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

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

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Abstract

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

Introduction

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

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

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

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

Materials and methods

Media and culture conditions

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

Antimicrobial susceptibility testing

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

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

Construction of *S. aureus* gene deletion mutants

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

deleting *blaZ* gene from *mecC* deletion mutants using plasmid pIMAY∆*mecC*∆*blaZ*.

Complementation of mutant strains

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

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

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

Cultures were then centrifuged at 2,500g for 10 minutes, and pellets resuspended in sterile phosphate buffered saline (PBS) to an OD_{595} of 0.2, giving approximately 1.3×10^6 CFU (range: 1.1 – 1.4 x 10^6) in 10 µl . For each strain, six groups of *G. mellonella* (n=10 in each group) were injected with 10 µl aliquots of resuspended culture between two posterior thoracic segments using a Tridak Stepper Pipette Dispenser (Dymax, UK). Groups of *G. mellonella* were treated by injection with 50 mg/kg vancomycin, 40 mg/kg cefoxitin, 20 mg/kg penicillin sodium salt, 20 mg/kg potassium clavulanate, 20 mg/kg penicillin sodium salt combined with 20 mg/kg potassium clavulanate or PBS at 2, 24 and 48 hours after inoculation. The treatments were given blind and the treatment identities not revealed until the experiment was completed. Larvae were considered dead when they did not respond to touch to the head. The experiment was performed twice with almost identical results, results of one experiment are presented in the text (Fig. 5), and the results for the second are presented in Fig. S3.

In vitro selection of penicillin resistance

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

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

Bioinformatics analysis

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

Results

PBP2c does not mediate resistance to penicillin

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

As PBP2c encodes low-level resistance to penicillins such as oxacillin and the SCC*mec* type XI *mec* complex included a novel type of *blaZ*, we investigated the relative contribution of *mecC* and $blaZ_{LGA251}$ to β-lactam resistance. We generated gene deletions of *mecC*, *blaZ* and both *mecC*/*blaZ* in two different *mecC*-MRSA strains; LGA251, a multilocus sequence type (ST)425 isolated from cattle in England and 02.5099.D, a ST1944 (CC130) isolate from human infection in Scotland (5). Deletion of *mecC* in LGA251 (LGA251Δ*mecC*) and 02.5099.D (02.5099.DΔ*mecC*) caused loss of resistance to cefoxitin as 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

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

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

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

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

To further understand the basis for the effect of clavulanic acid, we next tested the *mecC*/*blaZ* deletion mutant strains against the same range of β-lactams in disc diffusions assays, alone and in the presence of 15 µg/ml clavulanic acid (Table S1). The results showed that the effect of clavulanic acid on penicillin resistance was negated in both the LGA251Δ*blaZ* and 02.5099.DΔ*blaZ*, indicating that, as would be expected, the zone increases mediated by clavulanic acid were dependent on the $blaZ_{LGA251}$ encoded β-lactamase (Table S1). Interestingly, the presence of clavulanic acid alone in the media prevented growth of both the Δ*mecC* strains. Furthermore complementation with *mecC* on a plasmid failed to reverse this, nor was this effect seen in either of the Δ*blaZ* strains (LGA251Δ*blaZ* and 02.5099.D*ΔblaZ*) demonstrating that it was not a by-product of the mutant construction process *per se* but most likely due to the specific loss of the chromosomally encoded *mecC* (Table S1).

Selection of penicillin resistance in *mecC*-MRSA isolates

Next we sought to elucidate the molecular basis for why PBP2c was unable to mediate resistance to penicillin. We first attempted to identify *mecC* mutations that conferred resistance to penicillin by screening a collection of whole genome sequenced *mecC*-MRSA isolates (data not shown) to identify naturally occurring amino acid substitutions. We identified ten different amino acid substitutions present in PBP2c and tested representative isolates by penicillin disc diffusion with clavulanic acid to see if there was any effect on penicillin susceptibility (Table 1). None of the isolates tested showed any resistance to the combination of penicillin and clavulanic acid. We next sought to select mutants *in vitro* with PBP2c mutations conferring penicillin resistance. We grew two wildtype *mecC*-MRSA strains: LGA251 and 02.5099.D in gradually increasing concentrations of penicillin supplemented with clavulanic acid at 15 μg/ml for forty days (Fig. 4). We also grew the corresponding isogenic Δ*blaZ* mutants (LGA251Δ*blaZ* and 02.5099.DΔ*blaZ*) in penicillin alone (Fig. 4). At a number of points in the experiment, isolates were plated to single colonies and 2-3 individual colonies tested for penicillin and cefoxitin MIC and *mecC* sequenced to identify potential mutations mediating penicillin resistance (Fig. 4). The MIC testing of LGA251Δ*blaZ* from day 9 and 02.5099.DΔ*blaZ* from day 13 showed that as well as becoming penicillin resistant the strains had also substantially increased their resistance to cefoxitin (8 *cf.* ≥ 128 μg/ml), suggesting the change seen was a general increase in β-lactam resistance and not specific to penicillin (Table S3). Only colonies from wild-type strain 02.5099D grown in penicillin and clavulanic acid at day 40 (02.5099-D40-A) revealed the presence of G to A mutation at

position 1636 in *mecC* causing a Val546Ile substitution in the transpeptidase domain in PBP2c. None of the other strains screened had any *mecC* mutations, suggesting resistance was due to mutations elsewhere in the chromosome or upregulation of genes involved in resistance. Disc diffusion testing of two individual colonies (02.5099-D40-A-C1 and 02.5099-D40-A-C2) with the Val546Ile substitution showed that resistance had increased to all β-lactam antibiotics except for aztreonam, which the strains were already completely resistant, and to ceftaroline, the new anti-MRSA cephalosporin (Table S4). We cloned the mutated *mecC* gene (*mecC*^{Val546Ile}) from 02.5099-D40-A-C1 and 02.5099-D40-A-C2 into RN4220 and into *blaZ-mecC*–null strains LGA251Δ*blaZ*Δ*mecC* and 02.5099.DΔ*blaZ*Δ*mecC* and tested resistance to penicillin and cefoxitin using disc diffusion. We found that the strains with *mecC*^{Val546Ile} were equally susceptible to penicillin as the wildtype *mecC*, demonstrating that the Val546Ile substitution alone was not capable of mediating resistance (Fig. S2A and Table S4). Interestingly however, when we tested the *mecC*^{Val546Ile} strains for cefoxitin resistance, we found that the strains were not resistant to cefoxitin as measured by disc diffusion and had an MIC of 2 μg/ml in comparison to 16 μg/ml for strains expressing wildtype *mecC* (Fig. S2B and Table S4). Further disc diffusion testing against the full panel of ß-lactams, revealed that the Val546Ile substitution only effected resistance to cefoxitin (Table S4). This demonstrates the importance of valine at position 546 for specifically mediating cefoxitin resistance in PBP2c.

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

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

Discussion

Antibiotic resistance is major international problem and with few new antibiotics likely to be available in the immediate future. Novel methods to combat antibiotic resistance are therefore required, including repurposing older antibiotics. Here, we present evidence of one such case. We show that, the newly described *mecC* encoded PBP2c does not mediate resistance to penicillin and that the adjacently encoded type E *blaZ* gene is required for resistance to penicillin. Our data suggest that this biological difference can be exploited for treatment by combining penicillin and clavulanic acid, a β-lactam inhibitor, to block the action of the $blaZ_{LGA251}$-encoded β-lactamase. Importantly, we show that our *in vitro* data translate to successful treatment of experimental infections *in vivo* in a non-vertebrate model. The combination of penicillin and clavulanic acid was as effective as vancomycin in reducing the mortality of wax moth larvae infected with three different *mecC*-MRSA strains. We also observe *in vitro* activity of clavulanic acid in combination with amoxicillin – a combination that is already commercially available as Augmentin – suggesting that drugs already in clinical use might be successful in treating *mecC*-MRSA infections. Additionally, variable resistance to different cephalosporins has been reported previously for *mecC*-MRSA isolates, suggesting that certain cephalosporins might also be also be used for treatment (21). Our data for thirty *mecC*-MRSA isolates found uniform zones of inhibition (Fig. 3C) for all the cephalosporins tested, including susceptibility to ceftaroline (the new anti-MRSA cephalosporin). However given the reliance of cefoxitin/oxacillin testing for MRSA detection there is a lack of clinical 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 37º, 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 ß-lactam resistance
450 requires epistatic mutations (25-28). It remains to be seen what the distinct

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

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

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

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

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Author contributions

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

Conflict of Interest

The authors declare that they have no conflict of interest.

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Table 1: Locations of amino acid substitutions in PBP2c in wildtype strains 0820 'A' to 51618 and the location of the substitution found in strain 02.5099-D40-A. Residues likely in the transpeptidase domain (residues 324-665) 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

Figure legends

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

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

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

acid**.** The error bars represent the standard error. Two and three letter codes and concentrations of discs are shown below each class of β-lactam antibiotics. Note: results are shown for oxacillin on two different media; Iso-Sensitest and Mueller-Hinton agar with 2% NaCl at 30ºC (BSAC / June / 2014 – recommended media). Resistance breakpoints for penicillin, oxacillin, cefoxitin are 24 mm, 14 mm, 21 mm, respectively). BSAC MIC breakpoint for penicillin (Pen) and cefoxitin (Fox) are 0.12 and 4 µg/ml, respectively. Clavulanic acid was included in the media at 15 µg/ml.

Figure 4. *In vitro* selection of penicillin-resistance. Graphs show changes in subinhibitory concentrations for mutant *mecC*-MRSA strains LGA251*ΔblaZ* and 02.5099.DΔ*blaZ* (A) or wild type *mecC*-MRSA strains LGA251 and 02.5099.D (B) during the course of *in vitro* penicillin resistance selection (A) or penicillin and clavulanic acid selection (B) grown in continuous culture of Iso-Sensitest broth at 37^{o}C. Arrows indicates time points selected for *mecC* gene sequencing.

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