Published in final edited form as:

Sci Prog. 2002 ; 85(Pt 1): 57–72.

Methicillin resistance in *Staphylococcus aureus*:

mechanisms and modulation

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Abstract

Staphylococcus aureus is a major pathogen both within hospitals and in the community. Methicillin, a β -lactam antibiotic, acts by inhibiting penicillin-binding proteins (PBPs) that are involved in the synthesis of peptidoglycan, an essential mesh-like polymer that surrounds the cell. *S. aureus* can become resistant to methicillin and other β -lactam antibiotics through the expression of a foreign PBP, PBP2a, that is resistant to the action of methicillin but which can perform the functions of the host PBPs. Methicillin-resistant *S. aureus* isolates are often resistant to other classes of antibiotics (through different mechanisms) making treatment options limited, and this has led to the search for new compounds active against these strains. An understanding of the mechanism of methicillin resistance has led to the discovery of accessory factors that influence the level and nature of methicillin resistance. Accessory factors, such as Fem factors, provide possible new targets, while compounds that modulate methicillin resistance such as epicatechin gallate, derived from green tea, and corilagin, provide possible lead compounds for development of inhibitors.

Introduction

Staphylococcus aureus, a member of the family *Micrococcaceae*, is a Gram-positive coccus whose cells tend to occur either singly or if dividing cells do not separate, form pairs, tetrads and distinctive irregular “grape-like” structures. Humans are commonly colonised by *S. aureus* on external skin surfaces and the upper respiratory tract, particularly the nasal passages. Healthy individuals are usually unaware of staphylococcal carriage but they may suffer from minor skin infections such as boils and abscesses. However, *S. aureus* is an opportunist pathogen, and given the right circumstances can cause more serious infections. Burns and surgical wound infections are commonly invaded by *S. aureus*, where the production of toxins by *S. aureus* can e.g. give rise to toxic shock syndrome leading to fever, sickness and in some cases death. Infections caused by *S. aureus* include, pneumonia, (inflammation of lungs), mastitis (infection of the mammary glands), infections of skin (impetigo, cellulitis and staphylococcal scalded skin syndrome), osteomyelitis (infection of bone), endocarditis (infection of the endothelial lining of the heart and valves) and bacteremia (bacteria present in blood). *S. aureus* can also cause food poisoning, the result of enterotoxin production.

Treatment of *S. aureus* infections before the 1950s involved the administration of benzylpenicillin (penicillin G) (Figure 1), a β -lactam antibiotic, but by the late 1950s *S. aureus* strains resistant to benzylpenicillin were causing increasing concern. Resistant strains typically produced an enzyme, called a β -lactamase, which inactivated the β -lactam. Efforts were made to synthesise penicillin derivatives that were resistant to β -lactamase hydrolysis. This was achieved in 1959 with the synthesis of methicillin, which had the phenol group of benzylpenicillin disubstituted with methoxy groups (Figure 1). The methoxy groups produced steric hindrance around the amide bond reducing its affinity for staphylococcal β -lactamases. Unfortunately, as soon as methicillin was used clinically, methicillin-resistant *S. aureus* (MRSA) strains were isolated¹. Resistance was not due to β -lactamase production

but due to the expression of an additional penicillin-binding protein (PBP2a), acquired from another species, which was resistant to the action of the antibiotic¹. The use of different types of antibiotics over the years has led to the emergence of multi-resistant MRSA strains², the result of mutations in genes coding for target proteins and through the acquisition and accumulation of antibiotic resistance-conferring genes. We are now in a situation where, in some cases, the glycopeptide antibiotic vancomycin, is the only option for antimicrobial therapy. With the demonstration that vancomycin resistance conferring genes from other bacterial groups can be expressed in *S. aureus* and with the emergence of glycopeptide-intermediate resistant *S. aureus* strains² the search for new antistaphylococcal agents is a pressing issue. One approach to combat resistance is to find novel targets, while another is to find agents that can reduce or moderate resistance to an existing antibiotic. Investigations into methicillin resistance in *S. aureus* has led to the discovery of new proteins involved in cell wall synthesis that might act as targets and compounds that modulate methicillin resistance. This review focuses on both the mechanisms and the factors that modulate methicillin resistance in *S. aureus*.

Penicillin-binding proteins: the targets of β -lactam antibiotics

The staphylococcal cell is surrounded by a mesh-like structure 20-40 nm thick, called peptidoglycan, that is composed of a series of short glycan chains of approximately 20 alternating *N*-acetylmuramic acid and β -1-4-*N*-acetylglucosamine residues³. Attached to each *N*-acetylmuramic acid residue is a pentapeptide chain referred to as the stem peptide. The glycan chains in peptidoglycan are linked together via the last glycine residue of a pentaglycine cross-bridge attached to the L-lys residue (position 3) on one stem peptide and the D-Ala residue (position 4) on another (Figure 2). Pentaglycine cross-bridges are preformed in the cytoplasm by the FemX, FemA, and FemB proteins, which attach the glycine residues to the L-lysine residue of the stem peptides⁴. The cross-linking or transpeptidation reactions take place on the external surface of the cytoplasmic membrane in a reaction catalysed by penicillin-binding proteins (PBPs). There are four PBPs in *S. aureus*, PBP1, PBP2, PBP3, and PBP4. High molecular weight PBPs have two protein domains, one involved in transpeptidation (cross-linking) the other involved in transglycosylation (extending the glycan chain). The β -lactam antibiotics, which resemble the terminal D-alanyl-D-alanine bond of the stem peptide, inhibit the transpeptidation domain of PBPs (and carboxypeptidase activity of low molecular weight PBPs) thus interfering with the cross-linking reaction. Without cross-linking of the peptidoglycan, the cell wall becomes mechanically weak, some of the cytoplasmic contents are released and the cell dies³.

Methicillin resistance

Methicillin resistance in clinical isolates has been reported to arise from expression of a methicillin-hydrolysing β -lactamase⁵ and through the expression of an altered form of PBP2 that has a lower penicillin-binding affinity and higher rates of release of the bound drug compared to the normal PBP²⁶. However, the main mechanism of methicillin resistance in *S. aureus* is through the expression of a foreign PBP, PBP2a (not to be confused with PBP2), that is resistant to the action of methicillin but which can takeover the transpeptidation (cross-linking) reactions of the host PBPs. Synthesis of PBP2a is regulated and normally kept at low level, but the level of synthesis can be enhanced if mutations occur in the regulatory genes. PBP2a and the control of PBP2a synthesis are described below.

PBP2a

MRSA differ genetically from methicillin-sensitive *S. aureus* isolates by the presence, in the chromosome, of a large stretch of foreign DNA (40-60 Kb), referred to as the *mec* element, and the presence of the *mecA* gene that encodes the 76 KDa penicillin-binding protein,

PBP2a (also referred to as PBP2'). The *mecA* gene has been proposed to originate from *Staphylococcus sciuri*⁷. Although the mechanism of gene acquisition from this species is not known, two genes, *ccrA* and *ccrB*, present on the *mec* element from one isolate, have been shown to code for recombinase proteins that are capable of excising and integrating the *mec* element into the chromosome⁸. Examination of a large number of MRSA isolates has led to the conclusion that the original acquisition of the *mecA* gene has occurred once and that MRSA isolates are descendants of a single clone⁹. Although the arrangement and composition of the *mec* element may vary between isolates¹⁰, the *mecA* gene itself is highly conserved. In common with other PBPs, PBP2a has the common structural motifs that are associated with penicillin binding yet its affinity for β -lactam antibiotics is greatly reduced. Consequently, at therapeutic levels of methicillin that would inhibit the transpeptidational activities of other PBPs, PBP2a remains active ensuring the cross-linking of the glycan chains in peptidoglycan. PBP2a is not able to completely compensate for the other PBPs since cells grown in the presence of methicillin exhibit a marked reduction in the degree of cross-linking. However, the limited degree of cross-linking is enough to ensure survival of the cell.

Regulation of PBP2a expression

Adjacent to *mecA* on the staphylococcal chromosome are two genes, *mecR1* and *mecI*, that are co-transcribed divergently from *mecA* (Figure 3A). The *mecR1* gene encodes a membrane-bound signal transduction protein (MecR1) while *mecI* encodes a transcriptional regulator (MecI). Between *mecA* and *mecR1* are the promoters for these genes and an operator region that encompasses the -10 sequence of *mecA* and the -35 sequence of *mecR1* (Figure 3A)¹¹. MecR1 and MecI have high protein sequence homology with the proteins, BlaR1 and BlaI, respectively, that are involved in the inducible expression of the plasmid-mediated staphylococcal β -lactamase gene, *blaZ*. The arrangement of the genes coding for BlaR1 and BlaI resembles the *mecA* system suggesting that *mecA* may have acquired the regulatory genes from the *blaZ* system sometime in the past¹². The operator regions are similar enough to allow BlaI to regulate PBP2a expression¹³. Consequently, the presence of a plasmid carrying the *blaZ* regulatory genes can render PBP2a expression inducible under the control of BlaR1 and BlaI, a situation that commonly occurs in clinical isolates of MRSA¹⁴.

The nature of the signalling system for inducible β -lactamase expression has recently been elucidated¹⁵. BlaI, a DNA-binding protein, binds to the operator region as a homodimer and represses RNA transcription from both *blaZ* and *blaR1-blaI* (Figure 3B). Consequently, in the absence of a β -lactam antibiotic, β -lactamase is expressed at low levels. BlaR1, present in the cytoplasmic membrane, detects the presence of the β -lactam by means of an extracellular penicillin-binding domain and transmits the signal via a second intracellular zinc metalloprotease signalling domain (Figure 3B). Binding of a β -lactam to BlaR1 stimulates the autocatalytic conversion of the intracellular zinc metalloprotease domain of BlaR1 from an inactive proenzyme to an active protease¹⁵. The activated form of BlaR1 is thought to directly or indirectly cleave BlaI resulting in fragments that are incapable of forming dimers and binding DNA (Figure 3C)¹³. Without BlaI bound to the operator site, transcription of both *blaZ* and *blaR1-blaI* can commence and β -lactam resistance can be conferred through β -lactamase synthesis (Figure 3C). An additional gene product, BlaR2, also regulates β -lactamase synthesis, although the role of this protein has not been elucidated. Whether there are other proteins involved in the signalling system also remains to be determined.

Unlike β -lactamase synthesis, expression of PBP2a is not strongly inducible in isolates carrying the normal regulatory genes (*mecA* and *mecR1-mecI*) and induction is much slower (15 minutes for β -lactamase expression compared to up to 48 hours for PBP2a

synthesis). This is because MecI is a tight regulator of *mecA* transcription¹⁶ and most β -lactam antibiotics do not efficiently activate MecR1. Consequently, some isolates, referred to as pre-MRSA, are methicillin-sensitive despite carrying the *mecA* gene. However, selective pressure through antibiotic usage has promoted *S. aureus* isolates that have mutations or deletions in *mecI* or the *mecA* promoter/ operator region giving rise to an inactive repressor and constitutive PBP2a expression¹⁷. Isolates carrying these mutations can have one of two methicillin resistance phenotypes, homogeneous or heterogeneous, depending on the population structure of a given strain. Homogeneous resistance refers to a cell population where all cells are resistant to high concentrations of methicillin (> 128 mg/l), while heterogeneous resistance refers to a cell population where only a small minority of cells exhibit high-level methicillin resistance. The small minority of cells that exhibit high-level resistance in the heterogeneous population are due to an additional chromosomal mutation(s), designated *chr**, that occurs outside of the *mec* element¹⁸. The nature of the *chr** mutations that give rise to homogeneous methicillin resistance are not known but mutations in the newly described *hmr* loci may be responsible in some cases¹⁹.

Modulation of methicillin resistance

PBP2a synthesis is regulated by MecI and MecR1 proteins and, when present, the regulatory/ signalling proteins of the *blaZ* system. In addition, homogeneous resistance is dependent upon mutations at a separate genetic locus, *chr**. Other factors both internal and external also influence methicillin resistance.

Internal factors affecting methicillin resistance

Since PBP2a is essential in conferring methicillin resistance, any factor that interferes with the expression of the *mecA* gene or with the activity of PBP2a will affect methicillin resistance. Genetic and biochemical studies have established that PBP2a has strict substrate requirements. Consequently, any factors that influence formation of the substrate have the potential to perturb or modulate methicillin resistance. In this context, early inhibitors of cell wall synthesis such as fosfomycin, β -chloro-D-alanine, and D-cycloserine have been shown to reduce methicillin resistance²⁰. Studies have shown that PBP2a requires:

- i. *Glycan chains to be of certain lengths.* PBP2a is dependent upon the transglycosylase activity of PBP2. β -lactams inhibit the transpeptidase domain of high molecular weight PBPs but do not affect the transglycosylase domain. Inactivation of the transglycosylase domain of PBP2, results in an increase in glycan chains of shorter lengths and a marked decrease in methicillin resistance²¹. Therefore, compounds that target the transglycosylase domains of PBPs could serve as useful therapeutic agents.
- ii. *The stem peptide to have the normal peptide configuration.* The addition of glycine to the growth medium leads to stem peptides of peptidoglycan ending in two glycine residues instead of two alanine residues. This leads to a decrease in methicillin resistance and conversion of a highly resistant homogeneous strain to a heterogeneous phenotype²². Inactivation of *murE* (*femF*), the gene coding for UDP-N-acetylmuramyl tripeptide synthetase, also gives rise to a reduction in methicillin resistance. This results from a reduction of UDP-linked muramyl pentapeptides and accumulation of UDP-linked muramyl dipeptides in the cell wall precursor pool²³. These results illustrate that PBP2a requires the stem peptides to have the correct length and contain the normal series of peptides.
- iii. *Requires the pentaglycine cross-bridge to be intact.* FemA, FemB and FemX (FmhB) are involved in building the pentaglycine cross-bridges that link the glycan chains together⁴. FemX adds the first glycine, FemA adds glycines 2 and 3, and

FemB adds glycines 4 and 5. FemA and FemB are not interchangeable, consequently inactivation of the genes coding for either of these proteins results in cell walls that contain mono- or triglycine cross-bridges, respectively. Inactivation of either of the *femA* or *femB* genes is thought to be lethal but compensatory mutations can restore cell viability, although growth is severely affected⁴. Significantly, inactivation of either *femA* or *femB* also results in a large reduction in methicillin resistance. Consequently, the FemA and FemB proteins represent new targets for drug development²⁴. Inactivation of FemA and FemB has the added advantage in that interference with the cross-bridge length also affects the secretion of virulence factors which could limit the ability of the cell to cause infection²⁵. Incidentally, the Lif protein that adds serine residues in the cross-bridges of other staphylococcal species, complements the activity of FemB by incorporating serine residues at positions 4 and 526. Introduction of serine residues into the cross-bridge results in a reduction in methicillin resistance indicating that PBP2a can only handle cross-bridges containing glycine at positions 4 and 526. So it is possible that the development of inhibitors of FemA and FemB, might not be compromised by the acquisition of other cross-bridge building proteins from other species.

Peptidoglycan synthesis requires the coordinated activity of not only the biosynthetic enzymes but also the lytic enzymes that are involved in peptidoglycan remodelling and cell division. Methicillin resistance is affected by the inactivation of genes that affect the autolytic enzyme activities of the cell. Inactivation of the *llym* gene, coding for a protein of unknown function, converts a homogeneous strain to a heterogeneous phenotype and is associated with increased autolytic activity²⁷. The activities of the global regulator proteins such as Sar, Agr and SigmaB are also known to affect methicillin resistance⁴. Their affects are probably mediated through their control of genes involved in cell wall and exoprotein synthesis.

External factors that affect methicillin resistance

External factors that affect methicillin resistance include among others, salt concentration, pH, medium composition, osmolarity and temperature²⁸. Some of these external influences are exploited in the clinical laboratory to enhance the detection of strains exhibiting heterogeneous methicillin resistance; isolates are grown in the presence of high NaCl concentrations (2%) and at lower temperatures (30-35°C). Why NaCl concentration and temperature have this affect is not known.

Compounds that modulate methicillin resistance

Baicalin, a flavone compound isolated from the Chinese herb Xi-nan Huangqin (*Scutellaria amoena*)²⁹ and a tripeptide, LY301621 (carbobenzoxypheylalanine-proline-phenylalanine alcohol)³⁰, increase the susceptibility of MRSA isolates to β -lactams but the mechanism of how they achieve this is not understood. A few compounds are known to affect the expression of the *mecA* gene. For compounds such as the polyoxotungstates³¹ and totarol³², the synthesis of PBP2a is reduced but the mechanism of how this is achieved is not known. For others, such as polidocanol (a dodecyl polyethyleneoxide ether)³³, and glycerol monolaurate³⁴ the site of action is thought to be the cytoplasmic membrane where they interfere with either the signalling domain of MecR1 or some other protein involved in signal transduction. The non-ionic detergent Triton X-100 is also thought to target the cytoplasmic membrane but it does not interfere with PBP2a production. Strains grown in the presence of Triton X-100 have increased susceptibility to methicillin and have increased rates of bacteriolysis³⁵. MRSA grown in the presence of triton X-100 are converted from a homogeneous to a heterogeneous phenotype, although there appears to be strain-to-strain

variability. Inactivation (or partial disruption) of the *fmt* genes, *fmtA* and *fmtB*, have been shown to enhance triton X-100-mediated methicillin sensitivity. The FmtA protein is a membrane protein that has two of three conserved β -lactam binding motifs but it does not bind β -lactams³⁶. Inactivation of the *fmtA* gene affects the cell wall structure but the precise role of the FmtA protein has not been determined. How the inactivation of the *fmtB* gene results in a reduction in methicillin resistance is also not known, but methicillin resistance can be restored by the addition of cell wall precursors, glycosamine-1-phosphate and N-acetylglycosamine-1-phosphate³⁷.

Other compounds are known not to affect PBP2a synthesis and interfere with the activity of PBP2a, these include licoricidin, a phenolic compound isolated from commercial licorice³⁸, and the triazine dye, cibacron blue F3GA³⁹.

Epicatechin gallate (ECG) and epigallocatechin gallate (EGCG) (Figure 4) are polyphenolic compounds present in green tea (*Camellia sinensis*) that have activities against a wide range of bacteria⁴⁰. At high concentrations epicatechin gallate selectively inhibits the growth of methicillin-resistant *S. aureus*. Under the electron microscope cells treated with green tea extracts have gross morphological changes characterised by the clumping together of partial divided cells that have thickened internal cell walls⁴¹. Methicillin-sensitive strains appear to be unaffected by the extract. At low concentrations (25 mg/l), both ECG and EGCG can modulate methicillin resistance and act synergistically with β -lactams such as methicillin and oxacillin⁴². The action of ECG in combination with oxacillin is bactericidal⁴³. EGCG does not appear to affect transcription of the *mecA* gene and has been proposed to exert its effect through direct binding to peptidoglycan⁴⁴. However, other workers have shown that an extract of green tea (ECG) affects the quantities of PBP1, PBP3 and PBP2a but not PBP2, present in the cytoplasmic membrane, suggesting a more specific mode of action⁴². The effect on PBP3 expression might be significant since, cephradine (a type of β -lactam antibiotic) a relatively selective inhibitor of PBP3 has an inhibitory affect on methicillin resistance at sub-minimum inhibitory concentrations suggesting a role for PBP3 in methicillin resistance²⁰. ECG and EGCG have also been shown to damage bacterial membranes⁴⁵.

Green tea administered as a spray has been successfully used in the treatment of an MRSA infection of the trachea (windpipe)⁴⁶. Unfortunately, epicatechin gallate cannot be widely administered because it is broken down by esterases in the body to the inactive products, epicatechin and gallic acid. This prevents the oral or parenteral co-administration of ECG with oxacillin or methicillin. Recently, two other polyphenolic compounds, corilagin⁴⁷, extracted from the leaves of *Arctostaphylos uva-ursi* and tellimagrandin I⁴⁸ extracted from the petals of rose red (*Rosa canina* L.) have been reported. Both compounds act synergistically with oxacillin. Corilagin appears to interfere with PBP2a activity rather than PBP2a synthesis and has greater activity against MRSA than either ECG or tellimagrandin I⁴⁷. Further investigations into the mechanisms of action of these polyphenolic compounds combined with the synthesis of more stable derivatives are warranted.

Concluding remarks

Peptidoglycan synthesis is a complex process involving the coordination of both biosynthetic and degradative pathways. PBP2a is not native to *S. aureus*, and has to fit within this system. Unfortunately, PBP2a is less sensitive to the action of methicillin and is capable of conferring methicillin resistance. Fortunately, the substrate requirements of PBP2a are relatively strict and this is a weakness that can be exploited. By targeting and inhibiting the proteins, such as FemA and FemB, involved in the formation of these substrates, methicillin resistance can be modulated. Compounds such as epicatechin gallate and

corilagin are attractive compounds to develop into therapeutic agents since these compounds exist already and are known to modulate methicillin resistance.

Acknowledgments

The authors are grateful to the Medical Research Council for financial support.

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Benzylpenicillin

Methicillin

Fig. 1.
The chemical structures of β -lactam antibiotics benzylpenicillin and methicillin.

Fig. 2.
A schematic representation of the cross-linking of two glycan chains in peptidoglycan of *S. aureus*. MurNAc, N-acetylmuramic acid; GlcNAc, N-acetylglucosamine.

Fig. 3.

A, Schematic representation of the *mecA*-*mecR*-*mecI* coding region. Arrows indicate the relative directions of transcription of the *mecA* and *mecR1*-*mecI* genes. *B*, Repression of *blaZ* and *blaR1*-*blaI* transcription by *BlaI* in the absence of an inducer. *C*, Induction of β -lactamase synthesis in the presence of a β -lactam antibiotic (see text for details).

(-)-Epicatechin-3-Gallate

(-)-Epigallocatechin-3-Gallate

Fig. 4.

The chemical structures of the polyphenolic compounds, epicatechin gallate and epigallocatechin gallate present in green tea (Camellia sinensis).