Microbiology and Infectious Disease

Extensive re-modelling of the cell wall during the development of *Staphylococcus aureus* bacteraemia

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Abstract

Introductory Paragraph / Abstract

The bloodstream represents a hostile environment that bacteria must overcome to cause bacteraemia. To understand how the major human pathogen *Staphylococcus aureus* manages this we have utilised a functional genomics approach to identify a number of new loci that affect the ability of the bacteria to survive exposure to serum, the critical first step in the development of bacteraemia. The expression of one of these genes, *tcaA*, was found to be induced upon exposure to serum, and we show that it is involved in the elaboration of a critical virulence factor, the wall teichoic acids (WTA), within the cell envelope. The activity of the TcaA protein alters the sensitivity of the bacteria to cell wall attacking agents, including antimicrobial peptides, human defence fatty acids, and several antibiotics. This protein also affects the autolytic activity and lysostaphin sensitivity of the bacteria, suggesting that in addition to changing WTA abundance in the cell envelope, it also plays a role in peptidoglycan crosslinking. With TcaA rendering the bacteria more susceptible to serum killing, while simultaneously increasing the abundance of WTA in the cell envelope, it was unclear what effect this protein may have during infection. To explore this, we examined human data and performed murine experimental infections. Collectively, our data suggests that whilst mutations in *tcaA* are selected for during bacteraemia, this protein positively contributes to the virulence of *S. aureus* through its involvement in altering the cell wall architecture of the bacteria, a process that appears to play a key role in the development of bacteraemia.

eLife assessment

This **important** study uses an innovative GWAS approach and targeted testing to highlight *S. aureus* genes that modify susceptibility to serum, serum-derived antimicrobial products, and commonly used antibiotics. These findings are significant in that they highlight evidence of evolution of virulence determinants in the setting of exposure to host stressors expected to be present during bacteremia and antibiotic therapy. **Compelling** results build on a foundation of work attributing loss-of-function mutations in tcaA to glycopeptide non-susceptibility.

Main Text

Staphylococcus aureus is an important human pathogen and a significant global health concern[(1), (2)]. The most common interaction with its human host is as an asymptomatic coloniser; however, it frequently transitions to a pathogenic state with the ability to cause a wide range of diseases, ranging from relatively minor skin and soft tissue infections (SSTI) to more life-threatening incidents of endocarditis or bacteraemia[(1), (2)]. *S. aureus* is notorious for producing a plethora of virulence factors ranging from pore forming toxins to various immune evasion strategies[(3)]. The toxicity of *S. aureus* is generally accepted as playing an important role during infection, with high toxicity isolates typically causing more severe symptoms and disease progression[(4), (5)]. However, for the more invasive diseases such as bacteraemia and pneumonia, it has been shown that the causative isolates are often impaired in their toxin production and instead rely on alternative virulence approaches to cause disease, such as being able to better survive exposure to host immune defences[(6), (7)].

As the most severe type of infection caused by *S. aureus*, bacteraemia has a mortality rate of between 20 and 30%[(8), (9)], and despite infection control measures that have reduced the incidence of MRSA bacteraemia, the incidence of MSSA bacteraemia continues to increase in several countries, suggesting we have much to learn about this disease process[(10)]. Entry into the bloodstream is the first step in the development of bacteraemia and represents a major bottle neck for the bacteria, whether they seed from an infection elsewhere in the body, or take a more direct route through an intravenous device. As a heavily protected niche, the bloodstream contains numerous humoral immune features with potent anti-staphylococcal activity, including antimicrobial peptides (AMPs) and host defence fatty acids (HDFAs)[(11)–(13)]. However, the fact that cases of *S. aureus* bacteraemia occur demonstrates that these do not represent an impregnable force and that the bacteria can adapt to resist these defensive features.

In previous work we have pioneered the application of genome wide association studies (GWAS) to characterise bacterial virulence, where it has proven to be a powerful approach to define complex regulatory pathways[(7), (15)–(19)]. In related work we have also begun to characterise a new category of *S. aureus* genes we refer to as MALs (mortality associated loci) and found that many of these are involved in serum survival[(20)], with serum containing the bulk of the humoral immune response present in blood, but without the added complexity of the cellular components. Given the outstanding research questions surrounding the development of bacteraemia we performed a GWAS on a collection of 300 clinical isolates with respect to serum resistance. We found significant variability in how well individual isolates survive exposure to serum and identified seven novel effectors of this activity. Of particular note was the TcaA protein, which we have further characterised

and demonstrate that it is a critical protein for the bacteria involved in the remodelling of the bacterial cell wall during the development of bacteraemia.

Results

Survival of S. aureus upon exposure to human serum is a variable trait. The human bloodstream is a highly protected niche, and so to establish an infection in this environment the bacteria must evade many aspects of host immunity, such as the bacterial membrane damaging antimicrobial peptides (AMP) and host defence fatty acids (HDFAs) found in serum[(11)–(13), (21)–(24)]. To examine the level of variability that exists in serum-susceptibility in natural populations of *S. aureus*, we exposed 300 clinical isolates to pooled human serum and quantified the proportion of each culture that survived. These 300 isolates represent the two major clones, as defined by multi-locus sequence typing: clonal complex 22 (CC22) and clonal complex 30 (CC30), that are responsible for the majority of MRSA cases in the UK and Ireland[(16)]. The isolates were collected from 300 individual cases of bacteraemia. This collection is a unique resource as each strain has been sequenced, extensively phenotyped, and we have clinical metadata for many of the patients the bacteria were isolated from[(16)]. We found there to be similar levels of variability in the ability of the bacteria to survive exposure to human serum across the two CCs, both with a >10[(3)] fold difference between the most and least susceptible isolate with respect to the number of bacteria that survived (Fig. 1a &b).

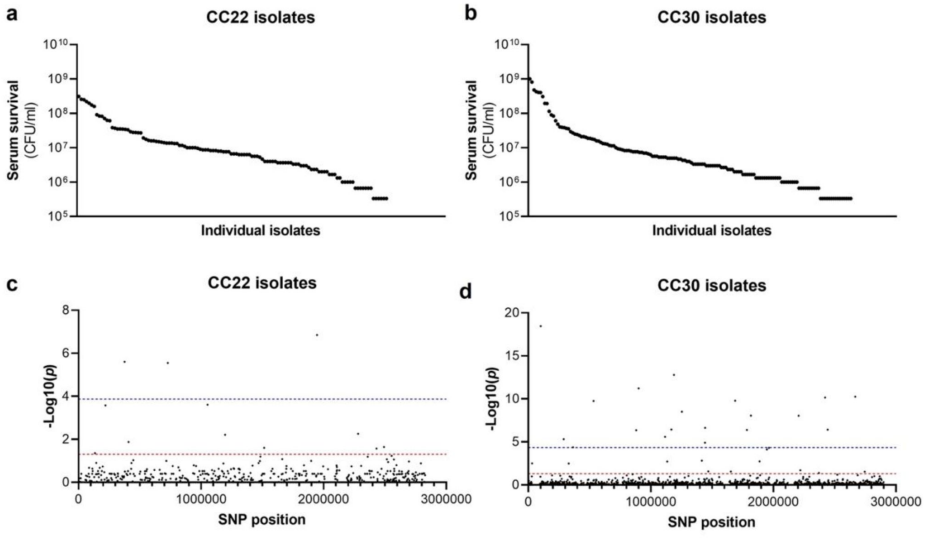

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Figure 1:

Serum susceptibility is multifactorial and varies widely across closely related *S. aureus* isolates. (**a & b**) The susceptibility of 300 bacteraemia isolates from clonal complexes CC22 and CC30 were exposed to human serum and their ability to survive this exposure quantified. The survival of each isolate was quantified in triplicate and dot represents the mean of these data. (**c & d**) Manhattan plots representing the statistical associations (on the y axis) between individual SNPs across the genome (on the x axis) and serum survival. The dotted red line represents the uncorrected significance threshold, and the dotted blue line represents the Sidak corrected (for multiple comparisons) threshold.

Survival of S. aureus upon exposure to human serum is a polygenic trait. As the genome sequence for each of the 300 clinical *S. aureus* isolates was available we performed a GWAS (genome wide association study) to identify polymorphic loci or single nucleotide polymorphisms (SNPs) that associated with the level of susceptibility of isolates to serum. For this, the data from the two distinct clones were analysed independently, with population structure within the clones being accounted for (Fig. 1c & d, Tables 1a & 1b). We applied both uncorrected and corrected (for multiple comparisons) significance thresholds to this analysis, as our previous work has demonstrated that the stringency of multiple correction approaches increases the likelihood of type II errors (i.e. false negative results). For the CC22 collection 12 loci were associated with serum survival and for the CC30s there were 32 associated loci (Fig. 1c & d, Tables 1a & 1b).

Tables 1:

Loci associated with serum survival in the CC22 (Table 1a) and CC30 (Table 1b) collections. The SNP position is relative to the origin of replication in the reference genomes: HO 5096 0412 (CC22) and MRSA252 (CC30). Locus tags and where available gene names or putative protein functions have been provided. NTML refers to the mutant available in the Nebraska transposon library.

Table 1a

SNP position	CC22 locus tag	Description	p Value	NTML
1945336	SAEMRSA15_RS09605	Intergenic	6.851546	N/A
375179	SAEMRSA15_03110	*metE*	5.606418	NE944
728154	SAEMRSA15_06310	*saeS*	5.553331	NE1296
1053036	N/A	Intergenic	3.60927	N/A
219764	SAEMRSA15_01760	putative oxidoreductase	3.575929	NE312
2280500	SAEMRSA15_20910	alcohol dehydrogenase	2.257841	NE1147
1196127	SAEMRSA15_10750	*yfhO*	2.212486	NE1597
408213	SAEMRSA15_03430	*guaA*	1.876101	N/A
2492381	SAEMRSA15_23120	*tcyB*	1.645759	NE228
1514441	SAEMRSA15_13420	hypothetical protein	1.600037	NE1349
2430758	SAEMRSA15_22540	*tcaA*	1.572702	NE285
134276	SAEMRSA15_01100	hypothetical protein	1.358386	NE599

Table 1b

SNP position	CC30 locus tag	Description	p Value	NTML
101174	SAR0097	putative DNA binding protein	18.466	N/A
1188605	SAR1143	*arcC*	12.786	NE1719
900409	SAR0856	phosphoglycerate mutase	11.226	NE1422
2667418	SAR2584	*gntR*	10.263	NE1124
2421610	SAR2347	putative membrane protein	10.160	N/A
1687821	SAR1615	*aroK*	9.793	N/A
532944	SAR0495	*veg*	9.751	NE1703
1252982	SAR1203	*recG*	8.502	NE1344
1816815	SAR1754	*clpX*	8.064	N/A
2206190	SAR2143	*ilvC*	8.050	NE1177
1443567	SAR1386	*trpA*	6.647	NE304
1167449	SAR1119	*uvrC*	6.439	NE1212
2442306	SAR2374	*ureC*	6.425	NE410
1784580	SAR1719	*tgt*	6.396	NE1885
880082	SAR0835	*est*	6.354	NE1122
1116463	SAR1070	*pdhD*	5.600	NE1610
289265	SAR0248	*tagB*	5.324	NE362
1442353	SAR1385	*trpB*	4.910	NE310
364861	SAR0319	NADH flavin oxidoreductase	4.373	N/A
1963781	SAR1877	*menE*	4.315	N/A
1946567	SAR1856	*arsB2*	4.122	NE1484
1416353	SAR1364	iron-sulfur cluster biosynthesis protein	2.829	NE1910
1887414	SAR1810	*fhs*	2.741	NE706
1133886	SAR0251	*tarF*	2.725	N/A
30969	SAR0023	*sasH*	2.507	NE295
330358	SAR0284	*essC*	2.486	NE1484
2220348	SAR2151	*tex*	1.704	NE1079
1468392	SAR1410	*hipO*	1.569	NE1688
1652613	SAR1582	*zwf*	1.556	N/A
2744774	SAR2656	*nrmA*	1.549	NE1223
2370812	SAR2287	*lacR*	1.392	NE346

Functional validation of the GWAS results identifies seven novel genes that affect serum survival. Of the 44 loci associated with serum survival, there were transposon mutants available for 32 in the Nebraska library[25]. To functionally verify our GWAS findings, each of these mutants were tested for their ability to survive exposure to human serum. We found seven mutants were significantly affected in this ability, and this effect was complemented by expressing the inactivated gene *in trans* (Fig. 2). The pRMC2 plasmid used to complement the mutated phenotypes requires the use of two antibiotics, one for selection of the plasmid and one for induction of gene expression[26]. These antibiotics can often affect bacterial gene expression. As such validation of complementation is based on the comparison between the empty and target gene containing plasmid which can be grown under the same conditions. The seven genes were *tcaA*, a gene associated with resistance to the antibiotic teicoplanin[27]; *tarK*, a gene involved in wall teichoic acid (WTA) biosynthesis[28]; *gntR*, which encodes gluconate kinase[29]; *ilvC*, which encodes ketol-acid reductoisomerase[30]; *arsB*, which is an efflux pump involved in arsenic resistance[31]; *yfhO*, which is involved in the glycosylation of lipoteichoic acid[32]; and *pdhD*, which encodes a lipoamide dehydrogenase[33].

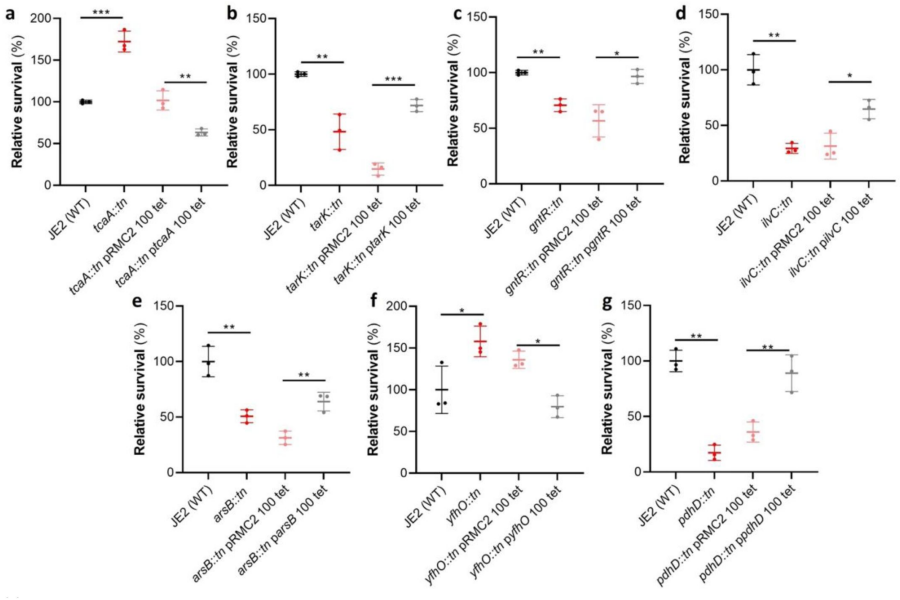

Figure 2:

Functional verification of loci involved in serum sensitivity of *S. aureus*. The effect of the inactivation of the genes associated serum sensitivity was examined using transposon mutants of strain JE2. Of the 32 mutants tested seven were significantly affected: (**a**) *tcaA*, (**b**) *tarK*, (**c**) *gntK*, (**d**) *ilvC*, (**e**) *arsB*, (**f**) *yhfO* and (**g**) *pdhD*. The effect of the mutations on serum sensitivity was complemented by expressing the gene from the expression plasmid pRMC2 (e.g. the *tcaA* complementing plasmid is called p*tcaA*). The dots represent individual data points, the bars the mean value, and the error bars the standard deviation. Significance was determined as * <0.05, ** <0.01, *** <0.001.

The SNPs in the tcaA *gene of the clinical isolates are associated with decreased serum sensitivity.* The *tcaA* gene has previously been reported to be involved in conferring increased sensitivity to the antibiotic teicoplanin[27], although the molecular detail of how it achieves this has yet to be determined, and our findings suggest it also confers increased sensitivity to serum (Fig. 2a). To visualise the signal detected by the GWAS for this gene we have mapped the position of the clinical isolates with the *tcaA* SNPs onto a figure displaying the range of serum sensitivities of the collection (Fig. 3a). The majority of the isolates with the SNPs were less sensitive to serum, suggesting the SNPs negatively affects the activity of the protein. The most common SNP in the *tcaA* gene amongst the clinical isolates conferred a Phe (290) to Ser change in the protein sequence. Using AlphaFold[34] we generated a structure for TcaA where it is predicted to be membrane bound with a single membrane spanning domain; to have a zinc finger presented intracellularly; with the majority of the protein including the Phe290 displayed extracellularly (Fig. 3b). Given that phenylalanines are frequently involved in protein-protein interactions[35] we examined the effect this change has on the activity of TcaA by cloning the *tcaA* gene with this SNP into the complementing pRMC2 plasmid and comparing its ability to confer serum resistance relative to that of the *tcaA* gene without the SNP. The TcaA protein with Ser at position 290 instead of Phe were slightly more resistant to serum killing, but not as affected as when the entire protein was inactivated, suggesting this change has a subtle effect on the serum sensitising activity of the protein (Fig. 3c).

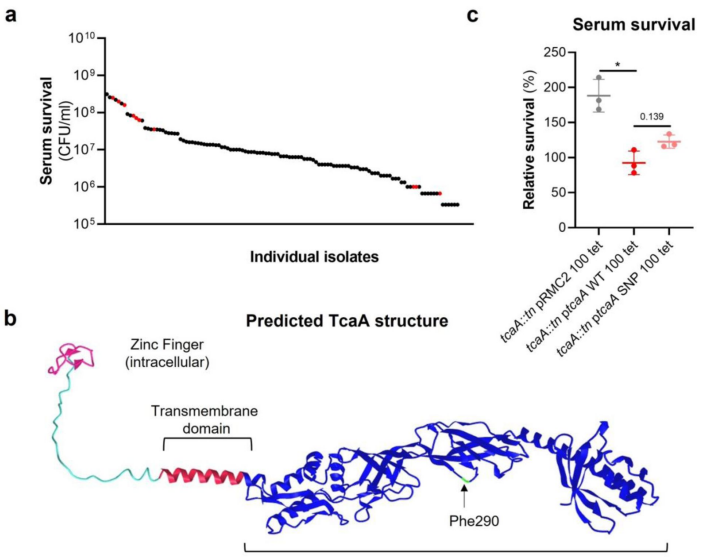

Figure 3:

The clinical tcaA *mutations associate with increased serum resistance*. **(a)** The individual clinical isolates with polymorphism in the *tcaA* gene are indicated (in red) on a graph displaying the range of serum survival of the collection. **(b)** The structure of the TcaA protein as predicted by AlphaFold. **(c)** The most common *tcaA* SNP decreases the sensitivity of *S. aureus* to serum. The dots represent individual data points, the bars the mean value, and the error bars the standard deviation.

TcaA confers increased sensitivity to multiple antibacterial components of serum. To examine whether the effect of TcaA on serum resistance was specific to the JE2 background we transduced the *tcaA::tn* mutation from JE2 into *S. aureus* strains SH1000 and Newman. Attempts were made to transduce this mutation into a clinical CC22 isolate, but this lineage was refractory to any of the available transducing phage[(36), (37)]. In all three backgrounds TcaA increased the sensitivity of the bacteria to serum (Fig. 4a) verifying that the effect was not limited to the JE2 background. To determine which aspects of human serum TcaA was conferring increased sensitivity to, we measured the relative ability of the wild type and mutant to survive exposure to some of the anti-bacterial factors found in serum: to the HDFA arachidonic acid, and to two AMPs: HNP-1 and LL37. The strains producing TcaA (i.e. the WT and complemented strain) were more sensitive to all three components of human serum (Fig. 4b-d). For the AMPs this sensitivity was significant after only 90 min exposure, whereas for arachidonic acid sensitivity the bacteria were incubated overnight, and differences in sensitivity determined by bacterial growth (OD_{600}).

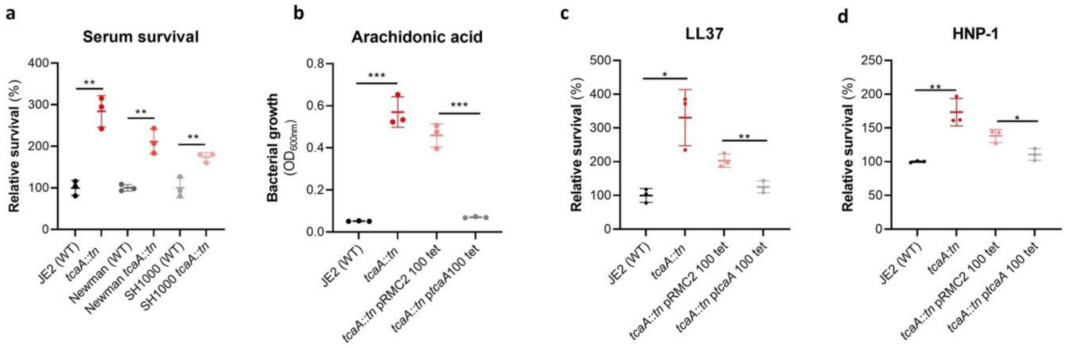

Figure 4:

TcaA production confers increased sensitivity to several antibacterial components of serum. **(a)** The inactivation of the *tcaA* gene decreases the sensitivity of three *S. aureus* strains (i.e., JE2, Newman and SH1000) to serum (90 min exposure). **(b)** TcaA production by the wild type and p*tcaA* complemented strain confers increased sensitivity to arachidonic acid (100μM) following overnight growth. **(c)** TcaA production by the JE2 wild type and p*tcaA* complemented strain confers increased sensitivity to LL37 (5μg/ml, 90 min exposure) and **(d)** TcaA production by the wild type and p*tcaA* complemented strain confers increased sensitivity to HNP-1 (5μg/ml, 90 min exposure). The dots represent individual data points, the bars the mean value, and the error bars the standard deviation. Significance was determined as * <0.05, ** <0.01, *** <0.001.

Although none of the individual antibacterial components of serum tested here are involved in *S. aureus* cell clumping, it is a possibility that some of the effects seen in serum is as a result of this activity, artificially amplifying the effect. To examine this the wild type and *tcaA* mutant were visually examined by microscopy in both PBS and serum where no differences in clumping was observed (Supplementary Fig. 1). Of the other GWAS identified genes, the *ilvC* mutant was less sensitive to arachidonic acid, the *yfhO* mutant was more resistant to HNP-1 and LL-37, while the *tarK, gntK, arsB and phdD* mutants were all more sensitive to HNP-1 and LL37 when compared to the JE2 wild type strain (Supplementary Fig. 2).

Wall teichoic acids contribute to the arachidonic acid sensitivity of TcaA producing strains. The antibacterial properties of HDFAs and AMPs share some common features in that they both penetrate through the cell wall and attack the bacterial membrane[(11)–(13), (21)–(23)]. AMPs are positively charged molecules that rely on the relatively negative charge across the bacterial cell envelope to penetrate, and thus resistance is frequently acquired by changing the charge across the cell wall such that the AMPs are repelled[(22), (23)]. Less is known about how HDFAs penetrate the cell wall; however, resistance is associated with changes in the abundance of wall teichoic acids (WTA) in the bacterial cell walls[(24)]. WTA are hydrophilic and when present in the cell wall they are believed to be protective by interfering with the penetration of the hydrophobic HDFAs through to the bacterial membrane[(24)]. It has also been shown that when the ligation of WTA to the cell wall is affected, such that they are instead released into the environment, this also decreases the sensitivity of the bacteria to HDFAs[(13)]. With distinct modes of action and means of accessing their target, it is intriguing to consider how TcaA may be contributing to increasing the sensitivity of *S. aureus* to both these types of anti-bacterial molecules.

To examine whether the TcaA associated increase in sensitivity to arachidonic acid is a result of an increase in the abundance of WTA in the bacterial cell we extracted and quantified WTA from the wild type and *tcaA* mutant, where we found that the cell wall of the wild type had significantly more WTA when compared to the *tcaA* mutant cell extract (Fig. 5a & b & c)). We next quantified WTA in the bacterial supernatant, as recent work on a *S. aureus lcpA* mutant demonstrated that another WTA related means of altering sensitivity to arachidonic acid is to release the WTA the bacteria surface[11]. We found significantly less WTA in the extract of the TcaA producing wild type strain compared to that of the *tcaA* mutant (Fig. 5a), which could explain the observed difference in sensitivity to HDFAs. It was unclear from the previous work on LcpA how the released WTA by the *lcpA* mutant affects arachidonic acid sensitivity, and here we demonstrate that this mutant, like the *tcaA* mutant, is also less sensitive to human serum (Fig. 5d). One hypothesis is that the WTA can either sequester or inactivate HDFAs in the environment surrounding the bacteria, thereby neutralising them. To test this, we harvested the supernatant of the wild type JE2, the JE2 *tcaA* mutant, and the JE2 *lcpA* mutant from overnight growth. These supernatants were used to supplement fresh broth (at 10%) into which the wild type JE2 strain was inoculated. In the absence of any arachidonic acid JE2 grew equally well regardless of the supernatant supplement (Fig. 5e). However, in the presence of the supernatant of both the *tcaA* and *lcpA* mutant (both of which contain an abundance of released WTA), JE2 was able to grow in the presence of arachidonic acid; whereas when the JE2 supernatant was used as the supplement the bacteria were unable to grow (Fig. 5e). To further verify that soluble WTA can neutralise arachidonic we performed WTA extractions from an isogenic wild type and mutant of the *S. aureus* strain LAC where the mutant has had the *tarO* gene deleted and consequently does not produce any WTA (Fig. 5e). The incorporation of the wild type LAC purified WTA extract to broth at 2% created an environment in which JE2 could grow in the presence of arachidonic acid, whereas the equivalent WTA extract of the *tarO* mutant, which contains no WTA, did not (Fig. 5e). Together these data demonstrate that WTA in the environment can neutralise arachidonic acid, and this provides a likely explanation for the increased sensitivity to HDFAs associated with TcaA production.

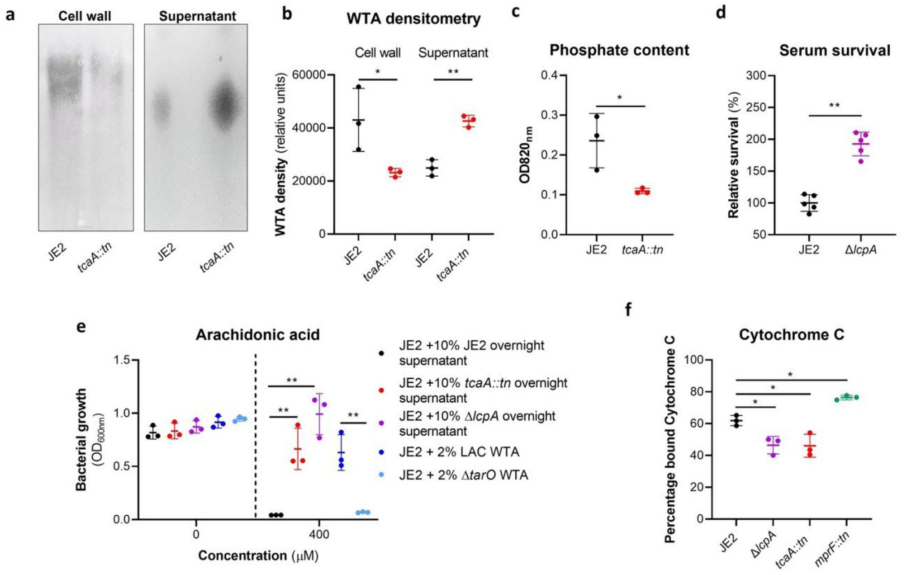

Figure 5:

Wall teichoic acids are released from the cell wall in the *tcaA* mutant to affect resistance to HDFAs and AMPs. **(a)** WTA was extracted from both cells and supernatant of the wild type and *tcaA* mutant of *S. aureus* and visualised on and SDS-PAGE gel stained with 1 mg/ml Alcian blue. The *tcaA* mutant had significantly less WTA in the cell wall but more in the supernatant. **(b)** The WTA extractions were performed on the wild type and tcaA mutant in triplicate the relative density of the WTA quantified by densitometry **(c)** The phosphate content of the cell wall WTA extracts was quantified, which verified that the *tcaA* mutant has significantly less WTA. **(d)** The inactivation of the *lcpA* gene increases the sensitivity of *S. aureus* to killing by serum. **(e)** The wild type strain JE2 was grown in broth supplemented (at 10%) with supernatant of either itself (JE2), with that from a *tcaA* mutant, or with that from a *lcpA* mutant. These

supernatants had no effect on the growth of JE2 in the absence of arachidonic acid. In the presence of arachidonic acid JE2 was unable to grow when supplemented with its own supernatant, however, when supplemented with the supernatant of the two mutants, which both contain soluble WTA, JE2 was able to grow. The addition of purified WTA extract (at 2%) from another *S. aureus* strain (LAC) also neutralised arachidonic acid, however an equivalent extract from an isogenic WTA mutant (LAC Δ*tarO*) did not. **(f)** The charge across the cell wall of the wild type and *tcaA* mutant was compared using cytochrome C, where the mutant was found to be less negatively charged. An *mprF* mutant has been included as a control. The dots represent individual data points, the bars the mean value, and the error bars the standard deviation. Significance was determined as * <0.05, ** <0.01, *** <0.001, **** <0.0001.

The reduced abundance of WTA in the bacterial cell wall contributes to the resistance of S. aureus to AMPs. In additional to being hydrophilic, WTA is also predominantly negatively charged[28]. Given that a change in charge across the bacterial cell wall is frequently associated with resistance to AMPs[21]–[23], and that the wild type strain has more WTA in its cell wall, this may explain the increased AMP sensitivity of the TcaA producing strain. To examine this, we incubated the bacteria with cytochrome C, which is positively charged and through its electrostatic-related ability binds to the bacterial cells, providing a measure of the charge across bacterial cell walls. Using this we found that the wild type strain bound significantly more cytochrome C and is therefore more negatively charged than the *tcaA* mutant, which explains the increase in AMP sensitivity we have found associated with TcaA production (Fig. 5e). Together these results explain how TcaA production affects the sensitivity of *S. aureus* to both HDFAs and AMPs. By retaining WTA within the cell wall, there is less released to sequester or inactivate the fatty acids from the environment, and due to their negative charge, increased amounts of WTA in the cell wall affects the charge across the cell wall and results in increased electrostatic attraction of the positively charged AMPs.

Serum induced tcaA expression increases both the abundance of WTA in the cell wall and the sensitivity S. aureus to teicoplanin. As mentioned previously, an additional interesting feature of the TcaA protein is that is has also been associated with changing the sensitivity of the bacteria to the antibiotic teicoplanin, although this has been reported to vary between strains[27], [38]. To examine this for our strains we determined the Minimum inhibitory concentrations (MICs) for three independent sets of isogenic wild type and tcaA mutants (ie in strains JE2, Newman and SH1000, all corresponding to CC8), where TcaA production resulted in increased sensitivity to this antibiotic across all three strains (Fig. 6a). To examine whether the clinical isolates with the SNPs in the tcaA gene associated with increased serum resistance also had altered teicoplanin sensitivity, we compared the ability of 12 clinical isolates containing the GWAS identified tcaA SNPs to grow in teicoplanin to that of 12 isolates from the same collection with the wild type gene sequence, i.e., without the polymorphism. The isolates with the polymorphism in the tcaA gene were on average less sensitive to teicoplanin (Fig. 6b). Using the pRMC2 complementing plasmid expressing either the wild type or SNP (conferring the Phe290-Ser substitution) containing tcaA gene, we demonstrated that this mutation significantly reduces the ability of the protein to sensitize the bacteria to teicoplanin (Fig. 6c), confirming that this mutation negatively affects the activity of the TcaA protein.

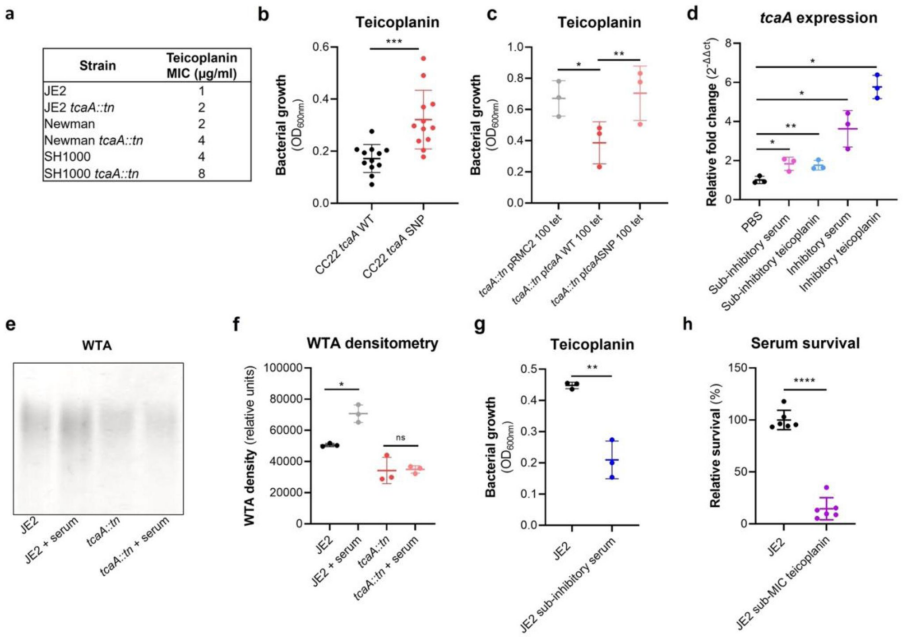

Figure 6:

TcaA confers increased sensitivity to teicoplanin. **(a)** The inactivation of the *tcaA* gene increases the MICs in three *S. aureus* backgrounds. **(b)** The clinical isolates with polymorphisms in the *tcaA* gene were on average less sensitive to teicoplanin (0.5µg/ml) than those with the wild type gene. **(c)** The most common *tcaA* SNP decreases the sensitivity of *S. aureus* to teicoplanin (0.5µg/ml). **(d)** The expression of the *tcaA* gene was quantified qRT-PCR in both subinhibitory and inhibitory concentrations of serum and teicoplanin. There was a dose dependent effect on *tcaA* induction for all concentrations used. (**e**) Exposure of JE2 to inhibitory concentrations of human serum resulted in an increase in WTA abundance in the bacterial cell wall, an effect not seen when the *tcaA* mutant was exposed to serum. (**f)** The WTA extractions were performed on the wild type and tcaA mutant in triplicate the relative density of the WTA quantified by densitometry. (**g**) Growth of JE2 in the presence of subinhibitory concentrations of teicoplanin (0.5 µg/ml) over a 24h period was quantified following pre-exposure to subinhibitory concentrations of human serum (2.5%), where preexposure to serum increased sensitivity to teicoplanin. (**h**) The ability of *S. aureus* to survive exposure to subinhibitory concentrations of serum (2.5%) following overnight growth in a subinhibitory concentration of teicoplanin (0.5 µg/ml), where preexposure to teicoplanin increases the sensitivity to serum. The dots represent individual data points, the bars the mean value, and the error bars the standard deviation. Significance was determined as * <0.05, ** <0.01, *** <0.001, **** <0.0001.

As TcaA has been shown to be upregulated when exposed to teicoplanin[27], [38], we examined whether serum would also induce its expression. The wild type JE2 strain was exposed to either sub-inhibitory (2.5% serum and 0.5µg/ml teicoplanin) or inhibitory (10% serum and 10µg/ml teicoplanin) concentrations of either serum or teicoplanin for 20 mins, total RNA was extracted and the transcription of the *tcaA* gene quantified by qRT-PCR. For both serum and teicoplanin, induction of expression of the *tcaA* gene was concentration dependent (Fig. 6d). Given the association of WTA abundance in the cell wall with *tcaA* expression described above (Fig. 5a-c) we next examined whether serum induced expression of *tcaA* would also result in an increase in abundance of WTA in the bacteria cell wall. Both the wild type and *tcaA* mutant were exposed to inhibitory concentrations of human serum for 90 mins and the WTA extracted from the cell walls, where serum exposure resulted in an increase in WTA for the wild type strain but not the *tcaA* mutant (Fig. 6e & f). To examine whether serum induced increased expression of *tcaA* would affect sensitivity to teicoplanin, we exposed the wild type bacteria to a sub-inhibitory concentrations of serum (2.5%) and then incubated these overnight in a subinhibitory concentration of teicoplanin (0.5µg/ml). Pre-exposure to serum induced increased sensitivity to teicoplanin suggesting that induction of *tcaA* expression by the initial serum exposure was sufficient to induce cross-sensitivity to the antibiotic (Fig. 6g). To put this sensitisation effect in the context of serum, the reverse experiment was also performed whereby the wild type strain was exposed to a sub-inhibitory concentration of teicoplanin (0.5µg/ml) overnight and a serum killing assay

performed following this incubation (Fig. 6h). Pre-exposure to teicoplanin also induced increased sensitivity to serum again highlighting this cross-sensitivity effect.

To verify this induction effect was not specific to the JE2 lineage a *tcaA::gfp* reporter plasmid was constructed and electroporated into JE2, SH1000, Newman and a clinical CC22 isolate EMRSA15. In each background serum, arachidonic acid and LL37 reduced *tcaA* expression with the exception of LL37 and the EMRSA15 strain (Supplementary. Fig. 3). These findings suggest that upon entry into the bloodstream and exposure to many of the antibacterial components of serum, expression of the *tcaA* gene is induced and this sensitizes *S. aureus* to killing by these components of serum.

The inactivation of tcaA *alters the antibiotic resistance profile of S. aureus.* There is emerging evidence that WTA play a role in cell division and peptidoglycan biosynthesis[39]. In support of this, it has been reported that a *tarO* mutant, devoid of WTA, confers sensitization to β-lactam antibiotics due to mislocalization of the PBPs[40]. As the target for teicoplanin is peptidoglycan, more specifically the terminal D-Ala-D-Ala residue of lipid II, we hypothesised that the increased ligation of WTA into the cell wall of the TcaA producing strain may affect peptidoglycan biosynthesis and assembly, and that this may be responsible for the change in sensitivity to teicoplanin. To test this further, we compared the sensitivity of the wild type and mutant to a range of peptidoglycan attacking antibiotics, where we found the TcaA producing wild type strain to be more sensitive to vancomycin (as shown previously[38]), oritavancin, but not dalbavancin (all alternative members of the glycopeptide class of antibiotics), and ramoplanin, a glycolipodepsipeptide antibiotic (Supp. Fig. 4). However, TcaA production conferred decreased sensitivity to oxacillin, a β-lactam antibiotic that targets penicillin binding protein 2 and moenomycin a phosphoglycolipid antibiotic that inhibits the transglycosylase PBP enzymes and prevents the formation of peptidoglycan polymers (Supp. Fig. 4). This suggests that TcaA production affects the overall composition and structure of peptidoglycan in the wildtype strain.

To examine whether the crosslinking of peptidoglycan is affected by TcaA production, we tested the susceptibility of the wild type and *tcaA* mutant to lysostaphin, which is a glycylglycine endopeptidase capable of cleaving the pentaglycine crosslinks of peptidoglycan[41]. We found that lysostaphin cleaved the cell wall of the wild type strain more slowly than for the *tca*A mutant (Fig. 7a). We next performed a triton X100 induced autolysis assay where the wild type exhibited a slower rate of autolysis compared to the mutant, suggesting that the cell wall is more robust due to higher levels of crosslinking when TcaA is produced (Fig. 7b). Together these data suggest that the integrity of the peptidoglycan, in particular its crosslinking, is increased when TcaA is produced. This provides a likely explanation for why the mutant is more resistant to teicoplanin, reduced crosslinking in the cell wall increases the number of off target D-ala D-ala which teicoplanin will bind to instead of reaching lipid II in the membrane and causing disruption to this. With changes to the quantity and composition of WTA and peptidoglycan associated with TcaA production, we sought to visualise the cell wall using transmission electron microscopy. We observed a clear difference in the density and structure of the outer layer of the bacterial cell wall between the wild type and *tcaA* mutant, confirming a role for TcaA in the structural integrity of the *S. aureus* cell wall (Fig. 7c, additional images in Supp. Fig. 5)).

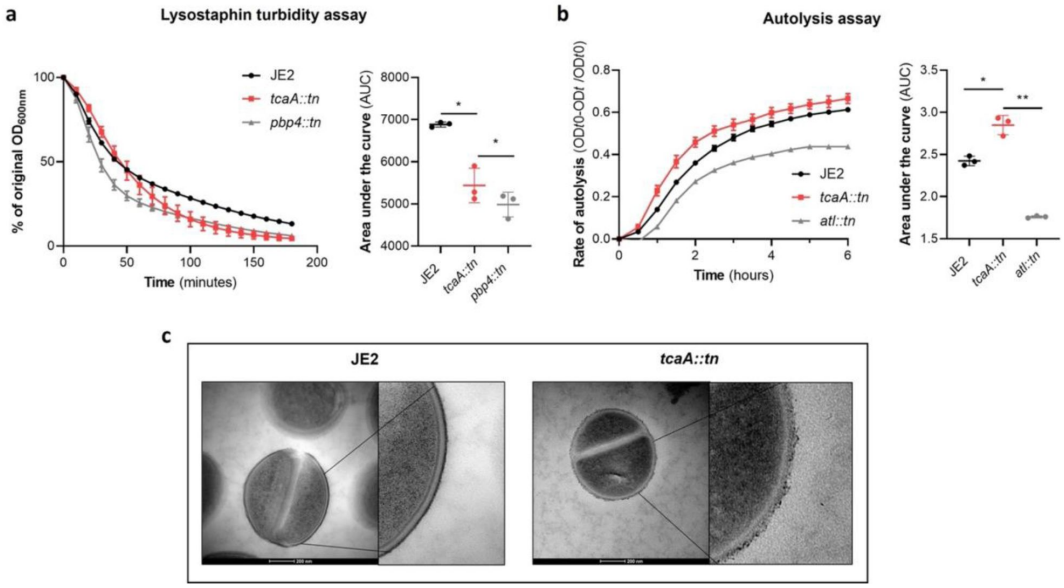

Figure 7:

TcaA alters the structure of the *S. aureus* cell wall. **(a)** The rate of lysis in the presence of lysostaphin was assayed, where the strain producing TcaA was lysed at a slower rate than the *tcaA* mutant. A *pbp4* mutant was included as a control. Turbidity (OD_{600}) and area under the curves (AUCs) of the OD_{600} data over time are presented. **(b)** The rate of autolysis of the bacteria in the presence of Triton x100 was assayed where the TcaA producing strain lysed at a slower rate relative to the *tcaA* mutant. An autolysin (*atl*) mutant was included as a control. Autolysis data and area under the curves (AUCs) of the autolysis data over time are presented. The dots represent individual data points, the bars the mean value, and the error bars the standard deviation. For both **(a)** and **(b)** significance was determined as * <0.05, ** <0.01 following area under the curve (AUC) analysis of three biological replicates **(c)** Transmission electron micrograph (TEM) of a wild type JE2 and *tcaA* mutant cell at two magnifications showing the smooth and consistent density of the cell wall when TcaA is produced, compared to the rough and patchy density of the mutant cell wall.

Once established, TcaA contributes positively to the development of bacteraemia. Our data shows that expression of *tcaA* is induced upon exposure to serum, that this can sensitize the bacteria to serum killing but also increase the abundance of WTA in the cell envelope. From a pathogenicity perspective these activities would appear to be in conflict, with one making it less likely for a bacterium to cause bacteraemia (increased serum sensitivity) and the other making it more likely for a bacterium to cause bacteraemia (increased WTA in the cell envelope[42], [43]). To understand this apparent dichotomy we first examined whether TcaA production only affected survival in serum, whereas in whole blood where other components of the human immune system may be present (e.g. complement, antibodies and phagocytes) that this effect may be lost. Using human blood from three donors, we demonstrated that TcaA production increases the sensitivity of the bacteria to whole blood killing (Fig. 8a). We next interrogated our human clinical data, where in addition to the 300 bacteraemia strains that form the basis of this study we have an additional 176 *S. aureus* isolates, which has allowed us to include more genetic diversity to this analysis (i.e. from CC22 (n=138), CC30 (n=162), ST8 (n=132) and ST93 (n=44)). The source of these isolates was also available to us (i.e. bacteraemia (n=341), SSTI (n=80) or carriage (n=55)), as well as their

genome sequences (Supplementary Table 1). We examined the distribution of nonsynonymous mutations in the *tcaA* gene across these, where it was present in 6% of the bacteraemia isolates compared with only 1.5% of non-bacteraemia isolates (n= 21/341 and 2/135 respectively; p = 0.03, in a Fisher's Exact test). However, when we examined the 30-day mortality rates for the 113 bacteraemia patients for whom we had this clinical data, the mortality rate of the proportion of patients infected with a *S. aureus* strain with the wild type *tcaA* gene was significantly higher compared to those infected with a mutated *tcaA* gene (Fig. 8b & c) (2-tailed chi squared test: p = 0.03). So, although the incidence of mutations in *tcaA* appear to be relatively rare, that isolates with variant *tcaA* genes are enriched amongst those causing bacteraemia suggests that a functional TcaA may limit the propensity of *S. aureus* to cause a bloodstream infection, such that it acts as a selective force for mutants of this gene. However, once the bacteraemia has become established, TcaA positively contributes to the infection process, likely due to the increased abundance of WTA in the cell envelope, and the greater structural integrity of the cell wall.

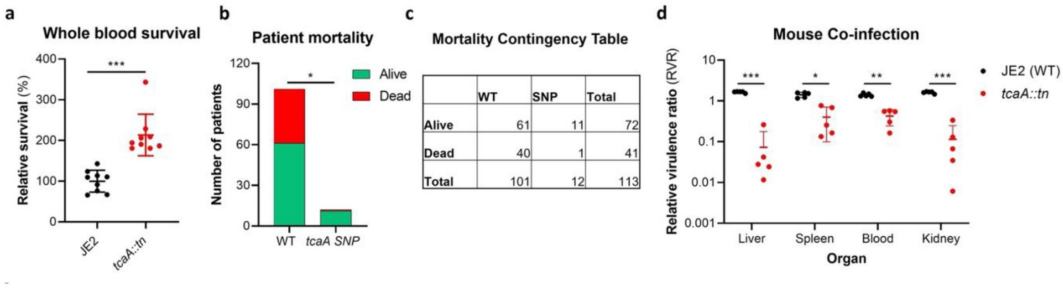

Figure 8:

TcaA contributes to increased disease severity in mice and humans. **(a)** Whole human blood survival. The wild type JE2 and tcaA mutant were incubated in human blood from three donors (each in biological triplicate) for 90 minutes and their relative survival was quantified. TcaA production renders that bacteria more susceptible to killing. The dots represent individual data points, the bars the mean value, and the error bars the standard deviation. **(b & c)** A graph and contingency table showing data from 113 patient with *S. aureus* bacteraemia. The 30-day mortality rate was significantly higher for those infected with a *S. aureus* strain with a wild type *tcaA* gene compared to those with a mutated *tcaA* gene. **(d)** Mice were injected intravenously with 100 µl of PBS containing an equal mixture of wild type and *tcaA* mutant (2 x $10^{(7)}$ CFUs of each). After 48 hrs, the ratio of the wild type and mutant were quantified and the relative virulence ratio calculated. In the blood and all organs tested the wild type bacteria had a competitive advantage over the mutant demonstrating its increased relative virulence. The dots represent individual data points (n=5 per group), the bars the mean value, and the error bars the standard deviation. Significance was determined as * <0.05, ** <0.01, and *** <0.001.

To experimentally test these human findings we utilised a mouse model of bacteraemia where the tail vein of C57/Bl6 mice are inoculated with 2 x $10^{(7)}$ colony forming units (CFU) of bacteria to establish a bloodstream infection, where they seed from there into organs such as the liver spleen and kidneys. We performed mixed infections, where the wild type and mutant bacteria had to compete during the development of the infection within the mouse. The mice were infected with equal quantities of both bacteria and the change in ratio of the bacteria in the mouse blood, liver, spleen and kidney recorded after 48hr, which allowed the relative virulence ratio (RVR) of each bacterium to be calculated[(44)]. The RVR of the TcaA

producing wild type strain was significantly higher than that of the mutant at a systemic level (Fig. 8d). As such, while the TcaA associated self-sensitization of the bacteria was not evident here, possibly due to the size of the inoculum level needed, once an infection has become established, TcaA contributes positively to disease severity as demonstrated by the wild type strain outcompeting the *tcaA* mutant.

Discussion

To develop novel therapeutic approaches for infectious diseases, we need to understand how microorganisms cause disease and evade the host's immune system. To address this, we have applied a population-based approach to analyse the pathogenicity of *S. aureus* and identified seven genes that affect the bacteria's ability to survive exposure to human serum, the first step in the development of bacteraemia. The dissection of the molecular detail of how these genes affect serum survival is underway to determine their potential for therapeutic intervention. However, our focus here has been on the *tcaA* gene where we show that its expression is induced upon exposure to serum, that it is involved in the ligation or retention of WTA in the cell wall, and that this renders the bacteria more susceptible to killing by both AMPs and HDFAs. We have also found that TcaA is associated with changes to the crosslinking of peptidoglycan, such that the bacteria are more susceptible to some antibiotics (e.g., teicoplanin) and less susceptible to others (e.g., oxacillin). It is interesting to note that our GWAS approach identified *tcaA* due to the presence of function altering SNPs amongst clinical isolates. Our human data suggests that bacteraemia and/or the antibiotics commonly used to treat it such as teicoplanin and vancomycin may impose a selective pressure on the bacteria to mutate this gene, due to the decreased sensitivity this confers to serum killing and to clinically relevant antibiotics. However, both our human and mouse data have confirmed, that once bacteraemia has become established, TcaA contributes positively to disease progression, likely due to its cell wall re-modelling activity. A graphical summary of this has been provided in Fig. 9.

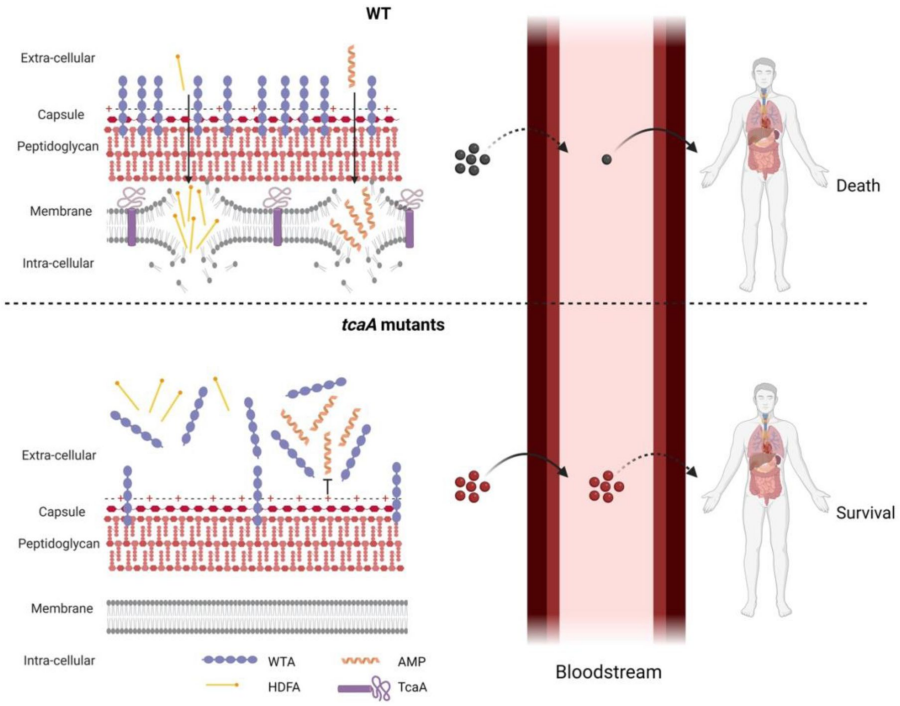

Figure 9:

Graphical summary of our hypothesis for TcaA activity**.** Upon exposure to serum the expression of *tcaA* is induced in the wild-type strain, resulting in an increased abundance of WTA in the cell wall and less in the extracellular milieu. This decreases the negative charge of the bacterial surface, rendering the bacteria more susceptible to AMPs. In addition, there is less WTA released into the extracellular milieu to sequester HDFAs, rendering the bacteria more susceptible to killing by these molecules. This self-sensitizing activity of TcaA limits the ability of *S. aureus* to establish a bloodstream infection. However, once established a strain with wild-type TcaA can cause more

severe disease. In *tcaA* mutants, there is lower abundance of WTA in the cell wall and a higher abundance in the extracellular milieu. This increases bacterial resistance to AMPs as it increases the charge of the bacterial surface, resulting in less electrostatic attraction. The release of WTA into the extracellular milieu also sequesters HDFAs, preventing them from reaching the bacterial surface. As a result, a *tcaA* mutant is more likely to establish a bloodstream infection. However, this infection is less likely to cause severe disease due to the importance of TcaA in remodelling the cell wall for the full virulence of *S. aureus*. (Image created using BioRender.com(http://biorender.com/)).

The *tcaA* gene is part of a three gene locus alongside the *tcaR* gene, which is predicted to encode a transcriptional regulator, and the *tcaB* gene which is predicted to encode an efflux pump[27]. This locus is also reported to be part of the *S. aureus* cell wall stimulon, following a number of studies that monitored the response of *S. aureus* to cell wall attack[45], [46]. It is interesting to note that the *tcaA* gene was not associated with serum resistance for the CC30 collection of isolates, however, there were very few isolates with variation in this gene amongst that collection such that they would have fallen below the minimum allele threshold. While work to understand the activity of each of the proteins encoded by the *tca* locus is underway, our data suggests that TcaA play a role in the ligation or retention of WTA within the cell wall, perhaps alongside the LcpA protein[13]. How this is achieved is yet to be determined. TcaA may play a direct role in ligating WTA to the cell wall; it may play a role in cell wall turnover and the subsequent release of WTA and other cell wall macromolecules into the external environment; or it may interact and subsequently interfere with other cell wall biosynthetic processes that are responsible for WTA ligation. It is interesting to note that *tarK*, which is one of the other six genes associated here with serum survival is also involved in the biosynthesis of WTA. Additionally, *yfhO* which encodes an enzyme that glycosylates lipoteichoic acid (LTA) is also involved in serum resistance[32], providing further evidence for the importance of teichoic acid associated molecules to this aspect of the biology and pathogenicity of *S. aureus*. For TcaR, we hypothesise that it likely controls the transcription of the co-transcribed *tcaA* and *tcaB* genes, and may directly respond to the external stimulus of teicoplanin and serum as reported here. What is less clear is what role TcaB plays, as efflux pumps are more typically associated with increasing the resistance of a bacterium to an antibiotic, or other toxic molecules.

In addition to *tcaA* this study has identified one other locus (*yfhO*) that negatively affects serum survival, and added a further five loci (*tarK, gntR, ilvC, arsB*, and *pdhD*) to the list of genes that contribute positively to serum survival by protecting the bacteria from HDFAs and AMPs. This leads us to the questions of why *S. aureus* has loci in apparent conflict with regards to the survival of the bacteria during bacteraemia. It is perhaps through the consideration of the other ways in which *S. aureus* interact with humans that we may understand this. The vast majority of interactions that occur between *S. aureus* and humans is as a commensal in the human nose from which it can readily transmit. The five identified protective genes may therefore also protect the bacteria during colonisation, which would confer a selective advantage, and it is just an unfortunate coincidence that they also confer increased survival in serum during the development of bacteraemia. While the focus of this work has been on the role of TcaA in serum, it is also worth considering what role it may have during colonisation of the nose, which involves the adherence of the bacteria to the nasal epithelium, to which WTA has a well-established role. It is therefore possible that TcaA enhances the ability of *S. aureus* cells to colonise the nose by retaining WTA within the cell wall. However, the role of TcaA in responsiveness to cell wall attack is as yet unclear, but perhaps it contributes to the competitiveness of *S. aureus* within the nasal microbiome in response to cell wall damage inflicted by competitors.

The evolution of the virulence of a pathogen is heavily dependent upon their mode of transmission, which for opportunistic pathogens raises interesting challenges. If within-host

invasiveness limits between-host fitness by preventing them from transmitting to a new host, there are likely selective pressures acting on bacterial populations to evolve means of limiting their ability to cause invasive disease. However, if increased invasiveness facilitates enhanced within-host fitness, as the bacteria multiply and spread throughout the body, the 'short-sighted evolution of virulence' hypothesis proposed by Levin and Bull[47] could apply and may explain situations like *S. aureus* bacteraemia. We propose that TcaA may represent a system at the cusp of both these scenarios, in that it responds to cell wall attack by serum by making itself more susceptible to killing by serum, thereby potentially limiting its ability to cause bacteraemia, which could have long-term between host benefits. However, once established in the bloodstream and TcaA has remodelled the cell wall, the short-sighted view of virulence hypothesis applies where the bacteria survive and thrive, allowing them to cause a successful bacteraemia.

Entry into the bloodstream to establish an infection has long been considered a significant bottle neck for the bacteria, but until now, the stringency of this bottleneck was believed to be a result of the onslaught of all the immune mechanisms present in blood. Here we suggest that *S. aureus* may contribute to this bottleneck by responding to serum induced cell wall damage by further sensitizing itself to this attack as part of the pathway towards increasing their pathogenic capabilities by remodelling the cell wall. Our findings presented here opens up an entirely novel aspect to the biology of a major human pathogen and brings into question the appropriateness of the term 'opportunistic' when we refer to such highly effective bacterial pathogens.

Materials and Methods

Bacterial Strains and Growth Conditions

A list of the genetically amenable bacterial strains and mutants can be found in Table 2, and the clinical strains are listed in Supplementary Table 1. All strains were cultured in Tryptic soy broth (TSB) for 18 h at 37°C with shaking. Nebraska transposon mutant library (NTML)[23] mutants were selected for using erythromycin (5 µg/ml). For the complemented pRMC2[45] strains, chloramphenicol (10 µg/ml) and anhydroustetracycline (100 ng/ml) were added to the media where indicated.

Table 2.

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	25
JE2 *tcaA:tn*	*tcaA* transposon mutant in JE2	25
JE2 *tcaA:tn* pRMC2	*tcaA* transposon mutant in JE2 transformed with empty pRMC2 vector	This study
JE2 *tcaA:tn* p*tcaA*	*tcaA* transposon mutant complemented with *tcaA* gene housed in pRMC2 expression plasmid	This study
JE2 *tarK:tn*	*tarK* transposon mutant in JE2	25
JE2 *tarK:tn* pRMC2	*tarK* transposon mutant in JE2 transformed with empty pRMC2 vector	This study
JE2 *tarK:tn* p*tarK*	*tarK* transposon mutant complemented with *tarK* gene housed in pRMC2 expression plasmid	This study
JE2 *gntR:tn*	*gntK* transposon mutant in JE2	25
JE2 *gntR:tn* pRMC2	*gntK* transposon mutant in JE2 transformed with empty pRMC2 vector	This study
JE2 p*gntR*	*gntK* transposon mutant complemented with *gntK* gene housed in pRMC2 expression plasmid	This study
JE2 *ilvC:tn*	*ilvC* transposon mutant in JE2	25
JE2 *ilvC:tn* pRMC2	*ilvC* transposon mutant in JE2 transformed with empty pRMC2 vector	This study
JE2 *ilvC:tn* p*ilvC*	*ilvC* transposon mutant complemented with *ilvC* gene housed in pRMC2 expression plasmid	This study
JE2 *arsB:tn*	*arsB* transposon mutant in JE2	25
JE2 *arsB:tn* pRMC2	*arsB* transposon mutant in JE2 transformed with empty pRMC2 vector	This study
JE2 *arsB:tn* p*arsB*	*arsB* transposon mutant complemented with *arsB* gene housed in pRMC2 expression plasmid	This study
JE2 *yfhO:tn*	*yfhO* transposon mutant in JE2	25
JE2 *yfhO:tn* pRMC2	*yfhO* transposon mutant in JE2 transformed with empty pRMC2 vector	This study
JE2 *yfhO:tn* p*yfhO*	*yfhO* transposon mutant complemented with *yfhO* gene housed in pRMC2 expression plasmid	This study
JE2 *pdhD:tn*	*pdhD* transposon mutant in JE2	25

JE2 *pdhD*:*tn* pRMC2	*pdhD* transposon mutant in JE2 transformed with empty pRMC2 vector	This study
JE2 *pdhD*:*tn* p*pdhD*	*pdhD* transposon mutant complemented with *pdhD* gene housed in pRMC2 expression plasmid	This study
RN4220	Restriction deficient mutant of 8325-4	48
DC10B	*Δdcm* mutant in *E. coli* DH10B (K12)	37
Mach1	One Shot™ Mach1™ T1 Phage-Resistant Chemically Competent *E. coli.*	Commercially available
SH1000	MSSA, laboratory strain, 8325-4 with a repaired rsbU gene; SigB positive.	49
SH1000 *tcaA*::*tn*	*tcaA* transposon mutant in SH1000	This study
Newman	MSSA, laboratory strain isolated from human infection	50
Newman *tcaA*::*tn*	*tcaA* transposon mutant in Newman	This study
tcaA::*tn* p*tcaA*SNP	*tcaA* transposon mutant complemented with *tcaA* gene harbouring F290S SNP housed in pRMC2 expression plasmid	This study
mprF::*tn*	*mprF* transposon mutant in Newman	25
Δ*lcpA*	In frame unmarked deletion of *lcpA* generated by allelic exchange	13
LAC	CA-MRSA USA300 type IV SCCmec	51
Δ*tarO*	*tarO* KO mutant in LAC	52
JE2 pSB2019:*tcaA*	JE2 transformed with the pSB2019:*tcaA* reporter fusion	This study
SH1000 pSB2019:*tcaA*	SH1000 transformed with the pSB2019:*tcaA* reporter fusion	This study
Newman pSB2019:*tcaA*	SH1000 transformed with the pSB2019:*tcaA* reporter fusion	This study
EMRSA15 pSB2019:*tcaA*	SH1000 transformed with the pSB2019:*tcaA* reporter fusion	This study

Serum & Blood Survival Assay

Normal human serum was prepared from blood obtained from 8 healthy volunteers using Serum CAT tubes (BD). On the day of collection the blood was allowed to clot for 30 mins at room temperature followed by 1h incubation on ice. Serum was extracted following two rounds of centrifugation at 700 x g at 4°C for 10 min. Serum from individual donors were pooled, aliquoted and immediately stored at -80°C, where each aliquot underwent only one round of freeze and thaw. The bacteria were grown overnight, and their density normalised to an OD_{600nm} of 0.1 and 20 µl used to incubate in 180 µl 10% pooled human serum (diluted in PBS) for 90 min at 37°C with shaking. Serial dilutions were plated on tryptic soy agar (TSA) to determine CFUs. The same number of bacterial cells inoculated into PBS, diluted, and plated acted as a control. Survival was determined as the percentage of CFU in serum relative to the PBS control. Relative survival was determined through normalisation to JE2,

which is presented ats 100% in the graphs. The survival of each bacterial isolate was measures in triplicate and the mean of these datal presented here.

Gwas

Genome-wide association mapping was conducted using a generalized linear model, with serum survival as the quantitative response variable. We accounted for bacterial population substructure by adding to the regression model the first two component from a *principal component decomposition* of SNP data for each set of clinical samples (CC22 and CC30). The first two components accounted for 32% and 40% of the total variance for CC22 and CC30, respectively. In both cases, three distinct clusters were identified. We further considered a third model where we used cluster membership as covariates in our regression model, where clusters were defined using K-means clustering analysis (setting K=3); this, however, yielded identical results to the one based on PCA components. In total, 2066 (CC22) and 3189 (CC30) unique SNPs were analysed, the majority of which were subsequently filtered out for exhibiting a minor allele frequency (maf) of <0.03, reducing the data to 378 and 1124 SNPs, respectively. The P-values reported in Tables 1a and b are not corrected for multiple comparison, however the Sidak method was used to correct for multiple comparisons and both significance thresholds are indicated in the Manhattan plots.

Genetic manipulation of bacterial strains

Wildtype genes were amplified by PCR from JE2 genomic DNA using the primers shown in Table 3 and KAPA HiFi polymerase (Roche). The PCR product was cloned into the tetracycline inducible plasmid pRMC2[26] using *Kpn*I and *Sac*I restriction sites and T4 DNA ligase (NEB). This was transformed into RN4220 and eventually into the respective NTML mutants through electroporation.

Table 3.

PCR primers used in this study.

Primer name	Primer sequence 5'-3'
tcaA FW	ataagcttgatggtaccaattgaagaggaacagaattgag
tcaA RV	tgaattcgagctcagatcatgacacatgcatcttatttaag
tarK FW	ataagcttgatggtacctttaataaaccgatggggg
tarK RV	tgaattcgagctcagatgcaagtaatatatgccaacattag
gntK FW	ataagcttgatggtaccgtatgtcgtgtaatgaaggagtg
gntK RV	tgaattcgagctcagatttctttctttcattttaattcaacc
ilvC FW	ataagcttgatggtacccgaaacacaaaatatttaattatttgg
ilvC RV	tgaattcgagctcagattaacaactcctcattgtaggtctatc
arsB2 FW	ataagcttgatggtaccgtcataaccttaagactggtgaatgc

arsB2 RV	tgaattcgagctcagatgagttgcctgtacatataaaataaattg
yfhO FW	ataagcttgatggtacctgaaagttgaggataatgttgtg
yfhO RV	tgaattcgagctcagatgtagcaacattatttttgtcttgc
pdhD FW	ataagcttgatggtaccgaattattattaatggaggggtaaaac
pdhD RV	tgaattcgagctcagatgcatgtcacatgttaacgatatctc

Site directed mutagenesis of the *tcaA* gene

Site directed mutagenesis was used to mutate the WT *tcaA* gene (Phe290) to the SNP *tcaA* (Ser290). Primers (5'' ttttcaagttTcaaaacgtatggtc 3", 5" atacgttttgaAacttgaaaatgcc 3") were designed to have a complementary overlap of 21 nucleotides at the 5' end, with the nucleotide to be mutated at the centre of the overlap (the primers had non complementary regions at the 3' end to facilitate annealing on the template). The p*tcaA* template was diluted to 33 ng/µl, and 1ul was amplified using these primers and Phusion DNA polymerase. The PCR product was subsequently checked by agarose gel electrophoresis and was treated with 1 µl of DpnI directly in the PCR mix for 1 h at 37°C. Then 1 µl of the mixture was used to transform One Shot™ Mach1™ T1 (ThermoFisher) competent cells by heat shock then into RN4220 and finally *tcaA::tn* by electroporation.

Phage transduction of the *bursa aurealis* transposon

The *tcaA bursa aurealis* transposon was phage transduced using the *S. aureus* phage Φ11[53]. The phage lysate of the donor strain (*tcaA::tn)* was prepared as follows: The donor strain was grown overnight in TSB for 18 h at 37°C with shaking. Two hundred microlitres of overnight culture was added to 25 ml of TSB containing 250 µl 1M $MgSO_4$, 250 µl 1M $CaCl_2$, 100 µl of Φ11 and left at 37°C with shaking until complete lysis was observed. The culture was subsequently pelleted at 12000 x *g* for 3 min. The supernatant containing the lytic phage was filtered sterilised and stored at 4°C. Next, recipient strains (Newman and SH1000) were grown overnight in 20 ml of LK broth (1% Tryptone 0.5% Yeast Extract, 0.7% KCl) and pelleted at 2500 x *g* for 10 min. The supernatant was removed, and the pellets resuspended in 1 ml of fresh LK broth. Two reactions were then set up, one containing phage and one as a non-phage control. To the tube containing the phage add: 250 µl of the recipient strain (SH1000 or Newman), 250 µl of phage lysate and 750 µl of LK broth containing 10 mM $CaCl_2$. To the tube containing the non-phage control add: 250 µl of the recipient strain (SH1000 or Newman) and 1 ml od LK broth containing 10 mM $CaCl_2$. Both reactions were incubated for 25 min at 37°C without shaking followed by 15 min at 37°C with shaking. Five hundred microlitres of ice cold 20 mM sodium citrate was added followed by centrifugation at 10000 x *g* for 10 min. Pellets were resuspended in 500 µl of 20 mM sodium citrate and incubated on ice for 2 h. One hundred microlitres of culture was plated out on TSA containing 5 µg ml^{-1} erythromycin to select for the transposon. For the SH1000 phage transduction 100 µl was also plated out on oxacillin TSA plates as an added control to ensure there was no contamination of the phage lysate with JE2 *tcaA::tn*.

Growth inhibition and MIC assays

To determine the relative inhibition of growth of the wild type and tcaA mutant by antibiotics a broth microdilution method was used[54]. Briefly, overnight cultures were normalised to an OD_{600nm} of 0.05 in cation adjusted Mueller Hinton broth (MHB++) and 20 µl of resultant suspension used to inoculate 180 µl of fresh MHB++ containing a 1:2 dilution

series of the respective antimicrobial agent. The ability of the bacteria to survive these 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 Incorporated, California, USA) and LL-37 (Sigma) susceptibility assays were performed as described previously[55]. Briefly, overnight cultures were normalised to an OD_{600nm} of 0.1 and incubated with 5 µg/mL of hNP-1 or LL-37 for 2 h at 37 °C. Serial dilutions were plated on tryptic soy agar (TSA) to determine CFUs. The same number of bacterial cells inoculated into PBS, diluted, and plated acted as a control. Survival was determined as the percentage of CFU on exposure to either LL-37 or HNP-1 relative to the PBS control. Relative survival was determined through normalisation to JE2.

mRNA extraction

Overnight cultures were back diluted to an OD_{600nm} of 0.05 in 50 ml of fresh TSB and grown to an OD_{600nm} of 2. Two hundred micro litres of either PBS, 25% serum (final concentration 2.5%, sub-inhibitory), 5 µg/ml teicoplanin (final concentration 0.5 µg/ml, sub-inhibitory), 100% serum (final concentration 10%, inhibitory), or 100 µg/ml teicoplanin (final concentration 10 µg/ml, inhibitory) was added to 1.8 ml of bacterial culture and incubated for 20 min. RNA was extracted by Quick-RNA Fungal/Bacterial Miniprep Kit (Zymo Research) according to the manufacturer's instructions. RNA integrity was checked by running 5 µl aliquot of the RNA on a 1% agarose gel and observing the intensity of the ribosomal RNA (rRNA). RNA samples were treated by TURBO™ DNase (Invitrogen) to eliminate any genomic DNA contamination. To verify that the samples were free from any DNA contamination, RNA samples were subjected to RT-qPCR alongside a no template control and 2.5 ng of a known JE2 genomic DNA and threshold rates compared.

qRT-PCR

To quantify the expression of the *tcaA* RT-qPCR was performed using the housekeeping *gyrB* gene as a control. Complementary DNA (cDNA) was generated from mRNA using qScript® cDNA Synthesis Kit (Quantabio). Following the manufacturers protocol the cDNA was used as a template for the RT-qPCR reaction. Primers used were as follows *gyrB* FW 5' ggtgactgcattagatgtaaac 3', *gyrB* RV 5' ctgcttctaaaccttctaatacttgtatttg 3', *tcaA* FW 5' tagtttgcgcttcaggtg 3', *tcaA* RV 5' tgtggacataaatttgatagtcgtc 3'. The RT-qPCR reaction was performed as follows: 10 µl 2x KAPA SYBR Mix, 1 µl of 10 µM forward primer, 1 µl of 10 µM reverse primer, 5 µl cDNA and RNase-free water up to a total of 20 µl volume. The RT-qPCR was performed on a Mic qPCR cycler (bio molecular systems) and the cycling conditions consisted of an initial denaturation step of 95°C for 2 min, followed by 40 cycles of two step cycling: 95°C 15 s, 60°C 1 min. RT-qPCR was carried out in technical triplicate for each sample and 3 biological repeats. The ratio of *tcaA* to *gyrB* transcript number was calculated using the using the $2^{-}(\Delta\Delta Ct)$ method[56].

WTA preparations

Crude WTA from murein sacculi was extracted for analysis by PAGE using adaptations of a previously described methodology[57], [58]. Overnight cultures were washed once in buffer 1 (50 mM MES, pH 6.5) followed by centrifugation at 5,000 g. Cells were resuspended in buffer 2 (4% [wt/vol] SDS, 50 mM MES, pH 6.5) and boiled for 1 h. Sacculi were centrifuged at 5,000g

and washed once in buffer 1, once in buffer 2, once in buffer 3 (2% NaCl, 50 mM MES, pH 6.5), once more in buffer 1 and finally resuspended in digestion buffer (20 mM Tris-HCl pH 8.0, 0.5% [w/v] SDS). To the digestion buffer suspension, 10 µl of proteinase K solution (2 mg/ml) was added and incubated on a heat block 50°C for 4 h at 1,400 rpm. Sacculi were centrifuged at 16,000 g and washed once in buffer 3, followed by 3 washes in dH_2O to removed SDS. Sacculi were responded in 0.1M NaOH and incubated for 16 h at room temperature at 1,400 rpm. Following the incubation, the sacculi were centrifuged at 16,000g, leaving the teichoic acids in the supernatant. 250 µl of 1 M Tris-HCL pH 6.8 was added to neutralise the NaOH and stored at -20°C.

WTA was also precipitated from the supernatant of overnight culture by adding 3 volumes of 95% ethanol and incubation at 4°C for 2 h. Precipitated material was separated by centrifugation at 16,000 g for 15 min, washed once in 70% ethanol and resuspended in 100 mM Tris-HCL (pH 7.5) containing 5 mM $CaCl_2$, 25 mM $MgCl_2$, DNase (10 µg/ml) and RNase (50 µg/ml) and incubated for 3 h at 37°C. The enzymes were heat inactivated at 95°C for 3 min. The supernatant WTA preparations were similarly stored at -20°C before being analysed by PAGE.

Wta page

WTA preparations were separated on tricine polyacrylamide gels using a BioRad tetra cell according to a previously described method[58]. The gels were separated at 4°C using a constant amperage of 40 mA under constant stirring until the dye front reached the bottom. Gels were washed 3 times in MilliQ H_2O followed by staining in 1 mg/ml Alcian blue overnight. Gels were subsequently destained in in MilliQ H_2O, until the WTA became visible and finally imaged.

Arachidonic acid supernatant conditioning

Overnight cultures of JE2, *tcaA*, and *lcpA* mutants were pelleted, and the supernatant saved. Growth inhibition assays were subsequently performed by adding 20 µl of OD_{600nm} 0.1 bacterial suspension to 180 µl 10% overnight supernatant diluted in fresh MHB++ containing 400µM of arachidonic acid (Sigma). Purified WTA extracts were also added to the growth media at a final concentration of 2%. The ability of the bacteria to survive the arachidonic acid was determined by quantifying bacterial growth (OD_{600nm}) following 24 h at 37°C using a CLARIOstar plate reader (BMG Labtech).

Cytochrome C binding assay

A cytochrome c binding assay was used to measure the relative surface charge of the bacteria[59]. Briefly, overnight cultures were normalized to an OD_{600nm} of 8. The bacterial suspensions were washed twice in MOPS buffer (20 mM pH 7.0) and finally resuspended in 200 µl of MOPS buffer. Samples were then combined with 50 µl of cytochrome C (equine heart Sigma, 2.5 mg/ml in MOPS buffer) and incubated for 10 min at room temperature. Finally, samples were pelleted (16,000 × g for 1 min) and 200 µl of supernatant read for absorbance at Abs_{530nm} using a SUNRISE Tecan microplate reader.

Construction of a TcaA reporter fusion

A *tcaA* promoter-*gfp* fusion (pSB2019:*tcaA*) was constructed to determine expression of *tcaA* in response to several antimicrobial components of serum. A region of 289 bp upstream of the *tcaA* gene[60] was amplified by PCR from JE2 genomic DNA using the primers

tcaA_promoter FW 5'-atatgaattcagtattagaagtcatcaatca -3' and *tcaA_promoter* RV 5'-atatccccgggtttcacctcaattctgttcct-3'. The pSB2019 vector was prepared by digesting pSB2031[61] from a previous study with EcoRI and SmaI to remove the RNAIII P3 promoter. The *tcaA* promoter region was also digested with EcoRI and SmaI and ligated into pSB2019 using T4 DNA ligase (NEB). This was transformed into DC10B[37], a strain of *E. coli* which mimics the adenine methylation profile of some *S. aureus* clonal complexes (including CC8 and CC22). The plasmid was then extracted and transformed into final strains via electroporation.

TcaA induction assay

S. aureus strains carrying the pSB2019:*tcaA* plasmid were grown overnight in TSB with 10ug/ml chloramphenicol. Each strain was then normalized to an OD_{600nm} of 0.05 in fresh TSB and subcultured to an OD_{600nm} of 0.5-0.6. Cultures were washed in PBS and concentrated to an OD_{600nm} of 1 in PBS. 100 ul of bacteria was combined with 100 ul of the appropriate antimicrobial compound (arachidonic acid or LL-37) in a black 96-well plate. GFP Fluorescence (485_{nm} excitation/520_{nm} emission/1000 gain) was measured in a PHERAstar FSX plate reader (BMG Labtech) over 2.5 h (readings every 30 min with 200 rpm shaking).

Lysostaphin turbidity assay

Overnight cultures were back diluted to an OD_{600nm} of 0.05 in 20 mL of fresh TSB and grown to an OD_{600nm} 0.5-0.7. Cultures were normalised to give 1 ml of OD_{600nm} 0.6. Lysostaphin was subsequently added to give a final concentration of 0.5 µg/mL. 200 µL of each strain was then added to a 96-well plate and incubated for 3 h at 37°C in a CLARIOstar plate reader. OD_{600nm} readings were taken every 10 min (500 rpm shaking before readings). Values were blank corrected according to 200 µL of PBS. The PBP4*::tn* mutant was included as a control.

Triton-X100 induced autolysis assay

Overnight cultures were back diluted to an OD_{600nm} of 0.05 in 20 mL of fresh TSB and grown to an OD_{600nm}0.5-0.7. Cells were washed once in ice cold water (13,000 rpm 1 min) and resuspended in autolysis buffer (water containing 0.1% Triton X-100) to give an OD_{600nm} of 1. 200 µL of each strain was then added to a 96-well plate alongside 200 µL of strains resuspended in water as a negative control and 200 ul of water as a blank. The *atl::tn* strain was used as a positive control. Strains were grown for 6 h at 37°C in a CLARIOstar plate reader. OD_{600nm} readings were taken every 30 min (500 rpm shaking before readings). The rate of autolysis was calculated as follows; (OD*t0*-OD*tn*/OD*t0*).

Transmission electron microscopy

S. aureus strains JE2 and the TcaA mutant were pelleted in microfuge tubes and fixed by resuspending in 2.5% glutaraldehyde in cacodylate buffer (pH 7.3) kept at fridge temperatures until fixation was complete and then stored in the fridge. Pellets were resuspended in a BSA/glutaraldehyde gel at 10-20°C which sets after the cells were re-centrifuged into a pellet. The pellets were postfixed with osmium ferrocyanide/osmium tetr oxide mix in cacodylate buffer, *en bloc* stained with uranyl acetate (2%) and Walton's lead aspartate solutions, prior to dehydration in ethanol and infiltration with propylene oxide and Epon resin mix. Embedded blocks were polymerised for 24-48 hours at 60°C. Sections (70nm) were cut on a Leica UC7 ultramicrotome and imaged using a FEI Tecnai T12 microscope.

In vivo intra-venous challenge model

For the mixed infection bacteraemia model, female, 7-8 week-old, C57BL/6NCrl mice (Charles River Laboratories) were used. Bacteria were prepared as described previously[62]. Briefly, 1:100 dilutions of overnight bacterial cultures were grown to mid-log phase (~2 h) in TSB with shaking at 180 rpm at 37 °C. Bacteria were then harvested, washed with PBS and adjusted spectrophotometrically at OD_{600nm} to obtain the desired bacterial concentration of ~4.8×10^{8} CFU/mL. A 50:50 mixture of JE2 and *tcaA::tn* were made and 100 µl was injected intravenously via the tail vein. The final CFU for each strain per mouse was ~2.4×10^{7} CFU. After 48 hours, terminal cardiac bleeds were performed on anesthetized mice and whole blood was transferred into heparin tubes (Sarstedt) and mixed to prevent coagulation. Mice were then euthanized by CO_2 inhalation. Organ homogenates of spleens, livers and kidneys were performed as previously described[63] and serial dilutions of each sample were plated onto TSA plates with and without erythromycin (5 µg/ml) for bacterial enumeration. The CFU of JE2 was calculated by subtracting CFU on TSA with erythromycin (*tcaA::tn* only) from CFU on TSA plates (JE2 and *tcaA::tn*).

Ethics statement

C57/Bl6 mice were bred in-house in Trinity College Dublin and C57BL/6NCrl mice were purchased from Charles River Laboratories. Mice were housed under specific pathogen-free conditions at the Trinity College Dublin Comparative Medicines unit and maintained under pathogen-free conditions in an Association for Assessment and Accreditation of Laboratory Animal Care (AAALAC)-accredited animal facility in NIAID, NIH, respectively. All mice were female and used at 6–8 weeks. All animal experiments were conducted in accordance with the recommendations and guidelines of the health product regulatory authority (HPRA), the competent authority in Ireland and in accordance with protocols approved by Trinity College Dublin Animal Research Ethics Committee and according to the regulations of NIAID's Division of Intramural Research Animal Care and Use Committee (DIR ACUC), animal study proposal LB1E. For the human serum collection, all healthy volunteers provided written informed consent and all methods and experimental protocols were carried out in accordance with the recommendations of the University of Bath, Research Ethics Approval Committee for Health. The present study was approved by the University of Bath, Research Ethics Approval Committee for Health [reference: EP 18/19 108].

Statistics

Paired two-tailed student t-test or One-way ANOVA (GraphPad Prism v9.0) were used to analyse the observed differences between experimental results. A p-value <0.05 was considered statistically significant and the Sidak method was used to correct for multiple comparisons.

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Conflict of Interests

we the authors declare no conflict of interest associated with the work described in this manuscript

Author Contributions

EJAD developed the methodology, conceptualized and performed the experiments, analysed data, provided supervisory support and contributed to writing the manuscript. NP developed methodology and performed experiments. TB developed methodology, performed experiments and provided supervisory support. DA performed experiments. ML provided materials, analysed data and provided supervisory support. MR developed methodology, analysed data and contributed to writing the manuscript. GYCC, RCH, RL performed serum resistance experiments with mouse serum and murine bacteraemia experiments and GYCC analyzed data. MO provided supervisory support and provided materials. EOB performed experiments and analysed data, RMM provided materials, analysed data and contributed to writing the manuscript. RCM conceptualized the project, provided materials, provided supervisory support, analysed data and contributed to writing the manuscript.

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Reviewer #1 (Public Review):

In this manuscript by Douglas et al, the investigative team seeks to identify Staphylococcus aureus genes (and associated polymorphisms) that confer altered susceptibility to human serum, with the hypothesis that such genes might contribute to the propensity of a strain to cause bacteremia, invasive disease, and/or death. Using an innovative GWAS-like approach applied to a bank of over 300 well-characterized clinical S. aureus isolates, the authors discover SNPs in seven different staphylococcal genes that confer increased survival in the setting of serum exposure. The authors then mainly focus on one gene, tcaA, and illustrate a potential mechanism whereby modification of peptidoglycan structure and WTA display leads to altered susceptibility to serum, serum-derived antimicrobial compounds, and antibiotics. One particularly significant finding is that the identified tcaA SNP is significantly associated with patient mortality, in that patients infected with the SNP bearing isolate are less likely to die from infection. It is therefore hypothesized that this SNP represents an adaptive mutation that promotes serum survival while decreasing virulence and host mortality. In a murine model of infection, the strain bearing the WT allele of tcaA is

significantly more virulent than the tcaA mutant, suggesting that the role of tcaA in bacteremia is infection-phase dependent.

This manuscript has many strengths. The triangulation of genomic analysis, patient outcomes data, and in vitro and in vivo mechanistic testing adds to the significance of the findings in terms of human disease. Testing the impact of mutating tcaA in multiple staphylococcal lineages and backgrounds also increases the rigor of the study. The identification of bacterial loci that impact susceptibility to both host antimicrobial compounds and commonly used antibiotics is also a strength of this work, given the evolutionary and treatment implications for such genes.

One moderate weakness is that the impact of the identified SNP in tcaA is only tested in some of the assays, whereas the majority of the testing is performed with a whole gene knockout. In some cases this results in more speculative conclusions that will require further testing to validate. All in all, this is an exciting manuscript that will be of interest to the broader research communities focused on staphylococcal pathogenesis, bacterial evolution, and host-pathogen interactions, as well as to clinicians who care for patients with invasive staphylococcal infection.

Reviewer #2 (Public Review):

The authors embarked on a study to identify SNPs in clinical isolates of S. aureus that influence sensitivity to serum killing. Through a phenotypic screen of 300 previously sequenced S. aureus bacteremia (SAB) isolates, they identified ~40 SNPs causing altered serum survival. The remainder of the study focuses of tcaA, a gene with unknown function. They show that when tcaA is disrupted, it results in increased resistance to glycopeptides and antimicrobial components of human serum.

They perform an elegant series of experiments demonstrating how a tcaA knockout is more resistant to killing by whole serum. arachadonic acid, LL-37 and HNP-1. They provide compelling evidence that in the absence of tcaA resistance to arachidonic acid is mediated through release of wall teichoic acids from the cell wall, which acts as a decoy and sequesters the fatty acid.

Similarly, they suggest that resistance to cationic antimicrobial peptides is through alteration of the net charge of the cell wall due to loss of negatively charged WTAs based on reduced cytochrome C binding.

They continue to show that tcaA is induced in the presence of human serum, which causes increased resistance to the glycopeptide teichplanin.

They propose that tcaA disruption causes altered cell wall structure based on morphologic changes on TEM and increased sensitivity to lysostaphin and increased autolysis via triton x-100 assay.

Finally, they propose that tcaA influences mortality in SAB based on raw differences in 30-day morality. Interestingly they do decreased fitness during murine bacteremia model compared to wild-type.

The strengths of this manuscript are that it is well written and the identification of SNPs leading to altered serum killing is convincing and valuable data. The mechanism for tcaA-mediated resistance to arachadonic acid and AMPs is compelling and novel. The murine infection data demonstrating that tcaA mutants exhibit reduced virulence is important data.

The weakness of this manuscript mainly concerns the proposed mechanism that tcaA mutants show reduced peptidoglycan crosslinking. This conclusion is based on qualitative TEM images and increased sensitivity to lysostaphyin/autolysis. While these data are suggestive. it is difficult to draw such a conclusion without analysis of the cell wall by LC-MS.

Overall, I think this is a good submission and the majority of their conclusions are supported by the data. The mechanism behind the clinically relevant tcaA mutation is important, given its known role in glycopeptide resistance and therefore likely clinical outcomes. This manuscript would benefit from the inclusion of some additional experiments to help support their finding.

Reviewer #3 (Public Review):

In this manuscript by Douglas et al., the authors used a functional genomics approach to understand how Staphylococcus aureus survives in the bloodstream to cause bacteraemia. They identified seven novel genes that affect serum survival. The study focused on tcaA, a gene associated with resistance to the antibiotic teicoplanin and is activated when exposed to serum and plays a role in producing a critical virulence factor called wall teichoic acids (WTA) in the cell envelope. This protein affects the bacteria's sensitivity to cell wall attacking agents, human defense fatty acids, and antibiotics, as well as autolytic activity and lysostaphin sensitivity. The data in this study suggested that TcaA play a role in the ligation or retention of WTA within the cell wall. However, more work is needed to clarify that part. Interestingly, despite making the bacteria more vulnerable to serum killing, tcaA contributes to S. aureus virulence by altering the cell wall architecture, as demonstrated by the wild type strain outcompeting the tcaA mutant in a Mouse Co-infection model. The study raises an important point that TcaA in S. aureus may represent a system balancing two scenarios: it makes the bacteria more susceptible to serum killing, potentially limiting bacteraemia and providing long-term benefits between hosts; however, once established in the bloodstream, the bacteria survive and thrive, causing successful bacteraemia, as per the short-sighted evolution of virulence hypothesis. This duality highlights the complex interplay between within-host and between-host fitness in bacterial evolution. I strongly suggest creating a graphical abstract to illustrate the complex relationship between within-host and between-host fitness scenarios involving TcaA. Having this visual representation in the discussion will enhance comprehension and provide a concise summary of the complex system for the reader.

In this manuscript, the authors achieved their aims, and the results support their conclusions. This work will be important for understanding this complex system and for developing novel therapeutics and vaccines for S. aureus.

Author Response:

The following is the authors' response to the original reviews.

We’d like to take this opportunity to thank the reviewers and editors for their consideration of our work. As detailed below, we have made the majority of the suggested corrections by the reviewers and believe these have greatly improved our manuscript. The reviewer’s comment are in blue font below and our response to each of these in black font.

> ***Reviewer #1 (Recommendations For The Authors):***
>
> *Suggestions to improve the manuscript:*
>
> *- Line 33 and 34: "This protein" is vague. Please reword to state whether you are referring to TcaA or to WTA*

This has been corrected in the revised manuscript (Line 33)

> *- Intro: It would be helpful to provide more rationale for testing serum as a surrogate to whole blood in the GWAS screen. Serum is obviously lacking components of the clotting cascade, and some of these components have antimicrobial functions. However, this is easily justified in the text- e.g. to avoid clumping during the screen, to focus only on serum-derived antimicrobial compounds, etc.*

This has been edited in the revised manuscript (Line 84-86)

> *- Line 120: Please state if the 300 clinical isolates represent 300 distinct patients, or if some of the isolates came from the same patient during sequential collections. If the latter, were there any instances in the which the tcaA SNP appeared during the course of infection?*

They each came from individual patients so we were unfortunately unable to look for within host events. This information has been added to the revised manuscript (line 104).

> *- Line 133: the closed parenthesis sign is missing after "CC22"*

This has been corrected in the revised manuscript (Line 135)

> *- Table 1a - NE1296 is misspelled as ME1296. Also there is a typo in the last entry of this table for the locus tag*

This has been corrected in the revised manuscript.

> *- Table 1b - the authors should comment (in the discussion) on the potential reasons why tcaA was not identified in the CC30 background.*

A comment to this effect has been added to the revised manuscript (Lines 553-59)

> *- Figure 2a - Why is the mutant with the empty complementation vector not significantly different from WT JE2?*

The most widely used and reliable expression plasmid for complementation of mutated phenotypes in S. aureus is the pRMC2 plasmid, which requires chloramphenicol selection and anhydrotetracycline to induce expression of the cloned gene. These antibiotics, and the presence of the plasmid often affect the expression of other genes by the bacteria (as noted by this reviewer). As such, to verify complementation of a mutation the comparison we make is between the strain containing the empty plasmid induced with anhydrotetracycline with a strain with the gene containing plasmid induced with anhydrotetracycline. In that situation, the only difference between those two strains under those conditions is whether the gene is expressed or not. A comment explaining this has been added to the revised manuscript (lines 149-153).

> *- Line 188: Statistical analyses should be applied to figure 3C, which also appears to be underpowered.*

P values have been added to this in the revised manuscript. We present data point of three biological replicates, which are the mean of three technical replicates, which we believe is sufficiently powers for this analysis.

> *- Figure 3 legend - Tecioplanin is mentioned in the title, but the data are not included here*

This legend title has been the revised (Line 193).

> *- Figure 4 - here is an example where testing the actual tcaA SNP could have been enlightening. For example, what if the selective pressure makes the SNP more relevant to a specific AMP or AA?*

While we agree that this would be an interesting experiment to perform, the complementing vector that we would need to use to compare the wild type and SNP contains gene requires antibiotics to select for the plasmid and another to induce expression. As such it becomes quite a complex and messy experiment where synergy between the antimicrobial agents would be likely, the results of which will be difficult to interpret.

> *- Lines 317-321 - Suggest moving this to discussion*

We have left this here as we felt it a necessary summation/explanation of the results described in that section. It is discussed again later in the discussion section.

> *- Line 341 - I believe "serum" should actually be "teicoplanin"*

This has been corrected in the revised manuscript (Line 342).

> *- Figure 6e - wouldn't it be more powerful to determine the WTA levels in the supernatants of these strains and conditions?*

We could have done this both ways, but we focussed here only on how TcaA ligates WTA into the cell wall in the presence of serum.

> *- Figure 6 - What is the explanation for the different growth yields for JE2 in tecioplanin in panel A versus panel F? Are these actually two different concentrations? If so, please update the figure legend and the methods.*

The concentration used for the A was inhibitory and for F sub-inhibitory. To improve the clarity of this we have now used a table displaying the MICs for the six strains as panel A. We have also included the concentration of teicoplanin used for each experiment in the legend.

> *- Line 413: Consider more precise language than "the cell wall is stronger". E.g. More crosslinks?*

This has been edited in the revised manuscript (Line 421)

> *- Line 415: Consider changing "altered" to a directional term such as increases. It can be difficult for the reader to follow the expected change when you are discussing how the lack of a gene versus the presence of a gene changes susceptibility in one direction and another phenotype in the opposite direction.*

This has been edited in the revised manuscript (Line 423).

> *- Figure 7: The conclusions made from panels A and B need to be supported by statistical analyses. It is unclear if these lines are truly different from one another.*

These have been included in the revised fig 7.

> *- Line 426: I believe "tcaA" is missing following "producing"*

This has been corrected in the revised manuscript (Line 434).

> *- Line 446: "increase" to "increases"*

This has been corrected in the revised manuscript (Line 460).

> *- Figure 8C: if one goal of the mouse experiment was to look at survival during transit in whole blood, earlier timepoints are indicated based on the described kinetics of bloodstream dissemination in this model.*

The primary goal of this experiment was to see if TcaA contributed positively or negatively to the development of the infection. Work on this protein is ongoing, and so we hope in coming years to be able to provide more detail on its activity in vivo.

> *- Line 506: "changes to the structural integrity of peptidoglycan" seems overstated without additional studies.*

This has been edited in the revised manuscript (Line 524).

> *- Line 564: "represents" to "represent"*

This has been corrected in the revised manuscript (Line 603).

> *- Line 588: The figures all refer to "100 net". Please confirm the concentration used.*

This has been corrected in the revised manuscript (Line 628).

> *- Line 609: This refers to capsule production? Is this a copy error from a prior paper?*

Yes it is, and has been corrected in the revised manuscript (Line 650).

> *- Line 763: Please provide the concentrations of arachidonic acid used for each experiment.*

This has been included in the revised manuscript (Line 805)

> *- Line 836 and 837: This mentions a time course for blood culture from the infected mice. Where are these data?*

Apologies, this is another cut and paste mistake from another paper, and had been removed.

> *- Line 870: please discuss how multiple comparisons testing was handled.*

This has been included in the revised manuscript (Line 908).

> *- Supplemental figure 5 - Please add statistical analyses to support the conclusions in the manuscript. For example, there appears to be no differences for dalbavancin. Please also italicize tcaA in the legend.*

These have been included and corrected in the revised manuscript.

Reviewer #2 (Recommendations For The Authors):

> *Line 65 - I would suggest adding the reference (doi: 10.1128/Spectrum.00116-21), which shows increased mortality in S. aureus bacteremia patients due to agr deficient isolates.*

The suggested manuscript shows this effect of Agr dysfunction to be limited to patients with moderate to severe SOFA scores. As such it would require a nuanced description here that we think will detract from the flow of the introduction.

> *Line 68 - Please add DOI: 10.1016/j.cmi.2022.03.015 as a reference to support the mortality rate in S. aureus bacteremia. A systematic review and meta-analysis provides the highest level of evidence, and this is a contemporary study performed in 2022*

This has been included in the revised manuscript (Line 68).

> *Line 70 - please add supporting reference for this statement*

This has been included in the revised manuscript (Line 70).

> *Figure 2 - This image is low quality and appears pixelated. Please revise*

This has been replaced with a higher resolution image in the revised manuscript.

> *Figure 3c Also appears slightly pixelated*

This has been replaced with a higher resolution image in the revised manuscript.

> *Line 173 - I think it would helpful to mention the catalytic activity encoded by tcaA (aside from mediating sensitivity to glycopeptides) is unknown.*

This has been included in the revised manuscript (Line 174)

> *Line 174 - also confers sensitivity to vancomycin 2023 eLife. https://doi.org/10.1128/AAC.48.6 .1953- 2023 eLife. 1959.2004(https://doi.org/10.1128/AAC.48.6.1953-1959.2004)*

This has been included in the revised manuscript, albeit at a later point than suggested here (Line 406)

> *Line 209 - did the authors test any other antimicrobial fatty acids such as palmitoleic acid? If common mechanism would also expect decreased sensitivity to other HDFA*

No, we focused on arachidonic acid as this is the most relevant antimicrobial fatty acid in serum and it is produced by neutrophils and macrophages during the inflammatory burst.

> *Figure 4a-D: it would be useful to know what the MIC to these different components is and how that MIC relates to the concentration in human serum*

We do not have MICs for all of these compounds tested here but can confirm that the concentrations used are physiologically relevant.

> *Figure 4b - Can you mention in the legend how the killing assays varied for arachadonic acid versus the other AMPs? I am not immediately clear how this experiment was performed, despite referring to methods*

This has been included in the text of revised manuscript (Line 211-213) and the figure legend.

> *Figure 5 - there is no panel D*

This has been corrected in the revised manuscript.

> *Figure 6a: Lines 328-329 state the experiment was performed in the MIC for each strain. The legend (line 374) states 0.5 ug/ml teicoplanin was used, which is below the MIC for all of the strains tested per supp table 2. Please correct this discrepancy.*

This figure has been revised and the additional information included to improve the clarity of this section in the revised manuscript.

> *Figure 6a: On line 328, the authors state that the tcpA knockout increases the MIC for teicoplanin in each background. Figure 6a is performed in the presence of teicoplanin at 1x the MIC of the wild type (which will be below the MIC for the knockout). Therefore, we know each tcpA mutant will be able to grow in the presence of sub-mic concentrations of teicoplanin. Would a more informative way of conveying this information be to have MIC on the Y axis and background on the X axis?*

This has been corrected and clarified in the revised manuscript with a table showing the MICs (fig. 6a).

> *Figure 6b-c: Similarly, would it be more helpful to show how the MIC varies with the different clinical isolate tcpA mutants?*

While MICs have uses in clinical setting, they are a relatively crude and binary (growth V no growth) way to measure and compare sensitivity. For these two groups of isolates the MICs did not vary, which is why we used a concentration that sat that the threshold and quantified growth of all the isolates in this. This information has been added to the legend.

> *Figure 6e: The figure legends instructs us to refer to supplemental figure 3 to see the densiometry results. However, Figure 6e appears to be 4 conditions (WT and mutant +/- serum) and only examines the cell wall, whereas the supplemental figure refers to two conditions (WT + mutant) and looks at the cell wall and supernatant. I would recommend providing the densitometry data associated with the conditions in figure 6e, especially as differences seem more subtle by eye.*

This has been included in the revised manuscript (fig. 6f)

> *Line 689-691 - description of teicoplanin concentrations used in figure 2. However, no teicoplanin was used in figure 2. Assume is referring to a different figure (figure 6?)*

This has been corrected and clarified in the revised manuscript. Line 724.

> *Please add a section in the methods describing how the MIC was determined for JE2, SH1000 and Newman. Was it performed in CA-MHB or the media that the experiment in figure 6a was performed in. Serum can alter the MIC of several antibiotics*

This has been corrected and clarified in the revised manuscript. Line 724-29.

> *Please add a section to the methods describing the whole blood killing assay, ideally describing how the blood was not frozen and used same day as venipuncture. This is important as freeze/thaw or time periods >12 hours are likely to severely effect the function of phagocytes, especially neutrophils.*

This has been corrected and clarified in the revised manuscript. Lines 635-639

> *Line 588: ng/ul should read ng/µl*

This has been corrected in the revised manuscript too ng/ml. Line 628

> ***Reviewer #3 (Recommendations For The Authors):***

We have now included a graphical abstract (Fig. 9)

> *Major:*
>
> *1- Line 102: I was not able to find the accession numbers of these 300 genomes, did the authors submit it to any public repository (e.g. NCBI)?*

These were submitted previously to a public repository and the associated reference cited, but we have provided these in supplementary Table 1.

> *Minor:*
>
> *1 - Typo in line 133. Fix parenthesis after CC22.*

Corrected.

> *2 - Typo: Fix figure 5 panels (5e should be 5d).*

Corrected.

> *3 - Line 276: It is not clear why the extract for this experiment was supplemented at 2% while the other part of the experiment was done with 10%. Clarification is needed.*

The experiments at 10% was using overnight supernatant, whereas those with 2% was a purified WTA extract. This has been clarified in the revised manuscript (lines 283 and in the figure legend)

> *4 - Line 278: Typo: Figure 6e should be figure 5d.*

Corrected. (Line 278)

> *5 - Figure 5f: There is no explanation in the text or in the figure legend what the purpose of using mprF was.*

A comment has been included in the figure legend.

> *6 - Line 328: It would be good if we the authors reports the CC of Newman and SH1000 for a better context for the readers.*

This has been added. (Line 332)

> *7 - Line 341: Did the authors mean less sensitive to teicoplanin?*

Corrected. (Line 342)

> *8 - Line 367: Dose dependent effect does not seem to be followed not only in panel H of Supp. Fig. 4(LL37 and EMRDA15) but also panels C, D and G.*

Corrected.

> *9 - Line 587: Typo: Table 2.*

These have all been corrected and/or clarified in the revised manuscript.