORF and its product have not been characterized, we analysed the predicted protein sequence to gain insight into its putative function.

We used BLASTP (Altschul *et al.*, 1997) to search for SA1233 homologues in a non-redundant database of protein sequences. We did not find any sequences that showed full-length similarity. However, a small portion of the sequence corresponding to residues 9 through 53 showed strong similarity to efflux and membrane proteins related to drug resistance. Similar results were obtained when BLAST searches were performed against the SwissProt Database (SP) and Protein Database (PDB). To confirm these results, we performed a multiple sequence alignment using the BLASTP hit sequences in the CLUSTALX environment (Thompson *et al.*, 1997). This analysis showed that the N-terminal region (55 residues) is conserved among many eubacterial species. We also did a conserved domain search on the N-terminal region against the PFAM database (Marchler-Bauer & Bryant, 2004). This search showed the presence of an ACR_tran domain (e-value 0.91), suggesting that SA1233 belongs to the integral membrane protein family AcrB/AcrD/AcrF (pfam00873) (Fig. 2b). Further analysis of the protein sequence supported these findings by revealing the presence of hydrophobic regions and three

Fig. 2. Bioinformatics characterization of *Msa* sequence. (a) Map of *msa* region. Sites for restriction enzyme *Hind*III are indicated (H) (b) Sequence alignment of the conserved domain of *Msa* and the position-specific scoring matrix (PSSM) for the *Acr*-*tran* domain. This domain is shared by the *AcrB/AcrD/AcrF* integral membrane proteins family (pfam00873). (c) Transmembrane regions of *Msa* identified using the SOSUI program (Hirokawa *et al.*, 1998). Primary transmembrane regions are predicted based on the hydropathy index of Kyte & Doolittle (1982). Secondary transmembrane regions are predicted based on the amphiphilicity index of Hirokawa *et al.* (1998).

potential transmembrane helices (Fig. 2c). These results suggest that SA1233 is a transmembrane protein that might be involved in drug resistance.

Impact of *msa* mutation on expression of *sarA*

The goal of this study was to find accessory elements that are necessary for the production or function of *SarA*. The reporter system (*cna-luxABCDE*) indicated that mutation of *msa* has a similar effect to mutation of *sarA* with respect to expression of *cna*. Specifically, mutation of both *sarA* and *msa* resulted in increased expression of *cna* (Fig. 1). To determine if the effect of *msa* on transcription from the *cna* promoter is mediated through an impact on *sarA* expression, we measured *sarA* transcription by qPCR in strains carrying the mutant *msa* gene. This analysis was done in the laboratory strain RN6390 and the clinical isolate UAMS-1 because previous studies showed that the regulatory functions of *sarA* are strain-dependent (Blevins *et al.*, 2002; Somerville *et al.*, 2002; Rice *et al.* 2004; Cassat *et al.*, 2005). Mutation of *msa* resulted in a twofold and 2.85-fold decrease in *sarA* expression in strains RN6390 and UAMS-1 respectively (Fig. 3). Complementation studies with

pMOE83 confirmed that the decrease was due to disruption of *msa* (Fig. 3). The decrease in *sarA* expression was observed in all three growth phases tested (mid-exponential, late-exponential and post-exponential) and confirmed by Western blot analysis (Fig. 4). This is consistent with the phenotype observed in the reporter strain and suggests that increased *cna* expression is correlated with derepression by *sarA*. These findings suggest that mutation of *msa* affects the expression of *sarA* at the transcriptional level in both RN6390 and UAMS-1.

Impact of *msa* mutation on expression of *agr*

One reported role of *sarA* is the activation of *agr*, which in turn controls expression of several virulence factors. Since *sarA* expression was affected by the *msa* mutation, we examined the expression of the two divergent *agr* operons (RNAIII and RNAII) in our mutant. In RN6390, RNAIII expression was fivefold lower in the *msa* mutant in the mid-exponential growth phase, but there was no significant change (less than twofold) in the late- and post-exponential growth phases (Fig. 5). In addition, mutation of *msa* caused a slight reduction in RNAII expression but the reduction was

Fig. 3. Relative expression levels of *sarA* in the *msa* mutation versus wild-type (*msa/wt*) and in the complemented mutant versus wild-type (*c/wt*) in RN6390 and UAMS-1. Values represent the mean ratio ($\pm$ SEM) for three independent experiments. Mid, late and post indicate the mid-exponential, late-exponential and post-exponential growth phases respectively.

not significant (less than twofold). The pattern of temporal expression of *agr* operons in the mutant and RN6390 was similar in that RNAPIII accumulated in the post-exponential growth phase. Complementation with pMOE83 restored expression of both RNAPIII and RNAPII (Fig. 5).

Mutation of *msa* in the clinical isolate UAMS-1 had the opposite effect on expression of *agr*. Specifically, the UAMS-1 *msa* mutant showed an increase in RNAPIII expression

during both the mid-exponential (2.24-fold) and late-exponential (10.40-fold) growth phases (Fig. 5). Expression of RNAPII was also significantly higher in the UAMS-1 *msa* mutant, but only in the late-exponential growth phase. Complementation with the functional copy of *msa* restored expression to levels similar to wild-type, indicating that the mutation of *msa* is responsible for the observed changes in expression of *agr* operons. These results demonstrate that the effect of the *msa* mutation on *agr* is strain-dependent. The amounts of RNAPIII and RNAPII transcripts produced by UAMS-1 were much lower than RN6390, which is consistent with previous findings (Blevins *et al.*, 2002). It should also be noted that *cna* is regulated by *sarA* in an *agr*-independent manner (Blevins *et al.*, 1999). Additionally, induction of *agr* is classically associated with decreased production of surface proteins (Novick *et al.*, 1993), but mutation of *msa* in UAMS-1 resulted in increased expression of *agr* and increased expression of the *cna-luxABCDE* reporter. Taken together, these results suggest that the differences we observed in expression of *agr* do not account for the increased expression of the *cna-luxABCDE* reporter in the *msa* mutant.

Impact of *msa* mutation on expression of virulence factors

We have shown that mutation of *msa* negatively affects transcription of *sarA*. We tested the effect of the *msa* mutation on expression of the genes encoding several virulence factors that are controlled by *sarA* (Tables 3 and 4). Dunman *et al.* (2001) developed a genome-scale transcription profile to identify genes regulated either directly or indirectly by *sarA*. We used this profile to select the *sarA*-regulated virulence genes to investigate in this study (Tables 3 and 4). Expression of virulence factors was measured in RN6390 and UAMS-1. In both strains, mutation of *msa* resulted in decreased expression of the genes encoding fibronectin-binding protein A (*fnbA*)

Fig. 4. Western blot analysis with anti-SarA polyclonal antibody in strains RN6390 and UAMS-1. Mid, late and post indicate the mid-exponential, late-exponential and post-exponential growth phases respectively. The number on the side represents the molecular mass in kDa.

Fig. 5. Relative expression levels of RNAII (a) and RNAIII (b) in the *msa* mutation versus wild-type (*msa*/wt) and in the complemented mutant versus wild-type (*c*/wt) in RN6390 and UAMS-1. Values represent the mean ratio ($\pm$ SEM) for three independent experiments. Mid, late and post indicate the mid-exponential, late-exponential and post-exponential growth phases respectively.

(Tables 3 and 4). On the other hand, mutation of *msa* resulted in increased expression of the genes encoding aureolysin (*aur*) and serine protease (*sspA*) in both strains (Tables 3 and 4). The fact that mutation of *msa* had a similar effect on expression of these genes in both RN6390 and UAMS-1, together with the observation that mutation of

msa had a similar effect on expression of *sarA* in both strains but an opposite effect on expression of *agr*, suggests that the altered transcription of these genes was mediated by the impact on expression of *sarA* rather than *agr*. However, the impact of the *msa* mutation on expression of other genes was strain-dependent. For instance, mutation of *msa*

Table 3. Relative expression of genes in *msa* mutant MOE41 and the complemented RN6390 strain (MOE43) versus wild-type strain RN6390

Gene	N315 ORF*	Function	agr effect†	sarA effect†	msa effect†	Fold change‡					
						MOE41 (<i>msa</i>) vs RN6390			MOE43 (pMOE83) vs RN6390		
						Mid	Late	Post	Mid	Late	Post
<i>hla</i>	SA1007	Alpha-toxin, haemolysin	+	+	+ ^{SD}	0.57	0.31	0.19	2.35	5.17	12.79
<i>aur</i>	SA2430	Zinc metalloproteinase	+	–	–	7.73	9.17	4.88	1.20	0.73	0.89
<i>sspA</i>	SA0901	Serine protease (V8)	+	–	–	1.22	29.50	6.57	1.08	1.41	0.34
<i>clfA</i>	SA0742	Fibrinogen-binding protein	–	+	No effect ^{SD}	1.26	1.24	0.70	0.80	0.93	0.59
<i>fnbA</i>	SA2291	Fibronectin-binding protein A	–	+	+	0.01	0.03	0.06	2.85	1.49	2.18
<i>spa</i>	SA0107	Protein A	–	–	+ ^{SD}	0.17	0.03	0.01	0.97	0.95	0.60
<i>lysA</i>	SA1232	Diaminopimelate carboxylase	Unknown	Unknown	+	0.1	0.5	1.2	0.8	0.6	0.5
<i>cspA</i>	SA1234	Major cold-shock protein	Unknown	No effect	+	<0.001	<0.001	<0.001	0.82	0.90	1.05

*Designation of gene in strain N315.

†Effect on gene transcription: +, activation; –, repression; ‘No effect’, no significant change in transcription (less than twofold).

‡Fold change in gene expression in the *msa* mutant (MOE41) or the complemented mutant (MOE43) relative to wild-type (RN6390). Values represent the mean ratio for at least two independent experiments. Expression measurements were done at mid-exponential, late-exponential and post-exponential growth phases.

^{SD}Strain-dependent effect.

Table 4. Relative expression of genes in *msa* mutant MOE54 and the complemented UAMS-1 strain (MOE163) versus wild-type strain UAMS-1

Gene	N315 ORF*	Function	<i>agr</i> effect†	<i>sarA</i> effect†	<i>msa</i> effect	Fold change‡					
						MOE54 (<i>msa</i>) vs UAMS-1			MOE163 (pMOE83) vs UAMS-1		
						mid	late	post	mid	late	post
<i>hla</i>	SA1007	Alpha-toxin, haemolysin	+	–	– ^{SD}	2.41	6.50	2.70	1.18	1.12	0.66
<i>aur</i>	SA2430	Zinc metalloproteinase	+	–	–	8.38	14.93	6.06	0.89	1.32	0.39
<i>sspA</i>	SA0901	Serine protease (V8)	+	–	–	1.35	11.85	42.22	0.95	2.35	0.54
<i>clfA</i>	SA0742	Fibrinogen-binding protein	–	+	+	0.85	0.68	0.31	0.83	1.52	0.60
<i>fnbA</i>	SA2291	Fibronectin-binding protein A	–	+	+	0.02	0.01	0.08	0.69	0.74	0.85
<i>spa</i>	SA0107	Protein A	–	No effect	No effect ^{SD}	1.27	1.15	1.27	0.69	0.90	0.88
<i>cna</i>	NA§	Collagen-binding adhesin	No effect	–	–	2.76	3.82	15.63	0.47	0.78	0.87
<i>lysA</i>	SA1232	Diaminopimelate carboxylase	Unknown	Unknown	+	0.78	0.33	0.23	0.87	0.83	0.49
<i>cspA</i>	SA1234	Major cold-shock protein	Unknown	No effect	+	<0.001	<0.001	<0.001	0.80	1.10	0.40

*Designation of gene in strain N315.

†Effect on gene transcription: +, activation; –, repression; 'No effect', no significant change in transcription (less than twofold).

‡Fold change in gene expression in the *msa* mutant (MOE54) or the complemented mutant (MOE163) relative to wild-type (UAMS-1). Values represent the mean ratio for at least two independent experiments. Expression measurements were done at mid-exponential, late-exponential and post-exponential growth phases.

§Strain N315 does not encode collagen-binding adhesin (*cna*).

^{SD}Strain-dependent effect.

resulted in reduced expression of alpha toxin (*hla*) in RN6390 but increased expression in UAMS-1. This is also consistent with earlier reports describing the impact of mutation of *sarA*. Similarly, while the *msa* mutation affected the transcription of clumping factor (*clfA*) in UAMS-1, it did not have a significant effect on expression of this gene in RN6390. Conversely, transcription of protein A (*spa*) was reduced by mutation of *msa* in RN6390 but was not significantly changed in UAMS-1. Complementation studies with pMOE83 confirmed that the *msa* mutation was responsible for all these transcriptional changes (Tables 3 and 4). The differences in transcription profiles between the two strains highlight the importance of examining the effect of regulatory genes such as *msa* in clinical isolates.

Phenotypic characterization of the *msa* mutant

To confirm our transcription data, we also used functional assays to examine the phenotype of the RN6390 and UAMS-1 *msa* mutants. As controls, we also examined the phenotype of the corresponding *sarA* and *agr* mutants. The *msa* mutation caused an increase in proteolytic activity (up to fivefold) in both strains (Fig. 6). Complementation with pMOE83 reduced protease activity to wild-type levels (Fig. 6). As previously shown in UAMS-1, the *sarA* mutant showed an increase in proteolytic activity (approx. ninefold), the *agr* mutant showed no significant change in proteolytic activity, whereas the *sarA/agr* double mutant showed an intermediate level (approx. fivefold) of proteolytic activity in comparison to the corresponding wild-type strain (Fig. 6). Previous results from our group

suggest that the serine protease encoded by *sspA* is the primary protease produced by UAMS-1 (Blevins *et al.*, 2002). However, the protease assay we used will detect the activity of aureolysin and serine (V8) protease, and the fact that expression of both of these genes was increased in the

Fig. 6. Protease activity in the *msa* mutant. Extracellular proteolytic activity was measured from post-exponential growth phase (12 h) by using azocasein. Total protease activity is shown as the percentage of activity relative to the parent strain. Regulatory mutants *sarA*, *agr* and *sarA/agr* were included as controls. Results represent the mean $\pm$ SEM. Student's paired *t*-test was used to compare each mutant to the corresponding parent strain (* denotes statistical significance of $P < 0.05$).

msa mutant suggests that both these proteases may contribute to this phenotype. Mutation of *msa* had a less dramatic impact on overall proteolytic activity than mutation of *sarA*, suggesting that altered expression of other loci that either encode additional proteases or encode regulatory factors that modulate expression of other protease genes may also be involved (Tables 3 and 4).

Haemolytic activity was reduced in the RN6390 *msa* mutant but was increased in the UAMS-1 *msa* mutant (Fig. 7). Complementation with pMOE83 returned haemolytic activity to near wild-type levels, indicating that the phenotype was due to mutation of *msa* (Fig. 7). These results are consistent with our transcriptional data (Tables 3 and 4) and with previous experiments demonstrating that mutation of *sarA* has a strain-dependent effect on haemolytic activity (Blevins *et al.*, 2002). However, UAMS-1 was recently shown to carry a nonsense mutation in *hla*, rendering it unable to make alpha-toxin (James E. Cassat, personal communication). This suggests that the increased haemolytic activity observed in the *msa* mutant is a function of some other haemolytic toxin.

We also assessed the ability of *msa* mutants to bind the host proteins fibronectin, fibrinogen and collagen. These assays revealed that, unlike the corresponding parent strains, neither *msa* mutant bound fibronectin or fibrinogen (Table 5). As was observed with changes in haemolytic and proteolytic activity, complementation with pMOE83 restored the ability to bind both proteins (Table 5). Results of binding assays from the corresponding *sarA* and *agr* mutants were consistent with previous studies (Blevins *et al.*, 2002). The decreased ability to bind fibronectin was consistent with our transcriptional analysis showing

Fig. 7. Haemolytic activity in the *msa* mutant. Haemolytic activity was measured from post-exponential growth phase (12 h) by using 1% rabbit blood. Total haemolytic activity is shown as the percentage of activity relative to the parent strain. Regulatory mutants *sarA*, *agr* and *sarA/agr* were included as controls. Results represent the mean $\pm$ SEM. Student's paired *t*-test was used to compare each mutant to the corresponding parent strain (* denotes statistical significance of $P < 0.05$).

decreased *fnbA* transcription in the *msa* mutants (Tables 3 and 4). However, the ability to bind fibronectin is very sensitive to protease degradation (McGavin *et al.*, 1997; Karlsson *et al.*, 2001), so the increased proteolytic activity observed in *msa* mutants is also likely to contribute to this phenotype. Results of fibrinogen-binding assays were consistent with transcription analysis in UAMS-1. However, in RN6390, the *msa* mutant failed to bind fibrinogen despite the fact that there was no significant change in *clfA* transcription in the RN6390 *msa* mutant. This is consistent with the hypothesis that increased proteolytic activity may also contribute to the reduced capacity to bind fibrinogen (McAleese *et al.*, 2001).

A collagen-binding assay was also done with the RN6390 and UAMS-1 *msa* mutants. However, since RN6390 does not contain the *cna* gene, we first transduced the *msa* mutation to strain UAMS-174, which is a derivative of RN6390 with a single copy of the *cna* gene integrated in the chromosome (Gillaspay *et al.*, 1998). Given the limited sensitivity of the microtitre plate assay employed in these experiments, we could not demonstrate collagen binding with UAMS-1 or UAMS-174. However, we did demonstrate that mutation of *msa* in both strains resulted a detectable level of collagen binding (Table 5). Once again, complementation with pMOE83 restored the wild-type phenotype (Table 5). These results are consistent with the results showing derepression of *cna* transcription in UAMS-1 in the post-exponential growth phase. They are also consistent with the fact that the *msa* mutant was isolated based on a phenotype defined using a reporter fusion with the *cna* promoter (*cna-luxABCDE*).

Temporal pattern of *msa* transcription

We also measured the temporal pattern of *msa* expression in both RN6390 and UAMS-1. The levels of transcription in all three growth phases for both strains was compared to that of strain RN6390 at mid-exponential phase. In both strains, the *msa* transcript level increased to a maximum in the transition from mid-exponential to late-exponential growth phase. As cells entered the post-exponential growth phase, the amount of *msa* transcript decreased to levels similar to mid-exponential phase. Strain RN6390 produced less *msa* transcript relative to UAMS-1 during mid-exponential and post-exponential growth phases but produced significantly more *msa* transcript during late-exponential growth phase. As expected, the *msa* mutants in both strains showed no transcription of *msa* at any growth phase (data not shown).

Impact of *msa* on expression of neighbouring genes

To characterize the potential effect of the mutation of *msa* on neighbouring genes, we analysed the expression of the two genes, *lysA* (SA1232) and *cspA* (SA1234), that flank *msa*. *lysA* is located downstream of *msa* and encodes diamino-pimelate decarboxylase. It is the last gene of the *dap* operon,

Table 5. Binding of host proteins in the *msa* mutant

	Wild-type		<i>msa</i>		<i>msa</i> pMOE83		<i>sarA</i>		<i>agr</i>		<i>sarA agr</i>	
	RN6390	UAMS-1	RN6390	UAMS-1	RN6390	UAMS-1	RN6390	UAMS-1	RN6390	UAMS-1	RN6390	UAMS-1
Fibronectin	+	+	–	–	+	+	–	–	+	+	–	–
Fibrinogen	+	+	–	–	+	+	–	–	+	+	–	–
Collagen*	–	–	+	+	–	–	ND	+	ND	–	ND	+

*Collagen binding in the RN6390 background was assayed with strain UAMS-174, a derivative of RN6390 that harbours a single copy of the *cna* gene integrated in the lipase gene (*geh*); ND, not determined.

which is involved in lysine biosynthesis and is transcribed in the opposite direction with respect to *msa* (Fig. 2; Wiltshire & Foster, 2001). The gene upstream of *msa*, *cspA* (SA1234), encodes a cold-shock protein and is transcribed in the same direction as *msa*. In both strains, transcription of *lysA* and *cspA* was negatively affected by *msa* mutation. Transcription of *cspA* was practically abolished in the *msa* mutants in both RN6390 and UAMS-1 (Tables 3 and 4). Complementation with the pMOE83 resulted in re-establishment of expression to near wild-type levels, indicating the *msa* mutation was responsible for the phenotype. The abolition of *cspA* transcription brings up the possibility that some or all the phenotypic changes observed in the *msa* mutation are mediated by the impact of *msa* on *cspA* transcription rather than a direct effect on *sarA*. Further analysis is necessary to evaluate the relative contribution of *cspA*; however, the facts that introduction of pMOE83 complemented the defect and that *cspA* is upstream of *msa* indicate that the change in expression of the neighbouring genes is not due to a polar effect resulting from the transposon insertion in *msa*.

DISCUSSION

S. aureus has the ability to control expression of genes encoding virulence factors in response to environmental changes inside the host during the infection process. Although an increasing number of loci have been identified that contribute to this regulatory control (see Cheung *et al.*, 2004; Bronner *et al.*, 2004 for reviews), the *agr* and *sarA* loci clearly play central roles. We are particularly interested in the *sarA* locus based on studies demonstrating that it is required for biofilm formation and contributes to virulence in several animal models of staphylococcal infection (Beenken *et al.*, 2003; Valle *et al.*, 2003; Blevins *et al.*, 2003; Booth *et al.*, 1997; Cheung *et al.*, 1994; Nilsson *et al.*, 1996; Sifri *et al.*, 2003). However, several lines of evidence suggest that *sarA* requires as yet unidentified accessory elements that modulate the transcription of *sarA* and/or the function SarA. For instance, mutation of *sarA* results in increased expression of some genes (e.g. *cna*) and decreased expression of others (e.g. *fmbA*). In addition, while mutation of *sarA* results in decreased expression of *agr*, a study using an *in vitro* transcription system showed that purified SarA represses transcription from *agr* promoters (Chakrabarti & Misra, 2000). This clearly suggests the existence of an

accessory factor that is present in growing cells but was not present in the *in vitro* transcription system. Finally, SarA is present in roughly equivalent amounts throughout the growth cycle but represses or activates its target genes in a temporal manner (Blevins *et al.*, 1999; Chien *et al.*, 1999; Manna *et al.*, 1998).

To identify genes that modulate the activity of *sarA*, we developed a luminescent reporter system (*cna-luxABCDE*) to screen for *sarA*-related defects. We used this reporter system to screen an RN6390 transposon-insertion library for mutants, and selected those mutants that showed a *sarA* phenotype in a *sarA*-positive background. We used the *cna* promoter as a reporter because regulation of the collagen-binding adhesin by *sarA* is well characterized and is independent of *agr*, thus limiting our search to genes that have a direct impact on *sarA* (Blevins *et al.*, 1999). We found that mutation of *msa* led to increased expression from the *cna* promoter despite the presence of a functional *sarA* locus. In addition, mutation of *msa* had a negative impact on *sarA* expression in the laboratory strain RN6390 and the clinical isolate UAMS-1, suggesting that *msa* is important for wild-type levels of *sarA* transcription. We used both strains in this study because it has become evident that regulation of virulence in RN6390 is not representative of clinical isolates (Blevins *et al.*, 2002; Somerville *et al.*, 2002; Rice *et al.*, 2004; Cassat *et al.*, 2005). We used the osteomyelitis isolate UAMS-1 because it is closely related to other prominent clinical strains and, unlike RN6390, has an *rsbU* locus and consequently a functional *sigB* regulon (Cassat *et al.*, 2005).

Since SarA activates transcription of *agr*, we expected that the decrease in *sarA* expression caused by mutation of *msa* would result in decreased expression of *agr*. However, while the onset of RNAPIII expression in the RN6390 *msa* mutant was delayed, the amount of RNAPIII in the post-exponential growth phase was comparable to that of the parent strain. In contrast, mutation of *msa* in UAMS-1 caused a transient increase in RNAPIII expression in the mid-exponential and late-exponential growth phases. The reasons for this strain-dependent difference are unclear. However, RN6390 produces significantly more RNAPIII than UAMS-1, and it is possible that the increase observed in UAMS-1 was simply masked in RN6390 given its already high level of *agr* expression. It is also unclear whether this difference is a

function of the impact of *msa* on *sarA* expression or involves additional differences between the two strains. For instance, it is well known that all 8325-4 derivatives, including RN6390, carry a defect in *rsbU*, which leads to reduced production of the alternative sigma factor B (σ^B) (Kullik *et al.*, 1998). Additionally, Cassat *et al.* (2005) have demonstrated that *sarT* and *sarU*, both of which are reported to influence *agr* expression via a *sarA*-dependent mechanism (Schmidt *et al.*, 2003), are absent in UAMS-1.

Given the impact of *msa* on the major global regulator *sarA*, we also examined the impact of mutating *msa* on expression of virulence factor genes known to be modulated by *sarA*. Expression of some of the virulence factors tested (*aur*, *sspA* and *fmbA*) showed a similar response to mutation of *msa* in both RN6390 and UAMS-1 while expression of other genes (*hla*, *clfA* and *spa*) was altered in a strain-dependent manner (Tables 3 and 4). As with *agr*, the reasons for these strain-dependent differences are not completely understood, but the results are generally consistent with previous findings showing that mutation of *sarA* has a disparate effect in RN6390 and clinical isolates including UAMS-1 (Blevins *et al.*, 2002; Somerville *et al.*, 2002; Rice *et al.*, 2004; Cassat *et al.*, 2005). A specific example is expression of *hla*, which is decreased in an RN6390 *sarA* mutant but increased in *sarA* mutants generated in UAMS-1 and other clinical isolates (Blevins *et al.*, 2002). The fact that mutation of *msa* resulted in reduced expression of *hla* in RN6390 but increased expression of *hla* in UAMS-1 is therefore consistent with the hypothesis that the impact of *msa* on *hla* expression is mediated through its impact on expression of *sarA*. The absence of *sarT* and *sarU* may also be relevant in that regard. Specifically, Schmidt *et al.* (2003) showed that SarA represses *sarT*, which in turn represses *hla*. Mutation of *sarA* in a strain that encodes *sarT* (e.g. RN6390) would therefore be expected to result in decreased expression of *hla* due to derepression of *sarT*. However, other investigators have reported that SarA is capable of binding the *hla* promoter directly (Chien *et al.*, 1999; Chan & Foster, 1998b) and it is possible that, in the absence of *sarT*, this binding results in repression of *hla* transcription (Cassat *et al.*, 2005). In this scenario, mutation of *sarA*, or a factor like *msa* that is required for induction of *sarA* expression, would result in increased rather than decreased transcription of *hla*.

The impact of *msa* on transcription of *spa* was also strain-dependent. Specifically, the RN6390 *msa* mutant failed to express *spa* in all growth phases, while in UAMS-1 the *msa* mutant showed no significant change (less than twofold) in *spa* expression in the post-exponential growth phase. It is also noteworthy that, in direct contrast to *agr*, *spa* expression was significantly higher in UAMS-1 (approx. tenfold) than in RN6390 (data not shown and Blevins *et al.*, 2002). This is perhaps to be expected in that *agr* represses *spa* transcription; however, regulation of *spa* transcription and protein A production is very complex. As a surface protein, it is preferentially produced in exponential growth and repressed as cultures enter the post-exponential growth

phase. In addition to *agr* and *sarA*, *spa* expression is controlled directly or indirectly by several regulators including σ^B , Rot, MgrA, ArlSR, SarS, SarT, SarU, SarA and RNAIII (Bronner *et al.*, 2004). In early growth, *spa* is up-regulated by SarS. As the cells enter post-exponential growth, the amounts of SarA and RNAIII increase. Both SarA and RNAIII repress *spa* directly and via *sarS*, leading to a shutdown in protein A production (Arvidson & Tegmark, 2001; Cheung *et al.*, 2004; Novick, 2003). It is important to note in that regard that all 8325-4 strains also carry a mutation in *tcaR*, which is an activator of *sarS* transcription (McCallum *et al.*, 2004). Indeed, we have confirmed that UAMS-1 has an intact *tcaR* locus and that it produces significantly higher levels of *sarS* as well as *spa* (data not shown). Whether this difference accounts for the different *spa* phenotypes observed in the RN6390 and UAMS-1 mutants remains to be determined.

Overall, our findings show that mutation of *msa* affects the expression of *sarA* and the genes encoding several virulence factors. To the extent that mutation of *sarA* has been shown to result in a reduced capacity to form a biofilm (Beenken *et al.*, 2003; Valle *et al.*, 2003) and reduced virulence in several animal models of *S. aureus* infection, this suggests that *msa* may also make an important contribution to staphylococcal pathogenesis. Interestingly, sequence analysis of Msa indicates that it is a membrane protein, which suggests that it might interact with the external environment. We hypothesize that Msa acts as a sensor for an external signal that modulates *sarA* expression and thereby regulates the expression of *S. aureus* virulence factors. Whether Msa affects transcription of *sarA* directly or via an intermediate protein is not yet clear. It is also unclear what environmental signals might influence *msa* transcription. The drastic impact of the *msa* mutation on transcription of the gene encoding the cold-shock protein CspA, and the close proximity of *msa* and *cspA* genes, suggest that the changes in expression of *sarA* and other virulence factors might be mediated through *cspA*. Indeed, Katzif *et al.* (2003) showed that, in addition to its role in cold-shock response, CspA regulates expression of at least 14 genes. CspA homologues from other bacteria have been shown to regulate genes at the transcriptional and translational level (Yamanaka, 1999). Interestingly, neither *cspA* nor *lysA* was found to be regulated by *sarA* in a previous transcriptional profiling study (Dunman *et al.*, 2001). Although these earlier experiments were done with the 8325 strain RN27 and an early generation gene chip that was limited to ~86 % of the genome of the single *S. aureus* strain COL, similar experiments with a UAMS-1 *sarA* mutant and a much more comprehensive gene chip also failed to identify *cspA* as part of the *sarA* regulon (James E. Cassat, personal communication). This suggests that the impact of *msa* on transcription of *cspA* and *lysA* regulation is independent of its impact on *sarA*.

Rossi *et al.* (2003) described another membrane protein, MsrR, that attenuates *sarA* and might be involved in sensing cell wall damage. The nature of the interaction of MsrR with

sarA has not yet been determined. To our knowledge, Msa and MsrR are the only membrane-associated proteins that have been shown to regulate *sarA* transcription. Given the complexity of virulence regulation in *S. aureus*, and the fact that *sarA* regulates a wide variety of genes that are not directly related to virulence, we expect that membrane proteins such as MsrA and putatively Msa (whether directly or through *cspA*) provide the *sarA* regulon with external stimuli that are necessary for its functions. Other accessory elements that are located in the cytoplasm (e.g. the *sarA* homologues) fine-tune *sarA*'s response to environmental conditions by activating or repressing different sets of genes.

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