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Review

# β-Lactam resistance in *Staphylococcus aureus*: the adaptive resistance of a plastic genome

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**Abstract**. Staphylococci have two mechanisms for resistance to β-lactam antibiotics. One is the production of β-lactamases, enzymes that hydrolytically destroy β-lactams. The other is the expression of penicillin-binding protein 2a (PBP 2a), which is not susceptible to inhibition by β-lactam antibiotics. Strains of *S. aureus* exhibiting either β-lactamase or PBP 2a-directed resistance (or both) have established a considerable ecological niche among human pathogens. The emergence and subsequent spread of bacterial strains designated as methicillin-resistant *S. aureus* (MRSA), from the 1960s to the present, has created clinical difficulties for nosocomial treatment on a global scale. The recent variants of MRSA that are resistant to glycopeptide antibiotics (such as vancomycin) have ushered in a new and disconcerting chapter in the evolution of this organism.

**Key words.** *Staphylococcus aureus;* beta-lactamase; *blaZ; blaI; blaR; mecR; mecI; mecA;* PBP 2a; MRSA; VRSA.

## Introduction

The timeline for bacterial evolution stretches far beyond the current era of antimicrobial therapy. During the past 60 years, humans have become the driving force behind the accelerated evolution of bacterial antimicrobial resistance [1]. Many chemotherapeutic treatments have been used during this brief time. Some are incredibly successful and offer life-saving chemotherapy, while others were used briefly and have since ceded into obsolescence. Nonetheless, the value of even the most effective of these treatments is threatened by bacterial acquisition and evolutionary perfection of resistance mechanisms. *Staphylococcus aureus*, the paradigm among the bacteria of this natural phenomenon, has always been a challenge for anti-microbial chemotherapy [2]. By virtue of its ability to overcome chemotherapy through acquisition of drug resistance, *S. aureus* continuously expands its ecological niche. Prior to the antibiotic era, the mortality of patients infected with pathogenic *S. aureus* exceeded 80%, and over 70% developed metastatic infections [3]. Survival was decidedly an uncertain outcome. The advent of penicillin in the 1940s immediately improved this prognosis, with over 94% of strains exhibiting susceptibility [4]. This was short-lived. The use of penicillin quickly selected *S. aureus* that were resistant as a result of β-lactamase expression [5]. Penicillin-resistant *S. aureus* emerged in hospitals around 1942, with ultimate proliferation in the community [6, 7]. By 1950, more than 50% of all staphylococcal isolates were resistant to penicillin. The pattern of resistance – first hospitals and then the community – is the common pattern for each new antibacterial [2, 8]. A semi-synthetic penicillin, methicillin, was introduced in 1959 in response to the challenge of β-lactamase-producing *S. aureus*. Shortly thereafter reports emerged (first from the United Kingdom, and then from elsewhere around the globe) of *S. aureus* with acquired methicillin

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resistance [9]. This new *S. aureus*, referred to as methicillin-resistant *Staphylococcus aureus* (MRSA), has since become one of the leading causes of bacterial infections and a global scourge [10, 11]. An irony of this trend toward escalating bacterial resistance is that it coincides with a period wherein the understanding of the mechanisms of resistance is increasing. The recent emergence of variants of MRSA resistant to vancomycin (the preferred agent for treatment of severe MRSA infections), due to the acquisition of the *vanA* gene from Enterococci, has created an unparalleled challenge in the evolution of this organism [12–14]. These isolates, referred to as vancomycin-intermediately-susceptible *Staphylococcus aureus* (VISA) and vancomycin-resistant *Staphylococcus aureus* (VRSA), portend a chemotherapeutic era in which the antibacterials of today may become ineffective. For this reason an important objective is to expand – or extend – the value of existing antibacterials. This review identifies the frontiers of our understanding of the mechanisms that underlie the gene regulation of β-lactam resistance, and the basis for the loss of β-lactam antibacterial efficacy in methicillin-resistant *S. aureus*, with emphasis on the past and present genomic determinants that have enabled the survival of this pathogen.

## The advent of staphylococcal resistance: β-lactamases

Antimicrobial resistance is associated with the perpetual change of a highly flexible bacterial genome that under pressure moves inexorably to further mechanistic perfection. *S. aureus* thrives as a result of its considerable genomic plasticity with respect to antibacterial resistance mechanisms. The Staphylococci have two primary resistance mechanisms with respect to the β-lactam antibiotics. One is the expression of β-lactamase enzymes (for *S. aureus*, the PC1 β-lactamase). β-Lactamases destroy β-lactams by hydrolysis, and are expressed by activation of the *blaZ* gene. Higher-level β-lactam resistance (MRSA) results from the acquisition of the *mecA* gene, which encodes the penicillin-binding protein 2a (PBP 2a). Strains of *S. aureus* exhibiting either β-lactamase- or PBP 2a-directed resistance (or both) have established a considerable presence in the exotic world of human pathogens [15]. β-Lactamase-dependent resistance is found currently in >95% of staphylococcal isolates [16], while MRSA constitutes 25–50% of clinical isolates in the North America, Europe and Asia [17]. Transcription of the β-lactamase and PBP 2a genes is controlled by the BlaR-BlaI-BlaZ and MecR-MecI-MecA regulatory systems, respectively. The two systems are remarkably similar in structure and function [18–21], yet still retain distinct identities [22, 23]. Of the two systems, the *S. aureus bla* regulatory system is the better characterized, as our understanding is complemented by that of a homologous system for β-lactamase expression found in the non-pathogenic Gram-positive bacterium *Bacillus lichenformis* [24–28]. The *mec* system is believed to parallel the better-characterized *bla* gene regulatory system, and therefore the *bla* system forms the working model for regulation of both components of resistant *Staphylococci*.

Kirby's discovery in the mid-1940s that penicillin was destroyed by penicillin-resistant strains of *S. aureus* [7] was followed by the identification of penicillinases – β-lactamases with high catalytic activity against penicillins – that mediate this resistance [29]. The resistance determinant was linked to β-lactamase expression, encoded by the *blaZ* gene maintained on a transposable element of a large plasmid [30–32]. The *bla* gene is linked to two adjacent regulatory genes, those of the antirepressor signal sensor/transducer *blaR1* (585 amino acids, $M_r$ = 69,246) and the repressor *blaI* (126 amino acids, $M_r$ = 14,876) [31, 33, 34]. The gene product of *blaZ* is the staphylococcal class A β-lactamase PC1 (281 amino acids, $M_r$ = 31,349) [35], which uses an active-site serine to hydrolyze the four-membered β-lactam ring. As is generally accepted with all serine β-lactamases, the β-lactamase has ancient origins, likely having evolved from the PBP enzymes [36, 37]. Expression of this β-lactamase is not constitutive but is induced following encounter of the *S. aureus* (possessing penicillinase plasmids) with β-lactams [38]. The initiating event for β-lactamase expression is an irreversible acylation, with concomitant opening of the β-lactam, of an active site serine in the sensor domain of cell surface BlaR1 protein (BlaR1 represents the transmembrane spanning and signal transducer domains; $BlaR1^S$ represents the truncated *C*-terminal sensor domain of signal transducer). This acylation is the first event in signal transduction. The second event of signal transduction is a discrete (zinc metalloprotease-dependent) autoproteolytic cleavage within the cytoplasmic domain of BlaR1/MecR1 [39, 40]. Autocleavage of the signal transducer is followed by proteolytic propagation, ultimately with proteolysis of the dimeric protein repressor, BlaI. Following this proteolysis, the repressor dissociates from its divergon binding site, enabling transcription of the *blaZ/blaR/blaI* (and in the case of MecR, the *mecA/mecR/mecI* genes). While structural [35, 41, 42] and kinetic [43, 44] studies have extensively characterized the *blaZ* β-lactamase, efforts to understand the regulatory system controlling its expression continue. Among the important questions concerning the operation of this gene system are the mechanism of BlaR1 acylation, the events of the signal transduction pathway, the proteolytic cleavage event of the BlaI repressor and the mechanism for re-repression once the threat of the β-lactam has passed.

## The origin of deregulation

The starting point for discussion of the regulatory systems controlling expression of the β-lactamase and PBP 2a is their respective signal-transducer proteins, BlaR1 and MecR1. After contentious debate [15, 22, 39, 40] several key mechanistic aspects have been settled. Both BlaR1 and MecR1 are transmembrane receptor proteins [45–47]. BlaR1 of *S. aureus* is a high molecular weight (HMW) penicillin-binding protein. The protein sequence from the gene [25, 46], and the crystal structure of the solubilized *C*-terminal domain, are known [25, 27, 48]. BlaR1 consists of two domains [37]. The first is an *N*-terminus domain of approximately 38 kDa, with α-helices crossing the membrane four times via four transmembrane segments (TM1, TM2, TM3, TM4) [45]. The transmembrane segments are interconnected by three loops (L1, L2, L3), where L1 and L3 are exposed to the cytoplasm and L2 is positioned on the outside of the cell (as shown in fig. 1). The second approximately 27-kDa domain consists of the *C*-terminus sensor of the plasma membrane surface. β-Lactams in the extracellular medium react with the active site serine of the *C*-terminal domain in the acylation event. The nucleophilic serine is $Ser^{389}$ for the *S. aureus* BlaR1 protein and $Ser^{402}$ for the *B. lichenformis* BlaR1 protein [27]. Acylation of this serine by a β-lactam initiates a signaling cascade, ultimately resulting in derepression of the β-lactamase-encoding gene. Both the 186-amino acid cytoplasmic segment (L3) and the 56-amino acid extracellular segment (L2) of the BlaR1 *N*-terminal domain are believed to be integral components of the signal cascade. The L3 segment is suggested to regulate, by responding to acylation of the *C*-terminal sensor domain, the autolytic zinc-metalloprotease activity of the *N*-terminal domain [45, 47]. The L2 loop and the $BlaR1^S$ bind non-covalently, and this interaction is altered by β-lactam acylation [25, 47]. While this proteolytic activity is surmised to play a fundamental role in signal transduction, the basis for the activation mechanism (such as might occur by conformational alteration of the L2 loop following β-lactam acylation of the serine) is not known with certainty.

The MecR1 protein is homologous to BlaR1. The similarities extend to mechanism, size and sequence (identity of sensor domains is 43%; of the protease domains is 33%; and of the full-length proteins is 34%) [25]. It is therefore highly probable that their tertiary structures and mechanism for gene derepression are similar. Nonetheless, there is currently no evidence to support overlap in the signal transduction: BlaR1 activation derepresses only the β-lactamase gene for β-lactamase expression, and MecR1 derepresses only the PBP 2a gene for PBP 2a expression. BlaR activation does not result in PBP 2a expression, nor does MecR activation result in β-lactamase expression [21, 22, 49–51]. Thus, the present understanding of BlaR1 activation and signal transduction is a cascade with a minimum of four events. The first is $Ser^{389}$ acyl-enzyme formation in the sensor domain [25, 52]. The second event is the alteration of protein conformation in response to stable acyl-enzyme formation, correlated to cross-membrane signal transduction. The third – and least well-understood event – is the cytoplasmic propagation of the signal. The fourth is the signal culmination resulting in gene derepression by the repressor proteolysis. The present understanding for each event is discussed.

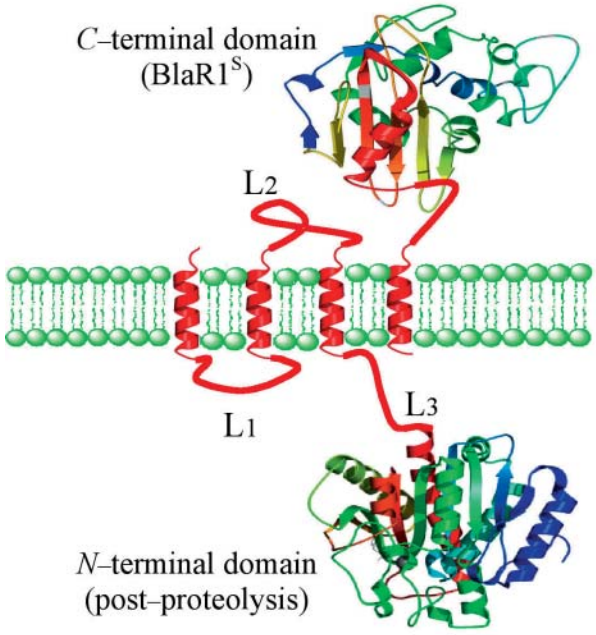



Figure 1. Schematic representation of the BlaR1 membrane protein that is involved in regulation of PBP 2a, where $BlaR1^S$ represents the sensor domain.

A broad spectrum of β-lactam structures is capable of successful acylation of staphylococcal $BlaR1^S$. Typical kinetic data ranges for staphylococcal $BlaR1^S$ are acylation rate constants ($k_2$) of 1–26 $s^{-1}$, $k_2/K_S$ values of 0.3–11 x $10^5$ $M^{-1}s^{-1}$ and deacylation constants ($k_3$) of 4–100 x $10^{-5}$ $s^{-1}$. The observed deacylation rate constants correspond to $t_{1/2}$ values of 10 to greater than 290 min. Complete deacylation exceeds the doubling time for *S. aureus* (20–30 min), indicating that acylation must be an irreversible event [52]. The rate constants determined for the sensor domain from *B. licheniformis* are similar [53]. Three conserved sequence motifs define the active site of all β-lactam serine transferases/hydrolases, including the class A, C and D β-lactamases and the cell wall transpeptidases [36]. These are an SXXK tetrad (that includes the nucleophilic serine), an SXN triad and a KTG triad. All are present in the BlaR1 and MecR1 sensor do-

main active sites. In BlaR1 of *S. aureus*, these motifs are found as $Ser^{389}$-X-X-$Lys^{392}$ (with $Ser^{389}$ as the nucleophile in acylation), $Ser^{437}$-X-$Asn^{439}$ (the sequence is $Ser^{450}$-X-$Thr^{452}$ in *B. licheniformis* [27]), and $Lys^{526}$-Thr-$Gly^{528}$. Moreover, $Ser^{389}$ is located at the *N*-terminus of a helix, which likely modulates its p$K_a$ to facilitate its activation as a nucleophile [26]. $Lysine^{392}$ is the general base that activates the serine for acylation in this motif [36, 52]. The serine β-lactam transferase/hydrolases have common mechanistic features to facilitate serine acylation by the β-lactam. Two of the most important are Lewis acid activation of the β-lactam carbonyl electrophilicity (the oxyanion hole), and the use of a general base for activation (by deprotonation) of the serine. Both features are in $BlaR1^S$. The $BlaR1^S$ oxyanion hole comprises the main-chain amide nitrogens of $Ser^{289}$ and $Thr^{529}$, and is organized similarly to that of the serine β-lactamases. While the general base used for serine activation varies among the three serine β-lactamase classes (A, C and D), the substantial spatial overlay of BlaR1 and the OXA-10 class D β-lactamase active sites suggest a shared mechanism for serine activation [36, 52, 54]. The activation of class D β-lactamases involves lysine carboxylation (that is, a lysine with a carbamate anion functional group obtained by reaction of the lysine ε-amino group with $CO_2$) as the serine base catalyst [52, 55]. The carboxylated lysine is the base for serine in both the acylation half-reaction [48, 52], and for water in the deacylation half-reaction [56]. The lysine in the OXA-10 β-lactamase is sequestered in an unusually hydrophobic environment (side chains of $Phe^{69}$, $Val^{117}$, $Phe^{120}$, $Trp^{154}$ and $Leu^{155}$) that is expected to lower the p$K_a$ of the lysine amino group such that it is in the free amine form necessary for reaction with $CO_2$ [55]. The existence of a similar hydrophobic environment has yet to be identified for $Lys^{392}$ of $BlaR1^S$ or *B. licheniformis*. In BlaR the position of this lysine with respect to $Ser^{437}$ is also appropriate for the carboxylated lysine acting as the requisite base for BlaR acylation [36, 52]. The participation of a $Lys^{392}$ $CO_2$-derived carbamate in the $BlaR1^S$ acylation is consistent with the observation that the acylation rate of the sensor domain increases in the presence of added bicarbonate [48, 52]. Moreover, the $^{13}C$ nuclear magnetic resonance (NMR) spectrum of the $BlaR1^S$ protein in the presence of $^{13}CO_2$ shows lysine carboxylation as evidenced by the diagnostic $^{13}C$ NMR resonance [52]. Therefore, $BlaR1^S$ is proposed to undergo carboxylation at $Lys^{392}$ (homologous to the $Lys^{70}$ for OXA-10) as a prerequisite for serine activation and for promoting acylation by β-lactams [48, 52, 55, 57, 58].

Kinetic study of the reaction of $BlaR1^S$ with β-lactams indicates that a small number of turnovers (with ceftazidime, six) are accomplished before the protein arrests as a stable acyl-enzyme [48]. The abrupt transition from hydrolytic turnover to stable $BlaR1^S$ acyl-enzyme is presumed to correlate with decisive alteration of protein structure. An observation may be relevant to this presumed alteration. A similar event occurs during OXA-10 β-lactamase catalysis, where the lysine carbamate can undergo spontaneous decarboxylation to arrest catalysis at the acyl-enzyme stage. Resumption of catalytic activity requires restoration of the lysine carbamate, by re-reaction with $CO_2$ [55, 59, 60]. It is not known whether the exceedingly slow deacylation of the $BlaR1^S$ acyl-enzyme involves a slow $CO_2$ reactivation step, followed by fast hydrolysis of the acyl-enzyme, or is simple bimolecular hydrolysis of the acyl-enzyme (which remains in the oxyanion hole and hence retains a portion of the active site activation for nucleophile addition). The lysine carbamate is absent in the X-ray structures for the apo-$BlaR1^S$ protein and for the β-lactam acylated-$BlaR1^S$ (both *S. aureus* and *B. licheniformis*) sensor domains [25, 27, 48]. The fundamental distinction between $BlaR1^S$ and class D β-lactamases is the inability of $BlaR1^S$ to accomplish deacylation. Structural evidence suggests a different role for the $Ser^{115}$ of the class D β-lactamases that is separate from the role of the homologous $Ser^{437}$ in $BlaR1^S$, as possibly accounting for this divergence. In class D β-lactamase catalysis the $Ser^{115}$ hydroxyl is believed to shuttle a proton to the tetrahedral intermediate on the acylation pathway, transferring the proton from the carboxylated lysine to the leaving group nitrogen of the tetrahedral intermediate and enabling productive collapse to the acyl-enzyme [55, 58]. In the case of $BlaR1^S$, a similar decarboxylation of $Lys^{392}$ would indeed entrap the acylated receptor species. This entrapment could coincide with the creation of an activated sensor state to initiate signal transduction [48].

## Signal transduction

The intermediate step between β-lactam acylation of $BlaR1^S$ and the ultimate derepression of the *bla* operon is that of signal transduction. Since β-lactams cannot permeate the cytoplasmic membrane, the presumption must be that sensor acylation is the initiating extracellular event. The current hypothesis proposes that signal propagation is mediated by an altered non-covalent interaction between the $BlaR1^S$ sensor domain and the L2 extracellular loop [25, 47]. The extracellular loop L2 (connecting the TM2-TM3 helices) has positively charged amino acids in non-covalent contact with the penicillin sensor domain. Phage display experiments with the *B. licheniformis* $BlaR1^S$ sensor domain implicate interruption of a non-covalent L2 loop interaction upon β-lactam acylation [47]. In both *B. licheniformis* and *S. aureus* this change is suggested to propagate through the transmembrane α-helices to alter, and thereby enable, activation of the metalloprotease cytoplasmic domain [25, 45, 47, 61].

An altered interaction could induce the conformational change presumed necessary for signal transduction [45, 61]. In the case of BlaR1 of *S. aureus*, a paired proline PXXP motif in the L2 loop is believed to be involved in the non-covalent interaction between this loop and the sensor domain [25]. Mutagenesis of these prolines, or the deletion of a 35 *C*-terminal amino acid segment in *S. aureus* BlaR1$^S$, results in constitutive β-lactamase synthesis [25, 62]. This observation does not reveal the proteolytic activation mechanism.

The *bla/mec* systems are distinctively different from all other bacterial signal transduction pathways [39, 40], which use a two-component, kinase-based system for signal transduction [63, 64]. If not this pathway, then what is the cytoplasmic signal transduction in BlaR1? One proposal is changed modality of integral membrane proteins, which exist either in anti-parallel β-barrel or α-helix bundle states [45]. This type of signal transduction involves realignment of the transmembrane helices. Circular dichroism (CD) analysis reveals a significant conformational change in the α-helices of the *S. aureus* BlaR1$^S$ domain upon β-lactam acylation [52]. However, direct evidence of a change in the transmembrane region upon acylation is lacking. Nor do the currently available X-ray structures for the apo- and penicillin-acylated-BlaR1$^S$ sensor domains of *S. aureus* and *B. licheniformis* suggest where a conformational change might occur [25, 27, 48]. A second signal transduction hypothesis is proteolytic cleavage of key signaling components [39]. This method is supported by the evidence for autocleavage of the cytoplasmic metalloprotease on the *N*-terminal of BlaR1. However, it does not address the events that occur in between the acylation of BlaR1 and the induction of metalloprotease cleavage [39]. All mechanisms should account for the observation that β-lactam acylation of BlaR1 initiates β-lactamase expression (minutes) significantly faster than MecR1 acylation initiates PBP 2a expression (hours) [22, 23]. While it is certain that the two signaling systems are fundamentally the same, what accounts for this difference? There is currently no answer.

## Repressor cleavage

The final steps leading to β-lactamase (and PBP 2a) expression are very similar. Intracellular metalloproteases, activated by the acylation event of the sensor domain, proteolytically cleave the dimeric repressor proteins, thus releasing them from their repressor sequence and allowing *blaZ* (or *mecA*) transcription. *S. aureus* BlaR1 and MecR1 (and BlaR1 of *B. licheniformis*) have (in their 186-amino acid cytoplasmic L3 domain) the sequence signature of a zinc metallopeptidase [39, 45, 65]. The signature is defined by a histidine sequence

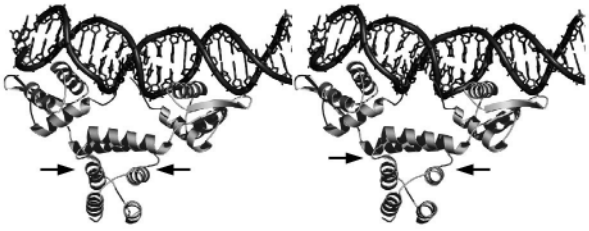


Figure 2. The three-dimensional structure of the complex of MecI with its operator DNA. The MecI dimer is shown in ribbon representation, while the DNA oligonucleotide is shown in capped-stick representation and a ribbon along the duplex backbone. The arrows point to the cleavage sites in the two monomers.

[as the Zn(II) ligands] and a glutamic acid, which is also required as the catalytic base for hydrolysis [66]. As zinc metalloproteases commonly autocatalytically self-activate by intramolecular cleavage, the same process is assumed here [67]. Although direct evidence for autocleavage within the BlaR1/MecR1 domains is lacking, one cleavage site is identified that upon proteolysis leaves the putative cytoplasmic protease tethered to the cytoplasmic membrane [39, 40]. The relationship of this tethered domain to the metalloprotease accomplishing the sequence-specific cleavage of the repressors is not known. An alternate theory suggests the inactivation of BlaI results from a non-covalent modification by a co-activator and the subsequent proteolysis may be a secondary phenomenon [68].

The 14-kDa BlaI and MecI repressor proteins consist of an 11-kDa DNA binding domain and a 3-kDa dimerization domain [49]. Deregulation involves three discrete events: cleavage of the repressor blocking gene transcription, divergent transcription of the genes encoding the regulated proteins and their cognate regulatory proteins, and re-repression of the gene (upon absence in the extracellular medium of β-lactam antibiotics). The metalloprotease cleaves, at a specific sequence, the two 14-kDa repressors into the DNA binding domain and dimerization domains. X-ray structure confirms a bound homodimeric repressor (fig. 2) [28, 69]. The *N*-terminal domain of each binds to the DNA with winged helix-turn-helix topology, independently binding to the DNA from its counterpart on the other monomer. The *C*-terminal dimerization domain has a spiral helical topology that intertwines its counterpart on the other monomer, giving stability to the dimer [28, 49, 69–71]. The structure of the repressor was determined with and without their cognate DNA substrate [28, 51]. The repressors in complex with their target DNA reveal a conserved protein-DNA interface between both *mec* and *bla* targets. Both recognize and specifically bind, by an α3 recognition helix on the *N*-terminal domain, to a conserved TACA/TGTA DNA motif [28]. Each repressor adopts a slightly different con-

formation when bound to either the *mec* or *bla* binding sites due to slight spatial differences between the DNA motifs [28]. MecI binds to either operon, and therefore blocks transcription of both the *mec* and *bla* operators. BlaI likewise binds to both the *mec* and *bla* operators [20, 50]. The interchangeability of BlaI and MecI is a consequence of their similarity. The *S. aureus* BlaI and MecI repressors have 125 amino acid residues with 61% identity to each other, and respectively 31 and 41% identity with *B. licheniformis* BlaI [51]. Nearly all of the residues that interact directly with DNA are conserved in the two repressors (the exception is $Asn^{65}$ in BlaI, which is replaced by $Lys^{65}$ in MecI). The repressor binds to the DNA with the *C*-terminal dimer domain oriented cytosolically, away from the *N*-terminal DNA binding region. The poorly accessible cleavage site is buried within the *C*-terminal domain. Proteolytic cleavage (at the $Asn^{101}$-$Phe^{102}$ for both BlaI and MecI) disrupts the dimer interface, causing the dimer to dissociate and release itself from the DNA. Gene transcription follows. It is also suggested that the metalloprotease works with another (currently unidentified) factor, involved in unraveling of the repressor structure to allow access of the metalloprotease to the cleavage site [27, 38]. Such a relationship remains unproven.

The operon encoding the regulated protein (β-lactamase or PBP 2a) and the regulatory proteins (BlaI/BlaR1 or MecI/MecR) overlap partially with the operator region to which the repressor binds. Binding of the repressor to the *bla* system results in repression of *blaZ*, *blaR1 and blaI*, along with autorepression of the operator region as well. Hence, removing the repressor results in both sets of genes being divergently transcribed with simultaneous expression of the β-lactamase (or PBP 2a) repressor and signal transducer. The repressors constrain transcription by binding two specific regions of dyad symmetry in the *bla* (*or mec*) operator, namely, the Z and R1 dyads in the intergenic region between the *blaZ* gene (or *mecA*) and the regulatory operon genes for *blaR1-blaI* (or *mecR1-mecI*). Cleavage of BlaI dissociates the dimer. Displacing the repressor from its intergenic operator site enables transcription of *blaZ*. Because BlaR1 is presumed to activate and signal transduce once, intact BlaR1 must be made continuously for sensing of the environmental β-lactam concentration. BlaI, BlaR1 (presumably including the metalloproteinase) and the β-lactamase are all expressed. BlaI increases its intracellular concentration about five-fold upon penicillin induction [70, 72]. Once the extracellular β-lactam antibiotic concentration diminishes, BlaR1 is no longer autoactivated. Proteolytic cleavage of MecI/BlaI ceases, and the intramolecular concentration of the repressor molecule increases. The repressors dimerize, bind DNA and re-suppress *mecA* and *blaZ* synthesis [22, 39].

## Methicillin resistance: PBP 2a as an enigma wrapped in a riddle

One strategy by which bacterial pathogens develop resistance to antibiotics is mutation of key residues within the target. This evolutionary adaptation of the target renders the antibiotic ineffective. While *S. aureus* has chosen, even after 50 years of selective pressure, not to acquire modified or new β-lactamases, it has acquired another (and highly successful) resistance mechanism. Methicillin, introduced in 1959 as the first semisynthetic penicillinase-resistant penicillin, was followed by the appearance in 1961 of the first MRSA strain [9]. By the 1980s MRSA was global [9]. The basis for the transformation to MRSA was the genomic acquisition of the *mecA* gene, encoding the PBP 2a enzyme. With the outbreak came clonal dissemination, leading to the divergence of MRSA into several dominant classes throughout the 1960s [11, 73–75]. MRSA is now a global scourge [76–80] causing a broad spectrum of infections such as superficial abscesses, septicemia, osteomyelitis, bacteremia, necrotizing pneumonia and endocarditis [16], refractory to almost all β-lactams [2]. Enright [81] has reviewed the path to clinical isolates from both hospital- and community-acquired infections. MRSA now appears in over 50% of the clinical strains in U.S. hospitals and is fatal in 20–40% of these cases [82]. The complex pathogenesis of *S. aureus* involves the strongly coordinated synthesis of cell wall-associated proteins and extracellular toxins. Vastly different from the highly disseminated strains of methicillin-susceptible *S. aureus* that cause infection, there are only a small number of clones responsible for the epidemic spread of MRSA [8]. Major MRSA clones repeatedly arise from successful epidemic MSSA strains. More distressing is the presence of isolates with decreased susceptibility to vancoymcin disseminating from MRSA. The trend toward increasing drug resistance reflects the ongoing pursuit of mechanistic perfection by a small number of successful *S. aureus* genotypes. Although some diversity exists, each clone preserves the *mecA* gene, often complemented by its regulatory genes *mecR1-mecI*. All are found on a mobile genomic island designated staphylococcal cassette chromosome *mec* (SCC*mec*) [83]. This chromosomal cassette combines the entire *mec* operon (approximately 28 kb) with the *ccr* gene, a complex encoding the site-specific recombinases responsible for SCC*mec* mobility [84]. This mobility is imperative for resistance, as MSSA must acquire SCC*mec* to form MRSA. However, introduction of SCC*mec* into MSSA is met with substantial cost, and many strains of MSSA select against it [85, 86]. Although SCC*mec* is found in many staphylococcal species, little is known about its origin. The atypical codon usage pattern and its GC content suggest acquisition from another bacterial species [87]. The closest possibility is a PBP from

*Staphylococcus sciuri*, a species that is considered to be taxonomically primitive among staphylococci and found predominantly in rodents and primitive mammals [88]. While the PBP of *S. sciuri* shares 88% amino acid homology with PBP 2a [89, 90], a definitive relationship is not proven. It is certain the SCC*mec* gene is transferred horizontally among staphylococcal species within its own genus, leading to significant clonal dissemination [83, 91, 92]. Different combinations of the *mec* gene complex and the *ccr* gene complex have led to five separate SCC*mec* lineages for *S. aureus*, each differing in size and composition [84, 91, 93]. It is believed that both the *mec* and *ccr* gene complexes continuously recombine and rearrange in the genomes of coagulase-negative staphylococci, perpetually forming new SCC*mec* elements. However, only a small fraction transfer to *S. aureus* strains isolated from the community [84]. Despite the continuous genomic reorganization, several genes are always preserved in each SCC*mec* element. The *mecA* gene (encoding PBP 2a) and its cadre of regulatory genes (*mecI* and *mecR1*) are found in each of the five SCC*mec* classes. Each component has been cloned and sequenced (MecI: 125 amino acids, $M_r$= 14,790, MecR1: 585 amino acids, $M_r$= 68,503 [94, 95]. As discussed previously, the regulatory system controlling transcription of the *mec* operon is remarkably similar to the *bla* system. There are, however, noteworthy differences [96]. Despite similar recognition sequences, the *bla* system induces *blaZ* transcription within minutes, while induction of *mecA* takes several hours [22, 23]. This difference may result from the difficulty that MecR1 has in sensing penicillins (such as methicillin and oxacillin) [97]. Additionally, MecR1 and BlaR1 are specific for their cognate repressors and cannot be functionally interchanged [22]. Furthermore, a chromosomally encoded factor (BlaR2) of unknown function is believed to have a role in BlaI cleavage and *bla* derepression [28, 38], while a similar factor has yet to be implicated for *mec*.

The resistance of MSSA to virtually all β-lactam antibiotics stems from the expression of penicillin-binding protein 2a (PBP 2a). PBP 2a is a high molecular weight (668 amino acids, $M_r$ = 76,102) membrane-bound transpeptidase belonging to the subclass B1 [95, 98, 99] family. In addition to its transpeptidase domain it also has a non-penicillin-binding domain of unknown function [59]. Methicillin-sensitive strains of *S. aureus* MSSA produce four PBPs that assemble and regulate the final stages of cell wall biosynthesis [100, 101]. As mentioned, acquisition of the *mec* operon by MSSA transforms it to the MRSA phenotype. In most (non-clinical) organisms, PBP 2a supplements the other PBPs rather than replacing their function. In other phenotypes, PBP 2a takes over transpeptidation [102, 103]. When the MRSA organism is subjected to β-lactam stress, PBP 2a confers resistance by contributing its transpeptidase activity (cell-wall cross-linking) to the transglycosylase function of native PBPs during cell wall synthesis [102, 104]. The following sections explore the important integral relationship PBP 2a has to staphylococcal resistance, from its response to β-lactam pressure to its role in cell wall synthesis.

## Basis of resistance to β-lactam antibiotics

An essential objective to the understanding of this pathogen is the precise role PBP 2a confers to the enigmatic ability of MRSA to circumvent the antibacterial effect of β-lactams. The many reviews [8, 81, 87, 96, 97, 105, 106] on this topic concur that the survival advantage of MRSA under β-lactam pressure correlates to the ability of PBP 2a to provide compensatory function. The PBPs are streamlined into a limited number of functions. Some exist in increased copy number, while other PBPs are altered by selection of mutant variants so as to diminish recognition of the β-lactam without compromise of the peptidoglycan role [106]. PBP 2a is the most abundant PBP of the MRSA microorganism at a copy number of 450–1200 per MRSA cell [107]. However, studies of heterogeneous populations of MRSA (in which the majority of cells are resistant to relatively low concentration of methicillin, while a much smaller proportion – $10^{-8}$ to $10^{-2}$ of the cells – are able to grow at higher methicillin concentrations) suggest that the PBP 2a abundance does not necessarily correlate with the level of resistance [108, 109].

The topology and mechanism (serine-derived acyl-enzyme) of the transpeptidase domain of PBP are similar to the serine β-lactamases (classes A, C and D) [36, 37], including the three active site signature sequences [SXXK, (S/Y)XN and KTS/KTG] [110]. Nonetheless, despite this similarity PBP 2a is different [85, 111–113]. Less is known about the PBP 2a reaction with β-lactams, and especially how it continues cell wall biosynthesis under conditions where the catalytic activity of the other *S. aureus* PBPs are compromised by β-lactam encounter [114, 115]. The antibiotic activity of the β-lactams is imputed to mimicry of an essential peptide motif of the bacterial cell wall, and to engage in confounding acylation of their PBP targets. The Tipper-Strominger hypothesis emphasizes the similarity between the β-lactam and the acyl-D-Ala-D-Ala moiety of the peptidoglycan, allowing irreversible β-lactam acylation resulting in loss of the transpeptidase and carboxypeptidase activities required for cell wall assembly [103, 116]. The resemblance of the β-lactam moiety to a dipeptide extends only to recognition. There can be no more certain a point of distinction than the contrast between the stable β-lactam-derived acyl enzyme, and the transient acyl-enzyme derived from D-Ala-D-Ala that effortlessly participates in cell wall construction. Thus, while transpeptidases turn over

multiple strands of peptidoglycan to accomplish cell wall synthesis, the β-lactam gives a stable acyl-enzyme that undergoes a very slow hydrolysis (a time scale greater than 30 min) [101]. The longevity of this acyl-enzyme corresponds to inactivation of the PBP enzyme, depriving the bacterium of the catalysts it needs to maintain its cell wall integrity. This deprivation ultimately results in cell death.

Kinetics powerfully describe the interaction between an enzyme and its substrate. The kinetic mechanism of PBP 2a is well approximated by the same three-step pathway that characterizes the other PBPs [98, 111, 117, 118]. From the non-covalent enzyme-β-lactam complex (described by dissociation constant $K_d$), a covalent acyl-enzyme forms (with rate constant $k_2$), and is followed by a slow hydrolytic deacylation step ($k_3$) releasing the deactivated β-lactam and regenerating the active enzyme. Recently published kinetics for PBP 2a, obtained using a chromogenic cephalosporin (nitrocefin) as a reporter substrate [117], correlate these kinetic values to relative β-lactam (penicillins, cephalosporins and a carbapenem) resistance [98]. The non-covalent PBP 2a pre-acylation complex $K_d$ values are in the millimolar range (ranging between 0.2 and 13 mM) for the spectrum of β-lactam structures that was evaluated [98, 119]. These values seemingly contrast with PBP 2, where significantly lower $K_d$ values are surmised [119-122]. A possible explanation for the higher $K_d$ values found with PBP 2a is diminished accessibility of the β-lactams to the PBP 2a active site. Indeed, even single mutations at the active site of the *Streptococcus pneumoniae* PBP 2x alter the topology (or change the polarity) such that entry to the active site is greatly affected [121, 123–127]. A no less important manifestation of resistance is the PBP 2a acylation rate constant ($k_2$), which attenuates three to four orders of magnitude over the corresponding rate constants for penicillin-sensitive PBPs [119, 120, 122]. The result is a 10,000-fold smaller $k_2/K_d$ ratio for PBP 2a (1–20 $M^{-1}s^{-1}$) compared with susceptible PBPs (200,000 $M^{-1}s^{-1}$) for many β-lactams. Another characteristic of PBP 2a is the extremely slow rate constants ($t_{1/2}$ of 26–77 h) for the deacylation ($k_3$) of the acyl-enzyme. However, most PBPs are notorious for their slow deacylation [98, 112, 122, 128], with PBP 5 of *Escherichia coli* as an exception ($t_{1/2}$ of < 10 min) [129]. Therefore, this does not explain the dramatically attenuated values for $k_2$, $K_d$ or $k_2/K_d$. Rather, these extraordinary differences evoke the image of a mechanistic or structural hindrance within PBP 2a that renders acylation problematic. Many early attempts were made to rationalize differential PBP 2a activity by sequence comparison with other PBPs [130–137], but always leading to the conclusion that PBP 2a was distinctively different. At last, some of the mystique enshrouding PBP 2a was removed with the disclosure of its soluble domain structure (determined by X-ray at 1.8 Å resolution) by Lim and Strynadka [138]. The soluble PBP 2a (644 amino acids, $M_r$ = 74, 000) has three domains. The domains are an *N*-terminal lobe (corresponding to an *N*-terminal extension or anchor, characteristic of the class B1 HMW PBP structural archetype) [59], a centralized non-penicillin binding domain of unknown function and a *C*-terminal transpeptidase domain. Of particular interest is the *C*-terminal transpeptidase domain, which has a folding pattern that is typical of the PBP transpeptidases and the serine β-lactamases [36, 139]. Nonetheless, PBP 2a possesses subtle structural deviations that contribute to its uniqueness [138]. The prominent difference is the active site motif of the nucleophilic serine ($Ser^{403}$-Thr-Gln-$Lys^{406}$) located on an α-helix sequestered within an extended narrow groove. Unlike typical PBPs, the groove impairs the accessibility of the active site serine to approaching β-lactams. A sense of this impairment is given by comparison (fig. 3A, C) of the apo-forms of PBP 2a ($Ser^{403}$) and PBP 5 ($Ser^{44}$). The groove is surmised to differentially limit access to β-lactams as compared with the cell wall substrate. Moreover, within the groove $Ser^{403}$ is less capable of nucleophilic approach to bound β-lactams (and quite possibly, also the peptidoglycan substrate). The implication that active site conformational change is needed, for both inactivation by β-lactam antibiotics and catalytic turnover of peptidoglycan substrates, was substantiated by comparison of the apo-PBP 2a and acyl-PBP 2a structures for three PBP 2a acyl-enzyme crystal structures (derived from nitrocefin, penicillin-G and methicillin) [138]. The conformational difference between the apo- and benzylpenicillin PBP 2a acyl-enzyme species is shown in fig. 3A and B. The implication is that PBP 2a conforms individually to each β-lactam (and substrate). Among the noticeable differences are the $Ser^{403}$ Cα, Cβ and Oγ locations. The difference in the location of these atoms between the acyl-enzyme and apo-structures is respectively 1.1, 1.4 and 1.8 Å. This suggests a $Ser^{403}$ conformation change preceding acylation [138]. Evidence supporting this conformational change is also provided by the PBP 2a CD spectra in the presence of oxacillin (a penicillin) and ceftazidime (a cephalosporin) [98]. The relatively slow kinetics for the interactions of β-lactam antibiotics with PBP 2a reveal decreased α-helix content (observed at minima 208 and 222 nm) upon exposure to the antibiotic, among a set of conformational changes seen within the first $t_{1/2}$ values for acylation (progressing to virtually complete protein acylation). These conformational changes continued for the duration of the monitoring over 3 days. In essence, the CD spectra indicate substantial conformational flexibility within the protein [98]. More important, this supports the suggestion from the X-ray data that PBP 2a undergoes conformational change in order to accommodate β-lactam binding. It remains unknown whether these changes are attributable

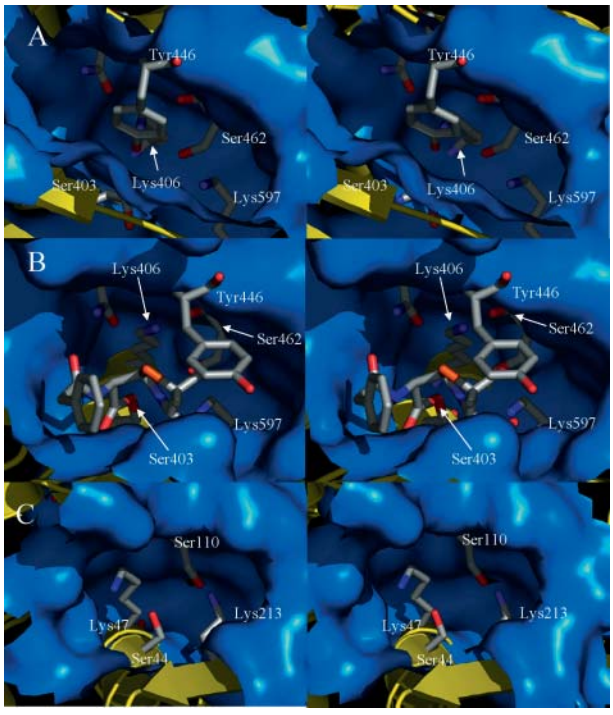


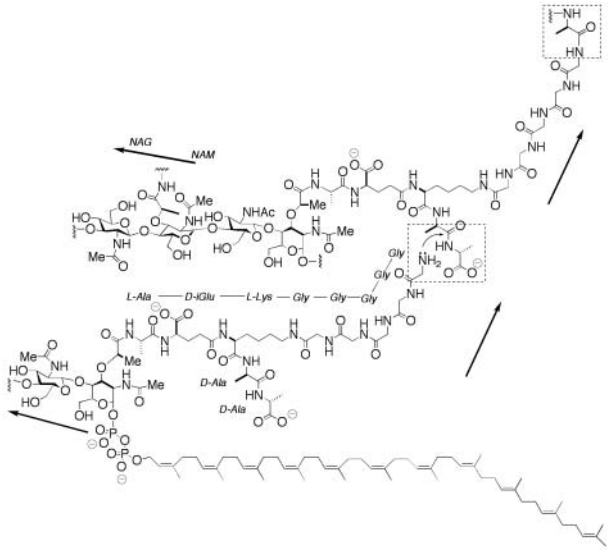

Figure 3. (A) Stereoview of the active site of the apo form of PBP 2a; (B) benzylpenicillin/PBP 2a acyl-enzyme species; (C) apo form of PBP5 from *E. coli*. Important residues are shown in capped-stick representation and colored according to atom type (white, blue and red representing carbon, nitrogen and oxygen, respectively). A Connolly solvent accessible blue surface is constructed around the residues. The protein backbone is shown in yellow ribbon representation.

to formation of the initial, non-covalent Michaelis complex or to the acylation.

PBP 2a manifests resistance to β-lactams in two ways: a sterically encumbered approach to the active site, quite possibly slowing non-covalent Michaelis-complex formation (as evidenced by the millimolar $K_d$ values for many β-lactams). Nucleophilic attack by the active site serine ($Ser^{403}$) on the β-lactam ring may also be impeded, with the slow acylation rate perhaps implicating a requirement for conformational change to expose the serine. Both factors make the formation of a PBP 2a acyl-enzyme difficult.

## The role of PBP 2a in cell wall synthesis

Not only is the bacterial cell wall indispensable for survival, it also contributes to infectivity and pathogenicity [140]. The cross-linked peptidoglycan (also referred to as the cell wall or sacculus) is a continuous elastic polymer surrounding the cytoplasm of virtually all bacteria. The peptidoglycan provides structural integrity to the cell, offers mechanical protection with respect to the high osmotic pressure of the cell [141] and withstands the restructuring that must accompany cell division [142, 143]. Within the stress-bearing Gram-positive peptidoglycan protective layer [144] are embedded teichoic acids [145] and many bacterial surface proteins [146, 147]. The peptidoglycan consists of a repeating β-1,4-linked *N*-acetyl-glucosamine-*N*-acetyl-muramic acid (NAG-NAM) disaccharide, where the NAM-pentapeptide stem (in *S. aureus*, NAM-L-Ala-γ-D-Glu-L-Lys-D-Ala-D-Ala) cross-links (with loss of the terminal D-Ala) to the adjacent peptidoglycan strand. In Gram-positive bacteria (such as *Enterococcus, Streptococcus* and *Staphylococcus*) this cross-link occurs to a peptidic chain attached to the ε-amine of the L-Lys [148]. In *S. aureus* the chain is a pentaglycyl (scheme 1). The cross-linking bridges the glycan and confers strength to the cell wall. The inside (newly synthesized) *S. aureus* peptidoglycan is highly cross-linked (estimated as 90%), whereas the outside (as a result of autolysin-controlled maturation) is less cross-linked [104, 149]. The penicillin-binding proteins are located on the extracellular surface of the cytoplasmic membrane, where they accomplish the final steps of cell wall assembly [36, 100, 150]. These steps include glycosyltransferase-catalyzed formation of the linear glycan chains and transpeptidase-catalyzed formation of the peptide cross-bridges. The transglycosylase and transpeptidase reactions are each catalyzed separately by the two domains of bifunctional penicillin-binding proteins (such as PBP 2). Thus, the MRSA PBP 2a transpeptidase activity complements the PBP 2 transglycosylase activity (which remains unaffected by the β-lactam inactivation of the PBP 2 transpeptidase). The *S. aureus* cell wall obtained from the cooperative PBP 2 and 2a catalysis is only very slightly different from the peptidoglycan made by PBP 2 (in the absence of β-lactams) [104]. Nonetheless, the assignment of a three-dimensional structure to the peptidoglycan remains elusive despite much effort over the past 30 years. Among the difficulties are the sacculus size (a single molecule per bacterium) and structural variability (as a result of autolysin maturation of the cell wall), and the extensive cross-linking. Substantial degradation is necessary to provide segments amenable to structural study. Moreover, continual cell wall synthesis and turnover present additional variability [149, 151, 152]. A recent model integrates the known data into a proposed scaffold structure for the *S. aureus* cell wall murein [149, 153]. Its notable departure from earlier proposals is a perpendicular – not parallel – orientation of the glycan to the membrane. In this model chains of glycan and oligopeptide both run in a plane perpendicular to the plasma membrane, with the oligopeptides maintaining a zigzag conformation and zippering adjacent glycan strands along their lengths [149]. This model accounts well for the high degree of peptidoglycan cross-linking. The transpeptidase and transglycosylase interaction




Scheme 1. The components in *S. aureus* cell wall biosynthesis. The undecaprenylpyrophosphate segment of lipid II inserts into the outer membrane leaflet, exposing the NAG-NAM disaccharide. Attached to the lactyl carboxylate of the NAM saccharide is the L-Ala-D-γ-glutamyl-L-Lys-D-Ala-D-Ala pentapeptide stem segment. The sequential action of three enzymes (FmhB, a glycyl transferase; and FemA and FemB, both diglycyl transferases) give the pentaglycyl peptide branch segment. The transglycosylase domain of PBP 2 polymerizes the NAG-NAM pair to give the repeating $[NAG\text{-}NAM]_x$ glycan strand. The arrows indicate the directionality of the glycan strands, suggested by Dmitriev et al. to have an orthogonal orientation relative to the membrane surface. The dashed box (center right) summarizes the reaction catalyzed by the transpeptidase domain of PBP 2 (and also by PBP 2a). In the first transpeptidase step, the active site serine is acylated by the D-Ala-D-Ala segment, with loss of the terminal D-Ala. In the second step the D-alaninyl group is transferred to the pentaglycyl amine giving the cross-link. A second dashed box (upper right) shows a completed cross-link. The arrows to the right indicate approximately an orthogonal plane of cross-links (relative to the glycan). The bending of the pentaglycyl branches is for legibility. The stem-branch cross-link will be stretched to its full length by the internal osmotic pressure of the bacterium. Dmitriev et al. propose a bifurcated interglycan and interlayer-linked Gram-positive scaffold [149].

determines the peptidoglycan structure. The *S. aureus* peptidoglycan is found in four important incarnations. These are MSSA, MRSA, VISA and VRSA. Each has a different transpeptidase/transglycosylase interaction yielding a unique cell wall phenotype.

Cell wall synthesis in MSSA is accomplished by four native staphylococcal PBPs (PBP1, PBP 2, PBP 3, PBP 4). Of particular interest is PBP 2, the only bifunctional (with separate transpeptidase and transglycosylase domains) PBP [59, 154]. Under ideal growth conditions (without β-lactam stress) the transpeptidase activities of PBP 2 and PBP 4 coordinate cell wall synthesis [104]. It is suggested that the transpeptidase activity of PBP 2 produces dimers, trimers and tetrameric muropeptides that are further cross-linked by PBP 4. The result is a strong, highly cross-linked peptidoglycan [155]. In the presence of β-lactams, the primary difference between MRSA and MSSA is the loss of the PBP 2 transpeptidase activity. MRSA peptidoglycan biosynthesis depends on the cooperative action of the transglycosylase domain of the native PBP 2 and the transpeptidase domain of PBP 2a [102]. The MRSA cell wall is less cross-linked (unlinked muropeptides with fewer dimers and trimers) [108, 114]. In studies where the PBP 2 transglycosylase is inactivated, *S. aureus* is unable to build the peptidoglycan and exhibits β-lactam sensitivity, even though the PBP 2a transpeptidase may be present [102, 156].

Both VISA and VRSA are characterized by irregular cell walls. VISA strains induce increased cell wall biosynthesis and decreased cross-linking, accumulating intact acyl-D-Ala-D-Ala termini, which result in non-productive vancomycin binding. Resistance is the outcome of thickened cell wall and changes to both the composition of peptidoglycan and expression of penicillin-binding proteins [157-163]. Recently emerged VRSA strains, having both the β-lactam resistance gene *mecA* and the vancomycin resistance gene *vanA* [164], synthesize a cell wall of unusual chemical composition with irregular cell wall precursors (i.e. muropeptide oligomers deficient of pentaglycine branches) [165, 166]. The *vanA* operon from enterococus allows synthesis of a cell wall precursor that ends in D-Ala-D-Lac rather than D-Ala-D-Ala. The D-Ala-D-Lac terminus has dramatically reduced affinity for vancoymcin. When challenged by vancomycin, this depsipeptide is synthesized and incorporated into the peptidoglycan [165, 166]. Inactivation of the *mec* gene does not reduce the vancomycin MIC for strain COLVA, indicating PBP 2a is not needed for biosynthesis of cell wall in VRSA. Rather, it was demonstrated that the native staphylococcal PBP 2 is essential for vancomycin resistance and for synthesis of the abnormally structured cell walls [165]. As demonstrated, both transpeptidases PBP 2 and PBP 2a exert considerable influence over the phenotypes of cell wall found in the four principal isolates of staphylococci. These interactions are complex and in most cases poorly characterized. What insight exists into the molecular events that transpeptidases accomplish when forming the cell wall?

The previous section introduced an important aspect of the of PBP 2a active site groove. While hindering β-lactam approach, it also may direct how the PBP interacts with the cell wall. This interaction is surmised to be dramatically different than in the native PBP 2 with its more exposed active site. Recently a model was proposed for the activation of catalysis in the transpeptidase PBP 2a, which reasoned that surface interactions between PBP 2a and a series of compounds mimicking its polymeric substrate would facilitate opening of the active site to facilitate entry of the peptidoglycan (or as in this case, the β-lactam) [167]. The experiment was simple: by use

of a nitrocefin-based chromogenic assay, the kinetics ($k_2$, $K_s$, $k_2/K_s$) of these compounds with PBP 2a were evaluated. The compounds mimic the *N*-acetylglucosamine (NAG)-*N*-acetylmuramic acid (NAM) disaccharide with the NAM pentapeptide (NAM-L-Ala-γ-D-Glu-L-Lys-D-Ala-D-Ala) appendage (but without the pentaglycyl segment, thus preventing turnover by PBP 2a). The outcome was surprising. As the concentration of the peptidoglycan fragment increased, the rate constant for PBP 2a acylation ($k_2$) by nitrocefin increased (by approximately 10–25-fold), and the dissociation constant ($K_s$) decreased (by approximately 2–4-fold). Bacterial cell wall also increased the deacylation rate constant ($k_3$) by approximately 3-fold. The binding of the cell wall surrogates was saturable, indicating the presence of a unique binding site. From these data the dissociation constants ($K_d$) for each fragment with PBP 2a (1–3 mM) were determined. It was previously argued that the effective concentrations of the cell wall components that the PBPs experience on the bacterial cytoplasmic membrane might be high [168]. Therefore, the millimolar $K_d$ values for the interactions between PBP 2a and the peptidoglycan fragments would seem reasonable. As such, there may not have been a compelling reason for evolutionary improvement of the cell wall affinity for the PBP. An assertion can also be made that real cell wall would have higher affinity, making the process more efficient in vivo, such that the many cross-linking events of the cell wall could proceed unencumbered within the 20–30 min required for doubling (under favorable conditions) of *S. aureus*. These findings imply a greater availability of the PBP 2a active site to the β-lactam antibiotic in the presence of increasing concentrations of the peptidoglycan fragments (as a result of a more stable non-covalent PBP 2a β-lactam-preacylation complex that shows more rapid enzyme acylation). X-ray structural evidence for other PBPs bound to peptidoglycan (pentapeptide) mimetics points to a new subsite that may function as a high-affinity anchor for the cell wall component [169–171]. A similar allosteric binding site for PBP 2a that facilitates access (for both substrate and β-lactam) to the active site may be implied. Although a binding site for cell wall has been documented, which interacts with PBP 2a in a saturable manner, the location of this binding site and how this binding event alters the protein structure is presently lacking.

## MSSA, glancing back . . . VRSA, looking ahead

Reflecting on the diverse events that have selected the highly resistant and pathogenic *S. aureus*, one appreciates the challenges it has overcome. *S. aureus* was exquisitely sensitive to penicillins in the early years of β-lactam use in the clinic, heralding what we now recognize as a temporary end to the era where *S. aureus* bacteremia proved lethal in more than 80% of cases [3]. To all good things must come an end. After MRSA was established as a significant nosocomial pathogen, vancomycin became the cornerstone of therapy [172–175]. Until the mid-1990s, clinical isolates of *S. aureus* remained fully susceptible to vancomycin treatment, with minimal inhibitory concentration (MIC) values around 1 μg/ml [176]. An exponential increase in the usage of vancomycin for treating infections caused by methicillin-resistant staphylococci (both coagulase-positive and -negative), *Clostridium difficile*, and enterococcal infections preceded the emergence of vancomycin-resistant staphylococci [175]. The first clinical strain (VISA) of *S. aureus* resistant to vancomycin (Mu50) was reported in 1997 from Japan [177]. As VISA is not clonal, most strains were initially characterized as containing a small number of vancomycin-intermediately resistant subpopulations (such as Mu50, with a vancomycin MIC of >8 μg/ml) at a frequency of 1 per 1,000,000. Heteroresistant strains of VISA exist and are susceptible to vancomycin. As such, it is hypothesized that VISA isolates can be selected among vancomycin-resistant subpopulations, similar to the way MRSA isolates can be selected from heterogenous MRSA [8, 177]. From this beginning, VISA has proliferated as measured by its frequency among worldwide clinical isolates [177–180]. Resistance in MRSA is manifested in the form of increased cell wall biosynthesis and decreased cross-linking of peptidoglycan [157]. The decreased cross-linking of peptidoglycan leads to the accumulation of D-Ala-D-Ala peptidoglycan termini. These bind vancomycin non-productively. The appearance of VISA in clinical isolates led to the growing concern that the enterococcal *vanA* gene could transfer to MRSA and work in conjunction with *mecA*. Such an event would render current antibiotics nearly impotent.

And this has happened. MRSA carrying the enteroccocal *vanA* gene complex, and expressing high-level resistance to vancomycin (MIC over 32 μg/ml), emerged in clinical strains from Michigan, Pennsylvania and New York [12–14]. These VRSA isolates demonstrate complete vancomycin resistance (MIC of >128 μg/ml). VISA resistance is chromosomally mediated, and VRSA acquires resistance by conjugal transfer of the *vanA* operon from *Enterococcus faecilis*. As MRSA, and moreover as VRSA, *S. aureus* has returned as a serious infectious agent. The need for novel antibiotics to treat this organism is as genuine today as at anytime in the past. This clinical urgency has been addressed in recent years by the introduction of Synercid (a combination of quinupristin and dalfopristin) [181], daptomycin [182] and linezolid (an oxazolidinone) [183] for the treatment of MRSA. However, resistance to all these agents exists, and the recent emergence of variants of MRSA resistant to linezolid [184] and glycopeptide antibiotics [12–14, 185] has created a situation in which certain strains of *S. aureus* are either treatable only

with a single class of antibiotics or simply not treatable. With respect to β-lactams, new structures (especially as fourth-generation cephalosporins) that are more capable of PBP 2a inactivation have been identified. A handful have advanced into clinical trial for MRSA treatment.

Similar to other organisms that must adapt to selective pressure, *S. aureus* has survived the eons of time. The development of new genetic capabilities occurs slowly or rapidly, and *S. aureus* exemplifies both. Its β-lactamase is an evolutionarily ancient response to β-lactams that was uncommon but is now common. The recent successive alterations to its cell wall – the phenotypic transition from MSSA to that of the pathogenically sublime vancomycin- and methicillin-resistant *S. aureus* – exemplifies extraordinary change over an incredibly short period of time. The future of this organism cannot be foretold. We can be certain, however, that the future will deliver surprise, and that the surprise may be unpleasant. The selection pressure that has driven these recent changes is fundamentally unchanged, and the remarkable facility with which *S. aureus* has adopted to this pressure leaves us at a loss to foretell its future phenotypic evolution.

This is not to imply that antibacterial drug design is bereft of direction, or that future antibacterial chemotherapy is bereft of hope. Our appreciation of the evolutionary genetics of the PBPs [186], of their cooperative participation in cell wall biosynthesis, [104] of their structures [130, 137, 138, 170] and of their basis for substrate recognition and catalysis [170, 187] is immeasurably better today than even 5 years ago. The realization that the catalytic activity of PBP 2 (and 2A) is influenced – indeed, likely even regulated – by the cell wall structure opens new opportunity for drug design. Pinho and Errington [188] have shown that only active PBP 2 is recruited to the *S. aureus* division site; Fuda et al. [167] have shown that the catalytic activity of PBP 2a is allosterically responsive to the cell wall structure; and Macheboeuf et al. [189] have visualized PBP active site restructuring (of the *S. pneumoniae* class A PBP1a) in response to substrate. Adversarial regulation of PBP 2 (whose transglycosylase activity remains essential to *S. aureus* [104]) differentiates glycopeptides effective against MRSA from those that are not [190, 191]. Moreover, a correlation between bacterial membrane depolarization and bacteriocidal activity has been demonstrated for both Telavancin and Daptomycin (respectively, a new lipid II-interacting semi-synthetic vancomycin glycopeptide and a lipopeptide) [192, 193]. All of these new correlations represent new hypotheses to guide discovery of new antibacterial leads, and to guide the development of new antibacterial synergies (as exemplified by the ability of gallate esters [194] and daptomycin [195, 196] to synergize β-lactams against MRSA). While the chemotherapy that will effectively counter the *S. aureus* phenotype of the future cannot be predicted, the truest sense of scientific optimism remains. *S. aureus* will always be a challenging pathogen, with the chemotherapy to vanquish it remaining for us to discover.

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