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The membrane protein PrsS mimics σ^S in protecting *Staphylococcus aureus* against cell wall-targeting antibiotics and DNA-damaging agents

Christina N. Krute, Harris Bell-Temin, Halie K. Miller, Frances E. Rivera, Andy Weiss, Stanley M. Stevens and Lindsey N. Shaw

Department of Cell Biology, Microbiology & Molecular Biology, University of South Florida, Tampa, FL, USA

Correspondence
Lindsey N. Shaw
shaw@usf.edu

Staphylococcus aureus possesses a lone extracytoplasmic function (ECF) sigma factor, σ^S. In *Bacillus subtilis*, the ECF sigma factor, σ^W, is activated through a proteolytic cascade that begins with cleavage of the RsiW anti-sigma factor by a site-1 protease (S1P), PrsW. We have identified a PrsW homologue in *S. aureus* (termed PrsS) and explored its role in σ^S regulation. Herein, we demonstrate that although a cognate σ^S anti-sigma factor currently remains elusive, *prsS* phenocopies *sigS* in a wealth of regards. Specifically, *prsS* expression mimics the upregulation observed for *sigS* in response to DNA-damaging agents, cell wall-targeting antibiotics and during *ex vivo* growth in human serum and murine macrophages. *prsS* mutants also display the same sensitivities of *sigS* mutants to the DNA-damaging agents methyl methane sulfonate (MMS) and hydrogen peroxide, and the cell wall-targeting antibiotics ampicillin, bacitracin and penicillin-G. These phenotypes appear to be explained by alterations in abundance of proteins involved in drug resistance (Pbp2a, FemB, HmrA) and the response to DNA damage (BmrA, Hpt, Tag). Our findings seem to be mediated by putative proteolytic activity of PrsS, as site-directed mutagenesis of predicted catalytic residues fails to rescue the sensitivity of the mutant to H_2O_2 and MMS. Finally, a role for PrsS in *S. aureus* virulence was identified using human and murine models of infection. Collectively, our data indicate that PrsS and σ^S function in a similar manner, and perhaps mediate virulence and resistance to DNA damage and cell wall-targeting antibiotics, via a common pathway.

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INTRODUCTION

Staphylococcus aureus is an opportunistic pathogen that colonizes up to 50 % of the human population (Diekema *et al.*, 2001). In addition to its lifestyle as a commensal, *S. aureus* is able to exist in almost every niche of the human body, causing diseases ranging from mild skin infections to life-threatening conditions. As such, *S. aureus* causes more morbidity and mortality than any other infectious agent and leads to more annual deaths than HIV/AIDS in the United States (Tonks, 2007; Klevens *et al.*, 2006, 2007; Moran *et al.*, 2006).

S. aureus is such a successful pathogen due to its many virulence determinants and the way they are regulated in order to establish and maintain infection. These virulence determinants include alternative sigma factors, of which *S. aureus* possesses three: σ^B, σ^H and σ^S (Shaw *et al.*, 2008; Tao *et al.*, 2010; Wu *et al.*, 1996). This latter component was discovered by our group, and is a member of the extracytoplasmic function (ECF) sigma factor family, which has been implicated in the response to cell envelope stress, DNA damage resistance and the progression of infection (Miller *et al.*, 2012; Shaw *et al.*, 2008).

ECF sigma factors are typically activated in response to numerous environmental conditions via regulated intramembrane proteolysis (RIP), which enables the dissociation of a sigma factor from its cognate anti-sigma factor (Brown & Hughes, 1995; Brown *et al.*, 2000; Flynn *et al.*, 2004; Helmann, 2002). RIP is particularly well characterized for σ^W of *B. subtilis*, which is involved in the response to cell envelope damage (Ellermeier & Losick, 2006; Heinrich & Wiegert, 2006; Helmann, 2006; Kunst *et al.*, 1997; Pietiäinen *et al.*, 2005). Upon sensing antimicrobial peptide stress or alkaline shock, σ^W is released from its cognate anti-sigma factor, RsiW, by sequential cleavage; first by the site-1 protease (S1P), PrsW, followed by RasP-mediated site-2 proteolysis (Ellermeier & Losick, 2006;

Abbreviations: ECF, extracytoplasmic function; RIP, regulated intramembrane proteolysis; Y2H, yeast two-hybrid.

Seven supplementary figures and five supplementary tables are available with the online Supplementary Material.

Heinrich & Wiegert, 2006; Schöbel *et al.*, 2004). Newly released σ^W subsequently impacts the transcription of more than 60 genes involved in maintaining cell envelope integrity (Cao *et al.*, 2002).

The role of PrsW has also recently been studied in *Clostridium difficile* (Ho & Ellermeier, 2011). This Gram-positive opportunistic pathogen has three ECF sigma factors, CsfT, CsfU and CsfV, along with cognate anti-sigma factors RsiT, RsiU and RsiV, respectively. CsfT is upregulated by the cell envelope-targeting agents bacitracin and lysozyme. The activation of CsfT is induced by RIP, whereby PrsW cleaves RsiT. This process was shown to be important in *C. difficile* virulence, as *prsW* mutants have a decreased ability to colonize the gastrointestinal tract of hamsters.

For *S. aureus*, a clear RasP/RseP homologue is present in the MEROPS protease database (http://merops.sanger.ac.uk/); however, no counterpart for PrsW has been annotated. In this work, we have investigated the function of a PrsW homologue in *S. aureus* (SAUSA300_0230), designated 'PrsS' for 'putative regulator of SigmaS'.

METHODS

Bacterial strains, plasmids and growth conditions. *S. aureus* and *Escherichia coli* strains used in this study are listed in Table 1. Strains were grown as previously described, unless otherwise indicated (Shaw *et al.*, 2008).

Construction of *lacZ* reporter fusion strains. In order to construct a *prsS-lacZ* reporter fusion strain, the *prsS* promoter region was amplified using primers OL888/OL887 (Table S1, available in the online Supplementary Material, for all oligonucleotides). This PCR product was cloned into the Gram-positive suicide vector pAZ106 (Kemp *et al.*, 1991; Salisbury *et al.*, 1972), creating pCNK622. *S. aureus* RN4220 was electroporated with pCNK622 as described previously (Shaw *et al.*, 2008), and the recombination event confirmed using gene-specific primer OL888 and plasmid-specific primer OL761, creating strain CNK622. This verified clone was used to generate a phage lysate to transduce strains 8325-4, SH1000 (Horsburgh *et al.*, 2002), Newman and USA300 (Kolar *et al.*, 2011) via Φ11-mediated transduction (Shaw *et al.*, 2008). The resulting strains CNK955, CNK661, CNK876 and CNK875, respectively, were confirmed by PCR using primers OL761/OL887. *sigS-lacZ* and *ctpA-lacZ* fusion strains were previously reported (Burda *et al.*, 2014; Carroll *et al.*, 2014).

Construction of mutant *sigS-lacZ* reporter fusion strains. To create *sigS-lacZ* reporter-fusions containing individual mutations of uncharacterized membrane-bound proteins, Φ11 lysates were generated using Network on Antimicrobial Resistance in *Staphylococcus aureus* (NARSA) transposon mutants NE1203 (SAUSA300_1495), NE1644 (SAUSA300_1788), NE1783 (SAUSA300_1684) and NE1942 (SAUSA300_0014) (Fey *et al.*, 2013). Strain CNK957 was transduced with individual Φ11 lysates, creating strains CNK1784, CNK1785, CNK1786 and CNK1787, respectively. Clones were confirmed as previously described (Bae *et al.*, 2004).

Construction of mutant strains. A *prsS* mutant (NE166) obtained from the NARSA transposon mutant library (Fey *et al.*, 2013) was used to generate a Φ11 lysate to transduce USA300. The resulting strain CNK1460 was confirmed by PCR using OL888, located upstream of *prsS*, and transposon-specific primer OL1472. A USA300 *sigS* mutant strain was constructed via Φ11-mediated transduction using a lysate from a previously constructed SH1000 *sigS* mutant (Shaw *et al.*, 2008). The resulting strain (HKM852) was confirmed by PCR using OL281, located upstream of *sigS*, and OL429, located downstream of *sigS*. A USA300 HOU *prsS sigS* double mutant was constructed via Φ11-transduction of CNK1460 using a lysate generated from the previously constructed SH1000 *sigS* mutant (Shaw *et al.*, 2008). The resulting strain (CNK1870) was confirmed as described above by PCR. An *rpoE* mutant was previously constructed (Weiss *et al.*, 2014).

Construction of *prsS* complement strains. A *prsS* complement strain was constructed by amplifying the *prsS* promoter and coding region using primers OL888/OL1469, and cloning this product into the Gram-positive shuttle vector pMK4 via transformation of DC10B (Monk *et al.*, 2012; Sullivan *et al.*, 1984), creating pCNK1461. *S. aureus* RN4220 was electroporated with pCNK1461, with clones verified using gene-specific primer OL888 and plasmid-specific primer OL1057, creating strain CNK1462. Clones were used to generate a Φ11 lysate for the transduction of a USA300 HOU *prsS* mutant. The resulting strain (CNK1467) was confirmed by PCR using primers OL888/OL1057. A *prsS* site-directed-mutant complement was constructed by performing targeting E215A/E216A mutagenesis in the *prsS* coding region. This was achieved by splicing by overhang extension (SOEing) PCR using primers OL888/OL2138 and OL2137/OL1469, which contained mutated nucleotide sequences. These products were cloned into pMK4, and verified by sequencing (MWG Operon), creating pCNK1871. *S. aureus* RN4220 was electroporated with this construct, with clones verified by PCR, creating strain CNK1872. Clones were used to generate a lysate for the transduction of a USA300 *prsS* mutant via Φ11, creating strain CNK1873.

5′-rapid amplification of cDNA ends (5′-RACE). 5′-rapid amplification of cDNA ends (5′-RACE) was performed as described previously (Carroll *et al.*, 2012) using RNA extracted during exponential growth (3 h) of USA300 WT. RNA extraction was performed using a Qiagen RNeasy kit, and 5′-RACE was performed using a Takara 5′-RACE kit, and primers OL968-72, according to the manufacturer's protocol. Amplified cDNA products were cloned into the pSC-A TA cloning vector, and 11 constructs were analysed by sequencing (MWG Operon).

β-Galactosidase assays. β-Galactosidase assays on tryptic soy agar (TSA) plates and in tryptic soy broth (TSB), RAW 264.7 murine macrophage-like cells, human serum and porcine serum were performed as previously described (Carroll *et al.*, 2012; Miller *et al.*, 2012; Shaw *et al.*, 2008). Minimal levels of native β-galactosidase activity were subtracted when calculating β-galactosidase activity in all assays.

MICs of cell wall-targeting antibiotics. The MICs of cell wall-targeting antibiotics were determined as previously described (Burda *et al.*, 2012).

Methyl methane sulfonate (MMS) and hydrogen peroxide (H_2O_2) survival assays. Survival assays were performed as previously described (Miller *et al.*, 2012). Briefly, exponentially growing USA300 WT, *prsS* mutant and *prsS* complement strains were washed three times with PBS. Cells were resuspended in PBS and MMS or H_2O_2 was added to a final concentration of 50 mM or 1.3 M, respectively. Cultures were incubated shaking at 37 °C, and the percent recovery determined by comparing pre-exposure c.f.u. ml^{-1} to final c.f.u. ml^{-1} after 30 min incubation with MMS, or 5 min incubation with H_2O_2. Data are presented from three independent experiments.

Hybrigenics ULTImate yeast two-hybrid (Y2H) screen. A Y2H screen was performed by Hybrigenics as described previously to identify an anti-sigma factor in *Helicobacter pylori* (Colland *et al.*,

Table 1. Strains and plasmids used in this study

Strain or plasmid	Genotype or description	Reference or source
E. coli		
DH5α	Cloning strain	Salisbury *et al.* (1972)
DC10B	Cloning strain	Monk *et al.* (2012)
S. aureus		
RN4220	Restriction-deficient transformation recipient	Lab stocks
8325-4	WT laboratory strain, *rsbU*$^-$	Lab stocks
SH1000	WT laboratory strain, *rsbU*$^+$	Horsburgh *et al.* (2002)
Newman	WT laboratory strain, human clinical isolate	Lab stocks
USA300	USA300-HOU MRSA isolate cured of pUSA300-HOU-MRSA	Kolar *et al.* (2011)
CNK622	RN4220 pAZ106 : : *prsS-lacZ*, *prsS*$^+$	This study
CNK955	8325-4 pAZ106 : : *prsS-lacZ*, *prsS*$^+$	This study
CNK661	SH1000 pAZ106 : : *prsS-lacZ*, *prsS*$^+$	This study
CNK876	Newman pAZ106 : : *prsS-lacZ*, *prsS*$^+$	This study
CNK875	USA300 pAZ106 : : *prsS-lacZ*, *prsS*$^+$	This study
NE166	USA300 JE2 *prsS* : : *bursa aurealis*, *prsS*$^-$	NARSA
CNK1460	USA300 HOU *prsS* : : *bursa aurealis*, *prsS*$^-$	This study
CNK1462	RN4220 pMK4 : : *prsS*, *prsS*$^+$	This study
CNK1467	USA300 HOU *prsS* : : *bursa aurealis* pMK4 : : *prsS*, *prsS*$^+$	This study
CNK1872	RN4220 pMK4 : : *prsS*^{E215A,E216A}	This study
CNK1873	USA300 HOU *prsS* : : *bursa aurealis* pMK4 : : *prsS*^{E215A,E216A}	This study
HKM852	USA300 HOU *sigS* : : *tet*, *sigS*$^-$	This study
HKM779	RN4220 pSC-A : : *tet* : : *sigS-lacZ*, *sigS*$^+$	Burda *et al.* (2014)
CNK957	USA300 HOU pSC-A : : *tet* : : *sigS-lacZ*, *sigS*$^+$	This study
CNK1870	USA300 HOU *prsS* : : *bursa aurealis sigS* : : *tet*, *prsS*$^-$, *sigS*-	This study
NE1203	USA300 JE2 SAUSA300_1495 : : *bursa aurealis*, SAUSA300_1495$^-$	NARSA
NE1644	USA300 JE2 SAUSA300_1788 : : *bursa aurealis*, SAUSA300_1788$^-$	NARSA
NE1783	USA300 JE2 SAUSA300_1684 : : *bursa aurealis*, SAUSA300_1684$^-$	NARSA
NE1942	USA300 JE2 SAUSA300_0014 : : *bursa aurealis*, SAUSA300_0014$^-$	NARSA
CNK1784	USA300 HOU pSC-A : : *tet* : : *sigS-lacZ*, *sigS*$^+$, SAUSA300_1495 : : *bursa aurealis*, SAUSA300_1495$^-$	This study
CNK1785	USA300 HOU pSC-A : : *tet* : : *sigS-lacZ*, *sigS*$^+$, SAUSA300_1788 : : *bursa aurealis*, SAUSA300_1788$^-$	This study
CNK1786	USA300 HOU pSC-A : : *tet* : : *sigS-lacZ*, *sigS*$^+$, SAUSA300_1684 : : *bursa aurealis*, SAUSA300_1684$^-$	This study
CNK1787	USA300 HOU pSC-A : : *tet* : : *sigS-lacZ*, *sigS*$^+$, SAUSA300_0014 : : *bursa aurealis*, SAUSA300_0014$^-$	This study
LNS1788	USA300 pAZ106 : : *ctpA-lacZ*, *ctpA*$^+$	Carroll *et al.* (2014)
JAI1287	USA300 HOU *rpoE* : : *bursa aurealis*, *rpoE* $^-$	Weiss *et al.* (2014)
Plasmid		
pAZ106	Promoterless *lacZ* suicide vector	Kemp *et al.* (1991)
pMK4	Gram-positive shuttle vector	Sullivan *et al.* (1984)
pCNK622	pAZ106 containing a 1.1 kb fragment of the *prsS* promoter	This study
pCNK1461	pMK4 containing a 1.7 kb fragment with the *prsS* promoter and coding region	This study
pCNK1871	pMK4 containing a 1.7 kb fragment with the *prsS* promoter and *prsS*^{E215A,E216A} mutations	This study
pSC-A	TA clone suicide vector	StrataClone

2001). We used a bait plasmid containing the coding region of *sigS* and an *S. aureus* prey library with over two million clones. Putative interacting proteins were assigned a 'predicted biological score' as an indicator of bait–prey interaction specificity, which was generated as an e-value dependent on the number of hits for a specific protein domain and data accumulated from previously performed Hybrigenics screens (Rain *et al.*, 2001). The predicted biological score ranges from A, indicating the highest probability of specificity, to E, indicating the lowest specificity between two proteins.

Proteomic analyses. Triplicate USA300 WT or *prsS* mutant strains were grown to post-exponential (5 h) or stationary phase (15 h) in TSB. To isolate membrane proteins, cultures were washed three times with PBS, and protoplasts generated by incubating cells with 100 μg of lysostaphin at 37 °C for 30 min. Membrane proteins were isolated from protoplasts as described previously (Eymann *et al.*, 2004). Cytoplasmic proteins were obtained by mechanical disruption of cells using 0.1 mm glass beads and a Biospec Mini BeadBeater-16. Membrane proteins were standardized to 200 μg, and cytoplasmic

proteins were standardized to 70 μg. Samples were then subjected to filter aided sample preparation and trypsin digestion, as described previously (Rivera *et al.*, 2012). The resulting peptides were de-salted and analysed by linear trap quadrupole tandem mass spectrometry (LTQ-MS/MS); and Mascot data files were interrogated and normalized using Scaffold software with parameters to allow a false protein discovery rate of <1 %. Ratios and fold changes of protein abundance were calculated as *prsS* mutant/WT spectral counts and significance was determined using Fisher's exact test ($P<0.05$).

Transmission electron microscopy (TEM). USA300 WT and *prsS* mutant cultures were grown to exponential phase, and prepared and photographed at the University of South Florida Microscopy Core Facility as previously described (Kolar *et al.*, 2011).

Quantitative real-time PCR (qPCR). qPCR was performed on USA300 WT, *prsS* mutant, and *sigS* mutant strains in triplicate as previously described (Kolar *et al.*, 2011) to analyse expression of *prsS*, *sigS*, *bmrA*, *ezrA*, *femB*, *hmrA*, *hpt* and *tag*. Control primers for 16S rRNA were used as previously described (Koprivnjak *et al.*, 2006).

Whole human blood survival assay. Human blood survival assays were performed as previously described (Kolar *et al.*, 2011). Briefly, exponentially growing USA300 WT, *prsS* mutant and complement strains were washed three times with PBS and resuspended in 1 ml of pooled whole human blood (Bioreclamation). Blood cultures were incubated with shaking at 37 °C for 3 h, and percent recovery determined by comparing initial c.f.u. ml^{-1} to final c.f.u. ml^{-1}. Data presented are from blood from five different people and three biological replicates.

Murine sepsis infection model. Murine sepsis infection models were performed using ten female 6-week-old CD-1 mice (Charles River) per strain, as described previously (Kolar *et al.*, 2013). Mice were infected via tail vein injection with 1×10^8 c.f.u. of USA300 WT or *prsS* mutant strains. After 7 days, mice were euthanized and brain, heart, lungs, liver, spleen and kidneys were harvested. C.f.u./organ loads were determined by homogenizing organs in PBS, serially diluting and plating on TSA. This study was performed in strict accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health. The protocol was approved by the Institutional Animal Care and Use Committee of the University of South Florida (Permit Number: A-4100-01).

Statistical analyses. All statistical analyses in this study were performed using SAS software (version 9.2; SAS Institute). The distribution of data was determined in SAS through tests for normality (*SAS proc univariate*) and equality of variance (*SAS proc ttest*). For parametrically distributed data, Student's *t*-test was used. The statistical significance of bacterial recovery from organs in the murine model of sepsis was evaluated using a Mann–Whitney Test. For all statistical analyses, the significance level was set at $\alpha=0.05$.

RESULTS

Identification of a PrsW homologue in *S. aureus*

A protein BLAST analysis of the *S. aureus* genome using the *B. subtilis* PrsW amino acid sequence identified SAUSA300_0230 as having 25 % identities and 47 % positives ($e=8\times10^{-8}$). The SAUSA300_0230 protein in *S. aureus* is 380 amino acids in length, which is longer than its homologue in *B. subtilis* at 218 amino acids and in *C. difficile* at 238 amino acids (Fig. S1). Alignment analysis reveals conservation of the proposed catalytic residues: E75, E76 and H175 in *B. subtilis*; E78, E79 and H184 in *C. difficile*; E215, E216 and H325 in *S. aureus* (Ellermeier & Losick, 2006; Heinrich & Wiegert, 2006). Interestingly, it appears that PrsW-like elements are also broadly conserved across a number of other bacterial species (Figs S2 and S3). For the purposes of this study, we termed the SAUSA300_0230 gene '*prsS*', for 'putative regulator of SigmaS'.

Yeast two-hybrid (Y2H) analysis to identify a σ^S anti-sigma factor

Given that S1P and S2P homologues are conserved in *S. aureus*, we sought to investigate whether a σ^S anti-sigma factor could be uncovered using a Y2H screen. Accordingly, we identified 13 proteins that putatively interact with σ^S including components of RNA polymerase and transcription factors, all of which are expected for a sigma factor (Table S2). Unexpectedly, however, no candidate anti-sigma factor was identified in this screen. As such, we set out to characterize the role and regulation of *prsS* in *S. aureus*, to assess whether it phenocopied our findings from studies with *sigS*.

Identification of the *prsS* transcriptional start site by 5′-RACE

In order to characterize the expression of *prsS* in *S. aureus*, we began by identifying its transcriptional start site. In total, 11 different 5′-RACE fragments were subject to Sanger sequencing, leading to the identification of one transcriptional start site at an adenine residue 115 bases 5′ of the *prsS* start codon. This putative promoter region comprises a −35 sequence of TgGAaA, followed by a 17 bp spacer, and −10 sequence of TAaAAT (lower case bases vary from σ^A consensus sequences). A putative Shine–Dalgarno sequence (aGAGG) is located 2 bp upstream of the ATG start codon. Collectively, these elements are potentially ideal for strong recognition by both σ^A and the ribosome.

prsS expression mirrors that of *sigS* and is upregulated by DNA damage and cell wall stress

We next sought to understand if *prsS* expression mirrored that of *sigS*. Accordingly, a *prsS-lacZ* reporter fusion in strains RN4220, 8325-4, SH1000, Newman and USA300 was analysed at hourly intervals. These experiments revealed that *prsS*, much like *sigS* (Miller *et al.*, 2012), is transcribed at low levels under standard laboratory conditions in all strains apart from RN4220, with an approximately 11.2-fold increase in RN4220, and peak expression occurring during exponential growth (Fig. 1a). To assess whether *prsS* expression, like *sigS*, is inducible in response to external stress, we employed a modified disk diffusion assay in conjunction with the USA300 *prsS-lacZ* reporter fusion strain and a library of stress chemicals. A variety of agents induced *prsS* expression, including those that elicit oxidative/DNA damage stress (ciprofloxacin, H_2O_2, MMS, peracetic acid and pyrogallol), as well as cell wall-targeting antibiotics (ampicillin, bacitracin, cefotaxime, oxacillin, penicillin-G, phosphomycin and

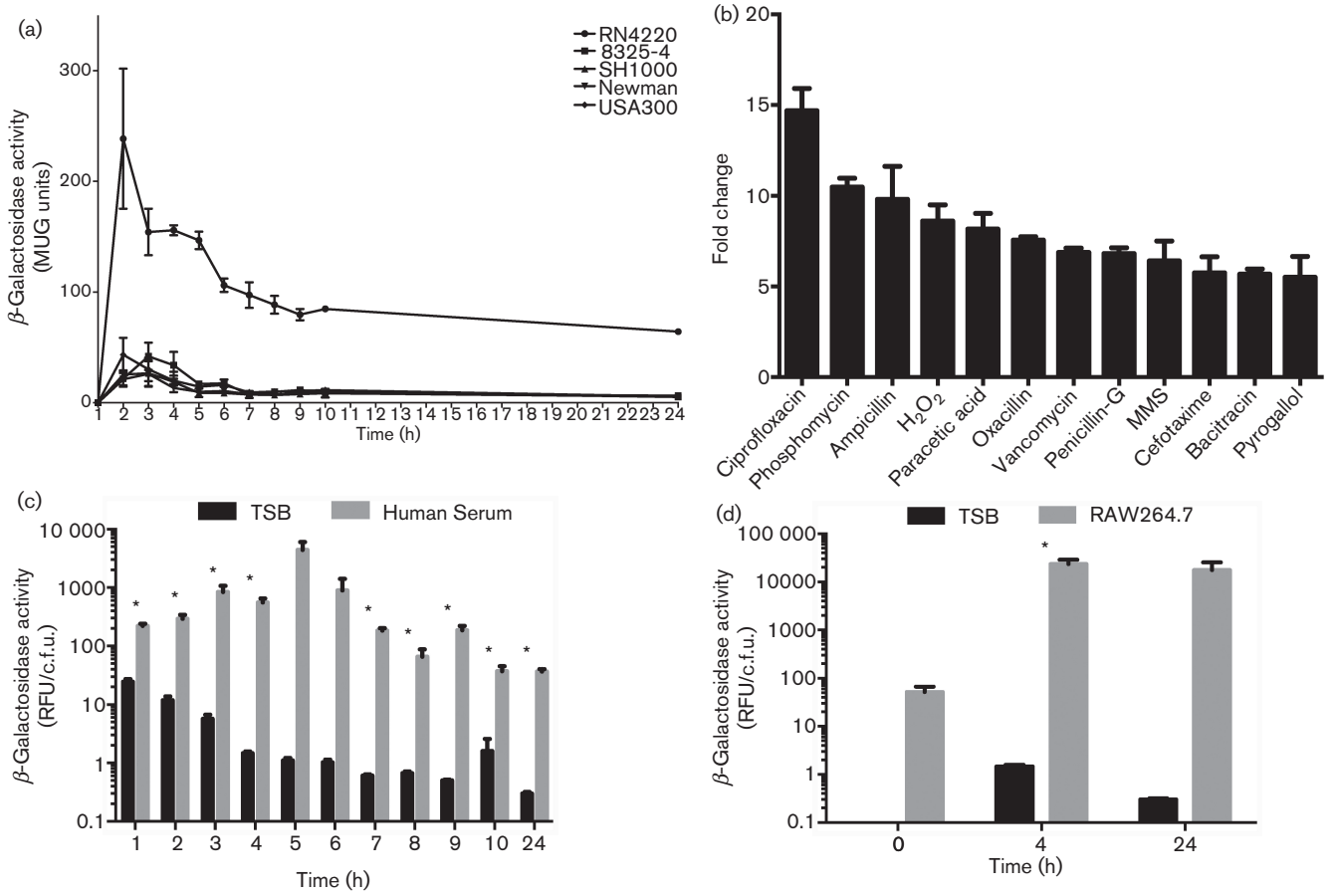

Fig. 1. Analysis of *prsS* transcription. β-galactosidase activity was measured as relative fluorescence units (RFU) of 4-methylumbelliferyl-β-D-galactopyranoside (MUG) activity for *prsS-lacZ* fusion strains in RN4220, 8325-4, SH1000, Newman and USA300 under standard conditions in TSB (a), in the presence of subinhibitory concentrations of the agents shown (b), in human serum (c) or in murine RAW 264.7 cells (d). Assays were performed on duplicate samples, and the values averaged (mean) from at least three independent experiments. Error bars are shown ± SEM; *$P<0.05$ using Student's *t*-test.

vancomycin). Most of these agents, including H_2O_2, MMS, ciprofloxacin, cefotaxime, ampicillin, oxacillin and phosphomycin, also elicit the same effects on *sigS* expression (Miller *et al.*, 2012). These findings were validated by β-galactosidase assays performed on USA300 *prsS-lacZ* cultures grown in TSB containing subinhibitory concentrations of these agents (Figs 1b, S4 and S5). As a control, to demonstrate that the effects observed are unique to *prsS* and *sigS*, we analysed expression of the membrane-associated protease, CtpA, using a *ctpA-lacZ* reporter fusion (Carroll *et al.*, 2014). We determined that no increases in expression were observed when this construct was tested in a similar fashion (data not shown). Collectively, these results mirror closely what we have previously shown for *sigS* expression (Miller *et al.*, 2012), and suggest that PrsS and σ^S may serve common functions in the *S. aureus* cell.

prsS expression is increased in human serum and murine macrophages

Previous studies have demonstrated that *sigS* expression is increased in both porcine serum and RAW 264.7 murine macrophage-like cells (Miller *et al.*, 2012). To examine if we observe similar alterations, the USA300 *prsS-lacZ* reporter fusion strain was incubated first in human serum, with expression measured every hour for 10 h and again at 24 h (Fig. 1c). We determined that *prsS* expression is increased in human serum compared to TSB at all hours, with the most significant increase (386-fold) occurring at 4 h. Similar increases in *prsS* expression were observed when this experiment was performed using *prsS-lacZ* fusions in RN4220, 8325-4 and SH1000 backgrounds (Fig. S6). Next, murine macrophages were infected with a USA300 *prsS-lacZ* reporter fusion strain, and *prsS* expression was measured at 4 h and 24 h post-infection (Fig. 1d). We observed increased *prsS* expression in RAW 264.7 cells compared to TSB at both time points post-infection, with a 16,180-fold increase at 4 h, and a 58,875-fold increase at 24 h. Similar increases in *prsS* expression were observed when these experiments were performed with fusions in RN4220, 8325-4 and SH1000 backgrounds (Fig. S7). Collectively, these data again closely mirror that which we have previously demonstrated for *sigS* (Miller *et al.*, 2012).

PrsS, like σ^S, protects *S. aureus* against killing by DNA-damaging and cell wall-targeting agents

Herein we show that *prsS* expression is induced by DNA-damaging inducing agents and cell wall-targeting antibiotics. Additionally, we have previously shown that *sigS* mutants are more sensitive to killing by these types of stress (Miller *et al.*, 2012). To investigate if, as with transcription, similar responses were observed between *sigS* and *prsS* mutants, we first exposed the *prsS* knockout to lethal concentrations of MMS (50 mM) and hydrogen peroxide (1.3 M). The mutant strain was found to be significantly more sensitive to killing by these types of stress (Fig. 2a). After incubation for 30 min with MMS, percent recovery of the WT strain was 2.7-fold greater than the *prsS* mutant. Likewise, *prsS* mutants show similar sensitivity to exposure to hydrogen peroxide; after 5 min, post-incubation percent recovery of the WT (7.9 %) is 2.4-fold higher than that of the *prsS* mutant (3.4 %) (Fig. 2b). Importantly, complementation of these phenotypes was achieved in both assays.

Previous studies on PrsW in *B. subtilis* have employed site-directed mutagenesis to confirm the proteolytic activity of this protein (Ellermeier & Losick, 2006; Heinrich & Wiegert, 2006). As such, we hypothesized that site-directed mutagenesis of the putative conserved catalytic amino acids (E215A, E216A; Fig. S1), would fail to rescue *S. aureus* from killing by MMS and H_2O_2 if PrsS also possesses protease activity. Accordingly, we complemented our *prsS* mutant with a *prsS* $^{E215A/E216A}$ variant carried on a plasmid. We then assessed the sensitivity of this strain to killing by both agents. Importantly, this complementation construct was unable to rescue sensitivity of the *prsS* mutant to either compound (Fig. 2a, b), suggesting that these conserved putatively proteolytic residues are necessary for the function of PrsS.

We next sought to determine if *prsS* mutants mirror those of *sigS* in their sensitivity to cell wall-damaging agents. We determined that the *prsS* mutant strain, just like *sigS* mutants (Miller *et al.*, 2012), had elevated sensitivity to β-lactam agents, as well as the peptide antibiotic bacitracin. Specifically, the ampicillin MIC for the WT was 102 μg ml^{-1}, which is 10.2-fold higher than that of the *prsS* mutant (10 μg ml^{-1}). More profoundly, the penicillin-G MIC for the WT is 617 μg ml^{-1}, which is 132.1-fold higher than that of the mutant (4.67 μg ml^{-1}). In the context of bacitracin, the MIC for the WT is 158 μg ml^{-1}, which is 4.8-fold higher than that of the *prsS* mutant (33.33 μg ml^{-1}).

To assess whether there were any additive effects during loss of both *prsS* and *sigS*, we constructed a double mutant, and repeated each of these assays. We determined that the *prsS sigS* double mutant behaved exactly like that of the individual mutants in these assays, revealing no additional effects (data not shown). These results indicate that, akin to σ^S, PrsS is involved in the response of *S. aureus* to DNA damage and cell wall-targeting antimicrobial agents.

Proteomic analysis to identify a candidate σ^S anti-sigma factor

Despite the fact that our Y2H screen failed to identify a σ^S anti-sigma factor, our transcription and phenotypic assays show that *prsS* and *sigS* function in similar fashions. As such, we used proteomic techniques in an attempt to identify an anti-sigma factor. Proteomic analyses of cells from post-exponential growth reveal a significant alteration in the stability of 14 membrane proteins in the mutant strain compared to the parent (Fig. 3a, Table S3). Of note, five of the eight proteins with increased abundance in the mutant strain are uncharacterized hypothetical proteins (SAUSA300_1684, SAUSA300_1351, SAUSA300_1788, SAUSA300_1495 and SAUSA300_0014). To assess if any of these proteins represented a putative anti-sigma factor, we individually introduced NARSA transposon mutations for four of these genes (no SAUSA300_1351 mutant was available) into a USA300 *sigS-lacZ* reporter fusion strain. If any of these genes encoded an anti-sigma factor, then one would observe a blue colouration in these strains, resulting from increased free

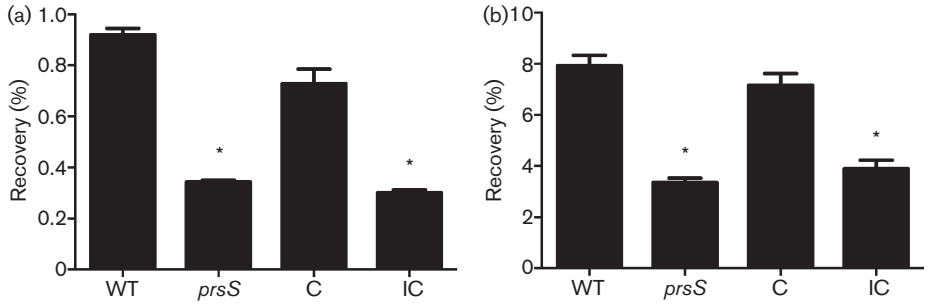

Fig. 2. PrsS, like σ^S, protects *S. aureus* against killing by DNA-damaging agents. Viability of the USA300 WT, *prsS* mutant (*prsS*), *prsS* complement (C), and *prsS*$^{E215A/E216A}$ inactive complement (IC) strains was assessed in the presence of 50 mM MMS (a) or 1.3 M hydrogen peroxide (b). C.f.u. counts were determined for strains pre- and 30 min (a) or 5 min (b) post exposure to these agents, and are averaged (mean) from three independent experiments. Error bars are shown ± SEM; *$P<0.05$ using Student's *t*-test.

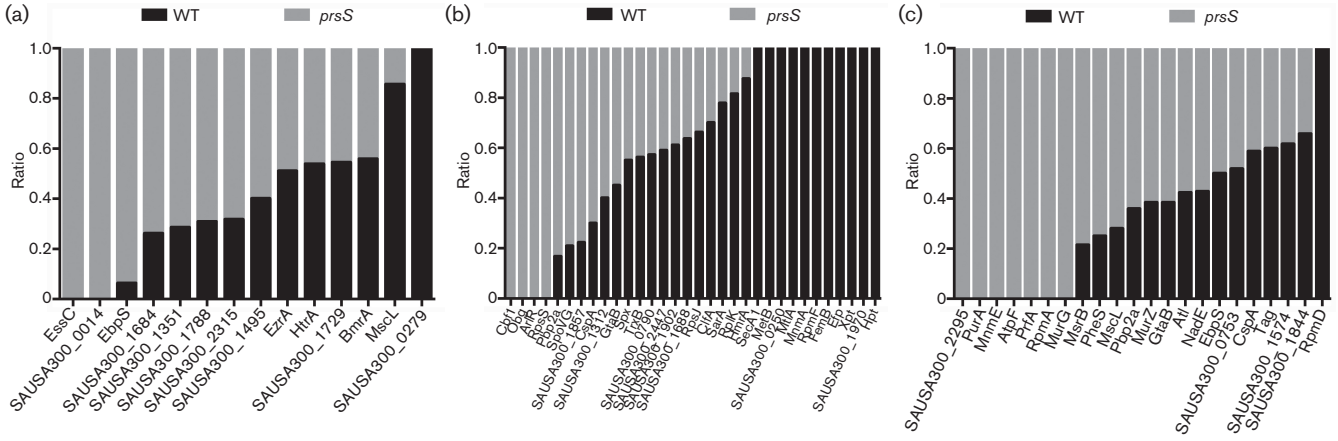

Fig. 3. Proteomic analyses of *prsS* mutant proteomes. Proteomic analyses were performed on WT and mutant strains to determine differences in membrane protein abundance during post-exponential growth (a) and cytoplasmic membrane protein abundance during post-exponential (b) and stationary (c) growth. Shown are the mean ratios of spectral counts for the *prsS* mutant / WT, and these are averaged (mean) from three independent replicates.

σ^S, and upregulation of the *sigS* gene via its auto-regulatory activity (Shaw *et al.*, 2008). In each case, disruption of these uncharacterized proteins did not induce *sigS* expression, suggesting that they are not σ^S anti-sigma factors. As a SAUSA300_1351 mutant is not present in the NARSA transposon mutant library, we performed a bioinformatic analysis on this protein using the sequences of known anti-sigma factors. Such comparison revealed that it does not share any homology with such proteins, suggesting that it is also not an anti-sigma factor.

prsS mutants have alterations in cell size and abundance of proteins involved in meticillin resistance

When examining other proteins altered in the *prsS* mutant membrane proteome, we observed a 2.04-fold reduction in the septation ring formation regulator EzrA. To determine if this alteration in EzrA had a similar effect to that previously reported for *ezrA* mutants (Jorge *et al.*, 2011), we performed TEM of *prsS* mutant cells. Upon deletion of *prsS*, we observed similar heterogeneity in cell size to that of *ezrA* mutants when compared to WT strains (Fig. 4), in which very large cells accounted for approximately 10 % of the total population.

In addition to decreased levels of EzrA, there was also increased abundance of penicillin-binding protein 2a (Pbp2a) in the cytoplasmic fractions of *prsS* mutant strains from both post-exponential (5 h) (Fig. 3b, Table S4) and stationary (15 h) (Fig. 3c, Table S5) cultures. Moreover, we also observed decreased accumulation of the meticillin resistance protein FemB in *prsS* mutants, as well as the HmrA protease, which is also required for resistance to β-lactam antibiotics (Botelho *et al.*, 2011; Henze *et al.*, 1993). Collectively, these data demonstrate that the decreased sensitivity of *prsS* mutants to β-lactam antibiotics is perhaps mediated by altered levels of key meticillin resistance proteins.

Proteome analyses demonstrate a decreased abundance of DNA damage repair proteins in *prsS* mutants

We also observed decreased abundance of several proteins in the *prsS* mutant that are involved in DNA damage repair, including a 2.26-fold decrease in the putative multi-drug resistant transport protein BmrA, which has been shown to export a number of agents that induce DNA damage (Orelle *et al.*, 2003; Steinfels *et al.*, 2004). Additionally, there were significant decreases in the mutant strain for xanthine phosphoribosyltransferase (Xpt, fourfold), and hypoxanthine phosphoribosyltransferase (Hpt, 4.67-fold). Both of these proteins are involved in nucleotide salvage pathways, which become highly important to cells during DNA damage repair (Christiansen *et al.*, 1997; Kilstrup *et al.*, 2005; Nilsson & Lauridsen, 1992). We also observed a 2.50-fold decrease in DNA-3-methyladenine glycosidase in the mutant, which is involved in the base excision repair pathway (Rain *et al.*, 2001), and a 2.92-fold decrease in a bacterioferritin comigratory protein SAUSA300_1844, which is involved in the response to oxidative stress (Jeong *et al.*, 2000). Similar to this latter protein, there was a decreased abundance in the mutant of three enzymes that have either a known or predicted role in oxidative stress resistance, including Spx, the Spx homologue SAUSA300_0790, and TrxB (Nakano *et al.*, 2003, 2005; Pamp *et al.*, 2006). Given that cells undergoing oxidative stress typically die from DNA damage, it appears that key components that facilitate the cellular response to DNA destabilization and reactive oxygen species have decreased accumulation in *prsS* mutant cells. Collectively, these proteomic findings seem to provide an explanation for the observed sensitivity of *prsS* mutants to this kind of stress.

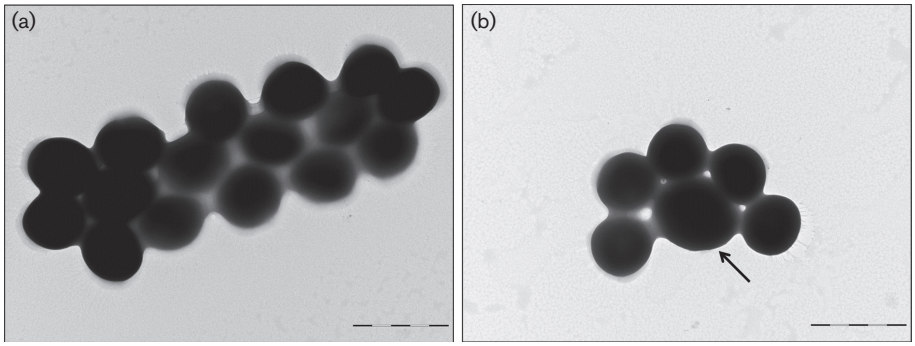

Fig. 4. *prsS* mutants display a larger cell size than WT strains. Transmission electron micrographs are shown for exponentially growing WT (a) and *prsS* mutant (b) cells. Images are representative of at least ten different frames, and are typical for each cell population. The black arrow highlights the increased cell size observed for *prsS* mutants. Bars, 1 μm.

The altered accumulation of stress resistance proteins in *prsS* mutants is mediated at the transcriptional level

PrsS, as with its counterparts from *B. subtilis* and *C. difficile*, appears to function as a protease in the cell, or at least conserved catalytic residues are required for its role in resistance to DNA damage and oxidative stress. To determine if the observed differences in protein accumulation in *prsS* mutants are mediated by transcriptional or post-translation regulation (e.g. proteolysis), we performed qPCR on *bmrA*, *ezrA*, *femB*, *hmrA*, *hpt* and *tag* using the WT strain, alongside *prsS* and *sigS* mutants (Fig. 5). Notably, we observed significantly decreased expression of all genes in both mutant strains. As a control, we also analysed the expression of these same six genes in an unrelated mutant (*rpoE*, the δ-subunit of RNAP; Weiss *et al.*, 2014) to ensure that the effects were specific to *sigS* and *prsS*, and found no significant changes in expression for any gene (data not shown). Taken together, these results suggest that the decreased abundance of proteins involved in resistance to cell wall-targeting antibiotics and DNA damage in *prsS* mutants results from transcriptional regulation, rather than direct PrsS proteolysis. Whilst these data do not directly implicate PrsS in controlling σ^S activity (putatively by cleaving an anti-sigma factor), they do suggest that both elements function in a similar manner within the cell.

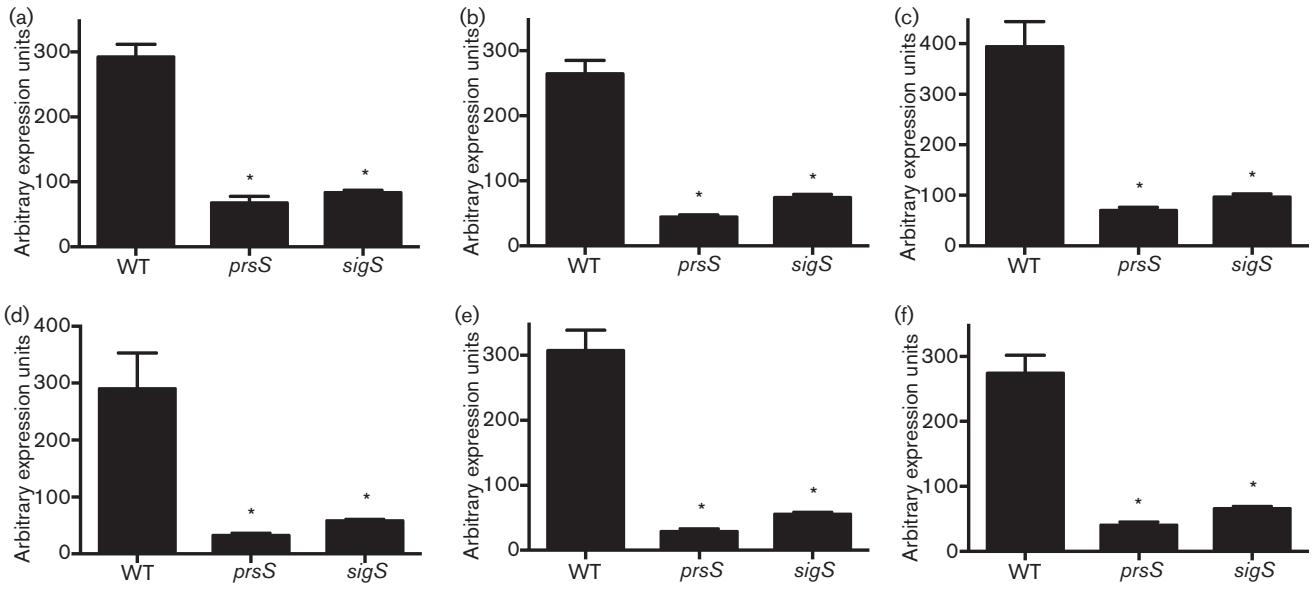

Fig. 5. The altered accumulation of stress resistance proteins in *prsS* mutants is mediated at the transcriptional level. Expression levels of *bmrA* (a), *ezrA* (b), *femB* (c), *hmrA* (d), *hpt* (e) and *tag* (f) were measured in WT, *prsS* and *sigS* mutant strains. Expression units are averaged (mean) from three independent replicates. Error bars are shown ± SEM; **P*<0.05 using Student's *t*-test.

PrsS is required for both *ex vivo* and *in vivo* infection

As *prsS* expression is increased in serum and murine macrophage-like cells, we assessed survival of *prsS* mutants using *ex vivo* and *in vivo* infection models. Firstly, exponentially growing WT USA300, *prsS* mutant and complement strains were incubated in whole human blood, and their percent recovery determined. After 3 h, the WT returned 30.6 % of the inoculum, whilst the *prsS* mutant returned only 8.5 %, representing a >3.5-fold change (Fig. 6). Importantly, *prsS* complementation restored survival to WT levels, with a recovery of 30.9 %. Next, we sought to determine if these *ex vivo* findings were recapitulated *in vivo* in a murine model of sepsis. Whilst we did not observe statistically significant dissemination to the brain, liver, spleen or kidneys, we did note a major impairment in the ability to infect the heart (8885-fold decrease, Fig. 7a) and lungs (55-fold decrease, Fig. 7b) by the *prsS* mutant strain. Collectively, these findings strongly implicate PrsS in the ability of *S. aureus* strains to cause disease in both human and murine systems, and mimic our findings with *sigS* mutants (Miller *et al.*, 2012; Shaw *et al.*, 2008).

DISCUSSION

In this study, we have identified a protein (PrsS) in *S. aureus* with homology to PrsW from *B. subtilis*. Interestingly, no anti-sigma has yet been identified for σ^S in *S. aureus*. Herein, we perform Y2H screens and proteomic studies, but do not identify an inhibitory protein that interacts with σ^S. As such, one might predict that this regulator falls into the small class of ECF sigma factors that do not possess an inhibitory counterpart, such as HrpL in *Pantoea stewartii* and *Erwinia herbicola* (Merighi *et al.*, 2003; Nizan-Koren *et al.*, 2003). However, we show in this study that the role and regulation of *prsS* phenocopy *sigS* in *S. aureus*. Whilst this does not definitively link PrsS to a role in directly controlling σ^S activity (putatively via proteolysis of an anti-sigma factor), it suggests that both elements function in a similar manner within the cell, perhaps via a common pathway. Interestingly, expression of *sigS* in *prsS* mutants is not altered in either standard conditions or *sigS*-inducting conditions, suggesting that PrsS mediated-control of σ^S occurs at the protein level (data not shown). Furthermore, we demonstrate that under standard conditions, *prsS* is lowly expressed in laboratory and clinical strains, with a strong increase in expression in the mutagenic strain RN4220, which mirrors that of *sigS* (Miller *et al.*, 2012).

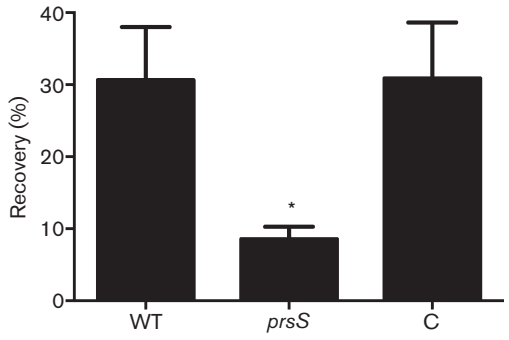

Fig. 6. PrsS provides a survival advantage in whole human blood. The viability of the USA300 WT, *prsS* mutant (*prsS*), and its complement (C) strain in whole human blood (pooled from five samples) was analysed. C.f.u. counts were determined for strains pre- and 3 h post-inoculation. Error bars are shown ± SEM; *$P<0.05$ using Student's *t*-test.

Although low levels of expression are observed for *prsS* during standard conditions, expression is elevated when exposed to stressors that elicit cell wall perturbations and DNA damage. In addition to increased expression for both of these conditions, we demonstrate that *prsS* mutants are more sensitive to DNA damage and certain cell wall-targeting antibiotics, which is again identical to *sigS* mutants (Miller *et al.*, 2012). Collectively, the mirroring of *sigS/prsS* expression patterns, and the phenotypes of their respective mutants, suggests that they may be involved in the same functional pathway. As such, we suggest that, despite not yet being identified, it is possible that PrsS may modulate σ^S activity. Indeed, such an observation is the mirror image of a study in *B. subtilis*, where Hastie *et al.* (2013) demonstrate RIP mediated degradation of the σ^V anti-sigma factor RsiV, yet the responsible S1P that initiates cleavage remains to be identified.

In efforts to understand the mechanism by which PrsS contributes to protection of the *S. aureus* cell against stress, we performed proteomic studies with the *prsS* mutant. Importantly, we observed altered accumulation of several proteins involved in the response to DNA damage and oxidative stress. Specifically, there is decreased abundance of the putative multi-drug resistance transport protein BmrA in the mutant strain, which has been shown to export DNA-damaging compounds ethidium bromide, Hoechst 33342, doxorubicin and 7-aminoactinomycin D in *B. subtilis* (Orelle *et al.*, 2003; Steinfels *et al.*, 2004). We also noted decreases in both Xpt and Hpt, each of which functions in nucleotide salvage pathways. Following conditions that elicit DNA damage, there is a major increase in demand for nucleotides within bacterial cells (Malachowa *et al.*, 2011); thus, an impaired ability to salvage such building blocks results in enhanced sensitivity to this type of stress. Finally, the base excision repair protein DNA-3-methyladenine glycosidase (Rain *et al.*, 2001) was also decreased in *prsS* mutants. The base excision repair pathway is a key mediator of the response to DNA damage, and is necessary to remove damaged or wrongly incorporated bases (Barnes *et al.*, 1993; Karran *et al.*, 1980). Importantly, *E. coli* mutants of this protein demonstrate increased susceptibility to alkylating agents (Karran *et al.*, 1980). Such sensitivity is mimicked by *prsS* mutants when exposed to the alkylating agent MMS. There was also decreased presence of a HtrA protease in the membrane, a protein with a demonstrated role in the

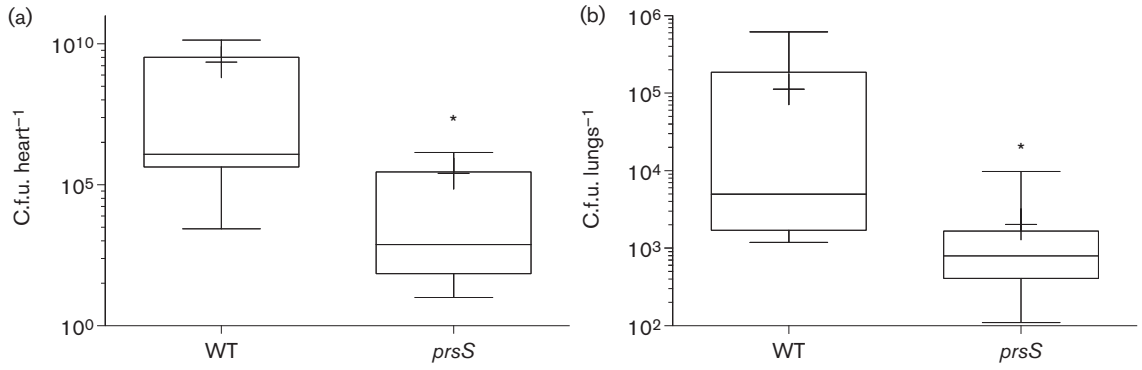

Fig. 7. PrsS is required for full virulence in murine models of infection. Bacterial loads in the heart (a) and lungs (b) are shown for mice infected with the WT or *prsS* mutant (*prsS*) strain. Box and whisker plots represent the minimum and maximum values (whiskers), as well as the 25th–75th percentile (boxes). The median c.f.u. organ^{-1} for each group is indicated as a solid horizontal black line, whilst the mean is indicated by +. Statistical significance was determined using a Mann–Whitney test; *$P<0.05$.

oxidative and DNA damage response in bacteria (Hansen & Hilgenfeld, 2013), although its precise role in *S. aureus* remains to be elucidated. Collectively, these alterations in proteome profile would appear to explain the sensitivity of *prsS* mutants to DNA-damaging stressors.

We also found that, as for *sigS* mutants, *prsS* mutants are more sensitive to ampicillin, bacitracin and penicillin-G. This also correlates with findings from the literature, which reveal *prsW* mutants of *C. difficile* are also sensitive to bacitracin (Ho & Ellermeier, 2011). Proteomic analyses performed herein revealed alterations in numerous proteins which contribute to antibiotic resistance in the mutant strain. Specifically, we noted an accumulation in the meticillin resistance protein Pbp2a in *prsS* mutant cytoplasm, coupled with a decrease in EzrA in the membrane of mutant strains. The alteration in abundance of these two proteins is of particular interest as *ezrA* mutants are incapable of properly localizing penicillin-binding proteins (Jorge *et al.*, 2011; Steele *et al.*, 2011). Further to this, we observed a decrease in the HmrA protease in *prsS* mutant strains, which has been shown to contribute to meticillin resistance by facilitating the activity of Pbp2a in *S. aureus* (Botelho *et al.*, 2011). Finally, we observed decreased abundance of the aminoacyltransferase FemB in the mutant strain. FemB is involved in the formation of pentaglycine interpeptide bridges, and cells lacking this enzyme display irregular septation patterns, and have increased susceptibility to meticillin (Henze *et al.*, 1993). Collectively, each of these observations begins to explain our findings that disruption of PrsS activity in *S. aureus* cells leads to increased sensitivity to β-lactam antibiotics and other cell wall-targeting agents.

Despite this information, it was not clear at what level these changes in protein abundance in *prsS* mutants were mediated, e.g. transcriptional or post-translation (i.e. putative proteolysis by PrsS). Using qPCR, we demonstrate that the alteration in abundance of key stress proteins actually occurs at the level of gene expression. Interestingly, transcription of these same genes is also decreased to similar levels in *sigS* mutant strains. Thus, the observed decrease in expression of these genes in both strains further demonstrates that *prsS* mutants behave like *sigS* mutants. Again, whilst this does not draw a direct connection between PrsS and σ^S activity, it does tend to suggest that these two elements function in a like manner to protect the cell from stress, putatively via a common pathway.

In the context of pathogenesis, we demonstrate that *prsS* expression is drastically increased *ex vivo* in human serum and RAW 264.7 murine macrophage-like cells. This would suggest that *prsS* is necessary to protect against the effects of the immune system during infection. We propose that this observation corroborates the observed upregulation of *prsS* in response to cell envelope and DNA damage stressors, as components of the immune system cause both of these kinds of stress (Cooke *et al.*, 2003; Hancock & Rozek, 2002; MacMicking *et al.*, 1997; O'Rourke *et al.*, 2003). In addition to these transcriptional effects, we also found that PrsS is required for survival in human blood and for dissemination to hearts and lungs during murine systemic infections. Of interest, PrsW proteins have previously been implicated in infection, as is the case in *C. difficile*, where this element is necessary for colonization of the caecum in a hamster model of infection (Ho & Ellermeier, 2011).

A possible explanation for the decrease in infectious capability of *prsS* mutants may be alterations in several proteins involved in type-VII secretion of virulence determinants. A previous study describes a regulatory network in which repression of the type-VII secretion system (T7SS) is mediated by SarA, whilst activation is controlled by ArlR and SpoVG (Burts *et al.*, 2005, 2008; Guinn *et al.*, 2004; Schulthess *et al.*, 2012). Interestingly, *prsS* mutants display alterations in all three of these proteins; specifically, we observed increases in both SpoVG and ArlR, and a decrease in the abundance of SarA. Additionally, *prsS* mutants display a decrease in EssC and an increase in EsaA, key components of this secretion

system (Anderson *et al.*, 2011; Burts *et al.*, 2005). Collectively, these alterations indicate that PrsS may have some role in the regulation of T7SS, which may explain its impaired virulence in both human and murine models of disease.

In summary, we present the characterization of the PrsS protein in *S. aureus*. Despite the absence of a candidate anti-sigma factor, there are key similarities between *sigS* and *prsS*. Specifically, the expression of *prsS* mirrors that of *sigS* in the context of strain variation, response to external stress and interaction with the innate immune system. In addition, *prsS* mutants phenocopy their *sigS* counterparts for sensitivity to DNA-damaging agents and cell wall-targeting antibiotics; and their role in *ex vivo* and *in vivo* survival. These findings appear to be, at least in part, the result of alterations in proteins involved in DNA damage repair and cell wall integrity upon *prsS* disruption, and are putatively mediated at the level of transcription, findings that are also mirrored in *sigS* mutants. Collectively, whilst our studies do not establish a direct link between PrsS function and σ^S activity (as would be predicted if RIP were occurring), they demonstrate a strong series of commonalities between these two factors. A further exploration of the relationship between these two proteins is currently underway in our laboratory.

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