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1 **Genomic identification of cryptic susceptibility to penicillins and  $\beta$ -lactamase**  
2 **inhibitors in methicillin-resistant *Staphylococcus aureus***

3

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35

36 **Abstract**

37 Antibiotic resistance in bacterial pathogens threatens the future of modern medicine.  
38 One such resistant pathogen is methicillin-resistant *Staphylococcus aureus* (MRSA),  
39 which is resistant to nearly all  $\beta$ -lactam antibiotics, limiting treatment options. Here,  
40 we show that a significant proportion of MRSA isolates from different lineages,  
41 including the epidemic USA300 lineage, are susceptible to penicillins when used in  
42 combination with  $\beta$ -lactamase inhibitors such as clavulanic acid. Susceptibility is  
43 mediated by a combination of two different mutations in the *mecA* promoter region  
44 that lowers *mecA*-encoded penicillin binding protein 2a (PBP2a) expression, and in  
45 the majority of isolates by either one of two substitutions in PBP2a (E246G or M122I)  
46 that increase the affinity of PBP2a for penicillin in the presence of clavulanic acid.  
47 Treatment of *S. aureus* infections in wax moth and mouse models demonstrate that  
48 penicillin/ $\beta$ -lactamase inhibitor susceptibility can be exploited as an effective  
49 therapeutic choice for 'susceptible' MRSA infection. Finally, we show that isolates  
50 with the PBP2a E246G substitution have a growth advantage in the presence of  
51 penicillin, but the absence of clavulanic acid, which suggests that penicillin/ $\beta$ -  
52 lactamase susceptibility is an example of collateral sensitivity (resistance to one  
53 antibiotic increases sensitivity to another). Our findings suggest that widely available  
54 and currently disregarded antibiotics could be effective in a significant proportion of  
55 MRSA infections.

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60

61 **Introduction**

62 The  $\beta$ -lactam family of antibiotics, is one of the most widely used and clinically  
63 important groups of antibiotics <sup>1</sup>. Resistance to  $\beta$ -lactam antibiotics in  
64 *Staphylococcus aureus* is either mediated by the acquisition of the *blaZ* gene  
65 encoding a  $\beta$ -lactamase, or in methicillin-resistant *S. aureus* (MRSA) from the  
66 acquisition of an alternative penicillin binding protein 2a (PBP2a) with a low affinity  
67 for  $\beta$ -lactam antibiotics, resulting in resistance to most  $\beta$ -lactams <sup>2</sup>. PBP2a is  
68 encoded by *mecA* or *mecC* (PBP2a'/c) carried on a mobile genetic element known as  
69 a staphylococcal cassette chromosome *mec* (SCC*mec*) <sup>3,4</sup>, and is regulated by two  
70 independent regulatory systems (*mecI-mecR-mecR2* and *blaI-blaR*) and multiple  
71 chromosomal genes <sup>5</sup>.

72  
73 Countering resistance to  $\beta$ -lactams was first achieved by the development of  $\beta$ -  
74 lactamase resistant  $\beta$ -lactams such as methicillin <sup>6</sup>, and subsequently by the  
75 development of  $\beta$ -lactamase inhibitors <sup>7</sup>. Unfortunately, MRSA is resistant to both  
76 methicillin antibiotics and insensitive to  $\beta$ -lactamase inhibitors. However, in the early  
77 1990s,  $\beta$ -lactams and  $\beta$ -lactamase inhibitors were tested against MRSA <sup>8-11</sup>, with  
78 success both *in vivo* and clinically <sup>12</sup>.

79  
80 Recently, different approaches to restore susceptibility to  $\beta$ -lactams have been  
81 investigated, including using multiple antibiotics to exploit collateral sensitivity  
82 (resistance to one antibiotic increases sensitivity to another) <sup>13-15</sup>. We recently  
83 demonstrated that the small subset of MRSA isolates with the *mecC* gene are  
84 susceptible to penicillins and  $\beta$ -lactamase inhibitors, as the *mecC* encoded PBP2c  
85 does not mediate resistance to penicillins <sup>16</sup>. Here, we demonstrate that  
86 unexpectedly, a significant proportion of all clinical *mecA*-positive MRSA isolates  
87 from diverse lineages are susceptible to penicillins and  $\beta$ -lactamase inhibitors. These

88 findings could provide the basis of new treatment options for susceptible MRSA  
89 infections using already licenced antibiotics.

90

## 91 **Results**

### 92 **Penicillin/ $\beta$ -lactamase inhibitor-susceptible MRSA**

93 As part of an earlier study <sup>16</sup>, we identified an *mecA*-positive MRSA isolate that  
94 exhibited increased susceptibility to penicillin in the presence of clavulanic acid (a  $\beta$ -  
95 lactamase inhibitor) (Fig 1a). We screened a selection of whole genome sequenced  
96 (WGS) MRSA isolates (n = 110) from different clinically relevant clonal lineages for  
97 the same increase in susceptibility ( $\geq 10$  mm increase in the zone of inhibition in the  
98 presence of 15  $\mu\text{g/ml}$  clavulanic acid, compared to penicillin alone). Fifty-five (50.0%)  
99 isolates from different lineages were susceptible to penicillin-clavulanic acid  
100 (Supplementary Table 1). We determined the minimum inhibitory concentration (MIC)  
101 for penicillin for a subset of isolates, which showed that the MIC was reduced below  
102 the European Committee on Antimicrobial Susceptibility Testing (EUCAST) / Clinical  
103 and Laboratory Standards Institute (CLSI) breakpoint ( $\leq 0.125$   $\mu\text{g/ml}$ ) <sup>17,18</sup> in ten of  
104 the fourteen susceptible isolates, and none of the ten resistant isolates (Fig. 1b). Two  
105 other  $\beta$ -lactam inhibitors, sulbactam and tazobactam also increased susceptibility to  
106 penicillin (Supplementary Fig. 1), suggesting that the effect of clavulanic acid was  
107 due to inhibition of the staphylococcal  $\beta$ -lactamase. Next, we evaluated if the  
108 increase in susceptibility was penicillin-specific by screening isolates against different  
109  $\beta$ -lactam antibiotics in the presence of clavulanic acid (Fig. 1c and d). This revealed  
110 that the penicillin-clavulanic acid susceptible isolates showed the greatest increase in  
111 susceptibility to penicillins (benzyl- and aminopenicillins), (Fig. 1d) and were broadly  
112 more susceptible to cephalosporins (except ceftaroline) and carbapenems than the  
113 resistant isolates (Fig 1c). In contrast, the presence of clavulanic acid produced only  
114 minor increases in susceptibility in the resistant isolates (Fig 1c).

## 115 Substitutions in PBP2a mediate increased penicillin susceptibility

116 Benzyl- and aminopenicillins bind reasonably well to PBP2a in comparison to the  
117 isoxazolyl penicillins (oxacillin) and cephalosporins<sup>19</sup>. As amino acid substitutions in  
118 PBP2a mediate resistance to fifth-generation cephalosporins<sup>20</sup>, in the absence  $\beta$ -  
119 lactamase activity, we hypothesised that inversely, increased susceptibility to  
120 penicillins might be mediated by PBP2a substitutions. We examined the PBP2a  
121 amino acid sequences of the 110 isolates (Supplementary Table 1). This revealed  
122 that 80.0% (44/55) of the penicillin-clavulanic acid susceptible isolates had either a  
123 E246G (PBP2a<sup>246G</sup>) (n=37) or a M122I (PBP2a<sup>122I</sup>) (n=7) substitution in the allosteric  
124 domain of PBP2a<sup>21</sup>. By contrast, only 29.1% (16/55) of the resistant isolates had the  
125 PBP2a<sup>246G</sup> substitution. Phylogenetic analysis of PBP2a indicated that 246E is  
126 present in the *S. aureus* COL genome (M122I was absent), an early MRSA strain  
127 isolated in the 1960s, with origins in the 1940s<sup>22,23</sup>, suggesting this might be the  
128 ancestral or 'wildtype' form (Supplementary Fig. 2).

129

130 Next, we tested the effect of the PBP2a substitutions experimentally. We deleted  
131 *mecA* (PBP2a) in isolates from two *S. aureus* lineages: ST398 (EC139<sup>24</sup>) and  
132 USA300/ST8 (BCVA289<sup>25</sup>) (both PBP2a<sup>246G</sup> and *blaZ* positive) and introduced  
133 plasmid-borne copies of three *mecA* alleles: the putatively resistant 'wildtype'  
134 *mecA*<sub>246E</sub> and the two alleles associated with susceptibility: *mecA*<sub>122I</sub> and *mecA*<sub>246G</sub>.  
135 All the complemented strains were resistant to penicillin alone (MICs >20 $\mu$ g/ml -  
136 breakpoint >0.125 $\mu$ g/ml) (Fig. 2a). For penicillin-clavulanic acid, the *mecA*<sub>246E</sub> strains  
137 were resistant with MICs of 1 $\mu$ g/ml (Fig. 2a). While the strains with *mecA*<sub>246G</sub> were  
138 susceptible, with MICs of 0.125 $\mu$ g/ml. Similarly strains with *mecA*<sub>122I</sub> had MICs of  
139 0.125 and 0.25 $\mu$ g/ml for USA300 and ST398 backgrounds, respectively. No  
140 difference in susceptibility was seen for cefoxitin (a cephalosporin), confirming that

141 the effect of the substitutions was limited to penicillin susceptibility (Supplementary  
142 Table 2).

143

144 **A combination of *mecA* promoter mutations and PBP2a substitutions mediate**  
145 **susceptibility**

146 We reasoned that the PBP2a substitutions might cause an increase in the affinity of  
147 PBP2a for penicillin. We tested PBP2a<sup>246E</sup>, PBP2a<sup>246G</sup> and PBP2a<sup>122I</sup> in a bocillin  
148 competition assay to determine their relative binding affinities for penicillin (Fig 2b).

149 This identified that all three variants had a similar affinity for penicillin, with 50%  
150 inhibitory concentrations (IC50) of 10.63 µg/ml (standard error of the mean ± 0.53) for  
151 PBP2a<sup>246G</sup> and 12.11(± 1.07) for PBP2a<sup>122I</sup> and 9.06 (± 2.70) for PBP2a<sup>246E</sup> (Fig 2b).

152 As susceptibility testing had been carried out in the presence of clavulanic acid, we  
153 repeated the bocillin binding assays with 15 µg/ml clavulanic acid. In the presence of  
154 clavulanic acid, the wildtype PBP2a<sup>246E</sup> had a two-fold higher IC50 of 32.07 (± 8.38)  
155 compared to that PBP2a<sup>246G</sup> 16.22 ± 3.19) and PBP2a<sup>122I</sup> (11.73 ± 4.50), which were  
156 virtually unaffected (Fig 2c).

157

158 To confirm the consistency of association between the two PBP2a substitutions and  
159 phenotypic susceptibility, we determined the penicillin and penicillin-clavulanic acid  
160 MICs for 274 WGS isolates from a range of *S. aureus* lineages (Supplementary  
161 Table 3). We then combined the data with the original 24 isolates with MIC data (Fig.  
162 1b) and plotted the frequency distributions of the penicillin and penicillin-clavulanic  
163 acid MICs (Fig. 2d and e). In the absence of a clinical breakpoint for penicillin-  
164 clavulanic acid we determined a tentative epidemiological (wildtype) cut-off (ECOFF)  
165 using a statistical method based on mixture models<sup>26,27</sup>. This supported setting the  
166 ECOFF between 2.0 and 3.0 µg/ml (2.449, rounded down to 2.0 µg/ml) for penicillin  
167 in the presence of 15 µg/ml clavulanic acid (Fig 2d). The same method supported an  
168 ECOFF between 0.19 and 0.25 µg/ml (0.21, rounded down to 0.19 µg/ml) for

169 penicillin alone, which is within one doubling dilution of the EUCAST clinical  
170 breakpoint (Fig 2e)<sup>18</sup>. Using the ECOFF of  $\leq 2.0$   $\mu\text{g/ml}$  as the cut-off, 213 (71.5%)  
171 isolates were classified as susceptible to penicillin-clavulanic acid, of which only 176  
172 (82.6%) had either a 246G or 122I substitution. This indicated that 37 (17.4%)  
173 isolates were susceptible despite having no PBP2a substitution. Conversely, only 37  
174 (43.5%) of the resistant isolates (MIC  $> 2$   $\mu\text{g/ml}$ ) had a 246G substitution.

175

176 Incomplete congruence between PBP2a substitutions and penicillin-clavulanic acid  
177 susceptibility led us to search for further mutations involved in susceptibility. We  
178 hypothesised that higher levels of PBP2a expression might overcome the effect of  
179 the PBP2a substitutions. We screened the same 298 isolates for mutations in the  
180 *mecA* promoter region, this identified two mutations that correlated with susceptibility.

181 The first mutation was a G to T transversion in the *mecA* ribosomal binding site  
182 (RBS), seven nucleotides upstream of *mecA* start codon (Fig 3a). Isolates with the  
183 *mecA*[-7]:T allele had a median penicillin-clavulanic acid MIC of 0.125  $\mu\text{g/ml}$  (range  
184  $< 0.016 - 6$ ) compared to a median of 8  $\mu\text{g/ml}$  (range: 0.023 – 96) for isolates with the  
185 ‘wildtype’ *mecA*[-7]:G. Previous work has demonstrated that despite being in the  
186 RBS, the T allele results in lower *mecA* transcript and PBP2a expression levels<sup>28</sup>.

187 We compared relative levels of *mecA* expression by reverse transcription quantitative  
188 polymerase chain reaction (RT-qPCR) in isolates from phylogenetically separate  
189 lineages with the *mecA*[-7]:G (n=7) allele to isolates with the *mecA*[-7]:T allele (n=6)  
190 (Fig 3b). Isolates with the T allele had a statistically significantly lower relative  
191 expression (mean relative expression: 3.24) than isolates with the G allele (mean:  
192 7.00) (P=0.0048) (Fig 3b). The second mutation was a C to T transition in the *mecA* -  
193 10 box, 33 nucleotides upstream of the *mecA* start codon (*mecA*[-33]:T) (Fig 3a).  
194 Isolates with this mutation had a median penicillin-clavulanic acid MIC of 0.047  $\mu\text{g/ml}$   
195 (range:  $< 0.016$  to 0.125). The C to T transition causes the generation of a perfect



196 palindrome within the Mecl-Blal binding site, which lowers *mecA* transcript and  
197 PBP2a expression levels<sup>28,29</sup>. Three isolates from distinct lineages with the *mecA*[-  
198 33]:T mutation had a mean relative expression rate of 0.27, suggesting the C to T  
199 transition results in very low levels of *mecA* expression in the tested isolates (Fig 3b).  
200  
201 When considering the two promoter mutations (*mecA*[-7]:G-T and *mecA*[-33]:C-T)  
202 together with the two PBP2a substitutions (PBP2a<sup>246G</sup> and PBP2a<sup>122I</sup>) (Fig 3a) we  
203 identified six genotypes. These were used to annotate the MIC distributions for  
204 penicillin and penicillin-clavulanic acid (Fig 3c and d). For penicillin-clavulanic acid,  
205 the genotypes split clearly into the bimodal distribution (Fig 3c). The majority of  
206 isolates with *mecA*[-7]:G (henceforth: Resistant 1) and *mecA*[-7]:G | E246G  
207 (Resistant 2) genotypes were found in the modal peak to the right with MICs above  
208 the ECOFF of  $\geq 2$   $\mu\text{g/ml}$  (Fig 3c). The majority of isolates with the other four  
209 genotypes: *mecA*[-7]:G-T (henceforth: Susceptible 1), *mecA*[-7]:G-T | E246G  
210 (Susceptible 2), *mecA*[-33]:C-T | *mecA*[-7]:G | E246G (Susceptible 3) and *mecA*[-  
211 7]:G | M122I (Susceptible 4) were located in the modal peak to the left with MICs  
212 below the ECOFF ( $\leq 2.0$   $\mu\text{g.ml}$ ). Isolates with susceptible genotypes had lower  
213 penicillin MICs in the absence of clavulanic acid than those with resistant genotypes  
214 (Fig 3c and d). The use of the six genotypes to predict susceptibility using the  
215 ECOFF as breakpoint was accurate in 94.6% (282/298) of isolates, with a 0.34%  
216 (1/298) very major error (VME) rate (defined as isolates that were phenotypically  
217 resistant but genotypically predicted to be susceptible) and a 5.0% (15/298) major  
218 error (ME) rate (phenotypically susceptible, genotypically predicted resistant).  
219  
220 Finally, we investigated if the presence of the six different types of class A  
221 staphylococcal  $\beta$ -lactamases (types A-F), or *blaZ* expression levels might affect  
222 penicillin-clavulanic acid susceptibility<sup>16</sup>. There was no association between the  $\beta$ -  
223 lactamase type and susceptibility that wasn't better explained by the six genotypes

224 (Supplementary Table 3 and Fig. 3a). Nor was there any significant association (P=  
225 0.43) between *blaZ* expression and susceptibility in twenty isolates (11 susceptible, 9  
226 resistant) as measured by RT-qPCR (Supplementary Fig. 3b).

227

## 228 **Clinical prevalence and epidemiology**

229 We sought to quantify the frequency of penicillin-clavulanic acid susceptibility in  
230 clinical MRSA isolates. We tested a collection of 270 *S. aureus* isolates (MRSA, n =  
231 218; MSSA isolates, n = 52) collected by the Danish Staphylococcus Reference  
232 Laboratory as part of bacteraemia surveillance<sup>30</sup> (Supplementary Table 4). Isolates  
233 were classified as resistant or susceptible based on the ECOFF of  $\leq 2.0$   $\mu\text{g/ml}$   
234 penicillin in the presence of 15  $\mu\text{g/ml}$  of clavulanic acid. All MSSA isolates, and  
235 84.9% (185/218) of the MRSA isolates had an MIC below the ECOFF. The MRSA  
236 isolates were from a variety of lineages as inferred from *spa*-typing including clonal  
237 complex (CC)1, CC5, CC8, CC30 and CC80. We then screened 2282 WGS MRSA  
238 isolates from Cambridge, UK for the six genotypes (Fig. 4a)<sup>31</sup>. None of the isolates in  
239 this collection had the Susceptible 4 genotype. Overall, 25.0% of isolates had one of  
240 the three remaining putative susceptible genotypes (Fig 4a). The dominant UK  
241 MRSA sequence type is ST22 (EMRSA-15), if the CC22 isolates (70.4% of isolates)  
242 were excluded then 82.8% (n=610) had one of three susceptible genotypes (Fig. 4a).  
243 Lineages with a high abundance of susceptible genotypes included CC1, CC5, CC8,  
244 CC30 and CC59 (Fig. 4a). Importantly, 56 of the CC8 isolates (from 24 patients)  
245 were USA300 which is the dominant clonal lineage in the United States (USA)<sup>32</sup>. All  
246 56 USA300 isolates had the Susceptible 2 genotype and a penicillin-clavulanic acid  
247 MIC below the ECOFF (Supplementary Table 3). We performed a phylogenetic  
248 analysis of 580 CC8 isolates, including 485 USA300 isolates (457 MRSA, 28 MSSA)  
249 from across the USA<sup>33,34</sup>. All 457 of the USA300 MRSA isolates had the Susceptible  
250 2 (*mecA*[-7]:G-T | E246G) genotype carried on a SCC*mec* type IVa (n=455) or IVb

251 element (n=1) (one isolate was non-SCC*mec* typeable), suggesting that the majority  
252 of the USA300 population is susceptible to penicillin-clavulanic acid (Fig 4b). We then  
253 screened 23 USA300 isolates, distributed across the phylogeny (isolated in New  
254 York<sup>34</sup>) for penicillin-clavulanic acid susceptibility (Fig. 4b). All had an MIC below the  
255 ECOFF (<2 µg/ml) (Supplementary Table 5).

256

### 257 **Heterogeneity of susceptible populations**

258 β-lactams resistance in most MRSA is heterogenous, meaning that while most cells  
259 in a population have low MICs, a fraction ( $10^{-4}$ – $10^{-8}$ ) can survive at much higher MICs  
260<sup>35</sup>. We tested representative isolates for all six penicillin-clavulanic acid susceptibility  
261 genotypes in a population analysis for their susceptibility profile to penicillin-  
262 clavulanic acid. Isolates representing the four susceptible genotypes all displayed  
263 heterogenous resistance to penicillin-clavulanic acid (Fig 5a). CFUs were drastically  
264 reduced at low concentrations of penicillin (0.12 to 1 µg/ml) and susceptible isolates  
265 had a median population MIC of 12 µg/ml (range 0.48 to 32) (Fig 5a). Notably, for  
266 four isolates from three different genotypes (Susceptible 1, 3, and 4) the entire  
267 population was completely inhibited by 4 µg/ml. The remainder of susceptible  
268 isolates had 'highly resistant cells' (cells capable of growing in concentrations greater  
269 than the ECOFF of  $\leq 2$  µg/ml) present at a frequency of  $10^{-5}$ – $10^{-8}$ . In contrast,  
270 resistant isolates displayed homogenous resistance to penicillin-clavulanic acid (Fig  
271 5a). CFUs of resistant isolates were unaffected at the lower penicillin concentration  
272 range (0.12 to 1 µg/ml) and had a higher median population MIC of 64 µg/ml (range  
273 64 to 128), greater than the susceptible isolates (Fig. 5a). Highly resistant cells (MIC  
274  $\geq 4$  µg/ml) were also present at higher frequencies ( $1$ – $10^{-4}$ ) than susceptible isolates.

275

### 276 **Penicillin-clavulanic acid is effective for MRSA treatment *in vivo***

277 We next sought to demonstrate efficacy in physiologically relevant infection models in  
278 which highly resistant cells would be present. First, we used a wax moth larvae

279 model of infection, larvae were infected with four different MRSA isolates, two with a  
280 resistant genotype (both ST22: Resistant 1) and two with a susceptible genotype  
281 (ST398 and ST8 (USA300): Susceptible 2). Treatment at approximate human  
282 dosages was with penicillin, penicillin-clavulanic acid, amoxicillin, amoxicillin-  
283 clavulanic acid, vancomycin or PBS (vehicle). Only vancomycin offered any  
284 improvement in survival for the two resistant isolates (Fig 5b and c). In contrast, for  
285 the two susceptible isolates both penicillin-clavulanic acid and amoxicillin-clavulanic  
286 acid increased survival in comparison to penicillin or amoxicillin alone, increasing the  
287 median survival times by 40 hours (Fig 5d and e). Given that amoxicillin-clavulanic  
288 acid is clinically available, we further tested its efficacy in a more physiologically  
289 relevant, higher infective dose ( $1 \times 10^7$  CFU) murine thigh infection model, with  
290 approximate human dosages using a USA300 strain (strain: MRSA 43484<sup>36</sup>  
291 (Susceptible 2, penicillin-clavulanic acid MIC: 0.19  $\mu\text{g/ml}$ , population analysis:  
292 Supplementary Fig. 4), USA300 phylogeny: Fig. 4b). Treatment with amoxicillin alone  
293 in a dose range of 10 – 100 mg/kg did not reduce the bacterial loads compared to  
294 vehicle treatment, whereas 100 mg/kg amoxicillin in combination with 20 mg/kg  
295 clavulanic acid significantly reduced the bacterial loads to a similar level as 40 mg/kg  
296 vancomycin (Dunnett's multiple comparisons test,  $p < 0.0001$ ) (Fig. 5f). Demonstrating  
297 the efficacy of amoxicillin-clavulanic acid as a treatment in a high dose infection  
298 model.

299

### 300 **PBP2a substitutions provide a growth advantage in the presence of penicillin**

301 As the acquisition of *mecA* (PBP2a) in a *SCCmec* element can exhibit significant  
302 fitness costs<sup>37</sup>, and affect toxicity<sup>38</sup> and biofilm formation<sup>39</sup>, we hypothesised that  
303 the PBP2a substitutions might confer a fitness advantage. We found no significant  
304 difference in biofilm formation or toxicity to human monocytic cells between any of  
305 the three PBP2a variants in two strain backgrounds (Supplementary Fig. 6). We then  
306 investigated the effect of the PBP2a substitutions on general fitness in three lineages

307 (ST22, USA300/ST8) and ST398) assayed by growth in a minimal medium, and in  
308 the presence of penicillin. In minimal medium, there were only minor differences (Fig  
309 6a, c, e), with the USA300 strain (BCV289) complemented with *mecA*<sub>246G</sub> growing  
310 marginally better in early exponential phase than *mecA*<sub>246E</sub> or *mecA*<sub>122I</sub> (Fig. 6a). In  
311 the ST22 (A75) background the *mecA*<sub>246E</sub> strain grew slower during exponential  
312 growth than the other backgrounds (Fig 6e). In the presence of penicillin, in all three  
313 strain backgrounds the isolates complemented with *mecA*<sub>246G</sub> grew better than the  
314 other two variants (Fig 6b, d and f). This was most pronounced in the ST398  
315 background (EC139), in which *mecA*<sub>246G</sub> strain grew considerably better in  
316 exponential phase, reached a higher optical density and grew with a reduced  
317 doubling time (dt) of 5.33 hours (95% confidence intervals (CI): 5.28 to 5.38)  
318 compared to either *mecA*<sub>246E</sub> (dt: 6.65, 95% CI: 6.62 to 6.69) or *mecA*<sub>122I</sub> (dt: 6.92,  
319 95% CI: 6.89 to 6.96) (Fig 6d). This demonstrated that complementation with  
320 *mecA*<sub>246G</sub> provided a growth advantage in the presence of penicillin, but the  
321 magnitude of this effect is influenced by strain background.

322

## 323 **Discussion**

324 We show that a significant proportion of clinical MRSA isolates are susceptible to a  
325 combination of penicillins and a  $\beta$ -lactamase inhibitor. Susceptibility is due to one of  
326 two different mutations in the *mecA* promoter region that both lower *mecA* (PBP2a)  
327 expression, and in the majority of isolates, by an additional substitution in PBP2a  
328 (E246G or M122I) that increases the affinity of PBP2a for penicillin in the presence of  
329 clavulanic acid. It is not clear how clavulanic acid causes the increased binding  
330 affinity of penicillin for PBP2a, as clavulanic acid binds to PBP2a poorly<sup>19</sup>. Modelling  
331 of PBP2a shows that position 246 is located near the allosteric site but does not  
332 indicate any clear mechanism for the increased affinity for penicillin (Supplementary  
333 Fig.7 and Supplementary Discussion). In some isolates, a RBS mutation alone  
334 appeared to be sufficient to mediate susceptibility, although given the complexity of

335 the regulation of  $\beta$ -lactam resistance in MRSA other genes might be involved <sup>5</sup>.  
336 Crucially, the PBP2a 246G substitution provides a fitness benefit for growth in  
337 presence of penicillin, suggesting that susceptibility to penicillin and  $\beta$ -lactamase  
338 inhibitors is a likely a case of collateral sensitivity <sup>40</sup>, which evolved due to selective  
339 pressure for maintaining the balance between fitness and resistance.

340

341 In our susceptibility assays we used Iso-Sensitest media (ISA) rather than Müller-  
342 Hinton agar (MHA) as currently recommended by CLSI and EUCAST <sup>18,19</sup>, because  
343 ISA was the recommended media for penicillin by the British Society for Antimicrobial  
344 Chemotherapy (BSAC) at the beginning of the study <sup>41</sup>. Comparison between  
345 susceptibility to penicillin-clavulanic acid on ISA and MHA revealed that a number of  
346 isolates that were susceptible on ISA remained resistant on MHA (Supplementary  
347 table 5), including isolates that responded to treatment *in vivo* (Fig 5b-e). This  
348 suggests that MHA is not the optimum media for the detection of susceptibility of  
349 penicillins and  $\beta$ -lactamase inhibitors, which is supported by a recent study which  
350 revealed that MHA failed to detect susceptibility to multiple antibiotic classes that  
351 were effective *in vivo* <sup>42</sup>. Our data also highlights the risk of using a single antibiotic  
352 (e.g. cefoxitin for MRSA) to determine resistance to an entire antibiotic class,  
353 potentially missing unexpected susceptibilities.

354

355 Both *in vitro* and *in vivo*, penicillins and clavulanic acid were efficacious at  
356 physiologically achievable concentrations <sup>43</sup>. In the absence of a clinical breakpoint,  
357 pharmacokinetic-pharmacodynamic (PK-PD) breakpoints can be used to infer  
358 susceptibility <sup>44</sup>. The tentative ECOFF wildtype cut-off of  $\leq 2.0$   $\mu\text{g/ml}$  penicillin in the  
359 presence of 15  $\mu\text{g/ml}$  of clavulanic acid, lies in the intermediate susceptibility  
360 category (susceptible  $\leq 0.25$   $\mu\text{g/ml}$ , resistant  $> 2$   $\mu\text{g/ml}$ ) of the EUCAST PK-PD  
361 breakpoint <sup>18</sup>. Large numbers of isolates had much lower MICs, and had amoxicillin-

362 clavulanic acid zone diameters greater than the breakpoint for other pathogenic  
363 species (Supplementary Table 1, 3 and 4) <sup>18</sup>. Previous studies have reported the  
364 successful use of penicillins and  $\beta$ -lactamase inhibitors for the treatment of MRSA in  
365 rabbits and rats, and for human infections <sup>11,45-47</sup>. This work, provides a mechanistic  
366 explanation for efficacy in these studies, although there have been previous  
367 conflicting reports <sup>9</sup>. While it is unlikely that penicillins and  $\beta$ -lactamase inhibitor  
368 combinations would be used as a monotherapy, they would be attractive additional  
369 therapeutic option for hard-to-treat infections such as multidrug-resistant MRSA <sup>48</sup>,  
370 particularly as  $\beta$ -lactams synergise with vancomycin and daptomycin <sup>49,50</sup>. PK/PD  
371 modelling studies, including an assessment of highly resistant cells during treatment  
372 is now needed to determine the optimum dosing strategy required for sustained  
373 efficacy before appropriate clinical trial could be conducted.

374

375 Our findings demonstrate that cryptic susceptibilities to already licensed and  
376 inexpensive antibiotics may emerge within constantly evolving bacterial populations,  
377 which then can be exploited for the treatment of antibiotic resistant pathogens.

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389 **Material and methods**

390 **Media and culture conditions**

391 Bacterial strains and plasmids used in this study are described in Supplementary  
392 Table 5 and 6. For routine culture, *Escherichia coli* (*E. coli*) was grown in Lysogeny  
393 broth (LB) or on LB-agar (Oxoid, UK) at 37°C. *S. aureus* was grown on tryptone soy  
394 agar (TSA), Columbia blood agar or in tryptone soy broth (TSB) (Oxoid, UK) at 28°C  
395 or 37°C accordingly. *E. coli* and *S. aureus* media were supplemented with 10 µg/ml  
396 chloramphenicol (Cm10) as appropriate. For growth curve studies, *S. aureus* strains  
397 were grown in SSM9PR minimal medium (1 × M9 salts, 2 mM MgSO<sub>4</sub>, 0.1 mM CaCl<sub>2</sub>,  
398 1% glucose, 1% casaminoacids, 1 mM Thiamine-HCl and 0.05 mM nicotinamide) at  
399 37°C<sup>51</sup>.

400

401 **Isolate selection**

402 For the 110 sequenced isolates in Supplementary Table 1, isolates were selected  
403 from sequenced isolates in our laboratory collections to provide a broad as possible  
404 selection of isolates as possible from different clinically relevant lineages (Multilocus  
405 sequence type (ST)1, 5, 8 22, 36, 45, 80, 88, 239, 398), with an obvious bias towards  
406 lineages that dominate in the UK (e.g. 40 isolates from ST22, which is the dominant  
407 lineage in the UK). For the additional 274 isolates that were combined with 24 of the  
408 original isolates with MIC data (from the previous 110), we again tried to select  
409 isolates from our laboratory collections that broadly covered a selection of clinically  
410 relevant MRSA lineages including clonal complex (CC) 1, 5, 8 (including 56 UK  
411 USA300 isolates), 22, 30, 45, 59, 72, 80, 97, 239, and 398 - amongst others). For  
412 lineages with multiple isolates (CC1, n=25, CC5, n=29, CC22, n=91, CC59, n=18,  
413 CC45, n=15, CC30, n=20) we used previously generated whole genome phylogenies  
414<sup>31</sup> to select isolates broadly across the phylogeny, as well as selecting isolates from  
415 the same clades.

416



417 **Antimicrobial susceptibility testing**

418 Inocula were prepared by the growth method. At least four morphologically similar  
419 colonies were touched with a sterile loop and transferred into Iso-Sensitest broth  
420 (Oxoid, UK). Inoculated broth was incubated at 37°C with 200 rpm shaking until the  
421 visible turbidity reached 0.5 McFarland standard. For disk diffusion and Etest method  
422 testing, the 0.5 McFarland standard broth was diluted 1:10 in distilled water before  
423 spreading onto agar plates. For Microbroth dilution for minimum inhibitory  
424 concentrations (MIC), the broth was diluted 1:100 in Iso-Sensitest broth for  
425 inoculation.

426

427 Disk diffusion susceptibility testing was carried out according to BSAC criteria (BSAC  
428 Methods for Antimicrobial Susceptibility Testing, version 14, January 2015).

429 Temocillin disks were purchased from MAST group, UK. All other antibiotic disks  
430 were purchased from Oxoid, UK. For the clavulanic acid assay, 15 µg/ml clavulanic  
431 acid (Sigma-Aldrich, UK) was added to Iso-Sensitest agar (ISA) or Müller-Hinton agar  
432 (MHA) (Oxoid, UK), as appropriate. After applying the antibiotic disks, all plates were  
433 incubated at 35°C for 20 hours before inhibition zones were measured. Oxacillin disk  
434 diffusion was also performed on MHA with 2% NaCl at 30°C for 24 hours. For disk  
435 testing with clavulanic acid, susceptibility was defined as ≥10 mm increase of the  
436 zone of inhibition in the presence of 15µg/ml clavulanic acid. For *mecA*  
437 complemented strains, ISA was supplemented with 200 ng/ml anhydrotetracycline  
438 (Atc) (Sigma-Aldrich, UK) to induce expression of *mecA* from pXB01, a modified  
439 tetracycline-inducible expression vector pRMC2<sup>16</sup>. Microbroth dilution for minimum  
440 inhibitory concentrations (MIC) was performed according to BSAC guidelines<sup>52</sup>. The  
441 antibiotic ranges were prepared in Iso-Sensitest broth a step higher than the final  
442 concentrations of 0.015-128 µg/ml for penicillin and 1-32 µg/ml for cefoxitin. A 96 well  
443 cell culture plate (Greiner Bio-One, CELLSTAR®) was loaded with appropriate  
444 antibiotic dilutions. For each test isolate, 75 µl of each antibiotic dilution were added

445 to a row of wells and 75 µl diluted test isolate culture was added into the wells. Each  
446 isolate was tested in triplicate. The 96 well plate was then placed in a plastic bag to  
447 minimize evaporation and was incubated at 35°C for 20 hours. Penicillin and  
448 cefoxitin Etest antimicrobial susceptibility testing on selected MRSA isolates was  
449 performed according to manufacturer's guidance. Medium used for Etest was ISA  
450 with or without 15 µg/ml clavulanic acid. Etest strips were purchased from bioMérieux  
451 UK Ltd.

452

### 453 **Construction of *S. aureus* gene deletion and complementation mutants**

454 *mecA* deletion mutants in *mecA*-MRSA strains (Supplementary Table 7) were  
455 generated by allelic exchange with the temperature-sensitive vector pIMAY, as  
456 described previously<sup>53</sup>. Primers used for *mecA* deletion are listed in Supplementary  
457 Table 8. Upstream sequence (AB) and downstream sequence (CD) of the *S. aureus*  
458 gene to be deleted were amplified with primers A/B or C/D using KOD Hot Start DNA  
459 Polymerase (Merck, UK). PCR products AB and CD were used as templates to  
460 obtain deletion construct AD with primers A/D in a splicing overlap extension (SOE)  
461 PCR. Product AD was digested with restriction enzymes KpnI and SacI and ligated to  
462 pIMAY digested with the same enzymes. The resulting plasmids were designated  
463 pIMAYΔ*mecA*. The plasmids were transformed into *E. coli* DC10B (a *dcm* deletion  
464 mutant of DH10B), allowing the plasmid to be directly transferred into *S. aureus*  
465 strains<sup>53</sup>. Plasmid DNA extracted from DC10B was then electroporated into recipient  
466 strains to create knockout mutants.

467

468 For complement expression of *mecA*, the genes were cloned into expression plasmid  
469 pXB01, a derivative of tetracycline-inducible expression vector pRMC2 with the *blaZ*  
470 gene deleted<sup>54</sup>. The *mecA* gene variants including the ribosome binding site were  
471 amplified from genomic DNA with primers: *mecA*-F-KpnI / *mecA*-R-SacI. PCR  
472 products were digested with KpnI and SacI and ligated with the pXB01 vector

473 cleaved with the same enzymes, generating plasmids *pmecA*<sub>246E</sub>, *pmecA*<sub>246G</sub>, and  
474 *pmecA*<sub>122I</sub>. The plasmids were transformed into *E. coli* DC10B, and plasmid DNA then  
475 extracted and electroporated into *mecA*-deletion strains for complementation with  
476 expression induced with 200 ng/ml An. Atc.

477

#### 478 **Antimicrobial susceptibility testing of Danish clinical isolates**

479 Antimicrobial susceptibility testing was performed on a selection of clinical isolates  
480 obtained from the Danish surveillance of MRSA and *S. aureus* causing bacteraemia.  
481 The selection was based on a total of 270 isolates including 100 isolates (52 MSSA,  
482 48 MRSA) from 2011 on which whole genome sequence data were previously  
483 obtained and 170 consecutively received non-CC398 MRSA isolates in 2016. *spa*  
484 types were obtained for all isolates. Antimicrobial susceptibility testing was performed  
485 as described above except that 0.5 McFarland inoculum was prepared using a  
486 densitometer and ISA plates with and without 15 µg/ml clavulanic acid was  
487 purchased as custom made plates (SSI Diagnostica, Hilleroed, Denmark).

488

#### 489 **Expression and purification of PBP2a variants in *E. coli***

490 PBP2A variants (PBP2a<sup>E246</sup>, PBP2a<sup>E246G</sup> and PBP2a<sup>M122I</sup> from MRSA strains RVC5,  
491 BCVA289 and ARARH150, respectively; residues 26-668, with a G26M mutation)  
492 were overexpressed using the auto-induction expression method at 25°C<sup>55</sup>. Cells  
493 were harvested by centrifugation after 20 hours of expression and lysed using  
494 Bugbuster (Novagen, Merck Millipore) containing 10 U/ml of benzonase nuclease  
495 (Novagen, Merck Millipore) and Protease Inhibitor Tablets, EDTA-free (Pierce  
496 Biotechnology, Thermo Fisher Scientific), following the manufacturer's instructions.  
497 After cell disruption, the lysates were cleared, and the soluble proteins were purified  
498 using HisPur Ni-NTA Resin columns (Pierce Biotechnology, Thermo Fisher Scientific)  
499 under native conditions, according to the manufacturer's instructions. The expression  
500 and purification yields were monitored by SDS-PAGE. The most concentrated elution

501 fractions were buffer exchanged to 20 mM sodium phosphate buffer pH 7.4 using  
502 PD-10 Desalting Columns (GE Healthcare Life Sciences), following the  
503 manufacturer's instructions. Protein concentrations were assessed using the BCA  
504 Protein Assay Kit (Pierce Biotechnology, Thermo Fisher Scientific).

505

#### 506 **Bocillin FL PBP2a and penicillin G/clavulanic acid binding assays**

507 The binding affinity of PBP2a for different antibiotics has been determined using a  
508 fluorescent penicillin reporter reagent, Bocillin FL<sup>56-58</sup>. In this study, the affinities of  
509 PBP2A variants for penicillin and clavulanic acid were determined using the same  
510 approach. Briefly, a reaction mixture containing 25 µg/ml of a purified PBP2a variant  
511 in 20 mM sodium phosphate buffer (pH 7.4) and various concentrations (0, 0.5, 1, 5,  
512 10, 20, 50, 100, 200, 500, 1000, 2000 µg/ml) of penicillin G or clavulanic acid was  
513 incubated at 37°C for 20 min. To test if the presence of clavulanic acid affected the  
514 affinity for penicillin G, increasing concentrations of penicillin G together with 15  
515 µg/ml of clavulanic acid were assayed. A final concentration of 20 µM Bocillin FL  
516 (13.3 µg/ml) was added to the reaction followed by 10 min incubation at 37°C. The  
517 reactions were quenched by adding SDS loading buffer and heating at 95°C for 10  
518 min. Samples were visualized using 10% Tris-Glycine-SDS PAGE. Protein gels were  
519 washed in distilled water for 10 min and scanned using a 473 nm laser of a Fuji  
520 Fluorescent Analyzer TLA-5100. Fluorescent intensity was quantified by ImageJ  
521 software and IC<sub>50</sub> was calculated from three independent assays using GraphPad  
522 Prism 5 software.

523

#### 524 **RNA isolation and quantitative real-time PCR (RT-qPCR)**

525 For each selected isolate, 15 ml of early log phase culture with an OD<sub>595nm</sub> of 0.3 was  
526 treated with 10 µg/ml oxacillin for 1 h at 37 °C to induce *mecA* expression and its  
527 respective untreated culture was used as a baseline control. After the induction, the  
528 OD<sub>595nm</sub> of the control culture was adjusted to be equal to that of the oxacillin induced

529 one if necessary and 10 ml of both cultures were spun for 10 min at 4,500 x g at 20  
530 °C. About 0.5 ml supernatant was left behind to re-suspend the pellet and 1 ml of  
531 RNAprotect Bacteria Reagent (Qiagen) was added and mixed immediately by  
532 vortexing. After incubation for 5 min at room temperature, cultures were spun for 5  
533 min at 10,000 x g at 4 °C to pellet the cells. Pellets were snap-frozen and stored at -  
534 80 °C until RNA isolation. Total RNA was isolated using Qiagen RNeasy Mini Kit  
535 (Qiagen) and an additional DNase treatment was performed with the Ambion  
536 TURBO DNA-free kit and cDNA was produced using QuantiTect Reverse  
537 Transcription Kit (Qiagen). RT-qPCR was performed using the SensiFast SYBR No-  
538 ROX Kit (Bioline) on a Rotor-Gene Q machine (Qiagen) using *mecA* and *blaZ*  
539 primers Gene expression fold-changes in induced cultures were calculated relative to  
540 control cultures using the  $\Delta\Delta C_t$  method<sup>59</sup> with *gyrB* as the reference.

541

#### 542 **Wax moth larva infection and treatment**

543 The wax moth larvae assay was based on that previously described by Desbois et al  
544 <sup>60</sup>. *Galleria mellonella* larvae were purchased in bulk from Livefood UK. Larvae were  
545 stored at 4°C upon arrival and kept at 37°C during the course of the assay. MRSA  
546 strains RVC5, 0081, EC139, BCVA289 were selected for evaluation of antimicrobial  
547 activities of penicillin and clavulanic acid in combination. Single bacterial colonies  
548 were picked to inoculate 5 ml of TSB, and cultures were grown overnight (~16 hours)  
549 at 37°C and 200 rpm shaking. Cultures were then diluted 1:100 into 5 ml of fresh  
550 TSB and grown for a further 4 hours at 37°C and 200 rpm shaking. Cultures were  
551 then centrifuged at 2,500g for 10 minutes, and pellets resuspended in sterile  
552 phosphate buffered saline (PBS) to an OD<sub>595</sub> of 0.2, giving approximately  $1.5 \times 10^8$   
553 CFU/ml. For each strain, six groups of *G. mellonella* (n=10 in each group) were  
554 injected with 10  $\mu$ l aliquots ( $\sim 1.5 \times 10^6$  CFU) of resuspended culture behind the rear  
555 thoracic segments using a Tridak Stepper Pipette Dispenser (Dymax, UK). Groups of  
556 *G. mellonella* were treated by injection with 50 mg/kg vancomycin, 20 mg/kg penicillin

557 sodium salt, 20 mg/kg clavulanic acid, 20 mg/kg penicillin sodium salt combined with  
558 20 mg/kg clavulanic acid, 20 mg/kg amoxicillin, 20 mg/kg amoxicillin combined with  
559 20 mg/kg clavulanic acid or PBS at 2, 24 and 48 hours after inoculation. The  
560 treatments were given blind and the treatment identities not revealed until the  
561 experiment was completed. Larvae were considered dead when they did not respond  
562 to touch to the head. Survival curves were generated and analysed using GraphPad  
563 Prism 6 software. Fig. 5 shows results of a single representative experiment, a  
564 replication experiment with broadly similar results is shown in Supplementary Fig. 5.

565

### 566 **Murine infection model**

567 Fresh overnight colonies from a 5% horse blood agar plate were suspended in saline  
568 to an OD<sub>546</sub> of 0.13, giving approximately  $2 \times 10^8$  CFU/ml. Mice (NMRI female mice,  
569 26 - 30 gram (Taconic, Denmark), 6-8 weeks old were inoculated intramuscularly  
570 with 0.05 ml of the suspension in the left thigh ( $1 \times 10^7$  CFU). Approximately 0.5 hrs  
571 before inoculation, mice were treated orally with 45  $\mu$ l Nurofen Junior (20 mg  
572 ibuprofen/ml - corresponding to 30 mg/kg) for pain relief. Four mice in each group  
573 were treated with a single subcutaneous dose of 0.2 ml with 10, 30 or 100 mg/kg  
574 amoxicillin (Amoxil, GlaxoSmithKline Middlesex UK) alone or in combination with 2, 6  
575 or 20 mg/kg clavulanic acid (Augmentin, Beecham Group Ltd, Middlesex, UK) or 40  
576 mg/kg vancomycin (Fresenius Kabi, Halden, Norway) or saline one-hour post  
577 infection. Mice were sacrificed at 1 hour for the start of treatment control group and at  
578 5 hours post infection for the treatment groups by cervical dislocation and thighs  
579 were collected and kept at -80°C. Each sample thigh was homogenized in 5 ml saline  
580 using a Dispomix® Drive, and serially diluted in saline and twenty microliter spots of  
581 serial dilutions were plated on blood agar plates. All agar plates were incubated for  
582 18 - 24 hrs at 35°C. Statistical comparison was carried out using a 1 way ANNOVA  
583 and Dunnett's multiple comparison, was performed for treatment groups comparing  
584 against the vehicle group in GraphPad Prism software. All animal procedures were

585 carried out at the Statens Serum Institute (SSI) and approved by the Danish Animal  
586 Procedure Inspectorate. Ethical approval was granted for the murine thigh infection  
587 model (2016-15-0201-01049). The SSI Animal Welfare Committee (SSI-AWC –  
588 equivalent an Institutional Animal Care and Use Committee (IACUC) requires that  
589 each experiment is further approved by the supervising laboratory animal  
590 veterinarians who are also part of the IACUC. All animals were randomised on arrival  
591 at SSI, and sample sizes were based on a combination of statistical analysis and the  
592 principles of 3R, that the minimum number of animals were used that were expected  
593 to provide statistically significant difference considering the expected intra-group  
594 variability of the infection model used.

595

#### 596 **Growth curves**

597 To assess the effect of different *mecA* variants on the growth of MRSA strains in  
598 liquid culture, Bioscreen C optical growth analyzer (Lab system, Finland) was used to  
599 monitor the growth rates of deletion mutants *A75ΔmecA*, *BCVA289ΔmecA* and  
600 *EC139ΔmecA* complemented with the three *mecA* variants. Briefly, overnight  
601 cultures were diluted 1/1000 into fresh SSM9PR minimal medium with or without 16  
602 µg/ml penicillin supplemented with 200 ng/ml Atc to induce the expression of  
603 plasmid-borne *mecA* gene. For each strain, 300 µl of inoculated medium was added  
604 into wells of the microplate in triplicate. Fresh medium with Atc was also added to  
605 three wells acting as blank controls. Cultures were incubated at 37 °C with  
606 continuous shaking for 24 hours and an optical density measurement at OD<sub>600nm</sub> was  
607 taken every 30 mins. Growth curves were analysed using the GraphPad Prism 6  
608 software and doubling time calculated using non-linear regression using an  
609 exponential growth equation with a least square fit, with Y0 constrained at the  
610 minimum optical density measured (an OD<sub>600nm</sub> of 0.069).

611

#### 612 **Toxicity and biofilm assays**

613 Immortalised human monocyte macrophage THP-1 cell lines were used as described  
614 previously<sup>61</sup>. Briefly, the cell line was grown in individual 30 mL suspensions of  
615 RPMI-1640, supplemented with 10% heat-inactivated fetal bovine serum (FBS), 1  $\mu$ M  
616 L-glutamine, 200 units/mL penicillin, and 0.1 mg/mL streptomycin at 37°C in a  
617 humidified incubator with 5% CO<sub>2</sub>. Cells were routinely viewed microscopically every  
618 48–60 h and harvested by centrifugation at 1,000 rpm for 10 min at room  
619 temperature and resuspended to a final density of 1–1.2 x 10<sup>6</sup> cells/mL in tissue-  
620 grade phosphate buffered saline. This procedure typically yielded >95% viability of  
621 cells as determined by trypan blue exclusion and easyCyte flow cytometry. To  
622 monitor *S. aureus* toxicity, 20  $\mu$ L of cells were incubated with 20  $\mu$ L of bacterial  
623 supernatant and incubated for 12 min at 37°C. For the USA300 strains, supernatants  
624 were diluted to 30% of the original volume in TSB as these isolates were  
625 considerably more toxic than the single-patient isolates. Cell death was quantified  
626 using easyCyte flow cytometry using the Guava viability stain according to  
627 manufacturer's instructions. Experiments were done in triplicate, and error bars  
628 indicate the average  $\pm$  the 95% confidence interval of multiple independent  
629 experiments.

630

631 Biofilm formation was quantified using a 1:40 dilution from overnight cultures into 100  
632  $\mu$ L of fresh TSB supplemented with 0.5% sterile filtered glucose (TSBG) in 96-well  
633 polystyrene plate (Costar). Perimeter wells of the 96-well plate were filled with sterile  
634 H<sub>2</sub>O and plates were placed in a separate plastic container inside a 37°C incubator  
635 and grown for 24 h under static conditions. For the transposon mutants, erythromycin  
636 (5  $\mu$ g/mL) was added to the growth medium. Semi-quantitative measurements of  
637 biofilm formation on 96-well polystyrene plates were determined based on the  
638 method of Ziebuhr et al<sup>23</sup>. Following 24-h growth, plates were washed vigorously five  
639 times in PBS, dried and stained with 150  $\mu$ L of 1% crystal violet for 30 min at room  
640 temperature. Following five washes of PBS, wells were re-suspended in 200  $\mu$ L of



641 7% acetic acid, and optical density at 595 nm was recorded using a Fluorimeter plate  
642 reader (BMG Labtech). For this experiment the assays were performed in triplicate  
643 on each plate and repeated three times.

644

#### 645 **ECOFF determination**

646 In order to help split isolates into phenotypically “wildtype” and “non-wildtype” based  
647 on MIC distributions, a series of mixture models were fitted to the data for each drug  
648 independently, ranging from 1 to 5 normal distributions. Each model was fitted to the  
649 distribution of log-transformed MIC by maximum likelihood. The likelihood function  
650 was based on a multinomial distribution over the set of intervals

651  $[0, x_1, \dots, x_n, \infty]$  where  $x_i$  stands for each of the tested concentrations for the drug  
652 considered. For each drug, the 5 models were compared using Akaike’s Information  
653 Criterion to determine the optimal number  $k$  of normal distribution components. If the  
654 best model was  $k=1$ , the distribution was labelled as unimodal, and no further  
655 analysis was made. For drugs with  $k \geq 2$ , the following analyses were then carried out:

- 656 1. Predicted distribution of MIC per component: for each component, we multiplied  
657 the probability mass at each concentration (= pdf integrated over the immediately  
658 lower interval) by the total number of isolates tested.
- 659 2. Direct classification of isolates by component: for each MIC, we split the isolates  
660 into the  $k$  components according to their relative probabilities at that point.
- 661 3. Optimal cut-off: we then sought to split each distribution into two modes. First, we  
662 estimated candidate cut-off points between each successive component of the  
663 mixture model. Each cut-off was computed as the concentration that minimised  
664 the risk of misclassification of isolates between the model components (achieved  
665 by minimising the difference between the cumulative density functions of the two  
666 components considered). For example, with  $k=3$ , there are three components  
667 centred at MIC values  $Y_1 < Y_2 < Y_3$ ; we then calculated two candidate cut-offs: one  
668 separating component 1 from 2+3, and the other separating 1+2 from 3. For each

669 cut-off, we then computed the number of isolates that would be misclassified,  
670 using the above direct classification as a reference: e.g. for the first cut-off, the  
671 number of isolates classified as 2 or 3 with MIC below the cut-off, plus the  
672 number of isolates classified as 1 with MIC above the cut-off.

673

## 674 **Bioinformatics**

675 Whole genome sequences were assembled using the pipeline described previously  
676 <sup>62</sup>. For each isolate the sequence reads were used to create multiple assemblies  
677 using VelvetOptimiser v2.2.5 <sup>63</sup> and Velvet v1.2 <sup>64</sup>. The assemblies were improved by  
678 scaffolding the best N50 and contigs using SSPACE <sup>65</sup> and sequence gaps filled  
679 using GapFiller <sup>66</sup>. Presence of PBP2a substitutions were identified by extracting the  
680 PBP2a sequence from the assembled genome sequences, aligning the PBP2a  
681 sequences using Muscle <sup>67</sup> in Seaview <sup>68</sup> and then identifying the presence of PBP2a  
682 substitutions using a custom python script. The *mecA* promoter mutations were  
683 identified using a similar approach using *in silico* PCR to identify the *mecA* promoter  
684 region and then aligning the *mecA* promoter sequence and identifying *mecA*  
685 mutations using a custom python script. The presence of the *blaZ* genes was  
686 confirmed using BLAST against assemblies, this identified that 273/298 of the  
687 previously screened WGS isolates had a single copy of *blaZ* (excluding *blaZ*-  
688 negative or isolates with two copies or truncated copies of *blaZ*). BlaZ amino acid  
689 sequences were then extracted, aligned and amino acids at positions 128 and 216  
690 compared to identify the BlaZ type (Type A: 128:T, 216:S, Type B: 128:K, 216:N,  
691 Type C: 128:T, 216:N, Type D: 128:A, 216:S, Type E (LGA251): 128:L, 216:S, Type  
692 F (a distinct type identified in this work): 128:A, 216:N) as previously described <sup>16,69,70</sup>.  
693 Phylogenetic analysis of the CC8 isolates was carried out as previously described <sup>33</sup>,  
694 briefly, sequence reads were mapped using SMALT v0.7.4  
695 (<http://www.sanger.ac.uk/science/tools/smalt-0>) to the *S. aureus* USA300\_FPR3757  
696 reference genome (accession: CP000255.1)<sup>71</sup>. A core genome alignment was

697 created after excluding mobile genetic element regions, variable sites associated with  
698 recombination (detected with Gubbins<sup>72</sup>) and sites with more than 5% proportion of  
699 gaps (i.e. sites with an ambiguous base). A maximum likelihood (ML) phylogenetic  
700 tree was generated with RAxML v8.2.8<sup>73</sup> based on generalised time reversible  
701 (GTR) model with GAMMA method of correction for among site rate variation and the  
702 phylogenetic tree annotated using Figtree (<http://tree.bio.ed.ac.uk/software/figtree/>).  
703 Phylogenetic analysis of PBP2a sequences was constructed by using PhyML v3.0 in  
704 Seaview with a Whelan and Goldman (WAG) substitution model and 100 bootstrap  
705 replicates<sup>74</sup>.

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#### 708 **Population analysis**

709 Population analysis was carried out as described by Kim et al<sup>75</sup> with minor  
710 modifications. Strains were grown overnight in tryptic-soya broth (TSB), and serial  
711 10-fold dilutions were plated in triplicate onto Iso-Sensitest Agar (Oxoid) plates  
712 containing varying concentrations of penicillin with 15 µg/ml clavulanic acid. Plates  
713 were incubated for 24 hours at 35°C. A mean of the three platings were plotted in  
714 Figure 5a. Biological replicates for strains BCVA289 and 0081 were generated and  
715 plotted in comparison to the original results in Supplementary Figure 4, showing  
716 broadly similar results.

717

#### 718 **Structural modelling of PBP2a**

719 Co-ordinates from representative structures of both the Gly246 (accession code  
720 3ZFZ,<sup>21</sup>) and Glu246 (accession code 1VQQ,<sup>76</sup>) forms of PBP2a were used to  
721 compare possible effects induced by sidechain alteration. Figures were prepared  
722 using Chimera<sup>77</sup> and Pymol (The PyMOL Molecular Graphics System, Version  
723 1.2r3pre, Schrödinger, LLC).

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999

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1015

1016 **Author contributions**

1017 Design of the study: EMH, XB, SJP, MAH. *mecA* deletion and complementation,  
1018 expression analysis, bocillin assays: XB. Antimicrobial susceptibility testing: XB, BB,  
1019 NG, KB. Biofilm and toxicity assays: HC and RM. Antimicrobial susceptibility testing  
1020 of Danish isolates: JL, ARL. Determination of the ECOFF: OR. Structural analysis of  
1021 PBP2a: AL. Infection and treatment experiments: EMH, XB, CVL. Bocillin binding  
1022 assays: IRG and RGS. Bioinformatics analysis of whole-genome sequence data:  
1023 EMH, FC, SR, DJ. Collection of USA300 isolates: ACU, FDL. Wrote bioinformatics  
1024 scripts: NG. Analysis and interpretation of the data: CUK, GP, MTGH, JP.  
1025 Coordinated the study and wrote the manuscript: EMH. Responsible for supervision  
1026 and management of the study: SJP and MAH. All authors read, contributed to and  
1027 approved the final manuscript.

1028 **Competing interests**

1029 CUK is a consultant for the World Health Organization (WHO) Regional Office for  
1030 Europe, QuantuMDx Group Ltd, and the Foundation for Innovative New Diagnostics,  
1031 which involves work for Cepheid Inc., Hain Lifescience and WHO. CUK is an advisor  
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1038 of scientific projects. SJP and JP are consultants to Next Gen Diagnostics Llc. SJP is  
1039 a consultant to Specific Technologies. All other authors declare no competing  
1040 financial interest

1041

1042 **Data availability**

1043 All data generated or analysed during this study are included in this published article  
1044 (and its supplementary information files).

1045

1046 **Figure Legends**

1047 **Figure 1: Penicillin susceptibility in the presence of clavulanic acid.** (a) Figure  
1048 shows a representative image of two MRSA isolates grown on Iso-sensitest agar or  
1049 Iso-sensitest agar with 15µg/ml of clavulanic acid with a penicillin E-test strip applied,  
1050 the upper susceptible isolate shows increased susceptibility to penicillin in the  
1051 presence of clavulanic acid, while the lower resistant isolate shows only a minor  
1052 effect. (b) E-test determination of minimum inhibitory concentration of penicillin alone  
1053 (black) and penicillin plus 15 µg/ml clavulanic acid (grey) of a selection of penicillin-  
1054 clavulanic acid susceptible (n=14) and resistant (n=10) MRSA isolates. The red line  
1055 indicates the current EUCAST clinical break point of  $\leq 0.125$  µg/ml. The effect of  
1056 clavulanic acid on susceptibility as measured by disk diffusion to different  $\beta$ -lactam  
1057 antibiotics in a panel of (c) penicillin-clavulanic acid resistant MRSA isolates (n=46),  
1058 and (d) penicillin-clavulanic acid susceptible MRSA isolates (n=32). Red bars  
1059 indicate isolates grown on Iso-Sensitest Agar (ISA) alone and blue bars indicate  
1060 isolates grown on ISA supplemented with 15µg/ml clavulanic acid. Y-axis indicates  
1061 the zone of inhibition in millimetres. Error bars indicate the standard deviation of the  
1062 mean. Note: OX(MHA) = Oxacillin performed on Müller-Hinton agar (recommended  
1063 media by EUCAST and CLSI).

1064

1065 **Figure 2: PBP2a substitutions mediating penicillin susceptibility.** (a) Minimum  
1066 inhibitory concentrations (MIC) of penicillin (black) or penicillin in the presence of 15  
1067 µg/ml clavulanic acid (grey) for wildtype strains EC139 (ST398) and BCVA289

1068 (USA300), and *mecA* mutants and complemented mutant strains with either an  
1069 empty vector (p) or one of the three different alleles of *mecA* (PBP2a<sup>246E</sup> (p246E),  
1070 PBP2a<sup>246G</sup> (p246G), and PBP2a<sup>1121</sup> (p1121)). Results presented are the mean of three  
1071 independent experiments. **(b)** Bocillin competition assay to determine the IC<sub>50</sub> of  
1072 penicillin for the three PBP2a variants (PBP2a<sup>246E</sup> – ‘WT’, PBP2a<sup>246G</sup> and  
1073 PBP2a<sup>M1221</sup>). **(c)** Bocillin competition assay to determine the IC<sub>50</sub> of penicillin in the  
1074 presence of 15µg/ml clavulanic acid for the three PBP2a variants (PBP2a<sup>246E</sup> – ‘WT’,  
1075 PBP2a<sup>246G</sup> and PBP2a<sup>M1221</sup>). The fluorescence intensity of the bands was plotted as  
1076 the percentage of unbound proteins as a function of penicillin G concentration, and  
1077 the IC<sub>50</sub> value was calculated from the plot. Data points represent the average of  
1078 three replicates and the curve is the predicted nonlinear regression result. **(d)** Mixture  
1079 models of MIC distributions of 298 MRSA isolates for penicillin in the presence of 15  
1080 µg/ml clavulanic acid, the best model is a mixture of 4 normal distributions, with a  
1081 proposed cut-off at 2.449, and **(e)** for penicillin alone, the best model is a mixture of 4  
1082 normal distributions, with a proposed cut-off at 0.218. The graphs show the proposed  
1083 categorisation of the MIC distribution into “wildtype” isolates (low MIC, green hues)  
1084 and “non-wildtype” isolates (high MIC, amber hues), and the proposed empirical cut-  
1085 off as the vertical dashed line. Amber isolates that fall below the cut-off and green  
1086 isolates that fall above the cut-off show the expected classification errors by applying  
1087 the cut-off.

1088

1089 **Figure 3: Genetic basis of MRSA penicillin/clavulanic acid susceptibility. (a)**

1090 Summary of PBP2a substitutions and *mecA* promoter mutations. Figure shows a  
1091 representation of the domain structure of the PBP2a protein and *mecA* promoter with  
1092 the location of the two PBP2a substitutions and *mecA* promoter associated with  
1093 penicillin susceptibility indicated and mean penicillin MIC in the presence of 15 µg/ml  
1094 of clavulanic acid for isolates with that genotype. **(b)** Relative *mecA* expression  
1095 measured by RT-qPCR. Figure shows the relative *mecA* expression after oxacillin

1096 induction, normalised to *gyrB* for isolates with *mecA*[-7]:T (n=3), *mecA*[-7]:G (n=7)  
1097 and *mecA*[-33]:T (n=6). Error bars indicate the standard deviation of the mean. Data  
1098 were analysed with a two-tailed, unpaired t-test. \*  $P = 0.0048$ , \*\*  $P = 0.0016$ . **(c)** MIC  
1099 distributions for penicillin (upper graph) and penicillin with 15 µg/ml clavulanic acid  
1100 (lower graph) with the number of isolates with each genotype combination of PBP2a  
1101 substitutions and *mecA* promoter mutations - indicated by colouring of the histogram  
1102 bars. **(d)** Joint distribution of MIC for 298 MRSA isolates, x axis shows penicillin MIC  
1103 and y axis shows penicillin MIC in the presence of 15 µg/ml clavulanic acid. Colour  
1104 and shape of the plot points indicate the genotype of the isolate.

1105

1106 **Figure 4: Prevalence and population genomics of penicillin-clavulanic acid.**

1107 **(a)** Graph shows the percentage abundance in the overall population and by clonal  
1108 complex (CC) of penicillin-clavulanic acid susceptible and resistant genotypes in  
1109 2282 clinical MRSA isolates from Cambridge, UK **(b)** Presence of SCC*mec* elements  
1110 in clonal complex (CC)8 and USA300 isolates. Figure shows a maximum likelihood  
1111 phylogenetic tree constructed from core genome SNPs of 580 CC8 isolates. USA300  
1112 isolates are indicated by blue colouring of the branches. Tips of the tree indicate the  
1113 SCC*mec* type (red = IVa, blue = IVb = orange = IVc, pink = IVd, purple = IVg, NT  
1114 (light blue) = non-typeable, - = negative for SCC*mec*). Isolates that were  
1115 phenotypically tested for penicillin-clavulanic acid susceptibility are indicated with an  
1116 asterix. Additionally, isolates: BCVA289, which was used for *in vivo* testing (wax  
1117 moth) and 43484, which was used for *in vivo* testing (murine thigh) are included for  
1118 comparison.

1119

1120 **Figure 5: Penicillins and clavulanic acid are efficacious for the treatment of**

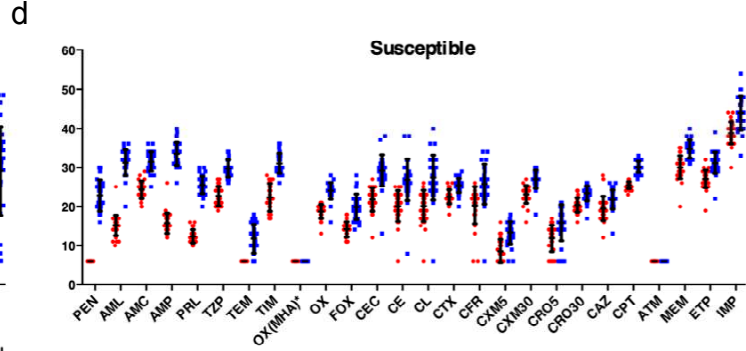
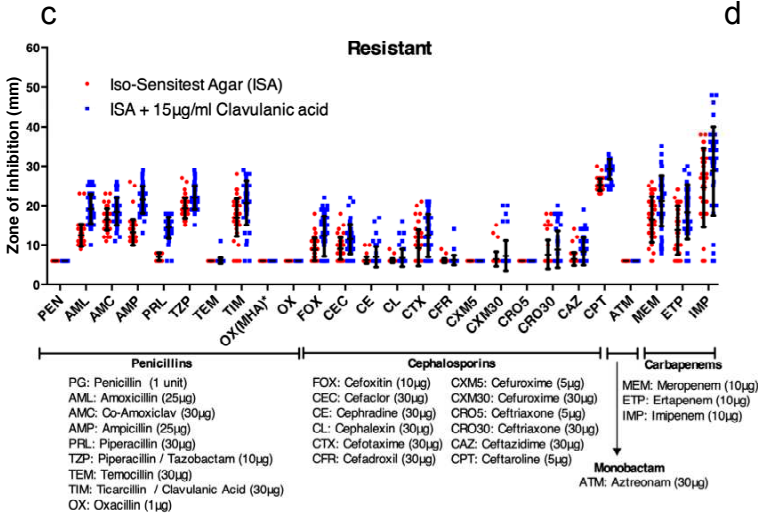
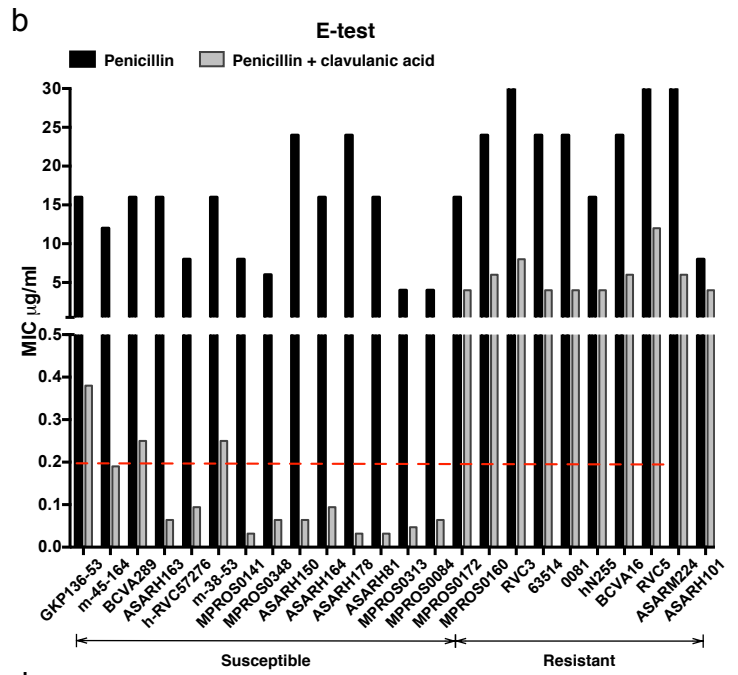
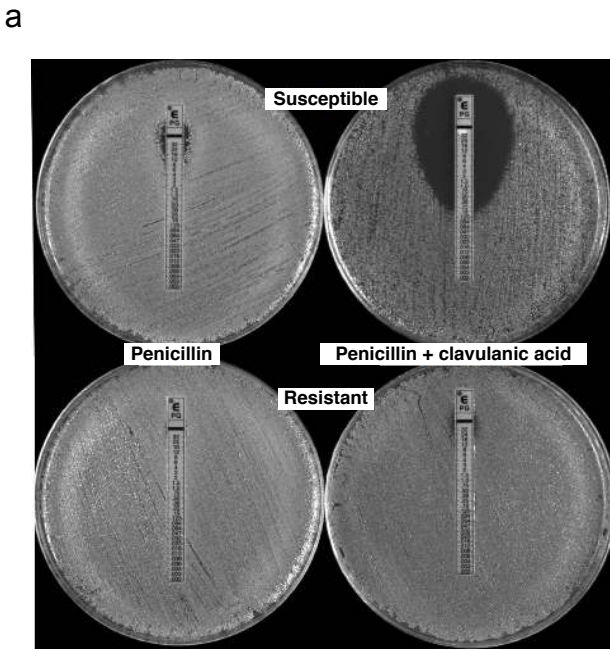
1121 **susceptible MRSA. (a)** Population analysis of resistance to penicillin and clavulanic  
1122 acid. Figure shows the log<sub>10</sub> CFU/ml of the different strains at various concentrations  
1123 of penicillin G (µg/ml) in the presence of 15 µg/ml clavulanic acid on Isosenstest

1124 plates. Plotted points are the mean of three technical replicates (biological replicates  
1125 for BCVA289 and 0081 shown in Supplementary Fig. 4). Survival curves for wax  
1126 moth larvae (*Galleria mellonella*) infected with  $\sim 1.5 \times 10^6$  CFU of: **(b)** 0081 (ST22)  
1127 (penicillin-clavulanic acid MIC = 4  $\mu\text{g/ml}$  - resistant), **(c)** RVC5 (ST22) (MIC = 12  
1128  $\mu\text{g/ml}$  - resistant), **(d)** BCVA289 (ST8 - USA300) (MIC = 0.25  $\mu\text{g/ml}$  - susceptible),  
1129 and **(e)** (EC139 (ST398) (MIC = 0.25 $\mu\text{g/ml}$  - susceptible). Ten larvae in each group  
1130 were experimentally infected and then treated at 2, 24, and 48 hours with  
1131 vancomycin (50 mg/kg), penicillin (20 mg/kg) clavulanic acid (20 mg/kg), penicillin-  
1132 clavulanic acid (1:1 ratio - 20 mg/kg), amoxicillin (20 mg/kg), amoxicillin-clavulanic  
1133 acid (1:1 ratio - 20 mg/kg) and PBS alone. Presented data are from a single  
1134 representative experiment, a replication experiment is presented in Supplementary  
1135 Fig. 5. **(f)** Effect of amoxicillin alone and in combination with clavulanic acid against  
1136 MRSA in a murine thigh infection model. Four mice in each treatment group were  
1137 inoculated with  $7 \log^{10}$  CFU of MRSA strain 43484 (USA300) and treated 1-hour post  
1138 inoculation with a single subcutaneous injection of either vancomycin (40mg/kg),  
1139 Amoxicillin (Amox) (10, 30, 100 mg/kg) alone or in combination (5:1 ratio) with  
1140 clavulanic acid (Clav) (2, 6, 20 mg/kg). The bar indicates the mean. \*\*\*\* indicates a  
1141 significant difference ( $p < 0.0001$ , Dunnett's multiple comparisons test) between  
1142 vehicle control. NS indicates there was no significant difference ( $p = 0.0982$ , Dunnett's  
1143 multiple comparisons test) between the combined amoxicillin 100 mg/kg: clavulanic  
1144 acid 20mg/kg and vancomycin 40 mg/kg.

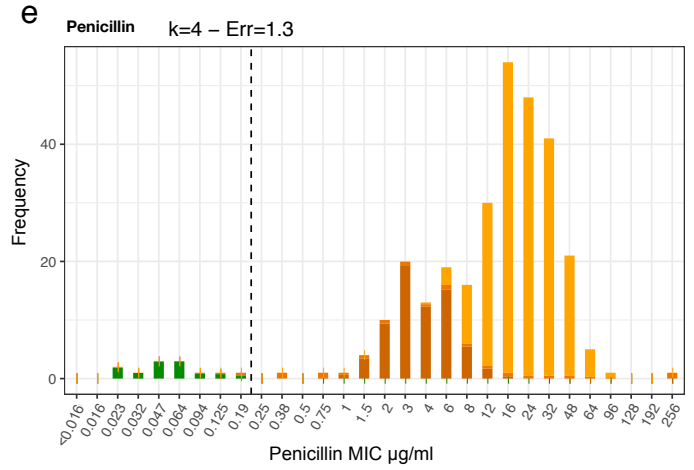
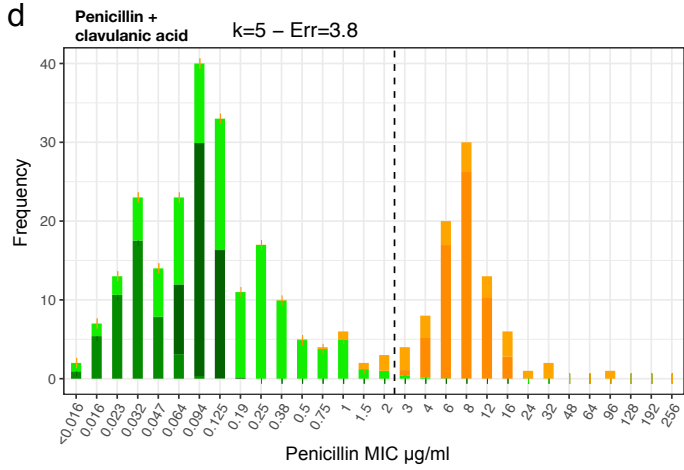
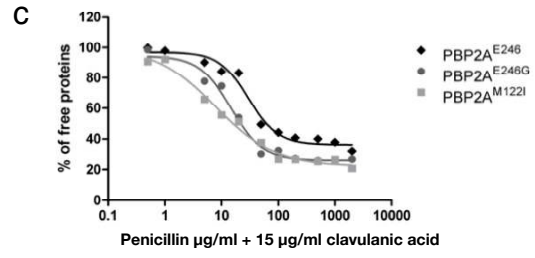
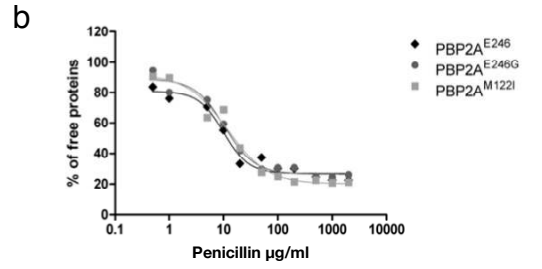
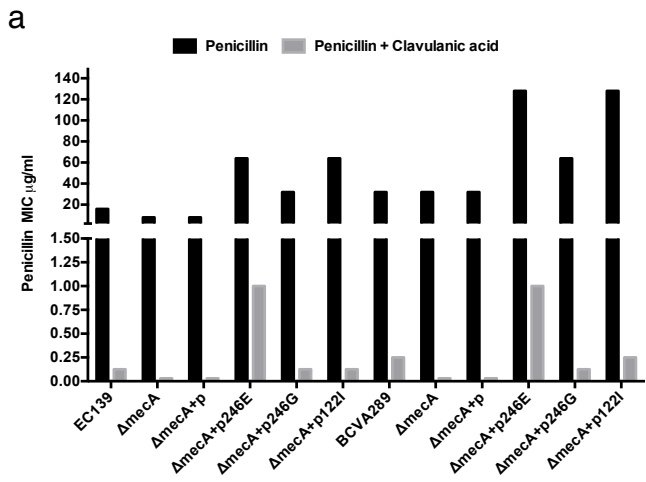
1145

1146 **Figure 6: PBP2a<sup>246G</sup> substitution provides an increased growth rate in the**  
1147 **presence of penicillin.** Figure shows growth curves for strain BCV289 $\Delta mecA$  grown  
1148 in **(a)** SSM9PR minimal medium and **(b)** SSM9PR with 16  $\mu\text{g/ml}$  penicillin, strain  
1149 EC139 $\Delta mecA$  grown in **(c)** SSM9PR minimal medium and **(d)** SSM9PR with 16  
1150  $\mu\text{g/ml}$  penicillin, and strain A75 $\Delta mecA$  grown in **(e)** SSM9PR minimal medium and **(f)**  
1151 SSM9PR with 8  $\mu\text{g/ml}$  penicillin. Lines are coloured depending on the vector the two

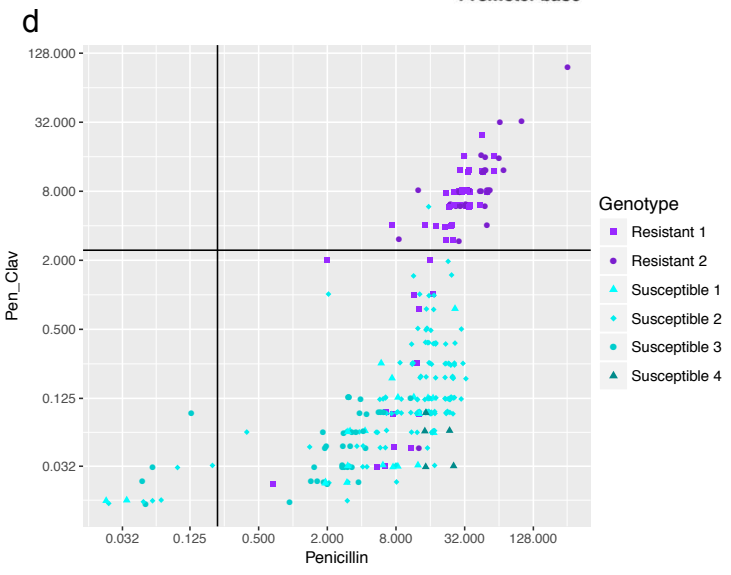
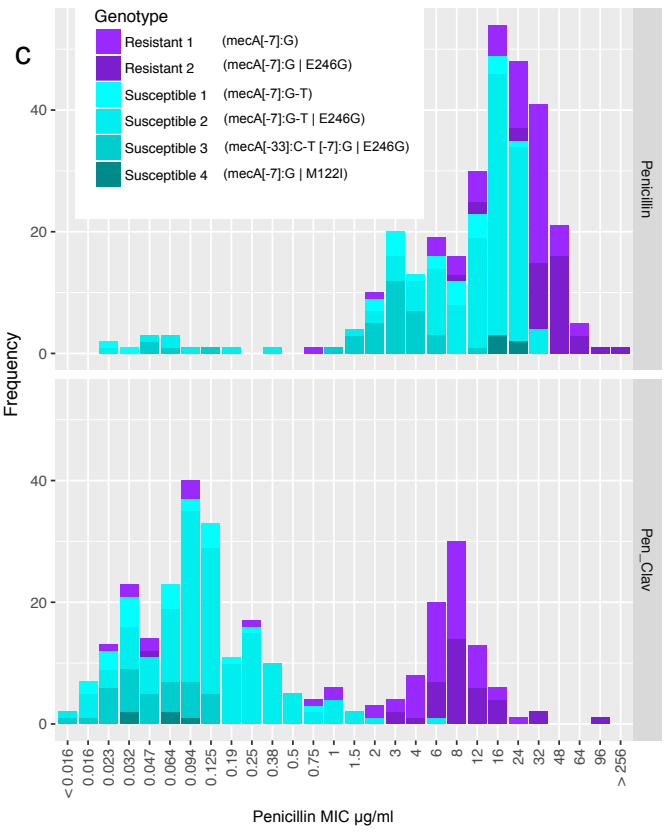
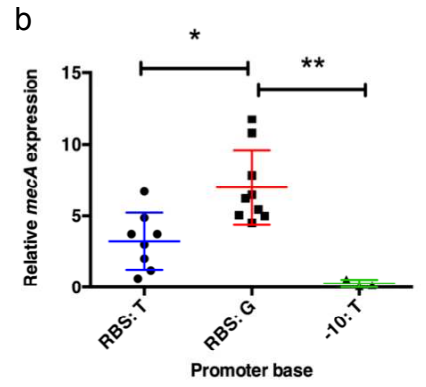
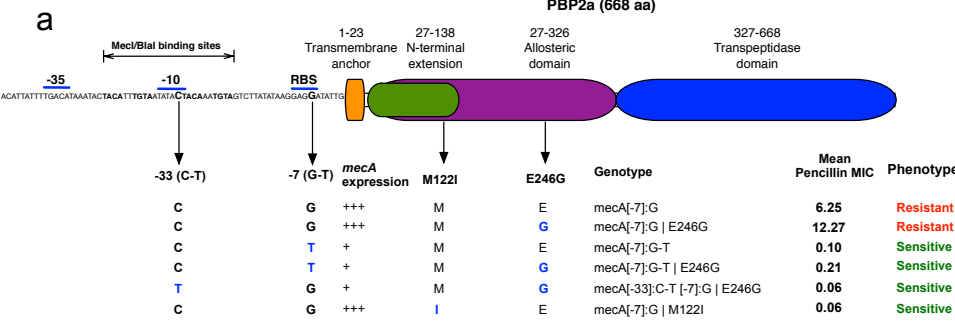
1152 strains were complemented with: orange = vector only control, green = vector  
1153 expressing *mecA*<sup>246G</sup>, blue = vector expressing *mecA*<sup>246E</sup>, black = vector expressing  
1154 *mecA*<sup>122I</sup>. The mean of a minimum of 6 independent replicates are plotted and error  
1155 bars indicate standard deviation.  
1156



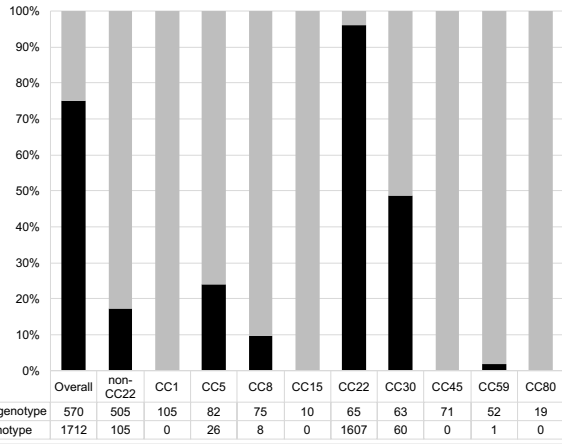




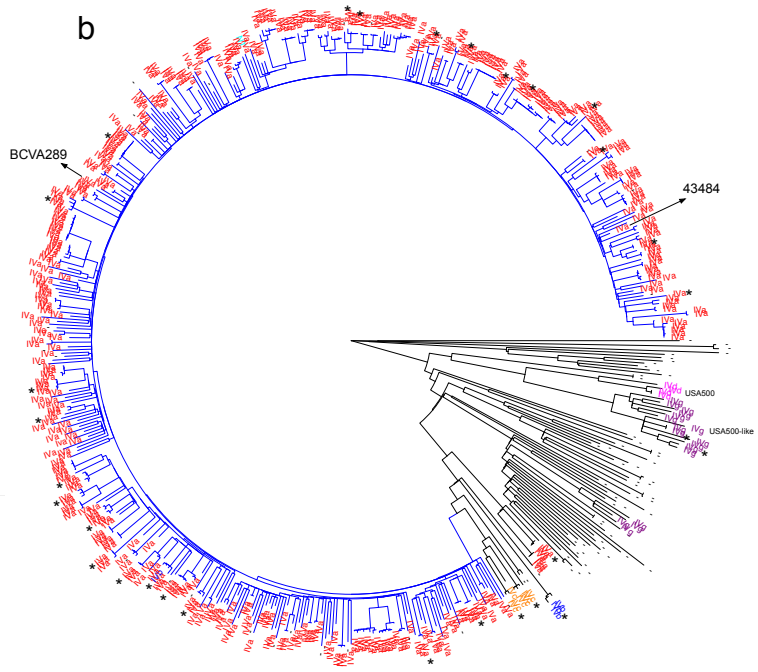
**PBP2a (668 aa)**

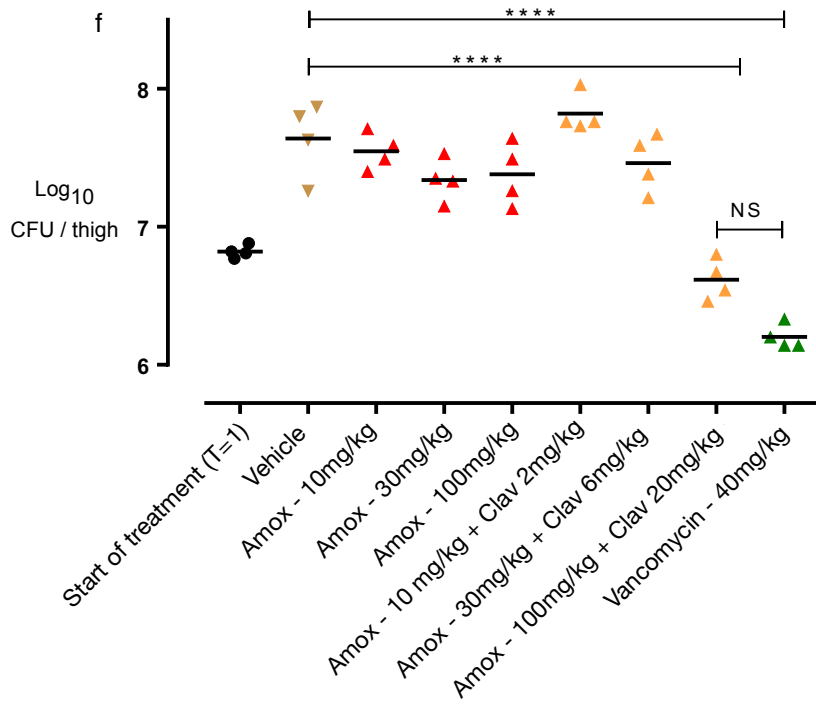
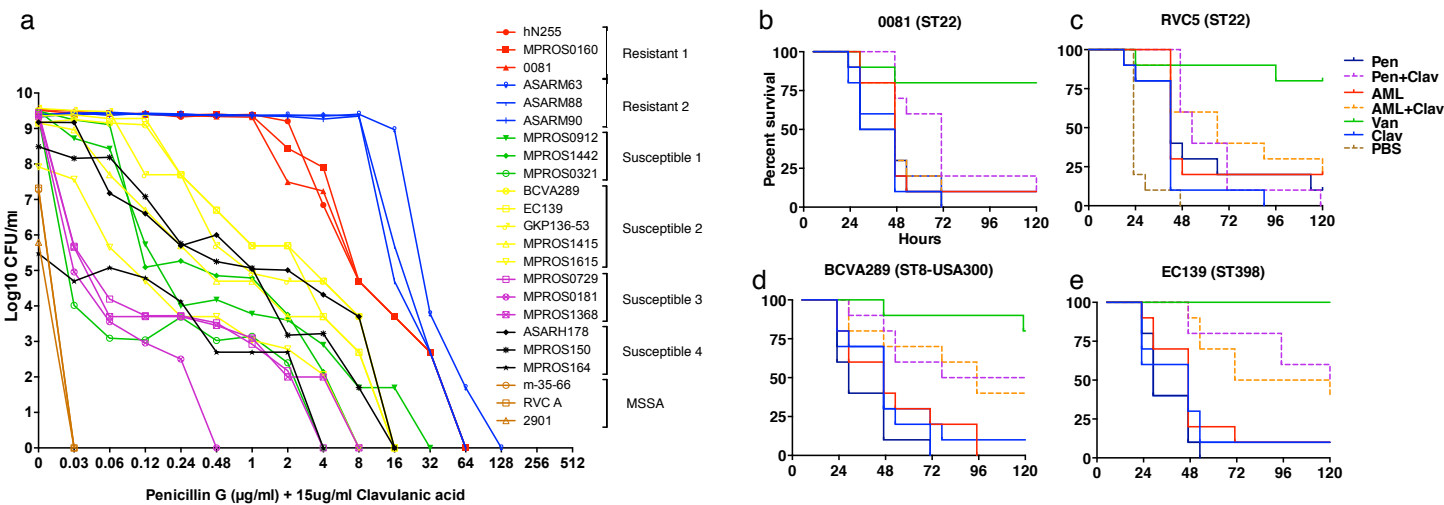


a

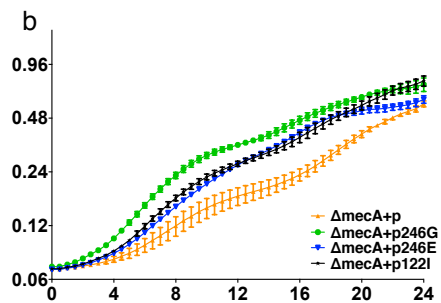
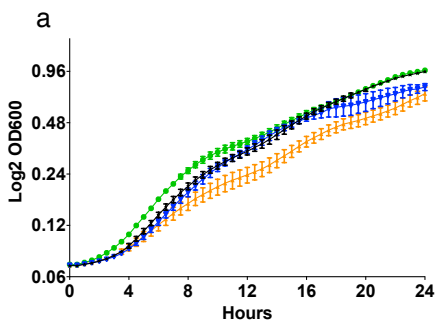


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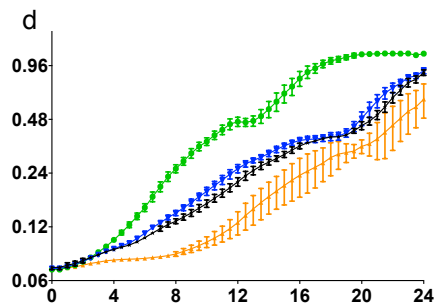
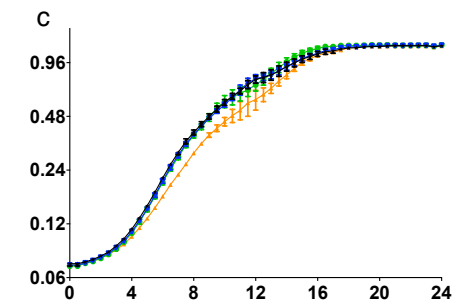




### BCVA289 (ST8-USA300)



### EC139 (ST398)



### A75 (ST22)

