

# NsaRS is a cell-envelope-stress-sensing two-component system of *Staphylococcus aureus*

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*Staphylococcus aureus* possesses 16 two-component systems (TCSs), two of which (GraRS and NsaRS) belong to the intramembrane-sensing histidine kinase (IM-HK) family, which is conserved within the firmicutes. NsaRS has recently been documented as being important for nisin resistance in *S. aureus*. In this study, we present a characterization of NsaRS and reveal that, as with other IM-HK TCSs, it responds to disruptions in the cell envelope. Analysis using a *lacZ* reporter–gene fusion demonstrated that *nsaRS* expression is upregulated by a variety of cell-envelope-damaging antibiotics, including phosphomycin, ampicillin, nisin, gramicidin, carbonyl cyanide *m*-chlorophenylhydrazone and penicillin G. Additionally, we reveal that NsaRS regulates a downstream transporter NsaAB during nisin-induced stress. *NsaS* mutants also display a 200-fold decreased ability to develop resistance to the cell-wall-targeting antibiotic bacitracin. Microarray analysis reveals that the transcription of 245 genes is altered in an *nsaS* mutant, with the vast majority being downregulated. Included within this list are genes involved in transport, drug resistance, cell envelope synthesis, transcriptional regulation, amino acid metabolism and virulence. Using inductively coupled plasma-MS we observed a decrease in intracellular divalent metal ions in an *nsaS* mutant when grown under low abundance conditions. Characterization of cells using electron microscopy reveals that *nsaS* mutants have alterations in cell envelope structure. Finally, a variety of virulence-related phenotypes are impaired in *nsaS* mutants, including biofilm formation, resistance to killing by human macrophages and survival in whole human blood. Thus, NsaRS is important in sensing cell damage in *S. aureus* and functions to reprogram gene expression to modify cell envelope architecture, facilitating adaptation and survival.

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**Abbreviations:** CCCP, carbonyl cyanide *m*-chlorophenylhydrazone; ICP-MS, inductively coupled plasma-MS; IM-HK, intramembrane-sensing histidine kinases; MU, 4-methyl umbelliferone; TCS, two-component system; TEM, transmission electron microscopy.

The dataset for the microarray carried out in this study has been deposited in the NCBI GEO database under accession number GSE27061.

Two supplementary tables are available with the online version of this paper.

## INTRODUCTION

*Staphylococcus aureus* is a highly successful and dangerous human pathogen that is a leading agent of both nosocomial and community-associated infections. It has an extremely diverse pathogenesis, causing infection in a plethora of ecological niches within the host. Typically these proceed from a localized site of infection (e.g. wound infection), through to systemic dissemination (bacteraemia) leading to metastatic disease (e.g. osteomyelitis, endocarditis and septic arthritis). The pathogenic success of *S. aureus* is

largely the result of its arsenal of virulence determinants, which include haemolysins, toxins, adhesins and other exoproteins, such as proteases, staphylokinase and protein A (Lowy 1998; Novick, 2006). These damaging virulence factors are subject to multi-level and multi-factorial regulation, both temporally and spatially, in response to the environments encountered during growth. Accordingly, *S. aureus* has a diverse and wide-reaching network of regulatory elements that serve to modulate gene expression (Cheung *et al.*, 1992; Fournier *et al.*, 2001; Giraud *et al.*, 1994; McNamara *et al.*, 2000; Novick *et al.*, 1995) and bring about infection. These include all of the classical regulators of microbial gene expression, including DNA-binding proteins, regulatory RNAs, alternative sigma factors and two-component systems (TCSs). This latter class is an elegant mechanism of modulating gene expression in response to external conditions. As the name suggests, they are composed of two elements: a membrane-associated histidine kinase and a cytoplasmically located response regulator. These systems function to sense change in the external environment and reprogram gene expression accordingly, to circumnavigate stress.

In *S. aureus*, there are 16 conserved two-component regulators encoded within the various genomes sequenced thus far. Of this number, there are those that control autolysis (ArlRS, LytRS), virulence (SaeRS, AgrCA) and cell wall synthesis/drug resistance (WalkR, GraRS, VraSR) (Brunskill & Bayles, 1996; Delaune *et al.*, 2011; Fournier *et al.*, 2001; Giraud *et al.*, 1994; Kuroda *et al.*, 2003; Meehl *et al.*, 2007; Novick *et al.*, 1995). There are yet others that sense external iron (HssRS), nitrogen (NreBC) or oxygen (SrrRS) conditions (Schlag *et al.*, 2008; Torres *et al.*, 2007; Yarwood *et al.*, 2001). Recent phylogenetic analysis of bacterial TCSs revealed a conserved group of regulators termed intramembrane-sensing histidine kinases (IM-HKs), which are largely confined to the firmicutes (Joseph *et al.*, 2002; Mascher, 2006). These unique TCSs are characterized as having very small histidine kinase proteins (less than 400 aa) with limited extracellular spanning domains (no more than 25 aa). The IM-HKs can be further divided into two major classes, those that are encoded upstream of a two-component ABC-type transport system, and those that are genetically linked to a conserved membrane protein. The latter class is only found in the low G + C Gram-positive bacteria and bears a HPK7 subfamily protein with multiple membrane-spanning domains. The best studied example of this class of regulators is VraRS from *S. aureus*, which is involved in resistance to cell-wall-targeting agents, and has been shown to protect against cell damage by modulating components of the peptidoglycan biosynthesis pathway (Gardete *et al.*, 2006; Kuroda *et al.*, 2003).

Quite distinct from the VraRS family is the second, and by far the most numerate, class of IM-HKs, which are defined by an HPK3i subfamily protein, containing only two membrane-spanning domains (Mascher, 2006). This family of regulators functions by sensing cell envelope

damage, and upregulating adaptation and survival pathways. This is mediated by a variety of processes, including remodelling of cell architecture, and detoxification via transport systems (Mascher, 2006). In *S. aureus* there are two members of this latter family, the relatively well-studied GraRS, and the recently named NsaRS (Blake *et al.*, 2011). GraRS has been described as being important in the resistance of *S. aureus* to antimicrobial peptides by a variety of mechanisms which remodel the cell wall (Herbert *et al.*, 2007; Li *et al.*, 2007). Reference to NsaRS has only recently appeared in the literature, where it was demonstrated to be important for nisin resistance in *S. aureus* (Blake *et al.*, 2011). In this study we present a characterization of the NsaRS system and reveal that, as with other IM-HK TCSs, it responds to disruption of the cell envelope and redirects gene expression to mediate resistance. Additionally, we demonstrate that NsaRS is required for full biofilm formation across a variety of *S. aureus* strains, and contributes to survival during challenge by components of the human innate immune system.

## METHODS

**Bacterial strains, plasmids and growth conditions.** *S. aureus* and *Escherichia coli* strains, along with the plasmids used in this study, are listed in Table 1. The strains were grown as described previously (Shaw *et al.*, 2008). Cultures for growth experiments were obtained by using the following protocol. Overnight *S. aureus* cultures (1 ml) were used to inoculate fresh medium and allowed to grow for 3 h. These exponentially growing cultures were used to seed new medium at OD<sub>600</sub> 0.05. Test cultures were allowed to grow for the time period required for the experiment. Where used, CLR medium consisted of CL (containing 400 µM magnesium) with the following metals at a final concentration of 0.2 µM: calcium chloride, copper sulfate, ferrous sulfate, manganese chloride, nickel sulfate and zinc sulfate. We deviated from published protocols (Horsburgh *et al.*, 2001a) by omitting molybdenum sulfate and including 0.2 µM cobalt sulfate.

**Construction of an *nsaRS* reporter fusion strain.** The putative promoter region of the *nsaRS* locus was amplified as a ~1.5 kb fragment (OL802/OL803) from *S. aureus* SH1000 genomic DNA. The reporter fusion was then constructed as described previously (Shaw *et al.*, 2004). Briefly, the PCR fragment was cloned into the Gram-positive suicide vector pAZ106 creating plasmid pSLK1. *S. aureus* RN4220 was transformed with the resulting plasmid, and clones were selected for using TSA containing erythromycin (plasmid-encoded). The resulting strains were confirmed by genomic DNA extraction and PCR analysis. Transformants were used to generate 80x phage lysates for transduction of *S. aureus* SH1000, with clones again confirmed by PCR analysis. This created strain SLK1.

**Construction of the *nsaS* mutant strain.** We generated an *nsaS* histidine kinase mutation to probe the role of this system as described previously (Shaw *et al.*, 2004). An 800 bp PCR fragment within the *nsaRS* locus was amplified (primers OL837/OL838) and cloned into the pAZ106 suicide vector, generating pSLK2, which was used to transform *S. aureus* RN4220 cells. A representative clone was used to generate a phage lysate for transduction of *S. aureus* SH1000, Newman and UAMS-1790 [USA300-HOU-MRSA (Highlander *et al.*, 2007) cured of pUSA300-HOU-MRSA, a gift from Dr Mark Smeltzer] using 80x. This created strains SLK2, SLK3 and SLK4, respectively. In this mutation, the forward primer (OL837) is located 191 nt downstream of the ATG of *nsaR*, whilst the reverse primer (OL838)

**Table 1.** Strains, plasmids and primers used in this study

Restriction sites are underlined. Primers are given in 5'–3' orientation.

Strain, plasmid or primer	Genotype or description	Reference or source
<b>Strains</b>		
<b><i>E. coli</i></b>		
DH5 $\alpha$	$\phi$ 80 $\Delta$ ( <i>lacZ</i> )M15 $\Delta$ ( <i>argF-lac</i> )U169 <i>endA1 recA1 hsdR17</i> ( $r_{\bar{K}}$ $m_{\bar{K}}$ ) <i>deoR thi-1</i> <i>supE44 gyrA96 relA1</i>	Lab stocks
<b><i>S. aureus</i></b>		
RN4220	Restriction-deficient transformation recipient	Lab stocks
8325-4	Wild-type laboratory strain <i>rsbU</i> mutant	Lab stocks
SH1000	Wild-type laboratory strain <i>rsbU</i> functional	Horsburgh <i>et al.</i> (2002)
Newman	Wild-type laboratory strain	Lab stocks
UAMS-1790	Sequenced USA300-HOU-MRSA isolate cured of pUSA300-HOU-MRSA	Highlander <i>et al.</i> (2007); from M. Smeltzer
SLK1	SH1000 <i>nsaRS-lacZ</i> fusion	This study
SLK2	SH1000 <i>nsaS</i> mutant	This study
SLK3	Newman <i>nsaS</i> mutant	This study
SLK4	USA300 <i>nsaS</i> mutant	This study
SLK5	SH1000 <i>nsaS</i> mutant complemented with pSLK3	This study
SLK6	Newman <i>nsaS</i> mutant complemented with pSLK3	This study
SLK7	USA300 <i>nsaS</i> mutant complemented with pSLK3	This study
<b>Plasmids</b>		
pAZ106	Promoterless <i>lacZ erm</i> insertion vector	Kemp <i>et al.</i> (1991)
pMK4	<i>cm</i> shuttle vector	Sullivan <i>et al.</i> (1984)
pSLK1	pAZ106 containing a 1.5 kb <i>nsaRS</i> promoter fragment	This study
pSLK2	pAZ106 containing an 800 bp mutagenic <i>nsaS</i> fragment	This study
pSLK3	pMK4 containing a 2.5 kb <i>nsaRS</i> complementation fragment	This study
<b>Primers</b>		
OL802	ATGGGATCCGTCACGTGTGCAGCATATGC	
OL803	ATGGAATTCAGGCGCGTCATGTTAACAGC	
OL837	ATGGGATCCCATTTGGTGTCAAGAAATCCGAA	
OL838	ATGGAATTCAGTTGTGCTGCAGTAATGGGTG	
OL931	ATGGAATTCGTGCTGTCGTAGCATTCAAAC	
OL1036	CCGCGCACATTTCCCGAAA	
OL1180	TGGTTACGCAAGGTGTTG	
OL1181	TCAACTGGTGAAGGACTG	
OL1222	GAAGCACAACATGGTGGT	
OL1223	TTGCTGCTACTCCACCA	
OL1224	AGATGAACTCGTCCA	
OL1225	GCACATCTGAAGGCG	
OL1226	CGTCATTGATGAGTGGTG	
OL1227	GGTACACTCCAAACATGC	
OL1333	CGGTGTTATTGTCGTTG	
OL1334	ACCATT0TAACGTTGGCA	
OL1335	TGCATGCCATGTTGCT	
OL1336	TTCACCAGCTCCAAC	

is located approximately one-third of the way into the *nsaS* coding region (~350 nt into the 942 nt gene). Accordingly, the recombination event results in a full-length *nsaR* along with approximately 350 nt of *nsaS* connected to the natural promoter. The remainder of the *nsaS* gene, including the histidine kinase region, is disrupted by the insertion event. To further verify the mutation, we conducted reverse-transcription studies, which confirmed, as predicted, that only ~350 bp of *nsaS* is transcribed with *nsaR*, and that no mRNA containing the last two-thirds of *nsaS* (including the histidine kinase domain) is produced (data not shown). To determine if mutation of *nsaS* affected *nsaR*, we conducted real-time PCR analysis for *nsaR* in

both the SH1000 wild-type and its *nsaS* mutant. We observed no alteration in expression of *nsaR* between the two strains (data not shown), indicating that *nsaS* mutation produced no unintended effects, such as compensatory *nsaR* overexpression.

**Construction of *nsaRS* complemented strains.** In order to construct a complementation vector the entire *nsaRS* locus was amplified (OL802/OL931) as a 2.5 kb fragment. *In silico* analysis reveals an additional gene (SACOL2647) upstream of *nsaR* that likely forms the first coding unit of this operon. Accordingly, the forward primer is located 703 nt upstream of SACOL2647, and the reverse

primer terminates downstream of *nsaS*. This fragment was cloned into the Gram-positive shuttle vector pMK4, creating pSLK3. *S. aureus* RN4220 was transformed with this construct, and clones were confirmed by genomic DNA extraction and PCR analysis, using a combination of gene- and vector-specific primers (OL802/OL1036). A representative isolate was selected to generate an 80 $\alpha$  phage lysate, to transduce the SH1000, Newman and UAMS-1790 *nsaS* mutants. Clones were again confirmed by PCR analysis, creating strains SLK5, SLK6 and SLK7, respectively.

**Plate-based assay to determine alterations in transcription resulting from external stress.** This assay was performed as described previously (Shaw *et al.*, 2008). Briefly, TSA plates were overlaid with TSB top agar (0.7% w/v) containing X-Gal, and seeded with exponentially growing SH1000 *nsaRS-lacZ* reporter-fusion cells. Sterile filter discs were overlaid onto these plates (three per plate) which were then inoculated with 10  $\mu$ l of the following stress inducing chemicals: 6 M HCl, 85% phosphoric acid, 100% TCA, 88% formic acid, 0.2 M acetic acid, 6 M sulphuric acid, 6 M nitric acid, 6 M sodium hydroxide, 2 M NaCl, 1 M glucose, 95% ethanol, 100% methanol, 100% 2-propanol, 10% SDS, 10% Triton X-100, 10% Tween-20, 1 M *N*-lauroyl sarcosine, 30% hydrogen peroxide, 1 M methyl viologen, 1% menadione, 2 mg pyrogallol ml<sup>-1</sup>, 1 M sodium nitroprusside, 1 M methyl-methane sulfonate, 5 mg penicillin G ml<sup>-1</sup>, 5 mg vancomycin ml<sup>-1</sup>, 2 mg phosphomycin ml<sup>-1</sup>, 5 mg spectinomycin ml<sup>-1</sup>, 100 mg ampicillin ml<sup>-1</sup>, 5 mg nisin ml<sup>-1</sup>, 5 mg gramicidin ml<sup>-1</sup>, 5 mg tetracycline ml<sup>-1</sup>, 50 mg kanamycin ml<sup>-1</sup>, 50 mg neomycin ml<sup>-1</sup>, 10 mg chloramphenicol ml<sup>-1</sup>, 20 mg puromycin ml<sup>-1</sup>, 2 mg bacitracin ml<sup>-1</sup>, 1 mg oxacillin ml<sup>-1</sup>, 1 mg cefotaxime ml<sup>-1</sup>, 5 mg D-cycloserine ml<sup>-1</sup>, 1 mg teicoplanin ml<sup>-1</sup>, 5 mg polymyxin B ml<sup>-1</sup>, 5 mg chlorpromazine ml<sup>-1</sup>, 0.1 mg carbonyl cyanide *m*-chlorophenylhydrazone (CCCP) ml<sup>-1</sup>, 1 mg valinomyacin ml<sup>-1</sup>, 2 mg mupirocin ml<sup>-1</sup>, 500 mM diamide, 12.8 mg berberine chloride ml<sup>-1</sup>, 4.21 M peracetic acid, 0.1 M EDTA, 1 M DTT. Plates were incubated for 24 h at 37 °C and screened for a blue halo around the perimeter of the filter discs, indicating expression.

**Kirby–Bauer sensitivity profiling of cell-wall-damaging agents.** Overnight broth cultures of the SH1000 wild-type and its *nsaS* mutant were diluted 1:1000 and added to 5 ml top agar; this was mixed and used to overlay TSA plates. Lawns were allowed to dry for 15 min and sterile filter disks were added to each plate. Penicillin G, oxacillin, ampicillin, cefotaxime, D-cycloserine, phosphomycin, gramicidin, teicoplanin, bacitracin or polymyxin B (all 10  $\mu$ l) was added to the filter disks from a 5 mg ml<sup>-1</sup> stock solution. Plates were incubated at 37 °C overnight and zones of inhibition were recorded.

**$\beta$ -Galactosidase assays.** Levels of  $\beta$ -galactosidase activity were measured as described previously (Shaw *et al.*, 2006, 2007). Fluorescence was measured using a BioTek Synergy 2 plate reader and calibrated with standard concentrations of 4-methyl umbelliferone (MU). One unit of  $\beta$ -galactosidase activity was defined as the amount of enzyme that catalysed the production of 1 pmol MU min<sup>-1</sup> (OD<sub>600</sub> unit)<sup>-1</sup>. Assays were performed on duplicate samples and the mean value was determined. The results presented here are representative of three independent experiments that showed less than 10% variability.

**Transcriptomic studies.** The SH1000 wild-type and *nsaS* mutant were grown for 3 h, which corresponds to a window of maximal *nsaRS* expression. After this time RNA was extracted, converted to fluorescently labelled cDNA and hybridized to *S. aureus* COL genome microarrays version 6.0, as described previously (Delgado *et al.*, 2008). Four hybridizations were performed for this study, including a biological replicate and a dye-swap experiment for each replicate to account for dye bias. Spots flagged as empty or bad were excluded and the raw data from each slide were normalized using the LOWESS

method, with background correction. Data from the replicates were combined (using the median value) and a one-sample *t*-test was performed. A volcano plot was used with a fold change cut-off of  $\geq 2$  and a *P*-value of  $< 0.05$  to filter the genes that were differentially expressed. ORF IDs in the list of differentially expressed genes were then mapped to *S. aureus* COL IDs where possible. The entire dataset for this study has been deposited in the NCBI GEO database under accession number GSE27061.

**Real-time PCR.** Quantitative real-time PCR analysis was conducted as described previously (Livak & Schmittgen, 2001; Riordan *et al.*, 2010) using primers specific for alanine dehydrogenase (OL1222/OL1223), Rex (OL1224/OL1225), NarI (OL1226/OL1227), L-lactate dehydrogenase (OL1180/OL1181), *nsaR* (OL1333/OL1334) and *nsaA* (OL1335/OL1336). The control primers were for the 16s rRNA gene, as described previously (Koprivnjak *et al.*, 2006).

**Thin-section electron microscopy.** Aliquots of bacteria grown in TSB or 10% NaCl for 3 and 6 h were processed and fixed as described previously (Shaw *et al.*, 2005). Samples were observed and photographed in an FEI Morgagni 268D (FEI) transmission electron microscope with an Olympus SIS MegaView III (ResAlta Research Technologies) camera at the Integrative Biology Electron Microscopy Core Facility at the University of South Florida.

**Negative staining electron microscopy.** A method of fixing bacteria in osmium tetroxide prior to pelleting was employed to stabilize cell walls. Osmium does not cross-link any protein in culture medium to bacterial cells, and allows preservation of the cell wall structure throughout centrifugation. This permits rinsing to remove culture media proteins prior to preparing cells for negative stain preparation and transmission electron microscopy (TEM) analysis. This method imparts electron density similar to that of uranyl acetate or other similar negative stains commonly used to observe bacteria via electron microscopy. Aliquots of cells grown in TSB for 3 and 6 h were initially fixed in an equal volume of 2% osmium tetroxide in distilled water for 1 h at 4 °C. Following fixation, cells were rinsed in distilled water and pelleted three times at 5000 r.p.m. for 10 min. Dilutions were performed to obtain approximately 2000–3000 cells per drop, before one drop of each sample was applied to a carbon-Formvar-coated copper grid. Grids were allowed to air dry and were visualized by electron microscopy.

**Biofilm formation assay.** Biofilm formation assays were performed as described previously (Beenken *et al.*, 2003). The absorbance of samples was read using a BioTek Synergy 2 plate reader.

**Metal ion profiling using inductively coupled plasma (ICP)-MS.** The SH1000 parental strain, its *nsaS* mutant and the complemented strain were grown in TSB and metal-limiting media for 4 h at 37 °C. Cells were harvested and their pellets were weighed and dried at 80 °C for 12 h. For hot acid digestion, filter pipette tips were rinsed with 2% nitric acid in ultrapure water. Dried pellets were allowed to incubate for 1 h at room temperature in 500  $\mu$ l ultrapure nitric acid OPTIMA. Each sample was then transferred to a borosilicate tube and placed in a silicone oil bath at 140–150 °C until  $\sim 200$ –250  $\mu$ l each sample remained. To each sample, 250  $\mu$ l of 30% hydrogen peroxide ULTREX II was added and incubated at room temperature for 5–10 min. Tubes were placed back into the oil bath at 120 °C and the hydrogen peroxide was allowed to evaporate until  $\sim 200$ –250  $\mu$ l remained. Tubes were removed from the water bath and ultrapure water was added to each sample up to 5 ml. For ICP-MS analysis, standards of the desired elements (1, 5, 10, 25, 100, 250 and 1000 p.p.b.) in 2% nitric acid were made to create a standard curve. The ICP mass-spectrometer was tuned with 10 p.p.b. of elements  $\geq$  Li for optimization of lenses, and the internal standard used was 10 p.p.b. Indium. A blank (2% nitric acid) standard curve and the

samples were loaded and processed using the Thermo PlasmaLab software. The amount of each metal was determined based on p.p.b. given and the dried cell weights. The data presented are derived from at least five independent cultures.

#### Macrophage culture and *S. aureus* intracellular survival assay.

The Newman wild-type and its *nsaS* mutant were analysed using a human macrophage model of survival and clearance, as described previously (Kubica *et al.*, 2008). The data presented were generated from six independent experiments. The data were analysed using Student's *t*-test with a 5% confidence limit to determine statistical significance.

**Whole human blood survival assay.** Exponentially growing cultures of the SH1000 wild-type, *nsaS* mutant and the complemented strain were washed three times with PBS and resuspended in 1 ml whole human blood. The initial inoculum of each strain was also determined at this time by serial dilution and plating on TSA. Blood cultures were incubated at 37 °C with shaking for 3 h, before the c.f.u. ml<sup>-1</sup> of each strain was determined, again by serial dilution and plating. Data are presented as percentage survival of each strain compared with initial inocula. These experiments were performed with two separate blood samples and represent five independent replicates.

## RESULTS

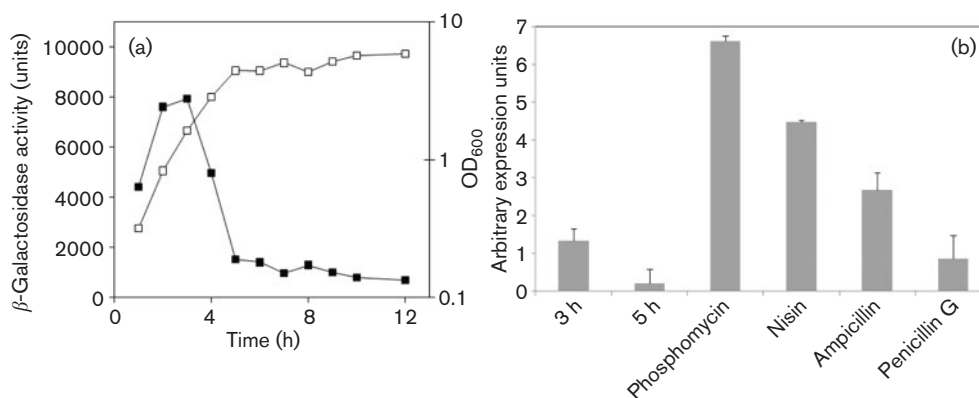
### Transcriptional profiling of the *nsaRS* TCS

We initially set out to investigate the role of *nsaRS* by analysing its transcription in the laboratory strain SH1000. A reporter–gene fusion, using a promoterless *lacZ* cassette, was created and monitored for transcription during growth. Analysis revealed that maximal transcription of *nsaRS* occurs during the exponential and post-exponential phases of growth (2–5 h) and declines into stationary phase (Fig. 1a). Given that TCSs function by sensing external stimuli, we sought to determine if the transcription of *nsaRS* could be modulated by the presence of chemical

stressor compounds. As such, a plate-based assay was employed, as described by Cao *et al.* (2002) and Shaw *et al.* (2008), containing a variety of stress-inducing compounds (see Methods) to mimic natural external conditions. What we found was that a variety of cell-envelope-targeting antibiotics upregulated transcription of *nsaRS*, including ampicillin, phosphomycin, D-cycloserine, gramicidin, nisin, CCCP and penicillin G (data not shown). To quantitatively confirm these observations, we conducted real-time PCR on *S. aureus* SH1000 grown in either TSB or TSB containing subinhibitory concentrations of ampicillin, phosphomycin, penicillin G and nisin (Fig. 1b). When analysed during a window of peak *nsaRS* expression (3 h) we found that phosphomycin had the most dramatic effect, increasing expression by approximately fivefold compared with standard conditions. Further to this, analysis using nisin revealed a 3.3-fold upregulation, whilst studies with ampicillin revealed a twofold increase in transcription. Finally, subinhibitory concentrations of penicillin G revealed no alteration in *nsaRS* expression, perhaps suggesting that greater concentrations of this agent, as used in the plate-based assay, are required to induce expression of this TCS.

### *NsaRS* regulates transcription of the downstream transporter *NsaAB* in response to nisin stress

As with other members of the IM-HK TCS family, *NsaRS* is encoded upstream of a transcriptionally distinct locus that specifies an ABC type transporter (termed *NsaAB*). The existing paradigm of this arrangement is that the transporter aids in detoxification of the cell in response to cell-envelope-damaging agents, and is commonly regulated by its cognate TCS (Staroń *et al.*, 2011). In order to ascertain if *NsaRS* regulates *NsaAB*, we conducted real-time PCR



**Fig. 1.** Transcriptional profiling of *nsaRS*. (a) A reporter *lacZ* fusion was created for *nsaRS* in the SH1000 background, and analysed using 4-MU. Cultures were grown in TSB and OD<sub>600</sub> (□) and  $\beta$ -galactosidase activity (■) was measured every hour throughout growth. Data shown are from at least three independent cultures that demonstrated less than 10% variability. (b) qRT-PCR was performed for *nsaRS* under standard conditions (3 and 5 h), and also following the addition of subinhibitory concentrations of cell-wall-damaging chemicals (3 h). The data presented are from at least three independent experiments; error bars, SEM.

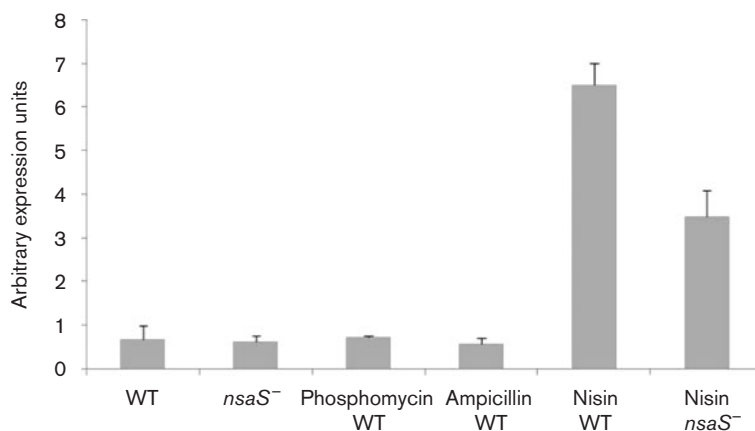
analysis on *nsaAB* in both the SH1000 wild-type and its *nsaS* mutant during a window of high NsaRS expression (3 h). Interestingly, we saw no alteration in *nsaAB* transcription between these two strains under standard conditions (Fig. 2). Given that these ABC transporters commonly function as detoxification units, we repeated *nsaAB* qRT-PCR analysis with cells grown under conditions shown to induce *nsaRS* expression. Accordingly, SH1000 was grown for 3 h in the presence of subinhibitory concentrations of phosphomycin, ampicillin and nisin, and analysed for *nsaAB* expression. Interestingly, whilst phosphomycin and ampicillin did not affect transcription of *nsaAB*, nisin resulted in an approximately tenfold upregulation of the transporter. To determine if the nisin-dependent stimulation of *nsaAB* expression was mediated via NsaRS, we repeated these analyses with the *nsaS* mutant. Compared with the wild-type strain we observed a twofold reduction in *nsaAB* expression in the *nsaS* mutant when grown in a subinhibitory concentration of nisin. Interestingly, the expression levels of *nsaAB* were still increased fivefold above that of the wild-type grown under standard conditions. This suggests that NsaRS plays a major role in regulating *nsaAB* in response to nisin stress but that other factors may also contribute to this process. These findings are consistent with existing literature on similar systems, which reveals that there is often discrepancy between IM-HK TCS induction and downstream transporter regulation (Staroń *et al.*, 2011).

### NsaRS has a significant role in the ability of *S. aureus* to develop resistance to bacitracin

In this study, we show that a number of cell-envelope-targeting antimicrobials upregulate *nsaRS* transcription. Additionally, others have shown the importance of NsaS in the development of resistance to nisin (Blake *et al.*, 2011). Finally, a previous study revealed that an *nsaS* mutant had a fourfold increase in sensitivity to bacitracin (Matsuo *et al.*, 2010). As such, we set out to assess the sensitivity of the *nsaS* mutant to a variety of cell-envelope-targeting antimicrobial compounds. Using a modified Kirby–Bauer

disk-diffusion approach we were unable to identify any increase in sensitivity of the mutant compared with the wild-type strain (see Methods for list; data not shown). Further qualification of this observation by determining minimum bactericidal concentration (MBC) also revealed no alteration between SH1000 and its *nsaS* mutant (data not shown). We suggest that these findings are consistent with the existing literature, which demonstrates that mutants of IM-HKs are themselves typically not sensitive to the antibiotics they sense (Staroń *et al.*, 2011). Moreover, it is mutants of the transporters they regulate that demonstrate this type of sensitivity (Staroń *et al.*, 2011). This observation is somewhat surprising, however, given that an *nsaS* mutant was previously reported as being more sensitive to bacitracin (Matsuo *et al.*, 2010). When performing MIC analyses, we found that the *nsaS* mutant was not altered for bacitracin in the SH1000 and USA300 backgrounds. The disparity between our findings, and those of Matsuo *et al.* (2010) is probably explained by bacitracin MICs of the strains chosen; our study used SH1000 and USA300, whilst the previous study used MW2. SH1000 and USA300 have an MIC of  $16 \mu\text{g ml}^{-1}$  for bacitracin, whilst MW2 has an MIC of  $64 \mu\text{g ml}^{-1}$ . The fact that Matsuo and colleagues reported an MIC of  $16 \mu\text{g ml}^{-1}$  for the *nsaS* mutant in MW2, which is that observed for our wild-types, suggests that intrinsic drug resistance mechanisms of MW2 may have some impact on this effect.

Given the recent documentation of the role of NsaS in mediating resistance to nisin, we next determined if the *nsaS* mutant was impaired in its ability to mediate resistance to other cell-wall-targeting compounds. As such, we analysed spontaneous mutation frequencies for D-cycloserine, phosphomycin, vancomycin, cefotaxime and bacitracin. When performing 13 replicates from five separate cultures, we were unable to find alterations in the spontaneous mutation frequency for D-cycloserine, phosphomycin, vancomycin or cefotaxime. We did, however, find a major discrepancy between the wild-type strain and *nsaS* mutant for bacitracin. Specifically, we obtained 886 mutants from a combined inocula of  $5.77 \times 10^{10}$  for



**Fig. 2.** Role of NsaRS in regulating the transporter *nsaAB*. Transcription of *nsaAB* was initially determined under standard conditions in SH1000 (WT) and its *nsaS* mutant grown for 3 h. These analyses were repeated using TSB containing subinhibitory concentrations of phosphomycin, ampicillin and nisin in SH1000 and its *nsaS* mutant (no change observed for phosphomycin and ampicillin, data not shown). The data presented are from at least three independent experiments; error bars, SEM.

SH1000 when plated on bacitracin agar plates containing  $3 \times \text{MIC}$ . In contrast, we were only able to obtain three mutants from a combined inocula of  $3.84 \times 10^{10}$  for the *nsaS* mutant strain. This results in a spontaneous mutation frequency of  $1.53 \times 10^{-8}$  for SH1000 and  $7.81 \times 10^{-11}$  for the *nsaS* mutant, representing a 196-fold decrease in spontaneous mutation frequency for the mutant strain.

### Profiling the NsaRS transcriptome

Given that TCSs function via modulation of gene expression, we set out to analyse those genes that fall either directly or indirectly under the control of NsaRS. As such, we analysed an *nsaS* histidine kinase mutant via microarray analysis. Cultures of both SH1000 and its *nsaS* mutant were grown in TSB for 3 h, which corresponds to a window of maximal *nsaRS* expression. Total RNA was converted to cDNA and analysed using *S. aureus* COL genome microarrays v. 6.0 as described previously (Delgado *et al.*, 2008). A total of 245 genes were found to be altered in transcription at a level of twofold or greater. Of these genes, 34 are negatively controlled by NsaRS (see Supplementary Table S1, available with the online version of this paper), whilst 211 are positively regulated (Supplementary Table S2), indicating that this TCS functions overwhelmingly as a positive regulator of gene expression. Of those genes found to be repressed, a large number were components that had been previously implicated in virulence, including  $\beta$ -haemolysin, hyaluronate lyase, fibronectin binding protein A, seven of the genes required for capsule formation and six of the ten extracellular proteases. This last category of enzymes was amongst the most upregulated in the *nsaS* mutant, with members of the *spl* operon transcribed at levels more than sixfold higher than in the parental strain. Perhaps unsurprisingly, of the 211 genes positively controlled by NsaRS, a large number specify proteins whose function is associated with the cell envelope. These include components involved in cell wall biosynthesis, surface proteins, cell-wall-anchoring enzymes and, most extensively, those classified as encoding transporters. Indeed, 55 such elements were identified of this latter class, encompassing those either previously shown or implicated in the transport of metal ions, amino acids, peptides, sugars and antimicrobial compounds. In addition to this, there were alterations in genes that function in the biosynthesis and metabolism of amino acids, including arginine, lysine, threonine, glutamate, aspartate, phenylalanine and methionine. Another major class of genes with altered expression included those involved in transcriptional regulation, with 14 such elements identified, including SarS and Rex. Other ontologies of function identified included metabolic enzymes, DNA-damage-repair-encoding genes and elements involved in nucleotide biosynthesis. Interestingly, there were also a large number of genes contained within the transcriptome that have previously been shown to function in anaerobic growth, including acetoin reductase, L-lactate dehydrogenase and D-lactate dehydrogenase (Pagels *et al.*, 2010).

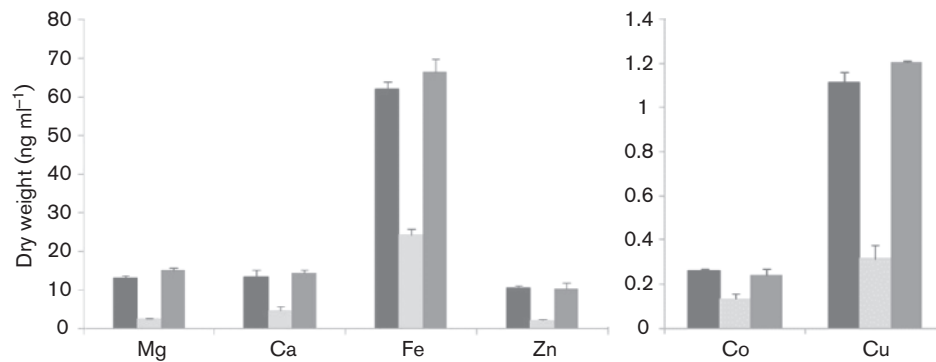
Confirmation of transcriptome data was performed using qRT-PCR analysis for selected genes identified in the dataset. Specifically, this was performed for alanine dehydrogenase, Rex, *narI* and L-lactate dehydrogenase, revealing 12.56-, 2.69-, 3.83- and 2.12-fold changes, respectively, in the *nsaS* mutant compared with the wild-type, akin to that seen in the transcriptome.

### ICP-MS profiling reveals that NsaRS is required for metal ion transport under limiting growth conditions

Transcriptomic analysis revealed a total of 55 transporters displaying decreased expression in the *nsaS* mutant. Of these elements, at least 20 appear to be specific to metal transportation, particularly with respect to divalent metal ions. Accordingly, we set out to analyse by ICP-MS the ability of the wild-type, *nsaS* mutant and complemented strains to import a variety of these ions. Quintuplet samples of each strain were grown in either TSB or metal-ion-limited media (CLR) supplemented with 400  $\mu\text{M}$  magnesium sulfate and 0.2  $\mu\text{M}$  calcium chloride, copper sulfate, ferrous sulfate, manganese chloride, nickel sulfate, zinc sulfate or cobalt sulfate. Samples were then analysed for the presence of the eight specific ions: Mg, Ca, Fe, Zn, Co, Ni, Cu, and Mn. We found that whilst little variation was observed during growth in replete media, the mutant strain displayed a marked decrease in its ability to import six of the eight metal ions tested when grown in limiting media (Fig. 3). Specifically, we determined a 2-fold decrease in cobalt, a 2.5-fold decrease in iron and calcium, a 3.5-fold decrease in copper and an approximately 5-fold decrease in both zinc and magnesium ions in the *nsaS* mutant strain. These findings suggest a role for NsaRS in metal ion import, particularly with respect to low-abundance growth conditions.

### Electron microscopy reveals alterations in the cell envelope of *nsaS* mutant cells

Given that our data thus far suggest a role for the NsaRS TCS in sensing cell envelope stress, we performed TEM analysis of the wild-type, mutant and complemented strains. When cells were grown in TSB for 3 h, we observed that the *nsaS* mutant had a diffuse and less defined/smooth cell wall compared with the parental and complemented strains (Fig. 4). Additionally, negative stain analysis revealed markedly increased encapsulation of the *nsaS* mutant after 3 h of growth, which became even more striking at later time points (data shown from 6 h). This finding is perhaps explained by the observed greater than twofold increase in transcription of seven of the ten genes required for capsule biosynthesis in the *nsaS* mutant. Finally, we also observed decreased transcription of a number of osmoprotectants (*cudT* and *opuCA*) in the *nsaS* mutant, along with a number of solute importers and general permeases. Accordingly, we grew the strains in TSB containing 10% NaCl to assess whether the *nsaS* mutant

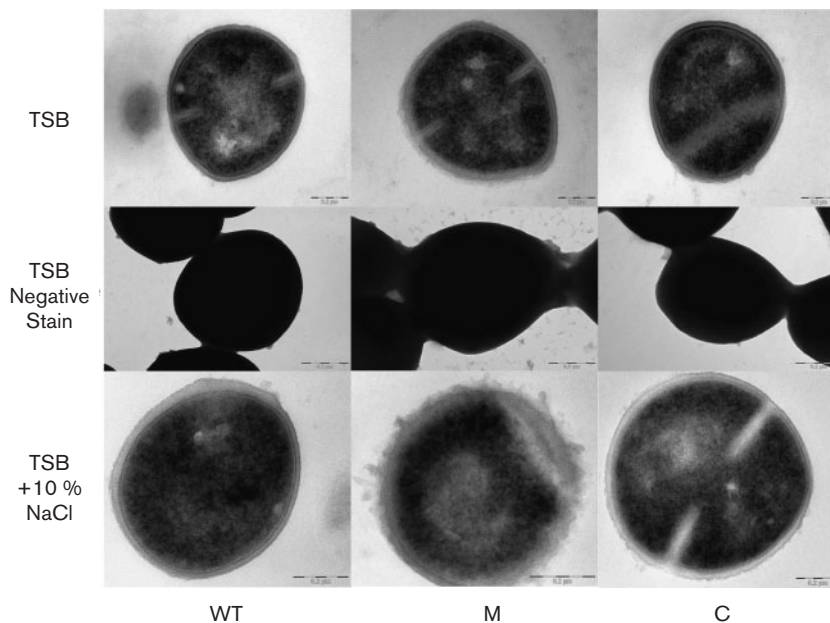


**Fig. 3.** The transport of divalent metal ions under low-abundance conditions requires a functional NsaRS. The SH1000 wild-type (black), its *nsaS* mutant (light grey) and the complemented strain (dark grey) were grown for 4 h in metal-ion-limiting media (CLR) containing 400  $\mu$ M magnesium sulfate and 0.2  $\mu$ M calcium chloride, copper sulfate, ferrous sulfate, manganese chloride, nickel sulfate, zinc sulfate and cobalt sulfate. Cells were harvested and analysed for the presence of the supplementary metal ions using ICP-MS. The data presented are from five independent cultures; error bars, SEM. Two graphs are presented because of the widely differing y-axis scales.

displayed impaired growth. Upon analysis we observed only minor defects in the *nsaS* mutant when compared with the wild-type strain (1.5-fold maximal decrease in viability at 6 h growth). However when these same cultures (6 h) were analysed by TEM we observed dramatic alterations in cellular appearance. Following the trend of TSB-grown cultures, mutant cells incubated with 10% NaCl showed highly irregular and diffuse cell walls compared with similarly grown wild-type and complemented cells. Taken together, these data suggest that NsaRS influences cell envelope architecture, particularly during times of stress.

### NsaRS contributes to biofilm formation in a variety of diverse *S. aureus* strains

NsaRS is an orthologue of GraRS in *S. aureus*, another IM-HK TCS involved in sensing disruption and instability of the cell wall (Li *et al.*, 2007; Kraus *et al.*, 2008). Previous work by other groups has revealed that GraRS mutants are impaired in their ability to form a biofilm (Boles *et al.*, 2010; Herbert *et al.*, 2007). In addition to this, we observed that a number of proteins required for biofilm formation display altered transcription in the *nsaS* mutant strain. Specifically, proteases are strongly upregulated in the *nsaS* mutant strain, which, as demonstrated by the recent work



**Fig. 4.** NsaRS mediates remodelling of cell envelope architecture. SH1000, its *nsaS* mutant and the complemented strain were grown in TSB for thin section (3 h cultures) and negative stain (6 h cultures) analyses. Additionally, thin section images were captured for strains grown in TSB containing 10% NaCl (6 h). WT, Wild-type; M, *nsaS* mutant; C, *nsaS* mutant strain complemented *in trans* within *nsaRS*. The images presented are representative of more than 10 frames from three independent cultures. Bars, 0.2  $\mu$ m.



by ourselves and others, would be counterproductive to biofilm formation (Beenken *et al.*, 2010; Boles & Horswill, 2008; Tsang *et al.*, 2008). Additionally, the *nsaS* mutant has reduced expression of a number of anaerobiosis genes, which should also negatively impact biofilm formation (Beenken *et al.*, 2004). Finally, the synthesis of the surface proteins SasG and surface protein A, which have previously been shown to be required for biofilm formation, are also reduced in the mutant (Corrigan *et al.*, 2007; Merino *et al.*, 2009). Analysis of the SH1000 *nsaS* mutant for biofilm formation (Beenken *et al.*, 2003) revealed a 3.7-fold reduction for the mutant strain compared with the wild-type (Fig. 5). This phenotype was reversed when the *nsaRS* locus was introduced into the mutant strain *in trans*. Given the importance of biofilm formation to *S. aureus* disease progression, we assessed whether this phenotype was reproducible in other strains. Accordingly, we tested *nsaS* mutants in strain Newman and a community-acquired MRSA USA300 isolate. Again, in each case we were able to demonstrate a decrease in biofilm formation (Newman, 2.7-fold; USA300, 3.9-fold) for *nsaS* mutant strains, which was restored to wild-type levels upon complementation. Thus, it would appear that NsaRS contributes to biofilm formation across diverse and clonally distinct *S. aureus* strains.

#### NsaRS functions in protecting *S. aureus* cells during interaction with components of the innate immune system

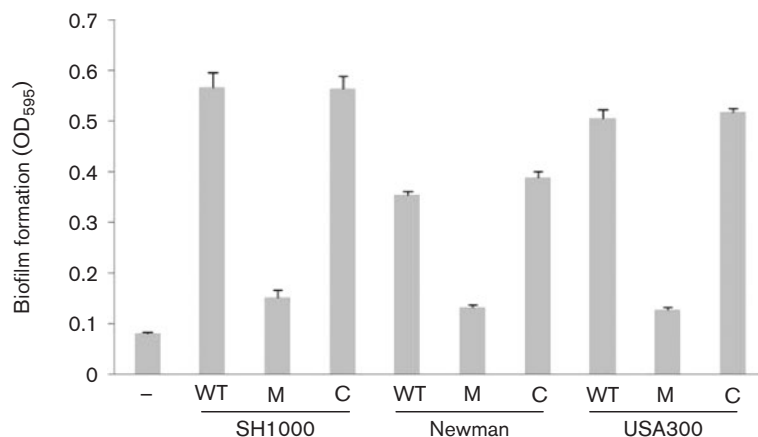
In order to determine the impact of NsaRS on *S. aureus* virulence, we examined interaction of the *nsaS* mutant with components of the innate immune system. This was performed using a human macrophage model of survival and clearance previously described by us (Koziel *et al.*, 2009; Kubica *et al.*, 2008). As such, the Newman wild-type and *nsaS* mutant were challenged with human monocyte-derived macrophages (hMdMs) at an m.o.i. of 1:50, and monitored for up to 5 days. Our use of Newman in these studies rather than SH1000, as in other studies, stems from the observation that SH1000 produces variable and inconsistent results in this model, whilst our experience

reveals that Newman generates far more reproducible data (Koziel *et al.*, 2009; Kubica *et al.*, 2008). As such, we observed a consistent and significant reduction in the number of *nsaS* mutant cells recovered from infected hMdMs over the course of the infection period (Fig. 6a). Specifically, we observed a 25-fold reduction in mutant cells after 24 h compared with the parental strain ( $P=0.060$ ). Furthermore, by the second day post-infection we observed a further 70-fold reduction in *nsaS* mutant cells ( $P=0.036$ ), and by the third day we were unable to recover any cells from *nsaS*-infected hMdM cells, whilst the parental strain still returned 100–1000 c.f.u. ml<sup>-1</sup> at this time.

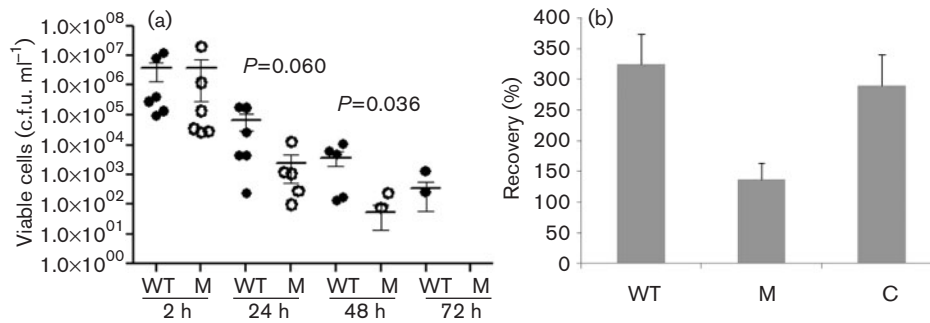
Further to this, we also used a whole human blood survival assay to examine the pathogenic fitness of the *nsaS* mutant. Accordingly, SH1000, its *nsaS* mutant and complemented strain were grown to exponential phase in TSB, before being inoculated into whole human blood. Cells were incubated for 3 h, and the survivability of each strain was determined and converted to per cent survival of the inoculum. An analysis with two different blood samples and five different replicates revealed that the wild-type and complemented strains not only were able to survive incubation in whole human blood but also continued to grow. Specifically, we were able to recover  $324 \pm 43.29\%$  (SEM) and  $290 \pm 31.29\%$  of the wild-type and complemented strain inocula, respectively, after 3 h incubation (Fig. 6b). In contrast, the *nsaS* mutant demonstrated a 2.4-fold decrease in bacterial load compared with the parental strain, returning only  $136 \pm 48.88\%$  of the inoculum upon incubation. Thus it would appear that the *nsaS* mutant strain is not only more rapidly cleared by hMdMs than the wild-type but also impaired in its ability to proliferate in human blood. As such, the NsaRS system seemingly has a significant role during interaction with components of the human innate immune system.

## DISCUSSION

In this study, we present a characterization of the TCS NsaRS from *S. aureus*. This TCS belongs to the IM-HK



**Fig. 5.** The NsaRS TCS contributes to biofilm formation in *S. aureus*. *nsaS* mutants in the SH1000, Newman and USA300 background were assayed for biofilm formation. WT, Wild-type; M, *nsaS* mutant; C, *nsaS* mutant strain complemented *in trans* within *nsaRS*. The negative control (-) was the non-biofilm-forming strain 8325-4. Data presented are from at least five independent cultures. Error bars, SEM.



**Fig. 6.** NsaRS plays an important role during the interaction of *S. aureus* with components of the human innate immune system. (a) Phagocytosis assays were carried out for the Newman strain (●) and *nsaS* mutant (○) at an m.o.i. of 1 : 50 (hMdmS). After challenge, cultures were maintained for the desired time before infected cells were lysed by treatment with ice-cold water. Lysates were serially diluted to determine the presence of viable staphylococci remaining within cells. The data presented were generated from six independent experiments and analysed for statistical significance using Student's *t* test. (b) Per cent recovery of SH1000 (WT), its *nsaS* mutant (M) and complemented strain (C) in whole human blood. Data presented are from two separate blood samples and five individual replicates; error bars, SEM.

family of regulators which commonly modulate cell envelope stability by sensing disruption of the cell wall and membrane (Joseph *et al.*, 2002; Mascher, 2006; Staroń *et al.*, 2011). We present evidence here that NsaRS fulfils a similar function in *S. aureus*, responding to cell envelope damage mediated by a variety of antimicrobial agents. A recent study demonstrated that development of spontaneous resistance to nisin in *S. aureus* is primarily mediated by NsaS (Blake *et al.*, 2011). Nisin is a polycyclic antibacterial peptide produced by *Lactococcus lactis* and is particularly effective against other Gram-positive bacteria. It functions by binding to the carbohydrate–phosphate moiety of the cell wall biosynthesis component lipid II (de Kruijff *et al.*, 2008; Schneider & Sahl, 2010). By binding and sequestering lipid II, nisin blocks cell wall biosynthesis and can lead to delocalization of biosynthetic components and aberrant septum formation (Hasper *et al.*, 2006; Hyde *et al.*, 2006). Additionally, nisin is also believed to use this docking event with lipid II to engineer pore formation in the membranes of target cells. In this study we provide corroborative evidence for the observations of Blake *et al.* (2011) by demonstrating that nisin results in elevated *nsaRS* expression and upregulation of the downstream NsaAB transporter, which is, at least in part, dependent on NsaRS.

Interestingly, in this study we demonstrate that a number of other antimicrobial agents that target the cell wall also upregulate *nsaRS* expression. Specifically, both phosphomycin and D-cycloserine, which affect enzymes involved in intracellular peptidoglycan assembly, upregulate expression of *nsaRS*. The observation with regard to phosphomycin supports that of a previous study, which demonstrates that both *nsaR* and *nsaS* are upregulated in *S. aureus* following phosphomycin challenge (Petek *et al.*, 2010). Furthermore, microarray analysis in this study reveals that NsaRS is responsible for regulation of another TCS, *yhcSR*. Previous

studies with cells depleted for *yhcSR* reveal elevated sensitivity to this antibiotic (Sun *et al.*, 2005), suggesting interplay between these two systems when sensing phosphomycin-mediated damage. Further to this, using a plate-based screening assay, we demonstrate that ampicillin and penicillin G, both of which are involved in extracellular peptidoglycan cross-linking, results in increased *nsaRS* expression. This information, in the context of our finding that *nsaS* mutants have an almost 200-fold decreased capacity for spontaneous bacitracin resistance, which blocks dephosphorylation and recycling of undecaprenyl-pyrophosphate, suggests a major role for NsaRS in sensing and responding to alterations in the cell wall. Indeed this latter finding corroborates a recent study which demonstrates that an NsaS mutant of MW2 has a fourfold increase in sensitivity to bacitracin (Matsuo *et al.*, 2010).

Thus it appears that NsaRS has a role in sensing and responding to perturbation of the cell wall biosynthesis pathway at each step in the cycle, from early, intracellular peptidoglycan subunit assembly (phosphomycin and D-cycloserine), to the transport and anchoring of these moieties via lipid II (nisin), followed by extracellular cross-linking of the peptidoglycan subunits (ampicillin and penicillin G), and finally re-entry of the bactoprenol carrier to continue the cycle (bacitracin). Interestingly, *nsaRS* does not appear to respond to every cell-wall-targeting antibiotic, as we observe no upregulation of *nsaRS* or sensitivity of the *nsaS* mutant to other drugs, including vancomycin. Additionally, we find no upregulation of *nsaRS* by, or sensitivity of the *nsaS* mutant to, a variety of antimicrobial peptides (data not shown). We do, however, show that certain cell-membrane-damaging agents, such as gramicidin, nisin and CCCP, upregulate expression of *nsaRS*. Furthermore, a previous study by Muthaiyan *et al.* (2008) reveals upregulation of *nsaR* in response to daptomycin. This indicates that this system responds to

perturbation of not only the cell wall but also the cell membrane. Thus, it appears that NsaRS has a general role in sensing and responding to disruption of the cell envelope in *S. aureus*. The fact that it does not respond to every cell-envelope-targeting compound is consistent with the literature from other IM-HK studies (Staroń *et al.*, 2011) and is perhaps explained in *S. aureus* by a number of other cell membrane or cell wall stress-responding TCSs, such as GraRS, VraRS and WalKR (Li *et al.*, 2007; Kraus *et al.*, 2008; Pietiäinen *et al.*, 2009; Delaune *et al.*, 2011).

In addition to findings focused on cell-envelope-targeting antibiotics, we also present electron microscopy data revealing that *nsaS* mutants have a more diffuse and less defined/smooth cell wall. This disparity from the parental and complemented strains is even further enhanced when cells are subject to salt stress, with *nsaS* mutant cells displaying striking and irregular cell wall appearances. This is perhaps explained by the observation that transcription of a number of osmoprotectants (*cudT* and *opuCA*) is repressed in the *nsaS* mutant, along with a number of solute importers and general permeases. These alterations in cell wall structure can perhaps be further explained by diminished expression in the *nsaS* mutant strain of genes previously shown to function in cell wall biosynthesis (e.g. *tagB*, *fmhA* or *scdA*). Additionally, we observed decreased expression of *glpQ* in the *nsaS* mutant, which is part of the glycerol utilization pathway (Nilsson *et al.*, 1994). Analysis of *S. aureus* cells that are deprived of their ability to utilize glycerol reveals major remodelling of the cell envelope and an accumulation of fatty acids in the cell resulting from a failure to incorporate them into the phospholipid membrane (Ray *et al.*, 1972). Pilot metabolic profiling conducted in our laboratory reveals that a large number of saturated fatty acids do indeed accumulate in *nsaS* mutant cells (S. L. Kolar and L. N. Shaw, unpublished observation).

Further electron microscopy studies using negative staining techniques reveal a significant alteration in encapsulation of the *nsaS* mutant compared with the wild-type and complemented strains. During growth in TSB and high salt concentrations (data not shown) we observed in the mutant strain a significantly increased association of cells in what appears to be an extracellular capsule. This is perhaps explained by microarray analysis, which reveals that *nsaS* mutant cells demonstrate a greater than twofold increase in transcription of seven of the ten capsule-encoding genes. In addition to this, we also noted a number of other genes in the transcriptome that have functions connected with cell envelope formation and architecture. These include membrane proteins, transporters, cell-wall-anchoring proteins and cell-wall-associated proteins. Thus, it is clear that, in addition to its role in sensing perturbation of the cell wall and membrane, NsaRS has a significant effect on remodelling cell envelope architecture during growth of *S. aureus*. This is perhaps not surprising given that many other IM-HK TCSs, including the NsaRS orthologue GraRS, have similar roles (Joseph *et al.*, 2002; Mascher, 2006; Li *et al.*, 2007).

In addition to this, and perhaps equally intriguing, is the number of elements identified in the transcriptome that have a role in transport. We identified that 55 such genes have a known or implied role in the import/export of sugars, metal ions, amino acids and antimicrobial compounds. Given this, it is perhaps not surprising that we demonstrate a role for NsaRS in the process of cellular import. When grown during metal-ion-limiting conditions, the levels of intracellular divalent metal ions in the *nsaS* mutant were significantly reduced. This is in contrast with our finding that the same strain grown in replete media has no such defect. As we have shown that there are alterations in the cell surface of the *nsaS* mutant, it is possible that these findings result from a decreased association of metal ions with the surface of mutant cells. However, this is perhaps unlikely given that under metal replete conditions we do not observe similar decreases in internal metal concentrations. Thus it would appear that NsaRS mediates internal metal ion pools, not during conditions of abundance but more specifically during times of scarcity. As such, *S. aureus* cells may utilize NsaRS as a modulator of low abundance metal ion import during times of starvation. It could be speculated that this would be of particular importance during life within the host, which is known to be an environment of famine for metal ion availability. Indeed, such a mechanism might perhaps contribute to the diminished virulence phenotype we observed during *ex vivo* testing with the *nsaS* mutant.

Of interest from the microarray studies was the observation that other TCSs are controlled by NsaRS. The positive regulation of SACOL0201/0202 by NsaRS is intriguing because, whilst this regulatory element has not formally been studied thus far, it has been suggested to play a role in the anaerobic response of *S. aureus* (Fuchs *et al.*, 2007). As such, the finding that a number of components involved in anaerobiosis have reduced expression in the *nsaS* mutant strain (such as *idhA*, *idh2*, acetoin reductase, *fdhD* and *aldI*) is reasonable, and could perhaps be mediated, at least in part, via control of SACOL0201/0202 by NsaRS. Further to this, we also identified decreased expression of the anaerobiosis repressor Rex in the *nsaS* mutant. This finding is somewhat at odds with our other data, as it could be predicted that a strain displaying reduced Rex expression would also demonstrate increased transcription of anaerobically involved loci (Fuchs *et al.*, 2007; Pagels *et al.*, 2010). However, it is tempting to speculate that SACOL0201/0202 and Rex have antagonizing roles in the process of controlling the anaerobic response, and that NsaRS mediates their interaction via regulatory control. It is also equally possible that NsaRS participates in upregulation of the anaerobic response directly, thus proving dominant to the inhibitory capacity of Rex.

We demonstrate that *nsaS* mutants are impaired in their ability to form biofilms across a variety of *S. aureus* strains. There are a number of regulatory elements that have been suggested as having roles in this process in *S. aureus*, including other TCSs and global regulatory loci such as *agr*,

*sarA* and  $\sigma^B$  (Beenken *et al.*, 2004). Recent work conducted by ourselves and others (Beenken *et al.*, 2010; Boles & Horswill, 2008; Tsang *et al.*, 2008) has focused not on these regulatory elements but rather on the physiological conditions that facilitate or prevent this aggregate lifestyle. As such, when the NsaRS regulon is analysed, it is perhaps no surprise that a mutant in this system would be impaired in forming and/or maintaining a biofilm. Specifically, the expression of a variety of extracellular proteases (including the V8 protease, staphopain B and a number of Spls), all of which have an important role in the breakdown of extracellular components required for biofilm formation, are increased in the *nsaS* mutant (Beenken *et al.*, 2010; Boles & Horswill, 2008; Tsang *et al.*, 2008). Further to this, the *nsaS* mutant has reduced expression of a number of anaerobiosis genes, which would again be counterproductive to this process (Beenken *et al.*, 2004). Finally, the *nsaS* mutant has diminished synthesis of the surface proteins SasG and surface protein A, which have been shown to be required for biofilm formation (Corrigan *et al.*, 2007; Merino *et al.*, 2009). Collectively, these observations probably explain the role of *nsaRS* in biofilm formation, a phenotype which is broadly maintained across a number of distinct *S. aureus* lineages.

Finally, we present data evaluating the role of *nsaRS* in the virulence of *S. aureus*. Using a human model of macrophage clearance and survival we observed a 25-fold reduction of mutant cells after 24 h, a 70-fold reduction after 2 days and a lack of mutant cells after this time. Thus it would appear the *nsaS* mutant is more rapidly cleared than the wild-type upon interaction with human macrophages. Additionally, when performing survival experiments using whole human blood, we observed a reduction in the capacity of the *nsaS* mutant to proliferate. Whilst the wild-type and complemented strains are able to survive and rapidly proliferate upon challenge, *nsaS* mutant growth seemingly stalls when faced with these hostile conditions. Human blood is replete with a variety of antimicrobial agents, including leukocytes, antimicrobial peptides/proteins and factors of the complement system (Levy, 2000). As such, whole blood survival assays corroborate the *ex vivo* macrophage survival and clearance data, and suggest an important role for NsaRS in mediating resistance of *S. aureus* to the human immune system. Additionally, transcriptome analysis reveals that the expression of a number of elements known to be involved in virulence (such as *essABC*, *sarS*, *spa*, *srtB*, *htrA*, *clpB* and *isaB*) is decreased in the *nsaS* mutant. Conversely a number of known virulence factors were also elevated in the transcriptome (such as the V8 protease, staphopain B, FnpB, Hlb, HysA). It is entirely possible that these increases in transcription present a situation akin to that of a *sarA* mutant, which overexpresses extracellular proteases and has attenuated virulence. Accordingly, this imbalance of virulence determinant expression would likely destabilize the pathogenic response of *nsaS* mutant cells and perhaps explains the phenotypes observed.

In summary, we present a characterization of the IM-HK family member and GraRS orthologue, NsaRS. NsaRS appears to have parallel roles to its better studied counterpart in a number of processes, including biofilm formation and impaired interaction with components of the innate immune system (Li *et al.*, 2007; Kraus *et al.*, 2008; Boles *et al.*, 2010; Cheung *et al.*, 2010). This is probably explained, in part, by the relatedness of these two systems, and the observation that *nsaR* has previously been shown to be subject to twofold repression by GraRS (Herbert *et al.*, 2007). Additionally, we demonstrate a number of unique NsaRS phenotypes, indicating its independent contribution to, and role in, sensing and adapting to cell-envelope-mediated stress. We also show that the NsaRS regulatory system appears to have an important role in the transport of metal ions, and is required for full biofilm formation across a number of genetically diverse *S. aureus* strains. Finally, the defects we present for the *nsaS* mutant when interacting with components of the innate immune system strongly argue for the importance of this TCS in the *in vivo* lifestyle of *S. aureus*.

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