Novel mutations in penicillin-binding protein genes in clinical *Staphylococcus aureus* isolates that are methicillin resistant on susceptibility testing, but lack the mec gene


1Department of Veterinary Medicine, University of Cambridge, Cambridge, UK; 2Scottish MRSA Reference Laboratory, NHS Greater Glasgow and Clyde, Stobhill Hospital, Glasgow, UK; 3Wellcome Trust Sanger Institute, Hinxton, UK; 4Department of Microbiology and Infection Control, Statens Serum Institut, Copenhagen, Denmark; 5Department of Clinical Medicine, University of Cambridge, Cambridge, UK

*Corresponding author. E-mail: mah1@cam.ac.uk
†Contributed equally.

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**Objectives:** Methicillin-resistant *Staphylococcus aureus* (MRSA) is an important global health problem. MRSA resistance to β-lactam antibiotics is mediated by the mecA or mecC genes, which encode an alternative penicillin-binding protein (PBP) 2a that has a low affinity to β-lactam antibiotics. Detection of mec genes or PBP2a is regarded as the gold standard for the diagnosis of MRSA. We identified four MRSA isolates that lacked mecA or mecC genes, but were still phenotypically resistant to pencillinase-resistant β-lactam antibiotics.

**Methods:** The four human *S. aureus* isolates were investigated by whole genome sequencing and a range of phenotypic assays.

**Results:** We identified a number of amino acid substitutions present in the endogenous PBPs 1, 2 and 3 that were found in the resistant isolates but were absent in closely related susceptible isolates and which may be the basis of resistance. Of particular interest are three identical amino acid substitutions in PBPs 1, 2 and 3, occurring independently in isolates from at least two separate multilocus sequence types. Two different non-conservative substitutions were also present in the same amino acid of PBP1 in two isolates from two different sequence types.

**Conclusions:** This work suggests that phenotypically resistant MRSA could be misdiagnosed using molecular methods alone and provides evidence of alternative mechanisms for β-lactam resistance in MRSA that may need to be considered by diagnostic laboratories.

**Keywords:** β-lactams, MRSA, mecA, mecC

**Introduction**

β-Lactam antibiotics work by acylation of the transpeptidase domain active site of penicillin-binding proteins (PBPs), blocking access to their substrate and preventing cross-linking of peptidoglycan strands during cell wall synthesis. Initially, resistance to penicillin in *Staphylococcus aureus* was mediated by the mecA or mecC genes, which encode an alternative penicillin-binding protein (PBP) 2a that has a low affinity to β-lactam antibiotics. Detection of mec genes or PBP2a is regarded as the gold standard for the diagnosis of MRSA. We identified four MRSA isolates that lacked mecA or mecC genes, but were still phenotypically resistant to pencillinase-resistant β-lactam antibiotics.

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Results

We identified four mecA-negative isolates (XB84, 85, 86, and 87) from the Scottish MRSA Reference Laboratory that exhibited resistance to penicillinase-resistant penicillins, but lack both meca and mecC. These isolates were tested for resistance to multiple -lactams, and phenotypic resistance to oxacillin and cefoxitin confirmed that they were mecA resistent, mecC-negative MRSA isolates. We conducted disc diffusion assays in the presence of clavulanic acid (CLA), and tested them for oxacillin and cefoxitin (OXA) resistance by using -lactamase inhibitor controls, respectively. mecA-negative and mecC-resistant isolates were tested for -lactamase production, respectively.

Table 1. Relevant phenotypic and genotypic characteristics of resistant isolates

<table>
<thead>
<tr>
<th>Isolate</th>
<th>Country</th>
<th>Origin</th>
<th>Isolated from</th>
<th>Year of isolation</th>
<th>MLST</th>
<th>spa type</th>
<th>OXA Etest (mm)</th>
<th>FOX Etest (mm)</th>
<th>PEN G Etest (mg/L)</th>
<th>PEN G CRO Etest (mg/L)</th>
<th>β-Lactamase resistance</th>
<th>blaZ gene</th>
<th>blaZ type</th>
</tr>
</thead>
<tbody>
<tr>
<td>XB84</td>
<td>Scotland</td>
<td>human wound</td>
<td>wound</td>
<td>2010</td>
<td>15</td>
<td>t084</td>
<td>11</td>
<td>17</td>
<td>2</td>
<td>&gt;32</td>
<td>+</td>
<td>+ C</td>
<td></td>
</tr>
<tr>
<td>XB85</td>
<td>Scotland</td>
<td>human wound</td>
<td>wound</td>
<td>2010</td>
<td>1</td>
<td>t127</td>
<td>0</td>
<td>18</td>
<td>4</td>
<td>&gt;32</td>
<td>+</td>
<td>+ C</td>
<td></td>
</tr>
<tr>
<td>XB86</td>
<td>Scotland</td>
<td>human wound</td>
<td>wound</td>
<td>2010</td>
<td>15</td>
<td>t907</td>
<td>0</td>
<td>17</td>
<td>3</td>
<td>&gt;32</td>
<td>-</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>XB87</td>
<td>Scotland</td>
<td>human wound</td>
<td>wound</td>
<td>2010</td>
<td>8</td>
<td>t008</td>
<td>0</td>
<td>12</td>
<td>4</td>
<td>&gt;32</td>
<td>+</td>
<td>+ A</td>
<td></td>
</tr>
</tbody>
</table>

For oxacillin: Etest MIC breakpoint is >2 mg/L; disc diffusion—susceptible ≥15 mm diameter, resistant ≤14 mm diameter.

For cefoxitin: Etest MIC breakpoint is >4 mg/L; disc diffusion—susceptible ≥22 mm diameter, resistant ≤21 mm diameter.

For penicillin G: Etest MIC breakpoint is >0.12 mg/L; disc diffusion—susceptible ≥25 mm diameter, resistant ≤24 mm diameter.
antibiotic: clavulanic acid. No major reduction was seen in the zones of inhibition of oxacillin, cefoxitin or penicillin in combination with clavulanic acid at either 2:1 or 1:1, suggesting that β-lactamase production was not mediating resistance (Table 1).

In addition to PBP2a and β-lactamase, five other proteins (PBP2, PBP4, GdpP, YjbH and AcrB) have been reported previously to be associated with β-lactam resistance in S. aureus. We screened the genome sequences of the four resistant isolates with a panel of susceptible isolates belonging to the same STs (ST1, 10 isolates; ST8, 4 isolates; and ST15, 2 isolates) (data not shown). In the comparison, we also included the two other endogenous PBPs (PBP1 and PBP3) on the basis that these are also bound by β-lactam antibiotics. We found a small number of amino acid substitutions in proteins PBP1, PBP2, PBP3 and YjbH in one or more of the resistant isolates, but absent in the susceptible counterparts (Table 2). Interestingly, we found three separate residues with substitutions that were present in at least two isolates from different STs, suggestive of homoplasy (independent evolution of the same trait). First, a His-499→Thr substitution in the transpeptidase domain of PBP1 was present in three isolates from different STs: XB85 (ST1), XB86 (ST15) and XB87 (ST8) (Table 2). Second, a Thr-552→Ile substitution in the transpeptidase domain of PBP2 was found in three isolates from two STs: XB84 (ST15), XB86 (ST15) and XB87 (ST8). Third, isolates XB84 (ST15), XB85 (ST1) and XB86 (ST15) all shared the same Ser-634→Phe substitution in the transpeptidase domain of PBP3 (Table 2). Fourth, we identified two different non-conservative amino acid substitutions at the same position in the transpeptidase domain of PBP1, a Tyr-336→Asn substitution in XB84 (ST15) and a Tyr-336→Cys substitution in XB85 (ST1). A number of single substitutions were also present in one of the resistant isolates that were absent in the susceptible isolates of the same ST (Table 2). Two further substitutions were present in PBP2: a Thr-31→Met substitution in XB85 and an Asp-156→Tyr substitution in the transpeptidase domain of isolate XB86. XB85 also had a Thr-371→Ile substitution in the transpeptidase domain of PBP1 (Table 2). Except for MRSA strain CM05 (accession: AMAB00000000), we were unable to find any genomes in the NCBI/EMBL databases containing the same substitutions.

Table 2. Locations of amino acid substitutions identified in methicillin-resistant isolates that were absent in susceptible isolates of the same ST

<table>
<thead>
<tr>
<th>Isolate</th>
<th>MLST</th>
<th>PBP1</th>
<th>PBP2</th>
<th>PBP3</th>
<th>YjbH</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Y336C</td>
<td>Y336N</td>
<td>T371I</td>
<td>H499Y</td>
</tr>
<tr>
<td>XB84</td>
<td>15</td>
<td>−</td>
<td>+</td>
<td>−</td>
<td>−</td>
</tr>
<tr>
<td>XB85</td>
<td>1</td>
<td>+</td>
<td>−</td>
<td>+</td>
<td>+</td>
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<tr>
<td>XB86</td>
<td>15</td>
<td>−</td>
<td>−</td>
<td>−</td>
<td>+</td>
</tr>
<tr>
<td>XB87</td>
<td>8</td>
<td>−</td>
<td>−</td>
<td>−</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td></td>
<td>T31M</td>
<td>D156Y</td>
<td>T552I</td>
<td>Y430D</td>
</tr>
<tr>
<td></td>
<td></td>
<td>−</td>
<td>+</td>
<td>−</td>
<td>−</td>
</tr>
<tr>
<td></td>
<td></td>
<td>+</td>
<td>+</td>
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<td>+</td>
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<td></td>
<td></td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>−</td>
</tr>
</tbody>
</table>

MLST, multilocus ST.

Discussion

In this work we have identified MRSA isolates belonging to three STs, which unlike BORSA strains are resistant to both oxacillin and cefoxitin. This resistance does not appear to be mediated by hyper-production of a β-lactamase and we have identified a number of novel substitutions in the transpeptidase domains of PBPs 1, 2 and 3 that we hypothesize could mediate this resistance. The transpeptidase domains of PBPs are the target of β-lactam antibiotics and substitutions in the transpeptidase domain of PBP2 have been shown previously to reduce the acylation efficacy of PBP2 by β-lactams and thus provide some degree of resistance. Given the small number of isolates in this study and that our targeted search for mutations might have excluded other genes involved in resistance, further experimental work is necessary to characterize the contribution of the novel PBP substitutions to β-lactam resistance. Mutations in PBP1 and PBP3 have not been implicated previously in S. aureus resistance; however, in Staphylococcus lugdunensis, a tetrapeptide duplication in the transpeptidase domain of PBP1A/B has been shown to be associated with increased β-lactam resistance. Currently, mec gene-negative MRSA isolates are not widely reported, but it is important to characterize the basis for resistance in these isolates, especially for clinical laboratories that rely on the molecular detection of mecA/C and/or PBP2a as the gold standard for MRSA detection. Furthermore, PBP2a has been highlighted as an attractive target for drug development and should PBP2a-targeted inhibitors become available, it is important to understand alternative mechanisms for S. aureus to develop resistance to β-lactams. Finally, it is clear that there are multiple distinct mechanisms for β-lactam resistance in S. aureus and these need to be taken into consideration by diagnostic laboratories.

Acknowledgements

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mec gene-negative MRSA

References