MICROBIOLOGY

SHORT COMMUNICATION

Douglas *et al.*, *Microbiology* 2021;167:001096

DOI 10.1099/mic.0.001096

MICROBIOLOGY SOCIETY

OPEN ACCESS

The MpsB protein contributes to both the toxicity and immune evasion capacity of *Staphylococcus aureus*

Edward J. A. Douglas[1]†, Seána Duggan[1]†, Tarcisio Brignoli[1] and Ruth C. Massey[1,2,*]

Abstract

Understanding the role specific bacterial factors play in the development of severe disease in humans is critical if new approaches to tackle such infections are to be developed. In this study we focus on genes we have found to be associated with patient outcome following bacteraemia caused by the major human pathogen *Staphylococcus aureus*. By examining the contribution these genes make to the ability of the bacteria to survive exposure to the antibacterial factors found in serum, we identify three novel serum resistance-associated genes, *mdeA*, *mpsB* and *yycH*. Detailed analysis of an MpsB mutant supports its previous association with the slow growing small colony variant (SCV) phenotype of *S. aureus*, and we demonstrate that the effect this mutation has on membrane potential prevents the activation of the Agr quorum sensing system, and as a consequence the mutant bacteria do not produce cytolytic toxins. Given the importance of both toxin production and immune evasion for the ability of *S. aureus* to cause disease, we believe that these findings explain the role of the *mpsB* gene as a mortality-associated locus during human disease.

INTRODUCTION

Staphylococcus aureus is a major cause of human disease ranging from mild skin and soft tissue infections to more severe and life-threatening infections such as bacteraemia and pneumonia [1]. The continuing emergence of antimicrobial resistance is reducing therapeutic options and increasing the burden of this debilitating disease [2]. *S. aureus* bacteraemia (SAB) is associated with significant morbidity and mortality, where the 30-day mortality rate for SAB of approximately 20% has not substantially changed in over two decades [3]. This would indicate that improvements to infection control and surveillance and changes to therapeutic approaches over this time period have been insufficient in controlling this important health problem and suggests that a greater understanding of this disease is needed. While SAB severity has been largely attributed to comorbidities of the host [4, 5], our understanding of the role of specific bacterial factors in infection severity and disease progression is growing [6] and is the focus of this study.

To gain a deeper understanding of the role the bacteria play in the development and severity of SAB we previously performed a study of 300 SAB isolates, where we identified a number of genes as contributing to patient outcome that we refer to as mortality-associated loci (MALs) [6]. In this study we examine whether these MALs contribute to the ability of *S. aureus* to survive in human serum, given the importance of this activity for their ability to cause SAB. We identified three MALs as contributing to serum resistance, with one gene, *mpsB*, being of particular interest due to its association with the persister or small colony variant (SCV) phenotype associated with chronic *S. aureus* infections [7]. The MpsABC system participates in cation translocation, and hence the generation of membrane potential, as well as CO_2 transport [7, 8]. Mutants of the *mpsABC* operon exhibited an SCV-like phenotype and have been shown as attenuated in membrane potential and oxygen consumption rates [7]. MpsABC represents an important functional system of the respiratory chain of *S. aureus* that acts as an electrogenic unit responsible for the generation of membrane potential [7]. In this short communication we examine the contribution MpsB makes to serum resistance and demonstrate that it also contributes to the ability of *S. aureus* to produce cytolytic toxins, where the effect the loss of this gene has on membrane potential is central to these key pathogenic capabilities.

Received 30 June 2021; Accepted 23 August 2021; Published 07 October 2021

Author affiliations: [1]School of Cellular and Molecular Medicine, University of Bristol, Bristol, BS8 1TD, UK; [2]Schools of Microbiology and Medicine, University College Cork and APC Microbiome Ireland, Cork, Ireland.

***Correspondence:** Ruth C. Massey, ruth.massey@bristol.ac.uk

Keywords: mpsB; *Staphylococcus aureus*; serum survival; small colony variants.

†These authors contributed equally to this work

Table 1. Strains used in this study

Strain	Description	Reference
JE2	USA300; CA -MRSA, type IV SCCmec; lacking plasmids p01 and p03; wild-type strain of the NTML	[11]
JE2 RNAIII:*gfp*	JE2 transformed with a GFP-tagged RNAIII fusion vector	[14]
mpsB::*tn*	*mpsB* transposon mutant in JE2	[11]
mpsB::*tn* pRMC2	*mpsB* transposon mutant in JE2 transformed with empty pRMC2 vector	This study
mpsB::*tn* p*mpsB*	*mps*B transposon mutant complemented with mpsB gene cloned into the pRMC2 expression plasmid	This study
mpsB::*tn* RNAIII:*gfp*	*mpsB* transposon mutant transformed with a GFP-tagged RNAIII fusion vector	This study
agrA::*tn* RNAIII:*gfp*	*agrA* transposon mutant transformed with a GFP-tagged RNAIII fusion vector	This study
hemB::*tn*	*hemB* transposon mutant in JE2	[11]
crtM::*tn*	*crtM* transposon mutant in JE2	[11]

METHODS

Bacterial strains and growth conditions

A list of bacterial strains can be found in Table 1. All strains were cultured in tryptic soy broth (TSB) for 18 h at 37 °C with shaking. The transposon mutants were selected for using erythromycin (5 µg ml^{-1}). For the complemented pRMC2 strains, chloramphenicol (10 µg ml^{-1}) and anhydrous tetracycline (45–180 ng ml^{-1}) were added to the media [9]. Carbonyl cyanidem-chlorophenylhydrazone (CCCP; Cayman Chemical) was added to growth media at a final concentration of 0–6.25 µM where indicated.

Genetic manipulation

The *mpsB* gene was amplified by PCR from JE2 genomic DNA using the primers *mpsB* FW 5′ atatagatctgaagaagtatttatag-gaggtgaaagg 3′ and *mpsB* RV 5′ tgaattcgagctcagatacttagcatcg-caacatatcatc 3′ and KAPA HiFi polymerase (Roche). The PCR product was cloned into the tetracycline inducible plasmid pRMC2 (a generous gift from Tim Foster [9]) using *Bgl*II and *Sac*I restriction sites and T4 DNA ligase (NEB), creating p*mpsB*. This was transformed into RN4220 and eventually into JE2 *mpsB*::*tn* through electroporation.

Serum resistance

Overnight cultures were standardized to an OD_{600nm} of 0.1 and incubated in 10% pooled human serum (Sigma Aldrich) diluted in phosphate-buffered saline (PBS) for 90 min at 37 °C with shaking. Serial dilutions were plated on tryptic soy agar (TSA) to determine colony-forming units (c.f.u.). The same number of bacterial cells inoculated into PBS, diluted and plated acted as a control. Survival was determined as the percentage of c.f.u. in serum relative to the PBS control. Relative survival was determined through normalization to JE2.

Minimum inhibitory concentrations (MICs)

MICs were performed according to the micro broth dilution method [10] using Corning Costar polystyrene 96-well plates. Briefly, overnight cultures were normalized to an OD_{600nm} of 0.1 in cation adjusted Mueller–Hinton broth (MHB++) and 20 µl of resultant suspension was used to inoculate 180 µl of fresh MHB ++ containing gentamicin (Sigma). A series of 1 in 2 dilutions were subsequently performed and incubated for 20 h at 37 °C without shaking. The ability of the bacteria to survive the antibiotics was determined by quantifying bacterial growth (OD_{600nm}) using a CLARIOstar plate reader (BMG Labtech).

Antimicrobial peptide susceptibility

Antimicrobial peptide susceptibility human neutrophil defensin-1 (hNP-1) (AnaSpec, Inc., CA, USA) and LL-37 (Sigma) susceptibility assays were performed as described previously [11, 12]. Briefly, overnight cultures were normalized to an OD_{600nm} of 0.1 and incubated with 5 µg ml^{-1} of hNP-1 or LL-37 for 2 h at 37 °C. Serial dilutions were plated on TSA to determine c.f.u. The same number of bacterial cells inoculated into PBS, diluted and plated acted as a control. Survival was determined as the percentage of c.f.u. in serum relative to the PBS control. Relative survival was determined through normalization to JE2.

Carotenoid pigmentation

Carotenoid pigment quantification was performed as described previously with minor modifications [12, 13]. Overnight bacterial cultures were used to inoculate 2 ml of fresh TSB in a 1:1000 dilution, which was subsequently grown for 24 h at 37 °C with shaking (180 r.p.m.). A volume of 850 µl of bacterial culture was centrifuged at 10000 ***g*** for 4 min, supernatant was discarded and cells were resuspended in 100% methanol. Cells were heated for 3 min at 55 °C in a water bath and centrifuged at 10000 ***g*** for 2 min to remove cell debris and the extraction was repeated twice. The absorbance of the methanol extracts was measured at 453 nm using a CLARIOstar plate reader. JE2 *crtM*::*tn*, devoid of staphyloxanthin, was used as a negative control.

Membrane potential

Overnight cultures were diluted to an OD_{600nm} of 0.05 in 20 ml of fresh TSB and grown to exponential phase. Cultures were subsequently normalized to 0.1 in 1 ml of PBS. Two sets of conditions were used for each strain. To one condition 10 µl of 500 µM CCCP was added. To both conditions 10 µl of 3 mM $DiOC_2(3)$ (ChemCruz) was added. Samples were incubated for 30 min at room temperature. Green fluorescence and red fluorescence were recorded using a CLARIOstar plate reader. Membrane potential was calculated according to the ratio of red : green fluorescence.

Growth curve

Overnight bacterial cultures were diluted 1:1000 in fresh TSB. Two hundred microlitres of suspension was monitored for growth for 24 h at 37 °C with 200 r.p.m. shaking. OD_{600nm} readings were taken every 30 min using a CLARIOstar plate reader.

Cytolytic activity

The monocytic THP-1 cell line (ATCC TIB-202) was used as previously described [12, 14]. Briefly, cells were grown in 30 ml of RPMI-1640, supplemented with heat-inactivated foetal bovine serum (10%), L-glutamine (1 µM), penicillin (200 units ml^{-1}) and streptomycin (0.1 µg ml^{-1}) (defined as complete medium) in a humidified incubator at 37 °C with 5% CO_2. For toxicity assays, cells were harvested by centrifugation at 400 ***g*** and resuspended to a final density of 1–1.5×10^6 cells ml^{-1} in tissue-grade PBS, typically yielding >95% viability. These were then incubated for 10 min in the harvested supernatant of bacteria grown for 18 h in TSB (at a dilution of 10% in TSB) and THP-1 cell death was quantified by trypan blue exclusion.

Agr activity

A plasmid containing the RNAIII promoter fused to the green fluorescent protein (GFP) was transformed by electroporation into JE2, *mpsB::tn* and *agrA::tn* [14]. Overnight cultures were diluted to an OD_{600nm} of 0.05 in fresh TSB. A 200 µl volume was then monitored for GFP fluorescence (485_{nm} excitation/520_{nm} emission/1000 gain) and OD_{600nm} over a period of 24 h (readings every 30 min with 200 r.p.m. shaking between readings) using a CLARIOstar plate reader.

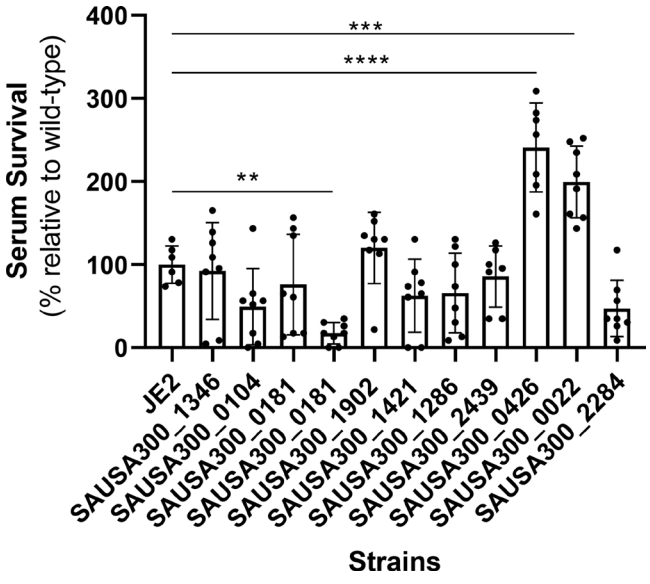

Fig. 1. Serum survival screen of MALs. Mutants in which MALS were inactivated by transposon insertion were incubated in human serum for 90 min, serially diluted and plated on TSA for enumeration. Colony-forming units (c.f.u.) in serum were compared to c.f.u. following a 90 min incubation in PBS. The dots represent biological replicates, the bars represent the mean of the replicates and the error bars represent the standard deviation. Statistics were performed using a one-way ANOVA. Significance was determined as <*0.01; **0.001; ***0.0001.

Table 2. *S. aureus* genes previously associated with patient mortality (MALs)

Locus tag	Putative function	Gene name	NTML identifier
SAUSA300_1346	Putative DnaQ family exonuclease/DinG family helicase	*dinG*	NE346
SAUSA300_0104	Transcriptional regulator, AraC family		NE78
SAUSA300_2360	Non-ribosomal peptide synthetase	*mdeA*	NE119
SAUSA300_1902	Conserved hypothetical protein		NE202
SAUSA300_1421	Conserved hypothetical protein		NE443
SAUSA300_1286	Aspartate kinase	*lysC*	NE579
SAUSA300_2439	galU UTP-glucose-1-phosphate uridylyltransferase	*galU*	NE614
SAUSA300_0426	Hypothetical protein	*mpsB*	NE1288
SAUSA300_0022	Hypothetical protein	*yycH*	NE1693
SAUSA300_2284	Hypothetical protein		NE1871

Statistics

Paired two-tailed Student's *t*-test or one-way analysis of variance (ANOVA) with a Dunnet's multiple comparisons test, with single pooled variance (GraphPad Prism v9.0), were used to analyse the observed differences between experimental results. A *P*-value <0.05 was considered statistically significant.

RESULTS

In previous work we identified a number of MALs, or genes associated with host mortality following SAB (Table 2) [6]. Given the importance of the ability of *S. aureus* to survive exposure to the many antibacterial factors found in blood for its ability to cause SAB, we hypothesized that some of these genes may contribute to this activity. To test this, we exposed an isogenic set of strains in which each of the 11 MALs were inactivated by transposon insertion to human serum (10%), and quantified their ability to survive relative to the wild-type strain. Three MALs were found to significantly affect the ability of *S. aureus* to serum survival: *mdeA*, which contributes positively to serum resistance, and *mpsB* and *yycH*, which contribute negatively to this (Fig. 1).

As part of the *mpsABC* locus, mutation of *mpsB* is likely to result in a change from the wild-type to the persister or SCV phenotype [7]. SCVs are typically auxotrophic for menadione, haemin or thymidine, but the *ΔmpsABC* mutant was

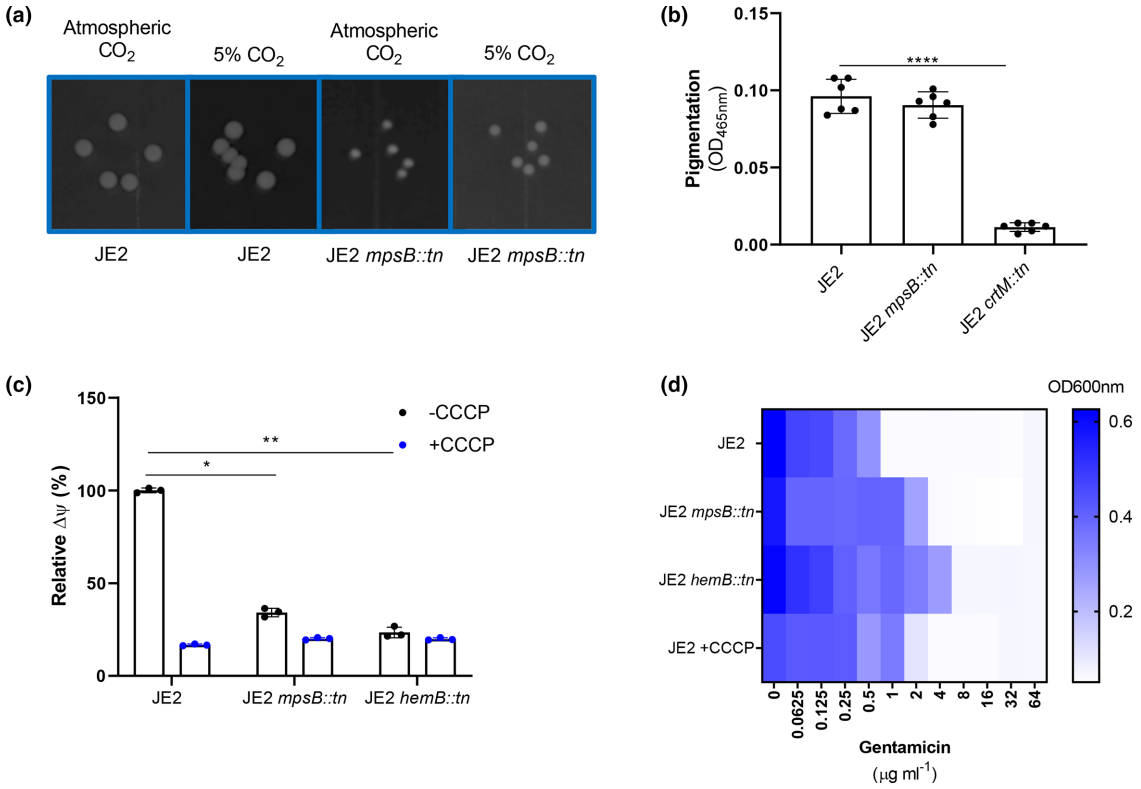

Fig. 2. The MpsB mutant displays many SCV associated phenotypes. (a) Photograph of colonies of wild-type *S. aureus* strain (JE2) and an isogenic MspB mutant grown with and without supplementation with 5% CO_2, demonstrating the CO_2-dependent effect on the growth of the mutant. (b) Staphyloxanthin biosynthesis is unaffected in the MpsB mutant when compared to the wild-type stain. A staphyloxanthin biosynthesis mutant (JE2 *crtM::tn*) is provided as a negative control. (c) Membrane potential was measured using the fluorescent dye $DiOC_3(3)$. The wild-type JE2 and a *hemB* SCV (i.e. JE2 *hemB::tn*) are provided as controls where the native and post-addition of CCCP membrane potential is shown. The MpsB mutant displays a similar reduction in membrane potential to the *hemB* SCV mutant. (d) Loss of membrane potential is associated with increased gentamicin resistance. When *mpsB* and *hemB* are inactivated the gentamicin MIC increases to 4 and 8 μg ml^{-1}, respectively, as shown by an OD heat map. Growth of JE2 in 0.5 μg ml^{-1} CCCP phenocopies the *mpsB:tn* and *hemB::tn* mutants and results in an increased gentamicin MIC. Significance was determined as <*0.01; **0.001; ****0.0001.

found to be a CO_2-dependent SCV and could be partially complemented using 5% CO_2 [7, 8]. To verify that our MpsB mutant displayed similar characteristics to those described previously we examined its growth on agar both with and without CO_2 supplementation, where the colony size was smaller when grown in air compared to the wild-type strain, but when grown in 5% CO_2, the colony size of the mutant increased (Fig. 2a). We also examined whether carotenoid or staphyloxanthin biosyntheis was affected in the MpsB mutant, but, unlike other SCV types, the MpsB mutant produced wild-type levels of this protective pigment (Fig. 2b). We next examined whether membrane potential was affected in our MpsB mutant using the fluorescent stain $DiOC_2(3)$. This membrane potential indicator dye emits green fluorescence when it enters bacterial cells, but as it increases in concentration within cells that are maintaining membrane potential, the dye self-associates, causing the fluorescence emission to shift to red [15]. Using this, we found that the membrane potential of the MpsB mutant was significantly reduced to a level similar to that of a wild-type strain exposed to CCCP (a chemical that increases proton permeability, thereby dissipating membrane potential) (Fig. 2c) [16]. The membrane potential of the MpsB mutant was also similar to that of a haemin auxotrophic SCV, where the *hemB* gene was inactivated (Fig. 2c). Another characteristic feature of SCVs is their enhanced resistance to the aminoglycoside antibiotic gentamicin [17]. To verify this for the MpsB mutant, we determined the gentamicin MIC for the wild-type JE2 strain, the MpsB mutant, the HemB mutant and the wild-type strain when grown in CCCP. The mutants and JE2 grown in CCCP were all more resistant to this antibiotic (Fig. 2d). Together, these data suggest that the MpsB mutant displays some of but not all the classic SCV phenotypic traits.

To ensure that MpsB alone was responsible for the increase in serum resistance we have observed for this mutant, and that there were no polar effects as a result of the transposon insertion into the *mpsB* gene, we cloned the gene and expressed it from an inducible promoter in the pRMC2 plasmid [9]. This complemented the serum resistance levels of the MpsB mutant back to wild-type levels (Fig. 3a). Of the antibacterial components of serum, the antimicrobial peptides (AMPs), given their reliance on intact membrane potential for

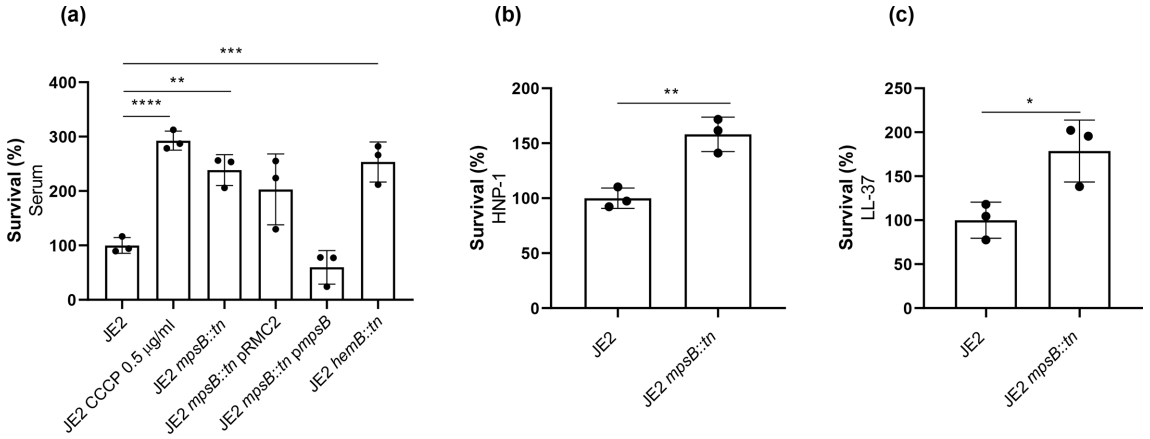

Fig. 3. The resistance of the MpsB mutant to serum is mediated by increased resistance to AMPs. (a) The effect that the inactivation of *mpsB* has on serum resistance was confirmed by complementing the mutant with the *mpsB* gene expressed from an inducible plasmid (p*mpsB*). The serum resistance of the wild-type JE2 exposed to CCCP and the *hemB* SCV is provided for comparison. (b, c) Loss of *mpsB* results in increased relative survival upon exposure to the AMPs HNP-1 and LL-37. The dots represent biological replicates, the bars represent the mean of the replicates and the error bars represent the standard deviation. Statistics were performed using a one-way ANOVA and paired *t*-tests. Significance was determined as <*0.05; **0.01; ***0.001, ****0.0001.

translocation, are most likely to be involved in the observed MpsB-associated sensitivity of the wild-type strain [18–20]. To test this, we exposed the wild-type and MpsB mutant to two AMPs, HNP-1 and LL-37, and in both cases the MpsB mutant was more resistant to these molecules when compared to the wild-type strains (Fig. 3b, c).

In addition to serucm resistance, mortality following SAB is significantly associated with the ability of the bacteria to secrete cytolytic toxins [6, 21]. SCVs are frequently described as being non-haemolytic [22], and we examined here whether the MpsB mutant was impaired in its cytolytic capability. To test this, we incubated the bacterial supernatant with THP-1 cells, which are an immortalized monocyte progenitor cell line that are sensitive to the majority of cytolytic toxins produced by *S. aureus* [21]. We found the MpsB mutant to be less cytolytic, and this effect was complemented by expressing

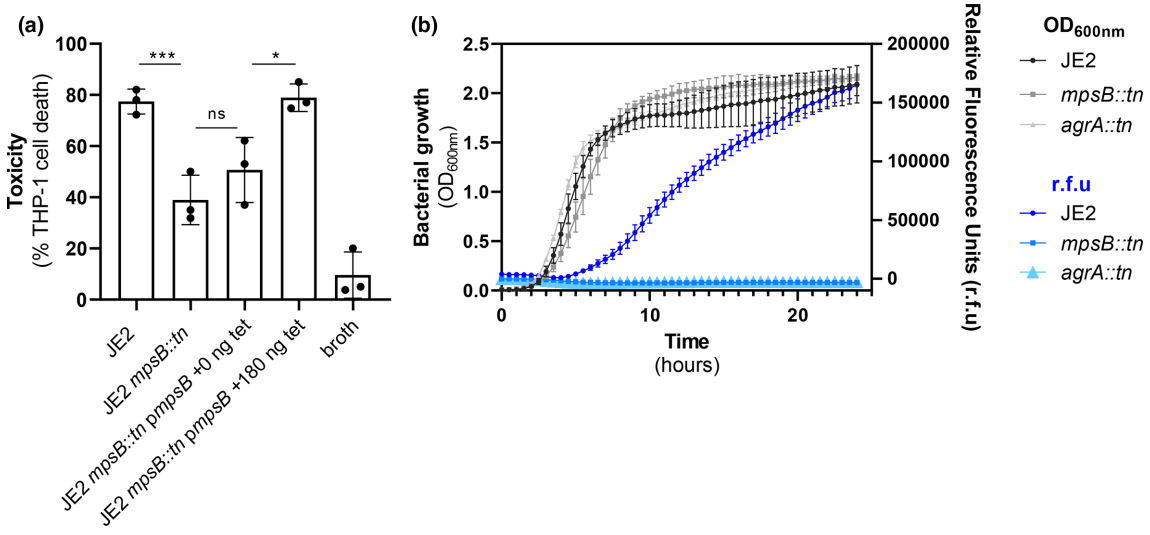

Fig. 4. The MpsB mutant is less cytolytic mediated through a lack of activation of the Agr system. (a) Inactivation of *mpsB* results in a significant decrease in the ability of *S. aureus* to lyse the human monocyte (THP-1) cell line. This effect was complemented using a strain expressing a functional copy of *mpsB* (p*mspB*). (b) Using an Agr fluorescence reporter system we found that when compared to the wild-type strain JE2, the Agr system does not become activated in the MpsB mutant. An AgrA mutant is provided for comparison. The dots represent biological replicates, the bars represent the mean of the replicates and the error bars represent the standard deviation. Statistics were performed using a one-way ANOVA and significance was determined as <*0.05; **0.01.

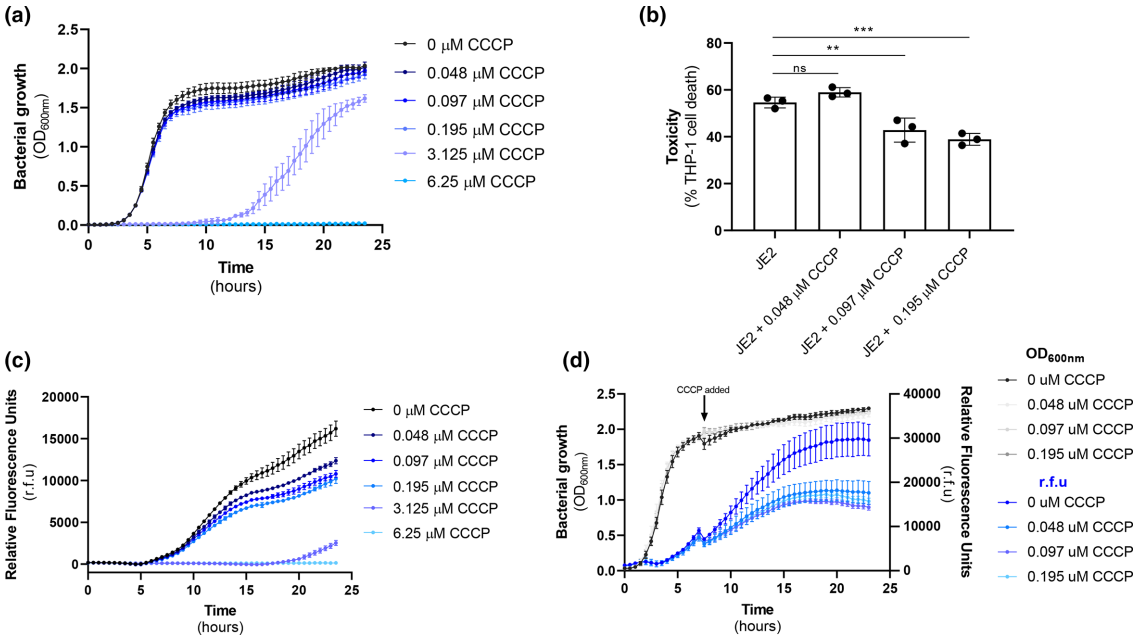

Fig. 5. Activation of the Agr system requires membrane potential. (a, b) The effect of a range of concentrations of CCCP on the growth (a) and Agr activity (b) of *S. aureus* JE2 was quantified using a fluorescence reporter over a 24 h period, where concentrations ranging from 0.048 to 0.195 µM were found to affect Agr activation but not growth. (c) CCCP at a range of concentrations (0.048–0.195 µM) was spiked into cultures of bacteria as they reached stationary phase of growth, and the effect of this on Agr activity was monitored. (d) The wild-type *S. aureus* strain JE2 was grown in CCCP at a range of concentrations (0.048–0.195 µM) and the effect hat this has on the cytolytic activity of the bacteria was quantified. The dots represent biological replicates, the bars represent the mean of the replicates and the error bars represent the standard deviation. Statistics were performed using a one-way ANOVA and significance was determined as <*0.05; **0.01.

the gene in *trans* (Fig. 4a). The major regulator of toxin production for *S. aureus* is the Agr quorum sensing system [23], and to test how active this system is in the MpsB mutant we introduced a plasmid containing an Agr activity reporter fusion (RNAIII::GFP) [14]. We monitored both growth and RNAIII::GFP expression over a 24 h period and demonstrated that in a similar manner to an Agr mutant, the Agr system does not get activated in the MpsB mutant, which explains the lack of cytolytic activity (Fig. 4b).

The activation of the Agr system is both density-dependent, and highly sensitive to the metabolic status of the bacterium. The slow growth rate and effect of mutations in genes involved in key metabolic processes has been proposed as the reason why other SCVs do not switch on their Agr system and remain non-cytolytic. Interestingly, while we see slower growth on agar (Fig. 1a), we do not see any growth defects for the MpsB mutant when grown in broth (Fig. 4b), suggesting that a difference in relative density of the bacteria does not account for the inactive Agr system. Given the critical role of two membrane proteins in the activation of the Agr system, AgrB and AgrC, we hypothesized that a change in membrane potential could directly impact on their activity and cause the lack of activation we have observed for the MpsB mutant. To test this, we sought to identify a range of concentrations of CCCP that affect membrane potential, but do not affect bacterial growth, which we found to be between 0.048 and 0.195 µM (Fig. 5a).

We examined the effect these concentrations of CCCP have on Agr activation using the reporter system, where there were significant decreases in Agr activation (Fig. 5b). To ensure that the effect of growth in CCCP on Agr activity was not due to effects on metabolic process throughout the growth curve, we also performed an experiment where we allowed the bacteria to grow to early stationary phase, monitoring Agr activation throughout. At hour 7, when the Agr system was becoming activated, we added CCCP and found that this addition, which would result in a sudden drop in membrane potential, had an immediate effect on Agr activity (Fig. 5c). We also performed THP-1 lysis assays on the bacteria following exposure to the low doses of CCCP, which confirmed that the effect we observed on Agr activity was sufficient to affect the cytolytic activity of the bacteria (Fig. 5d). With these controls, we show that the effects we observe are a direct consequence of altered membrane potential. This suggests that the Agr quorum sensing system requires a specific level of membrane potential for efficient activation.

DISCUSSION

In this study we identify three novel genes that contribute to the serum survival of *S. aureus*: *mdeA*, *yycH* and *mpsB*. We show that MdeA is involved in resisting the antimicrobial action of serum, whereas YycH and MpsB sensitize *S.*

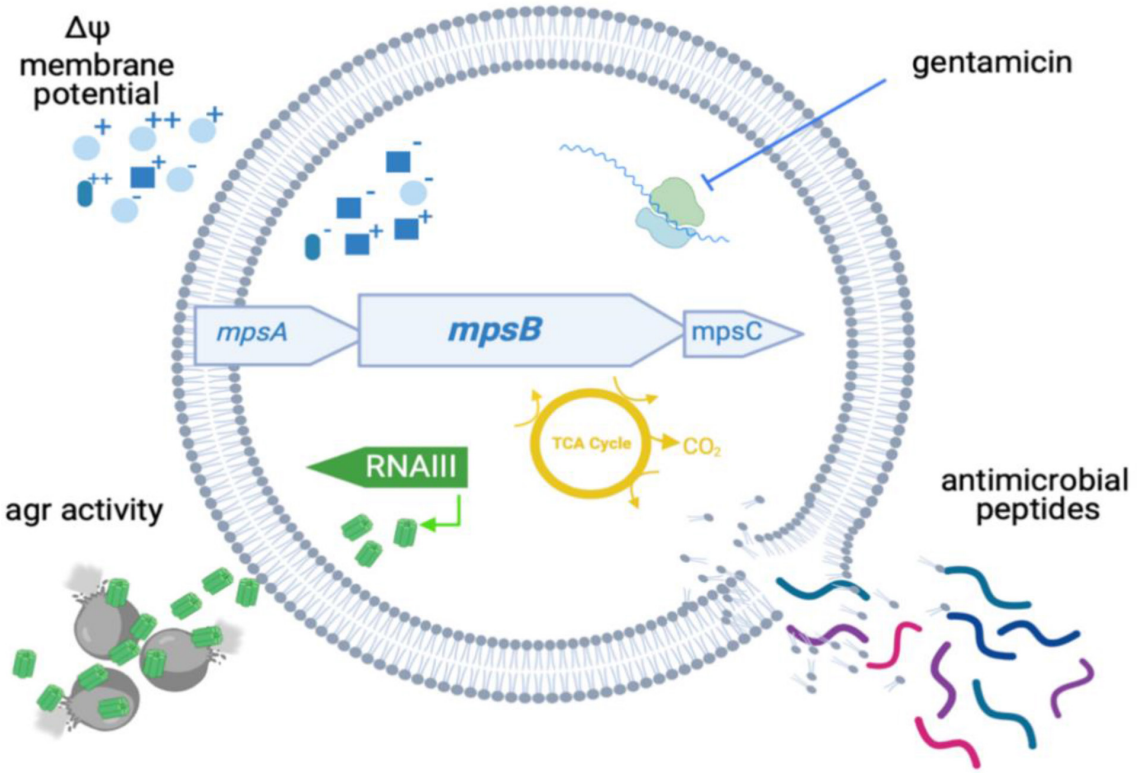

Fig. 6. Graphical summary of the effects of the loss of MpsB. Disruption of *mpsB* disrupts membrane potential (upper left quadrant), which leads to increased resistance to the protein synthesis inhibiting antibiotic gentamicin (upper right quadrant) and cationic antimicrobial peptides (lower right quadrant). Membrane potential also effects the activity of the Agr system (RNAIII), leading to reduced cytotoxicity. Image created using *Biorender*.

aureus to serum. MdeA is a chromosomally encoded efflux pump that has been shown to confer resistance to a range of antibacterial compounds, including quaternary ammonium compounds and the antibiotics novobiocin, mupirocin, fusidic acid and norfloxacin [24, 25], and we hypothesize that MdeA may also expel antimicrobial agents found in serum. YycH is an auxiliary regulator of the WalRK two-component system, where a *yycH* mutant has been shown to have decreased WalRK activity, leading to impaired autolytic activity [26]. It is currently unclear how loss of YycH causes increased serum survival, but recent work has shown that alterations to cell wall architecture leads to increased survival in macrophages [27]. It is therefore possible that the antibacterial factors common to both macrophage and serum may be involved here.

Previous work on the Mps system has focused on either *mpsA* or the entire *mpsABC locus* and shown that the deletion of this locus results in a CO_2-dependent SCV phenotype in the *S. aureus* HG001 background [7]. The SCV phenotype is widely accepted to be a strategy for protection against antibiotic therapy and aspects of the host immune system, and alongside their low-virulence phenotype this makes them particularly well adapted for persistence. Here, we focus on *mpsB*, and demonstrate that it directly contributes to the SCV-associated phenotypes previously reported for *mpsA* and *mpsABC* mutants [7, 8]. We show that disruption of *mpsB* in the JE2 background results in a partial SCV phenotype where we have a conditional effect on growth (slow on agar but normal in broth), reduced toxicity and increased resistance to the aminoglycoside gentamicin, to human serum and to antimicrobial peptides, but without the characteristic decrease in carotenoid pigment formation. Furthermore, we show that *mpsB* contributes to toxicity and the activity of the Agr system. A graphical summary of this is provided in Fig. 6.

The lack of toxin production by SCVs is well established, and is considered to be a result of a lack of activation of the Agr system due to the effects of the SCV conferring mutations on metabolism, and the associated reduction in cell growth. While the impact of *mpsB* inactivation was sufficient to switch off Agr activity, we did not observe an effect on growth in broth (Fig. 4b), which led us to consider whether membrane potential was directly involved in Agr activity. Using the membrane potential inhibitor CCCP, our findings suggest that this may be the case, as we see an immediate drop in Agr activity upon exposure to this chemical (Fig. 5c). While numerous factors have previously been shown to impact on Agr activity, a direct role for membrane potential has not been suggested prior to this study, where we hypothesize that the effect may be mediated

through the effect membrane potential may have on the activity of the two critical membrane-located proteins of the Agr system, AgrB and AgrC.

By focusing on data from human infections, our aim is to unravel the pathogenic mechanisms utilized by *S. aureus* to cause disease. This approach has led to the identification of a number of MALs, and here we present the characterization of the first three of these, where their contribution to serum resistance is established. For *mpsB* we present further detailed characterization of its activity, where its partial SCV phenotype allows it to survive the double edged-sword that human serum represents for bacteria – rich in metabolites while also highly immuno-protected [28, 29]. While further work is underway to understand the role of the other MALs, this work provides an explanation for why the *mpsB* gene was associated with patient outcome following *S. aureus* bacteraemia.

Funding information
This work was funded by a PhD studentship to E.J.A.D., funded by the School of Cellular and Molecular Medicine, University of Bristol, and a BBSRC grant awarded to R.C.M., while R.C.M. is a Wellcome Trust-funded Investigator (grant reference number: 212258/Z/18/Z).

Author contributions
E.J.A.D., developed the methodology, performed experiments, analysed data and contributed to writing the manuscript. S.D., developed methodology, performed experiments, provided supervisory support, analysed data and contributed to writing the manuscript. T.B., developed methodology, provided supervisory support and contributed to writing the manuscript. R.C.M., conceptualized the projects, provided supervisory support, analysed data and contributed to writing the manuscript.

Conflicts of interest
The authors declare that there are no conflicts of interest.

References

1. Lowy FD. *Staphylococcus aureus* infections. *N Engl J Med* 1998;339:520–532.
2. Gordon RJ, Lowy FD. Pathogenesis of methicillin-resistant *Staphylococcus aureus* infection. *Clin Infect Dis* 2008;46:S350-9.
3. van Hal SJ, Jensen SO, Vaska VL, Espedido BA, Paterson DL, *et al*. Predictors of Mortality in *Staphylococcus aureus* Bacteremia. *Clin Microbiol Rev* 2012;25:362–386.
4. Lamagni TL, Potz N, Powell D, Pebody R, Wilson J, *et al*. Mortality in patients with meticillin-resistant *Staphylococcus aureus* bacteraemia, England 2004–2005. *J Hosp Infect* 2011;77.
5. Kang C-I, Song J-H, KS K, Chung DR, Peck KR. Clinical features and outcome of *Staphylococcus aureus* infection in elderly versus younger adult patients. *Int J Infect Dis* 2011;15.
6. Recker M, Laabei M, Toleman MS, Reuter S, Saunderson RB, *et al*. Clonal differences in *Staphylococcus aureus* bacteraemia-associated mortality. *Nat Microbiol* 2017;2:1381–1388.
7. Fan S-H, Ebner P, Reichert S, Hertlein T, Zabel S, *et al*. MpsAB is important for *Staphylococcus aureus* virulence and growth at atmospheric CO2 levels. *Nat Commun* 2019;10:3627.
8. Mayer S, Steffen W, Steuber J, Götz F. The *Staphylococcus aureus* NuoL-like protein mpsa contributes to the generation of membrane potential. *J Bacteriol* 2015;197:794–806.
9. Corrigan RM, Foster TJ. An improved tetracycline-inducible expression vector for *Staphylococcus aureus*. *Plasmid* 2009;61:126–129.
10. Andrews JM. Determination of minimum inhibitory concentrations. *J Antimicrob Chemother* 2001;48.
11. Fey PD, Endres JL, Yajjala VK, Widhelm TJ, Boissy RJ, *et al*. A genetic resource for rapid and comprehensive phenotype screening of nonessential *Staphylococcus aureus* genes. *mBio* 2013;4:e00537.
12. Duggan S, Laabei M, Alnahari AA, O'Brien EC, Lacey KA. Small membrane stabilizing protein critical to the pathogenicity of staphylococcus aureus. *Infect Immun* 2020:88-e00162
13. Morikawa K, Maruyama A, Inose Y, Higashide M, Hayashi H, *et al*. Overexpression of sigma factor, ςB, urges *Staphylococcus aureus* to thicken the cell wall and to resist *β*-Lactams. *Biochem Biophys Res Commun* 2001;288:385–389.
14. Stevens E, Laabei M, Gardner S, Somerville GA, Massey RC. Cytolytic toxin production by *Staphylococcus aureus* is dependent upon the activity of the protoheme IX farnesyltransferase. *Sci Rep* 2017;7:13744.
15. Bunting JR, Phan TV, Kamali E, Dowben RM. Fluorescent cationic probes of mitochondria. Metrics and mechanism of interaction. *Biophys J* 1989;56.
16. Kasianowicz J, Benz R, McLaughlin S. The kinetic mechanism by which CCCP (carbonyl cyanidem-Chlorophenylhydrazone) transports protons across membranes. *J Membr Biol* 1984;82:179–190.
17. Massey RC, Buckling A, Peacock SJ. Phenotypic switching of antibiotic resistance circumvents permanent costs in *Staphylococcus aureus*. *Curr Biol* 2001;11:1810–1814.
18. Sadowska B, Bonar A, Eiff C, Proctor RA, Chmiela M, *et al*. Characteristics of *Staphylococcus aureus*, isolated from airways of cystic fibrosis patients, and their small colony variants. *FEMS Immunology & Medical Microbiology* 2002;32.
19. Gläser R, Becker K, von Eiff C, Meyer-Hoffert U, Harder J. Decreased susceptibility of *Staphylococcus aureus* small-colony variants toward human antimicrobial peptides. *J Invest Dermatol* 2014;134.
20. McNamara PJ, Proctor RA. *Staphylococcus aureus* small colony variants, electron transport and persistent infections. *Int J Antimicrob Agents* 2000;14:117–122.
21. Laabei M, Uhlemann A-C, Lowy FD, Austin ED, Yokoyama M, *et al*. Evolutionary trade-offs underlie the multi-faceted virulence of *Staphylococcus aureus*. *PLoS Biol* 2015;13:e1002229.
22. von Eiff C, Heilmann C, Proctor RA, Woltz C, Peters G, *et al*. A site-directed *Staphylococcus aureus* hemB mutant is a small-colony variant which persists intracellularly. *J Bacteriol* 1997;179:15.
23. Peng HL, Novick RP, Kreiswirth B, Kornblum J, Schlievert P. Cloning, characterization, and sequencing of an accessory gene regulator (agr) in *Staphylococcus aureus*. *J Bacteriol* 1988;170:4365–4372.
24. Yamada Y, Shiota S, Mizushima T, Kuroda T, Tsuchiya T. Functional gene cloning and characterization of MDEA, a multidrug efflux pump from *Staphylococcus aureus*. *Biol Pharm Bull* 2006;29:801–804.
25. Huang J, O'Toole PW, Shen W, Amrine-Madsen H, Jiang X, *et al*. Novel Chromosomally Encoded Multidrug Efflux Transporter MdeA in Staphylococcus aureus. *Antimicrob Agents Chemother* 2004;48:909–917.
26. Cameron DR, Jiang J-. H, Kostoulias X, Foxwell DJ, Peleg AY. Vancomycin susceptibility in methicillin-resistant *Staphylococcus aureus* is mediated by YycHI activation of the WalRK essential two-component regulatory system. *Sci Rep* 2016;6:30823.
27. Sutton JAF, Carnell OT, Lafage L, Gray J, Biboy J. Staphylococcus aureus cell wall structure and dynamics during host-pathogen interaction. *PLoS Pathog* 2021:e1009468.
28. Peschel A. How do bacteria resist human antimicrobial peptides? *Trends Microbiol* 2002;10:179–186.
29. Psychogios N, Hau DD, Peng J, Guo AC, Mandal R. The human serum metabolome. *PLoS ONE* 2011:e16957.