

1 **Old drugs to treat resistant bugs: methicillin-resistant *Staphylococcus***
2 ***aureus* isolates with *mecC* are susceptible to a combination of penicillin**
3 **and clavulanic acid.**

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5 Running title: *mecC* and *blaZ* mediated β -lactam resistance in *mecC*-MRSA.

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27 **Abstract**

28 β -lactam resistance in methicillin-resistant *Staphylococcus aureus* (MRSA) is
29 mediated by the expression of an alternative penicillin-binding protein 2a
30 (PBP2a, encoded by *mecA*) with a low affinity for β -lactam antibiotics.
31 Recently, a novel variant of *mecA* known as *mecC* was identified in MRSA
32 isolates (*mecC*-MRSA) from both humans and animals. In this study, we
33 demonstrate that the *mecC* encoded PBP2c does not mediate resistance to
34 penicillin. Rather, broad-spectrum β -lactam resistance in *mecC*-MRSA strains
35 is mediated by a combination of both PBP2c and the distinct β -lactamase
36 encoded by *blaZ*_{LGA251} which is part of the *mecC*-encoding SCC*mec* type XI.
37 We further demonstrate that *mecC*-MRSA strains are susceptible to a
38 combination of penicillin and the β -lactam inhibitor clavulanic acid *in vitro*, and
39 that the same combination is effective *in vivo* for the treatment of an
40 experimental *mecC*-MRSA infection in wax moth larvae. Thus we
41 demonstrate how the distinct biological differences between the *mecA* and
42 *mecC* encoded PBP2a/PBP2c has the potential to be exploited as a novel
43 approach for the treatment of *mecC*-MRSA.

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52 **Introduction**

53 Antimicrobial resistance is a global health problem of particular importance,
54 which has led to an urgent need for new antimicrobial drug development. An
55 alternative approach to this problem is to re-sensitize resistant bacteria to
56 existing antibiotics using novel inhibitors or synergistic combinations of
57 existing drugs that overcome the mechanism(s) of resistance (1). The
58 classical example of this is the combination of β -lactamase inhibitors such as
59 clavulanic acid or sulbactams and a β -lactam antibiotic. Following the
60 introduction of each generation of β -lactam, resistance has rapidly emerged.
61 In the case of *Staphylococcus aureus*, penicillin resistance mediated by a
62 *blaZ* encoded β -lactamase (penicillinase) was followed by the emergence of
63 methicillin-resistant *S. aureus* (MRSA) shortly after the introduction of
64 methicillin (a β -lactamase-resistant β -lactam) in 1961 (2, 3).

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66 Resistance to β -lactam antibiotics in MRSA is primarily mediated by the
67 acquisition of an alternative penicillin-binding protein 2 (PBP2a) encoded by
68 the *mecA* gene, which is carried on a mobile element known as a
69 staphylococcal cassette chromosome (*SCCmec*) (4). In 2011, a new type of
70 MRSA with a divergent *mecA* homologue known as *mecC* was described,
71 which like some other types of MRSA is associated with livestock (5, 6). It has
72 been demonstrated that *mecC* mediates resistance to cefoxitin and oxacillin in
73 a range of strain backgrounds and that *mecC* expression is inducible with
74 oxacillin (7). The *mecC* encoded PBP2a (PBP2c) shares only 63% amino acid
75 identity with the *mecA* encoded PBP2a (PBP2a). The difference in amino acid
76 identity is reflected in the distinct biochemical properties of PBP2c whereby it

77 shows a greater affinity for oxacillin than for ceftioxin and is less stable at
78 37°C (8). Furthermore, unlike PBP2a, PBP2c does not require the
79 transglycosylase (TGase) activity of the native PBP2 for high level resistance,
80 suggesting it may preferentially cooperate with an as yet unidentified
81 monofunctional TGase (8).

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83 Here we report that the biological differences between PBP2a and PBP2c
84 extend to the unexpected finding that PBP2c does not mediate resistance to
85 penicillin, and that expression of the β -lactamase – *blaZ* (*blaZ*_{LGA251}) located
86 adjacent to *mecC* on the SCC*mec* type XI element is required for broad-
87 spectrum resistance to β -lactams. We demonstrate that this singular property
88 of PBP2c can be exploited therapeutically for the treatment of *mecC*-MRSA
89 infections.

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102 **Materials and methods**

103 **Media and culture conditions**

104 Bacterial strains and plasmids used in this study are described in Table S5.
105 For routine culture, *Escherichia coli* was grown in Lysogeny broth (LB) or on
106 L-agar (Oxoid, UK) at 37°C. *S. aureus* was grown on tryptone soy agar (TSA),
107 Columbia blood agar or in tryptone soy broth (TSB) or (Oxoid, UK) at 28°C or
108 37°C accordingly. *E. coli* and *S. aureus* media were supplemented with 10
109 µg/ml chloramphenicol (Cm10) as appropriate.

110

111 **Antimicrobial susceptibility testing**

112 Antimicrobial susceptibility testing was performed using disc diffusion
113 susceptibility testing according to BSAC criteria (BSAC Methods for
114 Antimicrobial Susceptibility Testing, version 13, June 2014). All antibiotic discs
115 were purchased from Oxoid, UK. For the clavulanic acid assay, 15 µg/ml
116 potassium clavulanate (Sigma-Aldrich, UK) was added to Iso-Sensitest agar
117 (ISA) or Mueller-Hinton agar (MHA) (Oxoid, UK), as appropriate. Test isolates
118 were grown to 0.5 McFarland standard in Iso-Sensitest broth (Oxoid, UK) and
119 diluted 1:10 in distilled water before spreading onto agar plates with or without
120 potassium clavulanate. After applying the antibiotic discs to Iso-Sensitest agar
121 or Mueller-Hinton agar with 2% NaCl as appropriate, all plates were incubated
122 at 35°C for 20 hours before inhibition zones were measured. Microbroth
123 dilution for Minimum inhibitory concentrations (MIC) was performed according
124 to BSAC (9). The range for MIC determination was 0.015-128 µg/ml for
125 penicillin and 1-32 µg/ml for ceftiofur. For *mecC/blaZ* complemented strains,
126 Iso-Sensitest broth was supplemented with anhydrotetracycline (Atc) 200

127 ng/ml to induce expression of *mecC/blaZ* from pXB01, a modified tetracycline-
128 inducible expression vector pRMC2.

129

130 **Construction of *S. aureus* gene deletion mutants**

131 Oligonucleotide primer sequences are listed in Table S5. Using primer de-
132 *blaF* and de-*blaR*, inverse PCR was performed on pRMC2 using KOD Hot
133 Start DNA Polymerase (Merck, UK) following manufacturers instructions to
134 simultaneously remove the resistance gene *bla* and to introduce a NotI
135 restriction site at each end of the PCR product. After NotI digestion and self-
136 ligation, a modified tetracycline-inducible expression vector was obtained,
137 designated pXB01. *mecC* and *blaZ* deletion mutants in *mecC*-MRSA strains
138 were generated by allelic exchange with the temperature-sensitive vector
139 pIMAY, as described previously (10). Upstream sequence (AB) and
140 downstream sequence (CD) of the *S. aureus* gene to be deleted were
141 amplified with primers A/B or C/D using KOD Hot Start DNA Polymerase
142 (Merck, UK). PCR products AB and CD were mixed in a single PCR fused by
143 in splicing overlap extension (SOE) PCR using KOD Hot Start DNA
144 Polymerase (Merck, UK) and using primers A/D to obtain deletion construct
145 AD. Product AD was digested with restriction enzymes KpnI and SacI and
146 ligated to pIMAY digested with the same enzymes. The resulting plasmids
147 were designated pIMAY Δ *mecC* or pIMAY Δ *blaZ*. The plasmids were
148 transformed into *E. coli* DC10B (a *dcm* deletion mutant of DH10B, allowing
149 the plasmid to be directly transferred into *S. aureus* strains (10). Plasmid DNA
150 extracted from DC10B was then electroporated into recipient strains to create
151 knockout mutants. *mecC-blaZ* double deletion mutants were generated by

152 deleting *blaZ* gene from *mecC* deletion mutants using plasmid
153 pIMAY Δ *mecC* Δ *blaZ*.

154

155 **Complementation of mutant strains**

156 For complement expression of *mecC* and *blaZ*, the genes were cloned into
157 expression plasmid pXB01, a derivate of tetracycline-inducible expression
158 vector pRMC2 deleted *bla* gene (11). Both genes including their ribosome
159 binding site were amplified from LGA251 genome DNA with primers *mecC*-F-
160 KpnI / *mecC*-R-SacI and *blaZ*-F-KpnI / *blaZ*-R-SacI. PCR products were
161 digested with KpnI and SacI and ligated with the pXB01 vector cleaved with
162 the same enzymes, generating plasmids pXB01-*mecC* and pXB01-*blaZ*. The
163 plasmids were transformed into *E. coli* DC10B, and plasmid DNA then
164 extracted and electroporated into mutant strains for complementation with
165 expression induced with 200 ng/ml anhydrotetracycline (Sigma-Aldrich, UK).

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167 **Wax moth larvae (*Galleria mellonella*) infection and treatment assay**

168 The wax moth larvae assay was based on that previously described by
169 Desbois et al (12). *Galleria mellonella* larvae were purchased in bulk from a
170 commercial supplier: Livefood Ltd, UK. Larvae were stored at 4°C upon arrival
171 and kept at 37°C during the course of the assay. *mecC*-MRSA strains
172 LGA251, 02.5099.D and 71277 were selected for evaluation of antimicrobial
173 activities of penicillin and clavulanic acid in combination. Single bacterial
174 colonies were picked to inoculate 5 ml of TSB, and cultures were grown
175 overnight (~16 hours) at 37°C and 200 rpm. Cultures were then diluted 1:100
176 into 5 ml of fresh TSB and grown for a further 4 hours at 37°C and 200 rpm.

177 Cultures were then centrifuged at 2,500g for 10 minutes, and pellets
178 resuspended in sterile phosphate buffered saline (PBS) to an OD₅₉₅ of 0.2,
179 giving approximately 1.3×10^6 CFU (range: $1.1 - 1.4 \times 10^6$) in 10 μ l . For each
180 strain, six groups of *G. mellonella* (n=10 in each group) were injected with 10
181 μ l aliquots of resuspended culture between two posterior thoracic segments
182 using a Tridak Stepper Pipette Dispenser (Dymax, UK). Groups of *G.*
183 *mellonella* were treated by injection with 50 mg/kg vancomycin, 40 mg/kg
184 cefoxitin, 20 mg/kg penicillin sodium salt, 20 mg/kg potassium clavulanate, 20
185 mg/kg penicillin sodium salt combined with 20 mg/kg potassium clavulanate or
186 PBS at 2, 24 and 48 hours after inoculation. The treatments were given blind
187 and the treatment identities not revealed until the experiment was completed.
188 Larvae were considered dead when they did not respond to touch to the head.
189 The experiment was performed twice with almost identical results, results of
190 one experiment are presented in the text (Fig. 5), and the results for the
191 second are presented in Fig. S3.

192

193 ***In vitro* selection of penicillin resistance**

194 LGA251 and 02.5099.D were serially passaged for 40 days in sub-inhibitory
195 concentrations of penicillin in the presence of 15 μ g /ml clavulanic acid.
196 Briefly, strains were grown on Columbia blood agar and four single colonies
197 were used to make a 0.5 McFarland standard in Iso-Sensitest broth. This was
198 diluted to final 1:200 dilution in 2 ml of Iso-Sensitest broth with range of 2-fold
199 penicillin concentrations (0.03125 to 4 μ g/ml) and a fixed concentration of 15
200 μ g/ml clavulanic acid. After incubation for 24 hours at 37°C with 200 rpm
201 shaking, the culture with the highest antibiotic concentration showing clear

202 visible growth was adjusted back to 0.5 McFarland standard and used to
203 inoculate a fresh set of tubes as above and incubated for another 24 hours.
204 Once growth occurred at the 4 µg/ml concentration of penicillin, the increment
205 was changed from 2 fold increases to 2 µg /ml increases. At selected time
206 points, cultures were plated out and 2 - 3 resistant colonies were picked,
207 penicillin and ceftiofur MIC determined and the *mecC* gene amplified by PCR
208 using primers *mecCf*., *mecCm* and *mecCr* and sequenced (Source
209 Bioscience Sequencing, Cambridge, UK) (Table S5). Strains LGA251Δ*blaZ*
210 and 02.5099.DΔ*blaZ* were also serially passaged for 40 days in sub-inhibitory
211 concentrations of penicillin in the same manner but in the absent of clavulanic
212 acid.

213

214 **Bioinformatics analysis**

215 β-lactamase sequences for type A (accession: EVL36279), B (accession:
216 WP_020978264), C (accession: WP_015056218) and D (accession: Q53699)
217 were downloaded from the NCBI. Alignments were generated using Muscle in
218 Seaview (13, 14). For the phylogeny, *blaZ* from *Micrococcus caseolyticus*
219 (accession: WP_041636568) was included as an outgroup and *blaZ* from
220 *Staphylococcus xylosus* (accession: CCM44120) was included for comparison
221 (15). Maximum likelihood phylogenetic trees were constructed using PhyML
222 v3.0 in Seaview with a WAG substitution model and 100 bootstrap replicates
223 (16).

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226

227 **Results**

228 **PBP2c does not mediate resistance to penicillin**

229 Previously work by Kim *et al.* identified that the PBP2a encoded by *mecC*
230 (PBP2c) had a higher relative affinity for oxacillin compared to cefoxitin,
231 suggesting that PBP2c has a higher affinity for penicillins than cephalosporins
232 (8). The class E *mec* complex (*mecI–mecR1–mecC–blaZ*) present in the
233 SCC*mec* type XI contains a *blaZ* gene (henceforth: *blaZ*_{LGA251}) present
234 downstream of the *mecC* gene. The *blaZ*_{LGA251} is phylogenetically distinct from
235 other previously reported *blaZ* genes in *S. aureus* (Fig. 1A) and *mecC*-MRSA
236 strains with *blaZ*_{LGA251} don't harbor any other *blaZ* genes (data not shown).
237 Currently there are four types (A to D) of staphylococcal β -lactamases based
238 on the differences of amino acids at positions 128 and 216 (17, 18). At
239 position 216, *blaZ*_{LGA251} shares a serine with type A and D *blaZ* (Fig. 1B),
240 while at position 128 *blaZ*_{LGA251} uniquely has a leucine. Therefore, we propose
241 that *blaZ*_{LGA251} is a new staphylococcal *blaZ* type; a type E *blaZ* (Fig. 1).

242

243 As PBP2c encodes low-level resistance to penicillins such as oxacillin and the
244 SCC*mec* type XI *mec* complex included a novel type of *blaZ*, we investigated
245 the relative contribution of *mecC* and *blaZ*_{LGA251} to β -lactam resistance. We
246 generated gene deletions of *mecC*, *blaZ* and both *mecC/blaZ* in two different
247 *mecC*-MRSA strains; LGA251, a multilocus sequence type (ST)425 isolated
248 from cattle in England and 02.5099.D, a ST1944 (CC130) isolate from human
249 infection in Scotland (5). Deletion of *mecC* in LGA251 (LGA251 Δ *mecC*) and
250 02.5099.D (02.5099.D Δ *mecC*) caused loss of resistance to cefoxitin as
251 measured by disc diffusion and MIC, while complementation with *mecC*

252 restored resistance as previously reported (Fig. 2A and Table S1) (7).
253 However, deletion of *mecC* caused no reduction in penicillin resistance in
254 either LGA251 or 02.5099.D, demonstrating that *mecC* did not mediate
255 resistance to penicillin in either strain background (Fig. 2B and Table S1). In
256 contrast when the *blaZ*_{LGA251} gene was deleted, resistance to penicillin was
257 abolished in both strain backgrounds and the penicillin MIC decreased from 8
258 to <0.0075 and 32 to 0.0625 µg/ml in LGA251 and 02.5099.D, respectively
259 (Fig. 2B and Table S1). Complementation of the mutants with *blaZ* restored
260 penicillin MICs to wildtype levels in both backgrounds (Fig. 2B and Table S1).
261 These findings were confirmed by the creation of double *mecC/blaZ* deletion
262 strains (LGA251Δ*mecC*Δ*blaZ*) and (02.5099.DΔ*mecC*Δ*blaZ*), which were then
263 individually complemented with plasmid-borne copies of *mecC* and *blaZ*. In
264 each case it was the complementation with *blaZ* and not the complementation
265 with *mecC* that restored resistance to penicillin (Fig. 2B and Table S1).

266

267 ***mecC*-MRSA strains are susceptible to a combination of clavulanic acid** 268 **and penicillin *in vitro***

269 As penicillin resistance in *mecC*-MRSA strains is mediated by the *blaZ*_{LGA251}
270 alone, we tested if *mecC*-MRSA strains were susceptible to the combination
271 of penicillin and clavulanic acid (a β-lactamase inhibitor). We included
272 clavulanic acid at a clinically relevant concentration of 15 µg/ml in
273 bacteriological media and carried out penicillin and cefoxitin disc diffusion
274 assays according to BSAC criteria, against a panel of 30 *mecC*-MRSA
275 isolates (Fig. 3) (19, 20). This showed that clavulanic acid increased
276 susceptibility to penicillin in all strains tested (except strain Sa09315 which

277 was already susceptible due to a frameshift in *blaZ*) and more than doubled
278 the mean zone of inhibition (10 *cf.* 27 mm – resistance cut off = 24 mm) (Fig.
279 3). Furthermore, clavulanic acid reduced the penicillin MICs by a mean of 65-
280 fold and restored breakpoint susceptibility in 24 of 30 strains tested (Fig. 3A).
281 In the remaining 6, the MIC was reduced to the breakpoint (breakpoint 0.12
282 µg/ml) (Fig. 3A). In contrast, only minor reductions were seen for cefoxitin
283 when combined with clavulanic acid (Fig. 3B). In view of the relative instability
284 of PBP2c at higher temperatures (the assays were performed at 35°C), we
285 confirmed the effect of clavulanic acid on cefoxitin and penicillin at 25, 30 and
286 37°C. The results for cefoxitin showed a clear increase in the zone of
287 inhibition with increasing temperature, as previously reported, with no major
288 effect of clavulanic acid seen at any temperature (8) (Fig. S1). While for
289 penicillin there was only a minor effect with increasing temperature and similar
290 zones of inhibition in the presence and absence of clavulanic acid (Fig. S1).
291 Together these data demonstrate that the effect of clavulanic acid was not
292 due to the temperature-sensitive activity of PBP2c at 35°C.

293

294 **Role of *mecC* and *blaZ* in resistance against a broad range of β -lactam** 295 **antibiotics**

296 We next tested the effect of clavulanic acid on resistance against a broad
297 range of β -lactam antibiotics (8 penicillins, 12 cephalosporins, 1 monobactam
298 and 3 carbapenems) for the same panel of 30 *mecC*-MRSA strains. The
299 results showed that clavulanic acid increased susceptibility to all penicillins
300 tested except for oxacillin (Fig. 3C and Table S2). Oxacillin was tested on
301 Mueller-Hinton agar as recommended by BSAC, EUCAST and CLSI

302 guidelines, and also using Iso-Sensitest where there was a small decrease in
303 the presence of clavulanic acid (mean 20 *cf.* 24 mm), which was less than that
304 observed for other penicillins. The effect of clavulanic acid was most
305 pronounced for penicillin (mean 11 *cf.* 27 mm), amoxicillin (mean 22 *cf.* 34
306 mm), ampicillin (mean 24 *cf.* 36 mm), and temocillin (mean 7 *cf.* 17 mm) (Fig.
307 3 and Table S2). Only very small increases in susceptibility were seen in the
308 presence of clavulanic acid for the tested cephalosporins and carbapenems,
309 and no effect at all was seen for the one monobactam tested (aztreonam)
310 (Fig. 3 and Table S2).

311

312 To further understand the basis for the effect of clavulanic acid, we next
313 tested the *mecC/blaZ* deletion mutant strains against the same range of β -
314 lactams in disc diffusions assays, alone and in the presence of 15 μ g/ml
315 clavulanic acid (Table S1). The results showed that the effect of clavulanic
316 acid on penicillin resistance was negated in both the LGA251 Δ *blaZ* and
317 02.5099.D Δ *blaZ*, indicating that, as would be expected, the zone increases
318 mediated by clavulanic acid were dependent on the *blaZ*_{LGA251} encoded β -
319 lactamase (Table S1). Interestingly, the presence of clavulanic acid alone in
320 the media prevented growth of both the Δ *mecC* strains. Furthermore
321 complementation with *mecC* on a plasmid failed to reverse this, nor was this
322 effect seen in either of the Δ *blaZ* strains (LGA251 Δ *blaZ* and 02.5099.D Δ *blaZ*)
323 demonstrating that it was not a by-product of the mutant construction process
324 *per se* but most likely due to the specific loss of the chromosomally encoded
325 *mecC* (Table S1).

326

327 **Selection of penicillin resistance in *mecC*-MRSA isolates**

328 Next we sought to elucidate the molecular basis for why PBP2c was unable to
329 mediate resistance to penicillin. We first attempted to identify *mecC* mutations
330 that conferred resistance to penicillin by screening a collection of whole
331 genome sequenced *mecC*-MRSA isolates (data not shown) to identify
332 naturally occurring amino acid substitutions. We identified ten different amino
333 acid substitutions present in PBP2c and tested representative isolates by
334 penicillin disc diffusion with clavulanic acid to see if there was any effect on
335 penicillin susceptibility (Table 1). None of the isolates tested showed any
336 resistance to the combination of penicillin and clavulanic acid. We next sought
337 to select mutants *in vitro* with PBP2c mutations conferring penicillin
338 resistance. We grew two wildtype *mecC*-MRSA strains: LGA251 and
339 02.5099.D in gradually increasing concentrations of penicillin supplemented
340 with clavulanic acid at 15 $\mu\text{g/ml}$ for forty days (Fig. 4). We also grew the
341 corresponding isogenic $\Delta\textit{blaZ}$ mutants (LGA251 $\Delta\textit{blaZ}$ and 02.5099.D $\Delta\textit{blaZ}$)
342 in penicillin alone (Fig. 4). At a number of points in the experiment, isolates
343 were plated to single colonies and 2-3 individual colonies tested for penicillin
344 and cefoxitin MIC and *mecC* sequenced to identify potential mutations
345 mediating penicillin resistance (Fig. 4). The MIC testing of LGA251 $\Delta\textit{blaZ}$ from
346 day 9 and 02.5099.D $\Delta\textit{blaZ}$ from day 13 showed that as well as becoming
347 penicillin resistant the strains had also substantially increased their resistance
348 to cefoxitin (8 *cf.* $\geq 128 \mu\text{g/ml}$), suggesting the change seen was a general
349 increase in β -lactam resistance and not specific to penicillin (Table S3). Only
350 colonies from wild-type strain 02.5099D grown in penicillin and clavulanic acid
351 at day 40 (02.5099-D40-A) revealed the presence of G to A mutation at

352 position 1636 in *mecC* causing a Val546Ile substitution in the transpeptidase
353 domain in PBP2c. None of the other strains screened had any *mecC*
354 mutations, suggesting resistance was due to mutations elsewhere in the
355 chromosome or upregulation of genes involved in resistance. Disc diffusion
356 testing of two individual colonies (02.5099-D40-A-C1 and 02.5099-D40-A-C2)
357 with the Val546Ile substitution showed that resistance had increased to all β -
358 lactam antibiotics except for aztreonam, which the strains were already
359 completely resistant, and to ceftaroline, the new anti-MRSA cephalosporin
360 (Table S4). We cloned the mutated *mecC* gene (*mecC*^{Val546Ile}) from 02.5099-
361 D40-A-C1 and 02.5099-D40-A-C2 into RN4220 and into *blaZ-mecC*-null
362 strains LGA251 Δ *blaZ* Δ *mecC* and 02.5099.D Δ *blaZ* Δ *mecC* and tested
363 resistance to penicillin and cefoxitin using disc diffusion. We found that the
364 strains with *mecC*^{Val546Ile} were equally susceptible to penicillin as the wildtype
365 *mecC*, demonstrating that the Val546Ile substitution alone was not capable of
366 mediating resistance (Fig. S2A and Table S4). Interestingly however, when
367 we tested the *mecC*^{Val546Ile} strains for cefoxitin resistance, we found that the
368 strains were not resistant to cefoxitin as measured by disc diffusion and had
369 an MIC of 2 μ g/ml in comparison to 16 μ g/ml for strains expressing wildtype
370 *mecC* (Fig. S2B and Table S4). Further disc diffusion testing against the full
371 panel of β -lactams, revealed that the Val546Ile substitution only effected
372 resistance to cefoxitin (Table S4). This demonstrates the importance of valine
373 at position 546 for specifically mediating cefoxitin resistance in PBP2c.
374
375

376 **A combination of clavulanic acid and penicillin is effective *in vivo* for**
377 **treatment of *mecC*-MRSA infections**

378 Finally, we sought to determine if the effect of penicillin and clavulanic acid
379 seen *in vitro* could translate to therapeutic treatment of *S. aureus* infection *in*
380 *vivo*. We used the wax moth larvae model of infection with 3 different *mecC*-
381 MRSA strains belonging to different multi-locus sequence types; LGA251
382 (ST425), 02.5099.D (ST1944), 71277 (ST130) (12). We compared the effect
383 of penicillin / clavulanic acid (2:1) with penicillin, clavulanic acid, ceftiofur and
384 PBS against a gold standard treatment of vancomycin (Fig. 5). For both
385 LGA251 and 02.5099.D 10% of larvae treated with PBS or clavulanic acid
386 survived to 120 hours, while for 71277, larvae were all dead by 48 and 68
387 hours, respectively (Fig. 5). Treatment with penicillin alone led to a modest
388 improvement, with survival at 120 hours of 20% for LGA251, 30% in
389 02.5099.D and 20% in 71277 (Fig. 5). Despite the presence of PBP2c,
390 ceftiofur performed moderately better than penicillin and with survival at 120
391 hours to 40% in LGA251 and 71277 and to 50% in 02.5099.D. In contrast,
392 survival at 120 hours for penicillin and clavulanic acid was 90% for LGA251,
393 65% for 71277 and 70% for 02.5099.D (Fig. 5). While the 'gold standard'
394 treatment of vancomycin had survival at 120 hours of 90% for LGA251 and
395 80% for, 02.5099.D and 71277, respectively (Fig. 5). Statistical analysis
396 showed there was no significant difference between treatment with a
397 combination of penicillin and clavulanic acid and vancomycin (Log-rank
398 (Mantel-Cox) Test: LGA251; P = 0.970, 02.5099.D; P = 0.259, and 71277; P =
399 0.370). Repeat experiments showed broadly identical results (Fig. S3).

400

401 **Discussion**

402 Antibiotic resistance is major international problem and with few new
403 antibiotics likely to be available in the immediate future. Novel methods to
404 combat antibiotic resistance are therefore required, including repurposing
405 older antibiotics. Here, we present evidence of one such case. We show that,
406 the newly described *mecC* encoded PBP2c does not mediate resistance to
407 penicillin and that the adjacently encoded type E *blaZ* gene is required for
408 resistance to penicillin. Our data suggest that this biological difference can be
409 exploited for treatment by combining penicillin and clavulanic acid, a β -lactam
410 inhibitor, to block the action of the *blaZ*_{LGA251}-encoded β -lactamase.
411 Importantly, we show that our *in vitro* data translate to successful treatment of
412 experimental infections *in vivo* in a non-vertebrate model. The combination of
413 penicillin and clavulanic acid was as effective as vancomycin in reducing the
414 mortality of wax moth larvae infected with three different *mecC*-MRSA strains.
415 We also observe *in vitro* activity of clavulanic acid in combination with
416 amoxicillin – a combination that is already commercially available as
417 Augmentin – suggesting that drugs already in clinical use might be successful
418 in treating *mecC*-MRSA infections. Additionally, variable resistance to different
419 cephalosporins has been reported previously for *mecC*-MRSA isolates,
420 suggesting that certain cephalosporins might also be also be used for
421 treatment (21). Our data for thirty *mecC*-MRSA isolates found uniform zones
422 of inhibition (Fig. 3C) for all the cephalosporins tested, including susceptibility
423 to ceftaroline (the new anti-MRSA cephalosporin). However given the reliance
424 of ceftoxitin/oxacillin testing for MRSA detection there is a lack of clinical
425 breakpoints for most cephalosporins, therefore it is not clear if these zones of

426 inhibition would translate into clinical efficacy, further work is required to
427 address this question.

428

429 These findings suggest that penicillin can readily bind to PBP2c to prevent cell
430 wall biosynthesis, a notion supported by data from Kim *et al.*, which showed a
431 higher binding affinity between the PBP2c to penicillins than cephalosporins in
432 comparison to PBP2a (8). This is consistent with our finding that clavulanic
433 acid was effective at conferring increased susceptibility against all the tested
434 penicillins (except oxacillin) but had no effect against cephalosporins, It
435 remains to be seen if this property is conserved amongst the variants of *mecC*
436 identified in different coagulase negative staphylococci (15, 22-24).

437

438 Biologically, the finding that the PBP2c has evolved so as not to mediate
439 resistance to penicillin is of interest. The linkage of both *mecC* and *blaZ*_{LGA251}
440 on a single genetic element might have enabled PBP2c to evolve distinct
441 properties from PBP2a, without the constraint of having to mediate resistance
442 to penicillin. This might suggest that the selective pressure to mediate
443 resistance to cephalosporins or as yet unidentified β -lactam(s) under specific
444 conditions (temperature, pH, ion concentration, etc) has selected PBP2c to be
445 unable to mediate resistance to penicillin. Indeed, the previous demonstration
446 that PBP2c is unstable at 37^o, suggests that selection pressures might have
447 selected for function at lower temperatures (8). Equally, it is known that there
448 are specific fitness costs associated with expression of PBP2a including a loss
449 of toxicity and later biofilm formation, and that high level β -lactam resistance
450 requires epistatic mutations (25-28). It remains to be seen what the distinct

451 advantage of the PBP2c is in comparison to PBP2a or the selective pressures
452 that have driven this.

453

454 *mecC*-MRSA strains are commonly isolated from cattle (29), and it is possible
455 that the routine use of both 1st and 3rd generation cephalosporins for the
456 treatment and prevention of mastitis may have provided the selective
457 pressure that has driven the emergence of *mecC*-MRSA in dairy cows (30).
458 Future studies should be targeted to investigate this question to provide
459 insight for improvements in antibiotic stewardship in veterinary medicine.

460 We have also identified the first mutation associated with specific loss of
461 cefoxitin resistance in PBP2c. Comparison of PBP2c and PBP2a structures
462 suggests this substitution might effect lobe:lobe positioning; also, V546 is
463 adjacent to a beta strand “cascade” that likely packs differently in
464 PBP2c/PBP2a protein cores i.e. V546(c)/L549(a) sits adjacent to
465 I354(c)/L357(a) which could propagate all the way up to motif III (KSG) and
466 motif I (SXXK) at active site (Fig. S4) (31, 32). Further structural insights into
467 the difference between two proteins are required to shed further light on our
468 understanding of the distinct biological properties of PBP2a and PBP2c.

469

470 In conclusion, our findings further highlight how the limited functional
471 sequence space can be exploited for antibiotic drug design and synergistic
472 therapy. This is particularly important given the increasing challenges posed
473 by multidrug resistance and offers a paradigm for tackling an emerging,
474 resistant bacterial pathogen with an old antibiotic.

475

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482

483 **Author contributions**

484 X.B designed and carried out experimental work and analyzed the data and
485 contributed to the manuscript, E.M.H. designed and carried out experimental
486 work, bioinformatics, analyzed the data and wrote the manuscript. A.L.L
487 contributed to experimental design and carried out structural analysis. N.G
488 carried out experimental work. R.Z., J.P. and S.J.P. contributed to the
489 analysis and critically revised the manuscript. M.T.G.H. contributed to the
490 analysis and interpretation of the data and critically revised the manuscript.
491 G.K.P contributed to experimental design, analyzed the data and critically
492 revised the manuscript. M.A.H. coordinated the study and wrote the
493 manuscript.

494

495 **Conflict of Interest**

496 The authors declare that they have no conflict of interest.

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 633 physiological function. Proc Natl Acad Sci U S A **110**:16808-13.
 634

635 **Table 1:** Locations of amino acid substitutions in PBP2c in wildtype strains
 636 0820 'A' to 51618 and the location of the substitution found in strain 02.5099-
 637 D40-A. Residues likely in the transpeptidase domain (residues 324-665)
 638 based on alignment with PBP2a are highlighted in blue.

Representative strain	No. of isolates tested	Position of amino acid substitution										
		63	130	145	173	185	269	346	349	466	482	546
LGA251	1	N	V	K	N	R	Y	A	P	R	D	V
0820 'A'	1							S				
52902	2										N	
ST20120827	1			I								
77964	9				K							
Sa13307	4	K										
M4A	1								A			
71957	14									H		
m-40-71	1					L						
1198/2006	1		I									
51618	1						C					
02.5099-D40-A	1											I

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648 **Figure legends**

649 **Figure 1.** (A) Maximum likelihood tree generated from amino acid sequences
650 showing relationships of staphylococcal β -lactamases. Values above
651 branches indicate bootstrap support. (B) Alignment of the amino acid
652 sequence of representative type A-D β -lactamases in comparison to the
653 SCC*mec* type XI encoded type-E (LGA251) β -lactamase. Highlighted
654 residues indicate the amino acids at positions 128 and 216 used to type the β -
655 lactamase.

656

657 **Figure 2:** Effect of deletion of *mecC* and *blaZ* in strains LGA251 and
658 02.5099.D on the minimum inhibitory concentrations of: (A) cefoxitin; (B)
659 penicillin. For each strain background (LGA251 and 02.5099.D) the first strain
660 is the wildtype followed to the right by its various mutants and complemented
661 mutants. +p denotes complemented with the empty vector, +*pmecC*
662 complemented with a vector borne copy of *mecC*, +*pblaZ* with a vector borne
663 copy of *blaZ*.

664

665 **Figure 3: The effect of clavulanic acid on *mecC*-MRSA strains.** (A)
666 Penicillin MICs (B) and Cefoxitin MICs for individual isolates in the presence
667 (in red) and absence (in black) of clavulanic acid (C) Mean results for a panel
668 of 30 *mecC*-MRSA strains against a range of β -lactam antibiotics as
669 measured by disc diffusion assays in the presence and absence of clavulanic

670 acid. The error bars represent the standard error. Two and three letter codes
671 and concentrations of discs are shown below each class of β -lactam
672 antibiotics. Note: results are shown for oxacillin on two different media; Iso-
673 Sensitest and Mueller-Hinton agar with 2% NaCl at 30°C (BSAC / June / 2014
674 – recommended media). Resistance breakpoints for penicillin, oxacillin,
675 cefoxitin are 24 mm, 14 mm, 21 mm, respectively). BSAC MIC breakpoint for
676 penicillin (Pen) and cefoxitin (Fox) are 0.12 and 4 $\mu\text{g/ml}$, respectively.
677 Clavulanic acid was included in the media at 15 $\mu\text{g/ml}$.

678

679 **Figure 4.** *In vitro* selection of penicillin-resistance. Graphs show changes in
680 subinhibitory concentrations for mutant *mecC*-MRSA strains LGA251 Δ *blaZ*
681 and 02.5099.D Δ *blaZ* (A) or wild type *mecC*-MRSA strains LGA251 and
682 02.5099.D (B) during the course of *in vitro* penicillin resistance selection (A) or
683 penicillin and clavulanic acid selection (B) grown in continuous culture of Iso-
684 Sensitest broth at 37°C. Arrows indicates time points selected for *mecC* gene
685 sequencing.

686

687 **Figure 5: Experimental treatment of *Galleria mellonella* infected by**
688 ***mecC*-MRSA strains: (A) LGA251 (B) 02.5099.D (C) 71277.10.** Ten larvae
689 in each group were experimentally infected and then treated at 2, 24 and 48
690 hours with Vancomycin (50 mg/kg / 6.73×10^{-9} mol), Penicillin (20 mg/kg /
691 1.12×10^{-8} mol), Clavulanic acid (20 mg/kg / 1.69×10^{-8} mol), Penicillin /
692 Clavulanic acid, Cefoxitin (40 mg/kg / 1.78×10^{-8} mol), and PBS alone. Figure
693 shows data from a single experiment.