Whole-genome analysis of an oxacillin-susceptible CC80 mecA-positive Staphylococcus aureus clinical isolate: insights into the mechanisms of cryptic methicillin resistance

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Objectives: The mec and bla systems, among other genetic factors, are critical in regulating the expression of methicillin resistance in Staphylococcus aureus. We examined by WGS a naturally occurring oxacillin-susceptible mecA-positive S. aureus isolate to identify the mechanism conferring oxacillin susceptibility.

Methods: The mecA-positive oxacillin-susceptible S. aureus isolate GR2 (penicillin and oxacillin MICs 0.094 and 1 mg/L, respectively), belonging to clonal complex 80, was characterized. DNA fragment libraries were sequenced on Roche 454 and Illumina MiSeq sequencers and de novo assembly of the genome was generated using SeqMan NGen software. Plasmid curing was conducted by SDS treatment. Expression of mecA was quantified without/with β-lactam pressure.

Results: The genome of GR2 consisted of a 2 792 802 bp chromosome and plasmids pGR2A (28 889 bp) and pGR2B (2473 bp). GR2 carried SCCmec type IV, with a truncated/non-functional mecR1 gene and no mecI. A single copy of the bla system, with an organization unique for S. aureus, was found, harboured by plasmid pGR2A. Particularly, the blaZ gene was orientated like its regulatory genes, blaI and blalR1, and a gene encoding transposase IS666 was integrated between blaZ and the regulatory genes deleting the 5′-end of blaL; blal, encoding blaZ/mecA repressor, was intact. After plasmid loss, GR2 became penicillin and oxacillin resistant (MICs 0.5 and 6 mg/L, respectively).

Conclusions: We can conclude that after exposure to β-lactams, the non-functional BlaR1 does not cleave the mecA repressor Blal, derepression does not occur and mecA is not efficiently expressed. Removal of the bla system after curing of pGR2A allows constitutive expression of mecA, resulting in oxacillin and penicillin resistance.

Introduction

MRSA is a common pathogen causing in the community mostly skin and soft tissue infections and in medical facilities also severe infections, such as bacteremias, pneumonias and surgical site infections.2 Staphylococcus aureus has developed resistance to methicillin and other β-lactam antibiotics by acquiring the mecA or mecC gene, which encode a modified PBP2a. The mecA and mecC genes are carried on the staphylococcal cassette chromosome (SCC) mec element. mecA- or mecC-positive S. aureus isolates are methicillin resistant but may also exhibit oxacillin MICs in the susceptible range (≤2 mg/L).3 Most mecA-positive S. aureus isolates exhibit a degree of oxacillin heteroresistance. Such isolates can be improperly diagnosed and the use of β-lactams might lead to treatment failure.3 However, truly oxacillin-susceptible mecA-positive S. aureus isolates, which do not exhibit oxacillin heteroresistance, have also appeared.4 Recently, the emergence of oxacillin-susceptible mecA-positive isolates has been reported worldwide.4–8 These isolates pose a diagnostic challenge for routine clinical microbiology laboratories and for clinicians treating infections due to oxacillin-susceptible mecA-positive S. aureus.

There are genetically diverse MRSA strains in which mecA gene expression is modulated by a DNA-binding protein, MecI, and a signal transducer, MecR1. The corresponding genes mecI-mecR1 are organized in an operon and located upstream of mecA in the SCCmec element.9 In the absence of β-lactam antibiotics, a homodimeric methicillin repressor, MecI, represses the transcription of mecA and mecI-mecR1 by binding to two palindromes located within the promoter/operator region.10 Derepression occurs when an extracellular sensor domain of MecR1, which is a membrane protein, binds with β-lactam antibiotics. The structure of MecR1 is altered, leading to activation of the metalloprotease
domain of the protein, which faces the cytosol and in turn, directly or indirectly, causes cleavage of MecI.13 This cleavage inactivates the repressor and releases it from its DNA-binding site, leading to PBP2a synthesis.

Expression of the mecA gene can be additionally regulated by the bla system, which is homologous to the mec system. The blaI-blaR1 genes primarily regulate the synthesis of a β-lactamase (BlaZ), which confers resistance to penicillins in both MRSA and MSSA.12 The regulation of the bla system is similar to that of the mec system. The blaI- blaR1-blaZ genes are usually carried on a plasmid or located on a transposon. The operator regions of the mec and bla systems show substantial sequence similarity with each other, which allows mec-bl a cross-talk. Therefore, BlaI can bind to the operon region of mecA and repress its transcription. Repression is even stronger when both repressors, MecI and BlaI, are present together in a cell. 

The mecA gene can be additionally regulated by the mecI-MecR1 system. In the absence of functional mecI-mecR1, requiring minutes and several hours, respectively.13,14 In the absence of functional mecI-mecR1 genes, mecA gene expression is regulated only by the blaI-blaR1 system.

There are many other genetic factors unrelated to the mec and bla systems that influence methicillin resistance, are located on chromosome or plasmids and are probably pleiotropic.15 However, it seems that the mecI-mecR1 and bl aI-blaR1 genes play the critical role in regulating the phenotypic expression of methicillin resistance. We examined by WGS the nucleotide sequence of the genome of a truly oxacillin-susceptible mecA-positive S. aureus isolate of MLST clonal complex 80 (CC80). The aim of this study was to elucidate the mechanism underlying the oxacillin susceptibility of a naturally occurring mecA-positive S. aureus isolate.

Materials and methods

Bacterial isolates
A truly oxacillin-susceptible (oxacillin MIC 1 mg/L, no oxacillin heteroresistance) mecA-positive isolate from our laboratory collection, designated GR2, was tested in this study. The GR2 isolate was characterized by spa type T044 and belonged to MLST ST728, which is a single-locus variant of ST80, belonging to CC80, a European community-associated MRSA clone.

Phenotypic testing of mecA-positive isolates
The mecA-positive study isolate was tested for penicillin, oxacillin and fusidic acid MICs by broth microdilution according to CLSI recommendations and interpretative criteria16 and also by Etest (bioMérieux, Marcy-l’Etoile, France). The CLSI-recommended reference strain S. aureus ATCC 29213 was used as control with every set of tests. The Slide MRSA latex agglutination test (bioMérieux) was performed to detect PBP2a (Table S1, available as Supplementary data at JAC Online).

WGS
Genomic DNA was extracted using the DNeasy Blood and Tissue Kit (Qiagen) and purified DNA was quantified with a Qubit 2.0 Fluorometer (Life Technologies). A next-generation sequencing approach was undertaken commercially by Eurofins MWG Operon (Ebersberg, Germany) using a Roche 454 GS FLX+ system and non-cloned shotgun DNA libraries and also non-commercially on an Illumina MiSeq system with DNA fragment libraries prepared using a Nextera XT kit (Illumina) according to the manufacturer’s protocol.

Data analysis
Raw sff files (Roche 454 GS FLX+ system) with read length 700–1200 bases or FASTQ files (Illumina MiSeq) with read length 300 bases were de novo assembled into contigs using SeqMan NGen software. The resulting contigs were ordered by Mauve Contig Mover and alignment of contigs end to end to find overlap between adjoining contigs was achieved using SeqMan software. Sequencing yielded an average genome coverage of 50x or 133x using Roche 454 or Illumina MiSeq, respectively. The remaining gaps between contigs were closed by PCR amplification and Sanger sequencing. Automated genome annotation was performed using the RAST annotation server (http://rast.nmpdr.org/). Manual sequence editing was conducted using SeqBuilder software (version 10.1.2; DNASTAR). The DNA sequences were aligned using Mafft (version 10.1.2; DNASTAR) and BLASTn software. SNP analysis was performed using the CSI Phylogeny 1.0a server.17 SNPs were excluded when the distance between SNPs was <10 bp. Identification of acquired antimicrobial resistance genes was conducted by the ResFinder 2.1 server.18

Plasmid curing
Plasmid curing was done by treating the isolates with 0.002% SDS as described previously.19 Loss of plasmids was verified by WGS.

RNA isolation and gene expression analysis using reverse transcription and quantitative real-time PCR (RT-qPCR)
For RNA isolation, colonies from overnight blood agar cultures were used to inoculate Mueller–Hinton broth (MHB) with or without a β-lactam antibiotic at an OD560 of 0.05. Oxacillin and penicillin were added to MHB at concentrations of 0.25 and 0.5 mg/L and 0.0235 and 0.047 mg/L, respectively. Samples containing 5×10⁸ cells were taken from the exponential (3.5 h) and stationary (12 h) growth phases. To prevent degradation of RNA, one volume of bacterial culture was mixed with two volumes of RNAProtect Bacteria Reagent (Qiagen) and then incubated for 5 min at room temperature. Subsequently, the cell wall was mechanically disrupted using a TissueLyser (Qiagen) and glass beads (Sigma – Aldrich, Zwijndrecht, The Netherlands). To quantify expression of the mecA gene, total RNA was isolated using the RNeasy Mini Kit (Qiagen) and an on-column DNase treatment (RNase-Free DNase Set; Qiagen) according to the manufacturer’s recommended protocols. The purified RNA was quantified with a Qubit 2.0 Fluorometer (Life Technologies, Carlsbad, CA, USA) using a broad-range RNA quantification kit (Life Technologies). The RNA quality was measured by a RNA ScreenTape kit (Agilent Technologies, Palo Alto, CA, USA) in a 2200 TapeStation system (Agilent Technologies). Only samples with preserved 16S and 23S peaks and RNA integrity number values >7 were selected for gene expression analyses. For RT-qPCR analyses, an iTag™ Universal SYBR® Green One-Step Kit (Bio-Rad Laboratories) and 20 ng of template RNA were used. The mecA and gryB genes were detected using their respective specific primers as described before.20 A CFX96™ Touch Real-Time PCR detection system (Bio-Rad Laboratories) was used for the measurements. Fold changes in the expression levels of mecA were calculated in relation to the levels of gryB using the software CFX Manager version 3.1 (Bio-Rad Laboratories).

Nucleotide sequence accession numbers
The sequences of the chromosome of S. aureus GR2 and its two plasmids pGR2A and pGR2B have been deposited in GenBank under accession numbers CP010402, CP010403 and CP010404, respectively.
**Results**

**Genome features of the oxacillin-susceptible mecA-positive GR2 isolate**

The genome of the oxacillin-susceptible mecA-positive isolate designated GR2 consisted of a 2792802 bp chromosome and two plasmids, pGR2A and pGR2B, sized 28895 and 2473 bp, respectively. The chromosome showed a GC content of 32.9% and contained 2577 coding sequences and 74 RNAs. Plasmid pGR2A had a GC content of 29.6% and possessed 28 coding sequences, while plasmid pGR2B revealed a GC content of 30.8% and harboured 2 coding sequences.

**Comparison of the GR2 and 11819-97 genomes**

The genome sequence of GR2 was compared with the sequence of an oxacillin-resistant mecA-positive S. aureus isolate, designated 11819-97 (ST80-IV), that was determined previously and composed of 2846546 bp (GenBank accession number CP003194). Based on the CSI Phylogeny server, 2795582 positions were found in both analysed chromosomes, which reflected 98.21% of the 11819-97 chromosome covered by the two isolates. The SNP calling analysis revealed 145 SNPs within the core genome between the two isolates.

In comparison with isolate 11819-97, no additional genes were detected in the chromosome of isolate GR2 with the exception of SCCmec. Both isolates carried SCCmec type IV, which differed between them in the J2 region (Figure 1). This was caused by the integration of partial plasmid p18810-P03 into the SCCmec of isolate 11819-97. During the integration of plasmid p18810-P03, isolate 11819-97 acquired additional 18 genes including a copy of the bla system (Figure 1). Integration of plasmid p18810-P03 also caused deletion of five genes. Moreover, isolate GR2 did not possess an S. aureus φ37-like prophage, which had been reported in the chromosome of isolate 11819-97.

The GR2 and 11819-97 isolates differed also from each other by the plasmid content. A GenBank search using the BLASTn program showed that the nucleotide sequence of plasmid pGR2A was most similar to that of the 22317 bp plasmid of 11819-97 (GenBank accession number CP003193). The 5′-part of plasmid pGR2A of size 23050 bp was highly similar (99.9% identity) to almost the entire sequence of plasmid p11819-97 with the exception of a fragment encompassing the blaZ gene region (Figure 2).

Moreover, sequence analysis revealed an integrated 4896 bp plasmid pT49 (GenBank accession number KM281803) within the sequence of plasmid pGR2A (Figure 2).

Plasmid pGR2B did not have any counterpart in isolate 11819-97 and showed the highest nucleotide similarity to S. aureus plasmid pH19 (99.8% identity; GenBank accession number EU350089). Plasmid pGR2B in isolate GR2 harboured the ermA(C) gene conferring macrolide resistance.

![Figure 1. Comparison of the SCCmec region from the GR2 isolate (GenBank accession number CP010402) and the 11819-97 isolate (GenBank accession number NC_017351). The arrows represent the genes: green, common to both isolates; red, specific to the 11819-97 isolate; and blue, specific to the GR2 isolate. Only the following selected genes are annotated: the SCCmec cassette recombinases (ccrB2 and ccrA2); the determinant encoding resistance to methicillin (mecA) and its regulatory gene (mecR1); the determinant encoding resistance to penicillin (blaZ) and its regulatory genes (blaI and blaR1); and the transposases of IS431 and IS1272. The pairs of direct repeats (DRs) and inverted repeats (IRs) are present at the termini of SCCmec. The 116 bp inverted terminal repeats (TIRL and TIRR) of the transposon designated Tn5404 are represented by the vertical bars.](image-url)
Predicted antibiotic resistance based on the presence of acquired antimicrobial resistance genes

All detected resistance genes in the 11819-97 and GR2 genomes are summarized in Table 1. Both genomes possessed resistance genes that should have conferred phenotypic resistance to aminoglycosides, β-lactams, fusidic acid, macrolides and tetracyclines. In the case of macrolide resistance, isolate GR2 possessed the \( \text{erm(C)} \) gene located on plasmid pGR2B, while in isolate 11819-97 the resistance was conferred by the \( \text{mph(C)} \) and \( \text{msr(A)} \) genes harboured by plasmid p18810-P03 integrated into the SCC\( m\text{ec} \) region (Figure 1).

Sequence analysis of the mec gene complex

SCC\( m\text{ec} \) type IV possesses the class B \( m\text{ec} \) gene complex, which does not contain the \( m\text{ec1} \) gene and its \( m\text{ecR1} \) gene is truncated and non-functional. The 8114 bp \( m\text{ec} \) gene complex was almost identical in the GR2 and 11819-97 isolates. Only one SNP was found in this complex. Notably, this SNP was located in the \( m\text{ecA} \) gene and led to an amino acid change from isoleucine to threonine at position 314 (I314T) in PBP2a, which resides in the non-penicillin-binding domain and probably does not affect PBP2a function. BLAST searches in the GenBank database showed that the PBP2a amino acid sequence from the oxacillin-resistant \( m\text{ecA} \)-positive isolate 11819-97 (with the I314 position) was identical to those from other oxacillin-resistant \( S.\text{aureus} \) isolates. Using the PBP2a amino acid sequence from the oxacillin-susceptible \( m\text{ecA} \)-positive isolate GR2 (with the T314 position), BLAST analysis did not yield any identical amino acid sequence in the GenBank database.

<table>
<thead>
<tr>
<th>Predicted phenotype</th>
<th>Resistance gene</th>
<th>11819-97 genome location (position)</th>
<th>GR2 genome location (position)</th>
</tr>
</thead>
<tbody>
<tr>
<td>β-Lactam resistance</td>
<td>( m\text{ecA} )</td>
<td>chromosome (39686...41692)</td>
<td>chromosome (39686...41692)</td>
</tr>
<tr>
<td>Penicillin resistance</td>
<td>( \text{blaZ} )</td>
<td>chromosome (51659...52504)</td>
<td></td>
</tr>
<tr>
<td>Macrolide resistance</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Macrolide, lincosamide and streptogramin B resistance</td>
<td>( \text{mph(C)} )</td>
<td>chromosome (53170...54069)</td>
<td></td>
</tr>
<tr>
<td>Aminoglycoside resistance</td>
<td>( \text{ant(6)-Ia} )</td>
<td>chromosome (65794...66430)</td>
<td></td>
</tr>
<tr>
<td>Aminoglycoside resistance</td>
<td>( \text{aph(2')-III} )</td>
<td>chromosome (67050...67844)</td>
<td></td>
</tr>
<tr>
<td>Tetracycline resistance</td>
<td>( \text{tet(38)} )</td>
<td>chromosome (144850...146202)</td>
<td></td>
</tr>
<tr>
<td>Fusidic acid resistance</td>
<td>( \text{fusB} )</td>
<td>plasmid p11819-97 (1336...1977)</td>
<td>plasmid pGR2A (26021...27400)</td>
</tr>
<tr>
<td>Penicillin resistance</td>
<td>( \text{blaZ} )</td>
<td>plasmid p11819-97 (3371...4216)</td>
<td>plasmid pGR2A (3489...4334)</td>
</tr>
<tr>
<td>Tetracycline resistance</td>
<td>( \text{tet(K)} )</td>
<td>plasmid pT49 (216...1595)</td>
<td>plasmid pGR2B (1680...2414)</td>
</tr>
<tr>
<td>Macrolide resistance</td>
<td>( \text{erm(C)} )</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Figure 2. Structure comparison of plasmids: (a) pGR2A present in the GR2 isolate (GenBank accession number CP010403) and (b) p11819-97 (GenBank accession number NC_017350) and pT49 (GenBank accession number KM281803) present in the 11819-97 isolate. The arrows represent the genes: green, common to both isolates and possessing the same orientation; red, common to both isolates, but possessing opposite orientation; and black, specific to the GR2 isolate. Only the following selected genes are annotated: the determinant encoding resistance to penicillin (\( \text{blaZ} \)) and its regulatory genes (\( \text{blaI} \) and \( \text{blaR1} \)); the determinant encoding resistance to fusidic acid (\( \text{fusB} \)); the determinant encoding resistance to tetracycline (\( \text{tet(K)} \)); and the transposase of IS66.
blaR1. Organization of the bla system such as that in plasmid pGR2A is unique in S. aureus. The blaZ gene inversion and multiplication of the transposase gene resulted in loss of the original promoter/operator region of blaR1 in plasmid pGR2A (Figure 3). Moreover, the 5’-end of the blaR1 gene was deleted. These data overall indicated that non-functional N-terminally truncated BlaR1 sensor/transducer could be produced by the oxacillin-susceptible mecA-positive isolate. We checked if the inversion of blaZ also included its promoter/operator region. In Figure 4, for comparison purposes, the blaZ promoter/operator region from plasmid pGR2A is inverted. In the inverted region of plasmid pGR2A, the blaZ gene was preceded by a perfectly conserved sequence of the blaZ promoter/operator region, including ribosome-binding site sequences, promoter sequences and palindromic sequences to which the BlaI repressor binds. The nucleotide sequences of the blaZ and blaI genes were identical to corresponding sequences of the p11819-97 plasmid. Despite plasmid pGR2A possessing the intact sequence of the blaZ gene, which confers resistance to penicillin, the GR2 isolate showed a very low penicillin MIC (0.094 mg/L) within the susceptible range.

**Sequence analysis of other genes involved in the expression of methicillin resistance**

The sequences of genes that had been identified to impact the expression of methicillin resistance were compared between isolates GR2 and 11819-97. The following genes were analysed: murE (MS7_0974, locus in the 11819-97 genome), murF (MS7_2098), femA (MS7_1331), femB (MS7_1332), femX (MS7_2282), hmrA (MS7_2151), glmM (MS7_2176), ppb2 (MS7_1406), ppb4 (MS7_0693), fntA (MS7_1014), dacA (MS7_2178), sigB (MS7_2081), spoVG (MS7_0475), sarA (MS7_0669), xdrA (MS7_1852), ccpA (MS7_1742), secDF (MS7_1652), vraR (MS7_1919), vraS (MS7_
1920), agrA (MS7_2050) and agrB (MS7_2048). Identical sequences of these genes were shared between the two isolates.

Phenotypic results before and after plasmid curing
We grew the GR2 isolate in the presence of a plasmid curing agent, which resulted in loss of the two plasmids, as confirmed by WGS analysis. Isolate GR2, which before the experiment was oxacillin and penicillin susceptible (MICs 1 and 0.094 mg/L, respectively) and fusidic acid resistant (MIC 12 mg/L), was shown to be oxacillin and penicillin resistant (MICs 6 and 0.5 mg/L, respectively) and fusidic acid susceptible (MIC 0.25 mg/L) after plasmid curing.

Expression of the mecA gene
Gene expression analysis revealed that under the same growth conditions, mecA expression levels in the GR2 isolate with plasmids were always lower than in GR2 without plasmids (Figure 5). The smallest difference in expression of mecA (1.5-fold) was observed between GR2 with or without plasmids during the stationary growth phase in MHB without antibiotic (Figure 5b and d), while the greatest difference (13.8-fold) was detected during the exponential growth phase in MHB supplemented with penicillin at 0.047 mg/L (Figure 5c). The differences were higher during the exponential growth phase than during the stationary growth phase. Moreover, addition of β-lactam antibiotics at increasing concentrations amplified the expression of mecA during both exponential and stationary growth phases. In GR2 with plasmids, compared with MHB alone, addition of oxacillin at 0.5 mg/L had only a minor effect, increasing expression of the mecA gene by 1.4- and 1.5-fold during exponential (Figure 5a) and stationary (Figure 5b) growth phases, respectively; whereas in GR2 without plasmids the expression of mecA increased by 5.8- and 4.7-fold, respectively. Addition of penicillin at 0.047 mg/L substantially decreased mecA gene expression in GR2 with plasmids by 7.1- and 4.2-fold during exponential (Figure 5c) and stationary (Figure 5d) growth phases, respectively; whereas, in GR2 without plasmids only a slight decrease by 1.2-fold in the mecA expression

Figure 4. Organization of upstream regions of the blaZ gene in (a) plasmid pGR2A and (b) plasmid p11819-97. For comparison purposes, the upstream region of the blaZ gene from plasmid pGR2A is inverted. The nucleotide sequences that are common to both plasmids are indicated by the green colour. –35 and –10 represent the conserved promoter regions. The bla operator consists of the R1 dyad and Z dyad, the sites to which BlaI binds. Only the following selected genes are annotated: the determinant encoding resistance to penicillin (blaZ) and its regulatory genes (blaI and blaR1); the determinant encoding resistance to fusidic acid (fusB); the determinant encoding resistance to tetracycline (tetK); and the transposase of IS66.
amount was observed during the exponential growth phase, and expression of mecA remained almost steady during the stationary growth phase.

Discussion

During the last decade, numerous studies reporting on the detection of oxacillin-susceptible mecA gene-carrying S. aureus isolates have appeared in the literature, coming from geographically distant countries all over the world, including Europe (Germany, the UK and Greece), Asia (India, Taiwan and Japan) and the Americas (the USA and Argentina). These growing reports suggest that oxacillin-susceptible mecA-positive S. aureus isolates possibly exist everywhere and may indeed be quite common. Given the millions of clinical MRSA infections, the therapeutic implications of the phenotypic oxacillin susceptibility of mecA-positive S. aureus clinical isolates, irrespective of their actual incidence, are evident and require significant attention.

Many of the studies that reported on oxacillin-susceptible mecA-positive S. aureus isolates concluded that such isolates may be misidentified by routine clinical laboratories. In that respect, early and accurate detection of oxacillin resistance is a priority for successful treatment of infections caused by S. aureus. It can be achieved by conventional phenotypic testing, including the use of chromogenic media, antimicrobial susceptibility testing, detection of PBP2a by latex agglutination tests and by using molecular detection of the mecA gene. In addition, the use of direct molecular detection methods targeting the mecA gene alone, without using concomitant culture-based confirmation, would misidentify false positively these strains as MRSA. Culture-based identification is therefore always recommended in parallel to molecular direct detection methods. However, using phenotypic determination of methicillin resistance alone, oxacillin-susceptible mecA-positive S. aureus can escape routine diagnostic laboratory tests. This highlights the need to implement molecular testing into routine diagnostics, in order to detect mecA/mecC genes and avoid misidentifying oxacillin-susceptible mecA-positive S. aureus, and subsequently mistreating the respective infections.

The results of WGS analysis before and after plasmid curing clearly indicated that plasmid pGR2A with a fully functional BlaI repressor and non-functional BlaR1 sensor/transducer was responsible for the oxacillin susceptibility of the mecA-positive S. aureus isolate GR2. We can conclude that BlaI constitutively represses expression of mecA; however, in the presence of β-lactam antibiotics, derepression does not occur because there is no production of functional BlaR1 to cleave the promoter-bound BlaI. This explains the oxacillin- and penicillin-susceptible phenotype of the GR2 isolate, although resistance genes mecA and blaZ and their intact promoter/operator regions were present in the genomic DNA. When plasmid pGR2A was removed from isolate GR2 by curing, the regular constitutive expression of the mecA gene was allowed, apparently leading to the observed oxacillin MIC of 6 mg/L and penicillin MIC of 0.5 mg/L. Correspondingly, removal of the fusB gene, which was carried in pGR2A (Figure 2), by plasmid curing apparently caused conversion of the isolate from fusidic acid resistant to susceptible. It should be noted that both isolates, GR2 with plasmids and GR2 without plasmids (GR2-cured), were positive for PBP2a by the Slidex test (Table S1). Although this test is only qualitative, after serial dilution of the cell extracts we could detect enhanced production of PBP2a by GR2 without plasmids (data not shown). These observations overall
WGS for elucidating the mechanism of cryptic methicillin resistance

indicate that a baseline still detectable production of PBP2a might not suffice to confer oxacillin resistance or heteroresistance, as was observed in the clinical isolate GR2.

We were able to delete the plasmid pGR2A from the bacterial cells in vitro. Such plasmid loss can potentially happen in vivo during antibiotic therapy using β-lactams. All the above raise questions regarding the real prevalence of oxacillin-susceptible mecA-positive isolates, how many are undetected in routine diagnostics, their characteristics and risks for getting infections with these isolates.

As the studied isolate was MSSA phenotypically but would have been identified as MRSA based on the presence of the mecA gene, we propose to refer to these isolates in the future as oxacillin-susceptible mecA-positive S. aureus. Since very little is known about the biology of these isolates and their ability to regain full gene regulatory capacity in clinical settings, and since mechanistic evidence for mecA and PBP2a activity becomes available, we recommend that they should be regarded as MRSA isolates concerning clinical management and hygiene precautions. To tackle an uncontrolled spread of oxacillin-susceptible mecA-positive S. aureus isolates in hospitals as well as in the community, phenotypic tests (PBP2a detection) should be combined with genotypic methods (e.g. mecA gene PCR screening). Otherwise, conducting phenotypic tests alone could conceal truly mecA-positive S. aureus isolates whereas over-reliance on genotypic testing may deprive patients of potentially better treatment regimens.

We found a novel amino acid substitution (I314T) in the non-penicillin-binding domain of PBP2a. Amino acid exchanges in the non-penicillin-binding domain of the PBP2a protein have been previously described among MRSA isolates and did not affect methicillin resistance. The oxacillin-susceptible mecA-positive S. aureus isolate characterized in the current study had the blaZ gene, which should confer resistance to penicillin. The nucleotide sequences of the blaZ gene and its promoter/operator region of the GR2 isolate were identical to those from penicillin-resistant Staphylococcus isolates. Therefore, if the amino acid substitution in the PBP2a protein was responsible for oxacillin susceptibility and not the mutated bla system on plasmid pGR2A, the GR2 isolate should be resistant to penicillin. The penicillin and oxacillin resistance of the GR2 isolate after deleting the pGR2A plasmid and, thus, removing the bla operon provides evidence that the bla system with a truncated blaR1 gene is responsible for β-lactam susceptibility of the GR2 isolate.

To our knowledge, this is the first study elucidating a mechanism responsible for oxacillin susceptibility in a naturally occurring mecA-positive S. aureus clinical isolate. However, further studies are needed to identify additional genetic factors and mechanisms that could also contribute to the oxacillin susceptibility of such mecA-positive S. aureus isolates. Finally, many parameters still remain unknown regarding the dissemination, the virulence mechanisms, the clinical challenges and the appropriate antibiotic treatment approaches.

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Transparency declarations
None to declare.

Supplementary data
Table S1 is available as Supplementary data at JAC Online (http://jac.oxfordjournals.org/).

References