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

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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 this 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.

53 **Main Text**

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

66

67 As the most severe type of infection caused by *S. aureus*, bacteraemia has a mortality rate
68 of between 20 and 30%[8], and despite infection control measures that have reduced the
69 incidence of MRSA bacteraemia, the incidence of MSSA bacteraemia continues to increase
70 in several countries, suggesting we have much to learn about this disease process. Entry
71 into the bloodstream is the first step in the development of bacteraemia and represents a
72 major bottle neck for the bacteria, whether they seed from an infection elsewhere in the
73 body, or take a more direct route through an intravenous device. As a heavily protected
74 niche, the bloodstream contains numerous humoral immune features with potent anti-
75 staphylococcal activity, including antimicrobial peptides (AMPs) and host defence fatty acids
76 (HDFAs)[9-12]. However, the fact that cases of *S. aureus* bacteraemia occur demonstrates that
77 these do not represent an impregnable force and that the bacteria can adapt to resist these
78 defensive features.

79

80 In previous work we have pioneered the application of genome wide association studies
81 (GWAS) to characterise bacterial virulence, where it has proven to be a powerful approach
82 to define complex regulatory pathways[7, 13-17]. In related work we have also begun to
83 characterise a new category of *S. aureus* genes we refer to as MALs (mortality associated
84 loci) and found that many of these are involved in serum survival[18]. Given the outstanding
85 research questions surrounding the development of bacteraemia we performed a GWAS on
86 a collection of 300 clinical isolates with respect to serum resistance. We found significant
87 variability in how well individual isolates survive exposure to serum and identified seven
88 novel effectors of this activity. Of particular note was the TcaA protein, which we have further
89 characterised and demonstrate that it is a critical protein for the bacteria involved in the
90 remodelling of the bacterial cell wall during the development of bacteraemia.
91
92 **Results**
93 *Survival of S. aureus upon exposure to human serum is a variable trait.* The human
94 bloodstream is a highly protected niche, and so to establish an infection in this environment
95 the bacteria must evade many aspects of host immunity, such as the bacterial membrane
96 damaging antimicrobial peptides (AMP) and host defence fatty acids (HDFAs) found in
97 serum[9-12,19-22]. To examine the level of variability that exists in serum-susceptibility in natural
98 populations of *S. aureus*, we exposed 300 clinical isolates to pooled human serum and
99 quantified the proportion of each culture that survived. These 300 isolates represent the two
100 major clones, as defined by multi-locus sequence typing: clonal complex 22 (CC22) and
101 clonal complex 30 (CC30), that are responsible for the majority of MRSA cases in the UK
102 and Ireland[14]. This collection is a unique resource as each strain has been sequenced,
103 extensively phenotyped, and we have clinical metadata for many of the patients the bacteria
104 were isolated from[14]. We found there to be similar levels of variability in the ability of the
105 bacteria to survive exposure to human serum across the two CCs, both with a $>10^3$ fold
106 difference between the most and least susceptible isolate with respect to the number of
107 bacteria that survived (Fig. 1a &b).

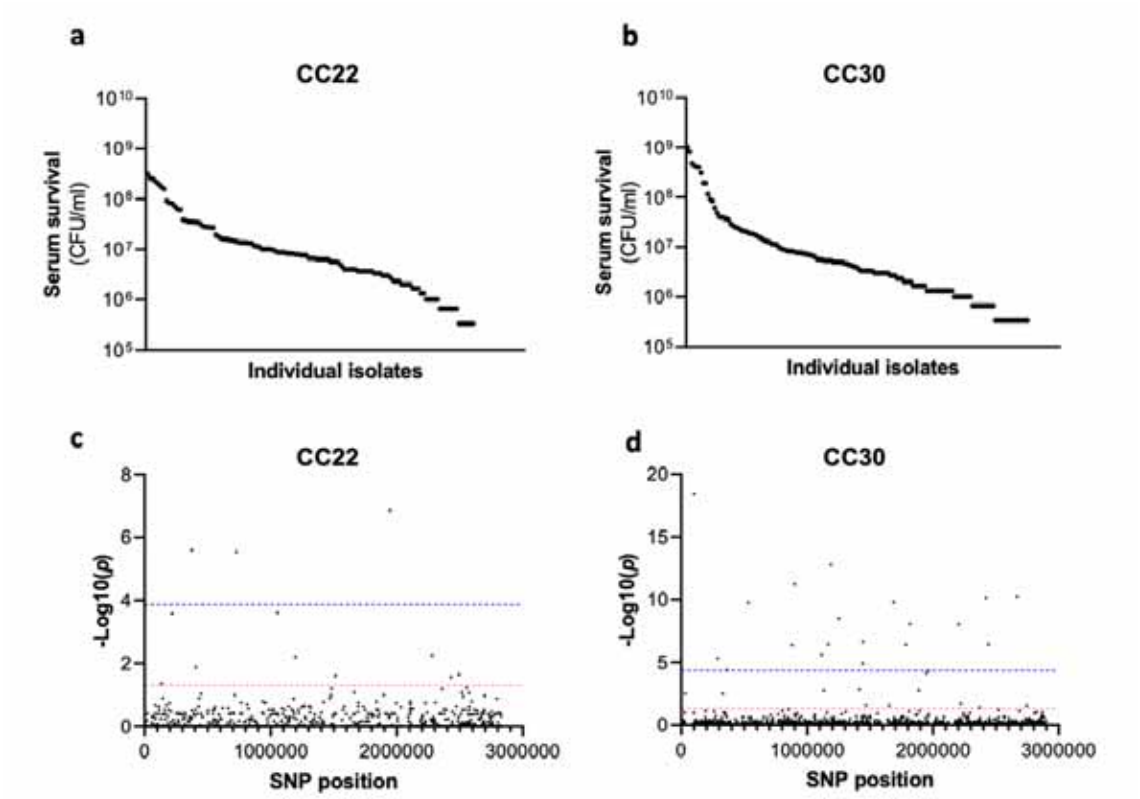

Figure 1: Serum susceptibility is multifactorial and varies widely across closely related *S. aureus* isolates. (**a & b**) The susceptibility of 300 bacteraemia isolates from clonal complexes CC22 and CC30 were exposed to human serum and their ability to survive this exposure quantified. The survival 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

125 both uncorrected and corrected (for multiple comparisons) significance thresholds to this
126 analysis, as our previous work has demonstrated that the stringency of multiple correction
127 approaches increases the likelihood of type II errors (i.e. false negative results). For the
128 CC22 collection 12 loci were associated with serum survival and for the CC30s there were
129 32 associated loci (Fig. 1c & d, Tables 1a & 1b).

130

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

137

138 **Table 1a**

SNP position	CC22 locus tag	Description	p Value	Ref base	SNP base	Mutation	NTML
1945336	SAEMRSA15_RS09605	Intergenic	6.854	A	G	N/A	N/A
375179	SAEMRSA15_03110	*metE*	5.606	C	A	Arg-Leu	NE944
728154	SAEMRSA15_06310	*saeS*	5.553	T	A	Thr-Ser	ME1296
1053036	N/A	Intergenic	3.609	A	T	N/A	N/A
219764	SAEMRSA15_01760	putatative oxidoreductase	3.576	G	A	Gly-Asp	NE312
2280500	SAEMRSA14_20910	alcohol dehydrogenase	2.257	A	T	Phe-Leu	NE1147
1196127	SAEMRSA15_10750	*yfhO*	2.212	C	T	Met-Ile	NE1597
408213	SAEMRSA15_03430	*guaA*	1.876	G	A	Ala-Thr	N/A
2492381	SAEMRSA15_23120	*tcyB*	1.645	A	G	Val-Ala	NE228
1514441	SAEMRSA15_13420	hypothetical protein	1.6	G	A	Thr-Ile	NE1349
2430758	SAEMRSA15_22540	*tcaA*	1.572	A	G	Phe-Ser	NE285
134276	SAEMRSA15)01100	hypothetical protein	1.358	A	g	N/A	NE599

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145 **Table 1b**

SNP position	CC30 locus tag	Description	p Value	Ref base	SNP base	Mutation	NTML
101174	SAR0097	putative DNA binding protein	18.466	G	A	Asp-Asn	N/A
1188605	SAR1143	*arcC*	12.786	C	A	Pro-His	NE1719
900409	SAR0856	phosphoglycerate mutase	11.226	C	T	Arg-Cys	NE1422
2667418	SAR2584	*gntR*	10.263	C	T	Arg-Gln	NE1124
2421610	SAR2347	putative membrane protein	10.160	C	T	Leu-Phe	N/A
1687821	SAR1615	*aroK*	9.793	C	A	Arg-Ile	N/A
532944	SAR0495	*veg*	9.751	G	T	Ala-Ser	NE1703
1252982	SAR1203	*recG*	8.502	C	T	Ala-Val	NE1344
1816815	SAR1754	*clpX*	8.064	C	T	Gly-Ser	N/A
2206190	SAR2143	*ilvC*	8.050	G	A	Gly-Asp	NE1177
1443567	SAR1386	*trpA*	6.647	G	T	Ser-Ile	NE304
1167449	SAR1119	*uvrC*	6.439	A	G	Glu-Gly	NE1212
2442306	SAR2374	*ureC*	6.425	C	A	Ser-Arg	NE410
1784580	SAR1719	*tgt*	6.396	C	T	N/A	NE1885
880082	SAR0835	*est*	6.354	G	C	Gly-Ala	NE1122
1116463	SAR1070	*pdhD*	5.600	C	T	Ala-Val	NE1610
289265	SAR0248	*tarK*	5.324	T	A	Stop	NE362
1442353	SAR1385	*trpB*	4.910	C	T	Stop	NE310
364861	SAR0319	NADH flavin oxidoreductase	4.373	C	A	Ala-Ser	N/A
1963781	SAR1877	*menE*	4.315	T	C	Ser-Gly	N/A
1946567	SAR1856	*arsB2*	4.122	G	C	Gly-Ala	NE1484
1416353	SAR1364	iron-sulfur cluster biosynthesis protein	2.829	C	A	Trp-Vys	NE1910
1887414	SAR1810	*fhs*	2.741	C	T	Ala-Thr	NE706
1133886	SAR1088	*pcyA*	2.725	A	G	N/A	N/A
30969	SAR0023	*sasH*	2.507	A	C	Asn-Thr	NE295
330358	SAR0284	*essC*	2.486	C	T	Ala-Val	NE1484
2220348	SAR2151	*tex*	1.704	C	A	Asp-Tyr	NE1079
1468392	SAR1410	*hipO*	1.569	G	A	Arg-His	NE1688
1652613	SAR1582	*zwf*	1.556	A	G	Ile-Val	N/A
2744774	SAR2656	*nrmA*	1.549	G	A	Ser-Ile	NE1223
2370812	SAR2287	*lacR*	1.392	T	C	Glu-Gly	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[23]. 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 seven genes were *tcaA*, a gene associated with resistance to the antibiotic teicoplanin[24]; *tarK*, a gene involved in wall teichoic acid (WTA) biosynthesis[25]; *gntR*, which encodes gluconate kinase[26]; *ilvC*, which encodes ketol-acid reductoisomerase[27]; *arsB*, which is an efflux pump involved in arsenic resistance[28]; *yfhO*, which is involved in the glycosylation of lipoteichoic acid[28]; and *pdhD*, which encodes a lipoamide dehydrogenase[30].

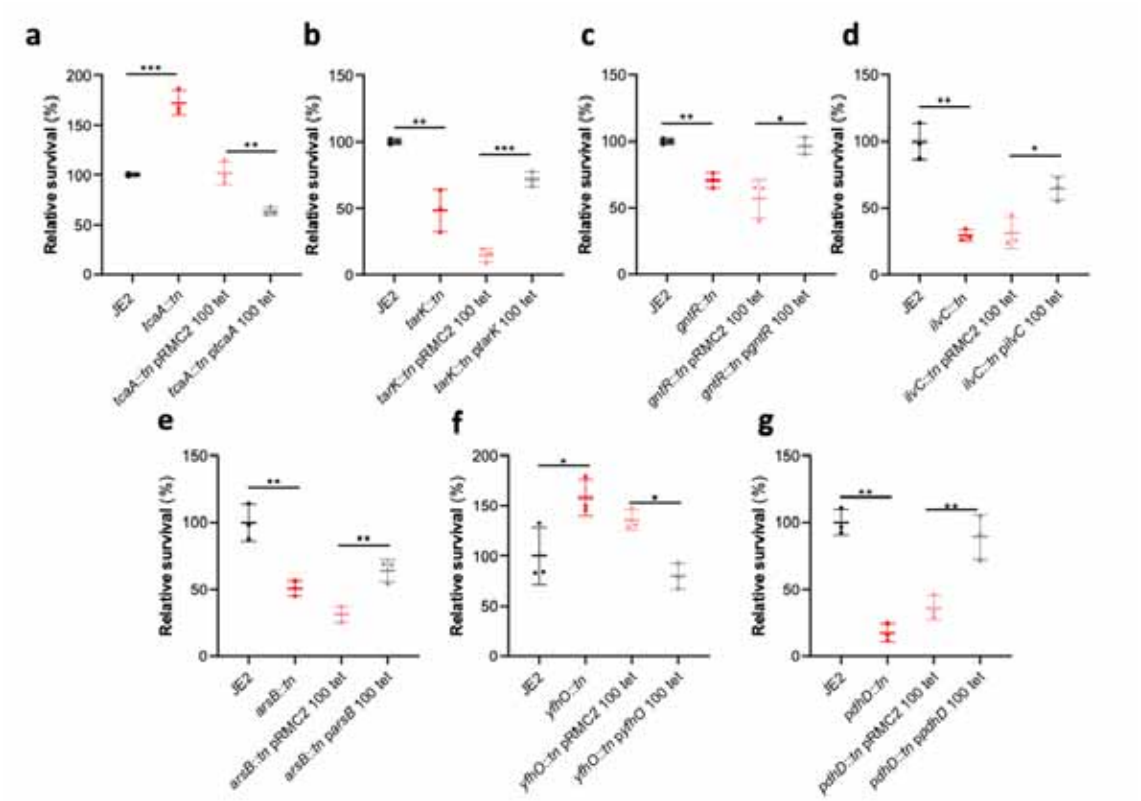
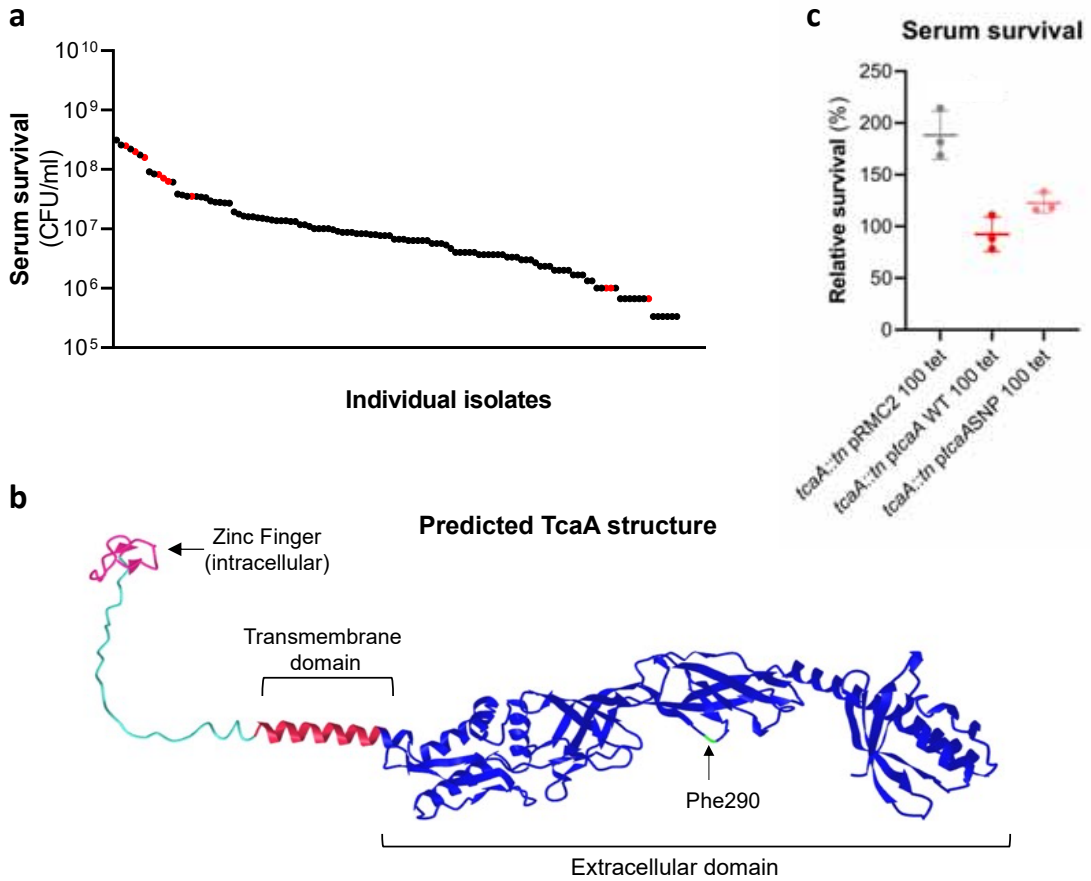


Figure 2: Functional verification of loci involved in serum sensitivity of *S. aureus*. The effect of the inactivation of the genes associated serum sensitivity was examined using transposon mutants of strain JE2. Of the 32 mutants tested seven were significantly affected: (**a**) *tcaA*, (**b**) *tarK*, (**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[24], 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

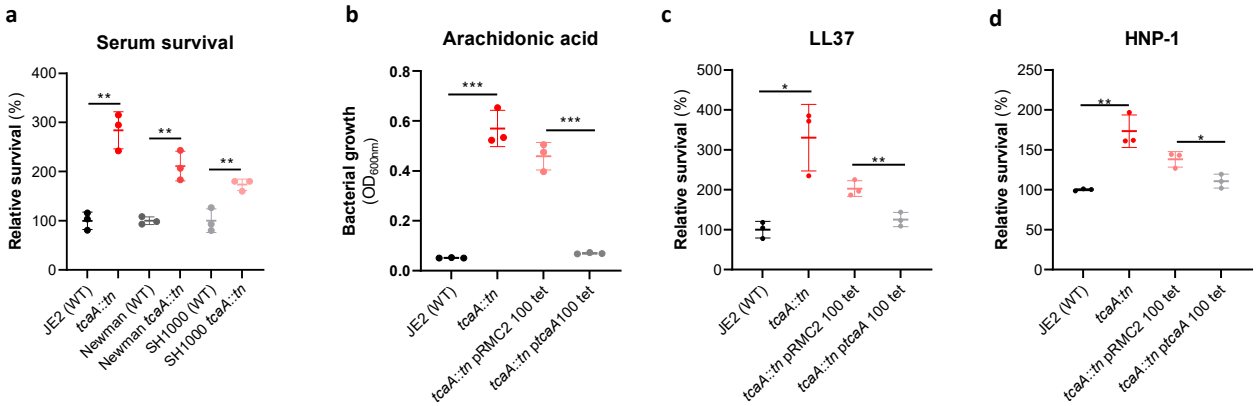
178 the isolates with the SNPs were less sensitive to serum, suggesting the SNPs negatively
179 affects the activity of the protein. The most common SNP in the *tcaA* gene amongst the
180 clinical isolates conferred a Phe (290) to Ser change in the protein sequence. Using
181 AlphaFold[31] we generated a structure for TcaA where it is predicted to be membrane bound
182 with a single membrane spanning domain; to have a zinc finger presented intracellularly;
183 with the majority of the protein including the Phe290 displayed extracellularly (Fig. 3b).
184 Given that phenylalanines are frequently involved in protein-protein interactions[32] we
185 examined the effect this change has on the activity of TcaA by cloning the *tcaA* gene with
186 this SNP into the complementing pRMC2 plasmid and comparing its ability to confer serum
187 resistance relative to that of the *tcaA* gene without the SNP. The TcaA protein with Ser at
188 position 290 instead of Phe were slightly more resistant to serum killing, but not as affected
189 as when the entire protein was inactivated, suggesting this change has a subtle effect on the
190 serum sensitising activity of the protein (Fig. 3c).



191

192 **Figure 3:** *TcaA mutations affect resistance to both teicoplanin and serum across*
193 *multiple S. aureus backgrounds*. **(a)** The individual clinical isolates with
194 polymorphism in the *tcaA* gene are indicated (in red) on a graph displaying the
195 range of serum survival of the collection. **(b)** The structure of the TcaA protein as
196 predicted by AlphaFold. **(c)** The most common *tcaA* SNP decreases the sensitivity
197 of *S. aureus* to serum. The dots represent individual data points, the bars the
198 mean value, and the error bars the standard deviation.
199
200 *TcaA confers increased sensitivity to multiple antibacterial components of serum.* To
201 examine whether the effect of TcaA on serum resistance was specific to the JE2 background
202 we transduced the *tcaA::tn* mutation from JE2 into *S. aureus* strains SH1000 and Newman.
203 Attempts were made to transduce this mutation into a clinical CC22 isolate, but this lineage
204 was refractory to any of the available transducing phage[33,34]. In all three backgrounds TcaA
205 increased the sensitivity of the bacteria to serum (Fig. 4a) verifying that the effect was not
206 limited to the JE2 background. To determine which aspects of human serum TcaA was
207 conferring increased sensitivity to, we measured the relative ability of the wild type and
208 mutant to survive exposure to some of the anti-bacterial factors found in serum: to the HDFA
209 arachidonic acid, and to two AMPs: HNP-1 and LL37. The strains producing TcaA (i.e. the
210 WT and complemented strain) were more sensitive to all three components of human serum
211 (Fig. 4b-d). For the AMPs this sensitivity was significant after only 90 min exposure, whereas
212 for arachidonic acid sensitivity was also evident after 90 minute, but was more prominent
213 after overnight incubation.
214
215 Although none of the individual antibacterial components of serum tested here are involved
216 in *S. aureus* cell clumping, it is a possibility that some of the effects seen in serum is as a
217 result of this activity, artificially amplifying the effect. To examine this the wild type and *tcaA*
218 mutant were visually examined by microscopy in both PBS and serum where no differences
219 in clumping was observed (Supplementary Fig. 1). Of the other GWAS identified genes, the
220 *ilvC* mutant was less sensitive to arachidonic acid, the *yfhO* mutant was more resistant to

221 HNP-1 and LL-37, while the *tarK, gntK, arsB and phdD* mutants were all more sensitive to
222 HNP-1 and LL37 when compared to the JE2 wild type strain (Supplementary Fig. 2).



223
224 **Figure 4:** TcaA production confers increased sensitivity to several antibacterial
225 components of serum. **(a)** The inactivation of the *tcaA* gene decreases the
226 sensitivity of three *S. aureus* strains (i.e., JE2, Newman and SH1000) to serum.
227 **(b)** TcaA production by the wild type and p*tcaA* complemented strain confers
228 increased sensitivity to arachidonic acid (100µM), **(c)** TcaA production by the JE2
229 wild type and p*tcaA* complemented strain confers increased sensitivity to LL37
230 (5µg/ml) and **(d)** TcaA production by the wild type and p*tcaA* complemented
231 strain confers increased sensitivity to HNP-1 (5µg/ml). The dots represent
232 individual data points, the bars the mean value, and the error bars the standard
233 deviation. Significance was determined as * <0.05, ** <0.01, *** <0.001.
234

235 *Wall teichoic acids contribute to the arachidonic acid sensitivity of TcaA producing strains.*
236 The antibacterial properties of HDFAs and AMPs share some common features in that they
237 both penetrate through the cell wall and attack the bacterial membrane[9-12,19-22]. AMPs are
238 positively charged molecules that rely on the relatively negative charge across the bacterial
239 cell envelope to penetrate, and thus resistance is frequently acquired by changing the
240 charge across the cell wall such that the AMPs are repelled[20,21]. Less is known about how
241 HDFAs penetrate the cell wall; however, resistance is associated with changes in the
242 abundance of wall teichoic acids (WTA) in the bacterial cell walls[22]. WTA are hydrophilic and
243 when present in the cell wall they are believed to be protective by interfering with the
244 penetration of the hydrophobic HDFAs through to the bacterial membrane[22]. It has also been

245 shown that when the ligation of WTA to the cell wall is affected, such that they are instead
246 released into the environment, this also decreases the sensitivity of the bacteria to HDFAs[11].
247 With distinct modes of action and means of accessing their target, it is intriguing to consider
248 how TcaA may be contributing to increasing the sensitivity of *S. aureus* to both these types
249 of anti-bacterial molecules.
250
251 To examine whether the TcaA associated increase in sensitivity to arachidonic acid is a
252 result of an increase in the abundance of WTA in the bacterial cell we extracted and
253 quantified WTA from the wild type and *tcaA* mutant, where we found that the cell wall of the
254 wild type had significantly more WTA when compared to the *tcaA* mutant cell extract (Fig. 5a
255 & b; densitometric analysis of triplicate extractions can be seen in Supp. Fig. 3). We next
256 quantified WTA in the bacterial supernatant, as recent work on a *S. aureus lcpA* mutant
257 demonstrated that another WTA related means of altering sensitivity to arachidonic acid is to
258 release the WTA the bacteria surface[11]. We found significantly less WTA in the extract of the
259 TcaA producing wild type strain compared to that of the *tcaA* mutant (Fig. 5a; densitometric
260 analysis of triplicate extractions can be seen in Supp. Fig. 3), which could explain the
261 observed difference in sensitivity to HDFAs. It was unclear from the previous work on LcpA
262 how the released WTA by the *lcpA* mutant affects arachidonic acid sensitivity, and here we
263 demonstrate that this mutant, like the *tcaA* mutant, is also less sensitive to human serum
264 (Fig. 5d). One hypothesis is that the WTA can either sequester or inactivate HDFAs in the
265 environment surrounding the bacteria, thereby neutralising them. To test this, we harvested
266 the supernatant of the wild type JE2, the JE2 *tcaA* mutant, and the JE2 *lcpA* mutant from
267 overnight growth. These supernatants were used to supplement fresh broth (at 10%) into
268 which the wild type JE2 strain was inoculated. In the absence of any arachidonic acid JE2
269 grew equally well regardless of the supernatant supplement (Fig. 5e). However, in the
270 presence of the supernatant of both the *tcaA* and *lcpA* mutant (both of which contain an
271 abundance of released WTA), JE2 was able to grow in the presence of arachidonic acid;
272 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 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. 6e). 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.

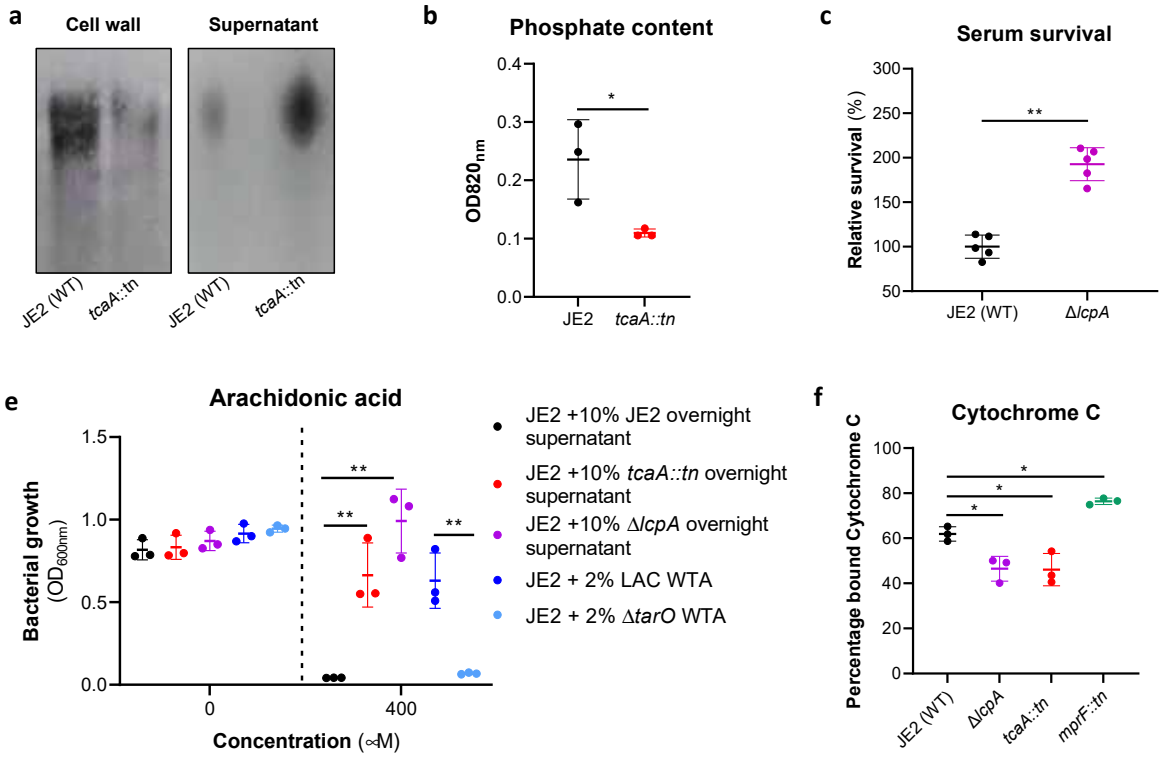


Figure 5: Wall teichoic acids are released from the cell wall in the *tcaA* mutant to affect resistance to HDFAs and AMPs. **(a)** WTA was extracted from both cells and supernatant of the wild type and *tcaA* mutant of *S. aureus* and visualised on and SDS-PAGE gel stained with 1 mg/ml Alcian blue. The *tcaA* mutant had significantly less WTA in the cell wall but more in the supernatant. **(b)** The phosphate content of the cell wall WTA extracts was quantified, which verified that the *tcaA* mutant

290 has significantly less WTA. **(c)** The inactivation of the *lcpA* gene increases the
291 sensitivity of *S. aureus* to killing by serum. **(d)** The wild type strain JE2 was grown
292 in broth supplemented with supernatant of either itself (JE2), with that from a *tcaA*
293 mutant, or with that from a *lcpA* mutant. These supernatants had no effect on the
294 growth of JE2 in the absence of arachidonic acid. In the presence of arachidonic
295 acid JE2 was unable to grow when supplemented with its own supernatant,
296 however, when supplemented with the supernatant of the two mutants, which both
297 contain soluble WTA, JE2 was able to grow. The addition of WTA extract from
298 another S. aureus strain (LAC) also neutralised arachidonic acid, however an
299 equivalent extract from an isogenic WTA mutant (LAC Δ*tarO*) did not. **(e)** The
300 charge across the cell wall of the wild type and *tcaA* mutant was compared using
301 cytochrome C, where the mutant was found to be less negatively charged. The
302 dots represent individual data points, the bars the mean value, and the error bars
303 the standard deviation. Significance was determined as * <0.05, ** <0.01, ***
304 <0.001, **** <0.0001.
305

306 *The reduced abundance of WTA in the bacterial cell wall contributes to the resistance of S.*
307 *aureus to AMPs.* In additional to being hydrophilic, WTA is also predominantly negatively
308 charged[25]. Given that a change in charge across the bacterial cell wall is frequently
309 associated with resistance to AMPs[19-21], and that the wild type strain has more WTA in its
310 cell wall, this may explain the increased AMP sensitivity of the TcaA producing strain. To
311 examine this, we incubated the bacteria with cytochrome C, which is positively charged and
312 through its electrostatic-related ability binds to the bacterial cells, providing a measure of the
313 charge across bacterial cell walls. Using this we found that the wild type strain bound
314 significantly more cytochrome C and is therefore more negatively charged than the *tcaA*
315 mutant, which explains the increase in AMP sensitivity we have found associated with TcaA
316 production (Fig. 5e). Together these results explain how TcaA production affects the
317 sensitivity of *S. aureus* to both HDFAs and AMPs. By retaining WTA within the cell wall,
318 there is less released to sequester or inactivate the fatty acids from the environment, and
319 due to their negative charge, increased amounts of WTA in the cell wall affects the charge

320 across the cell wall and results in increased electrostatic attraction of the positively charged
321 AMPs.
322
323 *Serum induced tcaA expression increases both the abundance of WTA in the cell wall and*
324 *the sensitivity S. aureus to teicoplanin.* As mentioned previously, an additional interesting
325 feature of the TcaA protein is that is has also been associated with changing the sensitivity
326 of the bacteria to the antibiotic teicoplanin, although this has been reported to vary between
327 strains[24,35]. To examine this for our strains we compared the ability of our three pairs of wild
328 type and *tcaA* mutants (in strains JE2, Newman and SH1000) to grow in the minimum
329 inhibitory concentration of teicoplanin for each wild type strain (Supp. Table 2), where TcaA
330 production resulted in increased sensitivity to this antibiotic across all three strains (Fig. 6a).
331 Minimum inhibitory concentrations (MICs) for teicoplanin have also been determined for
332 these, where in each case the production of TcaA halves the MIC of the antibiotic (Supp.
333 Table 2). To examine whether the clinical isolates with the SNPs in the *tcaA* gene
334 associated with increased serum resistance also had altered teicoplanin sensitivity, we
335 compared the ability of 12 clinical isolates containing the GWAS identified *tcaA* SNPs to
336 grow in teicoplanin to that of 12 isolates from the same collection with the wild type gene
337 sequence, i.e., without the polymorphism. The isolates with the polymorphism in the *tcaA*
338 gene were on average less sensitive to teicoplanin (Fig. 6b). Using the pRMC2
339 complementing plasmid expressing either the wild type or SNP (conferring the Phe290-Ser
340 substitution) containing *tcaA* gene, we demonstrated that this mutation significantly reduces
341 the ability of the protein to sensitize the bacteria to serum (Fig. 6c), confirming that this
342 mutation negatively affects the activity of the TcaA protein.
343
344 As TcaA has been shown to be upregulated when exposed to teicoplanin[24,35], we examined
345 whether serum would also induce its expression. The wild type JE2 strain was exposed to
346 either sub-inhibitory (2.5% serum and 0.5µg/ml teicoplanin) or inhibitory (10% serum and

347 10μg/ml teicoplanin) concentrations of either serum or teicoplanin for 20 mins, total RNA
348 was extracted and the transcription of the *tcaA* gene quantified by qRT-PCR. For both serum
349 and teicoplanin, induction of expression of the *tcaA* gene was concentration dependent (Fig.
350 6d). Given the association of WTA abundance in the cell wall with *tcaA* expression described
351 above (Fig. 5a-c) we next examined whether serum induced expression of *tcaA* would also
352 result in an increase in abundance of WTA in the bacteria cell wall. Both the wild type and
353 *tcaA* mutant were exposed to inhibitory concentrations of human serum for 90 mins and the
354 WTA extracted from the cell walls, where serum exposure resulted in an increase in WTA for
355 the wild type strain but not the *tcaA* mutant (Fig. 6e; densitometric analysis of triplicate
356 extractions can be found in Supp Fig. 3). To examine whether serum induced increased
357 expression of *tcaA* would affect sensitivity to teicoplanin, we exposed the wild type bacteria
358 to a sub-inhibitory concentrations of serum (2.5%) and then incubated these overnight in a
359 subinhibitory concentration of teicoplanin (0.5μg/ml). Pre-exposure to serum induced
360 increased sensitivity to teicoplanin suggesting that induction of *tcaA* expression by the initial
361 serum exposure was sufficient to induce cross-sensitivity to the antibiotic (Fig. 6f).
362
363 To verify this induction effect was not specific to the JE2 lineage a *tcaA::gfp* reporter plasmid
364 was constructed and electroporated into JE2, SH1000, Newman and a clinical CC22 isolate
365 EMRSA15. In each background serum, arachidonic acid and LL37 had a dose dependent
366 effect on *tcaA* expression with the exception of LL37 and the EMRSA15 strain
367 (Supplementary. Fig. 4). These findings suggest that upon entry into the bloodstream and
368 exposure to many of the antibacterial components of serum, expression of the *tcaA* gene is
369 induced and this sensitizes *S. aureus* to killing by these components of serum.

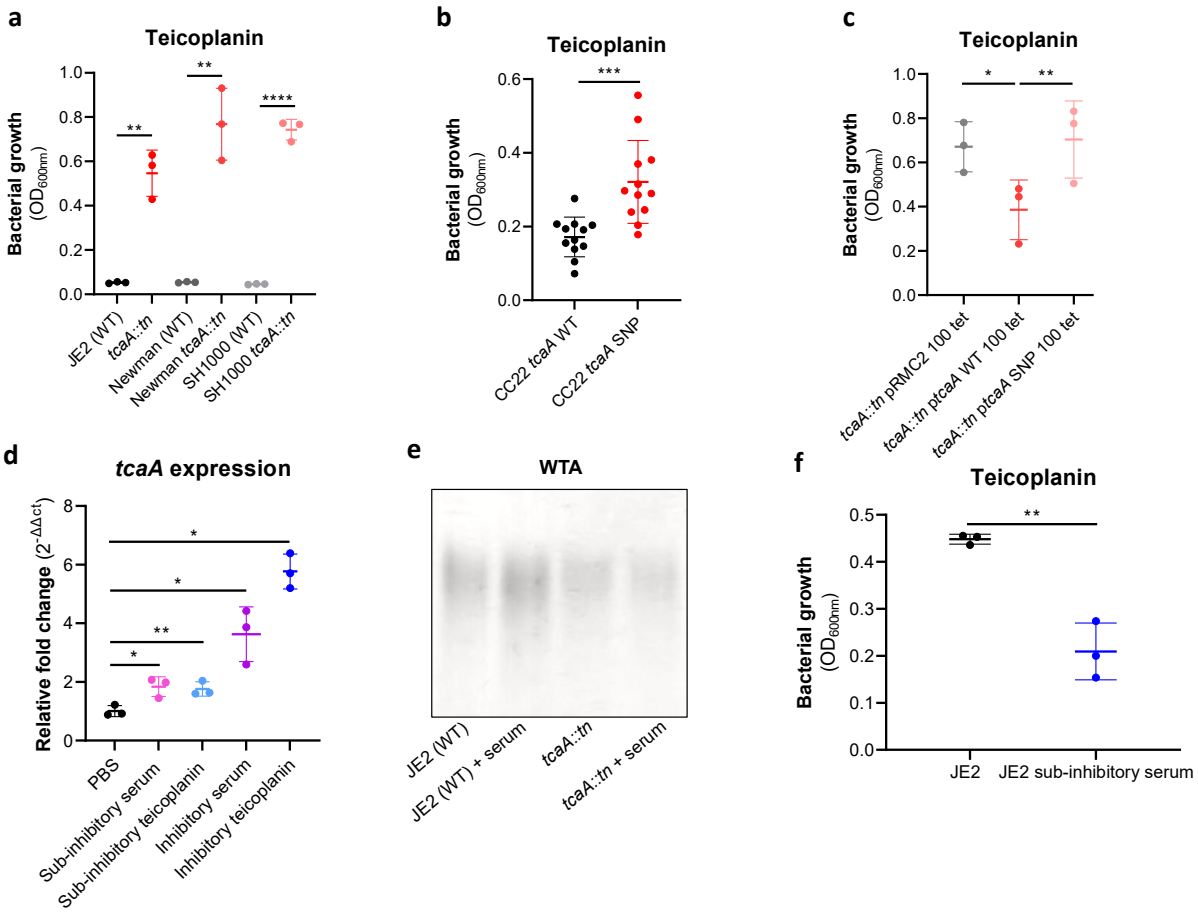


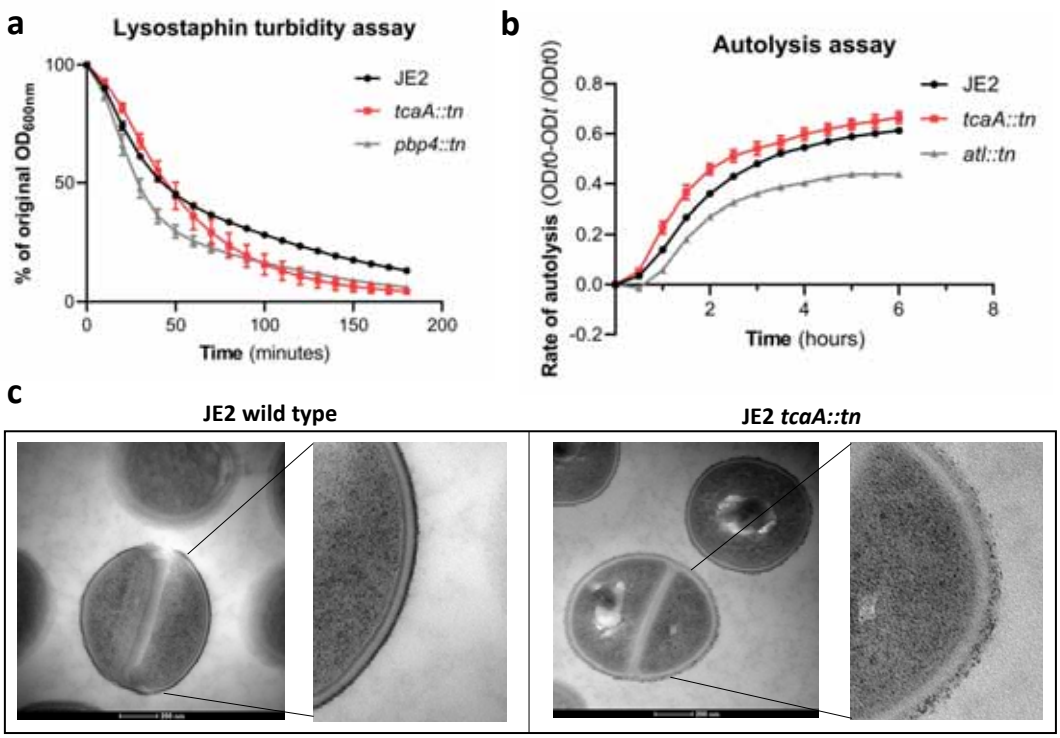
Figure 6: TcaA confers increased sensitivity to teicoplanin. **(a)** The inactivation of the *tcaA* gene decreases the sensitivity of three distinct *S. aureus* strains (i.e., JE2, Newman and SH1000) to teicoplanin (0.5µg/ml). **(b)** The clinical isolates with polymorphisms in the *tcaA* gene were on average less sensitive to teicoplanin than those with the wild type gene. **(c)** The most common *tcaA* SNP decreases the sensitivity of *S. aureus* to teicoplanin. **(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**) Growth of JE2 in the presence of sub-inhibitory 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. The dots represent individual

386 data points, the bars the mean value, and the error bars the standard deviation.
387 Significance was determined as * <0.05, ** <0.01, *** <0.001, **** <0.0001.
388

389 *The inactivation of* tcaA *alters the antibiotic resistance profile of S. aureus.* There is
390 emerging evidence that WTA play a role in cell division and peptidoglycan biosynthesis[36]. In
391 support of this, it has been reported that a *tarO* mutant, devoid of WTA, confers sensitization
392 to β-lactam antibiotics due to mislocalization of the PBPs[37]. As the target for teicoplanin is
393 peptidoglycan, more specifically the terminal D-Ala-D-Ala residue of lipid II, we hypothesised
394 that the increased ligation of WTA into the cell wall of the TcaA producing strain may affect
395 peptidoglycan biosynthesis and assembly, and that this may be responsible for the change
396 in sensitivity to teicoplanin. To test this further, we compared the sensitivity of the wild type
397 and mutant to a range of peptidoglycan attacking antibiotics, where we found the TcaA
398 producing wild type strain to be more sensitive to vancomycin, dalbavancin and oritavancin,
399 all alternative members of the glycopeptide class of antibiotic, and ramoplanin, a
400 glycolipodepsipeptide antibiotic (Supp. Fig. 5). However, TcaA production conferred
401 decreased sensitivity to oxacillin, a β-lactam antibiotic that targets penicillin binding protein 2
402 and moenomycin a phosphoglycolipid antibiotic that inhibits the transglycosylase PBP
403 enzymes and prevents the formation of peptidoglycan polymers (Supp. Fig. 5). This
404 suggests that TcaA production affects the overall composition and structure of peptidoglycan
405 in the wildtype strain.
406

407 To examine whether the crosslinking of peptidoglycan is affected by TcaA production, we
408 tested the susceptibility of the wild type and *tcaA* mutant to lysostaphin, which is
409 a glycylglycine endopeptidase capable of cleaving the pentaglycine crosslinks of
410 peptidoglycan[38]. We found that lysostaphin cleaved the cell wall of the wild type strain more
411 slowly than for the *tca*A mutant (Fig. 7a). We next performed a triton X100 induced autolysis
412 assay where the wild type exhibited a slower rate of autolysis compared to the mutant,
413 suggesting that the cell wall is stronger when TcaA is produced (Fig. 7b). Together these

414 data suggest that the integrity of the peptidoglycan, in particular its crosslinking, is
415 significantly altered when TcaA is produced. This provides a likely explanation for why the
416 mutant is more resistant to teicoplanin, reduced crosslinking in the cell wall increases the
417 number of off target D-ala D-ala which teicoplanin will bind to instead of reaching lipid II in
418 the membrane and causing disruption to this. With changes to the quantity and composition
419 of WTA and peptidoglycan associated with TcaA production, we sought to visualise the cell
420 wall using transmission electron microscopy. We observed a clear difference in the density
421 and structure of the outer layer of the bacterial cell wall between the wild type and *tcaA*
422 mutant, confirming a role for TcaA in the structural integrity of the *S. aureus* cell wall (Fig. 7c,
423 additional images in Supp. Fig. 6)).

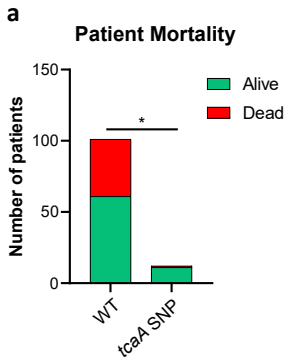


424

425 **Figure 7:** TcaA alters the structure of the *S. aureus* cell wall. **(a)** The rate of lysis
426 in the presence of lysostaphin was assayed, where the strain producing was
427 lysed at a slower rate than the *tcaA* mutant. A *pbp4* mutant was included as a
428 control. **(b)** The rate of autolysis of the bacteria in the presence of Triton x100
429 was assayed where the TcaA producing strain lysed at a slower rate relative to
430 the *tcaA* mutant. An autolysin (*atl*) mutant was included as a control. **(c)**

431 Transmission electron micrograph (TEM) of a wild type JE2 and *tcaA* mutant cell
432 at two magnifications showing the smooth and consistent density of the cell wall
433 when TcaA is produced, compared to the rough and patchy density of the mutant
434 cell wall.
435
436 *Once established, TcaA contributes positively to the development of bacteraemia.* Our data
437 shows that expression of *tcaA* is induced upon exposure to serum, that this can sensitize the
438 bacteria to serum killing but also increase the abundance of WTA in the cell envelope. From
439 a pathogenicity perspective these activities would appear to be in conflict, with one making it
440 less likely for a bacterium to cause bacteraemia (increased serum sensitivity) and the other
441 making it more likely for a bacterium to cause bacteraemia (increased WTA in the cell
442 envelope[39,40]). To understand this apparent dichotomy we first examined whether TcaA
443 production only affected survival in serum, whereas in whole blood where other components
444 of the human immune system may be present (e.g. complement, antibodies and
445 phagocytes) that this effect may be lost. Using human blood from three donors, we
446 demonstrated that TcaA production increase the sensitivity of the bacteria to whole blood
447 killing (Supp. Fig. 7). We next interrogated our human clinical data, where in addition to the
448 300 bacteraemia strains that form the basis of this study we have an additional 176 *S.*
449 *aureus* isolates, which has allowed us to include more genetic diversity to this analysis (i.e.
450 from CC22 (n=138), CC30 (n=162), ST8 (n=132) and ST93 (n=44)). The source of these
451 isolates was also available to us (i.e. bacteraemia (n=341), SSTI (n=80) or carriage (n=55)),
452 as well as their genome sequences (Supplementary Table 1). We examined the distribution
453 of nonsynonymous mutations in the *tcaA* gene across these, where it was present in 6% of
454 the bacteraemia isolates compared with only 1.5% of non-bacteraemia isolates (n= 21/341
455 and 2/135 respectively; p = 0.03, in a Fisher's Exact test). However, when we examined the
456 30-day mortality rates for the 113 bacteraemia patients for whom we had this clinical data,
457 the mortality rate of the proportion of patients infected with a *S. aureus* strain with the wild
458 type *tcaA* gene was significantly higher compared to those infected with a mutated *tcaA*
459 gene (Fig. 8a & b) (2-tailed chi squared test: p = 0.03). So, although the incidence of

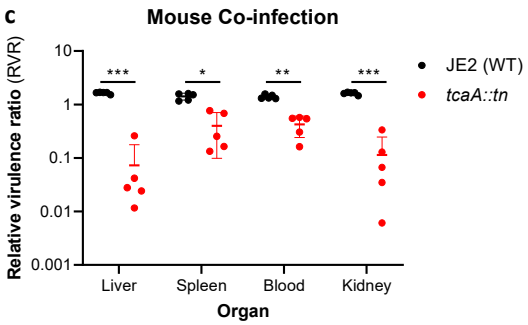
460 mutations in *tcaA* appear to be relatively rare, that isolates with variant *tcaA* genes are
461 enriched amongst those causing bacteraemia suggests that a functional TcaA may limit the
462 propensity of *S. aureus* to cause a bloodstream infection, such that it acts as a selective
463 force for mutants of this gene. However, once the bacteraemia has become established,
464 TcaA positively contributes to the infection process, likely due to the increased abundance of
465 WTA in the cell envelope, and the greater structural integrity of the cell wall.
466
467 To experimentally test these human findings we utilised a mouse model of bacteraemia
468 where the tail vein of C57/Bl6 mice are inoculated with 2 x 10^7 colony forming units (CFU) of
469 bacteria to establish a bloodstream infection, where they seed from there into organs such
470 as the liver spleen and kidneys. We performed mixed infections, where the wild type and
471 mutant bacteria had to compete during the development of the infection within the mouse.
472 The mice were infected with equal quantities of both bacteria and the change in ratio of the
473 bacteria in the mouse blood, liver, spleen and kidney recorded after 48hr, which allowed the
474 relative virulence ratio (RVR) of each bacterium to be calculated[41]. The RVR of the TcaA
475 producing wild type strain was significantly higher than that of the mutant at a systemic level
476 (Fig. 8c). As such, while the TcaA associated self-sensitization of the bacteria was not
477 evident here, possibly due to the size of the inoculum level needed, once an infection has
478 become established, TcaA contributes positively to disease severity as demonstrated by the
479 wild type strain outcompeting the *tcaA* mutant.
480



b

Mortality Contingency Table

	WT	SNP	Total
Alive	61	11	72
Dead	40	1	41
Total	101	12	113



481

482 **Figure 8:** TcaA contributes to increased disease severity in mice and humans. **(a**
483 **& b)** A graph and contingency table showing data from 113 patient with *S. aureus*
484 bacteraemia. The 30-day mortality rate was significantly higher for those infected
485 with a *S. aureus* strain with a wild type *tcaA* gene compared to those with a
486 mutated *tcaA* gene. **(c)** Mice were injected intravenously with 100 µl of PBS
487 containing an equal mixture of wild type and *tcaA* mutant (2 x 10^7 CFUs of each).
488 After 48 hrs, the ratio of the wild type and mutant were quantified and the relative
489 virulence ratio calculated. In the blood and all organs tested the wild type bacteria
490 had a competitive advantage over the mutant demonstrating its increased relative
491 virulence. Mean ± s.d. The dots represent individual data points (n=5 per group),
492 the bars the mean value, and the error bars the standard deviation. Significance
493 was determined as * <0.05, ** <0.01, and *** <0.001.
494

495 **Discussion**

496 To develop novel therapeutic approaches for infectious diseases, we need to understand
497 how microorganisms cause disease and evade the host's immune system. To address this,
498 we have applied a population-based approach to analyse the pathogenicity of *S. aureus* and
499 identified seven genes that affect the bacteria's ability to survive exposure to human serum,
500 the first step in the development of bacteraemia. The dissection of the molecular detail of
501 how these genes affect serum survival is underway to determine their potential for
502 therapeutic intervention. However, our focus here has been on the *tcaA* gene where we
503 show that its expression is induced upon exposure to serum, that it is involved in the ligation
504 or retention of WTA in the cell wall, and that this renders the bacteria more susceptible to
505 killing by both AMPs and HDFAs. We have also found that TcaA is associated with changes
506 to the structural integrity of peptidoglycan, such that the bacteria are more susceptible to
507 some antibiotics (e.g. teicoplanin) and less susceptible to others (e.g. oxacillin). It is
508 interesting to note that our GWAS approach identified *tcaA* due to the presence of function
509 altering SNPs amongst clinical isolates. Our human data suggests that bacteraemia and/or
510 the antibiotics commonly used to treat it such as teicoplanin and vancomycin may impose a
511 selective pressure on the bacteria to mutate this gene, due to the decreased sensitivity this
512 confers to serum killing and to clinically relevant antibiotics. However, both our human and

513 mouse data have confirmed, that once bacteraemia has become established, TcaA
514 contributes positively to disease progression, likely due to its cell wall re-modelling activity.
515
516 The *tcaA* gene is part of a three gene locus alongside the *tcaR* gene, which is predicted to
517 encode a transcriptional regulator, and the *tcaB* gene which is predicted to encode an efflux
518 pump[24]. This locus is also reported to be part of the *S. aureus* cell wall stimulon, following a
519 number of studies that monitored the response of *S. aureus* to cell wall attack[42,43]. While
520 work to understand the activity of each of the proteins encoded by the *tca* locus is underway,
521 our data suggests that TcaA play a role in the ligation or retention of WTA within the cell
522 wall, perhaps alongside the LcpA protein[11]. How this is achieved is yet to be determined.
523 TcaA may play a direct role in ligating WTA to the cell wall; it may play a role in cell wall
524 turnover and the subsequent release of WTA and other cell wall macromolecules into the
525 external environment; or it may interact and subsequently interfere with other cell wall
526 biosynthetic processes that are responsible for WTA ligation. It is interesting to note that
527 *tarK*, which is one of the other six genes associated here with serum survival is also involved
528 in the biosynthesis of WTA. Additionally, *yfhO* which encodes an enzyme that glycosylates
529 lipoteichoic acid (LTA) is also involved in serum resistance[29], providing further evidence for
530 the importance of teichoic acid associated molecules to this aspect of the biology and
531 pathogenicity of *S. aureus*. For TcaR, we hypothesise that it likely controls the transcription
532 of the co-transcribed *tcaA* and *tcaB* genes, and may directly respond to the external stimulus
533 of teicoplanin and serum as reported here. What is less clear is what role TcaB plays, as
534 efflux pumps are more typically associated with increasing the resistance of a bacterium to
535 an antibiotic, or other toxic molecules.
536
537 In addition to *tcaA* this study has identified one other locus (*yfhO*) that negatively affects
538 serum survival, and added a further five loci (*tarK, gntR, ilvC, arsB*, and *pdhD*) to the list of
539 genes that contribute positively to serum survival by protecting the bacteria from HDFAs and
540 AMPs. This leads us to the questions of why *S. aureus* has loci in apparent conflict with

541 regards to the survival of the bacteria during bacteraemia. It is perhaps through the
542 consideration of the other ways in which *S. aureus* interact with humans that we may
543 understand this. The vast majority of interactions that occur between *S. aureus* and humans
544 is as a commensal in the human nose from which it can readily transmit. The five identified
545 protective genes may therefore also protect the bacteria during colonisation, which would
546 confer a selective advantage, and it is just an unfortunate coincidence that they also confer
547 increased survival in serum during the development of bacteraemia. While the focus of this
548 work has been on the role of TcaA in serum, it is also worth considering what role it may
549 have during colonisation of the nose, which involves the adherence of the bacteria to the
550 nasal epithelium, to which WTA has a well-established role. It is therefore possible that TcaA
551 enhances the ability of *S. aureus* cells to colonise the nose by retaining WTA within the cell
552 wall. However, the role of TcaA in responsiveness to cell wall attack is as yet unclear, but
553 perhaps it contributes to the competitiveness of *S. aureus* within the nasal microbiome in
554 response to cell wall damage inflicted by competitors.
555
556 The evolution of the virulence of a pathogen is heavily dependent upon their mode of
557 transmission, which for opportunistic pathogens raises interesting challenges. If within-host
558 invasiveness limits between-host fitness by preventing them from transmitting to a new host,
559 there are likely selective pressures acting on bacterial populations to evolve means of
560 limiting their ability to cause invasive disease. However, if increased invasiveness facilitates
561 enhanced within-host fitness, as the bacteria multiply and spread throughout the body, the
562 'short-sighted evolution of virulence' hypothesis proposed by Levin and Bull[44] could apply
563 and may explain situations like *S. aureus* bacteraemia. We propose that TcaA may
564 represents a system at the cusp of both these scenarios, in that it responds to cell wall
565 attack by serum by making itself more susceptible to killing by serum, thereby potentially
566 limiting its ability to cause bacteraemia, which could have long-term between host benefits.
567 However, once established in the bloodstream and TcaA has remodelled the cell wall, the

568 short-sighted view of virulence hypothesis applies where the bacteria survive and thrive,
569 allowing them to cause a successful bacteraemia.
570
571 Entry into the bloodstream to establish an infection has long been considered a significant
572 bottle neck for the bacteria, but until now, the stringency of this bottleneck was believed to
573 be a result of the onslaught of all the immune mechanisms present in blood. Here we
574 suggest that *S. aureus* may contribute to this bottleneck by responding to serum induced cell
575 wall damage by further sensitizing itself to this attack as part of the pathway towards
576 increasing their pathogenic capabilities by remodelling the cell wall. Our findings presented
577 here opens up an entirely novel aspect to the biology of a major human pathogen and brings
578 into question the appropriateness of the term 'opportunistic' when we refer to such highly
579 effective bacterial pathogens.
580
581
582 **Materials and Methods**
583 **Bacterial Strains and Growth Conditions**
584 A list of the genetically amenable bacterial strains and mutants can be found in Table 3, and
585 the clinical strains are listed in Supplementary Table 1. All strains were cultured in Tryptic
586 soy broth (TSB) for 18 h at 37°C with shaking. Nebraska transposon mutant library (NTML)[23]
587 mutants were selected for using erythromycin (5 μg/ml). For the complemented pRMC2[45]
588 strains, chloramphenicol (10 μg/ml) and anhydrous tetracycline (200 ng/ul) were added to
589 the media where indicated.
590
591 **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	23

	NTML	
JE2 *tcaA:tn*	*tcaA* transposon mutant in JE2	23
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	23
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	23
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	23
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	23
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	23

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	23
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	46
DC10B	*Δdcm* mutant in *E. coli* DH10B (K12)	34
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.	47
SH1000 *tcaA::tn*	*tcaA* transposon mutant in SH1000	This study
Newman	MSSA, laboratory strain isolated from human infection	48
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	23
Δ*lcpA*	In frame unmarked deletion of *lcpA* generated by allelic exchange	11
LAC	CA-MRSA USA300 type IV SCCmec	49

Δ*tarO*	*tarO* KO mutant in LAC	50
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

592

593 **Serum & Blood Survival Assay**

594 Normal human serum was prepared from blood obtained from 8 healthy volunteers using
595 Serum CAT tubes (BD). Blood was allowed to clot for 30 mins at room temperature followed
596 by 1h incubation on ice. Serum was extracted following two rounds of centrifugation at 700 x
597 g at 4°C for 10 min. Serum from individual donors were pooled, aliquoted and immediately
598 stored at -80°C. The bacteria were grown overnight, and their density normalised to an
599 OD_{600nm} of 0.1 and 20 µl used to incubate in 180 µl 10% pooled human serum (diluted in
600 PBS) for 90 min at 37°C with shaking. Serial dilutions were plated on tryptic soy agar (TSA)
601 to determine CFUs. The same number of bacterial cells inoculated into PBS, diluted, and
602 plated acted as a control. Survival was determined as the percentage of CFU in serum
603 relative to the PBS control. Relative survival was determined through normalisation to JE2,
604 which is presented ats 100% in the graphs. The survival of each bacterial isolate was
605 measures in triplicate and the mean of these datal presented here.

606

607 **GWAS**

608 Genome-wide association mapping was conducted using a generalized linear model, with
609 capsule production as the quantitative response variable. We accounted for bacterial

610 population substructure by adding to the regression model the first two component from
611 a *principal component decomposition* of SNP data for each set of clinical samples (CC22
612 and CC30). The first two components accounted for 32% and 40% of the total variance for
613 CC22 and CC30, respectively. In both cases, three distinct clusters were identified. We
614 further considered a third model where we used cluster membership as covariates in our
615 regression model, where clusters were defined using K-means clustering analysis (setting
616 K=3); this, however, yielded identical results to the one based on PCA components. In total,
617 2066 (CC22) and 3189 (CC30) unique SNPs were analysed, the majority of which were
618 subsequently filtered out for exhibiting a minor allele frequency (maf) of <0.03, reducing the
619 data to 378 and 1124 SNPs, respectively. The P-values reported in Tables 1a and b are not
620 corrected for multiple comparison, however the Sidak method was used to correct for
621 multiple comparisons and both significance thresholds are indicated in the Manhattan plots.
622

623 **Genetic manipulation of bacterial strains**

624 Wildtype genes were amplified by PCR from JE2 genomic DNA using the primers shown in
625 table 4 and KAPA HiFi polymerase (Roche). The PCR product was cloned into the
626 tetracycline inducible plasmid pRMC2[45] using *Kpn*I and *Sac*I restriction sites and T4 DNA
627 ligase (NEB). This was transformed into RN4220 and eventually into the respective NTML
628 mutants through electroporation.
629

630 **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.

659 **Phage transduction of the *bursa aurealis* transposon**

660 The *tcaA bursa aurealis* transposon was phage transduced using the *S. aureus* phage
661 Φ11[51]. The phage lysate of the donor strain (*tcaA::tn)* was prepared as follows: The donor
662 strain was grown overnight in TSB for 18 h at 37°C with shaking. Two hundred microlitres of
663 overnight culture was added to 25 ml of TSB containing 250 µl 1M $MgSO_4$, 250 µl 1M $CaCl_2$,
664 100 µl of Φ11 and left at 37°C with shaking until complete lysis was observed. The culture
665 was subsequently pelleted at 12000 x *g* for 3 min. The supernatant containing the lytic
666 phage was filtered sterilised and stored at 4°C. Next, recipient strains (Newman and
667 SH1000) were grown overnight in 20 ml of LK broth (1% Tryptone 0.5% Yeast Extract,
668 0.7% KCl) and pelleted at 2500 x *g* for 10 min. The supernatant was removed, and the
669 pellets resuspended in 1 ml of fresh LK broth. Two reactions were then set up, one
670 containing phage and one as a non-phage control. To the tube containing the phage add:
671 250 µl of the recipient strain (SH1000 or Newman), 250 µl of phage lysate and 750 µl of LK
672 broth containing 10 mM $CaCl_2$. To the tube containing the non-phage control add: 250 µl of
673 the recipient strain (SH1000 or Newman) and 1 ml od LK broth containing 10 mM $CaCl_2$.
674 Both reactions were incubated for 25 min at 37°C without shaking followed by 15 min at
675 37°C with shaking. Five hundred microlitres of ice cold 20 mM sodium citrate was added
676 followed by centrifugation at 10000 x *g* for 10 min. Pellets were resuspended in 500 µl of 20
677 mM sodium citrate and incubated on ice for 2 h. One hundred microlitres of culture was
678 plated out on TSA containing 5 µg ml^{-1} erythromycin to select for the transposon. For the
679 SH1000 phage transduction 100 µl was also plated out on oxacillin TSA plates as an added
680 control to ensure there was no contamination of the phage lysate with JE2 *tcaA::tn.*

681

682 **Growth inhibition assays**

683 To determine the relative inhibition of growth of the wild type and tcaA mutant by antibiotics
684 a broth microdilution method was used[52]. Briefly, overnight cultures were normalised to an
685 OD_{600nm} of 0.1 in cation adjusted Mueller Hinton broth (MHB++) and 20 µl of resultant
686 suspension used to inoculate 180 ul of fresh MHB++ containing a 1:2 dilution series of either

687 arachidonic acid or teicoplanin (Sigma). The ability of the bacteria to survive the antibiotics
688 was determined by quantifying bacterial growth (OD_{600nm}) using a CLARIOstar plate reader
689 (BMG Labtech). For Fig. 2b 0.5µg/ml teicoplanin was used for the clinical CC22 isolates and
690 for Fig. 2d: 1µg/ml was used for the JE2 pair, 2 µg/ml for the Newman pair and 4 µg/ml for
691 the SH1000 pair.
692
693 **Antimicrobial peptide susceptibility**
694 Antimicrobial peptide susceptibility Human neutrophil defensin-1 (hNP-1) (AnaSpec
695 Incorporated, California, USA) and LL-37 (Sigma) susceptibility assays were performed as
696 described previously[53]. Briefly, overnight cultures were normalised to an OD_{600nm} of 0.1 and
697 incubated with 5 µg/mL of hNP-1 or LL-37 for 2 h at 37 °C. Serial dilutions were plated on
698 tryptic soy agar (TSA) to determine CFUs. The same number of bacterial cells inoculated
699 into PBS, diluted, and plated acted as a control. Survival was determined as the percentage
700 of CFU on exposure to either LL-37 or HNP-1 relative to the PBS control. Relative survival
701 was determined through normalisation to JE2.
702
703 **mRNA extraction**
704 Overnight cultures were back diluted to an OD_{600nm} of 0.05 in 50 ml of fresh TSB and grown
705 to an OD_{600nm} of 2. Two hundred micro litres of either PBS, 25% serum (final concentration
706 2.5%, sub-inhibitory), 5 µg/ml teicoplanin (final concentration 0.5 µg/ml, sub-inhibitory),
707 100% serum (final concentration 10%, inhibitory), or 100 µg/ml teicoplanin (final
708 concentration 10 µg/ml, inhibitory) was added to 1.8 ml of bacterial culture and incubated for
709 20 min. RNA was extracted by Quick-RNA Fungal/Bacterial Miniprep Kit (Zymo Research)
710 according to the manufacturer's instructions. RNA integrity was checked by running 5 µl
711 aliquot of the RNA on a 1% agarose gel and observing the intensity of the ribosomal RNA
712 (rRNA). RNA samples were treated by TURBO™ DNase (Invitrogen) to eliminate any
713 genomic DNA contamination. To verify that the samples were free from any DNA

714 contamination, RNA samples were subjected to RT-qPCR alongside a no template control
715 and 2.5 ng of a known JE2 genomic DNA and threshold rates compared.
716
717 **qRT-PCR**
718 To quantify the expression of the *tcaA* RT-qPCR was performed using the housekeeping
719 *gyrB* gene as a control. Complementary DNA (cDNA) was generated from mRNA using
720 qScript® cDNA Synthesis Kit (Quantabio). Following the manufacturers protocol the cDNA
721 was used as a template for the RT-qPCR reaction. Primers used were as follows *gyrB* FW 5'
722 ggtgactgcattagatgtaaac 3', *gyrB* RV 5' ctgcttctaaaccttctaatacttgtatttg 3', *tcaA* FW 5'
723 tagtttgcgcttcaggtg 3', *tcaA* RV 5' tgtggacataaatttgatagtcgtc 3'. The RT-qPCR reaction was
724 performed as follows: 10 µl 2x KAPA SYBR Mix, 1 µl of 10 µM forward primer, 1 µl of 10 µM
725 reverse primer, 5 µl cDNA and RNase-free water up to a total of 20 µl volume. The RT-
726 qPCR was performed on a Mic qPCR cycler (bio molecular systems) and the cycling
727 conditions consisted of an initial denaturation step of 95°C for 2 min, followed by 40 cycles of
728 two step cycling: 95°C 15 s, 60°C 1 min. RT-qPCR was carried out in technical triplicate for
729 each sample and 3 biological repeats. The ratio of *tcaA* to *gyrB* transcript number was
730 calculated using the using the $2^{-(\Delta\Delta Ct)}$ method[54].
731
732 **WTA preparations**
733 Crude WTA from murein sacculi was extracted for analysis by PAGE using adaptations of a
734 previously described methodology[55,56]. Overnight cultures were washed once in buffer 1 (50
735 mM MES, pH 6.5) followed by centrifugation at 5,000 g. Cells were resuspended in buffer 2
736 (4% [wt/vol] SDS, 50 mM MES, pH 6.5) and boiled for 1 h. Sacculi were centrifuged at
737 5,000g and washed once in buffer 1, once in buffer 2, once in buffer 3 (2% NaCl, 50 mM
738 MES, pH 6.5), once more in buffer 1 and finally resuspended in digestion buffer (20 mM Tris-
739 HCl pH 8.0, 0.5% [w/v] SDS). To the digestion buffer suspension, 10 µl of proteinase K
740 solution (2 mg/ml) was added and incubated on a heat block 50°C for 4 h at 1,400 rpm.
741 Sacculi were centrifuged at 16,000 g and washed once in buffer 3, followed by 3 washes in

742 dH_2O to removed SDS. Sacculi were responded in 0.1M NaOH and incubated for 16 h at
743 room temperature at 1,400 rpm. Following the incubation, the sacculi were centrifuged at
744 16,000g, leaving the teichoic acids in the supernatant. 250 µl of 1 M Tris-HCL pH 6.8 was
745 added to neutralise the NaOH and stored at -20°C.
746
747 WTA was also precipitated from the supernatant of overnight culture by adding 3 volumes of
748 95% ethanol and incubation at 4°C for 2 h. Precipitated material was separated by
749 centrifugation at 16,000 g for 15 min, washed once in 70% ethanol and resuspended in 100
750 mM Tris-HCL (pH 7.5) containing 5 mM $CaCl_2$, 25 mM $MgCl_2$, DNase (10 µg/ml) and RNase
751 (50 µg/ml) and incubated for 3 h at 37°C. The enzymes were heat inactivated at 95°C for 3
752 min. The supernatant WTA preparations were similarly stored at -20°C before being
753 analysed by PAGE.
754
755 **WTA PAGE**
756 WTA preparations were separated on tricine polyacrylamide gels using a BioRad tetra cell
757 according to a previously described method[56]. The gels were separated at 4°C using a
758 constant amperage of 40 mA under constant stirring until the dye front reached the bottom.
759 Gels were washed 3 times in MilliQ H_2O followed by staining in 1 mg/ml Alcian blue
760 overnight. Gels were subsequently destained in in MilliQ H_2O, until the WTA became visible
761 and finally imaged.
762
763 **Arachidonic acid supernatant conditioning**
764 Overnight cultures of JE2, *tcaA*, and *lcpA* mutants were pelleted, and the supernatant saved.
765 Growth inhibition assays were subsequently performed by adding 20 µl of OD_{600nm} 0.1
766 bacterial suspension to 180 µl 10% overnight supernatant diluted in fresh MHB++ containing
767 doubling concentrations of arachidonic acid (Sigma). Purified WTA extracts were also added
768 to the growth media at a final concentration of 2%. The ability of the bacteria to survive the

769 arachidonic acid was determined by quantifying bacterial growth (OD_{600nm}) following 24 h at
770 37°C using a CLARIOstar plate reader (BMG Labtech).
771
772 **Cytochrome C binding assay**
773 A cytochrome c binding assay was used to measure the relative surface charge of the
774 bacteria[57]. Briefly, overnight cultures were normalized to an OD_{600nm} of 8. The bacterial
775 suspensions were washed twice in MOPS buffer (20 mM pH 7.0) and finally resuspended in
776 200 µl of MOPS buffer. Samples were then combined with 50 µl of cytochrome C (equine
777 heart Sigma, 2.5 mg/ml in MOPS buffer) and incubated for 10 min at room temperature.
778 Finally, samples were pelleted (16,000 × g for 1 min) and 200 µl of supernatant read for
779 absorbance at Abs_{530nm} using a SUNRISE Tecan microplate reader.
780
781 **Construction of a TcaA reporter fusion**
782 A *tcaA* promoter-*gfp* fusion (pSB2019:*tcaA*) was constructed to determine expression of
783 *tcaA* in response to several antimicrobial components of serum. A region of 289 bp
784 upstream of the *tcaA* gene[58] was amplified by PCR from JE2 genomic DNA using the
785 primers *tcaA_promoter* FW 5'- atatgaattcagtattagaagtcatcaatca -3' and *tcaA_promoter* RV
786 5'-atatccccgggtttcacctcaattctgttcct-3'. The pSB2019 vector was prepared by digesting
787 pSB2031[59] from a previous study with EcoRI and SmaI to remove the RNAIII P3 promoter.
788 The *tcaA* promoter region was also digested with EcoRI and SmaI and ligated into pSB2019
789 using T4 DNA ligase (NEB). This was transformed into DC10B[34], a strain of *E. coli* which
790 mimics the adenine methylation profile of some *S. aureus* clonal complexes (including CC8
791 and CC22). The plasmid was then extracted and transformed into final strains via
792 electroporation.
793
794 **TcaA induction assay**
795 *S. aureus* strains carrying the pSB2019:*tcaA* plasmid were grown overnight in TSB with
796 10ug/ml chloramphenicol. Each strain was then normalized to an OD_{600nm} of 0.05 in fresh

797 TSB and subcultured to an OD_{600nm} of 0.5-0.6. Cultures were washed in PBS and
798 concentrated to an OD_{600nm} of 1 in PBS. 100 ul of bacteria was combined with 100 ul of the
799 appropriate antimicrobial compound (arachidonic acid or LL-37) in a black 96-well plate.
800 GFP Fluorescence (485_{nm} excitation/520_{nm} emission/1000 gain) was measured in a
801 PHERAstar FSX plate reader (BMG Labtech) over 2.5 h (readings every 30 min with
802 200 rpm shaking).
803
804 **Lysostaphin turbidity assay**
805 Overnight cultures were back diluted to an OD_{600nm} of 0.05 in 20 mL of fresh TSB and grown
806 to an OD_{600nm} 0.5-0.7. Cultures were normalised to give 1 ml of OD_{600nm} 0.6. Lysostaphin was
807 subsequently added to give a final concentration of 0.5 µg/mL. 200 µL of each strain was
808 then added to a 96-well plate and incubated for 3 h at 37°C in a CLARIOstar plate reader.
809 OD_{600nm} readings were taken every 10 min (500 rpm shaking before readings). Values were
810 blank corrected according to 200 µL of PBS. The PBP4*::tn* mutant was included as a control.
811
812 **Triton-X100 induced autolysis assay**
813 Overnight cultures were back diluted to an OD_{600nm} of 0.05 in 20 mL of fresh TSB and grown
814 to an OD_{600nm} 0.5-0.7. Cells were washed once in ice cold water (13,000 rpm 1 min) and
815 resuspended in autolysis buffer (water containing 0.1% Triton X-100) to give an OD_{600nm} of 1.
816 200 µL of each strain was then added to a 96-well plate alongside 200 µL of strains
817 resuspended in water as a negative control and 200 ul of water as a blank. The *atl::tn* strain
818 was used as a positive control. Strains were grown for 6 h at 37°C in a CLARIOstar plate
819 reader. OD_{600nm} readings were taken every 30 min (500 rpm shaking before readings). The
820 rate of autolysis was calculated as follows; (OD_{t0}-OD_{tn}/OD_{t0}).
821
822 **Transmission electron microscopy**
823 S. aureus strains JE2 and the TcaA mutant were pelleted in microfuge tubes and fixed by
824 resuspending in 2.5% glutaraldehyde in cacodylate buffer (pH 7.3) kept at fridge

825 temperatures until fixation was complete and then stored in the fridge. Pellets were
826 resuspended in a BSA/glutaraldehyde gel at 10- 20°C which sets after the cells were re-
827 centrifuged into a pellet. The pellets were postfixed with osmium ferrocyanide/osmium
828 tetroxide mix in cacodylate buffer, *en bloc* stained with uranyl acetate (2%) and Walton's
829 lead aspartate solutions, prior to dehydration in ethanol and infiltration with propylene oxide
830 and Epon resin mix. Embedded blocks were polymerised for 24-48 hours at 60°C. Sections
831 (70nm) were cut on a Leica UC7 ultramicrotome and imaged using a FEI Tecnai T12
832 microscope.
833
834 **In vivo intra-venous challenge model**
835 For survival studies, C57/Bl6 mice were injected with $5X10^7$ CFU of JE2 or *tcaA::tn* via the
836 tail vein. Mice were culled at specific time points post challenge and blood collected by
837 cardiac puncture. Blood was diluted in sterile PBS and plated on TSA to determine CFU/ml.
838 For the mixed infection bacteriemia model, female, 7-8 week-old, C57BL/6NCrl mice
839 (Charles River Laboratories) were used. Bacteria were prepared as described previously[60].
840 Briefly, 1:100 dilutions of overnight bacterial cultures were grown to mid-log phase (~2 h) in
841 TSB with shaking at 180 r.p.m. at 37 °C. Bacteria were then harvested, washed with PBS
842 and adjusted spectrophotometrically at OD_{600nm} to obtain the desired bacterial concentration
843 of ~4.8×10^8 CFU/mL. A 50:50 mixture of JE2 and *tcaA::tn* were made and 100 μl was
844 injected intravenously via the tail vein. The final CFU for each strain per mouse was ~$2.4 \times$
845 10^7 CFU. After 48 hours, terminal cardiac bleeds were performed on anesthetized mice and
846 whole blood was transferred into heparin tubes (Sarstedt) and mixed to prevent coagulation.
847 Mice were then euthanized by CO_2 inhalation. Organ homogenates of spleens, livers and
848 kidneys were performed as previously described[61] and serial dilutions of each sample were
849 plated onto TSA plates with and without erythromycin (5 μg/ml) for bacterial enumeration.
850 The CFU of JE2 was calculated by subtracting CFU on TSA with erythromycin (*tcaA::tn* only)
851 from CFU on TSA plates (JE2 and *tcaA::tn*).

852

853 **Ethics statement**

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

869

870 **Statistics**

871 Paired two-tailed student t-test or One-way ANOVA (GraphPad Prism v9.0) were used to
872 analyse the observed differences between experimental results. A p-value <0.05 was
873 considered statistically significant.

874

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890

891 **Author Contributions:** EJAD developed the methodology, conceptualized and performed
892 the experiments, analysed data, provided supervisory support and contributed to writing the
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894 methodology, performed experiments and provided supervisory support. DA performed
895 experiments. ML provided materials, analysed data and provided supervisory support. MR
896 developed methodology, analysed data and contributed to writing the manuscript. GYCC,
897 RCH, RL performed serum resistance experiments with mouse serum and murine
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903

904 **REFERENCES**

905 1. Gordon R.J. & Lowy F.D. Pathogenesis of methicillin-resistant Staphylococcus aureus
906 infection. *Clin Infect Dis* **5**, S350-9 (2008)

907 2. Lowy F.D. *Staphylococcus aureus* infections. *N Engl J Med* **339**, 520-32 (1998).
908 3. Powers M.E. &, Bubeck Wardenburg J. Igniting the fire: Staphylococcus aureus virulence
909 factors in the pathogenesis of sepsis. *PLoS Pathog*. **10**, e1003871 (2014).
910 4. Abdelnour A., Arvidson S., Bremell A., Ryden C. & Tarkowski A. The accessory gene
911 regulator (agr) controls Staphylococcus aureus virulence in a murine arthritis model. *Infect*
912 *Immun* **61**, 3879-85 (1993).
913 5. Cheung A.L. *et al*. Diminished virulence of a sar-/agr- mutant of Staphylococcus aureus in
914 the rabbit model of endocarditis. *J Clin Invest* **94**, 1815-22 (1994).
915 6. Rose H.R. *et al*. Cytotoxic Virulence Predicts Mortality in Nosocomial Pneumonia Due to
916 Methicillin-Resistant Staphylococcus aureus. *J Infect Dis* **211,** 1862-74 (2015).
917 7. Laabei M. *et al*. Evolutionary Trade-Offs Underlie the Multi-faceted Virulence of
918 Staphylococcus aureus. *PLoS Biol*. **13**, e1002229 (2015).
919 8. Kaash A.J. *et al.* Staphylococcus aureus bloodstream infection: a pooled analysis of five
920 prospective, observational studies. *J Infect* **68,** 242-51 (2014).
921 9. Levy O. Antimicrobial proteins and peptides of blood: templates for novel antimicrobial
922 agents. *Blood* **96**, 2664-72 (2000).
923 10. Kenny J.G. *et al*. The Staphylococcus aureus Response to Unsaturated Long Chain
924 Free Fatty Acids: Survival Mechanisms and Virulence Implications. *PLoS One* **4**, e4344
925 (2009).
926 11. Beavers W.N. *et al*. Arachidonic Acid Kills Staphylococcus aureus through a Lipid
927 Peroxidation Mechanism. mBio **10**, 5 (2019).
928 12. Das U.N. Arachidonic acid and other unsaturated fatty acids and some of their
929 metabolites function as endogenous antimicrobial molecules: A review. *J Advanc Res* **11** 57-
930 66 (2018).
931 13. Laabei M. *et al.* Predicting the virulence of MRSA from its genome sequence. Genome
932 Res. **24,** 839-49 (2014).
933 14. Recker M. *et al.* Clonal differences in Staphylococcus aureus bacteraemia-associated
934 mortality. *Nat Microbiol* **2**, 1381-1388 (2017).

935 15. Yokoyama M., *et al.* Epistasis analysis uncovers hidden antibiotic resistance-associated
936 fitness costs hampering the evolution of MRSA. *Genome Biol* **19**, 94 (2018).
937 16. Stevens E. *et al*. Targeted control of toxin production by a mobile genetic element in
938 Streptococcus pneumoniae. *Microb Genom* **8**, 000784 (2022).
939 17. Altwiley D. *et al*. A Functional Menadione Biosynthesis Pathway is Required for Capsule
940 Production by Staphylococcus aureus. *Microbiology* **167**, 001108 (2021).
941 18. Douglas E.J.A., Duggan S., Brignoli T. &, Massey RC. The MpsB protein contributes to
942 both the toxicity and immune evasion capacity of Staphylococcus aureus. *Microbiology* **167**,
943 001096 (2021).
944 19. Peschel A. *et al*. Inactivation of the dlt operon in Staphylococcus aureus confers
945 sensitivity to defensins, protegrins, and other antimicrobial peptides. *J Biol Chem* **274**, 8405-
946 10 (1999).
947 20. Ernst C.M. & Peschel A. Broad-spectrum antimicrobial peptide resistance by MprF-
948 mediated aminoacylation and flipping of phospholipids. *Mol Microbiol* **80**, 290-9 (2011).
949 21. Hwang-Soo J. & Otto M. Mechanisms of resistance to antimicrobial peptides in
950 staphylococci. *Biochimica et Biophysica Acta (BBA) – Biomembranes* **1848**, 3055-3061
951 (2015).
952 22. Kohler T., Weidenmaier C. & Peschel A. Wall Teichoic Acid Protects Staphylococcus
953 aureus against Antimicrobial Fatty Acids from Human Skin. *J Bacteriol* **191**, 4482–4484
954 (2009).
955 23. Fey P.D. *et al*. A genetic resource for rapid and comprehensive phenotype screening of
956 nonessential Staphylococcus aureus genes. *mBio* **4**, e00537-12 (2013).
957 24. Brandenberger M. *et al.* Inactivation of a novel three-cistronic operon tcaR-tcaA-tcaB
958 increases teicoplanin resistance in Staphylococcus aureus. *Biochim Biophys Acta*.
959 **1523**,135-9 (2000).
960 25. Xia G., Kohler T. & Peschel A. The wall teichoic acid and lipoteichoic acid polymers of
961 Staphylococcus aureus. *Int J Med Microbiol* **300**,148-54 (2010).

962 26. Fujita Y. & Fujita T. The gluconate operon gnt of Bacillus subtilis encodes its own
963 transcriptional negative regulator. *Proc Natl Acad Sci U S A* **84**, 4524-8 (1987).
964 27. Li K.H. *et al.* Biological Functions of ilvC in Branched-Chain Fatty Acid Synthesis and
965 Diffusible Signal Factor Family Production in Xanthomonas campestris. *Front Microbiol* **8**,
966 2486 (2017).
967 28. Shen Z., Han J., Wang Y., Sahin O. & Zhang Q. The Contribution of ArsB to Arsenic
968 Resistance in Campylobacter jejuni. *PLoS One* **8** e58894 (2013).
969 29. Rismondo J., Percy M.G. & Gründling A. Discovery of genes required for lipoteichoic
970 acid glycosylation predicts two distinct mechanisms for wall teichoic acid glycosylation. *J Biol*
971 *Chem* **293**, 3293–3306 (2018).
972 30. Hemilä H. Lipoamide dehydrogenase of Staphylococcus aureus: nucleotide sequence
973 and sequence analysis. *Biochim Biophys Acta* **1129**, 119-23 (1991).
974 31. Jumper J. *et al*. Highly accurate protein structure prediction with AlphaFold. *Nature* **596**,
975 583–9 (2021).
976 32. Ma B. & Nussinov R. Trp/Met/Phe hot spots in protein-protein interactions: potential
977 targets in drug design. *Curr Top Med Chem* **7**, 999-1005 (2007).
978 33. Waldron D.E. & Lindsay J.A. Sau1: a Novel Lineage-Specific Type I Restriction-
979 Modification System That Blocks Horizontal Gene Transfer into Staphylococcus aureus and
980 between S. aureus Isolates of Different Lineages. *J. Bacteriol* **188**, 5578-85 (2006).
981 34. Monk I.R. *et al*. Transforming the Untransformable: Application of Direct Transformation
982 To Manipulate Genetically Staphylococcus aureus and Staphylococcus epidermidis. *mBio* **3**,
983 e00277-11 (2012).
984 35. Maki H., McCallum N., Bischoff M., Wada A. & Berger-Bächi B. tcaA inactivation
985 increases glycopeptide resistance in Staphylococcus aureus. *Antimicrob Agents Chemother*
986 **48**, 1953-9 (2004).
987 36. Schlag, M., et al. Role of staphylococcal wall teichoic acid in targeting the major
988 autolysin Atl. *Mol Microbiol*. **75**, 864-73 (2010).

989 37. Brown S, et al. Methicillin resistance in Staphylococcus aureus requires glycosylated
990 wall teichoic acids. *Proc Natl Acad Sci U S A.* **109**, 18909-14 (2012).
991 38. Kurokawa K., *et al*. Glycoepitopes of staphylococcal wall teichoic acid govern
992 complement-mediated opsonophagocytosis via human serum antibody and mannose-
993 binding lectin. *J Biol Chem* **288**, 30956-68 (2013).
994 39. Wanner S., *et al.* Wall teichoic acids mediate increased virulence in Staphylococcus
995 aureus. *Nature Microbiol* **2**, 16257 (2017).
996 40. Weidenmaier C., *et al.* Lack of Wall Teichoic Acids in Staphylococcus aureus Leads to
997 Reduced Interactions with Endothelial Cells and to Attenuated Virulence in a Rabbit Model
998 of Endocarditis. *Journal Infect Dis* **191**, 1771–7 (2005).
999 41. Monk IR., Casey P.G., Cronin M., Gahan C.G. & Hill C. Development of multiple strain
1000 competitive index assays for Listeria monocytogenes using pIMC; a new site-specific
1001 integrative vector. *BMC Microbiol.* **13**, 8:96 (2008).
1002 42. Steidl R., *et al*. Staphylococcus aureus cell wall stress stimulon gene-lacZ fusion strains:
1003 potential for use in screening for cell wall-active antimicrobials. *Antimicrob Agents*
1004 *Chemother* **52**, 2923-5 (2008).
1005 43. Utaida S., *et al*. Genome-wide transcriptional profiling of the response of Staphylococcus
1006 aureus to cell-wall-active antibiotics reveals a cell-wall-stress stimulon. *Microbiology* **149**,
1007 2719-32 (2003).
1008 44. Levin BR & Bull JJ. Short-sighted evolution and the virulence of pathogenic
1009 microorganisms. *Trends Microbiol*. **2**, 76-81 (1994).
1010 45. Corrigan R.M. & Foster T.J. An improved tetracycline-inducible expression vector for
1011 Staphylococcus aureus. *Plasmid* **61**, 126-9 (2009).
1012 46. Kreiswirth, B. N. et al. The toxic shock syndrome exotoxin structural gene is not
1013 detectably transmitted by a prophage. *Nature* **305,** 709–712 (1983).
1014 47. Horsburgh, M. J. *et al.* σ^B Modulates Virulence Determinant Expression and Stress
1015 Resistance: Characterization of a Functional *rsbU* Strain Derived from *Staphylococcus*
1016 *aureus* 8325-4. *J Bacteriol* **184**, 5457–5467 (2002).

1017 48. Hawiger, J., Niewiarowski, S., Gurewich, V. & Thomas, D. P. Measurement of fibrinogen
1018 and fibrin degradation products in serum by staphylococcal clumping test. *J Lab Clin Med*
1019 **75**, 93–108 (1970).
1020 49. Diep, B. A. *et al.* Complete genome sequence of USA300, an epidemic clone of
1021 community-acquired meticillin-resistant Staphylococcus aureus. *The Lancet* **367**, 731–739
1022 (2006).
1023 50. Schuster, C. F. *et al.* High-throughput transposon sequencing highlights the cell wall as
1024 an important barrier for osmotic stress in methicillin resistant *Staphylococcus aureus* and
1025 underlines a tailored response to different osmotic stressors. *Mol Microbiol* **113**, 699–717
1026 (2020).
1027
1028 51. Krausz K.L. & Bose J.L**.** Bacteriophage Transduction in Staphylococcus aureus: Broth-
1029 Based Method. *Methods Mol Biol.* **1373**, 63-8 (2016).
1030 52. European Committee for Antimicrobial Susceptibility Testing (EUCAST) of the European
1031 Society of Clinical Microbiology and Infectious Diseases (ESCMID). Determination of
1032 minimum inhibitory concentrations (MICs) of antibacterial agents by broth dilution. *Clin*
1033 *Microbiol Infect* **9**, ix-xv (2003).
1034 53. Duggan S. *et al*. A Small Membrane Stabilizing Protein Critical to the Pathogenicity of
1035 Staphylococcus aureus. *Infect Immun* **88**:e00162-20 (2020).
1036 54. Livak, K. J. & Schmittgen, T. D. Analysis of Relative Gene Expression Data Using Real-
1037 Time Quantitative PCR and the 2−ΔΔCT Method. *Methods* **25**, 402–408 (2001).
1038 55. Kho, K. & Meredith, T. Extraction and Analysis of Bacterial Teichoic Acids. *Bio Protoc* **8**,
1039 (2018).
1040 56. Brignoli, T. *et al.* Wall Teichoic Acids Facilitate the Release of Toxins from the Surface of
1041 Staphylococcus aureus. *Microbiol Spectr* (2022) doi:10.1128/spectrum.01011-22.
1042 57. Douglas, E. J. A. *et al.* Antibacterial activity of novel linear polyamines against
1043 Staphylococcus aureus. *Front Microbiol* **13**, (2022).

1044 58. Dengler, V., Meier, P. S., Heusser, R., Berger-Bächi, B. & McCallum, N. Induction
1045 kinetics of the Staphylococcus aureus cell wall stress stimulon in response to different cell
1046 wall active antibiotics. *BMC Microbiol* **11**, 16 (2011).
1047 59. Qazi, S. N. A. *et al. agr* Expression Precedes Escape of Internalized *Staphylococcus*
1048 *aureus* from the Host Endosome. *Infect Immun* **69**, 7074–7082 (2001).
1049 60. Nguyen, T. H. *et al.* Rapid pathogen-specific recruitment of immune effector cells in the
1050 skin by secreted toxins. *Nat Microbiol* **7**, 62–72 (2022).
1051 61. Bae, J. S. *et al.* Contribution of Staphylococcal Enterotoxin B to *Staphylococcus aureus*
1052 Systemic Infection. *J Infect Dis* **223**, 1766–1775 (2021).
1053