The *mcsB* gene of the *clpC* operon is required for stress tolerance and virulence in *Staphylococcus aureus*

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The *clpC* operon in *Staphylococcus aureus* comprises four genes, denoted *ctsR*, *mcsA*, *mcsB* and *clpC*. A mutation within the *mcsB* gene resulted in hypersensitivity to heavy metal stress, temperature stress, osmotic pressure stress and oxidative stress. This mutation also resulted in sensitivity to variations in pH and lowered expression of the *clpC* operon under adverse extracellular conditions, as determined by quantitative real-time PCR (qRT-PCR). Additionally, virulence traits such as haemolytic activity, proteolysis, biofilm formation, and evasion from peritoneal fluid killing were substantially reduced in the $\Delta mcsB$ strain. Interestingly, mutated *mcsB* also caused a significant reduction in expression of virulence determinants *hla* and *saeS*. To be a successful pathogen, *S. aureus* must effectively overcome these types of stresses that are encountered within the host. These data show that an *S. aureus* strain lacking functional *mcsB* is stress hypersensitive and therefore less viable when introduced into hostile environments. For the first time, these studies have identified *mcsB* as a crucial and necessary component of stress and pathogenicity mechanisms.

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INTRODUCTION

Staphylococcus aureus is a notorious and troublesome pathogen known for its ability to survive and thrive in adverse extracellular conditions. Such changes in the environment can include oxidative stress, changes in pH, osmotic pressure, and exposure to heavy metals (Clements & Foster, 1999). To alleviate such stresses, the bacterium employs numerous and sometimes redundant mechanisms, including heavy metal efflux pumps, superoxide dismutases, and misfolded protein responses that trigger peptide degradation. To neutralize reactive oxygen species (ROS) generated by phagocytic cells, *S. aureus* utilizes catalase and alkyl hydroperoxide reductase enzymes. The production of staphyloxanthin, which provides the golden pigment of *S. aureus*, confers further antioxidant properties (Clauditz *et al.*, 2006). High levels of ROS can lead to protein misfolding, enzyme inactivation and eventual cell death.

To circumvent protein misfolding caused by oxidative stress, bacteria degrade damaged proteins prior to aggregation and precipitation. In both Gram-negative and Gram-positive bacteria, the Clp proteolytic complex plays a requisite role in cellular protein quality control and intracellular viability (Porankiewicz *et al.*, 1999; Frees *et al.*, 2004; Donegan *et al.*, 2010). This complex functions by degrading terminally nonfunctional proteins, commonly

utilizing energy derived from ATP hydrolysis involving AAA or AAA⁺ superfamily ATPases. Additionally, *clp* proteases have been shown to be essential for full expression of virulence traits in *S. aureus* (Frees *et al.*, 2003, 2004, 2005). These Clp proteins are encoded by five genes in *S. aureus*: *clpC*, *clpP*, *clpB*, *clpX* and *clpL*.

The *clpC* operon in *S. aureus* consists of four genes named *ctsR*, *mcsA*, *mcsB* and *clpC*. Three genes, *ctsR*, *mcsA* and *clpC* of the *clpC* operon, have been studied in *S. aureus* (Chastanet *et al.*, 2003; Sitthisak *et al.*, 2012; Frees *et al.*, 2004). CtsR in monomeric and dimeric forms binds directly to heptamer repeat sequences within promoter regions and negatively regulates the expression of the *clpC* operon. Numerous other stress operons, including *dnaK* and *groESL* of *S. aureus*, are also negatively regulated by CtsR (Derré *et al.*, 1999; Chastanet *et al.*, 2003). Though the *clpP* and *clpB* genes contain putative CtsR recognition sequences, there is no report that CtsR directly binds to these promoter regions (Chastanet *et al.*, 2003). We have recently characterized McsA as a redox-sensitive protein containing two Cys2–Cys2 zinc finger motifs which specifically bind copper, cobalt, cadmium and zinc ions (Sitthisak *et al.*, 2012). In addition to heavy metals, the zinc finger motifs of McsA may act as sensors of which the thiols become oxidized and induce activation of McsA under oxidative conditions (Elsholz *et al.*, 2011). ClpC has been well studied as an ATPase required for various physiological processes, including stress resistance, virulence and

Abbreviations: qRT-PCR, quantitative real-time PCR; ROS, reactive oxygen species.

metabolism (Chatterjee *et al.*, 2005, 2011; Luong *et al.*, 2011). To date, the *mcsB* homologue in *S. aureus* (SAR0527) has not been studied.

The McsB protein contains a highly conserved phosphotransferase domain from Asn¹¹⁰ to Lys²⁴³ (Krüger *et al.*, 2001). It is proposed that McsA and McsB are required to modulate the DNA-binding activity of CtsR, with the exception of heat-shock conditions, in which CtsR is able to modulate itself (Elsholz *et al.*, 2010). The sequence similarity of *mcsB* in *S. aureus* to *mcsB* in the non-pathogenic *Bacillus subtilis* has led us to hypothesize a role for McsB as a modulator of CtsR under oxidative and other stress conditions. As reported in *B. subtilis*, the stability of CtsR is dependent upon two proteins encoded within the *clpC* operon, namely McsA, a redox-sensitive protein, and McsB, a kinase (Kirstein *et al.*, 2005, 2007). Upon stress, activated McsA recruits McsB to target CtsR for degradation via ClpCP-dependent proteolysis. In turn, the CtsR regulon is derepressed and various stress tolerance genes are induced, including *clp*-encoded proteases. Disrupting the function of McsB might alter expression of the *clpC* operon, therefore leading to a stress-hypersensitive phenotype. It has been shown that a mutant in the *clp* operon is less viable in high-salt conditions (Frees *et al.*, 2004). Alterations of the expression of CtsR-regulated *clp* genes may also affect virulence determinants. Biofilm formation was decreased in *clpC* mutants but increased in *clpP* mutants of *S. aureus* (Frees *et al.*, 2004). Additionally, ClpXP is required for expression of the virulence-associated *agr* regulon in *S. aureus* (Frees *et al.*, 2003).

We hypothesized that strains lacking functional McsB would be unable to effectively induce the *clpC* operon even under adverse environmental conditions. The goal of this study was to determine the effect of a mutation at the *mcsB*

locus on transcriptional and phenotypic expression, and the pathogenicity of *S. aureus*. The results presented strongly suggest that *mcsB* is necessary for the stress-tolerance response and pathogenic traits of *S. aureus*.

METHODS

Bacterial strains and growth conditions. *S. aureus* and *Escherichia coli* strains used in this study are listed in Table 1. *S. aureus* strains were grown in tryptic soy broth (TSB) and *E. coli* strains were grown in Luria-Bertani broth (LB). The growth media were supplemented with kanamycin (25 µg ml⁻¹), carbenicillin (50 µg ml⁻¹) or chloramphenicol (10 µg ml⁻¹), when necessary. All cultures were grown at 37 °C unless otherwise noted.

Construction of an *mcsB* inactivation strain. The $\Delta mcsB$ strain was constructed using pTZ18R-mediated plasmid insertion as described by Singh *et al.* (2007). The 1.2 kb upstream and 1.1 kb downstream regions of the *mcsB* locus in *S. aureus* strain SH1000 were PCR-amplified using the primers *mcsB*-Up-F and *mcsB*-Up-R, and *mcsB*-Down-F and *mcsB*-Down-R, respectively. The two fragments were ligated together to produce a single 2.3 kb insert and ligated into the pTZ18R vector. The $\Delta mcsB$ -pTZ18R construct was linearized by restriction digestion at the site in which the upstream and downstream fragments were ligated, and a 0.8 kb kanamycin cassette was inserted to create the UKD-pTZ18R construct. The resulting plasmid was electroporated (Löfblom *et al.*, 2007) into the restriction minus/modification plus strain of *S. aureus* RN4220 (Kreiswirth *et al.*, 1983) and transformants were selected on kanamycin-containing medium. Since pTZ18R cannot replicate in Gram-positive bacteria, transformed plasmids integrated into the bacterial genome at the *mcsB* locus. This mutation was then transduced into *S. aureus* strain SH1000 by phage-80 α as described by Novick *et al.* (1986). The mutation was confirmed by PCR using *mcsB*-Up-F and *mcsB*-Down-R primers.

Construction of complemented $\Delta mcsB$ strain. The $\Delta mcsB$ strain was complemented *in trans* with an exogenous plasmid containing a

Table 1. Bacterial strains and plasmids used in this study

Cam^R, chloramphenicol resistance; Carb^R, carbenicillin resistance; Kan^R, kanamycin resistance.

Strain or plasmid	Relevant characteristics	Reference or source
<i>S. aureus</i> strains		
RN4220	Restriction-minus/modification-plus strain	Kreiswirth <i>et al.</i> (1983)
RN4220:: $\Delta mcsB$	Initial <i>mcsB</i> knockout strain, Kan ^R	This study
RN4220:: <i>mcsB</i> pLI50	Strain containing <i>mcsB</i> ⁺ construct, Cam ^R	This study
SH1000	NCTC 8325-4 with <i>rsbU</i> mutation repaired	Horsburgh <i>et al.</i> (2002)
SH1000:: $\Delta mcsB$	Transduced <i>mcsB</i> knockout strain, Kan ^R	This study
SH1000:: $\Delta mcsB$ ⁺	Complemented <i>mcsB</i> mutant, Kan ^R , Cam ^R	This study
<i>E. coli</i> strain		
Top10	<i>hsdR</i> , <i>mcrA</i> , <i>lacZ</i> Δ M15, <i>endA1</i> , <i>recA1</i>	Invitrogen
Plasmids		
pGEM-T	PCR TA-cloning vector; Carb ^R	Promega
pTZ18R	Gram-positive suicide vector; Carb ^R	Mead <i>et al.</i> (1986)
$\Delta mcsB$ -pTZ18R	pTZ18R containing 250 bp <i>mcsB</i> deletion	This study
UKD-pTZ18R	pTZ18R containing $\Delta mcsB$ and Kan ^R cassette	This study
pLI50	<i>E. coli</i> - <i>S. aureus</i> shuttle vector; Amp ^R , Cam ^R	Lee <i>et al.</i> (1991)
Pro- <i>mcsB</i> -pLI50	pLI50 containing <i>mcsB</i> complementation insert	This study

copy of the *mcsB* gene. Since the *mcsB* gene resides within the *clpC* operon, the *ctsR* promoter was cloned in front of the *mcsB* gene. The –500 to –1 genomic region of the *ctsR* operon was PCR-amplified using primers *mcsB*pro-F and *mcsB*pro-R (Table 2). Additionally, the 1,011 bp region of the *mcsB* gene was also PCR amplified using primers *mcsB*orf-F and *mcsB*orf-R (Table 2). The two fragments were ligated together to create the 1511 bp Pro-*mcsB* insert. This ligated insert was PCR-amplified and ligated into a shuttle vector, pLI50 (Lee *et al.*, 1991). The Pro-*mcsB*-pLI50 construct was introduced into *E. coli*. The resulting plasmid was then isolated from *E. coli* and introduced into *S. aureus* RN4220 and finally into the SH1000:: $\Delta mcsB$ strain by electroporation so as to complement the *mcsB* mutation *in trans*.

Growth measurement. To determine the effect of *mcsB* mutation on bacterial growth, OD₆₀₀ was measured under various environmental conditions. Overnight cultures of the wild-type and *mcsB* inactivation strains were diluted 1:100 in defined media (Townsend & Wilkinson, 1992) and grown for 1.5 h to early exponential phase/OD₆₀₀ 0.1. To induce stress, the following supplements were added: CuSO₄ (200 μ M), CoCl₂ (100 μ M), ZnSO₄ (100 μ M), CdCl₂ (25 μ M), NaCl (500 mM) and H₂O₂ (1 mM). Additional temperature stress was introduced by incubating the cultures at high (42 °C) and low (28 °C) temperatures. Variations in pH were created by pelleting the cells grown to OD₆₀₀ 0.1, decanting the media, and suspending the cells in media of which the pH has been adjusted to 6.0 and 8.0. Growth of each strain was measured every hour for up to 12 h.

Measurement of gene expression by quantitative real-time PCR (qRT-PCR) analysis. The effect of various oxidative stress-inducing agents on the expression of the *ctsR* operon and critical virulence genes was profiled by qRT-PCR. *S. aureus* strains SH1000, $\Delta mcsB$ and $\Delta mcsB^+$ were grown overnight at 37 °C in defined media. The next day, the cultures were diluted 1:100 in fresh defined media and grown to OD₆₀₀ 0.4. CuSO₄ (200 μ M), ZnSO₄ (100 μ M), CoCl₂ (100 μ M), CdCl₂ (25 μ M) or paraquat (1 mM) was added and the cells were grown for an additional 1 h. One culture was grown without stress-inducing agents. After 1 h, the cells were collected and total RNA was extracted using the RNeasy Protect Bacteria Mini kit (Qiagen) and quantified by A₂₆₀ (Nanodrop, Thermo Scientific). Five hundred nanograms of total RNA was converted to cDNA using the High Capacity RNA-to-cDNA kit (Applied Biosystems) following manufacturer's protocols. qRT-PCR was performed using the DyNamo Flash SYBR Green qPCR kit (Thermo Scientific) using gene-specific primers (Table 2) and data were collected using the ABI 7300 Real-Time PCR system (Applied Biosystems). Up- or down-regulation was normalized against the 16S rRNA and then against the uninduced cultures. All reactions were run in triplicate on the same cultures. All experiments were performed at least twice.

Haemolytic, proteolytic and hydrogen peroxide susceptibility assays. To examine the effect of *mcsB* mutation on haemolysis, an agar plate-based method was utilized as described by Frees *et al.* (2003). Five microlitres of SH1000, $\Delta mcsB$ and $\Delta mcsB^+$ knockout strains was patched onto nutrient agar containing 10% sheep's blood

Table 2. Primers used in this study

Endonuclease recognition sites are underlined.

Primer	Sequence (5'–3')
<i>mcsB</i> -UP-F	GGGGCTGCAGCAATAAAATGGAAGGGCTAACATC
<i>mcsB</i> -Up-R	GGGGGGATCCTTACTTTTCATCCATTGGCTGA
<i>mcsB</i> -Down-F	GGGGGGATCCTTGATTAGTCCTGAACATAAAACAACC
<i>mcsB</i> -Down-R	GGGGGAGCTCTAAAATATGTTCCGTTCCAACAAA
KanR-F	AAAGGATCCATGGCTAAAATGAGAATATCACC
KanR-R	AAAGGATCCCTAGGTACTAAAACAATTTCATCCAG
qRT16S-F	TCGAAGCAACGCGAAGAAC
qRT16S-R	CCATGCACCACCTGTCACTT
qRT <i>ctsR</i> -F	AACACGATTCACTAATGAACATGG
qRT <i>ctsR</i> -R	ATAACCTGTTCATCTTTATTTTCAAT
qRT <i>mcsA</i> -F	AATTAATTTTCAAGAAGTAGCGTTTAA
qRT <i>mcsA</i> -R	TTAGCACACCCAAATTTACCAAC
qRT <i>mcsB</i> -F	TCAAGCTATGGGAAGTACACG
qRT <i>mcsB</i> -R	AACCAAGTTGTTCATCATAACTTATATCA
qRT <i>clpC</i> -F	TAAAAGATAAGCGTGTTATGTCTTTAGA
qRT <i>clpC</i> -R	GTTGGATTTCTTCCATAACCTTTT
qRT <i>hla</i> -F	TAAAAAACTGCTAGTTATTAGAACGAA
qRT <i>hla</i> -R	GATATTTGAGCTACTTCATTATCAGG
qRT <i>sarA</i> -F	CAATTAGCTTTGAAGAATTCGCT
qRT <i>sarA</i> -R	CGAAGTAATCTTCTTGAGATAAAATTT
qRT <i>agrD</i> -F	CTTATTTTGTGATTTTATTACTGGGAT
qRT <i>agrD</i> -R	TTTTGGTACTTCAACTTCATCCATT
qRT <i>saeS</i> -F	ATCCGAACAACAAGAAAAAACAG
qRT <i>saeS</i> -R	TGATTATACCATCACGTAGTCCTTCA
<i>mcsB</i> Pro-F	ATGCGGTACCGAAGAATGGTGTTGCTTAGGATCG
<i>mcsB</i> Pro-R	ATGCCTCGAGGTATATCACCCCTTTTGGACCTTC
<i>mcsB</i> orf-F	ATGCCTCGAGATGATGACGCATAATATTCATGATAAT
<i>mcsB</i> orf-R	ATGCGGTACCCTACTTTATATGTTCTCTTAGTATATCTGC

(Remel), and the diameters of zones of clearing were recorded after 16 h. The effect of *mcsB* mutation on total proteolytic activity was measured by plating 2 μ l of wild-type and mutant cultures onto tryptic soy agar (TSA) containing 5% skimmed milk (Frees *et al.*, 2003). Variations in protein degradation were measured after 72 h. To determine the susceptibility of the *mcsB* mutant to hydrogen peroxide, disk diffusion was used as described by Frees *et al.* (2003). One hundred microlitres of mid-exponential phase culture was spread evenly on a TSA plate and allowed to dry. A sterile filter paper disk containing 5 μ l 1% H_2O_2 was placed on the plate. Diameters of zones of inhibition of growth were measured after 16 h incubation at 37 °C.

Opsonophagocytic killing assay. The ability of *S. aureus* strains SH1000, $\Delta mcsB$ and $\Delta mcsB^+$ to evade peritoneal lavage cell-mediated killing was assayed as described by Liebl & Martin (2009), with slight modifications. Briefly, 20 μ l peritoneal lavage was mixed with 20 μ l PBS (137 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4 , 2 mM KH_2PO_4 , pH 7.4). Fifteen microlitres of *S. aureus* culture at $\sim 10^5$ cells ml^{-1} was added to the peritoneal fluid mixture and incubated at 37 °C for 1 h. A 250 μ l volume of TSB was then added and incubated for 16 h at 37 °C. A control containing only PBS and bacterial culture was also prepared. After 16 h, growth of bacterial cells was measured by OD_{600} . The percentage of viable cells was calculated and normalized against *S. aureus* cells that did not receive peritoneal exudate. The experiment was run twice and in triplicate.

Biofilm assay. The ability of *S. aureus* to form biofilms was assayed as described by Cramton *et al.* (1999). Bacterial strains SH1000, $\Delta mcsB$ and $\Delta mcsB^+$ were grown overnight in TSB supplemented with 0.5% glucose. Next day, the cultures were diluted 1:100 in fresh TSB supplemented with 0.5% glucose and incubated overnight in tissue-treated 96-well microtitre plates at 37 °C without shaking (Thermo Scientific). The following day, the wells were washed three times with PBS (7 mM Na_2HPO_4 , 3 mM NaCl, pH 7.4). The biofilms were then dried thoroughly and stained with 0.1% crystal violet (Thermo Scientific), and solubilized in 30% acetic acid, and the absorbance was measured at 570 nm. All the experiments were repeated three times and in triplicate.

RESULTS AND DISCUSSION

mcsB inactivation reduces growth under stress conditions

Due to its highly conserved kinase domain, we had proposed that McsB of *S. aureus* functions as a modulator of *ctsR* expression as it does in the related yet non-pathogenic bacterium *B. subtilis* (Kirstein *et al.*, 2005). As CtsR regulates several stress tolerance genes, such as *dnaK*, *groESL* and *clpC*, we hypothesized that inactivation of *mcsB* might result in a strain that would not induce the *clpC* operon via McsB. This reduction of CtsR-regulated gene expression might result in hypersensitivity to extracellular stress. To test this hypothesis, growth curves using the *S. aureus* strains SH1000, $\Delta mcsB$ and $\Delta mcsB^+$ were carried out in defined media with and without the addition of various stress-inducing agents. The wild-type, *mcsB* mutant and $\Delta mcsB$ complemented strains grew similarly in defined media alone. However, upon stress, the $\Delta mcsB$ strain was significantly less tolerant, grew more slowly, and reached a lower cell density in the presence of heavy metals (Fig. 1b),

Fig. 1. Growth of the *mcsB* mutant and parent strains in defined media with and without the introduction of various stressors. The *mcsB* mutant strain shows hypersensitivity to each adverse condition tested. (a) SH1000, (b) $\Delta mcsB$, (c) $\Delta mcsB^+$ with the addition of heavy metal salts. (▲) $CoCl_2$, (■) $ZnSO_4$, (◆) $CuSO_4$ and (×) $CdCl_2$.

osmotic pressure (Fig. 2a), oxidative stress (Fig. 2b), changes in pH (Fig. 2c) and high temperature (Fig. 2d). These observations clearly show that *mcsB* has a critical role in mediating the stress tolerance response.

The *clpC* operon and virulence genes *hla* and *saeS* are repressed in the $\Delta mcsB$ strain

To determine whether *mcsB* is required for effective induction of the *clpC* operon under heavy metal stress, the changes in expression of the four genes of the *clpC* operon were investigated using qRT-PCR. Previously, our

Fig. 2. Growth curves of *S. aureus* SH1000 and *mcsB* strains under various stress-inducing conditions: (a) 500 mM NaCl; (b) 1 mM H₂O₂; (c) pH 6.0; (d) 42 °C. (◆) SH1000, (■) $\Delta mcsB$. Error bars, SD of two independent experiments run in triplicate.

laboratory showed induction of the *clpC* operon in the presence of copper and cadmium salts, as shown in Table 3 (Baker *et al.*, 2010; Sitthisak *et al.*, 2012). When the *mcsB* mutant strain was profiled, no significant induction of

genes within the *clpC* operon was observed (Table 3). The complemented *mcsB* mutant showed induction of the *clpC* operon similar to that of wild-type (Table 3). As CtsR is known to repress the *clpC* operon in *S. aureus*, these data identify McsB as a modulator of the repressor activity of CtsR upon heavy metal and oxidative stress caused by these metal ions.

The *clp* proteolytic genes have been implicated as having roles in virulence, including biofilm formation, oxidative stress resistance, and expression of virulence regulators such as *sarA* and *hla* (Michel *et al.*, 2006; Xiong *et al.*, 2006). The *clpC* gene is the most downstream locus of the *clpC* operon. Since we observed low expression of the *clpC* operon in the mutant *mcsB* strain, we examined the expression of the virulence genes *hla*, *sarA*, *saeS* and *agrD* by qRT-PCR in the wild-type and $\Delta mcsB$ strains with and without the addition of paraquat, which is known to induce oxidative stress. In defined media alone, the mutant *mcsB* strain showed significant reduction in expression of *hla* (downregulated 20.1 ± 1.4 -fold) and *saeS* (downregulated 2.33 ± 1.2 -fold) as compared with the wild-type (Table 4). A similar dramatic reduction in expression of *hla* has been recorded in a *clpP* mutant, which coincides with our data (Frees *et al.*, 2003). Comparable reduction of *hla* and *saeS* expression was observed after exposure to paraquat. However, no significant variations in expression of *sarA* or *agrD* were observed between wild-type and mutant *mcsB* strains grown either in defined media or under oxidative stress conditions, which corroborates with previous studies (Frees *et al.*, 2004). Since *agr* expression is not affected by the *mcsB* inactivation, our data show that decrease of expression of *hla* is not due to reduced *agr* expression, but possibly rather a reduction in degradation of Rot (repressor of α -toxin). Potentially, expression of *clpP*, which is proposed to be within the CtsR regulon, is also reduced in the *mcsB* knockout strain. In turn, accumulation of Rot, which is a target for degradation by ClpP, likely leads to overall repression of *hla* expression (Frees *et al.*, 2005). Additionally, reduction in *saeS* expression likely results in inhibition of expression of *hla* (Giraud *et al.*, 1997).

To provide evidence that the mutation at *mcsB* did not cause polar effects, transcriptional analysis of the *saeS* gene among SH1000, SH1000:: $\Delta mcsB$ and SH1000:: $\Delta mcsB^+$ strains was measured by qRT-PCR. ClpC has been reported to activate the transcription of the *sae* operon (Luong *et al.*, 2011). As shown in Table 4, the changes in *saeS* expression were similar for the *mcsB* mutant, the complemented strain and their isogenic parent strain. If there had been a polar effect of the *mcsB* mutation, the expression of *saeS* in the mutant and its complemented strain might have differed significantly from that of the parent strain.

Lower *hla* expression leads to diminished haemolytic activity in the *mcsB* mutant strain

To determine whether lower expression of stress tolerance genes such as *clpC* and virulence factors such as *hla* and *saeS*

Table 3. Transcriptional analysis of the *clpC* operon of *S. aureus*

After exposure to various heavy metal salts, up- or downregulation of the genes of the *clpC* operon was identified. SD values are based on two independent experiments run in triplicate.

Gene	Induction (fold) in the presence of:			
	CuSO ₄	ZnSO ₄	CdCl ₂	CoCl ₂
Strain SH1000				
<i>ctsR</i>	↑ 9 ± 2.5	↑ 1.8 ± 0.3	↑ 34 ± 3.8	↑ 1.5 ± 0.4
<i>mcsA</i>	↑ 10 ± 2.8	↑ 1.8 ± 0.4	↑ 31 ± 9.2	↑ 1.3 ± 0.1
<i>mcsB</i>	↑ 7 ± 4.7	↑ 1.6 ± 0.2	↑ 29 ± 6.0	↑ 1.6 ± 0.1
<i>clpC</i>	↑ 4.5 ± 1.2	↑ 1.4 ± 0.8	↑ 13 ± 2.2	↑ 1.0 ± 0.6
Strain SH1000::Δ<i>mcsB</i>				
<i>ctsR</i>	↑ 3.1 ± 1.1	↑ 1.1 ± 1.0	↑ 2.1 ± 1.0	↑ 1.4 ± 1.2
<i>mcsA</i>	↑ 3 ± 1.3	↑ 1.6 ± 1.1	↑ 2.6 ± 0	↑ 2.7 ± 1.3
<i>mcsB</i>	↑ 3.2 ± 1.2	↑ 2.1 ± 1.4	↑ 2.1 ± 1.1	↑ 1.6 ± 1.0
<i>clpC</i>	↑ 3 ± 1.1	↑ 2.8 ± 1.2	↑ 2.5 ± 2.0	↑ 2 ± 1.1
Strain SH1000::Δ<i>mcsB</i>⁺				
<i>ctsR</i>	↑ 5 ± 1.1	↑ 1.1 ± 1.2	↑ 11 ± 1.0	↑ 1.3 ± 1.4
<i>mcsA</i>	↑ 6.5 ± 1.2	↑ 1.2 ± 1.9	↑ 14 ± 1.1	↑ 1.1 ± 1.1
<i>mcsB</i>	↑ 5.5 ± 1.5	↑ 1.4 ± 1.0	↑ 14 ± 1.3	↑ 1.1 ± 1.2
<i>clpC</i>	↑ 4.9 ± 1.1	↑ 1.2 ± 1.3	↑ 9.6 ± 1.2	↑ 1.1 ± 1.1

might result in diminished virulence characteristics, the haemolytic activity of the Δ*mcsB* strain was measured. A major cytotoxic protein, α-toxin, is encoded by *hla*, which forms pores in the host cells that eventually lead to cell death (Bhakdi & Tranum-Jensen, 1991). The significant reduction in *hla* expression led us to investigate the haemolytic activity of the mutant *mcsB* strain against that of the wild-type by measuring the zone of clearing on blood agar. In agreement with the qRT-PCR data, the *mcsB* mutant strain did not produce any measurable zone of clearing (Fig. 3a). In contrast, the wild-type and *mcsB* complemented mutant strain showed substantial β-haemolytic activity.

Mutation at *mcsB* impairs proteolysis and resistance to hydrogen peroxide

CtsR has been shown to negatively regulate the ATPase gene *clpC*, and has been predicted to regulate *clpP* and *clpB*, as they are both preceded by the conserved CtsR heptamer recognition sequence (Chastanet *et al.*, 2003). Clp proteases are critical for both survival and virulence, as they are involved in degradation of misfolded proteins, breakdown of virulence regulators, and degradation of wound-healing proteins such as fibrinogen and fibronectin (Michel *et al.*, 2006). Since the *mcsB* mutant showed reduced expression

Table 4. Transcriptional analysis of virulence genes of *S. aureus*

The variation in expression is shown of several pathogenicity genes when comparing SH1000::Δ*mcsB* and SH1000 with and without oxidative stress. SD values are based on two trials run in triplicate. In the lower part of the table, changes in *saeS* expression are shown among SH1000, SH1000::Δ*mcsB* and SH1000::Δ*mcsB*⁺ strains grown with and without 1 mM paraquat. SD values are based on two independent experiments run in triplicate.

Gene	Change in gene expression SH1000::Δ <i>mcsB</i> vs SH1000	
	Defined medium (DM)	DM + 1 mM paraquat
<i>hla</i>	↓ 20 ± 1.4	↓ 18 ± 2.1
<i>sarA</i>	↓ 1.4 ± 1.1	↓ 1.3 ± 1.9
<i>agrD</i>	↓ 1.1 ± 1.6	↓ 1.1 ± 1.4
<i>saeS</i>	↓ 2.33 ± 1.2	↓ 1.9 ± 1.0
Strain	Change in <i>saeS</i> expression with and without 1 mM paraquat	
SH1000	↓ 2.88 ± 0.01	
SH1000::Δ <i>mcsB</i>	↓ 2.17 ± 0.12	
SH1000::Δ <i>mcsB</i> ⁺	↓ 2.24 ± 0.48

Fig. 3. (a) Haemolytic activity of *S. aureus* strains on blood agar plates. (1) *S. aureus* SH1000, (2) *S. aureus* $\Delta mcsB$, (3) *S. aureus* $\Delta mcsB^+$. (b) Total proteolytic activity as assayed on 5% milk agar plates. (1) SH1000, (2) SH1000:: $\Delta mcsB$, (3) SH1000:: $\Delta mcsB^+$. (c) Disk diffusion method to determine susceptibility to H_2O_2 . (1) SH1000, (2) SH1000:: $\Delta mcsB$, (3) SH1000:: $\Delta mcsB^+$.

of the *clpC* operon, we aimed to determine whether the *mcsB* mutation resulted in lower whole-cell proteolytic activity. As shown in Fig. 3(b), the wild-type and complemented $\Delta mcsB$ strains showed noticeable proteolysis on the milk agar plate. Conversely, the $\Delta mcsB$ strain showed no discernible zone of proteolysis. These data agree with earlier studies that show a decrease in total proteolytic activity in *clpP* mutants (Frees *et al.*, 2003).

The formation of ROS by H_2O_2 can induce serious DNA damage, protein misfolding and aggregation, which if not addressed can become toxic to the cell (Repine *et al.*, 1981; Imlay *et al.*, 1988; Cabiscol *et al.*, 2000). Clp proteases degrade misfolded proteins prior to aggregation to reduce toxicity (Krüger *et al.*, 2000). The susceptibility of each strain to H_2O_2 was also examined to determine any phenotypic effects caused by mutation at *mcsB*. The parent strain showed sensitivity to oxidative stress only at high concentrations, and a 12 mm zone of inhibition was measured (Fig. 3c). As the *mcsB* mutant strain does not have adequate expression of *clpC*, it showed decreased resistance to H_2O_2 , resulting in a zone of inhibition of 22 mm. The *mcsB* complemented mutant strain showed increased tolerance to H_2O_2 , with a zone of inhibition measuring 17 mm.

Inactivation of *mcsB* increases susceptibility to peritoneal fluid-mediated killing

Macrophages are central to the innate immune response as they engulf foreign antigens and sequester them to the

peroxisome for degradation. Strains that can tolerate high levels of hydrogen peroxide can evade this defence mechanism, while sensitive strains are more effectively destroyed (Mandell, 1975). The notable rise in hydrogen peroxide susceptibility in the $\Delta mcsB$ strain led us to carry out a bacterial killing assay using the wild-type, *mcsB* mutant and complemented mutant strains. As shown in Fig. 4, in the presence of murine peritoneal lavage the growth of the *mcsB* mutant strain was only 5% when compared with the parent strain, which was 43%. The resistance to peritoneal lavage was recovered by complementation. Thus we observed that the *mcsB* mutant strain is substantially more susceptible to killing by macrophages and neutrophils present in murine peritoneal fluid as compared with the parent and complemented $\Delta mcsB$ strains (Fig. 4a). These data support the hypothesis that disruption of functional McsB results in a reduced potential for *S. aureus* infection.

Biofilm formation and attachment are disrupted in the $\Delta mcsB$ strain of *S. aureus*

Functional *clp* genes are required for successful biofilm formation and attachment (Frees *et al.*, 2004). Deletions in *clpC* show sensitivity to high temperatures along with lowered virulence factors such as biofilm adherence and decreased replication within MAC-T cells (Frees *et al.*, 2004). Since a reduction in *clpC* expression was observed in the *mcsB* mutant strain, we carried out biofilm assays in the wild-type, $\Delta mcsB$ and $\Delta mcsB^+$ strains. Our data

Fig. 4. (a) Cell viability after exposure to murine peritoneal lavage. OD₆₀₀ values denote cell proliferation with (+) and without (-) addition of peritoneal fluid. Mean values from two trials run in triplicate are given; error bars, SD. (b) Biofilm attachment was measured in the SH1000, Δ*mcsB* and Δ*mcsB*⁺ strains. Error bars, SD values based on two independent experiments run in triplicate.

show a consistent 25 % reduction in biofilm attachment in the *mcsB* mutant strain as compared with the parent strain SH1000 (Fig. 4b), which correlates with earlier Δ*clpC* studies (Frees *et al.*, 2004). The complemented *mcsB* strain showed biofilm adherence similar to that of the wild-type.

Bacteria, as single, planktonic organisms, are highly susceptible to changes in the environment. Adverse conditions such as variations in pH, osmotic pressure, heat shock and high concentrations of inorganic molecules can cause significant cellular damage and may even lead to cell death if not addressed. Effective pathogens must be able to sense and respond to changing environmental conditions in order to colonize a host. Virulence factors are necessary to effectively evade the host defences and colonize host tissue. In summary, the data from these studies have demonstrated that *mcsB* plays critical roles in multiple aspects of physiology in *S. aureus*. Dysfunction in McsB leads to stress hypersensitivity, decrease of biofilm adherence, and transcriptional and phenotypic reduction in virulence determinants. Ultimately, a strain lacking functional *mcsB* is more susceptible to the changing environmental conditions that the bacteria will encounter during the process of infection. We have postulated that mutation at *mcsB* results in continual repression of the CtsR regulon, which encompasses various stress-induced operons. Future studies will

confirm the biochemical properties of McsB in regard to its modulation of CtsR.

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