

Mechanisms of Methicillin Resistance in *Staphylococcus aureus*

Sharon J. Peacock¹ and Gavin K. Paterson²

¹Department of Medicine, University of Cambridge, Cambridge CB2 0QQ, United Kingdom; email: sjp97@medschl.cam.ac.uk

²School of Biological, Biomedical, and Environmental Sciences, University of Hull, Hull HU6 7RX, United Kingdom

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Abstract

Staphylococcus aureus is a major human and veterinary pathogen worldwide. Methicillin-resistant *S. aureus* (MRSA) poses a significant and enduring problem to the treatment of infection by such strains. Resistance is usually conferred by the acquisition of a nonnative gene encoding a penicillin-binding protein (PBP2a), with significantly lower affinity for β -lactams. This resistance allows cell-wall biosynthesis, the target of β -lactams, to continue even in the presence of typically inhibitory concentrations of antibiotic. PBP2a is encoded by the *mecA* gene, which is carried on a distinct mobile genetic element (SCC*mec*), the expression of which is controlled through a proteolytic signal transduction pathway comprising a sensor protein (MecR1) and a repressor (MecI). Many of the molecular and biochemical mechanisms underlying methicillin resistance in *S. aureus* have been elucidated, including regulatory events and the structure of key proteins. Here we review recent advances in this area.

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THE EMERGENCE OF METHICILLIN-RESISTANT *Staphylococcus aureus*

Staphylococcus aureus can cause a wide range of infections in humans. The most common sites affected are skin and soft tissue; manifestations of infections in these sites include folliculitis, furuncles and carbuncles, impetigo, mastitis, wound infections, and staphylococcal scalded skin syndrome. More serious infections include bacteremia, pneumonia, endocarditis, bone and joint infections, and toxic shock syndrome. *S. aureus* can also be responsible for outbreaks of food poisoning. The versatility of *S. aureus* as a pathogen also extends to its host range, which includes domestic cats and dogs, horses, goats, sheep, cattle, rabbits, pigs, and poultry. Various infections have been reported in these species, but the most economically significant are mastitis in dairy cattle and other ruminants, lethal systemic infections in farmed rabbits, and bumblefoot (ulcerative pododermatitis) in poultry.

The mortality of patients with *S. aureus* bacteremia in the preantibiotic era exceeded 80% (1). The introduction of penicillin G in the early 1940s dramatically improved prognosis, but resistant strains were recognized as soon as 1942 (2). Their mechanism of resistance was a penicillinase/ β -lactamase enzyme that hydrolyzed the β -lactam ring and inactivated the drug (3, 4). This enzyme is encoded by *blaZ*, which typically resides on a large transposon on a plasmid. The rate of resistance to penicillin is now greater than 90% in human *S. aureus* isolates, rendering the use of penicillin essentially useless to treat these infections.

A semisynthetic penicillinase-resistant β -lactam named methicillin was developed in response to the emergence and spread of penicillin resistance. Methicillin (marketed as Celbenin) was introduced into the clinic in 1959, rapidly followed by the first report of resistant isolates cultured between July and October 1960 from three individuals in the same hospital in Guildford near London, United Kingdom (5). The isolates had the same phage type and included isolates taken on the same day from a patient and a nurse on the same ward. This discovery forewarned the subsequent emergence of methicillin-resistant *S. aureus* (MRSA) as a nosocomial pathogen. None of the three individuals had received methicillin. Another English report of two methicillin-resistant isolates a year later (6) was followed by the geographical spread of MRSA in Britain and increasing recognition in hospitals (7, 8). Elsewhere, MRSA emerged throughout the 1960s in many countries, including those where methicillin was not available, and it is now ubiquitous worldwide (9–12). In the United States alone, MRSA was estimated to cause more than 80,000

invasive infections in 2011 (13), and up to 53 million people globally may be colonized with MRSA and therefore pose a risk of infection to themselves and others (11). The morbidity and mortality caused by MRSA result in significant economic and societal costs (14, 15).

Although methicillin is no longer used clinically or even produced commercially, the term MRSA has persisted. Furthermore, the term methicillin resistance manifests as resistance to virtually all β -lactams with the exception of the latest generation of cephalosporin β -lactams. MRSA can also acquire resistance to multiple alternative antimicrobials, further complicating treatment of infection (16, 17). This resistance encompasses vancomycin (18), considered one of the last treatment options for severe MRSA infections (19), and relatively new agents such as linezolid and daptomycin (20).

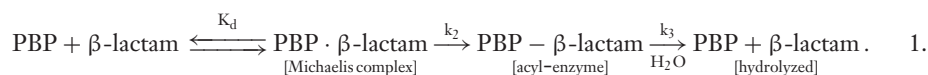
MECHANISM OF RESISTANCE IN METHICILLIN-RESISTANT *Staphylococcus aureus*

β -Lactam antimicrobial drugs target and inhibit bacterial cell-wall biosynthesis. Peptidoglycan is the main structural component of the cell wall, and it consists of glycan strands made of repeating *N*-acetylglucosamine and *N*-acetylmuramic acid disaccharides linked by peptide cross-links between *N*-acetylmuramic acid moieties on adjacent strands. Many of the steps in cell-wall biosynthesis are similar between diverse bacteria and have been reviewed elsewhere (21), including specifically in staphylococci (22). In brief, *N*-acetylglucosamine and *N*-acetylmuramic acid disaccharides are attached via a β -1,4-glycosidic bond to the reducing end of the growing peptidoglycan chain in a transglycosylation reaction. The newly incorporated repeating unit is cross-linked by a transpeptidation reaction to a stem peptide in an adjacent peptidoglycan strand. Both transglycosylation and transpeptidation are carried out by penicillin-binding proteins (PBPs); the latter reaction is the specific target of β -lactams. Transpeptidation is a two-step reaction beginning with active-site serine-mediated acylation of the position 4 D-Ala carbonyl in the stem peptide. Deacylation of the resultant intermediate follows via nucleophilic attack of a side chain in the amino group in the third position of the stem peptide on an adjacent peptidoglycan strand. The stem peptide composition varies between bacterial species, but is typically L-Ala- γ -D-Glu-diaminopimelate-D-Ala-D-Ala in gram-negative bacteria and L-Ala- γ -D-Glu-L-Lys-D-Ala-D-Ala in gram-positive bacteria such as staphylococci. In the case of *S. aureus*, cross-linking occurs between the position 4 D-Ala on one peptidoglycan strand and a pentaglycine extension attached to the position 3 L-Lys of the peptide stem of another (22).

Unlike the resistance of *S. aureus* to penicillin, methicillin resistance is not mediated by a plasmid-borne β -lactamase (23) and was referred to as intrinsic resistance in some of the early literature (23–26). Similar intrinsic resistance to β -lactams in other bacterial pathogens were found to be associated with alterations in PBPs, either in their amount or in their affinity for β -lactams, so attention turned to PBPs in MRSA as the likely mechanism. Alterations in PBPs specific to MRSA were observed, but it was unclear whether these related to overexpression and/or modification of existing PBPs or to the presence of a new PBP (26, 27). Hartman & Tomasz (28) resolved the major difference between isogenic methicillin-resistant and -susceptible strains by demonstrating the presence of a new PBP, termed PBP2a, with reduced affinity for β -lactams. This finding was confirmed shortly after by Utsui & Yokota (29) who referred to the new PBP as PBP2'. This protein is also referred to as MRSA PBP in the early literature (30). In contrast to β -lactamase, the gene responsible for methicillin resistance (initially referred to as *mec*^r for methicillin resistance) was located on the chromosome (31), which was traced to a region of “foreign” DNA present in resistant strains but absent in susceptible strains (32). Cloning and expression of the responsible gene in *Escherichia coli* resulted in the heterologous expression of PBP2a (33). This event was

subsequently replicated in a *S. aureus* background (34), and Song et al. (30) sequenced the gene [later named *mecA* (35)] and showed that it encodes a PBP. Confirmation of the role of *mecA* in conferring methicillin resistance came from transfer and transposon mutagenesis experiments performed at the Tomasz laboratory (36, 37).

β -Lactams inhibit the transpeptidation step of cell-wall biosynthesis by acting as substrate analogs of the D-Ala-D-Ala peptidoglycan side chain upon which PBPs act (38). A long-lived covalent acyl-enzyme complex forms between the β -lactam and the nucleophilic serine of the PBP active site, which inhibits cell-wall transpeptidation. Deacylation of this complex, as occurs during normal turnover, is impeded because the region of the active site that accommodates the deacylating acceptor moiety or a potential hydrolyzing water molecule is occupied by the β -lactam ring structure. Regeneration of the PBP is so slow relative to cell division that the enzyme is effectively irreversibly inactivated. The consequential loss of cell-wall cross-linking leads to defective cross-wall formation during cell division, followed by cell death. The exact mechanisms leading to this cell death are poorly understood. A role in some bacterial species is observed for autolytic enzymes causing cell-wall degradation and lysis (39–42). However, mechanistic understanding is complicated by variable responses to the same β -lactam by different bacterial species, as well as variable responses within the same bacterial species to different β -lactams (39, 40). In the case of staphylococci, there is evidence that the high internal pressure causes cytoplasm leakage through the weakened peripheral wall, leading to death (22). Reaction 1 shows the interaction of PBPs with β -lactam antibiotics:



Resistance conferred by PBP2a manifests through both (a) a reduced rate of β -lactam-mediated enzyme acylation (k_2) compared with that of native PBPs and (b) an absence of high affinity for β -lactam in the first instance (K_d). The second-order rate constant k_2/K_d is taken as a measure of the inhibitory potential of β -lactams against PBP. For PBP2a, this rate constant varies between different β -lactams and experimental approaches, but ranges from 1 to 19 M/s (43–45) and is two to three orders of magnitude lower than that observed for β -lactam-susceptible PBPs, including native PBP2 from *S. aureus* (46). Once acylated, PBP2a undergoes deacylation at a slow rate, comparable to β -lactam-susceptible PBPs (44, 45, 47), with a half-life value for the acyl-enzyme of up to 77 h (47).

Crystal structures of PBP2a from MRSA have been resolved, providing the structural basis for resistance. Overall, PBP2a forms an elongated protein with a transpeptidase domain (residues 327–668) and what was referred to in the first crystal structure report as a non-penicillin-binding domain (residues 27–138) (48), which contains an allosteric site (**Figure 1**) (49). The full-length protein also possesses a transmembrane (TM) domain (residues 1–23), which is typically removed to produce a soluble protein that is amenable to study. Significantly, with regard to resistance, the active-site serine of PBP2a is less accessible to β -lactams than with susceptible PBPs because of its location in a narrow extended cleft. The inaccessibility of the active site means that the slow deacylation of the inactivated acyl-enzyme intermediate that is displayed by PBP2a and shared with β -lactam-susceptible PBPs does not affect the clinical resistance of MRSA, given that this intermediate does not form at the concentrations of antibiotic reached in vivo. Furthermore, acylation appears to require conformational changes that make this reaction less favorable than in susceptible enzymes (48).

Another significant structural and functional feature of PBP2a that distinguishes it from other PBPs is that it is under allosteric control (49). Allostery was initially predicted from kinetic studies

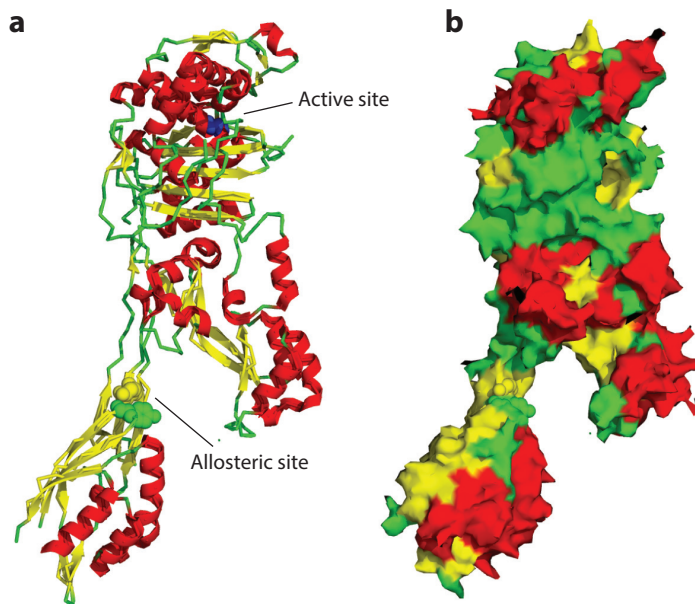


Figure 1

Structure of penicillin-binding protein 2a (PBP2a) from methicillin-resistant *Staphylococcus aureus* (MRSA). (a) View of a monomer of PBP2a. Active-site serine (position 403) is shown as blue spheres. The allosteric site is denoted by the highlighting of two of the residues that are important in its interaction with ceftaroline: Y105 (green spheres) and Y297 (yellow spheres) (49). (b) Surface topology view of the same molecule. Both structures are colored according to secondary structure: helix, red; sheet, yellow; loop, green. Figure generated from Protein Data Bank code 1VQQ using PYMOL (<http://www.pymol.org/>).

showing an increased rate of PBP2a acylation in the presence of synthetic fragments of peptidoglycan (47). Allosteric control of PBP2a was subsequently confirmed by structural analysis, and the allosteric site was identified within the non-penicillin-binding domain (49). Binding of nascent peptidoglycan at this allosteric site, 60 Å removed from the active site, stimulates conformational changes through a series of salt-bridge interactions that open the active site to facilitate substrate access (49). Binding of peptidoglycan at the allosteric site appears to occur through recognition of the D-Ala–D-Ala terminus of the pentapeptide stem and, thus, appears to open the active site for transpeptidation only in the presence of the nascent peptidoglycan substrate (49).

SCC mec

Sequencing of the region containing *mecA* revealed a distinct mobile genetic element named the staphylococcal chromosome cassette (SCC mec) that is present in MRSA but absent in methicillin-susceptible *S. aureus* (MSSA) (50). SCC mec elements are highly diverse, with 11 types (I to XI) recognized to date (51). Despite their diversity in size (from ~21 kb to 67 kb) and gene content, they all share important defining characteristics. In all cases, SCC mec is integrated into the *S. aureus* genome at an *attB* integration site sequence present at the 3' end of the *orfX* gene. Despite the significance of *orfX* as the site of SCC mec integration, its function was unresolved for many years until an analysis of its crystal structure revealed structural homology to ribosomal methyltransferase of the RlmH type (52). Insertion of SCC mec into *orfX* does not alter *orfX*

expression because the terminal amino acids and stop codon at the insertion site are unchanged, even though the DNA sequence is slightly altered (52). The second feature shared among the SCC*mec* elements is that they contain a *mec* gene complex comprising *mecA* and its regulatory genes *mecI* and *mecR* (although *mecI* and *mecR* are not intact in some SCC*mec* classes), a cassette chromosome recombinase (*ccr*) gene complex containing one or two site-specific recombinase genes responsible for movement of the SCC*mec*, and typically three J regions. Originally designated junkyard regions due to the presence of pseudogenes and truncated copies of transposons and insertion sequences, these J regions are now commonly referred to as joining regions because they can encode important functions such as resistance to additional antibiotics and to heavy metals. The third shared feature of SCC*mec* elements is their demarcation by specific inverted repeats and direct repeats containing the insertion site sequence recognized by the *ccr*-encoded recombinases.

SCC*mec* typing is widely used for epidemiological surveillance of MRSA and classifies SCC*mec* elements on the basis of their combination of *mec* gene and *ccr* gene complexes, with further subtyping based on the J regions (53). Two distinct *ccr* complexes have been described to date. The first comprises *ccrA* and *ccrB*, and the second consists of a single *ccrC* gene. Sequence variation among *ccrA* and *ccrB* defines several allotypes (in which nucleotide identity of <50% defines a new gene and novel allotypes of *ccr* genes are designated if their DNA sequence identities are between 50% and 84%, with an allele sharing $\geq 85\%$ nucleotide identity). The combination of *ccrA* and *ccrB* defines the *ccr* gene complex type, designated as type 1 (*ccrA1B1*), type 2 (*ccrA2B2*), type 3 (*ccrA3B3*), type 4 (*ccrA4B4*), type 7 (*ccrA1B6*), or type 8 (*ccrA1B3*). In contrast, reported *ccrC* variants are very similar, and only one allotype of *ccrC1* has been defined so far that constitutes *ccr* gene complex type 5. In addition to *mecA* and its regulatory genes, the *mec* gene complex includes associated insertion sequences; currently, five *mec* gene complex types are recognized largely on the basis of the presence and location of these insertion sequences. An International Working Group on the Classification of Staphylococcal Cassette Chromosome Elements (IWG-SCC) was established to provide consensus guidelines on this nomenclature (53; see http://www.sccmec.org/Pages/SCC_HomeEN.html). Various polymerase chain reaction (PCR)-based protocols that are currently used for SCC*mec* typing are likely to be superseded by whole-genome sequencing methods.

RECOMBINASES AND SCC*mec* MOBILITY

Excision and integration of SCC*mec* are mediated by the *ccr*-encoded recombinases located on SCC*mec*. They belong to the so-called large serine family of site-specific DNA recombinases, which include many bacteriophage integrases and transposases and are so named for their relatively large C-terminal domain and active-site serine nucleophile. During recombination, a recombinase tetramer synapses the two DNA substrates, followed by nucleophilic attack by the active-site serine of the recombinase to create double-strand breaks in the DNA (54). With each 5' DNA end covalently attached to a recombinase subunit, DNA strand exchange is thought to occur following 180° rotation of two subunits relative to the other two, so that subsequent religation through the reverse of the cleavage reaction results in a recombinant configuration (54). Integration of SCC*mec* occurs through the recombination of the *att*SCC site on a circular SCC*mec* and the *attB* site at the 3' end of *orfX*. This process results in the insertion of SCC*mec* into the chromosome flanked by new sites *attL* (within *orfX*) and *attR*. Excision is the reverse of these steps; it restores the chromosomal *attB* site and produces an excised circular SCC*mec* element with an intact *att*SCC site. Recognition and recombination of these sites by CcrA and CcrB have been demonstrated in a variety of in vitro and in vivo assays (55–57), and the overexpression of *ccrAB* in MRSA triggers the excision of SCC*mec* as a circular DNA molecule (58). Unlike other serine

recombinases, which do not act in concert, effective recombination and recognition of the widest panel of target sites require both CcrA and CcrB (55, 56). Differential binding to the target sites likely contributes to this cooperative activity (55), and yeast two-hybrid assays have shown that the two recombinases physically interact (56). Whereas CcrB can perform recombination of some of these sites alone, CcrA appears to have no or low recombinase activity. Nonetheless, the active-site serine residue of CcrA is still required when acting with CcrB, implying that it participates in the recombination reaction rather than simply contributing to substrate recognition (55). CcrA and CcrB are somewhat promiscuous with regard to substrate recognition, and they can act upon not only the canonical pairs of recombination sites described above but also several other non-canonical ones, such as *attSCC* and *attL*, and *attSCC* and *attR* (55). Such activity, again, differs from that of typical large serine recombinase family members and likely contributes to the gene plasticity of *SCCmec* elements. For example, the arginine catabolic mobile element (ACME), a virulence factor among the virulent USA300 clone, is integrated into the *SCCmec* at the *attR* site, whereas tandem arrangements of different *SCCmec* elements may have been generated by the promiscuous activity of CcrA and CcrB.

CcrA and CcrB, encoded by *SCCmec* types I to IV, can excise *SCCmec* elements belonging to each of these *SCCmec* types, showing that their recombinase activity is not specific for their associated *SCCmec* types (58). In contrast, CcrC appears to be specific to its cognate *SCCmec* type V (59), but there are few published data on how it mediates *SCCmec* excision and integration. Further understanding of the functioning of the Ccr recombinases may be important in explaining the host range of *SCCmec* among *S. aureus* lineages and other staphylococci, and may offer the opportunity for interventions or therapies based either on preventing the acquisition of *SCCmec* or on promoting its excision and the reversion of MRSA to MSSA.

Methicillin resistance can be transferred between *S. aureus* strains in the laboratory by transduction, albeit at low frequencies (32, 60, 61). The conditions required for transduction have recently been clarified using the bacteriophages 80 α and 29 to transduce *SCCmec* types IV and I into USA300 MSSA strains (62). Consistent with earlier findings, low frequencies of transduction were observed (10^{-9} to 10^{-10}), and a β -lactamase plasmid was required in the recipient strains (62). The basis for this requirement is unclear but is consistent both with the epidemiological association that most clinical strains of MRSA also produce β -lactamase (63) and with experimental work on the expression and stability of plasmid-expressed *mecA* (64). In the latter study, strains that had previously harbored *SCCmec* were able to maintain and express *mecA* along with high-level methicillin resistance, whereas *SCCmec*-naïve host strains expressed a lower level of *mecA* and resistance and selected for transformants with mutations in *mecA* that impeded resistance (64). The presence of a β -lactamase plasmid, and more specifically *blaR*, stabilized *mecA* integrity and the expression of resistance, an effect presumed to be related to the need to prevent deleterious overexpression of *mecA*.

Acquisition of *SCCmec* by *S. aureus* is largely restricted to a limited number of *S. aureus* lineages; multilocus sequence type clonal complexes (CCs) CC1, CC5, CC8, CC22, CC30, and CC45 predominate among MRSA isolates (65–67). The generation of resistance lineages from originally susceptible strains may occur through multiple independent transfers of resistance in different countries. In the case of CC5, at least 23 *SCCmec* acquisition events have been described (68), although it seems likely that the evolutionary history of *SCCmec* acquisition varies greatly between different lineages. The apparent ability of only a limited number of lineages to acquire *SCCmec* may be due to restriction-modification systems that block horizontal gene transfer into *S. aureus* in a lineage-specific manner (69), whereas sequence variation around the *attB* site may impede the integration of *SCCmec* into the chromosome of some strains (70). The effect of host strain background on the stability of *mecA* may also play a role in the distribution of *SCCmec* among

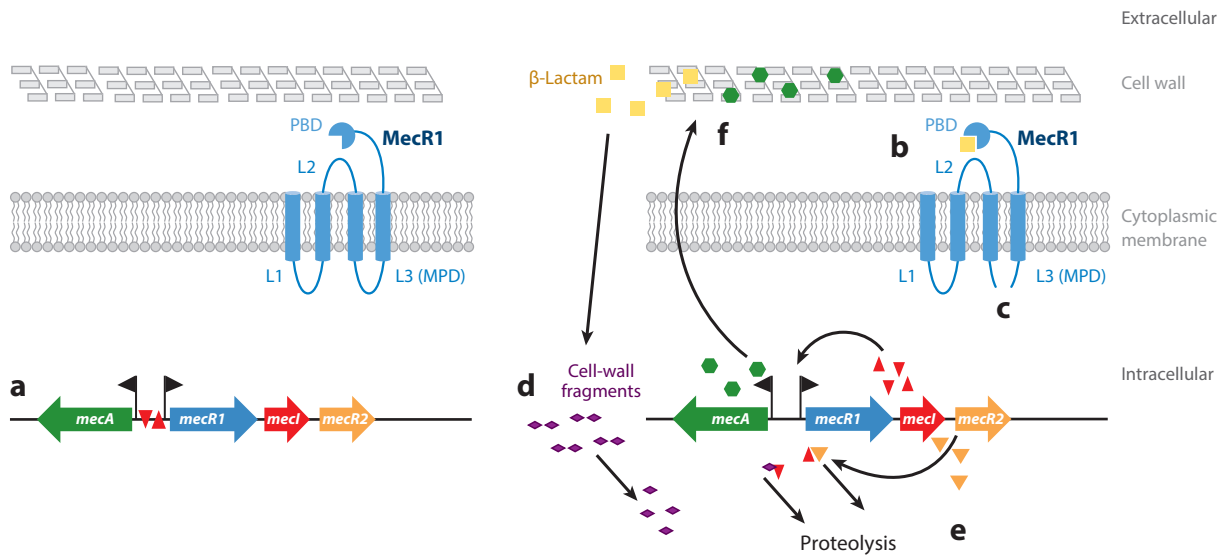


Figure 2

Model of the salient features of *mecA* regulation. (a) In the absence of β -lactams, transcription from the *mec* operator is prevented by the binding of the repressor MecI to this region. (b) β -Lactams are detected by their binding to the penicillin-binding domain (PBD) of MecR1. (c) This interaction triggers the autolytic activation of the intracellular metalloproteinase domain (MPD) of MecR1, located within L3. Whether the subsequent proteolysis of MecI is mediated directly by the activated MecR1 MPD is unclear. (d) Cytoplasmic cell-wall fragments, presumably generated by the disruption of cell-wall biosynthesis by β -lactams, act as a coactivator that binds MecI, disrupts its association with the *mec* operator, and promotes its proteolytic degradation. How these dipeptide cell-wall fragments, specifically γ -D-Glu-L-Lys, are generated in the cytoplasm is unclear but may involve the activated MPD of MecR1. (e) A second antirepressor, encoded by *mecR2* within the *mec* complex, is transcribed in the presence of β -lactams, and the resultant protein, MecR2, also binds to MecI, leading to its proteolysis. (f) The degradation of MecI causes *mecA* transcription, the production of PBP2a, and the expression of methicillin resistance.

S. aureus lineages (64) and might relate to the potential fitness costs associated with SCC*mec* (71, 72) and differences in the ability of lineages to mitigate them.

REGULATION OF METHICILLIN RESISTANCE

Transcription of *mecA* is induced in the presence of β -lactams by a signal transduction system encoded from the *mec* gene complex. This system is composed of an integral-membrane zinc-dependent sensor (MecR1) and a transcriptional repressor (MecI), which are located adjacent to *mecA* but are transcribed divergently from it (Figure 2).

The system is homologous to the *blaR1-blaI-blaZ* and *blaR-blaI-blaP* systems controlling β -lactamase expression in *S. aureus* and *Bacillus licheniformis*, respectively. Dimeric MecI represses transcription of *mecA* and *mecR1-mecI* by binding to a region of SCC*mec* comprising a 30-bp palindrome with 15 bp of dyad symmetry spanning the *mecA*-10 promoter sequence and the *mecR1*-35 sequence (73). BlaI and MecI are functionally interchangeable; each is able to bind and suppress transcription of the other's cognate promoter (73, 74). The signaling cascade leading to induction of methicillin resistance is largely inferred from studies on the *blaR1-blaI-blaZ* and *blaR-blaI-blaP* systems and involves proteolytically based signaling, in contrast to the more common signaling cascades in bacteria employing phosphorylation or methylation (75, 76). MecR1 is a TM metalloendopeptidase zymogen that detects the presence of β -lactams via its extracellular

penicillin-binding domain (PBD). Upon β -lactam-mediated acylation, a conformational change induces autocatalytic cleavage of the intracellular sensor domain, which in turn either directly or indirectly leads to cleavage of MecI. This process impedes MecI binding to the promoter region and induces *mecA* expression and methicillin resistance. The three-dimensional structure of MecI reveals a dimer consisting of two independent winged-helix domains and two dimerization domains that intertwine in a spiral-staircase architecture and are held together by a hydrophobic core (77, 78). Each of the winged-helix domains binds a palindromic DNA-operator half site (recognizing the consensus motif 5'-TACA/TGTA-3') in the *bla* and *mec* operators. DNA footprint studies show that a 45-bp length of DNA in the *mec* operator is protected by MecI (79). This observation agrees with structural studies showing MecI dimers binding to a 42-bp region of the *mec* operator containing four consensus motifs (78). This binding results in adjacent up and down MecI dimers binding to the *mec* operator (**Figure 3**) (78). Cleavage of MecI occurs within the dimerization domains, between Asn101 and Phe102, and is predicted to result in loss of the dimer interaction surface, dissociation, and repressor release, which trigger *mecA* transcription (77, 80, 81). Structural

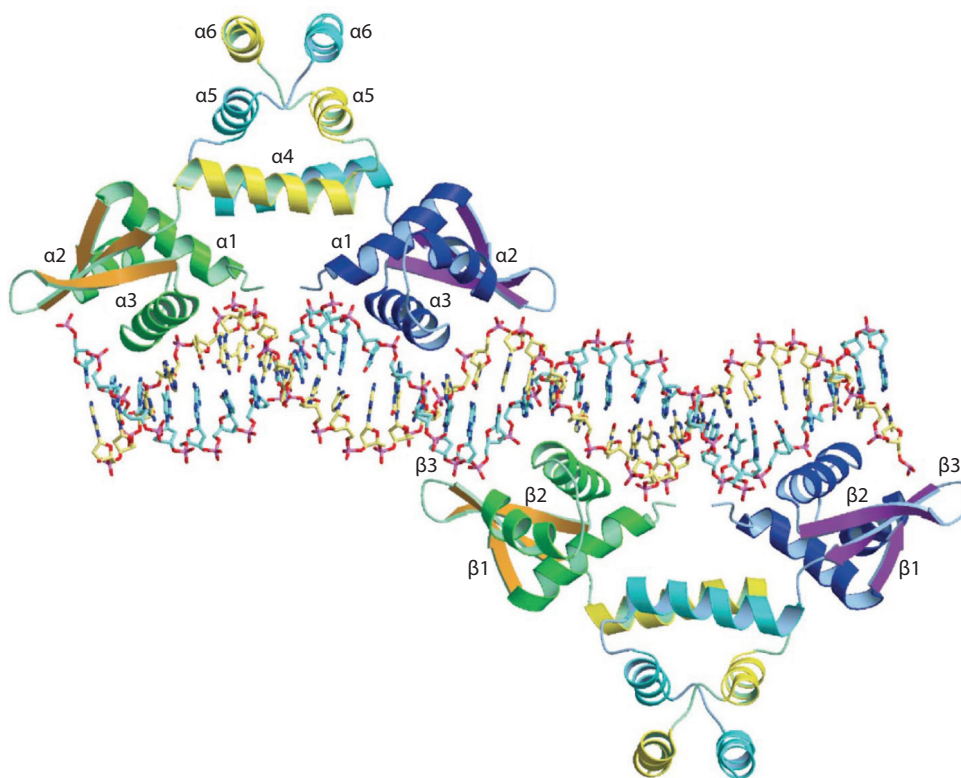


Figure 3

Structure of the MecI-DNA complex. Crystal structure of two MecI homodimers bound to the *mec* operator in a one-up-one-down pattern. The N-terminal domain comprises three α -helices ($\alpha 1$ -3) and three β -strands typical of winged-helix DNA-binding proteins. Protein-DNA interactions are determined largely by helix $\alpha 3$ (the so-called recognition helix). The scissile bond cleaved during lactam signaling, between Asn101 and Phe102, is located within helix $\alpha 5$, and its cleavage is proposed to destabilize dimerization and *mec* operator repression. Reproduced with permission from Reference 78 (<http://dx.doi.org/10.1107/S1744309106009742>).

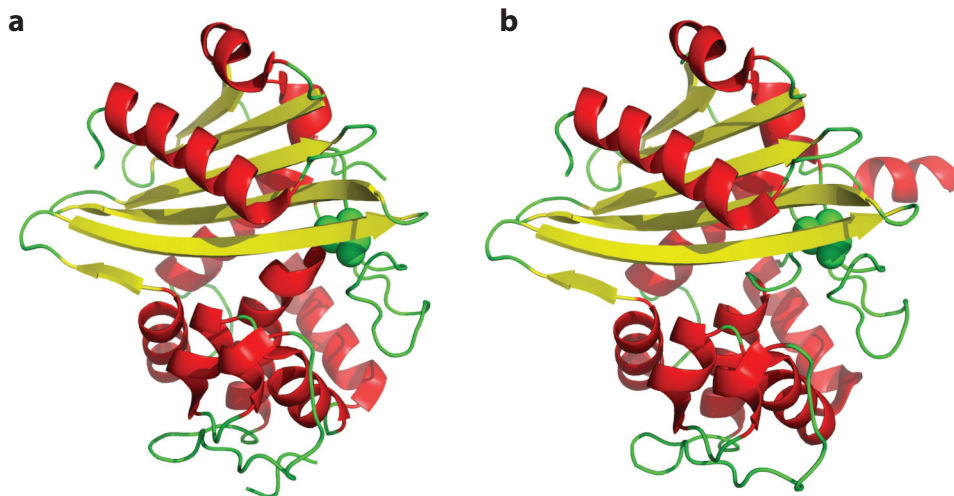


Figure 4

Structure and activation of MecR1. Crystal structure of the MecR1 penicillin-binding domain, both (a) unbound and (b) following acylation with penicillin. The colors correspond to secondary structure: helix, red; sheet, yellow; loop, green. The active-site serine is depicted as spheres. The two structures show that acylation with β -lactam has little impact on conformation, suggesting a role in signaling for other portions of MecR1. Figure generated from Protein Data Bank codes 2IWB and 2IWC using PYMOL (<http://www.pymol.org/>).

analyses of free and DNA-bound MecI and BlaI indicate that the C-terminal proteolytic cleavage site is more accessible when the repressors are bound to DNA than when they are in solution, suggesting that the proteolytic activation targets bound rather than free repressor (82). Whereas MecI and BlaI show cross-reaction in terms of repressor activity and the scissile bond in each is conserved, activation is specifically induced by their cognate signal transducer (73).

The crystal structure of the PBD of MecR1 reveals that it forms a two-domain structure of α/β -type fold, resembling the structure of PBPs and β -lactamases, with a catalytic serine residue as the site for β -lactam binding (**Figure 4**) (75). MecR1-PBD shares the same overall fold as the BlaR and BlaR1 proteins, with strong goodness-of-fit Z values of 35.3 to 37.8 (83). Analyses of covalent complexes with benzylpenicillin and oxacillin provide evidence that serine acylation of MecR1-PBD does not entail significant structural changes (**Figure 4**) (83). For instance, a comparison between the bound and unbound structures produces a root-mean-square deviation for the common carbon atoms of 0.48 Å (unbound versus penicillin-bound) and 0.50 Å (unbound versus oxacillin-bound) (83). On the basis of secondary-structure predictions and topological studies with the homologous BlaR protein (84), Marrero et al. (83) suggest that MecR1 displays a similar structure. This structure comprises a fourfold TM-helix bundle with a cytoplasmic loop (L1) connecting helices TM1 with TM2 and an extracellular loop (L2) connecting helices TM2 and TM3. The intracellular domain (L3) located between TM3 and TM4 is responsible for metalloproteinase activity, with the PBD located at the extracellular C terminus (**Figure 2**) (83). The lack of structural change within MecR1-PBD following β -lactam acylation suggests that another extracellular component of MecR2 contributes to signal transduction. This component appears to be L2, given that it is predicted to be in close proximity to the PBD of MecR1 (83) and that alanine substitution of two conserved proline residues in L2 of BlaR1 abolishes *blaZ* expression (85). Furthermore, direct interaction between L2 and the PBD of BlaR has been observed in phase

display experiments, and this interaction is impeded by the presence of penicillin (86). Thus, investigators proposed that in the basal state, L2 interacts with a region of MecR1-PBD including the active-site cleft, providing a stabilizing interaction maintaining the metalloproteinase domain (MPD) in a latent state in the absence of β -lactams (83). When β -lactams are present in the extracellular environment, they compete with L2 for binding to the PBD. Because the reaction of the β -lactam with the catalytic serine residue is covalent and the product is refractory to hydrolysis, the binding equilibrium would be displaced toward the acyl-enzyme complex and L2 forced out of its basal position around the MecR1-PBD active site. However, NMR spectroscopy studies of the L2 interaction with the PBD of BlaR1 show that L2, while binding to the sensor domain, does so proximal to the acylation site, rather than occluding it (87).

Consistent with this localization, penicillin G did not disrupt the interaction between L2 and the PBD (87). The exact molecular events by which L2 propagates the β -lactam-induced signal across the bacterial membrane are not clear. However, movement generated by L2 in response to β -lactam binding likely generates a structural motion across the membrane to the cytosol, enabling the autolytic cleavage and activation of the intracellular MPD. This autolytic cleavage of MecR1-MPD (L3) is predicted to occur within a consensus sequence conserved between BlaR, BlaR1, and MecR1: Baa-Lys-Arg/Glu(-)Arg-Baa-Xaa-Xaa-Baa (where Baa refers to bulky hydrophobic residues) (77). In the case of BlaR from *B. licheniformis*, experiments have shown that L3 is a zinc metalloproteinase, activated by self-proteolysis (88). Substitution of the residues predicted to be involved in zinc metalloproteinase activity, such as the His-Glu-Xaa-Xaa-His zinc-binding signature or cleavage site, reduced the induction of BlaP and are residues that are conserved in MecR1 (88). It is not clear, however, whether this autolytic activation occurs in an intramolecular or intermolecular fashion.

This activation of MecR1 in turn leads, directly or indirectly, to the downstream cleavage and inactivation of MecI and the transcription of *mecA*. As MecR1 and MecI are turned over by signal transduction their synthesis is also induced by β -lactam signaling, allowing return to a basal state when β -lactams are removed. The inactivation cleavage site of MecI and its BlaI relatives also shows a strong consensus, but one that is distinct from that observed in the cognate sensor proteins. The repressor cleavage site consensus follows the pattern Baa-Oaa-Oaa-Asn(-)Phe-Oaa-Glu/Lys-Xaa-Xaa-Xaa-Oaa (where Oaa refers to apolar residues) (77). Despite the differences in the cleavage sites, BlaR1 can indeed cleave directly BlaI (89), although this finding does not exclude the possibility that additional factors contribute to these events. However, direct proteolysis of MecI by MecR1 has yet to be demonstrated.

A second signal leading to inactivation of BlaI and MecI is generated by cell-wall dipeptides, likely produced from cell-wall perturbation in the presence of β -lactams (90). In the case of MecI, γ -D-Glu-L-Lys derived from *S. aureus* peptidoglycan binds to the C-terminal domain of the repressor, impairs its binding to the *mecA* operator, and enhances its susceptibility to proteolytic degradation (90). It is not clear how such peptide coactivators are generated and made available within the cell to have this effect, although researchers have hypothesized that the protease activity of activated BlaR and MecR may contribute to their generation (90).

Unexpectedly, overexpression of *mecI* had no effect on the expression of methicillin resistance in many *S. aureus* strains, suggesting the involvement of an additional regulatory factor (91). This function is provided by *mecR2*, a previously unrecognized gene located downstream of *mecI* (92). Cotranscribed from the *mecR1* promoter along with *mecR1* and *mecI*, *mecR2* encodes an antirepressor that binds MecI, disrupting its binding to the *mecA* promoter and facilitating its proteolysis (92). This proteolysis of MecI, independent of MecR1 and therefore presumed to occur through native cytosolic proteases, is essential for optimal methicillin resistance; expression of a proteolytic resistant MecI decreases methicillin resistance (81). The crystal structure of

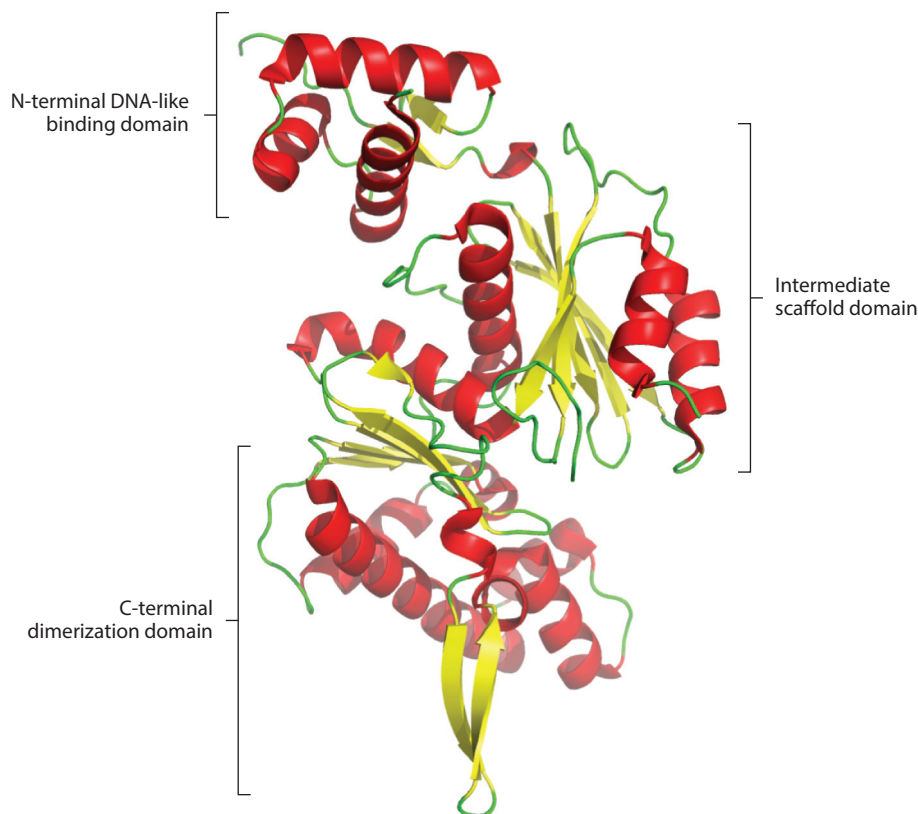


Figure 5

Structure of MecR2. The monomer showing the protein domains is colored according to secondary structure: helix, red; sheet, yellow; loop, green. Figure generated from Protein Data Bank code 41JA using PYMOL (<http://www.pymol.org/>).

MecR2 has been resolved, revealing that MecR2 is structurally similar to the ROK (repressors, open reading frames, and kinases) protein family and that it comprises three domains, an N-terminal DNA-binding-like domain and a C-terminal dimerization domain separated by an intermediate scaffold domain (**Figure 5**) (93). Consistent with the presence of a DNA-binding domain, MecR2 shows strong but nonspecific DNA-binding activity. This activity contributes to the expression of methicillin resistance; a deletion mutant in this domain shows significantly diminished antirepressor activity (93). Likewise, a MecR2 mutant in which an 11-residue stretch in the intermediate scaffold domain was replaced by 4 glycine residues also had reduced antirepressor activity, suggesting that this domain is also relevant to the function of MecR2 (93).

The binding of MecI and BlaI to both the *mec* and *bla* operators has been examined by fluorescence anisotropy, which has indicated that the four sets of interactions are similar to one another. Both monomers and dimers of each repressor bound to each operator with similar affinities (94, 95). In the cases of MecI binding to the *mec* operator and BlaI binding to both the *mec* and *bla* operators, the DNA–repressor complex is dominated by monomers, followed by dimers, with sequential monomer binding observed only at much higher repressor concentration. In contrast, all three binding events appear to compete with one another in the case of MecI binding

to the *bla* operator. Thus, there are subtle differences in the binding events, although how these influence the regulation and expression of methicillin resistance is not clear. An estimation of the concentrations of BlaI and MecI in MRSA cells found that the in vitro experiments and modeling were relevant for the repressor concentrations observed in living bacteria (94). This research also enabled the estimation that in a population of bacterial cells, 0.7–2.4% of individual cells have unrepressed *mecA* (94). This proportion ensures that most of the population is not affected by any deleterious effects of *mecA* expression in the absence of β -lactams, but it also guarantees that at least some of the population can survive any future exposure to these antibiotics (94). MecI–BlaI heterodimers have also been demonstrated (73, 79), and the proteolytic cleavage of BlaI in these heterodimers by BlaR1 may allow the *bla* system to indirectly regulate *mecA* expression (96).

Although investigators originally believed that acylation of BlaR1 by β -lactams cause its activation by autoproteolytic processing and subsequent BlaI inactivation, the heterologous expression of BlaR1 in *E. coli* suggests that this may not be the entire story. When expressed in *E. coli* and, therefore, in the absence of any other *S. aureus* proteins, BlaR1 shows constitutive autolytic activation and proteolysis of BlaI (89). These activities were not enhanced by acylation by β -lactams, as would be predicted from studies in *S. aureus*. Mutation of the penicillin-binding site serine (S398A) in BlaR1 had no impact on autoactivation or BlaI degradation, confirming that this activation is independent of β -lactams (89). Thus, an as-yet-unidentified mediator that is present in *S. aureus* but absent in *E. coli* may act to enhance the rates of signaling in the presence of β -lactams (89). If such a mediator exists, it may also act on the MecR1/MecI pathway.

The reversal of the induction of resistance is presumably important to *S. aureus* fitness because it removes the costs of resistance when not required in the absence of antibiotics. Again, the process in the case of *mecA* is not well characterized but is inferred from research on the *bla* system. Here, BlaR1 undergoes specific proteolysis at two sites, the cytoplasmic MPD and a second extracellular site, resulting in the shedding of the sensor domain (97). Cleavage in the proteolytic domain is thought to be autolytic, with type I signal peptidase responsible for the shedding of the sensor domain (97). Both events occur even in the absence of β -lactams and likely contribute to the reversal of the induction of resistance (97).

HETEROLOGOUS RESISTANCE

A notable feature of most MRSA isolates is that resistance to β -lactams is expressed in a heterotypic or heterogeneous fashion. This means that an inoculum, even from a single colony, produces cultures in which most of the cells exhibit only low levels of resistance, sometimes barely above that displayed by susceptible isolates. However, a minority of the cultured cells, usually 0.01–0.1%, display higher levels of resistance. Less common are isolates showing uniform high-level resistance, referred to as homotypic or homogeneous resistance. This characteristic of MRSA was recognized even among the earliest isolates and continues to be observed today.

Conversion from heterogeneous to homogeneous resistance can be induced by exposure to β -lactams in the laboratory and is associated with mutations, rather than adaptation, in the presence of antibiotic through alterations in gene expression (98, 99). A model system employing the introduction of plasmid-borne *mecA* into an MSSA strain reproduces the phenomenon, yielding a heteroresistant derivative in which most bacteria display a relatively low oxacillin minimum inhibitory concentration (MIC) value (0.75 $\mu\text{g}/\text{mL}$); a subpopulation of highly resistant mutant bacteria exhibit a higher oxacillin MIC (800 $\mu\text{g}/\text{mL}$) at a frequency of $\sim 10^{-4}$ (100). Genome sequencing has revealed that this highly resistant subpopulation is associated with a nonsense mutation in *relA*, predicted to result in a nonfunctional protein giving rise to constitutive (p)ppGpp production and the induction of the stringent response (100). Indeed, introduction of plasmid-borne *mecA* into

a *relA* mutant caused increased (p)ppGpp production compared with the introduction of *mecA* into the parental strain and the production of a homogeneous and high-level resistance in the *relA* mutant (100). Similar results are also observed with the introduction of not only *mecA* on a plasmid, but also an entire SCC*mec* element into an MSSA strain (101). Likewise, induction of the stringent response through exposure to subinhibitory concentrations of mupirocin induced high-level homogeneous resistance among a panel of diverse clinical isolates (101). High-level resistance in clinical isolates may, therefore, be caused by mutations inducing the stringent response, such as *relA*. However, these mutations have yet to be defined in these strains (101), given that any number of independent mutations affecting metabolism also induce the stringent response and homotypic resistance. A role for multiple independent mutations is compatible with the fact that the prevalence of the highly methicillin-resistant subpopulation can be high (10^3) and log units greater than that observed for the spontaneous mutation of a single gene (10^6 to 10^9) (98). Interestingly, under the stringent response, *mecA* transcription and PBP2a production are increased despite the ongoing stringent response, whereby metabolism and protein synthesis are greatly reduced. It thus appears that, through as-yet-unknown mechanisms, *mecA* is exempt from this cellular “shutdown” and that this upregulation is probably the mechanism underlying the shift in resistance (101).

Missense mutations in the RNA polymerase β -subunit gene *rpoB* are also implicated in the conversion from heterogeneous to homogeneous resistance, as well as in a third form of resistance known as Eagle-type resistance (102). Eagle-type MRSA is rare and shows the peculiar property of being resistant to high concentrations but susceptible to lower concentrations of methicillin. This outcome is due to the strong repression of *mecA* transcription by *mecI*, which is overcome at high but not low methicillin concentrations (103, 104). These missense mutations in *rpoB* alter the expression of genes involved in autolysis, resulting in reduced autolytic activity. In the case of Eagle-type resistance, this effect may help allow cells to tolerate, at least temporarily, the levels of methicillin required to induce *mecA* expression; in the case of homotypic resistance, it may combine with the effects of *mecA* to cause high-level resistance (102).

Genome sequencing of laboratory-isolated, highly resistant subpopulations from heterogeneous populations and comparison with their parental strain have revealed that at least 44 different mutations in 27 genes and 3 intergenic regions are implicated in the development of homotypic resistance (105). Reinforcing the role of the stringent response is the finding that many of the mutated genes are directly linked to that response and are predominantly involved in guanine metabolism, transcription, and translation. In agreement with previous research, mutations were observed in *relA* (100) and *rpoB* (102), and mutations in 17 different genes were sufficient to confer high-level homogeneous resistance (105). Correlated with resistance was an increase of at least twofold in PBP2a levels in the cell membranes of these high-resistance isolates (105).

The spontaneous development of resistant subpopulations of MRSA is observed in relation to intermediate resistance to vancomycin, which also targets cell-wall biosynthesis (106). The development of so-called vancomycin-intermediate *S. aureus* from a susceptible parental population, in the absence of antibiotic, appears to be driven by intraspecies competition within biofilms (106). Similar events may influence the heterologous expression of methicillin resistance.

CHROMOSOMAL GENES AFFECTING METHICILLIN RESISTANCE

Whereas *mecA* is essential for resistance, discussion of heterogeneous resistance reveals that it does not function in isolation and that “chromosomal” (i.e., non-SCC*mec*) genes influence the expression of resistance. Indeed, investigators have long known that variation in the level of resistance between strains does not always correlate with the level of PBP2a expression; strains

with MIC values as diverse as 1.5 µg/mL to 1.5 mg/mL express comparable amounts of *mecA* and PBP2a, which led to the suggestion that additional factors (e.g., factor X) are required for optimal resistance (28). Early research, often using the screening of transposon mutant libraries, identified a range of genes initially referred to as *fem* (factors essential for methicillin resistance), *aux* (auxiliary factors), or *bmt* (high methicillin resistance), and numerous genes that are unlinked to *mecA* and SCC*mec* but influence methicillin resistance are now recognized (107–110). Many are involved in cell-wall biosynthesis and contribute to methicillin resistance by ensuring the provision of adequate levels of wild-type cell-wall precursors for proper cell-wall synthesis, with changes in cell-wall substrates dramatically lowering the efficacy of cell-wall biosynthesis in the presence of β-lactams. These genes are covered in detail in excellent reviews by Roemer et al. (111) and Berger-Bächi & Rohrer (112).

Methicillin resistance mediated by PBP2a requires expression of the native and β-lactam-susceptible PBP2. Transposon inactivation of the latter gene results in a several hundred-fold reduction in methicillin MIC (113). Subsequent studies with site-directed mutants showed that it is the β-lactam-insensitive transglycosylation, not the transpeptidation activity of PBP2, that contributes to methicillin resistance (114). MRSA strains grown in the presence of β-lactams produce peptidoglycan with reduced cross-linking, and the transglycosylation activity provided by PBP2, resulting in longer glycan strands, may be important for compensating for this decreased cross-linking (114).

METHICILLIN RESISTANCE IN OTHER STAPHYLOCOCCI AND THE ORIGIN OF METHICILLIN-RESISTANT *Staphylococcus aureus*

The genus *Staphylococcus* comprises more than 50 species that are able to colonize humans and/or a variety of animal species. Whereas *S. aureus* is the most significant cause of human disease, several other species are clinically relevant. Often referred to collectively as coagulase-negative staphylococci to differentiate them from the coagulase-producing *S. aureus* (although a small number of non-*S. aureus* species do produce coagulase), these species include *Staphylococcus epidermidis*, *Staphylococcus lugdunensis*, *Staphylococcus haemolyticus*, and *Staphylococcus schleiferi*. *mecA* and SCC*mec* elements have been reported to be present in a range of staphylococcal species isolated from carriage and disease in humans and a variety of other host species. The origin of *mecA* has been proposed to be *Staphylococcus sciuri* (115, 116), but more recent data indicate that *Staphylococcus fleuretti* may be the origin (117). Both are abundant animal-associated staphylococci, and although the exact steps in the evolution of *mecA* and SCC*mec* have not yet been elucidated, SCC and *mecA* may have existed as individual components that originated and evolved in animal staphylococci before the emergence of SCC*mec* and its transfer into *S. aureus*.

Typing of SCC*mec* in staphylococci other than *S. aureus* poses a problem because current SCC*mec* typing focuses on the SCC*mec* types in *S. aureus*. Specific SCC*mec* types are often present in MRSA as well as other staphylococci and are named accordingly, but novel and nontypable SCC*mec* types are also found in non-*S. aureus* species and are not covered by the current typing scheme (118–120). The apparent importance of non-*S. aureus* staphylococci as a reservoir for SCC*mec* elements and their role as pathogens would make the systematic typing of these elements an important tool for understanding their evolution and epidemiology.

NON-*mecA* GENE-MEDIATED METHICILLIN RESISTANCE

Borderline oxacillin-resistant *S. aureus* (BORSA) strains are so called because they display low-level resistance, especially to oxacillin, with an MIC around the breakpoint. First described in 1986, this resistance is often associated with enhanced β-lactamase activity (121, 122). The relative

susceptibility of oxacillin to β -lactamase-mediated hydrolysis compared with related β -lactams probably explains why resistance is typically against oxacillin only. A membrane-bound methicillinase activity has also been proposed to be involved but has not been defined further (123). Among phage group 94/96 isolates, penicillinase A encoded by pBW15 is needed for borderline resistance, but is insufficient alone to reproduce the phenotype in a different strain background, suggesting that the specific background also contributes to the resistance phenotype (124).

BORSA isolates appear to be rare—a survey of 1,895 *S. aureus* clinical isolates reported this phenotype in only 23 (1.2%) (125)—although they are probably underreported due to the increased use of cefoxitin for screening, which does not detect such isolates. Although these isolates have been associated with disease and outbreaks (126, 127), the clinical significance of their resistance is uncertain. Animal model experiments indicate that BORSA strains are susceptible to treatment with oxacillin in vivo (128–130), and there have been no reports of treatment failure with penicillinase-resistant β -lactams in infections with such isolates (131).

Another *mec*-independent form of resistance is demonstrated by MODSA isolates, so called because they have modifications in their native PBP2s (132). Various point mutations in PBP2 and other PBP2s, often in the transpeptidase domain targeted by β -lactams, have been observed in such clinical isolates (132–135). There are few data on the prevalence of such isolates, but anecdotally they appear rare and their level of resistance low compared with that of *mecA* MRSA. They are important to consider, however, because of their potential to be overlooked when using *mecA* or PBP2a-based diagnostic assays. A case report of cloxacillin treatment failure in a patient with a *mecA*-negative, β -lactamase-negative infection may relate to such a MODSA isolate (136). Furthermore, the introduction of new β -lactams such as ceftobiprole and ceftaroline, which are active against MRSA, may provide increased selective pressure for the development of MODSA-like resistance.

In retrospect, many of the strains presumed to be MODSA by clinical microbiology laboratories may have been *mec*-positive MRSA encoding a divergent variant of *mecA*, *mecC* (137, 138). Discovered following the whole-genome sequencing of a phenotypically resistant but *mecA*-negative MRSA strain from bovine mastitis in England, and originally called *mecA*_{LGA251}, this allele shares 69% homology with *mecA* and is not detected by *mecA*-based PCR or PBP2a slide agglutination (137). It has since been described throughout Europe and in a wide range of host animal species. The oldest known isolate dates to 1975 (139), and it is found predominantly in CC130 and ST425 (138). The designation *mecC* was chosen because another *mecA* variant had already been described in *Macrococcus caseolyticus* (140) and was named *mecB* to reflect the order of discovery (51). *mecC* MRSA strains are relatively rare; their prevalence rate among MRSA isolates is 0.06% in Germany (141) and 0.46% in England (142) but reached 2.8% in Denmark in 2011, having increased since 2009 (143). Commercial and in-house PCR assays are being modified to allow simultaneous detection of *mecA* and *mecC* MRSA (139, 144–147), and their epidemiology will be better understood as more laboratories test for *mecC*.

Mutagenesis and cloning experiments have confirmed that *mecC* confers methicillin resistance in different *S. aureus* strain backgrounds (148). The PBP2s encoded by *mecA* and *mecC* show important differences, although their structural and evolutionary basis is not yet understood (149). Recombinant PBP2a_{*mecC*} has a higher affinity for oxacillin than for cefoxitin, whereas PBP2a_{*mecA*} shows less difference between the two β -lactams (149). This difference is significant in antimicrobial susceptibility testing; *mecC* MRSA typically displays an unusual profile of susceptibility to oxacillin and resistance to cefoxitin when tested using the Vitek 2 system (150). *mecA* MRSA

typically shows resistance to both β -lactams, which may offer a useful tool to differentiate the two (150). The two proteins also differ in their thermostability and temperature optima: PBP2_{*mecC*} is less stable at 37°C than PBP2_{*mecA*} (149). Another distinction between the two proteins is that whereas high-level oxacillin resistance mediated by PBP2_{*mecA*} requires the presence of native PBP2, as discussed above, this is not true of PBP2_{*mecC*}. *mecC* confers high-level resistance in the absence of PBP2; therefore, it presumably interacts not with PBP2 but with monofunctional glycotransferases(s) (149).

As with *mecA*, *mecC* is encoded within a SCC_{*mec*} element (137), albeit a distinct type associated only with *mecC* to date. The detection of *mecC* in other species of staphylococci [*Staphylococcus xylosus* (151), *Staphylococcus stepanovicii* (152), *Staphylococcus sciuri* (153), and *Staphylococcus saprophyticus* (154)] indicates that, as with *mecA*, *mecC* may also originate in coagulase-negative staphylococci.

NEW β -LACTAMS ACTIVE AGAINST PBP2a

Despite the broad-spectrum resistance to β -lactams conferred by PBP2a, some newer cephalosporin β -lactams are effective against MRSA. In particular, ceftaroline and ceftobiprole have broad-spectrum activity against a range of gram-positive and gram-negative bacteria, including MRSA, and are beginning to be used clinically to combat these infections (155, 156). Both are effective against MRSA in vitro and have significantly lower MIC values compared with those of other β -lactams. These lower MIC values are associated with the fact that ceftaroline and ceftobiprole have a significantly higher affinity for PBP2a (157–159). However, resistance to ceftaroline has already been observed at a low frequency among MRSA isolates (160, 161) and will need to be monitored closely in the future. Biochemical and structural studies have shown that ceftaroline is an allosteric activator of PBP2a in that it binds and activates the allosteric site by virtue of its D-Ala–D-Ala mimicry, thereby promoting the active-site binding of a second ceftaroline molecule (49, 162). Whether ceftobiprole also acts in the same way is unclear, and the structural basis for allosteric activation by ceftaroline but not other β -lactams remains to be resolved. Ceftaroline resistance is associated with mutations in the PBP2a transpeptidase domain, which presumably act to lower the binding of ceftaroline (160, 161). However, mutations in the allosteric domain have also been observed (160–163). Two in particular, N146K and E150K, have been characterized structurally and functionally and are known to disrupt the salt bridges needed for the allosteric response of PBP2a (163). This alteration of allosteric signaling represents a novel mechanism of antimicrobial resistance.

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